6 Meta-analysis of the restrictive impact of IFITM3 on a spectrum of pathogens.

6.1 Introduction

Cells possess a vast array of proteins to detect and restrict invading pathogens in order to prevent the onset of infection. In Chapter 1, a small proportion of these proteins were discussed in the context of preventing viral infection; however, cells must possess a far broader repertoire of anti-microbial defences to combat the wealth of micro-organisms that could potentially cause disease. Such defences must include the ability to detect the presence of the pathogen, as well as react to mount an immune response to remove the pathogen from the host.

Some host defence proteins are multi-faceted and are employed in the immune response to an array of pathogens of different origins. This is especially true of pathogen recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs). PAMPs are conserved features of pathogens, such as lipids, proteins and nucleic acids, which are recognised as being foreign by the host immune system. This ability allows the host to detect bacterial, viral, fungal and protozoan pathogens, amongst others (Takeuchi and Akira 2010). For instance, TLR4 alone is capable of detecting Gram-negative bacteria, fungi, trypanosomes and surface proteins on several viruses (Akira *et al.* 2006). Similarly, certain anti-microbial proteins, such as defensins, possess similar cross-kingdom defensive abilities and are capable of curtailing infection by bacteria, viruses and fungi (Ganz 2003).

IFITM3 has been identified as a potent antiviral protein, acting as both an intrinsic and innate immune defence protein. As previously discussed, it was initially identified as playing a developmental role in germ cell homing, but was later shown to have a role in the restriction of a small number of viruses, including influenza and dengue viruses (Brass *et al.* 2009). However, since 2009, the number of viruses restricted by the IFITM family has expanded considerably. Indeed, many of these studies have shown that IFITM3 is capable of preventing infection by enveloped viruses that enter the cell through the late endosomal pathway (Diamond and Farzan 2013). This has led to the generation of hypotheses on how the IFITM family achieves

restriction; namely through preventing the fusion of viral and cellular membranes (John *et al.* 2013).

Recently, the role of IFITM3 has been expanded somewhat by the discovery that it was capable of restricting reoviruses (Anafu *et al.* 2013), which is novel, as these viruses are nonenveloped. This has important implications, as non-enveloped viruses do not rely on membrane fusion to gain release from the endosomes. Instead, it is hypothesised that these viruses may physically disrupt the endosomal membrane through their surface proteins (Chandran *et al.* 2002; Wiethoff *et al.* 2005). This therefore widens the scope of the actions of IFITM3 beyond enveloped viruses and may also include other non-viral pathogens.

The aim of the current study was to analyse the effects of the loss of Ifitm3 *in vivo*, using the *Ifitm3*$^{-/-}$ mouse model, on the restriction of a range of pathogens[2]. It was hoped that doing so would further help to define the extent of the antiviral activities of IFITM3, and examine whether it could also prevent infection by non-viral pathogens.

6.1.1 Pathogens

6.1.1.1 *Salmonella* Typhimurium

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an intracellular bacteria typically used in mice to mimic the effects of typhoid fever in humans. *S.* Typhimurium enters cells through phagocytosis or by a bacterial triggered entry mechanism and replicates within endosomal-like structures known as Salmonella-containing vacuoles (SCVs) (Dandekar *et al.* 2012), which act as a protective niche shielding the bacteria from cellular killing mechanisms. It is therefore plausible to hypothesise that Ifitm3 may interact with *S.* Typhimurium during its invasive stages, owing to the presence of Ifitm3 on the cell surface, and within the endosomal pathway. Previously, a study showed how siRNA-transfected murine epithelial fibroblasts (MEFs) that had their expression of Ifitm3 knocked down, were no more susceptible to *Salmonella* infection than control cells (Yount *et al.* 2012), but the current study will show for the first time whether the loss of Ifitm3 has an impact *in vivo*.

[2] Non-influenza pathogen raw experimental data was collected by the following individuals: *Salmonella* and *Citrobacter*: Simon Clare, Leanne Kane; *Mycobacterium tuberculosis*: Douglas Young, Angela Rodgers; *Plasmodium*: Oliver Bilkner, Ashraful Haque; Respiratory syncytial virus: John Tregoning, Jacqueline McDonald.

In the C57BL/6 mouse, the attenuated *S.* Typhimurium are typically restricted to the gut and is cleared over a two week period. However, hyper-susceptible mice show evidence of bacteraemia associated with virulent strains, with colonisation of the spleen and liver, which can ultimately result in mortality (Santos *et al.* 2001). Therefore, *Ifitm3*$^{-/-}$ mice will be assessed accordingly for signs of morbidity, mortality and bacterial invasion.

6.1.1.2 *Citrobacter rodentium*

Citrobacter rodentium is a non-invasive, Gram-negative bacterium used in mice to model the pathogenesis caused by *E. coli* in humans, including enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* (Mundy *et al.* 2005; Clare *et al.* 2013). *C. rodentium* differs from *Salmonella* species insofar that it induces its pathological damage from outside the cell. Although it does not normally enter the host cell, a potential interaction between Ifitm3 and *C. rodentium* can be drawn through the intermediate of osteopontin, which interacts with IFITM3, as discussed in Chapter 4. *C. rodentium* infections are reduced 8- to 17-fold in mice that lack Opn (Wine *et al.* 2010); suggesting that the protein is required for attachment and subsequent colonisation of the gut by the bacteria. Therefore, the loss of Ifitm3, which has been shown to increase Opn expression (Figure 4.14), could hypothetically increase colonisation by *C. rodentium* in the gut.

Typically, challenge of adult C57BL/6 mice with *C. rodentium* results in a non-lethal infection, with bacteria colonising the caecum and colon and being shed in the faeces, before being cleared by day 21 post-infection. Knockout mice can show a variety of phenotypes that differ from wild type mice, including 1) failing to clear the infection; 2) showing reduced pathological damage; and 3) showing enhanced pathological damage (Mundy *et al.* 2005).

6.1.1.3 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), which is the second largest cause of pathogen-induced mortality after HIV (WHO 2013). *M. tuberculosis* is an intracellular respiratory bacterium that replicates primarily within macrophages and dendritic cells, before forming latent granulomas in the infected organs (Flynn and Chan 2001). Should these foci of infection become reactivated, potentially through immune-suppression, the

outgrowth of bacteria can cause pulmonary necrosis and severe pathological damage; thus permitting aerosol transmission to other hosts (van Crevel *et al.* 2002; North and Jung 2004).

Multiple factors would suggest that there may be an interaction between Ifitm3 and *M. tuberculosis*. Firstly, upon infection, *M. tuberculosis* triggers a substantial type II interferon response. The increase in IFNγ production is crucial for restriction of *M. tuberculosis* in both mice and in humans (Newport *et al.* 1996; North and Jung 2004), which would also up-regulate the expression of *Ifitm3*. Furthermore, *Ifitm3* expression is high in macrophages (Lattin *et al.* 2008; Wu *et al.* 2009): the primary site of replication for *M. tuberculosis*, which would again suggest a correlation between the bacterium and IFITM3 occurrence. Recently, a study has implicated a SNP (rs3888188) in the promoter of *IFITM3* with susceptibility to TB (Shen *et al.* 2013), wherein the minority rs3888188-G allele was significantly overrepresented in patients with TB compared to healthy controls in a Han Chinese population. Taken together, these factors make it possible to hypothesise that Ifitm3 may impact upon control of the bacterial infection *in vivo*.

6.1.1.4 *Plasmodium*

Plasmodium are protozoan parasites that are the causative agents of malaria, spread by the *Anopheles* species of mosquito. In humans, a number of species of *Plasmodium* can elicit malaria, although *P. falciparum* is the primary species causing morbidity and mortality; accounting for over 1 million malaria-related deaths annually (Liu *et al.* 2010a). In mice, malaria challenges can be conducted using *P. berghei*, which is a natural rodent pathogen. Such challenges have been used to inform host-parasite interactions (Franke-Fayard *et al.* 2004; Amino *et al.* 2006) and trial anti-malarial treatments (Kaiser *et al.* 2006).

Malaria infection can elicit a number of disease outcomes, varying from fever and malaise to lethal bouts of cerebral malaria and anaemia (Miller *et al.* 2002). Transmitted by mosquitoes into the host, the parasites travel in the bloodstream to the liver where they infect hepatocytes before transferring into the blood stage of their lifecycle where they infect and destroy red blood cells; causing morbidity. The presence of the parasite in the host elicits strong type I and type II IFN responses, which impact on the severity of infection (Hunt and Grau 2003; Haque *et al.* 2011),

with IFNα and IFNγ contributing to lethality in murine models. Furthermore, eight SNPs in the IFN receptor, *IFNAR1*, have been associated with the development of cerebral malaria in children; a finding that is corroborated in *Ifnar*$^{-/-}$ mice, which also do not develop cerebral malaria (Ball *et al.* 2013).

Interestingly, it has been reported that *IFITM3*, along with several other ISGs, is significantly up-regulated in patients that have become infected with *P. falciparum* (Sharma *et al.* 2011). It was shown that deletion of several of these ISGs, including *Tbk1*, *Irf3* and *Irf7* prevented mice from developing lethal cerebral malaria. Ifitm3 may therefore play a role in the pathogenesis of the disease, either to the benefit or detriment of the host.

6.1.1.5 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is one of the leading respiratory pathogens in children that necessitates hospitalisation (Hall 2001); accounting for three times more admissions to hospital than influenza viruses (Hall *et al.* 2009). RSV, like influenza virus, is an enveloped virus that initially causes a mild upper respiratory tract infection. This can develop and manifest as a lower respiratory tract infection that ultimately causes bronchiolitis and respiratory distress, at which point the disease presents the greatest risk of mortality in infants (Openshaw and Tregoning 2005).

Murine models have been employed in understanding the dynamics underpinning RSV infection. Similar to influenza virus infections, inbred strains of mice show a range of susceptibility to infection, with C57BL/6 mice representing one of the most resistant strains in terms of peak viral burden and weight loss over the course of challenge (Stark *et al.* 2002). However, the use of knockout mouse models has provided insight into the host factors influencing the severity of disease in humans, and conversely, mouse models have also been used to ratify genetic differences observed in humans (Collins and Graham 2008; Tregoning and Schwarze 2010).

Recently, it has been shown that IFITM3 may be involved in the control of RSV infection in both mice and humans (Janssen *et al.* 2007; Pennings *et al.* 2011; Ioannidis *et al.* 2012; Bucasas *et al.* 2013), owing to its up-regulation during periods of infection. Similarly, other members of

the Ifitm family have also shown RSV-linked up-regulation, including *Ifitm1* (Ravi *et al.* 2013). Therefore, it could be hypothesised that Ifitm3 may impact upon restriction in the murine model.

6.2 Results

6.2.1 Ifitm3 expression pattern

The pathogens used in the current study infect a variety of organs throughout the body, unlike influenza virus, which is predominantly restricted to the respiratory system. Tissue was therefore collected and sectioned from a number of locations affected by the multi-pathogen challenge, including lymph node, lung, spleen, liver and intestine. The expression of Ifitm3 was confirmed to be ablated in all *Ifitm3*$^{-/-}$ mouse organs, but was shown to be highly constitutively expressed in all wild type organs (Figure 6.1).

In wild type mice, the expression pattern of Ifitm3 was noteworthy. The spleen and lymph nodes indicated that Ifitm3 was predominantly expressed in the red pulp, but was absent from the white pulp. Similarly, intestinal staining revealed Ifitm3 expression to be high in the lamina propria, but not on the villus epithelium. Conversely, lung and liver showed ubiquitous expression of Ifitm3 throughout the tissues.

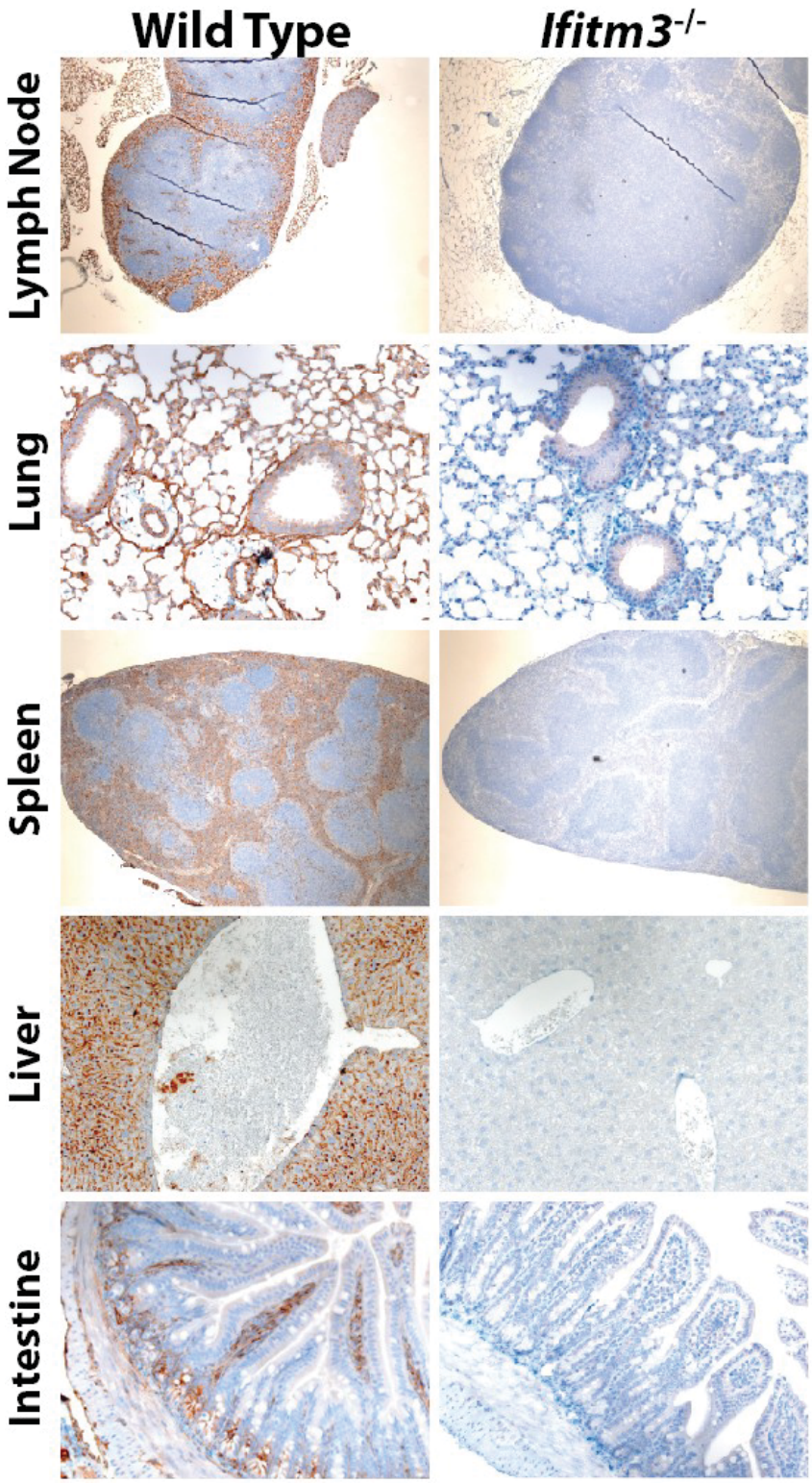

Figure 6.1: Expression of Ifitm3 at the predominant sites of pathogen infection. Paraffin-embedded sections from wild type and *Ifitm3*$^{-/-}$ mice were cut and stained for expression of Ifitm3 (brown), and counterstained with hematoxylin (blue). Original magnification of lymph node and spleen 10×; lung and colon 20×; liver 40×.

6.2.2 *Salmonella* challenge

Wild type and *Ifitm3*$^{-/-}$ mice were intravenously dosed with 1 × 10^6 CFU of *S.* Typhimurium M525 bacteria and observed for 28 days post-infection for signs of morbidity and weight loss (Figure 6.2a). All mice survived the challenge and gained weight over the time course of the study. *Ifitm3*$^{-/-}$ mice gained weight more slowly following challenge, which is due to them being ~5g heavier on the day of infection; thus making proportional increases in weight appear smaller.

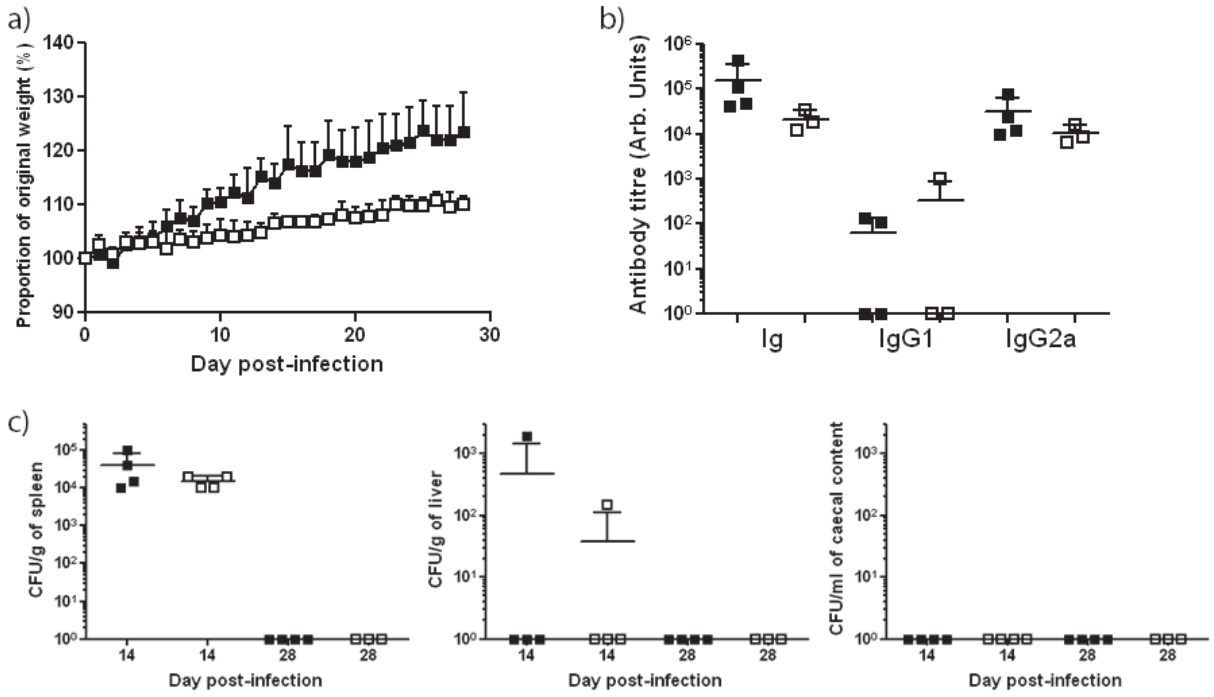

Figure 6.2: *S.* Typhimurium challenge of wild type and *Ifitm3*$^{-/-}$ mice. Mice were intravenously injected with *S.* Typhimurium and observed for weight loss for 28 days post-infection (a). Mice were killed on day 28 post-infection to assess neutralising antibody titre against *S.* Typhimurium (b). Spleen, liver and caecal contents were analysed and bacterial contents titred on days 14 and 28 post-infection to assess the colonisation of the bacteria (c). ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 3).

On day 28 post-infection anti-*S.* Typhimurium antibody titres were determined from the sera of wild type and *Ifitm3*$^{-/-}$ mice, which indicated that both genotypes of mice produced similar antibody profiles (Figure 6.2b), although *Ifitm3*$^{-/-}$ mice had a non-significant trend towards less total Ig. Further to this, bacterial load was determined in the spleen, liver and faceal contents (Figure 6.2c). Similarly, bacterial counts revealed no significant differences between wild type

and *Ifitm3*$^{-/-}$ mice; suggesting that Ifitm3 does not play a role in resistance to murine *Salmonella* infection.

6.2.3 *Citrobacter* challenge

Wild type and *Ifitm3*$^{-/-}$ mice were orally gavaged with 1×10^9 CFU of *C. rodentium* bacteria and monitored for 28 days post-infection for signs of morbidity. Weight loss profiles revealed that neither wild type nor *Ifitm3*$^{-/-}$ mice showed any overt signs of illness over the course of infection (Figure 6.3a). Bacteria shed in the faeces of these mice also revealed no significant differences between the genotypes, with clearance of infection achieved by day 25 post-infection in *Ifitm3*$^{-/-}$ mice (Figure 6.3b).

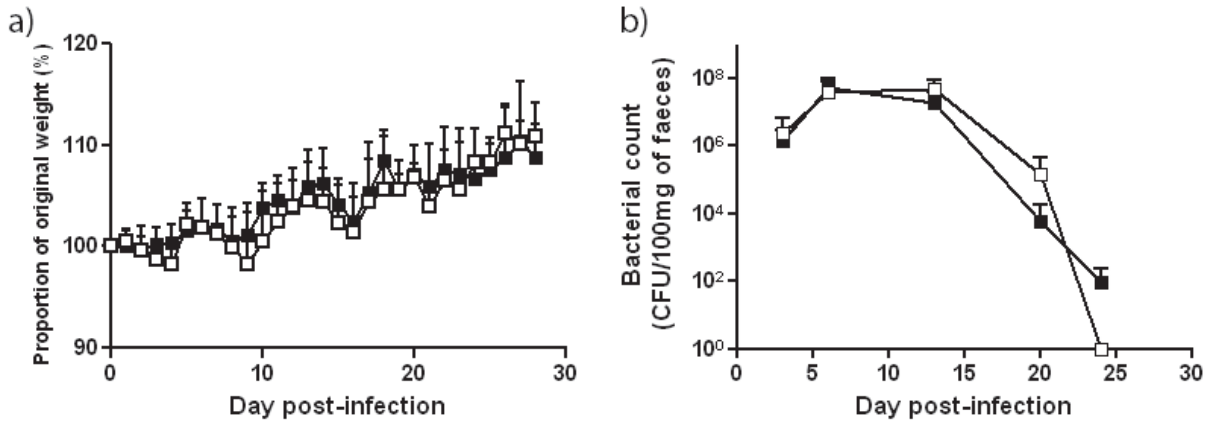

Figure 6.3: Weight loss and bacterial shedding of wild type and *Ifitm3*$^{-/-}$ mice infected with *C. rodentium*. Mice were orally infected with *C. rodentium* and weighed daily to monitor morbidity (a). Faecal samples were taken over the course of infection (b), and were homogenised, diluted and plated to count the number of colony forming units (CFU) shed over the course of the challenge. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 8).

Mice were sacrificed on days 14 and 28 post-infection to determine whether there were any differences in the bacterial burden between wild type and *Ifitm3*$^{-/-}$ mice (Figure 6.4). Counts in the caecum (total, caeceal patch and contents) and colon showed no significant differences in bacterial colonisation and clearance. Similarly, analysis of the liver and spleen revealed no instances of bacteraemia in either wild type of *Ifitm3*$^{-/-}$ mice. Taken together, these data suggest Ifitm3 does not impact on *C. rodentium* infection.

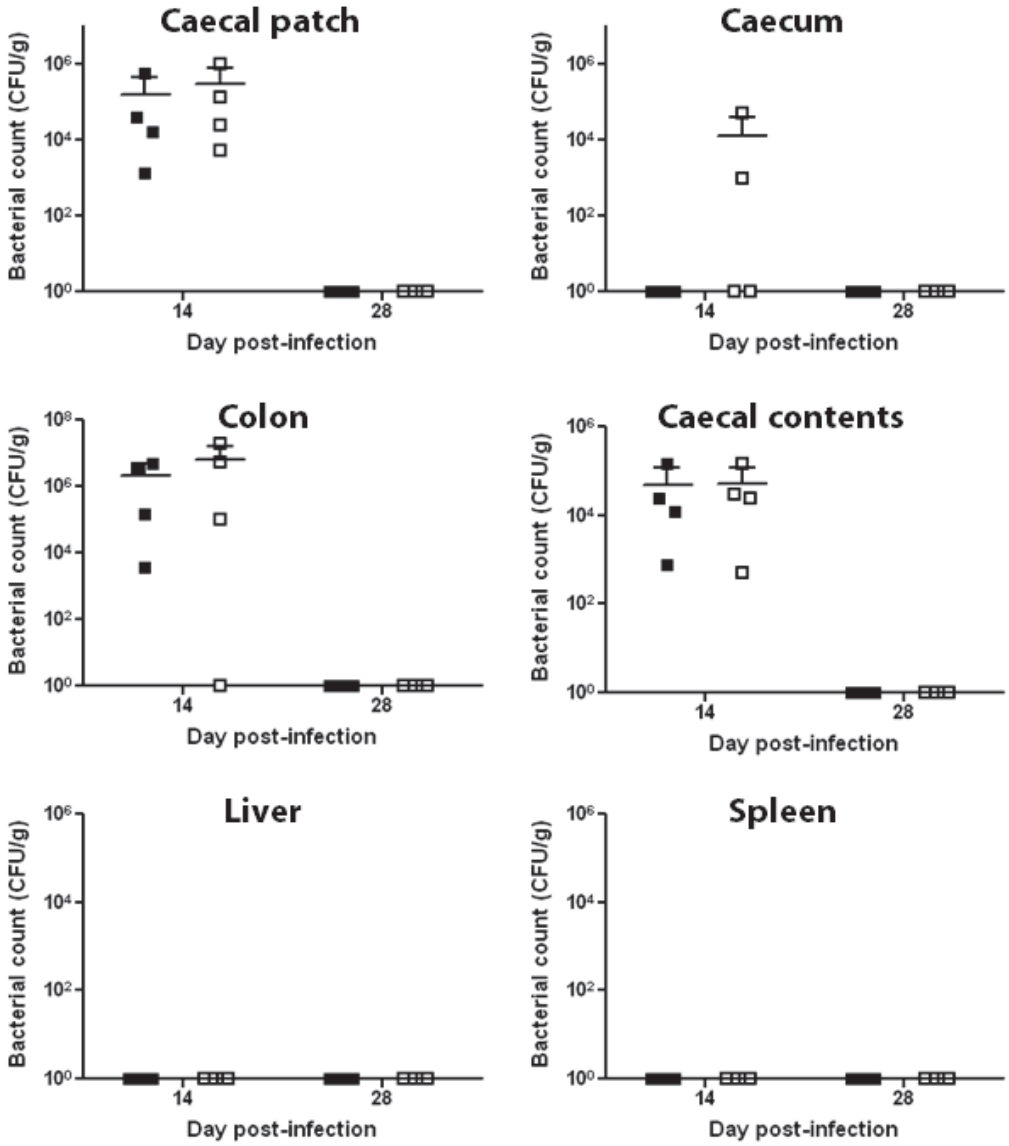

Figure 6.4: Bacterial counts of wild type and *Ifitm3*$^{-/-}$ mice infected with *C. rodentium* over the course of infection. Mice were killed on days 14 and 28 post-infection and the indicated organs were excised, homogenised and plated to count the number of colony forming units of *C. rodentium*. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 4).

6.2.4 *Mycobacterium* challenge

Wild type and *Ifitm3*$^{-/-}$ mice were intranasally infected with an aerosolised dose of approximately 100 CFU of H37Rv *M. tuberculosis* bacteria and monitored for signs of morbidity for the following 28 days. To determine whether Ifitm3 was involved in the control of the bacterial infection, mice were killed on days 0, 7, 14 and 28 post-infection to calculate the bacterial

burden in the lungs. As shown in Figure 6.5, there were no significant differences between wild type and *Ifitm3*$^{-/-}$ mice, with bacterial growth kinetics indicating that Ifitm3 does not impact on *M. tuberculosis* infection.

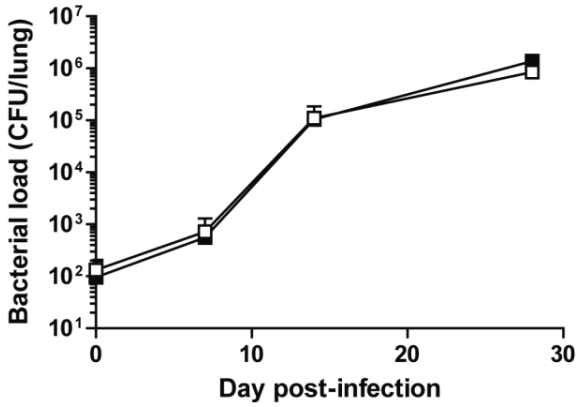

Figure 6.5: Bacterial growth kinetics of *M. tuberculosis* in the lungs of wild type and *Ifitm3*$^{-/-}$ mice. Mice were killed over the course of infection with H37Rv *M. tuberculosis* to determine the bacterial load within their lungs. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 5).

6.2.5 *Plasmodium* challenge

Mice were intraperitoneally injected with 5 × 10^5 red blood cells infected with a *P. berghei* ANKA reporter line, PbGFP-LUC$_{CON}$ (RMgm-28), which constitutively expresses a fusion protein of GFP and Firefly Luciferase (Franke-Fayard *et al.* 2005). IFNγ receptor knockout mice (*Ifngr*$^{-/-}$) mice were included to act as control, as these mice do not succumb to lethal episodes of cerebral malaria (CM). The experimental challenge revealed there to be no deviations from the phenotype observed with wild type C57BL/6 littermate controls, with both showing no resistance to CM (Figure 6.6a). The ~50% survival of wild type mice falls within acceptable boundaries owing to inherent inefficiencies in the delivery of parasites into the mice (personal communication from Ashraful Haque was involved in these experiments). Therefore, differences in survival shown in Figure 6.5a are non-significant. In contrast, *Ifngr*$^{-/-}$ mice infected in parallel were fully protected from infection. Analysis of parasite burden revealed that all mice were infected with *P. berghei* (Figure 6.6b), but with no significant differences. Additionally, levels of the inflammatory cytokines IFNγ, TNFα and MCP-1 were also analysed by cytometric bead array, which showed no significant differences between wild type and *Ifitm3*$^{-/-}$ mice (Figure 6.6c).

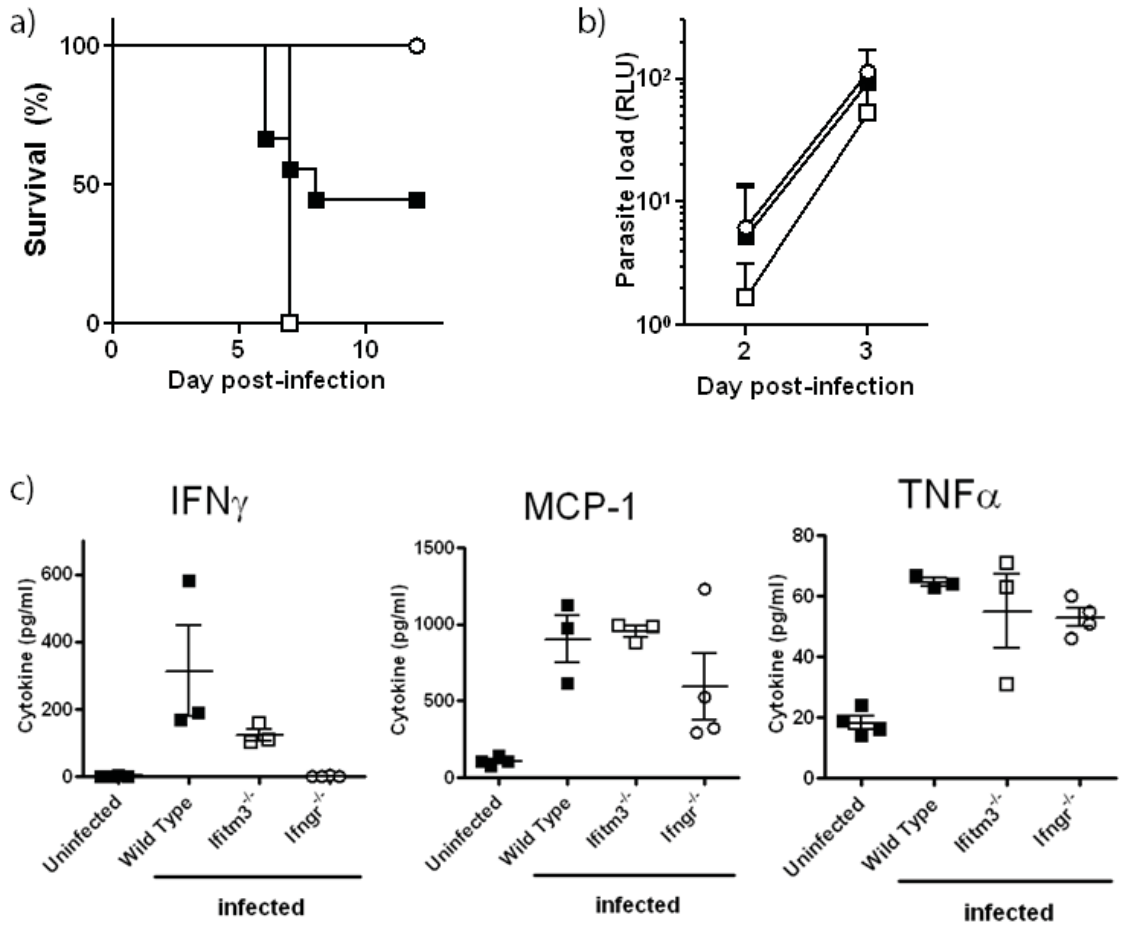

Figure 6.6: Malarial challenge of wild type and *Ifitm3*$^{-/-}$ mice with *P. berghei* ANKA. Mice were intravenously injected with red blood cells containing *P. berghei* ANKA and were monitored for survival for 12 days post-infection (a). Parasite burden was measured by luminescence readings from blood collected from the tail vein on days two and three post-infection (b), and cytokine dysregulation was measured from the sera on day three post-infection by cytometric bead array (c). ■: wild type, □: *Ifitm3*$^{-/-}$, ○: *Ifngr*$^{-/-}$. Results show means ± S.D. (n > 2).

6.2.6 Respiratory syncytial virus challenge

Wild type and *Ifitm3*$^{-/-}$ mice were intranasally infected with 5×10^5 PFU of RSV-A (A2 strain) and were monitored for weight loss for seven days post-infection. Cohorts of mice were killed on days four and seven post-infection to quantify viral burden and immunological changes over the course of the challenge.

As shown in Figure 6.7, *Ifitm3*$^{-/-}$ mice showed a highly significant amount of weight loss on days six and seven post-infection compared to wild type littermates. Furthermore, *Ifitm3*$^{-/-}$ mice

showed a higher peak in viral burden on day four post-infection, which remained significantly higher until day seven post-infection ($p = 0.005$).

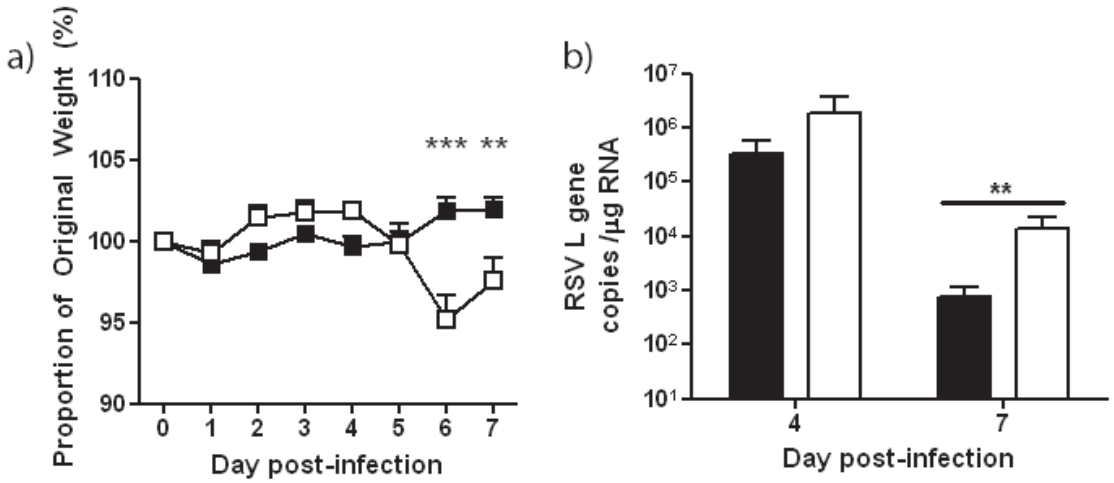

Figure 6.7: Weight loss and viral load associated with RSV infection of wild type and *Ifitm3*$^{-/-}$ mice. Mice were intranasally infected with RSV-A and weighed for seven days post-infection (a). Cohorts of mice were killed on days four and seven post-infection and viral titres calculated by RT-qPCR (b). ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. ($n > 5$). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Cellular infiltrate was quantified over the course of infection, which showed a significant increase in cells resident in the lungs on day seven post-infection in *Ifitm3*$^{-/-}$ mice (Figure 6.8a) and a similarly significant increase in cellular infiltrate in the BAL fluid on day four post-infection (Figure 6.8b). Flow cytometry revealed an increase in all cellular sub-populations in *Ifitm3*$^{-/-}$ mice relative to wild type littermates on day seven post-infection. In particular, numbers of CD3+ and CD8+ T-cell populations were significantly higher, as were NK cells in the lungs (Figure 6.8c) and granulocytes in the BAL (Figure 6.8d). Analysis of inflammatory cytokines, including IFNγ, IL-6 and IL-1β revealed perturbations in their levels between genotypes of mice in the lungs and BAL on day seven post-infection (Figure 6.9), with significantly higher levels of IFNγ and IL-1β in *Ifitm3*$^{-/-}$ mice relative to wild type controls.

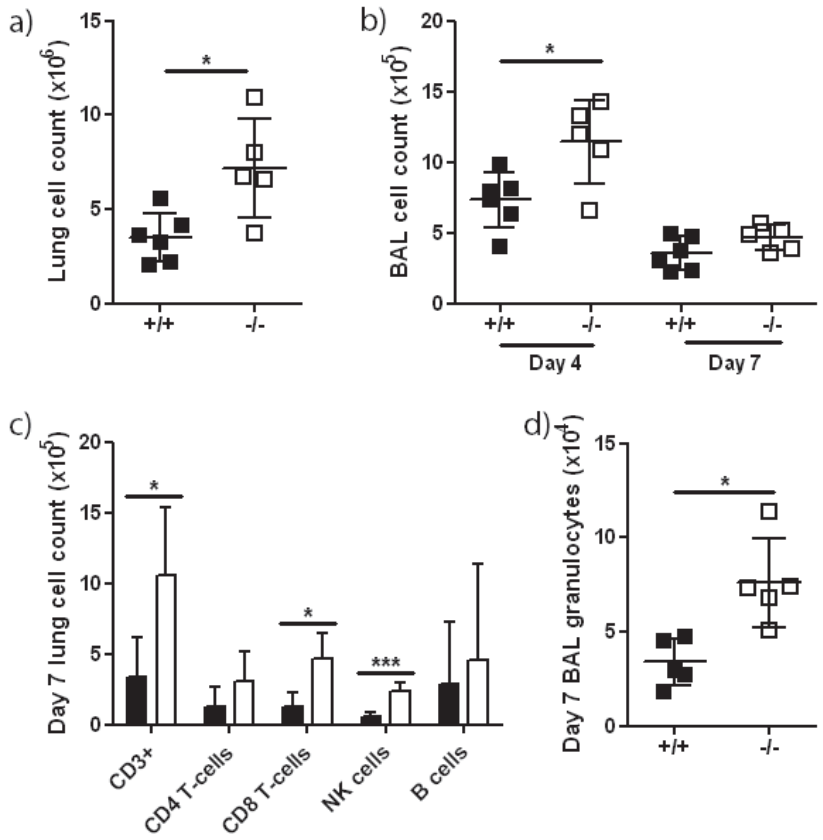

Figure 6.8: Cellular response of wild type and *Ifitm3*$^{-/-}$ mice to RSV infection. Total viable cell counts were calculated from the lungs on day seven post-infection (a) and from broncho-alveolar lavage (BAL) on days four and seven post-infection (b). On day seven, cellular sub-populations were identified by flow cytometry in the lungs (c) and BAL fluid (d). ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 5). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, ***: $p < 0.001$).

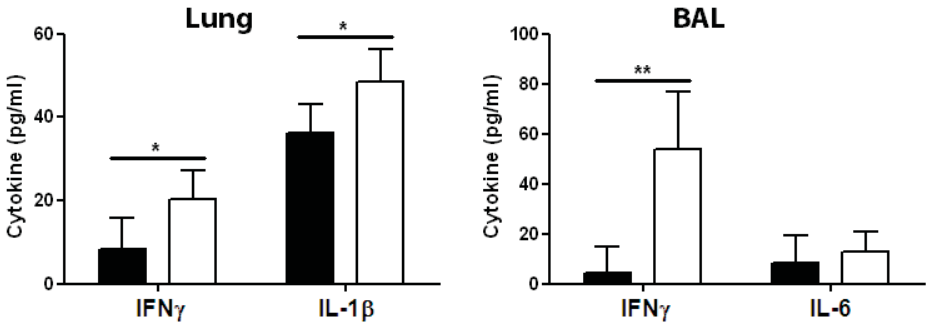

Figure 6.9: Inflammatory cytokines in the lungs and BAL on day seven post-infection in RSV-infected wild type and *Ifitm3*$^{-/-}$ mice. Levels of IFNγ, IL-1β and IL-6 were quantified by ELISA on day seven post-infection. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 5). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$).

6.3 Discussion

This study has expanded and defined the scale of restriction that Ifitm3 exerts over invading pathogens *in vivo*, using the *Ifitm3*$^{-/-}$ murine model. Although a clear phenotypic difference was only observed during challenge with RSV, the study is important considering the fact that the literature implicates IFITM3 in the restriction of several of the pathogens included here when tested *in vitro*.

The discovery that RSV is restricted by Ifitm3 *in vivo* is novel and ratifies associations between the protein and RSV *in vitro* (Janssen *et al.* 2007; Pennings *et al.* 2011; Ioannidis *et al.* 2012; Bucasas *et al.* 2013); therefore adding to the current base of knowledge regarding the viral specificity of Ifitm3. What is most striking about the restriction of RSV by Ifitm3 is the proposed route by which the virus gains access to the cell; it is unlike other viruses that have shown Ifitm3-based restriction, as it does not require the endosomal pathway. It is currently thought that RSV enters airway epithelial cells using nucleolin, which is situated in cholesterol rich microdomains/lipid rafts (San-Juan-Vergara *et al.* 2012; Lay *et al.* 2013). RSV is proposed to bind to nucleolin using its F protein, which initiates hemifusion of the RSV envelope with the cell membrane (Lay *et al.* 2013); thus delivering the viral content directly into the cytoplasm without the need for endosomes.

Recently, Li and colleagues (2013) suggested that the IFITM family of proteins was capable of restricting viral hemifusion and the formation of syncytia. Similar to other published studies, they suggested that the presence of IFITM proteins in the membrane reduced fluidity (Amini-Bavil-Olyaee *et al.* 2013; John *et al.* 2013); therefore making the energy demands required for fusion a barrier for the pathogen. The loss of Ifitm3 in mice therefore removes a block to viral entry; thus leading to an increase in viral load. Further to this, the higher viral burden would increase the prevalence of viral antigen, which would elevate the immune response from the host. This study suggests that the increase in viral burden and subsequent immune dysregulation results in the heightened morbidity of *Ifitm3*$^{-/-}$ mice during infection with RSV. Although both of these traits were seen with influenza virus infection, the phenotype seen in the RSV challenge is not as striking as with influenza in wild type or *Ifitm3*$^{-/-}$ mice (Everitt *et al.* 2012). As discussed previously, mouse background has a strong influence on viral susceptibility, with

C57BL/6 mice being particularly resistant to RSV infection (Stark *et al.* 2002). Furthermore, similar to influenza virus, differences exist in the virulence of RSV strains in mice, with some inducing far milder pathogenesis than others (Bem *et al.* 2011); hence explaining the reduced severity seen in the significant phenotype.

The distribution and specificity of Ifitm3 within cells may also account for the mild, but significant phenotype observed in the current study. It has been shown that Ifitm3 associates with the cellular membrane, but is primarily distributed intracellularly on endosomal membranes (Jia *et al.* 2012). Of the Ifitm family members, Ifitm1 is primarily localised to the cell surface (John *et al.* 2013), which is where RSV fuses with the cell. It is possible therefore that Ifitm1 may provide the strongest block to RSV infection. Previous studies have shown a degree of overlap of function between IFITM1, -2 and -3, but with certain members showing specificity for restricting particular viruses (Brass *et al.* 2009; Huang *et al.* 2011). Thus, although Ifitm3 is exerting a degree of restriction over RSV, Ifitm1 may be more capable of restriction owing to its cellular localisation. It has been shown that modification of the Y20 residue of IFITM3 results in an altered cellular distribution pattern, wherein the protein associates with the cell membrane and not the endosomes (Jia *et al.* 2012; John *et al.* 2013). This surface-localised form of IFITM3 can potently restrict HIV-1 virus (Jia *et al.* 2012), which like RSV fuses at the plasma membrane.

Indeed, *Ifitm1* has been shown to be up-regulated during RSV infection (Ravi *et al.* 2013), which would lend credence to the hypothesis of Ifitm1-mediated restriction of RSV. This could be further tested by gene knockdown *in vitro*, or through the generation of an *Ifitm1* knockout mouse line. The use of the *Ifitm*^{del} mice that show a deletion of all *Ifitm* genes may elucidate the role further (Lange *et al.* 2008). However, the current study suggests that Ifitm3 plays an important role in the control of RSV infection; something which may be true of the Ifitm family in general.

Despite evidence in the literature that suggests a role for IFITM3 in restriction of *Plasmodium* (Sharma *et al.* 2011) and *M. tuberculosis* (Shen *et al.* 2013), the current study showed there to be no obvious effect of Ifitm3 on either pathogen in murine models. *Plasmodium* infection has been shown to induce strong type I and II IFN responses in the host (Haque *et al.* 2011), which

subsequently signals for the activation of the ISG cascade. Although roles have been uncovered for Irf3 and Irf7 (Sharma *et al.* 2011), which are up-regulated by IFN, the current study suggests that the up-regulation of Ifitm3 has no large impact on the development of cerebral malaria. Similarly, the current study shows no evidence for control of *M. tuberculosis* bacterial burden, despite the fact that the pathogen triggers a type I IFN response (Novikov *et al.* 2011), and a SNP in IFITM3 has been reported to associate with TB severity (Shen *et al.* 2013). However, it should be noted that the current study only assayed for bacterial burden. Although no evidence was seen of morbidity, the complexities associated with the bacteria in terms of its heterogeneous disease outcomes (dormancy / activation, asymptomatic/symptomatic etc.) could not be accounted for in the murine model.

Similarly, the study revealed no role for Ifitm3 in the restriction of *Salmonella* or *Citrobacter* infections, despite the abundant expression of the protein in key organs that are colonised by the bacterial species (Figure 6.1). This study advances previous investigations with *Salmonella* that showed that Ifitm3 does not restrict the bacterium *in vitro* (Yount *et al.* 2012), by utilising an *in vivo* model as ratification.

In conclusion, the study has demonstrated the specificity of the IFITM family for viral pathogens, whilst simultaneously expanding the field by showing that RSV is restricted by Ifitm3 *in vivo*. Furthermore, the lack of phenotype elicited by pathogens that have been reported to trigger an up-regulation of IFITM3 highlights an issue with interpretation of data associated with IFN induction. Host cells are essentially 'blind' to the type of invading pathogen and as such trigger a broad-ranging immune response in order to combat the infection. Therefore, it is unsurprising that they in turn produce type I and II IFN, which may be an appropriate response. However, the production of IFN will subsequently up-regulate a large cascade of ISGs; not all of which will be relevant to the pathogen. Therefore, the observation that certain genes are up-regulated at the RNA level is not always indicative that the translated protein will be used to combat infection.