5 Final discussion

The data obtained in the present study have demonstrated that miR-155 is important for controlling a mucosal *C. rodentium* infection and for the development of full vaccine-induced resistance to infection with virulent *Salmonella*. Furthermore, we have shown that miR-155 is intrinsically required by B cells for the production of pathogen-specific antibody following both mucosal and systemic infections.

In recent years, miR-155 has been highlighted as an important miRNA after it was shown to be expressed in a number of different innate and adaptive immune cells following their activation. Furthermore, mice lacking miR-155 are immunodeficient and display defective T and B cell responses in addition to impaired DC-mediated T cell activation. However, the role of miR-155 in control of infection, in the context of the overall immune response, still remained to be elucidated. Thus, in this present body of work we have utilised mice with a targeted deletion of the miR-155 gene and miR-155-deficient, μMT-deficient chimeras to examine what effect the loss of miR-155 would have on disease progression, immunity and pathogenesis.

The innate immune system is responsible for providing an immediate response against invading organisms before the generation of pathogen-specific lymphocytes. While a number of previous studies have speculated that miR-155 may participate in the function of various innate immune cells including macrophages and dendritic cells, the data obtained from the above study suggests that miR-155 is not obviously essential for the early innate control of bacteria. Following infection with *C. rodentium,* we observed that miR-155-deficient germline mice and control C57BL/6 mice exhibited comparable numbers of bacteria in gastrointestinal tissues during the early and middle phases of infection, which are highly dependent upon innate immune mechanisms. Similarly, a number of innate immune factors play a vital role in controlling bacterial growth during the early stages of infection with *S.* Typhimurium. We established that the pathogen burden in systemic tissues

from unimmunised miR-155-deficient mice infected with virulent and attenuated *S.* Typhimurium did not differ significantly from controls throughout infection. Previous analyses have shown that macrophage activation and recruitment plays a decisive role in controlling the net growth of *Salmonella* within the reticuloendothelial system of infected mice. Histologically, we observed no overt abnormalities in the appearance or structure of granulomatous lesions in infected miR-155-deficient mice thus implying that macrophage function is not visibly affected in the absence of miR-155. Given that granuloma formation is a highly dynamic process that depends upon the balanced action of a number of cytokines including TNF-α, IFN-γ, IL-4, IL-12, IL-15 and IL-18, we further speculate that early cytokine production is not significantly affected in miR-155-deficient mice, such a view is contradictory to what has previously been suggested[28, 31, 33, 176, 180].

Additionally, we provide evidence that during infection with *C. rodentium* the deficiency in miR-155 results in severe damage to the colonic epithelium and may lead to the development of a polymicrobial infection. We found that coincident with the peak of infection, miR-155-deficient mice developed grossly exaggerated *C. rodentium*-induced colonic hyperplasia. Colons from miR-155-deficient mice weighed significantly more than colons from similarly infected C57BL/6 mice and were visibly thickened as a result of increased proliferation of the colonic epithelia, dilation and thickening of the colonic mucosa and considerable polymorphonuclear infiltrate. Furthermore, closer examination of colons from infected mice revealed that whilst the colonic epithelium remained grossly intact in C57BL/6 mice, miR-155 knockout mice exhibited considerable damage to luminal colonocytes and developed frequent breaks in the epithelial integrity. We subsequently isolated considerable numbers of *C. rodentium* in the livers, spleens and mLNs of several miR-155-deficient mice indicating that bacteria are able to disseminate through the damaged epithelia. In addition, the spread of bacteria to systemic sites implies that T cell-dependent antibody responses may be impaired in the absence of miR-155. The increased severity of hyperplasia observed in miR-155-deficient mice could be as a result of significant changes in metabolic,

catabolic, biosynthetic and ion transport pathways. Considerable shifts in nutrient range and availability in the gut can allow the outgrowth of bacterial species such as *C. rodentium,* which grow at high rates on these substrates. A role for diet and gut flora in transmissible murine colonic hyperplasia has previously been suggested but to the best of my knowledge has not been analyzed quantitatively and this may be something that can be performed.

During histopathological analysis we additionally noted the presence of microcolonies of coccus-shaped bacteria intimately attached to the epithelial surface of miR-155-deficient mice. Thus we tentatively suggest that infection with *C. rodentium* in miR-155-deficient mice may lead to a polymicrobial infection. Nevertheless, we have yet to identify the spatial and temporal changes in the gut microbiome of miR-155-deficient and control mice during infection with *C. rodentium* and thus we cannot speculate further. However, we are currently performing 16S ribosomal RNA (16S rRNA) sequence analysis. The 16S rRNA is a component of the 30S subunit of prokaryotic ribosomes which is highly conserved between different bacterial species and is highly species-specific. Therefore, 16S rRNA gene sequencing provides us with a rapid and accurate method for bacterial identification[206, 207].

Whilst the innate immune response is highly successful in controlling the initial growth of bacteria, the adaptive immune system, including T and B cells, plays an essential role in the eventual eradication of infection. This study highlights the importance of miR-155 for the development of acquired immune responses, in particular for the production of pathogen-specific antibody. Following infection with *C. rodentium,* we observed that miR-155-deficient mice displayed significantly greater pathogen loads in gastrointestinal tissues during the later stages of infection and remained chronically infected for a significant period of time after control animals had achieved complete clearance. These data suggest that miR-155 is important for the development of full protective immunity. When we studied serum antibody responses in mice infected with *C. rodentium* it became apparent that miR-155-deficient mice mount a blunted humoral immune response and produce significantly reduced levels of pathogen-specific Ig, IgG and IgA. We

were subsequently able to show that the impaired antibody responses observed in knockout mice was accompanied by defective germinal centre formation in mLNs. Additionally, we found preliminary evidence that germinal centre responses may also be affected in the caecal patch. This assumption was based on the presence of markedly fewer tingible body macrophages, a characteristic feature of germinal centres within the lymphoid tissue of the caecal patch. TBMs are thought to be responsible for removing apoptotic centrocytes thus their absence may possibly indicate that there is not a high death rate among miR-155-deficient plasmablasts as has been previously suggested[102, 210]. More likely the loss of miR-155 prevents the majority of B cells from differentiating into plasmablasts. In support of this, mir-155 has been shown to be expressed following BCR cross-linking and our microarray analysis identified that BCR signalling was significantly associated with down-regulated genes in miR-155-deficient mice. Furthermore, we noted that amongst the genes significantly down-regulated in miR-155-deficient caecal patches on day 4 pi, there was an over-representation of genes involved, at some stage, in the differentiation and function of B cells.

Microarray analysis additionally revealed that the expression of MMP3 and CXCR3 was significantly reduced in the caecal patches of knockout mice on day 4 and 14 pi, respectively. Both these genes are known to be involved in leukocyte trafficking and accordingly, KEGG testing for overrepresentation showed that the leukocyte transendothelial migration pathway was significantly associated with down-regulated genes. Therefore, we cautiously hypothesize that lymphocyte migration may possibly be affected in miR-155-deficient mice. Nevertheless, we have not studied the movement of different cell populations within *C. rodentium*-infected tissues and further investigation would be required to establish if lymphocyte migration is perturbed. This will be the next challenge.

Our studies have shown that miR-155-deficient germline mice are hyper-susceptible to infection due to impaired humoral immune responses and our data strongly points towards the defect being predominately within miR-155-

deficient B cells. However, the development of pathogen-specific humoral immune responses requires cross-talk between T cells and B cells. To assess the intrinsic requirement of miR-155 for B cell function we utilised mixed chimeras reconstituted with either wild-type or miR-155-deficient B cells. miR-155-deficient, μMT-deficient mice orally infected with *C. rodentium* successfully resolved infection but did so in a significantly delayed fashion compared to control chimeras. Similar to that observed in miR-155-deficient germline mice, chimeric mice with miR-155-deficient B cells remained heavily infected for an extended period of time after wild-type, μMT-deficient mice had cleared infection. Additionally, we observed that several miR-155-deficient, μMT-deficient mice demonstrated considerable levels of *C. rodentium* in systemic tissues such as the liver, spleen and mLNs indicating that they are highly susceptible to systemic infection. Examination of serum antibody responses in chimeric mice infected with *C. rodentium* showed that there was a general trend towards the reduced production of EspA-specific Ig and IgG in miR-155-deficient, μMT-deficient mice as a result of impaired germinal centre formation. However, it should be noted that in chimeras possessing only miR-155-deficient B cells, we observed a slight improvement in disease severity compared with miR-155-deficient germline mice. The increased susceptibility of miR-155-deficient germline mice could suggest that not only B cells are affected by the loss of miR-155. In support of this, previous studies have shown that miR-155-deficient mice exhibit defective B and T cell immunity as well as abnormal function of antigen-presenting cells and correspondingly, our microarray analysis identified that a number of diverse immune response pathways are under-represented in miR-155-deficient mice during infection with *C. rodentium* but, these were not significant after correction for multiple testing[31, 33, 34].

As discussed previously, miR-155-deficient mice infected intravenously with a live, attenuated *aroA S.* Typhimurium displayed no obvious differences in the rate or time of clearance compared to C57BL/6 mice. Eventual eradication of attenuated *Salmonella* from the reticuloendothelial system critically depends on the presence of *Salmonella*-specific $CD4^+$ T cells. Thus

these results imply that T cell function is not obviously affected in the absence of miR-155. Rodriguez *et al* showed however that miR-155-deficient mice whilst able to clear infection with attenuated *S*. Typhimurium produce significantly reduced amounts of IgM and switched antigen-specific antibodies following vaccination and are consequently less readily protected when challenged with a virulent *Salmonella* strain[31]. Consequently we wished to study humoral immune responses and the level of protection in miR-155-deficient, μMT-deficient mice following immunisation. We first immunised chimeras with *S*. Typhimurium SL3261 expressing TetC and serum TetC-specific antibody titres were subsequently measured. Despite exhibiting similar bacterial counts throughout infection, chimeric mice with miR-155-deficient B cells were found to produce significantly less Salmonella-specific Ig, IgG and IgM as well as considerably lower levels of IgG1 and IgG2a subclasses thus indicating that humoral immune responses are impaired. However, following challenge with virulent *S*. Typhimurium C5 even though there is some level of protection compared to naive C57BL/6 mice, both miR-155-deficient, μMT-deficient and wild-type, μMT-deficient mice were not fully protected with the majority of mice rapidly succumbing to infection. Although it is not entirely apparent at this time why wild-type, μMT-deficient chimeras are susceptible to challenge regardless of their robust production of *Salmonella*-specific antibodies, we next intend to determine whether serum antibody produced during immunisation can protect experimentally injected animals, independently.

Because the majority of phenotypic alterations observed in germline mice were recapitulated in chimeric mice with miR-155-deficient B cells we propose that miR-155 is required intrinsically by B cells for the production of pathogen-specific antibody and that the impaired humoral immune response observed in miR-155-deficient mice is B cell autonomous.