4 Immune response to *S.* Typhimurium in miR-155-deficient mice

4.1 General introduction

Unlike *C. rodentium* which causes a mucosal infection restricted to the intestinal tract, *S.* Typhimurium can establish a systemic infection in mice, which has superficial similarities to typhoid fever in humans.

Components of the immune mechanisms contributing to the control and clearance of *S.* Typhimurium infection in mice have previously been elucidated[175, 176]. For example, it is known that during the early phase of infection, cells of the innate immune system such as macrophages and neutrophilic granulocytes can be decisive for controlling the net growth, with a large fraction of *Salmonella* being killed by these cells, perhaps in association with complement and other serum factors[178]. The innate immune response, whilst successful in controlling initial growth of *S.* Typhimurium, is normally inadequate for achieving full protective immunity, particularly if higher doses of *Salmonella* or innately susceptible *Nramp1* defective mice are used[176]. Eventual eradication of bacteria during the late phases of infection (3-4 weeks post-inoculation) requires the development of a *Salmonella*-specific lymphocyte-associated response[178]. In particular, the induction of IFN-γ-producing $CD4^+$ T cells are critical for the resolution of infection[182, 187]. In contrast, B cell-mediated immune responses are generally regarded as having a less important role during the primary immune response but have been shown to contribute to the development and expression of full protective immunity[181, 187, 188].

4.2 Results

4.2.1 Infection with virulent *S*. Typhimurium strain SL1344

Full vaccine-induced resistance to infection with virulent *Salmonella* in mice is dependent on a combination of B and T cell responses, including the presence of specific antibody[187]. Virulent *Salmonellae* can replicate extremely rapidly in vivo and consequently bacterial numbers can reach a critical load threshold (>10^8 CFU per gram tissue) before an effective $CD4^+$ T cell or B cell response can be generated in unimmunised animals. *Salmonella*-specific $CD4^+$ T cell responses generally begin to be detectable by ~7 days post infection. Thus, vaccine induced antibody is thought to delay the growth of bacteria or facilitate clearance, allowing time for $CD4^+$ T cells and macrophages to become activated[179, 187].

We wished to ascertain the impact of the lack of miR-155 production during infection with *Salmonella* infection. To this end, naive miR-155-deficient and innately susceptible C57BL/6 mice were infected intravenously with 1 x 10^2 CFU of virulent *S*. Typhimurium SL1344. Spleens and livers were collected from infected mice at different time points after infection, and the size of pathogen burden was determined by viable count. Throughout infection the pathogen burden of *S*. Typhimurium SL1344 in systemic tissues of miR-155-deficient mice did not differ significantly from controls (Figure 46). From day 2 to day 4 pi, bacterial numbers in the liver and spleen increased by 2-3 logs and were beginning to near the critical load threshold (Figure 46). By 5 day pi, all remaining miR-155-deficient and C57BL/6 mice succumbed to infection (data not shown), thus demonstrating that both miR-155-deficient and control mice are similarly susceptible to infection with virulent *Salmonella*. This is consistent with previously published reports showing that unvaccinated miR-155-deficient and wild-type control mice infected orally with 1 x 10^8 CFU of SL1344, died within 7 days after infection[31]. Furthermore, this study subsequently went on to show that following *S*. Typhimurium *aroA* vaccination, miR-155-deficient mice were

less readily protected than controls when challenged with virulent *S.* Typhimurium.

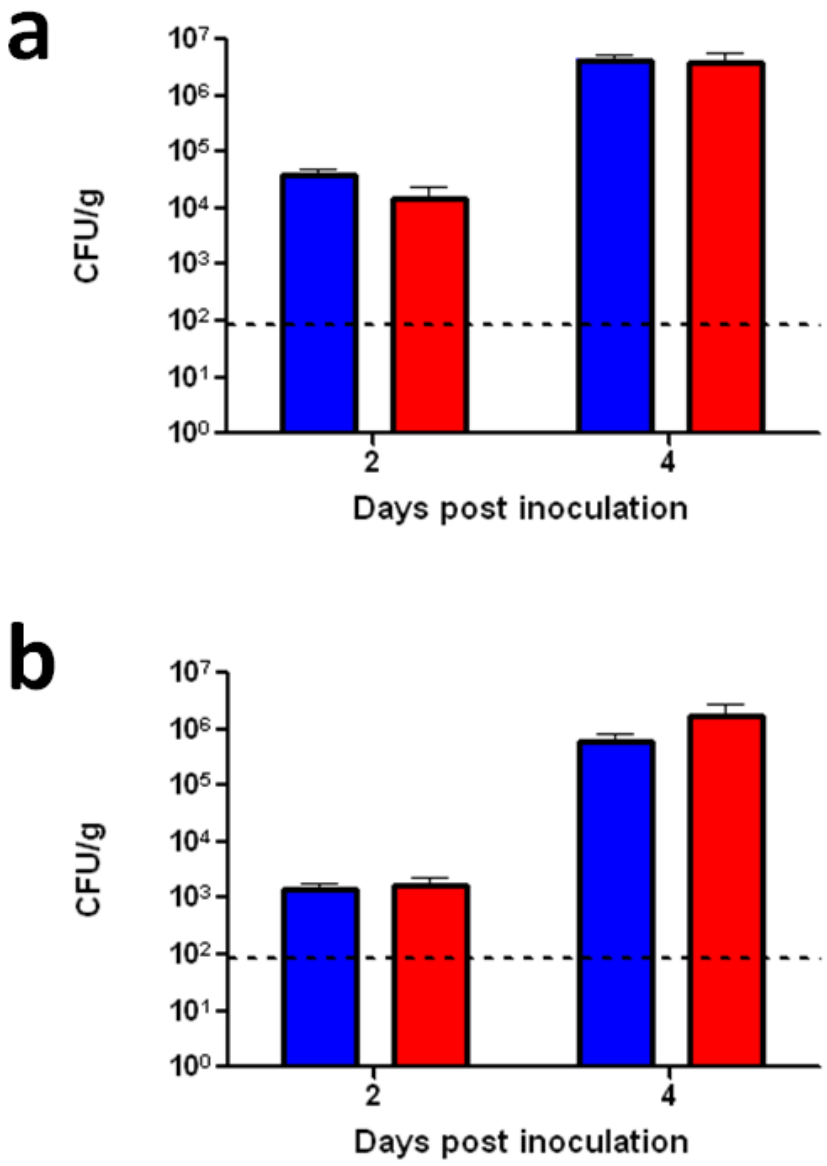

Figure 46. CFU of *S.* Typhimurium SL1344 in systemic tissues of miR-155-deficient and C57BL/6 mice

Control C57BL/6 (blue bars) and miR-155-deficient (red bars) mice were infected intravenously with 1 x 10^2 CFU of virulent *S.* Typhimurium SL1344. On days 2 and 4 pi, mice were sacrificed and numbers of *S.* Typhimurium (±SEM) in systemic tissue; (a) spleen, and (b) liver were enumerated, n=3 mice per group. Black broken lines indicate the detection level of the assay.

4.2.2 miR-155 is not required for the formation of pathological lesions at infection foci during infection with virulent *S*. Typhimurium

Following passage through the FAE and colonisation of the Peyer's patches, bacteria are believed to subsequently move predominantly into the mLNs via the lymphatic system. From there bacteria are able to disseminate to other tissues of the reticuloendothelial system, such as the liver and spleen. During the early stages of infection, control of bacterial growth in the reticuloendothelial system depends on a number of innate immune factors including the gene *Nramp1* (expressed by cells of the monocytes/macrophage lineage) and the production of IFN-γ, IL-12, IL-18, TNF-α and nitric oxide[187]. Suppression of bacterial growth also coincides with the formation of granulomatous lesions within the liver and spleen. Such pathological lesions consist of infected macrophages organised into discrete foci, surrounded by normal tissue. Mastroeni *et al* have suggested that the formation of these lesions confine bacteria to localised foci and in doing so prevent uncontrolled spread of bacteria throughout the body[176]. In fact, failure to form granulomas can result in abnormal growth and dissemination of the bacteria within infected tissues[176]. Lesion formation has been shown to be a highly dynamic process involving cell adhesion molecules such as ICAM1 and the balanced action of TNF-α, IFN-γ, IL-4, IL-12, IL-15 and IL-18[176, 180].

Whilst previous studies have focused on the ability of miR-155-deficient mice to resist oral challenge with virulent *S*. Typhimurium, none have looked at the distribution of *Salmonella* within infected tissues. Therefore, we next examined the formation of granulomatous lesions in miR-155-deficient and C57BL/6 mice following infection with SL1344. No obvious overt abnormalities were observed in the appearance or structure of granulomas in infected miR-155-deficient mice (Figure 47). By day 2 pi, organised granulomas had begun forming in the liver of miR-155-deficient and control mice (Figure 47b and e). We observed that as bacterial numbers in the liver increased from day 2 to day 4 after infection, there was a corresponding

increase in the size and number of pathological lesions (Figure 46 and 47). This is consistent with recently published data showing that the number of infected lesions increases in parallel with net bacterial growth rate, with a small increase in the size of the lesion and in the numbers of infected cells per lesion[176]. On day 4 pi, miR-155-deficient and C57BL/6 mice demonstrated widespread granulomatous lesions as well as areas of necrosis (Figure 47c and f). These data thus suggest that there is no failure to form pathological lesions in the absence of miR-155. Additionally, it indicates that macrophage function is not adversely affected by the loss of miR-155.

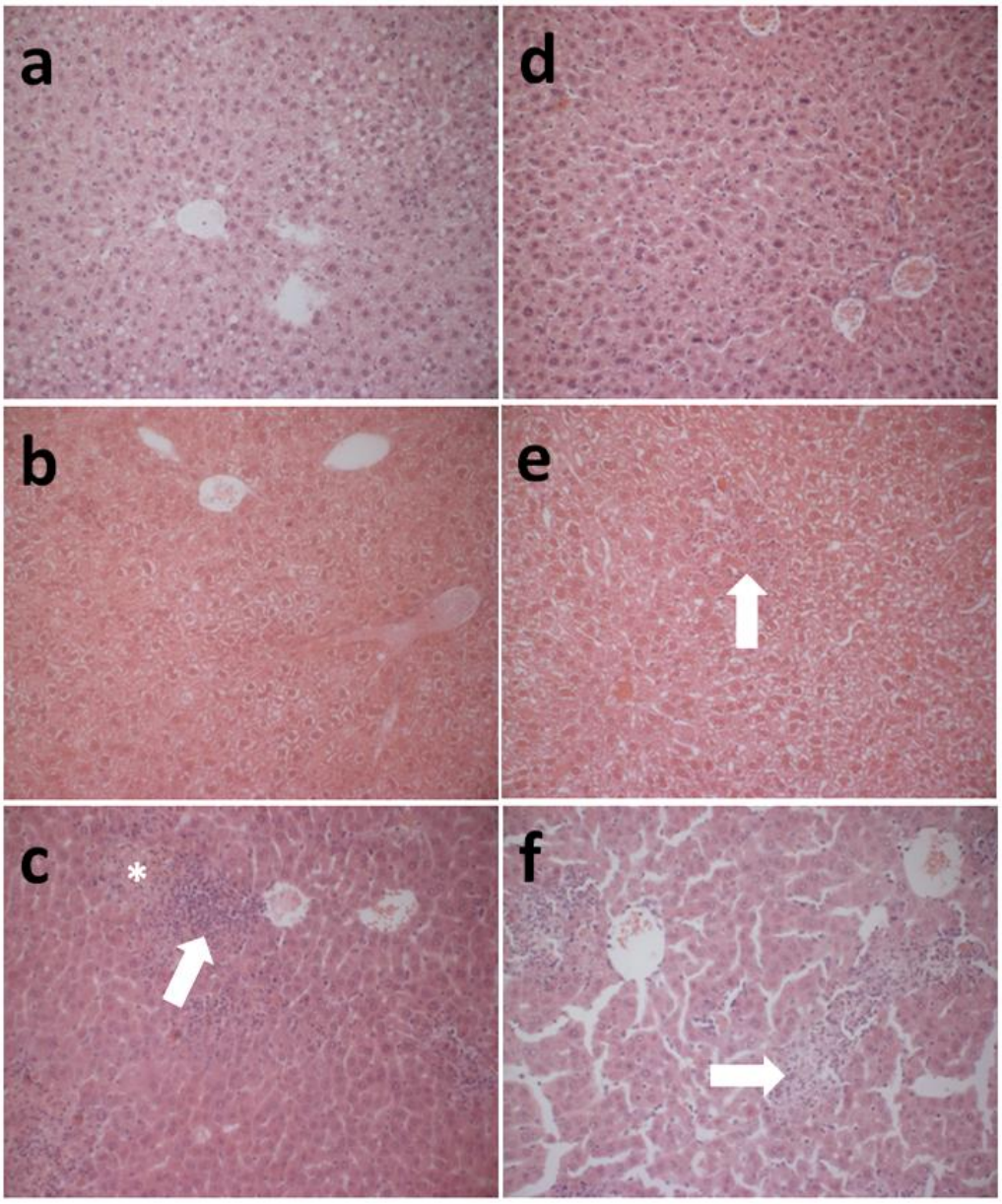

Figure 47. Granuloma formation in miR-155-deficient and C57BL/6 mice following infection with *S*. Typhimurium SL1344

Histopathological analyses (haematoxylin and eosin-stained sections; original magnification, x20) of liver sections from naive C57BL/6 (a) and naive miR-155-deficient mice (d), infected C57BL/6 (b) and infected miR-155-deficient mice (e) on day 2 pi and infected C57BL/6 (c) and infected miR-155-deficient mice (f) 4 days after infection, images are representative of 3 mice per group. By day 2 pi, granulomas (arrow) had begun forming in miR-155-deficient and control livers. 4 days after infection we observed numerous large granulomatous lesions in the liver sections as well as signs of necrosis (asterisk).

4.2.3 miR-155 not essential to resolve infection with attenuated *S*. Typhimurium

Effective clearance of a primary infection with attenuated *S*. Typhimurium is critically dependent on T cells. In contrast, B cells are not so important for the resolution of primary or secondary infection with attenuated *Salmonella*. This may be explained by the fact that attenuated *Salmonellae,* while able to replicate in vivo, do so at a much reduced rate than virulent strains thus, allowing a $CD4^+$ T cell response to develop before excessive bacterial growth can occur[187].

Whilst we know that miR-155-deficient mice are capable of effectively clearing an infection with attenuated *S*. Typhimurium, we do not know if they exhibit differences in the bacterial load within infected tissues. For that reason, we infected miR-155-deficient and C57BL/6 mice intravenously with 1×10^5 CFU of a live, attenuated *aroA S*. Typhimurium vaccine strain and spleen and liver counts of viable bacteria were assayed thereafter. There were no obvious disease related mortalities amongst either group of mice and all miR-155-deficient and C57BL/6 mice effectively eliminated the bacteria from the reticuloendothelial system[31]. Throughout infection there were no significant differences in bacterial counts in either the spleen or liver between miR-155-deficient and control mice (Figure 48). On day 14 pi, both groups of mice had efficiently controlled the initial growth of bacteria and had begun clearing the inoculum, as indicated by a log decrease in bacterial numbers between days 14 and 21 (Figure 48). By 6 weeks after infection, all mice sampled were found to be clear of this strain (data not shown). These results indicate that miR-155-deficient mice show no defects in the rate of clearance of bacteria from the reticuloendothelial system and that miR-155 is not essential for the effective resolution of infection with attenuated *S*. Typhimurium.

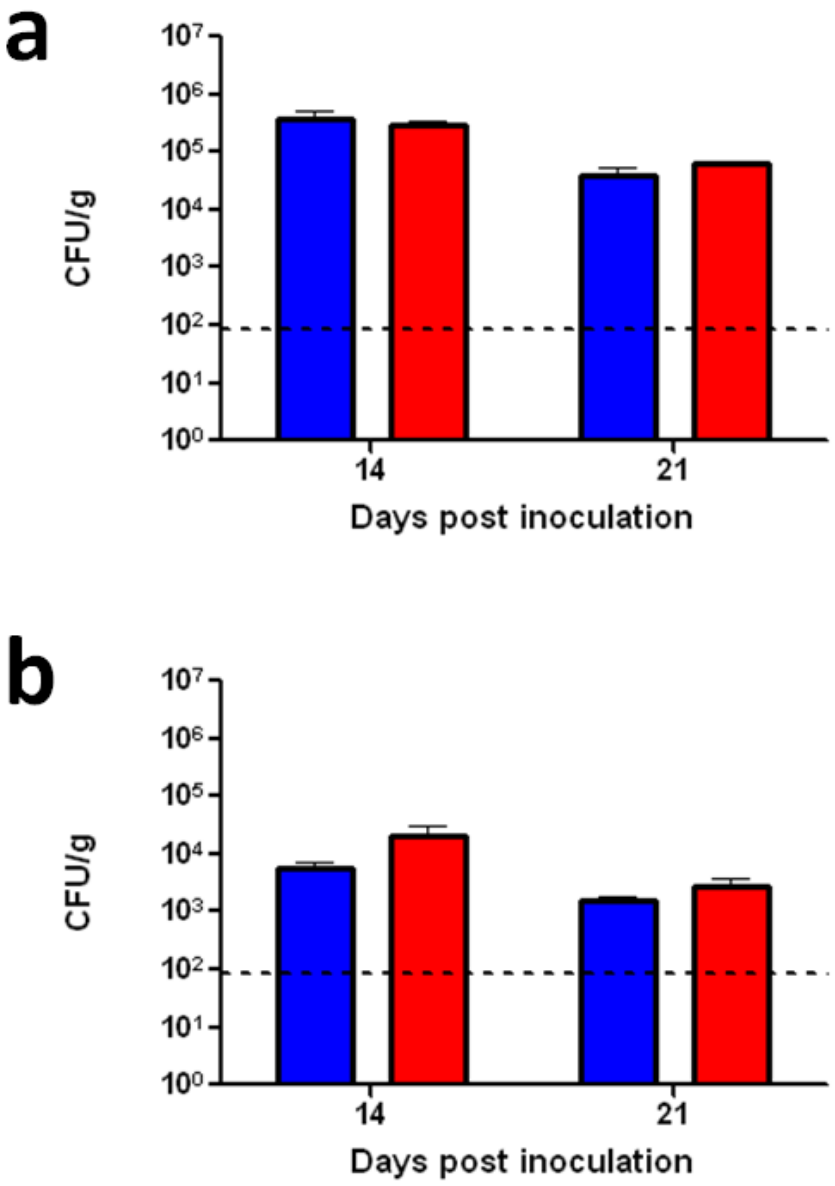

Figure 48. CFU of *S*. Typhimurium SL3261 in systemic tissue from miR-155-deficient and C57BL/6 mice

Control C57BL/6 (blue bars) and miR-155-deficient (red bars) mice were infected intravenously with 1 x 10^5 CFU of a live, attenuated aroA *S*. Typhimurium vaccine strain, SL3261. On days 14 and 21 pi, mice were sacrificed and numbers of *S.* Typhimurium (±SEM) in systemic tissue; (a) spleen, and (b) liver were enumerated, n=3 mice per group. Black broken lines indicate the detection level of the assay.

4.3 Chimeras with miR-155-deficient B cells have impaired humoral immune responses following vaccination

Previous studies have shown that miR-155-deficient mice produce significantly reduced amounts of IgM and switched antigen-specific antibodies after *aroA* vaccination and are consequently less readily protected when challenged with virulent *Salmonella*[31]. Development of pathogen-specific humoral immune responses requires cross-talk between T cells and B cells. Since the original study used miR-155-deficient germ-line mice we could not decipher whether the defect in humoral immunity was intrinsic to miR-155-deficient B or T cells. To this end, we utilised miR-155-deficient, μMT-deficient and wild-type, μMT-deficient chimeric mice possessing either miR-155-deficient or wild-type B cells, respectively. We first immunised mice intravenously with *S.* Typhimurium SL3261 (pnir15TetC) expressing TetC, the non-toxic protective C-terminal domain of tetanus toxin. TetC is a good model antigen through which to study antigen-specific responses, in vivo[180]. On day 20 pi, spleens and livers were collected from infected mice and the size of pathogen burden was determined by viable count. There were no significant differences in bacterial counts in either the spleen or liver of miR-155-deficient, μMT-deficient and wild-type, μMT-deficient mice (Figure 49). However, when serum TetC-specific antibody titres in infected mice were assessed on days 21 and 56 post immunization we found that immunisation of chimeric mice with miR-155-deficient B cells yielded significantly reduced production of TetC-specific Ig, IgG and IgM compared to immunized controls (Figure 50 and 51). In addition, we observed a general trend toward reduced production of TetC-specific IgG1 and IgG2a subclasses in miR-155-deficient, μMT-deficient chimeras (Figure 50 and 51). Thus it would appear that the impaired humoral immune response observed in miR-155-deficient mice is B cell autonomous.

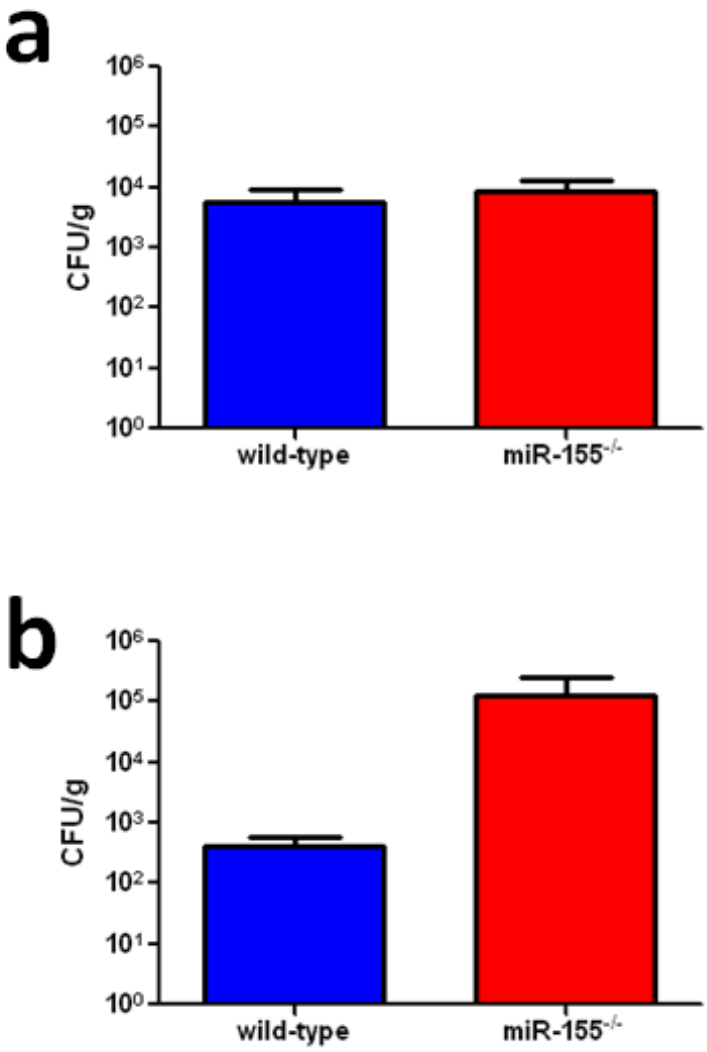

Figure 49. CFU of *S*. Typhimurium SL3261 in systemic tissue from miR-155-deficient, µMT-deficient and wild-type, µMT-deficient mice at day 20 pi

Control wild-type, µMT-deficient (blue bars) and miR-155-deficient, µMT-deficient (red bars) mice were infected intravenously with 1 x 10^5 CFU of a live, attenuated aroA *S.* Typhimurium vaccine strain, SL3261. On day 20 pi, mice were sacrificed and numbers of *S.* Typhimurium (±SEM) in systemic tissue; (a) spleen, and (b) liver were enumerated, n=6 mice per group.

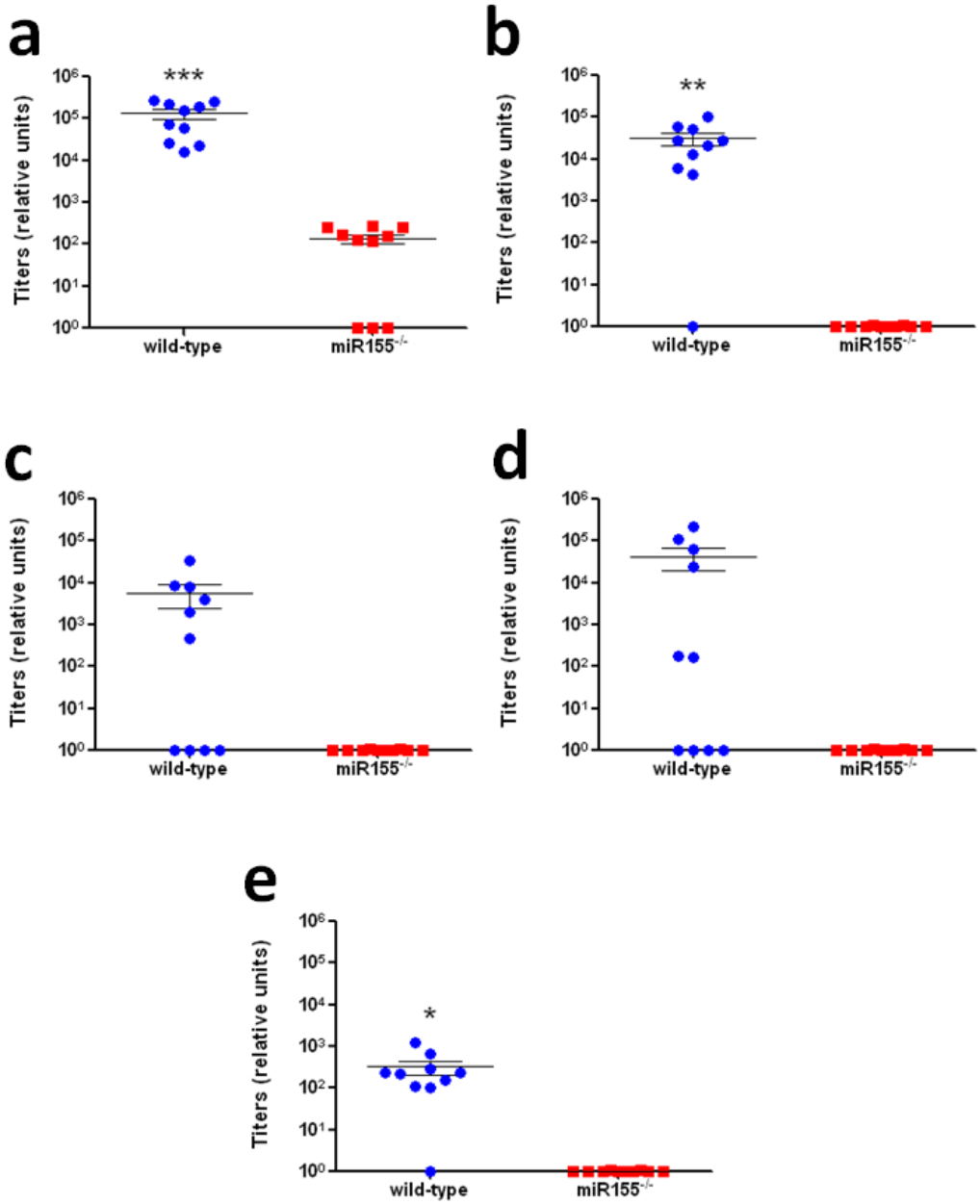

Figure 50. TetC-specific Ig levels from miR-155-deficient, µMT-deficient and wild-type, µMT-deficient chimeras infected with attenuated *Salmonella*, 21 days after infection

Serum antibody responses against TetC in wild-type, µMT-deficient (blue circles) and miR-155-deficient, µMT-deficient (red squares) mice at 21 days pi with attenuated *Salmonella*. Relative titres of anti-TetC serum Ig (± SEM) (a), IgG (b), IgG1 (c), IgG2 (d), and IgM (e) at day 21 pi were calculated.

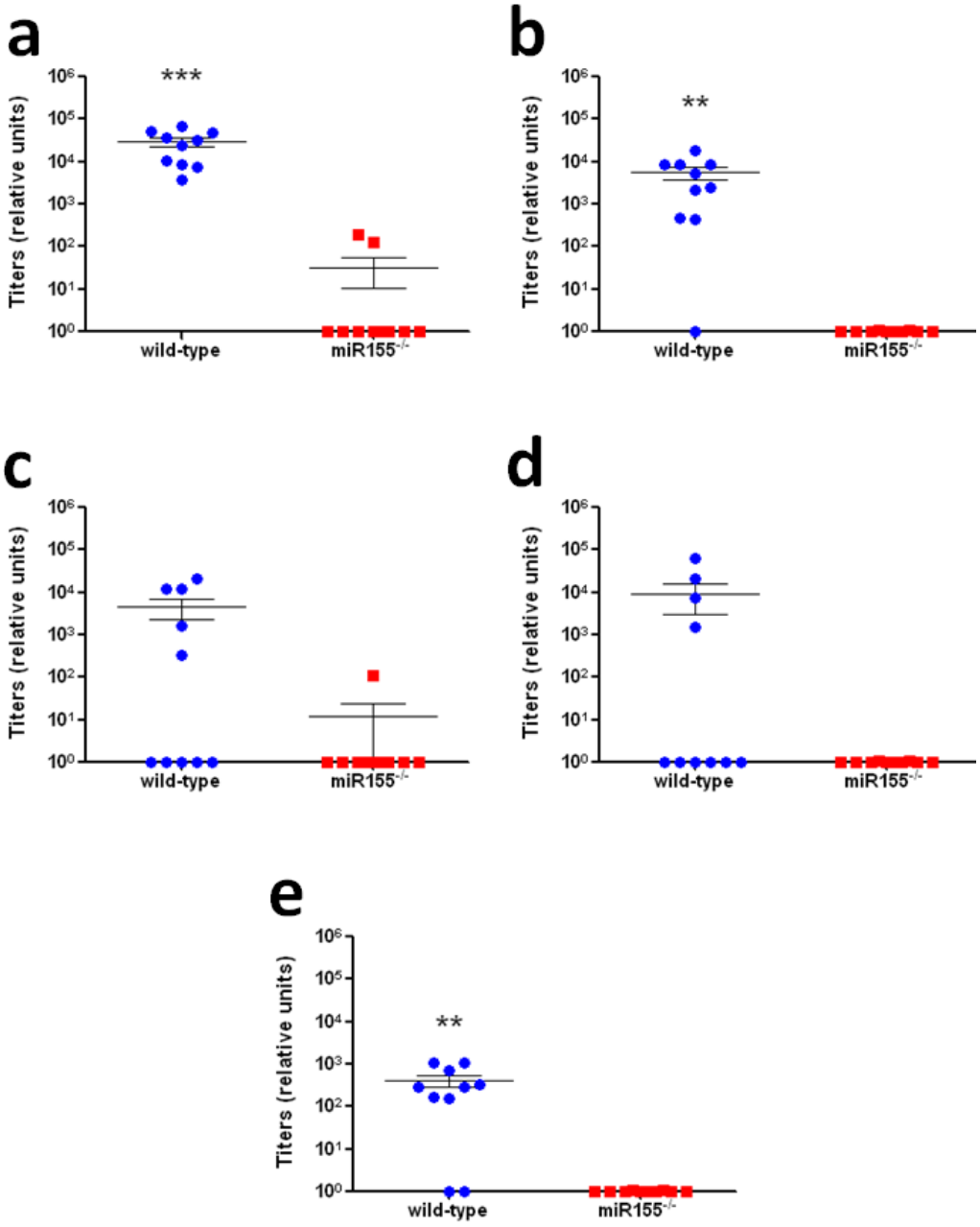

Figure 51. TetC-specific Ig levels from miR-155-deficient, µMT-deficient and wild-type, µMT-deficient chimeras infected with attenuated *Salmonella*, 56 days after infection

Serum antibody responses against TetC in wild-type, µMT-deficient (blue circles) and miR-155-deficient, µMT-deficient (red squares) mice at 56 days pi with attenuated *Salmonella*. Relative titres of anti-TetC serum Ig (± SEM) (a), IgG (b), IgG1 (c), IgG2 (d), and IgM (e) at day 21 pi were calculated.

4.3.1 Immunised miR-155-deficient, μMT-deficient mice are less readily protected when challenged with virulent *Salmonella*

Specific antibody can play a critical role in vaccine-induced protection against virulent bacteria thus we hypothesised that chimeric mice with miR-155-deficient B cells would be less readily protected after immunisation with a *Salmonella*-based vaccine. miR-155-deficient, μMT-deficient and wild-type, μMT-deficient chimeric mice together with miR-155-deficient germline mice were infected intravenously with 1×10^5 CFU of *S.* Typhimurium SL3261 live vaccine. After 3 months, livers and spleens from a sample of mice from each group were checked for the presence of the inoculating strain and found to be completely clear (data not shown). Subsequently, all remaining immunised mice were challenged with virulent *S.* Typhimurium C5. Naive unimmunised C57BL/6 mice were simultaneously similarly challenged. 100 % of naive C57BL/6 mice succumbed to infection by day 7 pi and in agreement with previously published reports immunised miR-155-deficient germline mice exhibited reduced protection against challenge with the majority of mice (5 out of 7; n = 7) succumbing to challenge by 21 days after infection[31] (Figure 52). We found that 8 out of 12 (n = 12) miR-155-deficient, μMT-deficient mice were unable to control a virulent challenge following immunisation (Figure 52). However, unexpectedly the majority of chimeric mice with only wild-type B cells also succumbed to challenge, despite their robust production of *Salmonella*-specific antibody (Figure 52). Because it was not possible to include a control group of immunised C57BL/6 mice we were unable to determine whether this is a problem with the vaccination or whether another cell type was inadvertently affected during the generation of chimeras. Such experiments are long term, labour intensive and expensive so to date we have been unable to repeat this experiment. Consequently we have planned a related experiment involving the passive transfer of antibody raised in these mice to naive germline miR-155-deficient mice. This will involve administering immune antibody and appropriate controls to mice and then

challenging them intraperitoneally with small doses of virulent *S.* Typhimurium to carefully monitor any passive protective effect.

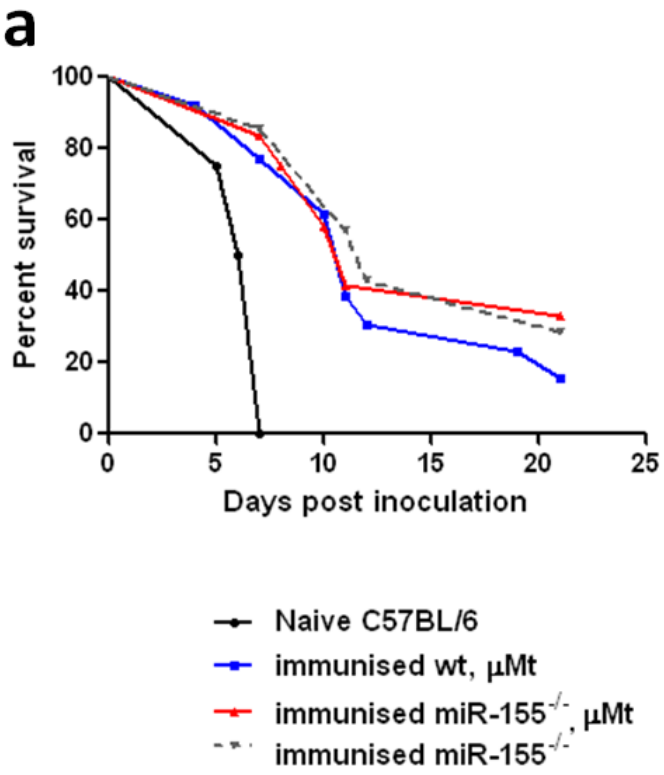

Figure 52. Survival of immunised mice challenged with virulent *S.* Typhimurium

miR-155-deficient, µMT-deficient and wild-type, µMT-deficient and miR-155-deficient germ-line mice were infected intravenously with 1 x 10^5 CFU of *S.* Typhimurium SL3261. After 3 months, remaining immunised mice were challenged orally with 1 x 10^{10} CFU of virulent *S.* Typhimurium C5. Naive unimmunised C57BL/6 mice were simultaneously challenged. Immunised miR-155-deficient, µMT-deficient mice n = 12, immunised wild-type, µMT-deficient mice n = 13, immunised germline miR-155-deficient mice n = 7 and naive unimmunised C57BL/6 mice n = 4.

4.4 Discussion

The results presented here highlight the importance of miR-155 during the development and generation of protective immunity against murine Salmonellosis. We provide further evidence that the susceptibility of miR-155-deficient mice and defective antibody responses is intrinsic to miR-155-deficient B cells.

Like C57BL/6 mice, unimmunised naive miR-155-deficient mice are highly susceptible to infection with virulent *Salmonella* and rapidly succumb to infection. During the early stages of infection, control of bacterial growth depends on the formation of pathological lesions (known as granulomas) within the liver and spleen which prevent the uncontrolled spread of bacteria throughout the body. Lesion formation depends on a variety of factors including the production of numerous cytokines and the expression of a variety of cell adhesion molecules such as ICAM-1[180]. The liver granulomas formed in miR-155-deficient mice were histologically indistinguishable from those in control mice. Additionally, we were unable to detect any obvious differences in bacterial counts or the rate of clearance between naive miR-155-deficient and C57BL/6 mice challenged with attenuated aroA *S.* Typhimurium. The innate immune system is essential for suppressing initial growth of *Salmonella* until an effective T and B cell response can be generated. During the late phases of infection, effective control and eventual eradication of bacteria critically depends on the presence of *salmonella*-specific $CD4^+$ T cells. Thus the results of our study indicate that both the innate immune and T cell responses function in the absence of miR-155, although we do not know if other defects are present that were not detected in these challenges.

However, previous studies have shown that germline miR-155-deficient mice whilst able to control infection with an attenuated strain of *Salmonella* are less readily protected following immunisation due to severely diminished pathogen-specific antibody production. Serum antibody produced by B cells during the initial infection is critical for protection from virulent *Salmonellae*

in immune hosts and requires the participation of B and T cells. Thus we wanted to ascertain whether the impaired antibody production in the absence of miR-155 is B or T cell autonomous, complementing our related studies with *C. rodentium* described previously. To this end we exploited chimeras possessing either miR-155-deficient or wild-type B cells. Key phenotypic alterations observed in germline miR-155-deficient mice were recapitulated in chimeric mice with only miR-155-deficient B cells. miR-155-deficient, μMT-deficient chimeras immunised with attenuated *S.* Typhimurium produced dramatically less *Salmonella*-specific Ig, IgG and IgM as well as IgG1 and IgG2a compared to control mice. This suggests that B cells require miR-155 for the production of pathogen-specific antibody and that the impaired humoral immune response observed in miR-155-deficient mice is B cell autonomous. However, for unknown reasons we found that following immunisation miR-155-deficient, μMT-deficient and wild-type, μMT-deficient mice alike were unable to control challenge with virulent *Salmonella* and due to time and practical constraints we have been unable to repeat this demanding experiment. Consequently, we will determine the protective capacity of serum antibody produced during immunisation, independently. This can be performed by injecting fractionated serum antibody intraperitoneally into naive mice and monitoring their survival following an intraperitoneal injection with exponentially growing *Salmonella*, as previously described[215].