6 Signatures of the streptomycin mouse model: investigating host susceptibility using mutant mice

6.1 Introduction

6.1.1 Systematic screening of knockout mice for the detection of novel phenotypes in infection susceptibility

The International Knockout Mouse Consortium (IKMC) was established in 2006 with the goal of mutating all protein-coding genes in the mouse, and making a library of mutant embryonic stem (ES) cells available to the scientific community [321]. The generation of mutant ES cell lines is based on a targeted approach using homologous recombination to introduce the desired mutation into the genome. The main strategy adopted in the IKMC is introduction of a DNA fragment containing a *lacZ* marker gene directly upstream of the critical exons of the targeted gene, thereby impeding gene function without deleting the targeted gene (Figure 6.1). The allele disrupted as described is referred to as the 'tm1a' allele. Breeding of mouse tm1a lines with lines expressing Flp and Cre recombinases can be used to either knock out the gene entirely or inactivate the gene in a tissue-specific manner through generation of a conditional line [322].

Mutant mouse lines are systematically screened for phenotypes at centres in the International Mouse Phenotype Consortium (IMPC). The Wellcome Trust Sanger Institute (WTSI) is a founding member of the IMPC and has made a substantial contribution to the colossal task of screening the ~ 20,000 mutant lines which represent all known and predicted mouse genes. Indeed in 2013 production of more than 900 mutant lines by the WTSI mouse genetics project (MGP) was reported, with close to 500 lines screened for viability and fertility, and 250 tested for adult phenotypes [323].

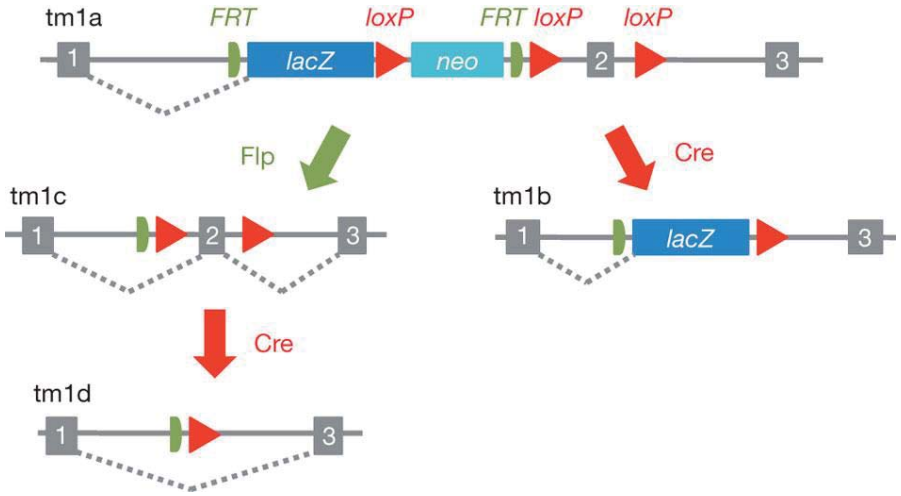


Figure 6.1. Mutant allele design used in the IKMC programme to mutate all protein coding genes in the mouse strain C57BL/6N. Generated by homologous recombination the tm1a allele contains a *lacZ* cassette and floxed cassette for neomycin resistance inserted into the intron preceding the critical exon (here exon 2). Conversion of a tm1a allele to a conditional allele with restored gene activity (tm1c) is performed with Flp recombinase. Crossing tm1c lines with lines in which expression of Cre recombinase is driven by a tissue-specific promoter results in frameshift mutations, forming the allele tm1d in these specific tissues. Alternatively Cre recombinase can be used to delete the neo cassette from the tm1a allele to generate a *lacZ*-tagged knockout allele (tm1b). Image taken from [322].

MGP phenotyping involves a comprehensive array of tests to examine many major organ systems of the adult mouse. Testing of the immune system is an important component of the screening process and is coordinated by the Infection, Immunity and Immunophenotyping (3i) consortium (http://www.immunophenotyping.org/). Both 'observational' and 'challenge' elements contribute to the overall assessment of immune function. In observational tests the immune compartment of organs in healthy animals is examined in flow cytometric analysis of spleen, lymph nodes, bone marrow and peripheral blood. The challenge component investigates the response of mutant mice to a range of infectious organisms and chemical stresses. At the time of writing 115 mutant lines have been screened for susceptibility to the parasitic worm *Trichuris muris*, 437 screened with DSS, 385 with influenza virus and 1,153 lines with *S.* Typhimurium M525 (TetC).

The immune challenges of the 3i program were selected to evaluate the function of broad aspects of innate and adaptive immunity. Multiple phenotypes can be interrogated using different challenges and the inclusion of pathogen challenges can identify distinct aspects of

the immune system that are involved in the responses to these infections. Also, a consideration involved in the design of the individual challenges was minimising variability in the measured outcomes between wild type control mice. Limited variability permits the use of small numbers of mice in screening; reducing the time and cost given the large number of lines, and in keeping with the principles of the 'three Rs' framework for the humane use of animals in research (replacement, reduction, refinement).

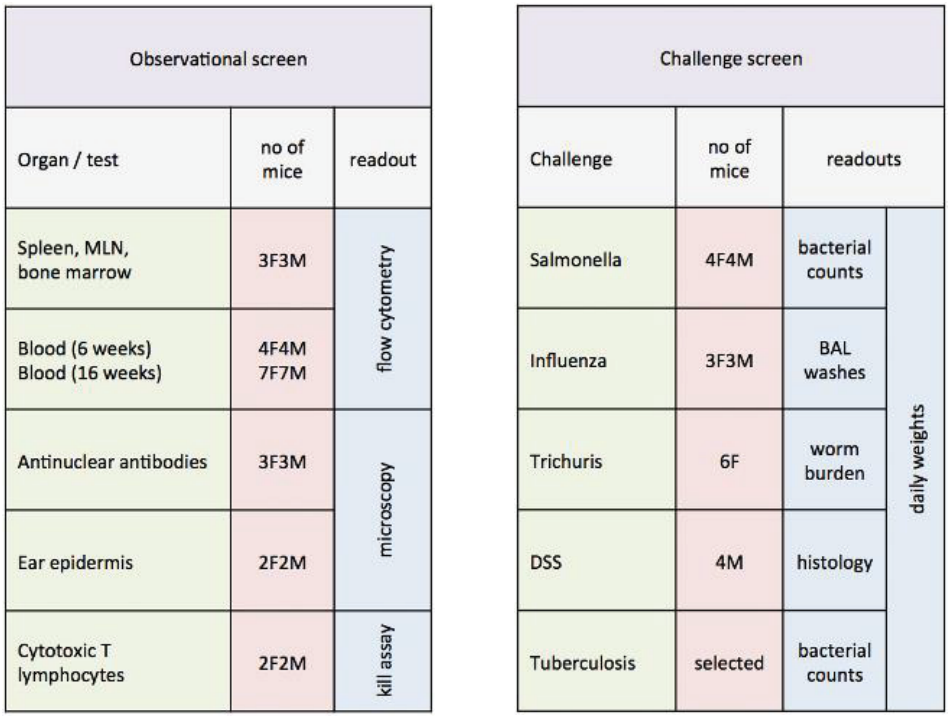


Figure 6.2. Summary of current screening for phenotypes relating to infection and immunity directed by the 3i consortium. Image taken from http://www.immunophenotyping.org/methods.

The MGP infection challenges have successfully recognised phenotypes in genes with previously published roles in infection such as *MyD88* and *NF-κB*, [130, 324, 325]. Many genes with no prior involvement in immunity have also been implicated, and for some mechanistic details have been uncovered. For example a phenotype in the *Salmonella* challenge was identified for *Mysm1* mutant mice with further investigation finding Mysm1 plays an important role in maintenance of bone marrow stem cell function and haematopoiesis [326]. In addition to defective bone formation mutants in gastric intrinsic factor (*Gif*) were found to exhibit an altered susceptibility to both *C. rodentium* and *S.* Typhimurium

challenges, with further secondary phenotyping demonstrating metabolic and immunological defects associated with vitamin B12 deficiency [327].

The MGP *Salmonella* challenge tests the involvement of host genes in protection in an *S.* Typhimurium typhoid model. Four female and four male mice around eight weeks of age are infected by intraperitoneal (IP) injection of 10^5 *S.* Typhimurium strain M525 expressing the tetanus toxin fragment C (TetC). At days 14 and 28 PI half of the infected mice are culled and the liver is removed for bacterial enumeration and histopathological examination. A blood sample is collected and plasma prepared for analysis of titres of antibody (total Ig, IgG1 and IgG2a) against TetC by ELISA.

We proposed that a *S.* Typhimurium infection challenge, which employs the streptomycin model of *S.* Typhimurium gastroenteritis, might provide complementary information on the role of genes in host protection against *Salmonella* infection. Host genes have been reported previously which affect susceptibility to infection via the oral route exclusively [328]. Such genes may not be identified in the existing *Salmonella* IP challenge. Streptomycin pre-treatment and *S.* Typhimurium infection could be performed as part of secondary phenotyping of lines alongside phenotyping involving other tests of immunity. Additionally the streptomycin-*Salmonella* challenge might be performed on mice with mutations in genes which were prioritised for gene targeting following identification in, for example, GWAS studies of IBD.

In this work tm1a mouse lines with mutations in *IL10rb, IL22ra1* and *BC017643* were selected for oral challenge with *S.* Typhimurium on the basis of phenotypes observed in infection with *C. rodentium* and *Salmonella* at the WTSI, and in the case of *IL10rb* and *IL22ra1* due to the known importance of these genes in immunity. In all tests of the primary phenotyping pipeline *IL10rb*$^{tm1a/tm1a}$ mice were comparable to wild type mice with the exception of infection with *C. rodentium* where the *IL10rb* mutants displayed delayed bacterial shedding and increased colon weights at day 28 PI. *IL22ra1*$^{tm1a/tm1a}$ mice were phenotypically normal in all tests with exception of the skin epidermal structure, as assessed by whole-mount staining of the tail, and the *C. rodentium* challenge [329]. *BC017643*$^{tm1a/tm1a}$ displayed a number of phenotypes in the primary screening pipeline, most notable of these was strongly increased susceptibility to infection in the *Salmonella* challenge. At day 3 PI *BC017643* mutant mice showed severe clinical signs of illness, with *Salmonella* CFU in the liver and spleen around three orders of magnitude greater compared with wild type mice.

These findings suggest a critical role for this unpublished gene in defence against bacterial infection. Using the streptomycin model we sought to investigate a potential role for BC017643 in mucosal bacterial defence. The functions of the cytokine receptor subunits IL10rb and IL22ra1 in host protection are introduced here briefly.

6.1.2 The role of IL10rb in infection and immunity

The IL10 family of cytokines consists of interleukins including 10, 20, 22, 24, 26, 28 (α and β) and 29, and is defined based on the structure and location of the genes which encode them, the structures of these proteins, and the receptor complexes they activate. While these features are relatively conserved across all family members the functional roles they perform are diverse. All IL10 family cytokines induce activation of target cells through heterotetrameric receptor complexes and all may activate cells through complexes which contain a common IL10rb subunit combined with cytokine-specific partners. Accordingly *IL10rb* is constitutively expressed in most cell types, while individual partner subunits display more restricted expression [330].

Produced by a wide range of immune cell types, IL10 is a major anti-inflammatory cytokine, guarding against excessive immune responses and limiting the extent of tissue damage during infection. Its many functions include supressing the secretion of pro-inflammatory cytokines such as IL1, IL6, IL12 and TNFα, and Th1 cytokines such as IL2 and IFNγ, and controlling differentiation and proliferation of macrophages and B and T lymphocytes [331]. Figure 6.3 summarises the major points of action of this cytokine.

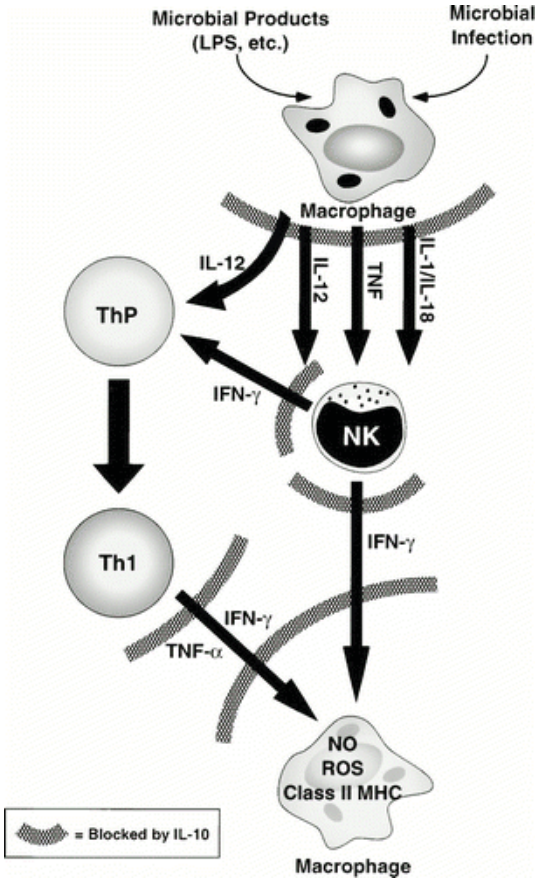


Figure 6.3. IL10 inhibits the major steps in activation of an inflammatory immune response. The detection of microbial molecules by macrophages and monocytes induces a combination of cytokines, which stimulate IFNγ production by NK cells, and polarisation of T cells to the Th1 subtype. Th1 cell cytokines as well as IFNγ from NK cells induce microbial killing mechanisms in macrophages including production of reactive oxygen and nitrogen species, and phagocytosis. IL10 inhibits this response system at all the sites indicated by thick grey curved lines. The combined effect of inhibition at all these individual sites is a large suppression of the overall inflammatory response. Image taken from [332].

The importance of IL10 and receptors in controlling inflammation, particularly in the intestinal mucosa, is illustrated by inactivating mutations which cause monogenic autosomal recessive IBD. Intestinal inflammation resulting from *IL10* mutation is early in onset and only successfully treated by haematopoietic stem cell transplantation to provide immune cells with a functional IL10 signalling pathway [331, 333]. The consequences of human *IL10* mutations are mirrored in mice; a spontaneous colitis phenotype is observed in lines carrying inactivating mutations in *IL10rb* and *IL10* [334, 335].

Whilst the involvement of IL10 pathway mutations in autoimmunity is unequivocal their impact upon control of infection is variable and context dependent; both protection and increased susceptibility have been reported. Suppression of IL10 activity is beneficial during systemic infection of mice both with *Listeria monocytogenes* and *S.* Choleraesuis [336, 337]. IL10-deficient mice display less acute infection and associated inflammation in infection with *C. rodentium*, surprising given the importance of IL10 in restricting inflammatory Th1 and Th17 responses. A second interesting outcome of *C. rodentium* infection of IL10-deficient mice is protection against the onset of spontaneous colitis [338]. Conversely in other cases of

pathogen-induced intestinal inflammation continued Th1 responses in the absence of IL10 can generate severe intestinal pathology; IL10-deficient mice show increased intestinal inflammation and mortality in *Trichuris muris* infection, and loss of IL10 also increases susceptibility to *Helicobacter hepaticus* [339, 340]. During co-infection of mice with malaria and *S.* Typhimurium the parasite infection induces IL10, resulting in reduced inflammatory pathology yet increased systemic *Salmonella* dissemination. This demonstrates how malaria can increase the risk of iNTS infection in Africa through a mechanism reliant upon IL10 [341]. Together these findings demonstrate that the impact of IL10 is highly dependent on factors such as the severity and location of infection, and the specific host factors involved in the response.

6.1.3 The role of IL22ra1 in the intestinal mucosa

IL22 signalling is important for induction of tissue repair and host defence at barriers including the skin, and the mucosae of the airways and intestine. By promoting epithelial proliferation IL22 restores barrier integrity to damaged epithelia. Interaction of antigen presenting cells with microorganisms induces production of IL23, which stimulates a variety of immune cells including IELs, ILCs and Th17 cells to produce IL17 and IL22. IL22ra1 accompanies IL10rb to form the receptor complex for IL22, and is present almost exclusively in non-haematopoietic cells; major targets of IL22 signalling are epithelial cells and keratinocytes. An important function of IL22 in the intestinal mucosa is restriction of commensal bacteria to the intestinal lumen by inducing the production of antimicrobial peptides including Reg3β and γ, lipocalin-2, and S100A8 and S100A9 to form calprotectin, also by the stimulation of production of mucins within goblet cells [342, 343].

Like IL10, IL22 signalling has been reported to be both detrimental and protective in different infections. Intestinal pathogens such as *Salmonella* which induce an upregulation of antimicrobial peptides via IL22 signalling, exploit this pathway to assist them in overcoming colonisation resistance and therefore IL22-deficient mice are protected in *Salmonella* infection. As described in Chapter 1, *S.* Typhimurium possesses a number of resistance mechanisms for avoidance of the harmful effects of antimicrobial peptides. Indeed *IL22* was one of the most highly induced genes in a previous study of mucosal *S.* Typhimurium infection and was found to be highly induced in infection in the work described here in section 3.3.6 also (log2 fold change = 4.26) [152].

Conversely, and again due to effects of IL22 signalling on the microbiota, IL22ra1-deficient mice produced at the WTSI were shown to be highly susceptible to infection with *C. rodentium*. Here IL22-induced production of the fucosyltransferase Fut2 is required for generation of oligosaccharides to feed the microbiota during the period of poor nutrient availability, which occurs during infection. In the absence of fucosylation the opportunistic bacterium *Enterococcus faecalis* is able to outgrow the microbiota, damage the epithelium and cause systemic infection [329, 344]. Similarly IL22-deficient mice also display systemic bacterial burden, increased epithelial damage and mortality [345].

Though classically IL22 is thought to protect against infection at body barriers, a recent paper has described a role for IL22 in protection during systemic infection through induction of hepatic C3, and potentially C3 induction at non-hepatic sites also. It is likely that further roles of IL22 remain undiscovered [346, 347].

6.2 Aims of the work described in this chapter

The potential of oral *S.* Typhimurium infection following streptomycin pre-treatment as a secondary phenotyping test in the MGP mutant mouse screening pipeline was examined. Three mutant lines were selected for investigation on the basis of phenotypes observed in primary phenotyping challenges. Mutant mice were investigated for differences in *Salmonella* burden in caecum, colon, liver and spleen, with further phenotyping performed for the *IL22ra1*$^{tm1a/tm1a}$ mutant line. To gain insight into processes controlled by the targeted genes during infection RNAseq was used to investigate the caecal tissue transcriptome of mutant lines.

6.3 Results

6.3.1 Expression of *IL22ra1* and *IL10rb* in mice homozygous for the tm1a mutant allele

In contrast to *IL10rb*$^{-/-}$ mice reported previously, spontaneous development of colitis was not observed in the *IL10rb*$^{tm1a/tm1a}$ mutant line produced at the WTSI [334]. As explained in the introduction section of this chapter, in tm1a mutant alleles the gene of interest is interrupted rather than deleted. Where the splice acceptor in the gene trap cassette is not 100% efficient alternative splicing can result in ‘skipping’ of the gene trap cassette, giving rise to ‘leaky’ expression of the wild type transcript [323]. We hypothesised that the *IL10rb*$^{tm1a/tm1a}$

allele may be hypomorphic, with sufficient functional *IL10rb* transcripts produced by unwanted splicing events to prevent colitis in mutant mice. Such an effect may be tissue-specific in distribution. Initially we attempted to investigate the extent of IL10rb-deficiency in the *IL10rb*$^{tm1a/tm1a}$ mouse line by Western blotting for IL10rb in cultured macrophages from *IL10rb*$^{tm1a/tm1a}$ and wild type mice. Unfortunately despite efforts with two antibodies directed against mouse IL10rb we were unsuccessful in detection of a polypeptide of the appropriate size for this protein even in wild type mice.

Expression of *IL10rb* and *IL22ra1* in their respective tm1a mutant mouse lines was therefore examined by qPCR and RNAseq. In *IL10rb*$^{tm1a/tm1a}$ mice the *IL10rb* gene is interrupted by insertion of the targeting cassette in the intron 5' to the critical third exon. Commercially available qPCR primers were selected to investigate levels of RNA for regions upstream (exons 1 & 2) and downstream (exons 3 & 4) of the inserted cassette in a range of tissues (Figure 6.4A). Signal from both regions of *IL10rb* was reduced in RNA samples from *IL10rb*$^{tm1a/tm1a}$ mice compared with wild type controls, with a markedly greater reduction in the region of exons 3 & 4. However consistent detection of signal from the region of exons 3 & 4 in RNA samples from *IL10rb*$^{tm1a/tm1a}$ mice indicates potentially incomplete abolition of full-length protein-coding *IL10rb* transcripts in the mutant line.

For further investigation RNA was extracted from bone marrow and peritoneal macrophages obtained from *IL10rb*$^{tm1a/tm1a}$ and wild type mice (n = 3) and qPCR was performed to assess the reduction in RNA in the region of exons 3 & 4 of *IL10rb*. *IL10rb*$^{tm1a/tm1a}$ BMDM displayed a 274-fold reduction in RNA from exons 3 & 4 (normalised against expression of the housekeeping gene *Gapdh*), while a 381-fold reduction was observed in peritoneal macrophages.

Further, differential expression analysis was performed on caecal transcriptome profiles from *S.* Typhimurium-infected wild type and *IL10rb*$^{tm1a/tm1a}$ mice (day 4 PI) generated by RNAseq (n = 5) (further detail in section 6.3.4). An average normalised read count across all samples of 2,873 for *IL10rb*, relative to the median across all genes of 357, indicates *IL10rb* is highly expressed in the infected caecum. *IL10rb* transcripts were decreased in *IL10rb*$^{tm1a/tm1a}$ *S.* Typhimurium-infected caecal samples compared with wild type controls with a log2 fold change of -4.32, equivalent to a ~ 20-fold reduction, with a p-value of 10^{-243} following correction for multiple testing.

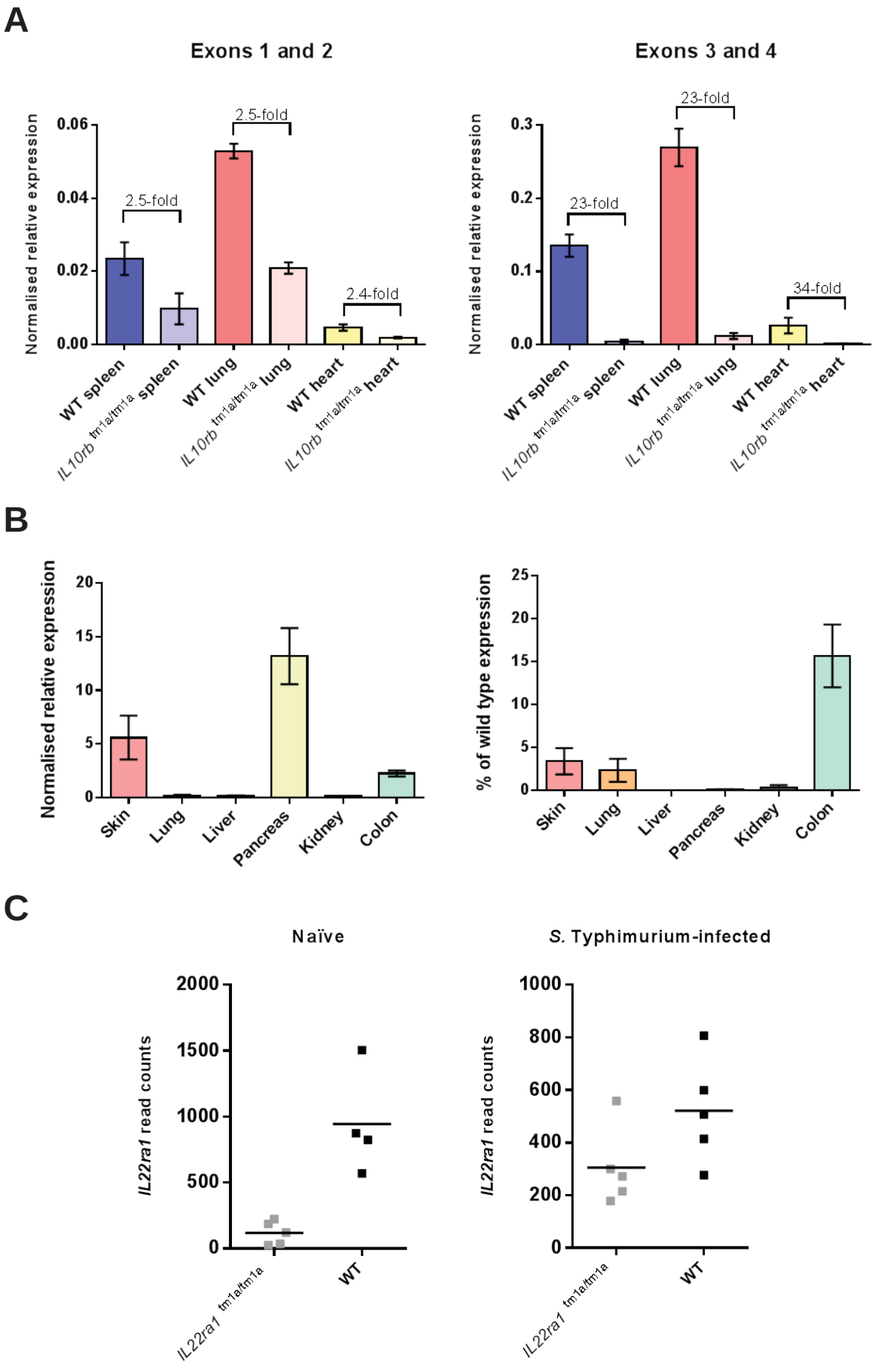
In summary these findings indicate 'leaky' expression of wild type *IL10rb* occurs in *IL10rb*$^{tm1a/tm1a}$ mice, with levels of full length transcripts controlled by cell- or tissue-dependent factors. Further work is needed to determine how conditions such as infection impact upon levels of full length *IL10rb* transcripts in these mice.

Possible production of full length *IL22ra1* transcripts in *IL22ra1*$^{tm1a/tm1a}$ mice was investigated with qPCR with primers for the amplification of a region spanning the fourth and fifth exons of the mouse *IL22ra1* gene. A range of tissues were selected for qPCR to assess level of *IL22ra1* expression in healthy wild type and *IL22ra1*$^{tm1a/tm1a}$ mice (Figure 6.4B). No obvious relationship was found between organ levels of *IL22ra1* expression in wild type mice and the reduction in expression in these organs in *IL22ra1*$^{tm1a/tm1a}$ mutants. *IL22ra1* transcripts were virtually undetectable in RNA from the liver and pancreas of *IL22ra1*$^{tm1a/tm1a}$ mice. However in several tissues expression of *IL22ra1* in the mutant line was considerable; for example in the colon expression in samples from *IL22ra1*$^{tm1a/tm1a}$ mice was 15.7% of the wild type level.

RNA extracted from wild type and *IL22ra1*$^{tm1a/tm1a}$ naïve and *S.* Typhimurium-infected (day 4 PI) caecal samples were sequenced. For both the naïve and infected samples, which were analysed in separate sequencing runs, the normalised read count for *IL22ra1* across all samples was greater than the median for all genes, indicating *IL22ra1* expression is substantial. Differential expression analysis of *IL22ra1*$^{tm1a/tm1a}$ and wild type naïve samples produced a log2 fold change in *IL22ra1* of -1.6 (approximately a 3-fold reduction in the mutant) with an adjusted p-value of 1.6×10^{-8}. In contrast analysis of the transcriptional profiles from *S.* Typhimurium-infected tissue showed *IL22ra1* was not significantly reduced in the mutant (log2 fold change = -0.44, adjusted p-value = 0.248). The read counts from the individual samples used in this analysis are displayed in Figure 6.4C.

These findings indicate that expression of *IL22ra1* is incompletely abolished in *IL22ra1*$^{tm1a/tm1a}$ mice, and that levels of residual expression are both tissue-dependent, and involve other factors including infection. For detailed investigation of the role of *IL22ra1* the tm1a allele should be converted to a tm1b allele in which the critical exon is deleted. Although we observed substantial levels of residual *IL22ra1* expression in *IL22ra1*$^{tm1a/tm1a}$ mice this has not precluded detection of phenotypes previously, as demonstrated by the increased susceptibility of this line in *C. rodentium* infection [329]. In addition, the majority of mutant animals which are currently phenotyped in the MGP pipeline contain a tm1a allele

in the targeted gene. For these reasons and due to the absence of the time required for production of full knockout mice the tm1a line was studied throughout this work.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2.5-fold
2.4-fold
23-fold
23-fold
34-fold
WT spleen
IL10rb tm1a/tm1a spleen
WT lung
IL10rb tm1a/tm1a lung
WT heart
IL10rb tm1a/tm1a heart
B
Normalised relative expression
% of wild type expression
Skin
Lung
Liver
Pancreas
Kidney
Colon
C
Naïve
S. Typhimurium-infected
IL22ra1 read counts
IL22ra1 tm1a/tm1a
WT

Figure 6.4. Analysis of *IL10rb* and *IL22ra1* expression levels in tm1a mutant mouse lines. (A) Relative expression of regions of the *IL10rb* gene spanning the first and second, and third (critical) and fourth exons, normalised against expression of the housekeeping gene *Gapdh*. Tissue pieces were taken from two wild type (WT) and two *IL10rb*$^{tm1a/tm1a}$ mice. All amplification reactions were performed in duplicate. Error bars display standard error on the mean. Fold reduction between the average expression for wild type and *IL10rb*$^{tm1a/tm1a}$ is displayed for each tissue. (B) Left - Relative expression of *IL22ra1* normalised against expression of the housekeeping gene *Gapdh* in a range of tissues from wild type mice (n = 3). Amplification reactions were performed in triplicate. Right - Percentage of wild type normalised expression levels detected in samples from *IL22ra1*$^{tm1a/tm1a}$ mice (n = 3). Error bars indicate the standard error on the percentage. (C) *IL22ra1* read counts from RNAseq of naïve and *S.* Typhimurium-infected caecal tissue. Naïve and infected samples were sequenced in separate sequencing runs and therefore cannot be compared directly.

6.3.2 *S.* Typhimurium colonisation and dissemination in *IL10rb*$^{tm1a/tm1a}$ mice following streptomycin pre-treatment

IL10rb$^{tm1a/tm1a}$ mice were treated with streptomycin 24 h prior to oral infection with *S.* Typhimurium SL1344 in two separate experiments. Representative data for *Salmonella* CFU in the spleen, liver, colon and caecum are presented in Figure 6.5. The results of both experiments suggest control of *Salmonella* in *IL10rb*$^{tm1a/tm1a}$ mice is comparable to wild type animals.

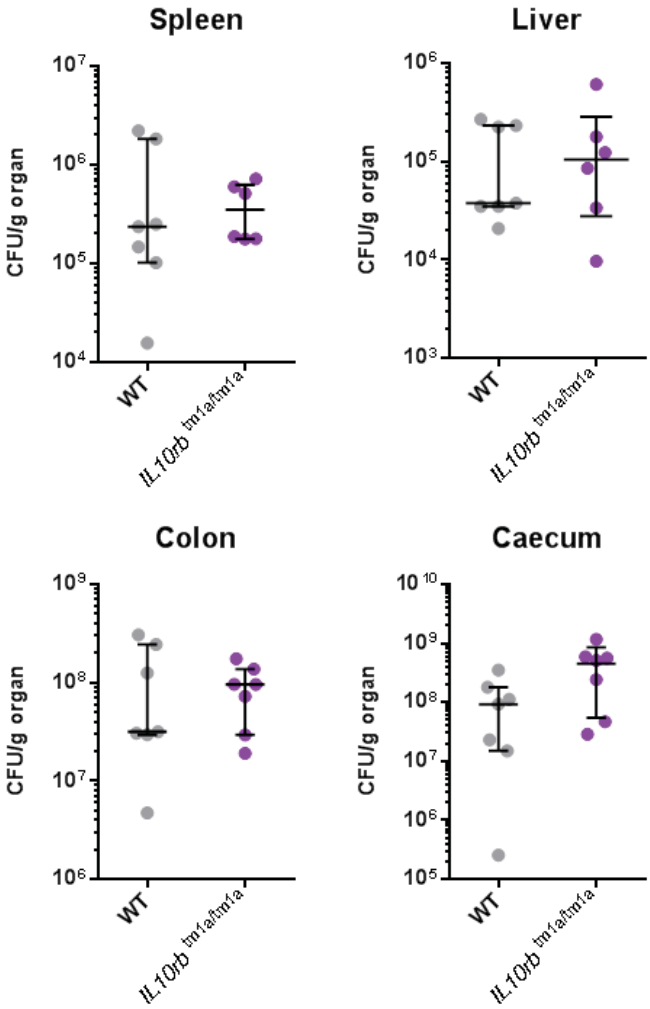


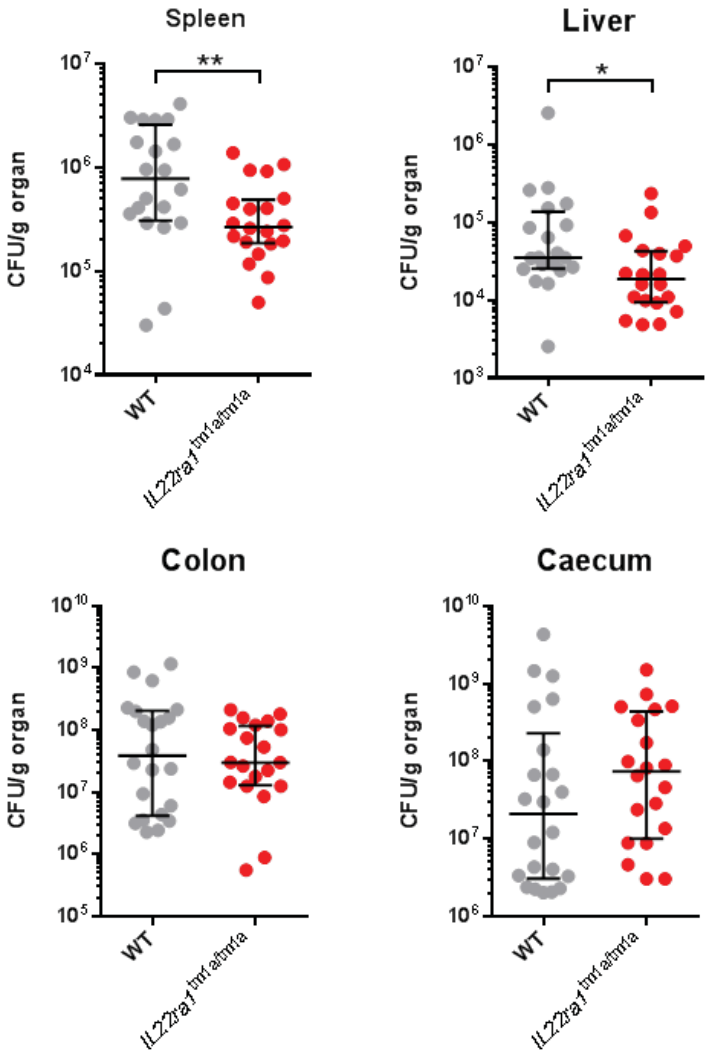
Figure 6.5. *Salmonella* burden in *IL10rb*$^{tm1a/tm1a}$ mice at day 4 PI with *S.* Typhimurium following streptomycin treatment. *Salmonella* organ CFU from oral infection of wild type (WT) and *IL10rb*$^{tm1a/tm1a}$ mice with *S.* Typhimurium SL1344 (1.3 x 10^4 CFU). Bars show median and interquartile range. Significance testing was performed with a Mann Whitney U test.

6.3.3 *S.* Typhimurium infection of *IL22ra1*$^{tm1a/tm1a}$ mice in the streptomycin model

IL22ra1$^{tm1a/tm1a}$ mice were treated with streptomycin 24 h prior to oral infection with *S.* Typhimurium SL1344 in three separate experiments. Reduced *Salmonella* colonisation of the liver and spleen in *IL22ra1*$^{tm1a/tm1a}$ mice was observed in multiple experiments, whilst CFU in the intestinal tissues did not appear to be affected by host genotype. Figure 6.6A shows the combined *Salmonella* CFU from the three experiments. Overall *Salmonella* counts in the spleen and liver were significantly reduced with p-values of 0.006 and 0.0142 respectively. *IL22ra1*$^{tm1a/tm1a}$ mice displayed reduced weight loss compared with wild type mice; a representative weight loss curve is presented in Figure 6.6B. Scoring to reflect the

severity of inflammation in H & E stained tissues from *IL22ra1*$^{tm1a/tm1a}$ and wild type mice indicated no significant difference in tissue pathology between mice of different genotypes (n = 6) (Figure 6.6C).

A


Spleen
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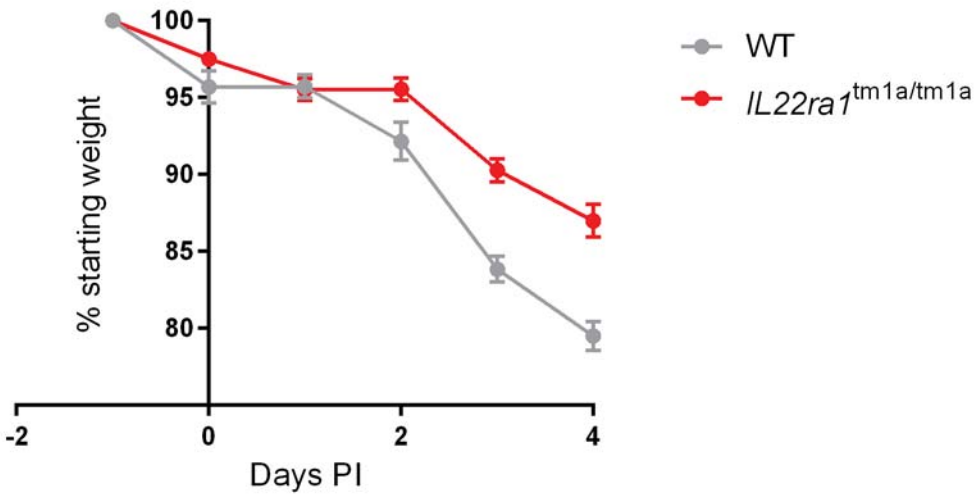
IL22ra1tm1a/tm1a

B


% starting weight
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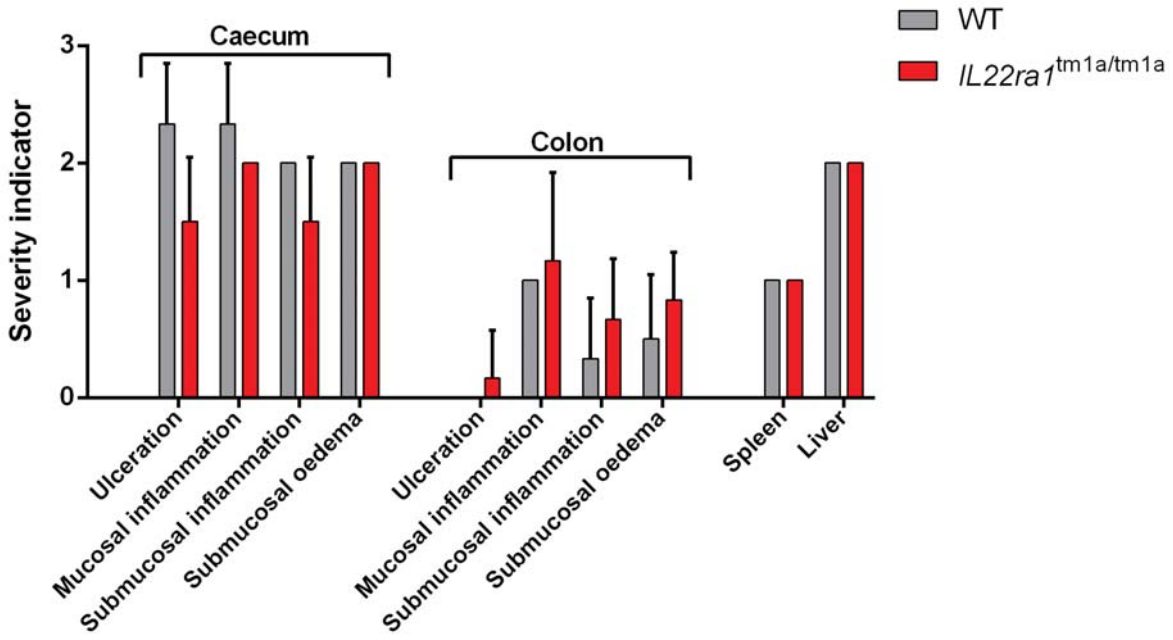

Figure 6.6. Burden of *Salmonella* in organs, weight loss, and intestinal inflammation in *IL22ra1*$^{tm1a/tm1a}$ mice at day 4 PI with *S.* Typhimurium following streptomycin treatment. (A) *Salmonella* organ counts data from infection of wild type (WT) and *IL22ra1*$^{tm1a/tm1a}$ mice with *S.* Typhimurium SL1344. Data is pooled from three individual experiments with oral delivery of a bacterial suspension of 1.2 x 10^4, 1.4 x 10^4 and 1.2 x 10^4 CFU. Bars show median and interquartile range. Significance was assessed with a Mann Whitney U test. (B) Weight curve for wild type (WT) and *IL22ra1*$^{tm1a/tm1a}$ mice infected with *S.* Typhimurium. Points indicate the mean of the mouse weights and error bars the standard error of the mean. Wild type: n = 7, *IL22ra1*$^{tm1a/tm1a}$: n = 8. (C) H & E-stained caecum, colon, liver and spleen from wild type (WT) and *IL22ra1*$^{tm1a/tm1a}$ mice infected with *S.* Typhimurium were examined for indicators of inflammation. Caecum and colon were assigned scores to reflect the severity of ulceration, mucosal and submucosal inflammation and submucosal oedema. Liver and spleen were assigned a single score to reflect the degree of inflammation (0, 1, 2, or 3 for absent, mild, moderate & severe), n = 6.

6.3.4 The transcriptome of *IL22ra1*$^{tm1a/tm1a}$ and *IL10rb*$^{tm1a/tm1a}$ mice in *S.* Typhimurium infection

Analyses described in the previous two sections addressed potential phenotypes of *S.* Typhimurium infection in the *IL22ra1*$^{tm1a/tm1a}$ and *IL10rb*$^{tm1a/tm1a}$ mutant mouse lines. To examine possible differences in molecular signatures of infection between mutant and wild type mice RNA extracted from *S.* Typhimurium-infected caecal tissue was analysed by

RNAseq. *IL22ra1*$^{tm1a/tm1a}$, *IL10rb*$^{tm1a/tm1a}$ and wild type mice were pre-treated with streptomycin and infected with *S.* Typhimurium SL1344 (n = 5). Samples of caecal tissue were collected at day 4 PI for RNA extraction followed by sequencing. RNA extracted from naïve *IL22ra1*$^{tm1a/tm1a}$ and wild type mice (oral treatment with streptomycin followed by PBS only) were also sequenced in a separate sequencing run (n = 4). Differential expression analysis was performed to detect abnormalities in the response to infection in the mutant lines at the level of the transcriptome, and differences in basal gene expression in naïve *IL22ra1*$^{tm1a/tm1a}$ mice. Fold change and p-value thresholds used to define differentially expressed genes were as previously used in Chapter 3 (log2 fold change < -1 or > 1, adjusted p-value < 0.05).

In samples from *S.* Typhimurium-infected *IL10rb*$^{tm1a/tm1a}$ mice 83 genes were significantly increased and 77 significantly decreased compared with samples from wild type mice. A larger number of differentially expressed genes were detected for *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$ mice; 219 genes were upregulated and 190 downregulated relative to wild type controls. In contrast a relatively small number of differentially expressed genes were detected in naïve *IL22ra1*$^{tm1a/tm1a}$ caeca compared with wild type controls; 15 genes were upregulated and 20 downregulated. Surprisingly little overlap was observed between differentially expressed genes for naïve and infected *IL22ra1* mutant mice; as few as 9 genes significantly altered in naïve *IL22ra1* mutants were also altered in the *S.* Typhimurium-infected animals relative to wild type controls with equal treatment. The 50 most highly increased and decreased genes for each comparison (*S.* Typhimurium-infected *IL10rb*$^{tm1a/tm1a}$ verses wild type, *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$ verses wild type, and naïve *IL22ra1*$^{tm1a/tm1a}$ versus wild type) are listed with corresponding transcript abundances, fold changes and associated p-values in Appendix 6.

Genes differentially expressed in *IL22ra1*$^{tm1a/tm1a}$ mice were compared with a list of 41 genes reported to be regulated by IL22 signalling in previously published studies [152, 329, 348-353]. Eight genes reported to be upregulated by IL22 were found to be significantly decreased in *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$ mouse caecum compared with wild type mice. These were genes encoding the antimicrobial peptides Reg3β, Reg3γ and lipocalin-2, the antimicrobial chemokine CXCL1, the cytokine IL10, the fucosyltransferase Fut2, a phospholipase of the antibacterial phospholipase A2 family (Pla2g5), and Socs3 - part of a negative feedback system to limit cytokine signalling through the JAK/STAT pathway

[354]. In naïve *IL22ra1*$^{tm1a/tm1a}$ caecum only one of the 40 genes, *Reg3β* was differentially expressed. Further many members of protein families implicated as targets of IL22 signalling were downregulated in the *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$ samples; for example expression of three matrix metallopeptidases, three members of the serum amyloid A family and six serine or cysteine peptidase inhibitors (SERPINs) were decreased in *IL22ra1*$^{tm1a/tm1a}$ caecum relative to wild type control samples. Interestingly, while mucins are known to be upregulated by IL22, three mucins (2, 4, and 20) were upregulated in *IL22ra1*$^{tm1a/tm1a}$ infected caecum relative to wild type controls, perhaps suggesting compensatory mechanisms may support mucin gene expression in the absence of IL22, or the cytokine may play different roles in regulation of the many members of this protein family [350]. The presence of known targets of IL22 signalling amongst the transcripts downregulated in *IL22ra1*$^{tm1a/tm1a}$ mice validates the ability of this approach to detect targets of IL22 in infection.

Genes differentially regulated in *IL10rb*$^{tm1a/tm1a}$ mice were also compared with published signalling targets; initially a list of over 40 genes previously reported to be regulated by the IL10 signalling pathway, many of which were supported by evidence from several studies [355-358]. Unexpectedly there was no overlap between the differentially expressed genes in *IL10rb*$^{tm1a/tm1a}$ mice and the published genes. As IL10rb is an essential component of the IL22 receptor complex, possible overlap with the 41 previously reported IL22-regulated genes was also examined. Four genes induced by IL22 were found to be downregulated in the caeca of *IL10rb*$^{tm1a/tm1a}$ mice; *Reg3β*, *Reg3γ*, *Pla2g5* and *Fut2*.

6.3.4.1 Signatures of T cell activation in the caecum of *IL10rb* mutant mice during *S.* Typhimurium infection

Despite the lack of changes in known IL10-regulated genes in the caecum of *Salmonella*-infected *IL10rb* mutants we observed a large number of genes important in T cell activation to be upregulated. T lymphocytes are one of the many cell types regulated by IL10; in particular IL10 is an important inhibitor of cytokine production by the Th1 subset and is essential for regulatory T cell function [359, 360]. We observed many proteins of the TCR/CD3 complex; the surface receptor complex for T cell activation by antigen and coupling to cellular signal transduction; were upregulated. Specifically eight proteins of the TCR including constant, variable and joining regions, and the δ, ε, and γ chains of CD3 were all significantly upregulated in *S.* Typhimurium-infected *IL10rb*$^{tm1a/tm1a}$ caecum relative to wild type controls. Many other genes involved in T cell signalling pathways were also

increased, including *Zap70*, Linker for activation of T cells (*Lat*), Inducible T cell co-stimulator (*Icos)*, and *IL2ra*. *Zap70* is important for signal transduction from the TCR and *IL2ra* encodes a chain of the receptor for IL2, a cytokine which acts primarily on T cells and promotes differentiation into effector and memory cell types. Upregulation of *CD40LG*, a T cell surface protein which regulates B cell function, and the B lymphocyte kinase *Blk*, also indicates possible effects on B cells in the *IL10rb* mutants.

6.3.4.2 Common genes are differentially expressed in *S.* Typhimurium-infected caecum from *IL10rb* and *IL22ra1* mutant mice

Given the involvement of IL10rb in the signalling receptor complex for IL22 we anticipated an overlap between genes differentially expressed in *IL22ra1* and *IL10rb* mutants in comparison with wild type mice. Indeed approximately half of transcripts significantly downregulated in caecal tissue from *IL10rb*$^{tm1a/tm1a}$ were also significantly downregulated in the caeca of *IL22ra1*$^{tm1a/tm1a}$ mice; the common genes accounting for one fifth of genes downregulated in the *IL22ra1* mutants. 28% of transcripts upregulated in *IL10rb* mutant tissue were similarly regulated in *IL22ra1* mutants, the common genes making up 11% of transcripts upregulated in the *IL22ra1* mutants. The numbers of genes which display common regulation are reported in Figure 6.7.

The greater overlap in downregulated genes in the two mutants is accompanied by a tighter correlation between fold changes for downregulated compared with those for upregulated genes; Pearson's r for the fold changes of the common downregulated genes was 0.54 compared with 0.21 for the upregulated genes. Notably transcripts encoding the serine protease Prss27, G-protein Gna14 and antimicrobial peptide Reg3γ, were in the top five most downregulated genes for both mutants, with the absolute reduction in transcript levels ranging from a factor of 7.8 to 5.1.

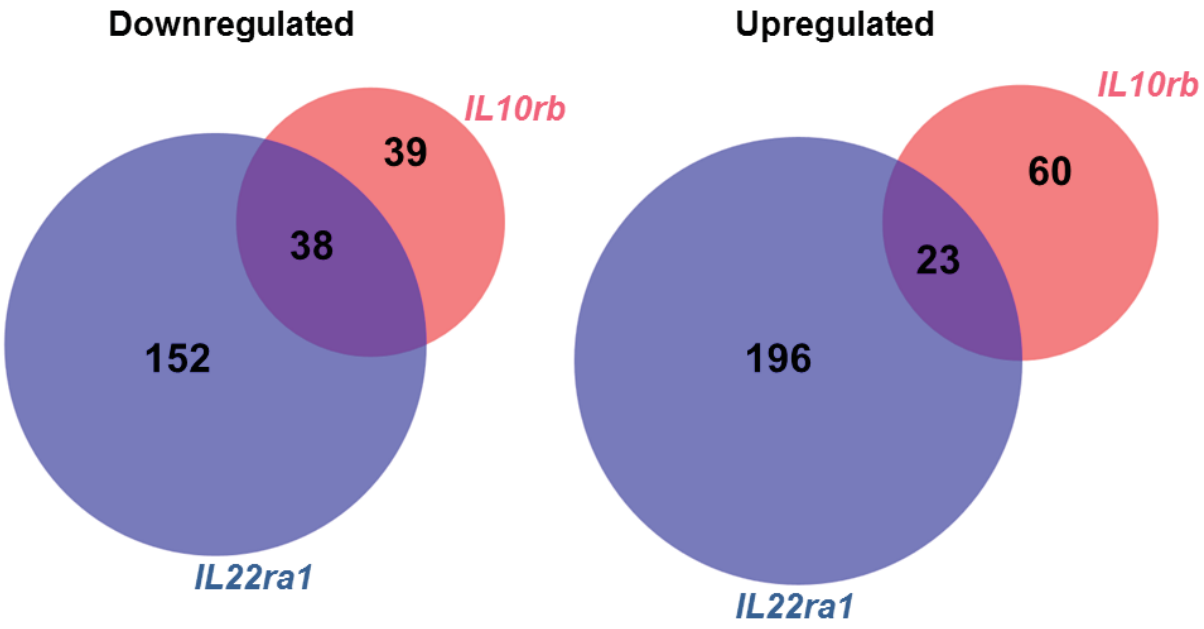

Figure 6.7. Overlap between genes differentially expressed in caecal tissue from *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$ and *IL10rb*$^{tm1a/tm1a}$ mice compared with wild type controls. RNA was extracted from caecal tissue of *S.* Typhimurium-infected *IL22ra1*$^{tm1a/tm1a}$, *IL10rb*$^{tm1a/tm1a}$ and wild type mice at day 4 PI and analysed by RNAseq. DESeq2 was used to perform differential expression analysis to identify genes expressed at different levels in mutant and wild type mice. Transcripts with a log2 fold change < -1 or > 1 and adjusted p-value < 0.05 are considered differentially expressed. Collections of genes with different expression in the *IL22ra1* and *IL10rb* mutant lines are indicated here by circles, with the circle size proportional to the number of genes it represents.

6.3.5 *S.* Typhimurium infection of *BC017643*$^{tm1a/tm1a}$ mice in the streptomycin model

As mentioned in section 6.1.1, *BC017643*$^{tm1a/tm1a}$ mice display dramatically increased susceptibility to *S.* Typhimurium following intraperitoneal delivery of the *S.* Typhimurium strain M525. Investigation of the protein encoded by *BC017643* has indicated an important role in generation of reactive oxygen species through positive regulation of levels of endoplasmic reticulum NADPH oxidase complex components (D. Thomas and S. Clare, unpublished). In this work we sought to investigate how deficiency in BC017643 affects mice orally infected with *S.* Typhimurium in the streptomycin model of gastroenteritis.

24 h post-treatment with streptomycin *BC017643*$^{tm1a/tm1a}$ and wild type control mice were infected with *S.* Typhimurium SL1344 (n = 7). At day 3 PI *BC017643*$^{tm1a/tm1a}$ mice displayed overt signs of illness and consequently all mice were culled. Organ plating was performed for the liver, spleen, colon and caecum for elucidation of *Salmonella* counts.

Relative to wild type controls *BC017643*$^{tm1a/tm1a}$ mice displayed highly increased *S.* Typhimurium CFU in the liver and spleen (both p = 0.0006), and significantly increased counts in the caecum (p = 0.011) (Figure 6.8). These findings indicate that the defect in BC017643 has an effect both in intestinal tissue, and in the liver and spleen following systemic dissemination of *S.* Typhimurium.

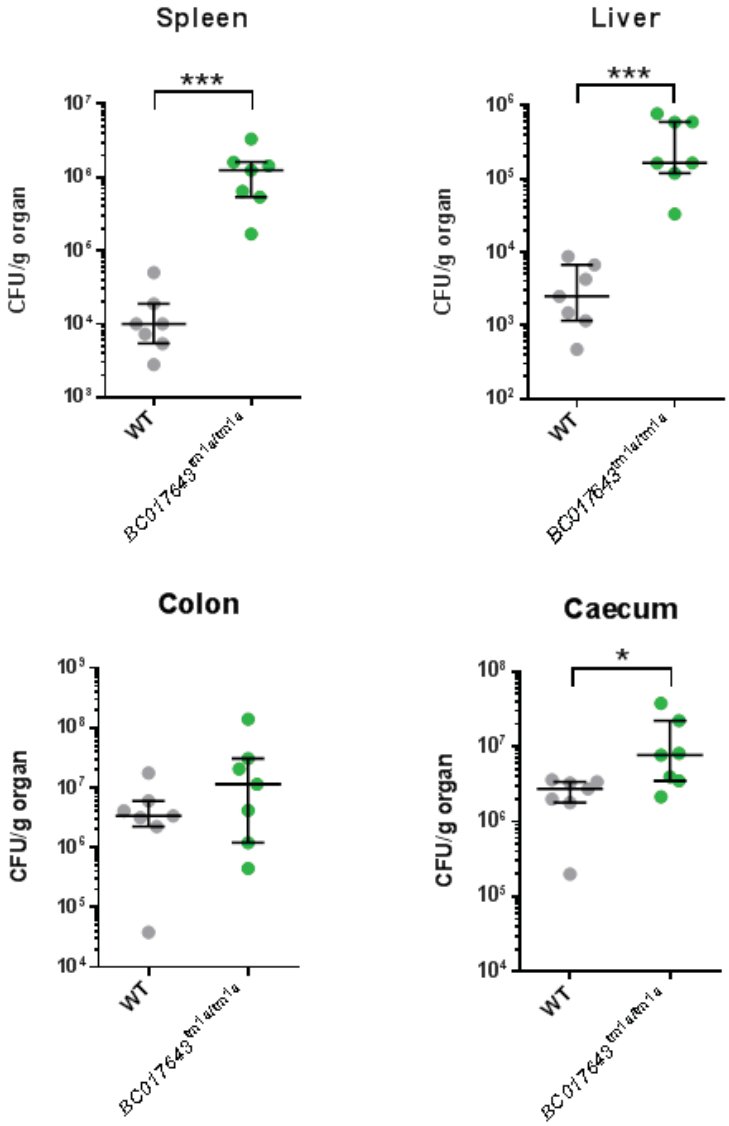

Figure 6.8. Burden of *Salmonella* in organs of *BC017643*$^{tm1a/tm1a}$ and wild type mice at day 3 PI. *Salmonella* organ counts from mice infected with *S.* Typhimurium SL1344 (9 x 10^3 CFU) following streptomycin treatment. Bars show median and interquartile range. Statistical significance was assessed with a Mann Whitney U test.

6.4 Discussion

IL10rb$^{tm1a/tm1a}$ mice failed to develop spontaneous colitis as observed in *IL10rb* and *IL10* mutant lines by others previously, despite the dramatic reduction in *IL10rb* transcripts in macrophages and *S.* Typhimurium-infected caecal tissue from *IL10rb*$^{tm1a/tm1a}$ mice relative to wild type controls [334, 335]. Several non-mutually exclusive possibilities might contribute to this difference. For example, despite the reduced level of *IL10rb* expression in mutant mice the leaky tm1a allele may give rise to sufficient IL10rb activity to avert development of obvious intestinal inflammation. As demonstrated in Chapter 4 correlation between transcript and protein abundance is modest, and therefore the dramatic reduction in transcript levels may have a smaller effect on the level of IL10rb protein. In addition, although a large reduction in *IL10rb* expression relative to wild type controls was demonstrated for isolated macrophages and *Salmonella*-infected caecum from *IL10rb*$^{tm1a/tm1a}$ mice, expression in naïve caecum or colon was not investigated here.

Further, both the IL10rb- and IL10-deficient lines described previously were in a different mouse background strain to the *IL10rb*$^{tm1a/tm1a}$ mice described in this thesis [334, 335]. Background strain can cause wide variations in the effect of inactivating mutations in mice, and therefore differences between the strains might be a factor in the different phenotypes observed here [361]. Finally, differences in the conditions under which mice were housed may also have a role. IL10-deficient mice maintained under SPF conditions were reported to show reduced levels of colitis compared to conventionally-housed mice [335]. A study in which IL10-deficient mice were shown to be protected from spontaneous colitis following *C. rodentium* infection also demonstrates the importance of environmental exposure to different bacteria in the development of colitis [338].

Previous studies addressing the effects of IL10 signalling upon outcomes in *Salmonella* infection support a detrimental impact of IL10 upon host defences [337, 341]. However, no significant difference in intestinal colonisation or systemic dissemination by *S.* Typhimurium was observed between wild type and *IL10rb*$^{tm1a/tm1a}$ mice. As relatively small numbers of mice were used in these experiments and bacterial counts displayed substantial variability this limited our ability to detect a phenotype. However similar to the absence of spontaneous colitis in *IL10rb*$^{tm1a/tm1a}$ mice it is again possible that leaky expression of *IL10rb* may have maintained sufficient signalling receptor activity to prevent detection of a phenotype. Alternatively, IL10-induced suppression of inflammatory and antimicrobial

pathways may not have a substantial effect on the course of this infection. Also important to consider is the role of IL10rb in the receptor complex activated by other IL10 family cytokines. While reduced IL10 signalling in isolation may be protective in *Salmonella* infection, the combined reduction in signalling by the entire array of IL10 cytokine family members may lead to more complex effects on the host response.

In contrast to *IL10rb*$^{tm1a/tm1a}$ mice for which qPCR and RNAseq supported a substantial reduction in target gene expression but no phenotype was observed in *S.* Typhimurium infection, in *IL22ra1*$^{tm1a/tm1a}$ mice the reverse was observed. Analysis of *IL22ra1* transcript abundances in naïve and *Salmonella*-infected caecal tissue suggested that whilst *IL22ra1* expression is significantly reduced in the caecum of naïve *IL22ra1*$^{tm1a/tm1a}$ mice relative to wild type controls, at day 4 PI mutant expression is comparable to wild type levels. Although *IL22ra1* transcript levels in naïve caecal tissue were just 3-fold lower than wild type levels this seemingly modest reduction must be sufficient to give rise to the phenotypic and molecular differences observed here in *S.* Typhimurium infection, and the susceptibility phenotype of *IL22ra1*$^{tm1a/tm1a}$ mice in infection with *C. rodentium* [329]. While the RNAseq evidence suggests that *IL22ra1* expression in the caecum is restored to wild type levels upon infection a delay in restoration during the earlier stages of infection may give rise to the observed differences. An alternative possibility is that whilst splicing in the tm1a allele successfully excludes the inserted reporter cassette, the full-length *IL22ra1* transcript produced in *IL22ra1*$^{tm1a/tm1a}$ mice contains a mutation, which either partially or completely inactivates the function of the encoded protein. To investigate this possibility an end-point reverse transcription (RT) PCR should be performed to amplify the whole transcript, followed by PCR and sequencing.

The detection of reduced systemic dissemination in *IL22ra1*$^{tm1a/tm1a}$ mice, despite the absence of significant differences in intestinal pathology and colonisation, is an interesting finding. As IL22 is reported to be important for tissue repair and epithelial barrier maintenance, mice deficient in the cytokine receptor might be expected to display increased systemic *Salmonella* dissemination. However a study of *IL22*$^{-/-}$ mice in the streptomycin model published last year demonstrated a significant reduction in *Salmonella* colonisation of the colon. The reduction in intestinal *Salmonella* was attributed to the failure of antimicrobial peptide induction in these mice, to the benefit of commensal bacteria susceptible to these molecules [152]. Although we did not detect a significant difference in *Salmonella*

colonisation of the intestine we observed reduced CFU in the liver and spleen. One possibility for the difference between our findings and the *IL22*$^{-/-}$ study is that accurate enumeration of tissue-associated bacterial counts is more difficult in the intestinal tissues than in the liver and spleen due to wide-ranging levels of contamination with adherent faecal material. Therefore reduced *Salmonella* in the gut may become apparent only as reduced systemic dissemination of *Salmonella* to the liver and spleen. Indeed interquartile ranges in CFU were observed to be much smaller in liver and spleen than the intestinal organs. To further investigate the possibility of increased colonisation resistance in *IL22ra1*$^{tm1a/tm1a}$ mice it would be interesting to perform 16S rRNA gene sequencing of intestinal content during *S.* Typhimurium infection similar to the *IL22*$^{-/-}$ study [152].

The possibility of IL22 effects on systemic control of bacteria should also be considered. However the finding that *IL22ra1*$^{tm1a/tm1a}$ mice display reduced *Salmonella* in the liver and spleen lies at odds with the finding that IL22 induces hepatic complement protein production resulting in enhanced opsonization of systemic bacteria [346].

Far fewer differences between the transcriptome profiles of *IL22ra1*$^{tm1a/tm1a}$ and wild type caecum were observed in naïve mice compared with mice infected with *S.* Typhimurium. Although fewer naïve mice were sequenced this is unlikely to account for the dramatic difference observed. The greater differences in the transcriptomes of infected mice were observed despite a significant difference in *IL22ra1* expression exclusively in the naïve condition. A likely explanation is that in the naïve state IL22ra1 signalling activity is limited in comparison to the infected state. Therefore during infection defective IL22 signalling is less well tolerated, resulting in larger effects on genes downstream of this cytokine. It was surprising to find that just one quarter of DE genes in naïve *IL22ra1*$^{tm1a/tm1a}$ mice were also DE during *S.* Typhimurium infection. This suggests that the combinatorial effect of activation of other pathways alongside IL22 signalling during infection results in effects on genes more broadly and different to those in the naïve condition where less immune signalling pathways are active.

Based on the broader role of IL10rb compared with IL22ra1, IL22ra1-deficient mice might be predicted to display a subset of the gene expression differences observed in IL10rb-deficient mice during infection. However we observed a greater number of DE genes in the *IL22ra1*$^{tm1a/tm1a}$ mutant mice. A complex interplay between cytokine signalling pathways during infection might account for this result. For further investigation of the individual

effects of IL10 and IL22 signalling during gastrointestinal infection with *S.* Typhimurium it would be interesting to repeat infections with knockout mice for the cytokines themselves.

The failure to identify differential expression of specific genes reported to be regulated by IL10 in *S.* Typhimurium-infected *IL10rb*$^{tm1a/tm1a}$ mice was potentially concerning, although again the difference may be a consequence of the broader role of IL10rb in signalling by other cytokines. However finding downregulation of several genes activated by IL22 in these mice was a reassurance that the DE genes we detected included those resulting specifically from IL10rb deficiency. The finding of upregulation of many genes relating to activation of T cells through the TCR complex in *S.* Typhimurium-infected *IL10rb*$^{tm1a/tm1a}$ mice was interesting and is worthy of more detailed investigation in the context of current knowledge on the effects of IL10 on T cells. The first step in this investigation would be to profile the transcriptome of naïve *IL10rb*$^{tm1a/tm1a}$ mouse caecum in order to determine whether this effect is present in the absence of *Salmonella*.

In summary the transcriptome analysis of mutant mice successfully identified genes known to be involved in the targeted pathways, demonstrating the approach can effectively identify genes regulated in infection by these targeted genes. Also detected were many genes with no obvious link to IL10 and IL22 pathways, and many predicted genes and genes of unknown function. These results implicate these genes as potential targets of the IL10 and IL22 pathways, although these may be indirect, and provide a starting point for further investigation of these links. Further work could involve more complex analysis to consider the individual and combined effects of *Salmonella*-infection and host genotype upon gene expression.

Detection of an infection susceptibility phenotype in *BC017643*$^{tm1a/tm1a}$ mice demonstrates the utility of screening approach for gaining insight into genes of unknown function. Prior work identified an important role for the product of gene *BC017643* in the control of systemic *S.* Typhimurium infection. The results presented in this chapter confirm this finding and suggest the gene is also important for control of *S.* Typhimurium in caecal tissue. *BC017643* has been shown to positively regulate proteins involved in the phagocyte NADPH oxidase complex, with the highest levels of expression in human blood cell subsets displayed by neutrophils and monocytes. As neutrophil-mediated killing of *Salmonella* in the intestinal mucosa is one of the major arms of defence at this site the increased *Salmonella* CFU in intestinal tissue of *BC017643*$^{tm1a/tm1a}$ is in keeping with the proposed role for the

BC017643 protein. The observation of more dramatic differences in CFU between *BC017643*$^{tm1a/tm1a}$ and wild type mice in the liver and spleen is interesting. On the one hand this might indicate that the activity of BC017643 is of greater importance in the control of disseminated bacteria. Alternatively this might reflect a complex relationship between control of *Salmonella* colonisation of the gut and systemic dissemination.