5 Investigating the impact of loss of IFITM3 on vaccination and medical therapies.

5.1 Introduction

Annual influenza epidemics are associated with morbidity and mortality, particularly in the elderly around the world (Molinari *et al.* 2007; Xue *et al.* 2010). For the majority of people that contract the virus, influenza virus infection results in a relatively short period of illness, after which a full recovery is made. However, others are more severely impacted by the virus and the infection becomes life-threatening.

Typically, those requiring hospitalisation and antiviral treatments are defined to be in "high risk" groups, namely the elderly, young children and those with pre-existing medical conditions that predispose them to severe viral infections (Bautista *et al.* 2010; Van Kerkhove *et al.* 2011). However, as discussed in section 1.3.3, the recent 2009 H1N1 pandemic resulted in an altered disease profile, with individuals that were previously regarded as "low risk" succumbing to infection, despite the fact that the virus itself had relatively low virulence (Donaldson *et al.* 2009). Analysis revealed that these severe cases were not due to a more virulent strain of the virus emerging. In Chapter 4, I discussed how some of these cases may be explained by undiagnosed host genetic factors, such as polymorphisms in IFITM3, which show no obvious phenotypic traits unless the individual becomes infected with a virus.

Traditional "at risk" groups have been the target of prioritisation for annual vaccination against influenza to lower the risk of infection and disease complications. Currently, two routes of administration are used for the delivery of influenza vaccine: intramuscular and intranasal, as discussed in section 1.4.5. Briefly, intramuscular vaccines use inactivated viruses or viral proteins to induce protection, whilst intranasal vaccines rely on live-attenuated viruses that replicate in the upper respiratory tract. Although intranasal vaccines have been shown to elicit a superior protective effect against influenza viruses (Fleming *et al.* 2006; Osterholm *et al.* 2012), they also could present additional risk to those that are immune-compromised or suffering from lung conditions. Such individuals are consequently prescribed the intramuscular vaccine.

As the use of genomics is incorporated into the disease diagnosis and therapies, it is appropriate to consider the impact of human genetic polymorphisms and their associated impact on human health under certain physiological conditions, such as viral exposure. Although I have previously shown that IFITM3 mutations and ablations have a dramatic impact on the health of mice and humans when exposed to influenza virus, there may also be other situations or practices where IFITM3 deficiency may have an impact, such as vaccination with live attenuated virus or certain therapies. Such considerations are especially important when one factors in the reported ethnic differences in the frequencies of polymorphisms such as SNP rs12252, which are far more abundant in Asian populations than they are in Europeans (Zhang *et al.* 2013b). This would potentially make the possibility of a "rare" complication stemming from an individual possessing rs12252-CC in European populations more common in those of Asian descent.

5.1.1 Influenza vaccine

Vaccination is the primary medical intervention used to lower the risk of contracting potentially life-threatening influenza virus infections in "at risk" groups, such as the young and the elderly. Additionally, it is also used to immunise proposed reservoirs of the virus, such as school-age children, in order to prevent community-level spread and consequently reduce the influenza-related morbidity of the population in general (Piedra *et al.* 2005; King *et al.* 2006; Grijalva *et al.* 2010). Indeed, in the United Kingdom, school-age influenza vaccination programmes are to be rolled out from 2013 in pre-school and primary school age children and 2015 in secondary schools, using live attenuated intranasal vaccines (Zosia 2013).

One of the aims of the current study was to use the *Ifitm3*$^{-/-}$ mouse to act as a model to test the safety and efficacy of the influenza vaccine, using commercially available intranasal vaccine. Potentially, live attenuated influenza vaccines (LAIVs) represent a form of vaccine with the highest theoretical risk to individuals with sub-optimally functioning IFITM3, owing to their ability to replicate in the hosts' respiratory tract; thus meriting the use of a pre-clinical model to assess their safety.

5.1.2 AmBisome

Amphotericin B (AmphoB) is a routinely used antifungal drug delivered by intra-venous infusion to combat systemic fungal infections, such as aspergillosis (Cornely *et al.* 2007; Moen *et al.* 2009), and protozoan-borne diseases such as leishmaniasis (Croft and Coombs 2003). It is also used prophylactically in patients admitted into hospital with a critical illness such as cancer, or if they are in an immunocompromised state, to reduce the risk of fungal complications (Walsh *et al.* 1999). AmBisome is a lipid-based forumulation of AmphoB that greatly reduces the nephrotoxicity and damage caused by traditional formulations of the drug (Coukell and Brogden 1998; Walsh *et al.* 1999); thus allowing sustained therapeutic treatment in patients. AmphoB is thought to function through its binding ability with the fungal membrane component ergosterol. This interaction results in the formation of pores in the membrane, which permits ion transport from the cells to induce death (Palacios *et al.* 2011).

In collaboration with Abraham L. Brass, the aim of this component of the study was to characterise the effects of AmBisome in wild type and *Ifitm3*$^{-/-}$ mice in relation to their influenza susceptibility. This was conducted following observations *in vitro* that administration of AmphoB to A549 cells expressing IFITM3 resulted in abrogation of the restrictive effects of IFITM3. These *in vitro* results, in addition to the *in vivo* findings, are presented and discussed here.

5.2 Results

5.2.1 The role of Ifitm3 in intranasal vaccination against influenza virus

Wild type and *Ifitm3*$^{-/-}$ mice were intranasally (i.n.) inoculated with FluMist vaccine (MedImmune): a trivalent LAIV containing recombinants of A/California/07/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010. After 21 days, mice were boosted with the same amount of vaccine. They were subsequently challenged 21 days after boost with 2000 PFU of A/England/195 H1N1 influenza virus; representing a 10× lethal dose for *Ifitm3*$^{-/-}$ mice. This represented a homologous challenge, owing to the similarities to A/California/07, which is present in the vaccine formulation.

Additionally, the experiment was repeated under the same conditions, but using delNS1 PR/8 influenza in place of FluMist as a replication competent, but attenuated virus, and a dose of 5000 PFU of PR/8 virus in the live challenge, which represents a 100× lethal dose for *Ifitm3*$^{-/-}$ mice.

5.2.1.1 Vaccine tolerance

Mice were inoculated with either what was termed a "normal" dose (1/10th human dose) of 20µl (Sun *et al.* 2011) or "high" dose (1/5th human dose) of 50µl of FluMist vaccine (MedImmune). Animals were subsequently observed and weighed for 10 days post-vaccination to record any adverse effects associated with LAIV use. The study showed no significant weight loss from either genotype of mouse, nor was there any evidence of morbidity (Figure 5.1). Additionally, mice were also weighed following dosing with delNS1 PR/8 virus, and also showed no adverse effects (Figure 4.7).

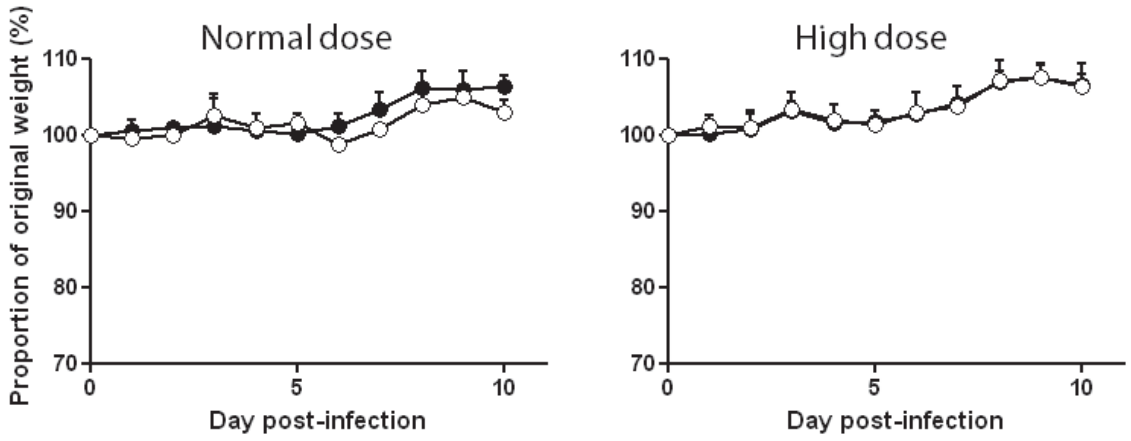

Figure 5.1: Tolerance of the live attenuated influenza vaccine, FluMist, in wild type and *Ifitm3*$^{-/-}$ mice. Mice were either administered intra-nasally with 1/10th human dose (normal) or 1/5th human dose (high) of FluMist and were weighed for 10 days post-vaccination. ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 5).

5.2.1.2 Vaccine efficacy: weight loss

Mice were primed and boosted with either 1/10th human dose of FluMist or 1000 PFU of delNS1 PR/8 virus and were subsequently challenged with lethal doses of their respective virulent homologous viruses. All vaccinated mice, regardless of genotype, survived the lethal challenge with influenza virus, whilst all unvaccinated mice succumbed to infection or surpassed 25%

weight loss by day six post-infection (Figure 5.2). Interestingly, vaccinated *Ifitm3*$^{-/-}$ mice showed mild signs of illness with a small, but significant, loss of weight on days four and five post-infection when challenged with Eng/195 (d4: p = 0.03, d5: p = 0.04), and on days five and six post-infection when challenged with PR/8 (d5: p = 0.03, d6: p = 0.02), compared with vaccinated wild type littermates.

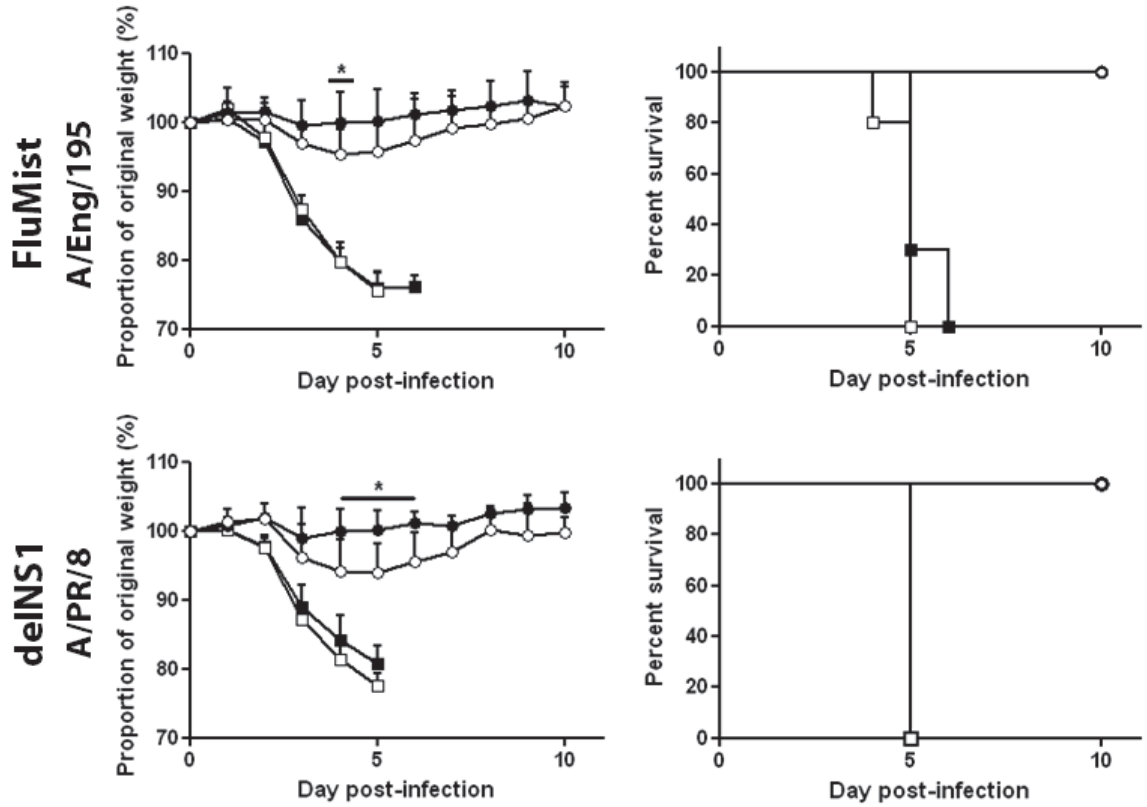

Figure 5.2: Efficacy of live attenuated influenza vaccines in terms of weight loss and survival of wild type and *Ifitm3*$^{-/-}$ mice. Mice were primed and boosted with either FluMist or PR/8 delNS1 influenza virus before challenge with 10 lethal dose of virulent virus. Animals were weighed daily and clinical symptoms recorded. Mice showing severe symptoms of illness or those that had surpassed 25% weight loss were killed in accordance with UK Home Office guidelines. ■: wild type, □: *Ifitm3*$^{-/-}$, ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 5). Statistical significance was assessed by ANOVA (*: p < 0.05).

5.2.1.3 Vaccine efficacy: viral kinetics

Lungs were taken from mice that were immunised with FluMist and subsequently challenged with Eng/195 influenza virus on days three and five post-infection to quantify viral load. Titres

of live virus were calculated by $TCID_{50}$ assay, owing to the inability of Eng/195 to form plaques. The assay showed that the viral kinetics observed in unvaccinated mice were similar to typical sub-lethal doses of A/X-31 influenza virus, with a persistent 10× higher viral load in *Ifitm3*$^{-/-}$ mice on day five post-infection ($p = 0.04$). By the same time point, virus was undetectable in vaccinated wild type and *Ifitm3*$^{-/-}$ mice. Furthermore, vaccination significantly reduced viral load on day three post-infection in wild type and *Ifitm3*$^{-/-}$ mice ($p = 0.02$).

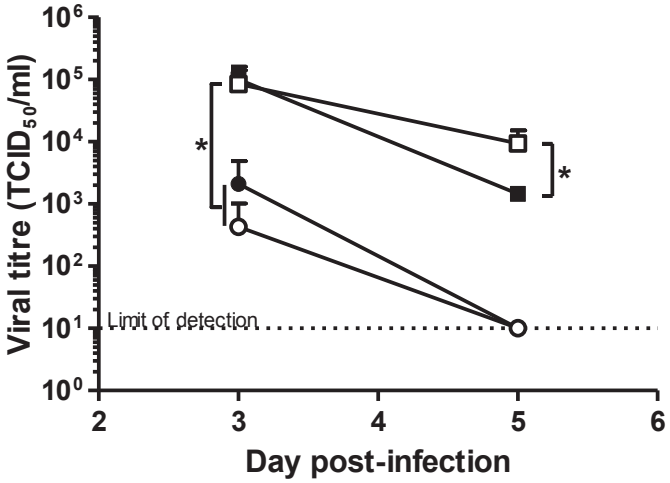

Figure 5.3: Effect of FluMist vaccination on influenza A viral kinetics in wild type and *Ifitm3*$^{-/-}$ mice. Results show the viral replication kinetics of Eng/195 influenza virus, as assessed by $TCID_{50}$ assay, in vaccinated and unvaccinated mice. ■: wild type, □: *Ifitm3*$^{-/-}$, ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. ($n > 5$). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$).

5.2.1.4 Antibody response to vaccination

Mice were bled by cardiac puncture 21 days after their immune boost with FluMist vaccine (42 days after the initial priming dose). Additionally, lungs were extracted from these mice, homogenised and the protein fraction retained for assays. Sera and lung homogenate were analysed for levels of non-influenza-specific immunoglobulin (Ig) subclasses through the use of Luminex assays to quantify the local and systemic responses to vaccine in the lungs and blood, respectively. As shown in Figure 5.4, the Ig profile of both genotypes of mice is broadly similar, but is higher in all subclasses in *Ifitm3*$^{-/-}$ mice. In particular, *Ifitm3*$^{-/-}$ mice showed significantly higher levels of IgM in their blood ($p = 0.002$), as well as significantly elevated levels of IgA ($p = 0.02$), IgG2a ($p = 0.02$) and IgG2b ($p = 0.01$) in their lungs.

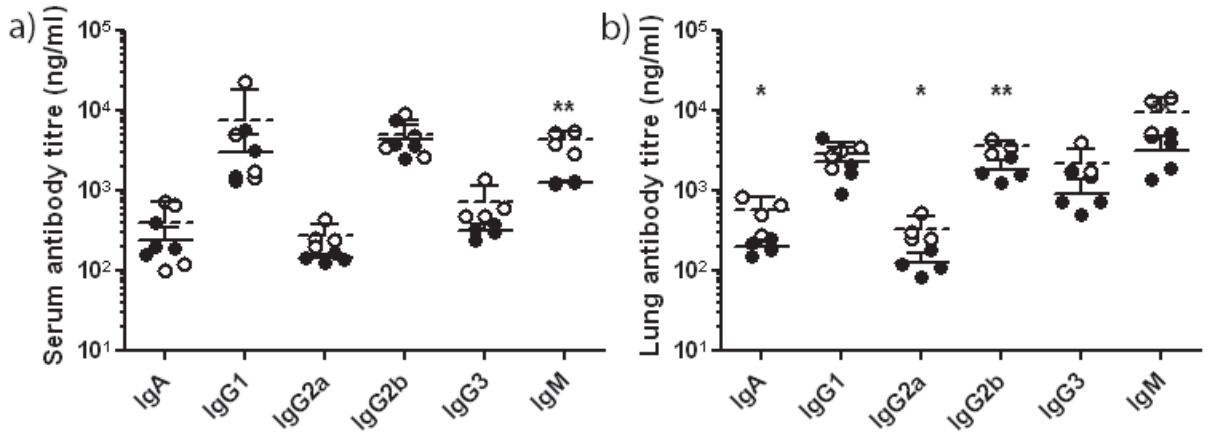

Figure 5.4: Immunoglobulin profile of the blood and lungs of wild type and *Ifitm3*$^{-/-}$ mice following immunisation with FluMist vaccine. Antibodies in the sera (a) and lung homogenate (b) of immunised, but uninfected, mice were quantified by bead-based array. ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n = 4), where solid lines indicate wild type mean, and dashed lines indicate *Ifitm3*$^{-/-}$ mean. Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$).

The influenza-binding antibodies in the sera were quantified through the use of A/Eng/195 HA-specific ELISA, which measures the ability of the antibody to bind influenza HA, and microneutralisation (MN) assay, which measures the capacity of the antibody to neutralise influenza virus and therefore prevent cell infection (Figure 5.5). Cell infection was measured by immunostaining infected cells with a monoclonal antibody to NP. Results showed that the MN titre, defined as the dilution at which influenza NP expression is reduced by >50% (50% infectivity in Figure 5.5a), for wild type mice averaged 1:130, whilst the *Ifitm3*$^{-/-}$ sera titre averaged 1:60; thus suggesting that antibodies from wild type mice neutralised influenza virus twice as well as *Ifitm3*$^{-/-}$ mice. ELISA showed the same pattern, with binding occurring to dilutions of 1:2560 in wild type mice and 1:1280 in *Ifitm3*$^{-/-}$ mice; suggesting that immune serum from wild type mice contained two-fold more influenza HA-specific antibodies than serum from *Ifitm3*$^{-/-}$ mice following FluMist vaccination.

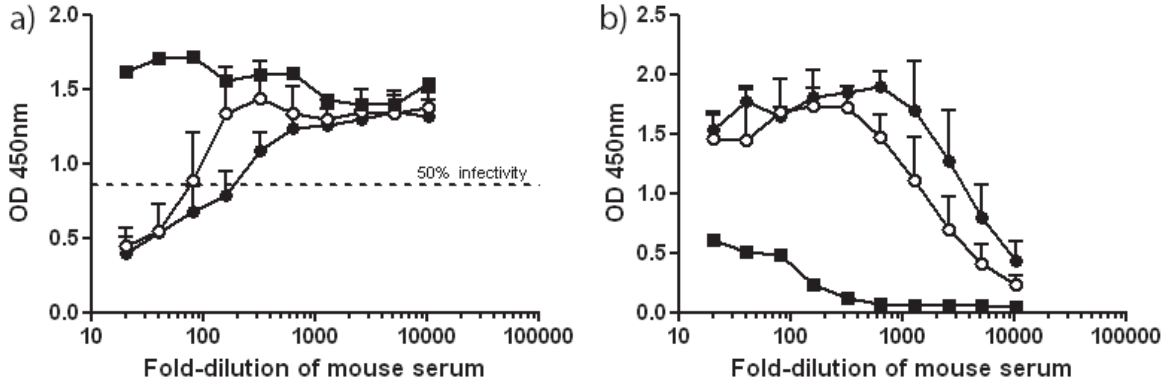

Figure 5.5: Influenza-binding capacity of wild type and *Ifitm3*$^{-/-}$ antibodies following FluMist immunisation. The neutralising capacity of sera antibodies were determined by microneutralisation (MN) assay (a), and influenza-binding capacity was measured by ELISA (b). MN titres were determined to be the final dilution of serum that caused >50% reduction in NP expression; therefore reducing cellular infection by >50%. ELISA titres were calculated to be the last dilution to give >50% of the plateau value, wherein binding of free virus to the plate was reduced by 50%. MN assays were stained for NP expression through the use of a secondary HRP-conjugated anti-NP antibody and absorbance read at 450nm to indicate the level of cellular infection. ELISA values were determined through incubation with anti-mouse HRP-conjugated secondary antibody to detect the level of influenza virus bound to the HA-specific ELISA plate, which was shown through absorbance readings at 450nm. ■: unvaccinated wild type control serum, ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n = 4).

5.2.1.5 Pathology

Lungs were excised on day five post-infection from vaccinated and unvaccinated mice to assess the impact of vaccination on preventing pathological damage. Gross observation of the pleural surfaces showed a dramatic reduction in the number of lesions on the surface of wild type and *Ifitm3*$^{-/-}$ mice following vaccination (Figure 5.6). Unvaccinated *Ifitm3*$^{-/-}$ mice displayed the widespread damage seen previously (Figure 4.10), whilst the unvaccinated wild type mice displayed sporadic lesions on their surface; owing to the high infectious dose of virus used in this experiment.

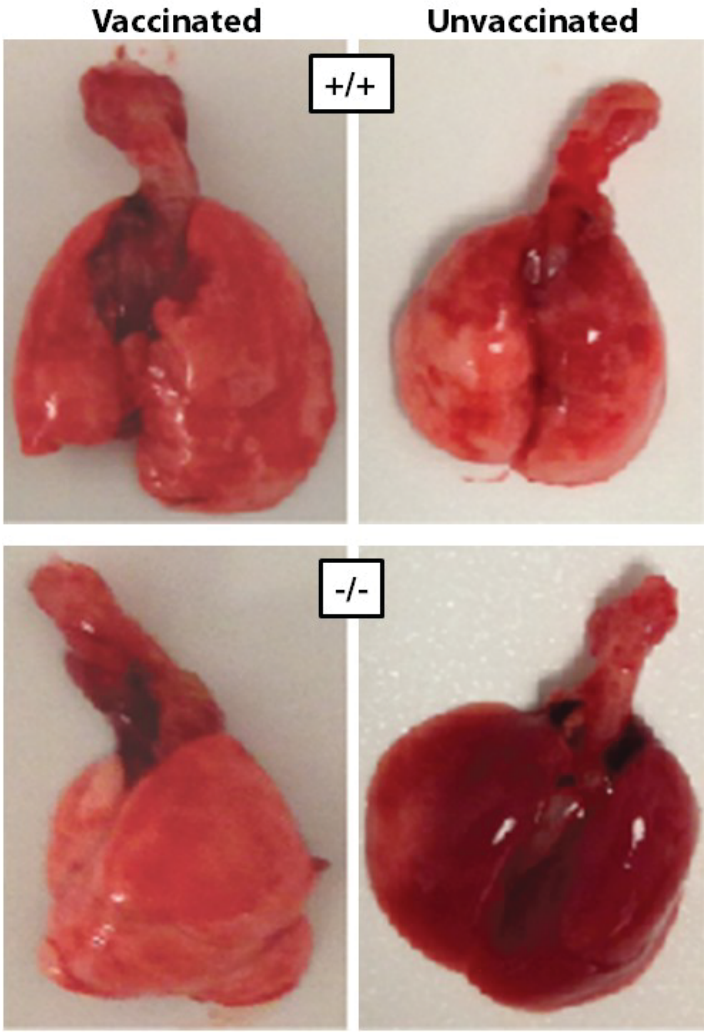

Figure 5.6: Effect of vaccination on pathological damage to the respiratory system following a lethal influenza A challenge. Vaccinated and unvaccinated wild type (+/+) and *Ifitm3*$^{-/-}$ (-/-) mice were challenged with a 10× lethal dose of Eng/195 influenza virus and their lungs were excised on day five post-infection to determine the extent of pathological damage.

Sectioning of the lungs revealed extensive cellular infiltrate, oedema and inflammation in all challenged unvaccinated mice (Figure 5.7). However, challenged vaccinated wild type and *Ifitm3*$^{-/-}$ mice showed very mild-to-negligible inflammation, with alveoli and bronchi free of cellular infiltrate; resembling a healthy, uninfected respiratory system.

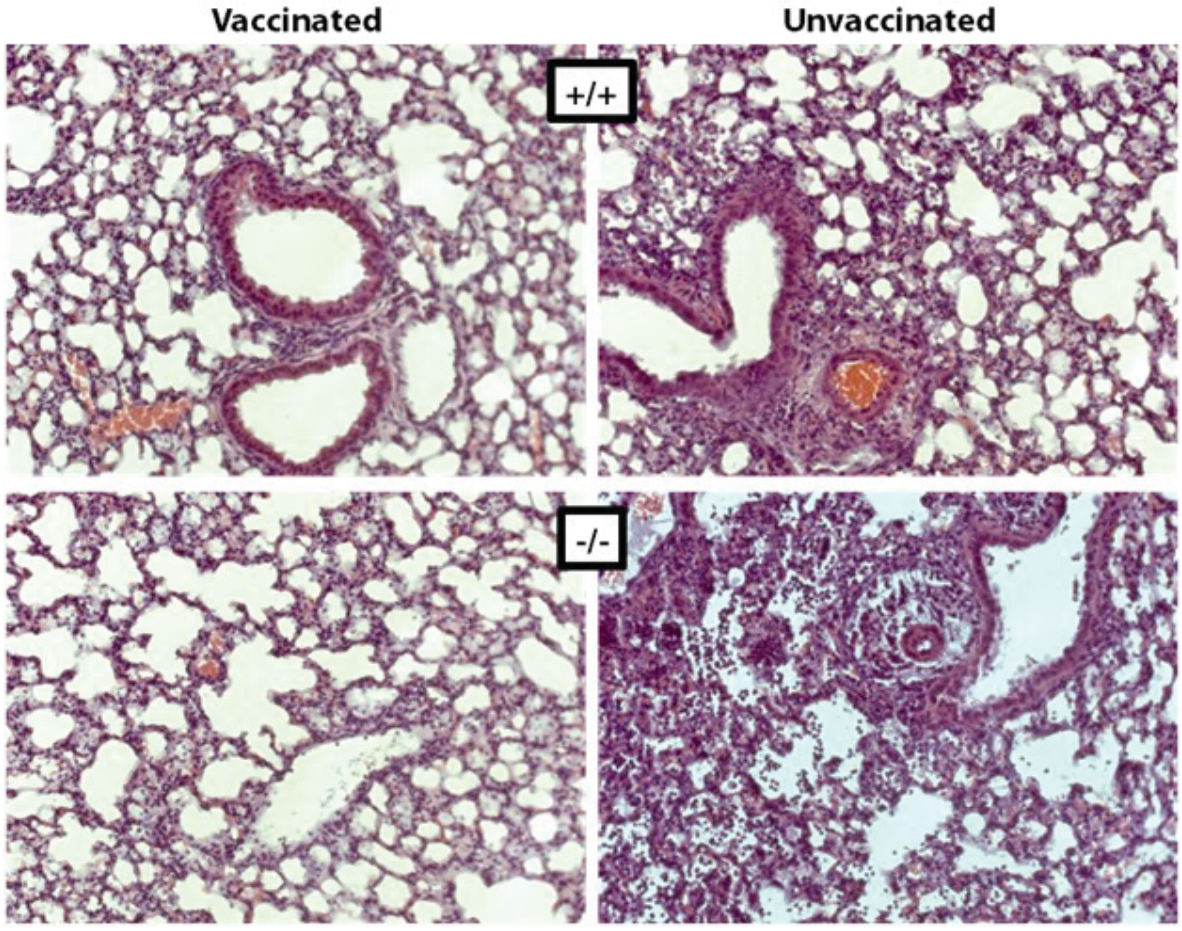

Figure 5.7: Histological impact of vaccination in wild type and *Ifitm3*$^{-/-}$ mice lungs following a lethal influenza A infection. Images show the extent of inflammation and damage in murine lungs at day five post-infection with a 10× lethal dose of Eng/195 influenza. Original magnification 20×.

5.2.1.6 Cellular response

In order to quantify the relative contributions of various immune cells to the inflammation observed without vaccine and to understand the adaptive immune response in vaccinated mice, lungs were extracted, homogenised and analysed by flow cytometry. Analysis showed that at both days three and five post-infection, vaccinated wild type and *Ifitm3*$^{-/-}$ mice showed a significantly higher proportion of CD8 and activated CD8 T-cells (d3: CD8+ $p = 0.001$, CD69+ $p = 0.002$; d5: CD8+ $p < 0.001$, CD69+ $p = 0.01$) in their lungs (Figure 5.8), with no significant changes in CD4+ T-cell populations. B-cells were also recorded as significantly lower in vaccinated Ifitm3-/- mice compared to unvaccinated *Ifitm3*$^{-/-}$ mice on day three post-infection. Granulocytes and macrophages were similarly affected, with a significant reduction in the

number of infiltrating macrophages (d3: p = 0.04; d5: p = 0.0002) and neutrophils (d3: p = 0.0001; d5: p = 0.0005) in vaccinated *Ifitm3*$^{-/-}$ mice throughout the infection.

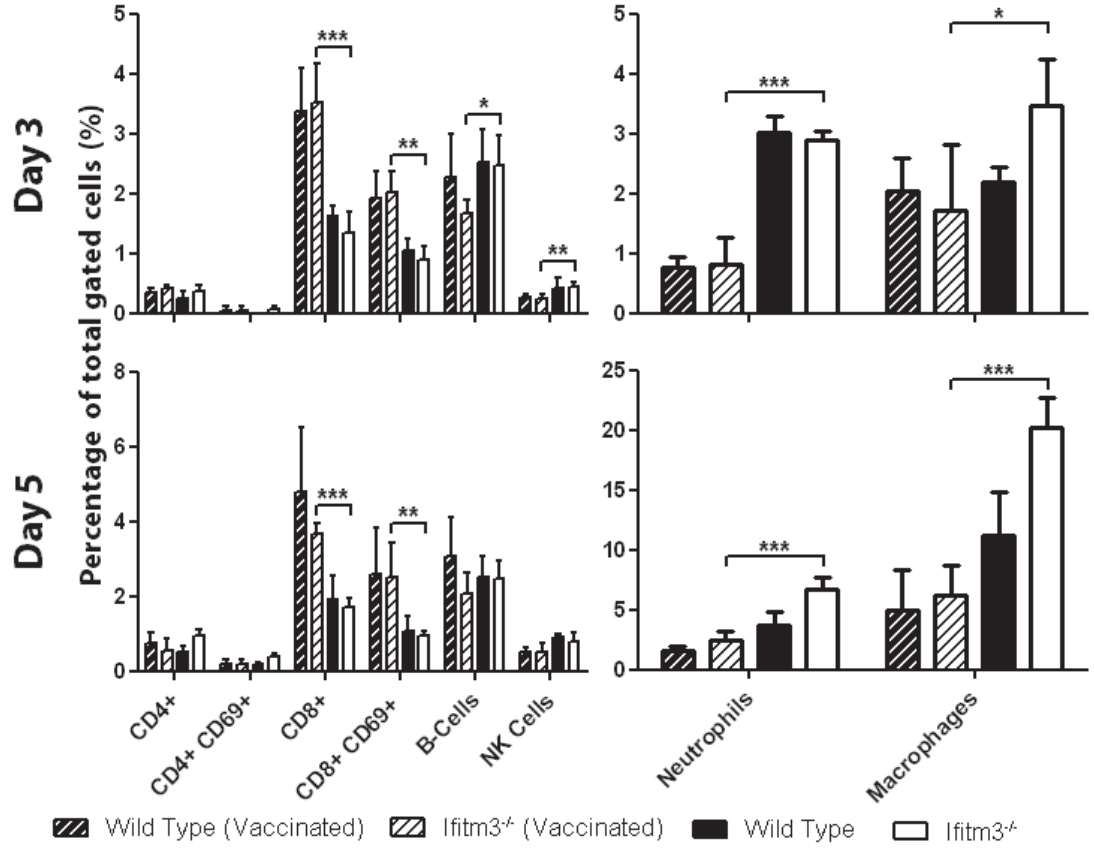

Figure 5.8: Impact of vaccination on immune cell populations within the lungs of wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Leukocytes were isolated from total lung homogenates and analysed by flow cytometry to quantify the effects of vaccination. Legend is shown in the Figure. Results show means ± S.D. (n = 4). Statistical significance was assessed by Student's *t*-test (*: p < 0.05, **: p < 0.01, ***: p < 0.001).

Interestingly, no significant differences were observed between vaccinated wild type and vaccinated *Ifitm3*$^{-/-}$ mice in their immune cell populations, although B-cell populations were marginally lower throughout the course of infection and CD8+ T-cells were at reduced levels on day five post-infection in vaccinated *Ifitm3*$^{-/-}$ mice compared to vaccinated wild type controls.

5.2.1.7 Cytokine response

Lung homogenates from days three and five post-infection were analysed by bead-based assay to quantify the levels of the key inflammatory cytokines MCP-1, G-CSF, IL-6 and TNFα. Results showed that vaccination significantly reduced the levels of all of these cytokines in wild type and

Ifitm3$^{-/-}$ mice throughout the course of infection (Figure 5.9). Remarkably, vaccination generally nullified the gross overproduction of these inflammatory cytokines in *Ifitm3*$^{-/-}$ mice to similar levels to wild type mice. However, it was notable that on day three post-infection, all cytokines were expressed at a higher level in vaccinated *Ifitm3*$^{-/-}$ mice compared with vaccinated wild type mice, with levels of G-CSF being significantly higher ($p = 0.04$). Similarly, unvaccinated mice displayed the same trend, with *Ifitm3*$^{-/-}$ mice showing an exaggerated profile compared to unvaccinated wild type mice, with the exception of TNFα on day five post-infection wherein wild type mice showed heightened levels.

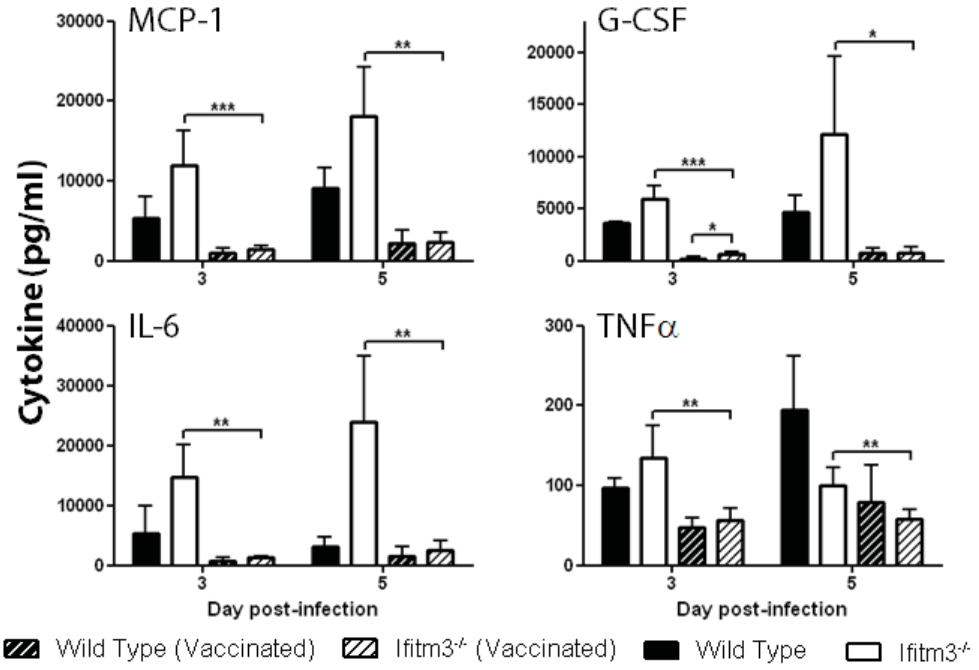

Figure 5.9: Effect of vaccination on inflammatory cytokine production in wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Lung homogenate was analysed for the levels of MCP-1, G-CSF, IL-6 and TNFa; all of which were significantly up-regulated in non-vaccine-based challenges (section 4.2.3). Legend is shown in the Figure. Results show means ± S.D. (n = 4). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

5.2.2 The role of Ifitm3 in intra-muscular vaccination against influenza virus

In addition to trialling intra-nasal vaccination regimens, wild type and *Ifitm3*$^{-/-}$ mice were intra-muscularly (i.m.) primed and boosted with Fluvirin vaccine (Novartis): a HA subunit-based trivalent seasonal vaccine, at the same time points as the i.n. immunisations. The immunising dose was also the same as FluMist: 1/10th human dose, as this dose had been previously utilised

by others (Easterbrook *et al.* 2011). Mice were subsequently challenged with 2000 PFU (10× lethal dose) of A/England/195 H1N1 influenza virus.

Of note, this experiment was only conducted as a pilot study once in isolation; therefore the results in this section are preliminary and are not discussed in-depth. No further vaccine could be sourced from suppliers or Novartis, owing to an industry-wide shortage in the 2012-2013 influenza season, which prevented repetition of the experiment.

5.2.2.1 Vaccine efficacy: weight loss

Mice were immunised twice (d0 and d21) and challenged on day 42, with Eng/195 influenza virus. Mice were monitored and weighed for 15 days post-infection for signs of severe illness and to determine the extent of morbidity through weight loss. All mice, regardless of vaccination state, lost weight over the course of infection (Figure 5.10). However, all vaccinated wild type mice survived the challenge with modest, transient weight loss, whilst 60% of vaccinated *Ifitm3*$^{-/-}$ mice survived, as too did 40% of unvaccinated wild type mice. All unvaccinated *Ifitm3*$^{-/-}$ succumbed to the infection or lost >25% of their body weight by day nine post-infection.

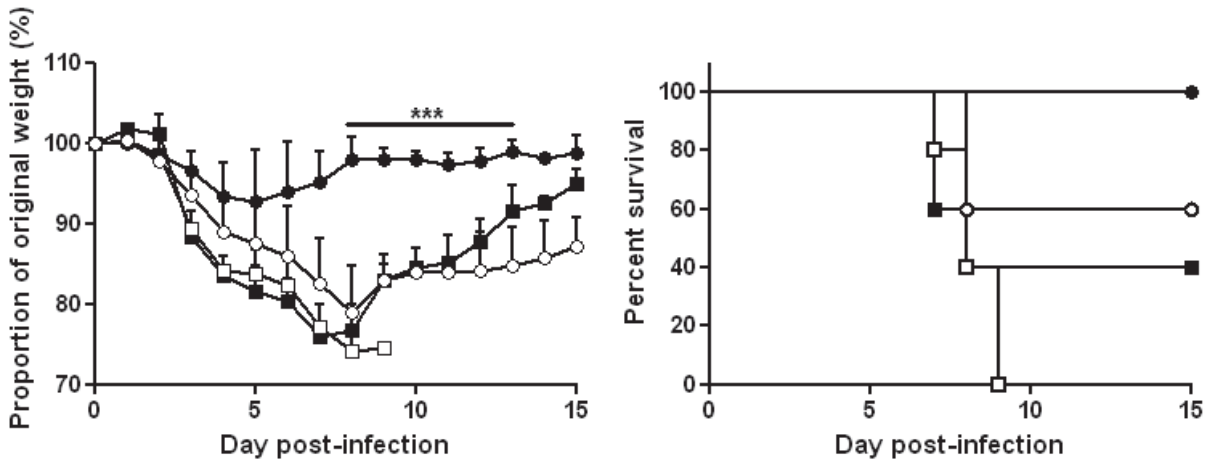

Figure 5.10: Effect of intra-muscular influenza vaccination on the weight loss and survival of wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Mice were weighed daily and monitored for signs of severe illness; those exceeding 25% weight loss were killed in accordance with Home Office guidelines. ■: wild type, □: *Ifitm3*$^{-/-}$, ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n = 5). Statistical significance was assessed by Student's *t*-test (***: $p < 0.001$).

5.2.2.2 Vaccine efficacy: viral kinetics

Lungs were excised and homogenised on days two and six post-infection and viral load quantified by $TCID_{50}$ assay. Results showed that vaccination elicited a mild 2.5-fold reduction in peak viral titre on day two post-infection in *Ifitm3*$^{-/-}$ mice, and a 6-fold reduction in wild type mice. All vaccinated mice showed a large, but non-significant reduction in viral load by day six post-infection, at which point virus was still present and detectable in the lungs.

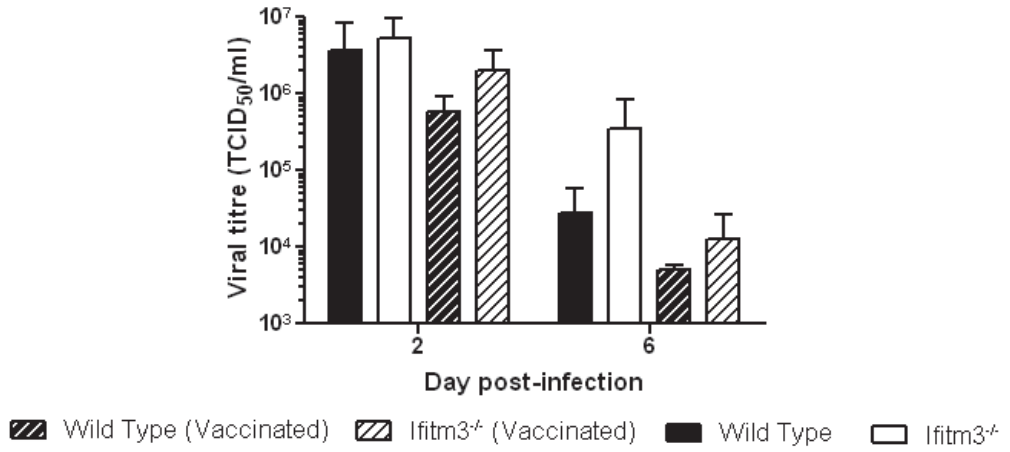

Figure 5.11: Effect of intra-muscular vaccination on viral load in the lungs of wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Lungs were excised and homogenised on days two and six post-infection to quantify viral load by $TCID_{50}$ assay. Legend is shown in the Figure. Results show means ± S.D. (n = 4).

5.2.2.3 Antibody response to vaccination

Sera and lungs were removed from mice 21 days post-second immunisation with Fluvirin vaccine and assessed for their total Ig profile by bead-based array on a Luminex FlexMAP3D. Results showed that on average, *Ifitm3*$^{-/-}$ mice had higher quantities of all Ig subclasses in both sera and lungs, with significantly higher levels of IgG2a and IgG2b in lungs ($p = 0.01$ and 0.004, respectively). However, influenza neutralising capacity of these antibodies was not determined owing to the preliminary nature of this pilot study.

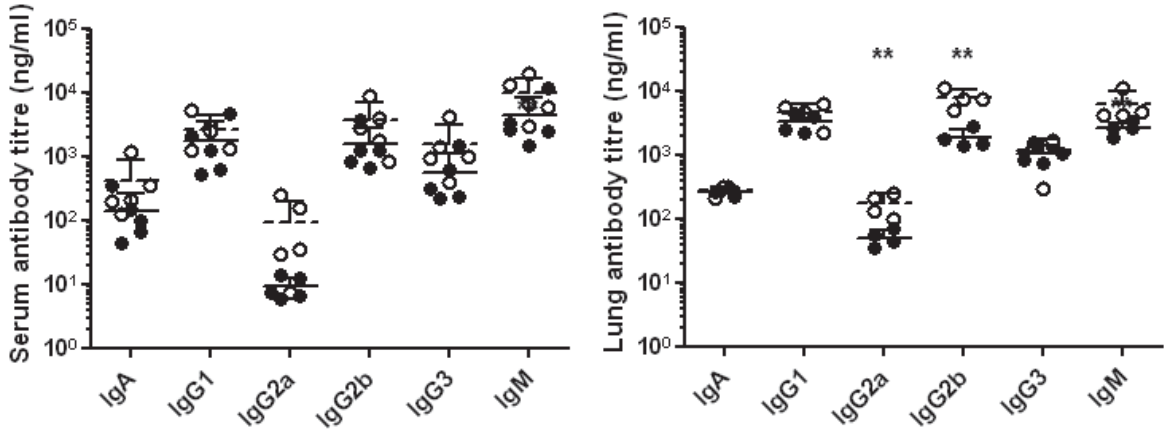

Figure 5.12: Immunoglobulin profile of the blood and lungs of wild type and *Ifitm3*$^{-/-}$ mice following immunisation with Fluvirin intra-muscular vaccine. Antibodies in the sera (a) and lung homogenate (b) of immunised, but uninfected, mice were quantified by bead-based array. ●: vaccinated wild type, ○: vaccinated *Ifitm3*$^{-/-}$. Results show means ± S.D. ($n > 4$), where solid lines indicate wild type mean, and dashed lines indicate *Ifitm3*$^{-/-}$ mean. Statistical significance was assessed by Student's *t*-test (**: $p < 0.01$).

5.2.2.3 Pathology

Respiratory systems were removed on day six post-infection and immediately photographed to assess gross pathological damage on the pleural surface. As shown in Figure 5.12, unvaccinated mice of both genotypes showed evidence of severe damage, with large hemorrhagic lesions on their surfaces. However, vaccination dramatically reduced this damage, with wild type and *Ifitm3*$^{-/-}$ lungs showing minor discolouration, but were ultimately healthy in appearance.

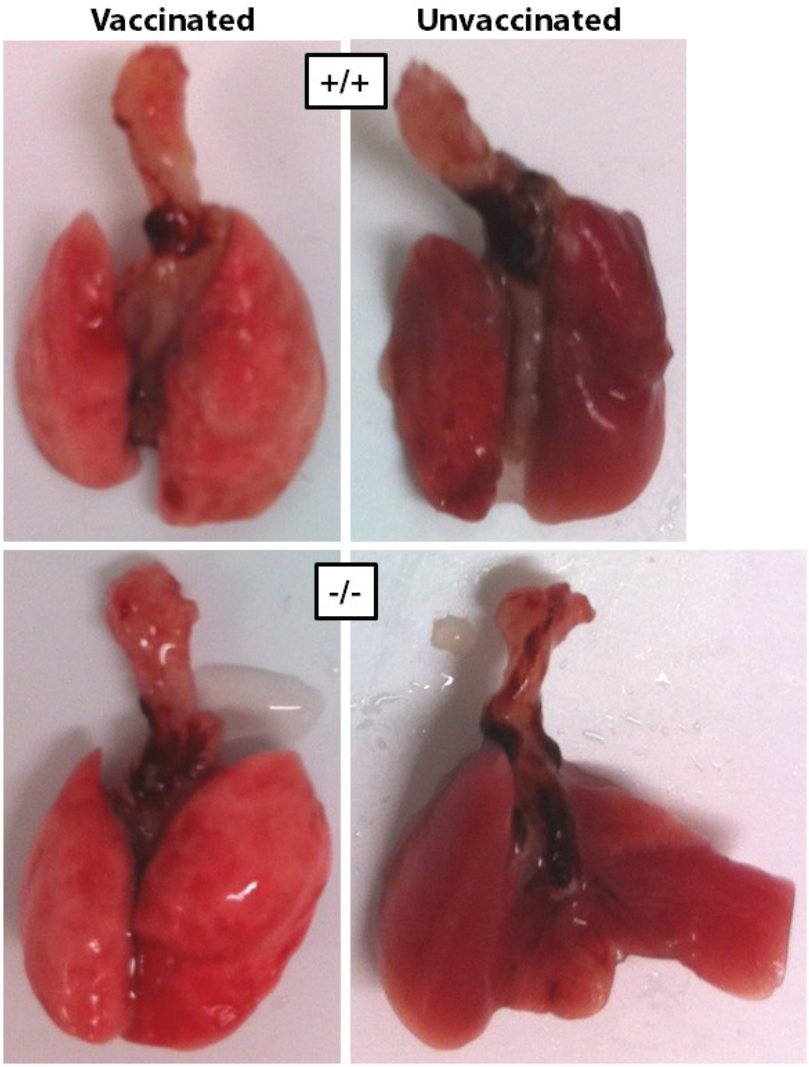

Figure 5.13: Impact of intra-muscular vaccination on pathological damage caused by a lethal challenge with influenza A virus. Vaccinated and unvaccinated wild type (+/+) and *Ifitm3*$^{-/-}$ (-/-) mice were challenged with a 10× lethal dose of Eng/195 influenza virus and their lungs were excised on day six post-infection to assess pathological damage.

5.2.2.4 Cellular response

Leukocytes were isolated from total lung homogenate on days two and six post-infection and were stained to quantify the level of individual cell populations during influenza infection. As shown in Figure 5.14, results from day three post-infection showed that vaccination resulted in significantly higher numbers of CD4 and CD8 T-cells ($p = 0.004$ and 0.002, respectively), as well as elevated levels of macrophages ($p = 0.04$) in vaccinated *Ifitm3*$^{-/-}$ mice compared with

unvaccinated *Ifitm3*$^{-/-}$ littermates. Vaccination also resulted in a significant reduction in the number of neutrophils in the lungs on day three post-infection in vaccinated *Ifitm3*$^{-/-}$ mice relative to unvaccinated *Ifitm3*$^{-/-}$ mice. There were no significant differences recorded between any subsets of mice on day six post-infection.

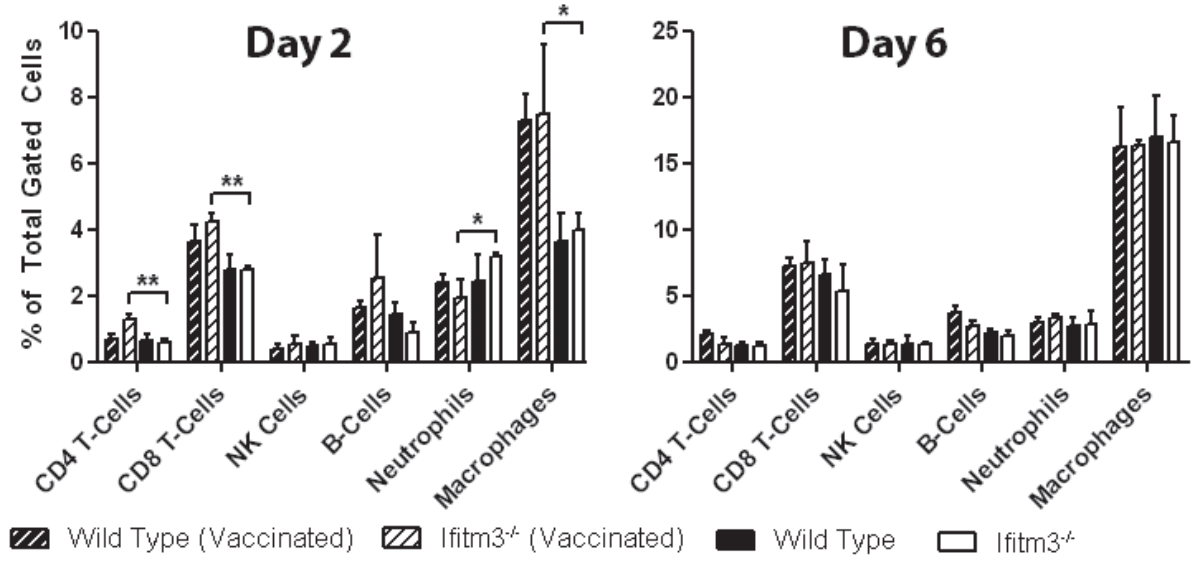

Figure 5.14: Effect of intra-muscular vaccination on leukocyte populations in the lungs of wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Lungs were removed and cell populations analysed by flow cytometry on days two and six post-infection. Legend is shown in the Figure. Results show means ± S.D. (n > 3). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$).

5.2.3 The effects of AmBisome on Ifitm3 functionality

My work investigating the effects of amphotericin B (marketed as AmBisome) on IFITM3 function was conducted in collaboration with Abraham L. Brass and members of his laboratory at Harvard University. Brief discussion of their *in vitro* work is included here to provide context and rationale for the *in vivo* work in wild type and *Ifitm3*$^{-/-}$ mice.

5.2.3.1 *In vitro* effects

Amphotericin B (AmphoB) was investigated for its effects on IFITM3 during influenza virus infection. As shown in Figure 5.15, the administration of AmphoB or the liposomal AmBisome resulted in abrogation of IFITM3 function. In Figure 5.15a, it can be seen that under normal non-AmBisome conditions, IFITM3-overexpressing A549 cells restrict influenza infection over a

range of MOIs, but this is entirely removed by the addition of AmBisome to the media. Further to this, the addition of AmphoB to HeLa cells results in cells becoming infected to a similar level as those that have had IFITM3 knocked down by specific shRNAs (Figure 5.15b), whilst the dosing of over-expressing IFITM3 HeLa cells with AmphoB results in a similar loss of restriction of influenza virus (Figure 5.15c).

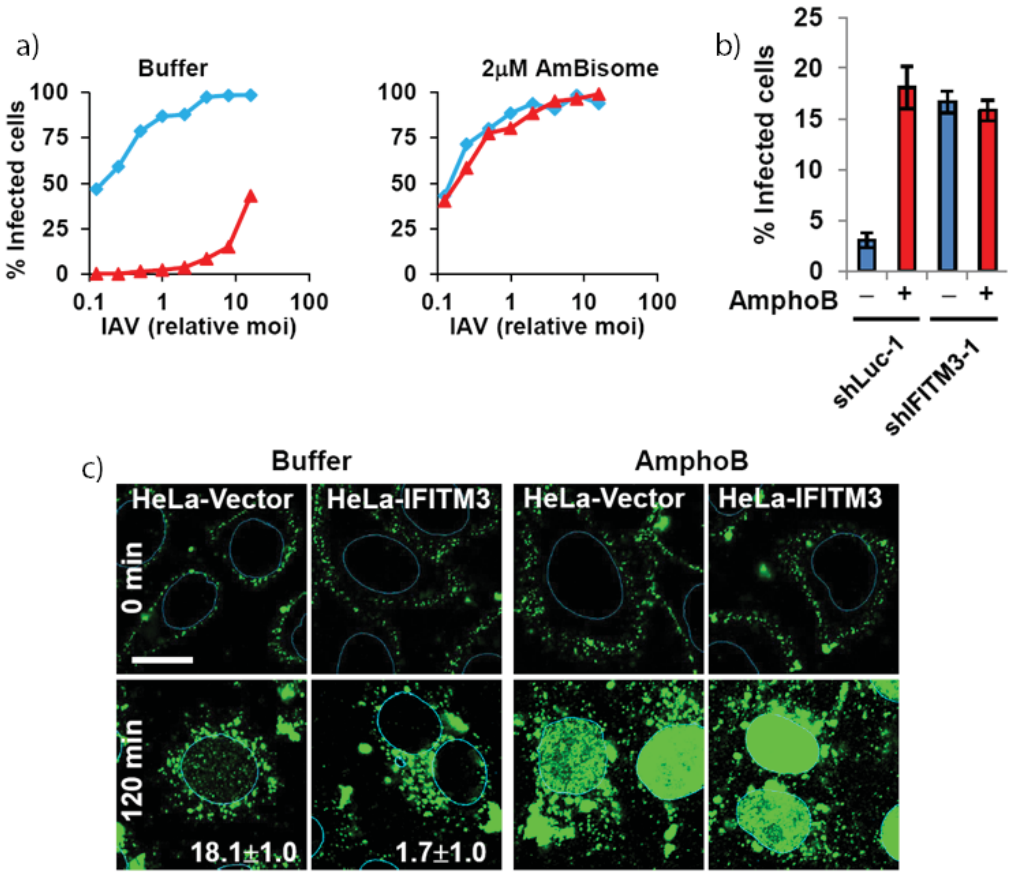

Figure 5.15: *In vitro* effects of amphotericin B on IFITM3-mediated restriction of influenza virus. (a) A549 cells expressing no (blue) or full-length IFITM3 (red) were incubated in the absence or presence of 2μM AmBisome and infected at the indicated range of MOIs with WSN/33 influenza. (b) HeLa cells were transfected with either a control shRNA specific for firefly luciferase (shLuc-1) or shRNA specific for IFITM3 (shIFITM3-1) to knockdown its expression. Cells were subsequently infected at an MOI of 0.1 PFU/cell with WSN/33 influenza in the presence of absence of amphotericin B (AmphoB) to measure relative infectivity. (c) HeLa cells either expressing regular (Vector) or amplified levels of IFITM3 (IFITM3) were infected with WSN/33 influenza in the presence of absence of amphotericin B and incubated for two hours before fixing and staining for viral NP expression (green) inside the nuclei of cells (blue circles). Results show means ± S.D. All data courtesy of Dr. Abraham L. Brass.

5.2.3.2 *In vivo* effects

The *Ifitm3*$^{-/-}$ mouse model was employed to investigate the effects of AmBisome administration *in vivo*. Mice were dosed with AmBisome at a concentration of 3mg/kg, which has shown to be both clinically relevant and non-cytotoxic (Proffitt *et al.* 1991; Wingard *et al.* 2000; Takemoto *et al.* 2004). To mimic the effects of human intravenous infusion, mice were given either two (two hours prior to infection and two days post-infection), or three (a further dose on day four post-infection) intravenous injections of the drug formulation, whilst being challenged with a non-lethal dose of X-31 influenza virus.

The cytotoxicity of the dose level was checked and showed no ill effects in either genotype of mice, either in terms of weight loss or morbidity (Figure 5.16a). Mice were initially trialled with two doses of AmBisome to test for phenotypic effects. The drug resulted in accelerated weight loss in both genotypes of mice (Figure 5.16b), with wild type mice showing the same weight loss profile as a untreated *Ifitm3*$^{-/-}$ mice and AmBisome-treated *Ifitm3*$^{-/-}$ mice showing a further acceleration of weight loss. However, all wild type mice survived the challenge (Figure 5.16c); recovering weight from day six post-infection. Analysis of the lungs on day three post-infection showed that AmBisome-treated wild type and *Ifitm3*$^{-/-}$ mice had a trend towards higher viral titres than untreated wild type mice at the same time point, and were more closely aligned to untreated *Ifitm3*$^{-/-}$ mouse titres (Figure 5.15d). Analysis of the brains, sera and spleens of all mice by qPCR for viral RNA showed no evidence of virus outside of the respiratory tract.

Mice were further treated with a three dose course of AmBisome to include a third infusion on day four post-infection. All *Ifitm3*$^{-/-}$ mice lost weight at the same rate as those of the two dose regimen (Figure 5.15e). However, AmBisome-treated wild type mice failed to recover weight and exhibited a terminal decline, which resulted in 100% mortality on day seven post-infection (Figure 5.15f).

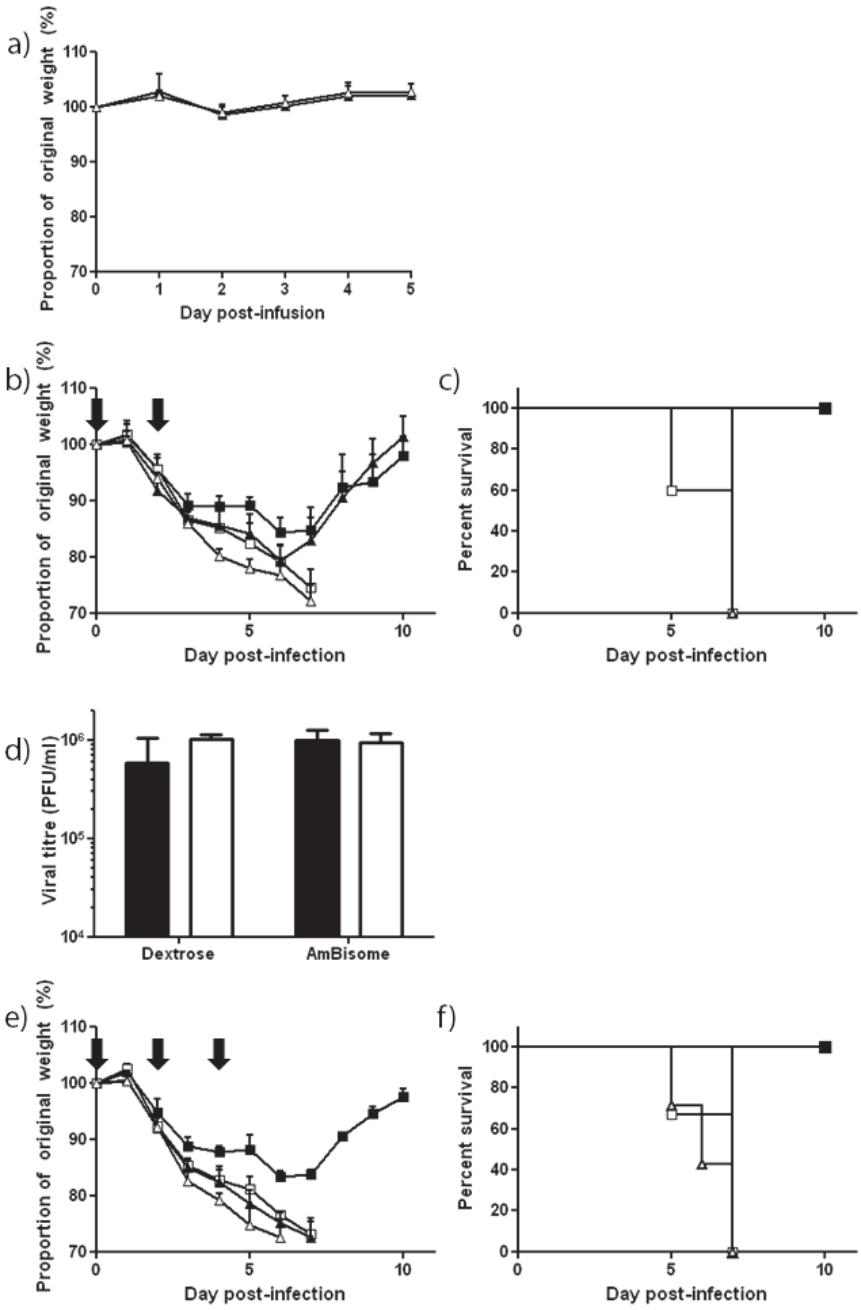

Figure 5.16: Effects of AmBisome on wild type and *Ifitm3*$^{-/-}$ mice during influenza A infection. Mice were injected intravenously with either 5% dextrose or 3mg/kg AmBisome in 5% dextrose and monitored for signs of weight loss or morbidity (a). With AmBisome yielding no adverse effects, mice were injected with two or three doses of AmBisome at the indicated time points (arrows) during challenge with X-31 influenza virus and monitored for weight loss (b,e) and survival (c,f). Lungs were excised on day three post-infection, following two doses of AmBisome, and were quantified for viral load (d). Mice exceeding 25% weight loss were killed in accordance with UK Home Office guidelines. ■: dextrose-treated wild type, □: dextrose-treated *Ifitm3*$^{-/-}$, ▲: AmBisome-treated wild type, Δ: AmBisome-treated *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 3).

Lungs were excised from untreated and treated mice on day four post-infection and their histology was analysed. As shown in Figure 5.17, AmBisome greatly amplified the pathological damage in wild type mice, with far higher cellular infiltrate and inflammation in the bronchi and alveoli. Similarly, AmBisome-dosed *Ifitm3*$^{-/-}$ mice showed qualitatively more inflammation than mice that were untreated.

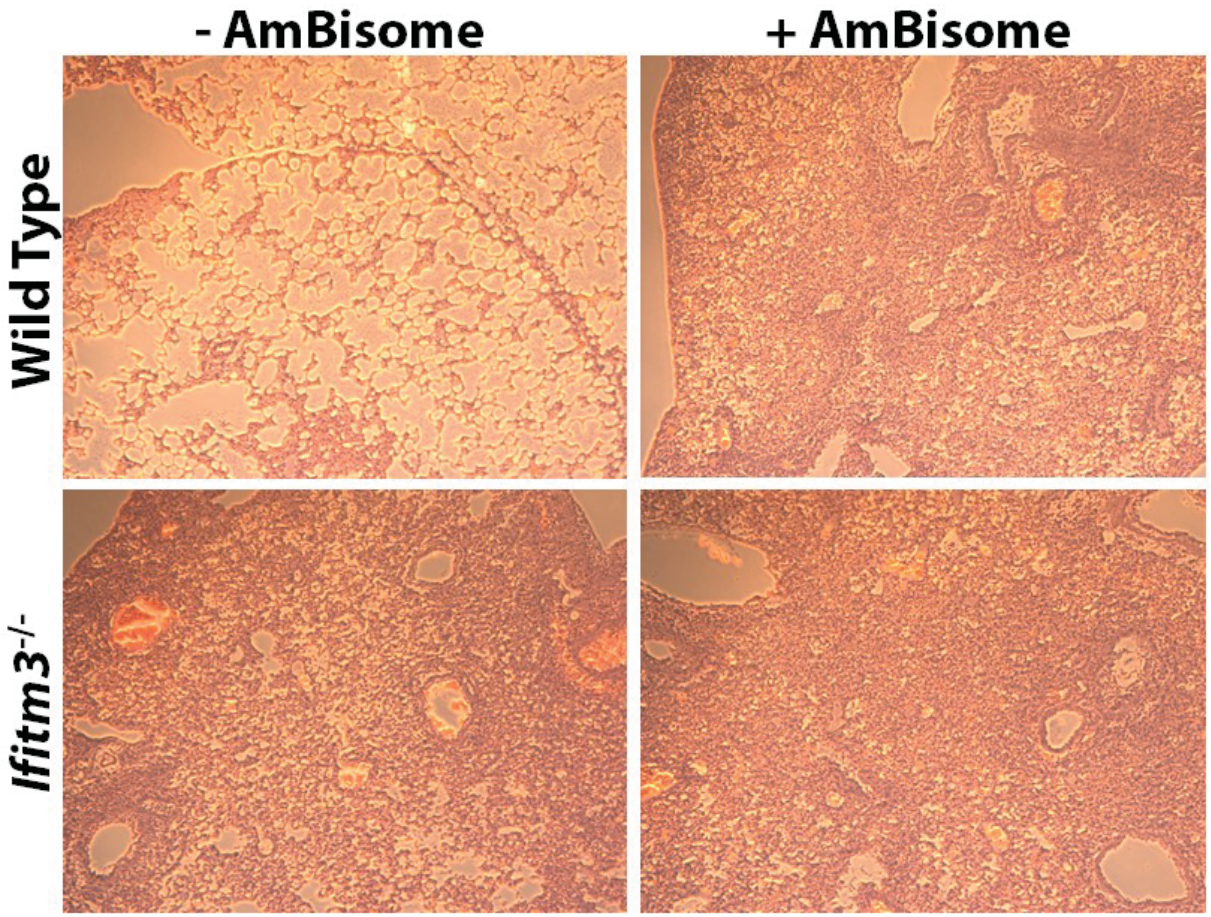

Figure 5.17: Effect of AmBisome on lung histology during influenza virus infection. Lungs were excised on day four post-infection with A/X-31 influenza. Mice had either received two infusions of dextrose (- AmBisome) or 3mg/kg AmBisome (+ AmBisome) on the day of infection and two days post-infection. Original magnification 10×.

5.3 Discussion

This study has showed that the loss of Ifitm3 functionality does not greatly impact on successful vaccination in the context of the parameters measured, both in terms of the safety of intra-nasal live attenuated formulation and the ability to mount a successful immune response to overcome a

subsequent lethal challenge with influenza virus. Furthermore, this study has also revealed how AmBisome, a routinely administered drug in hospitals, may bypass the restrictive effects of Ifitm3 to essentially render the patient susceptible to a potentially lethal viral infection.

5.3.1 Discussion: effect of vaccination in *Ifitm3*$^{-/-}$ mice

LAIVs were chosen for this study owing to the ability of the vaccine formulation to replicate in the upper respiratory tract. Previous *in vitro* studies into the loss of IFITM3 have shown that influenza virus replicates to higher titres in cells not expressing the protein (Brass *et al.* 2009; Feeley *et al.* 2011). Similarly, *in vivo* studies have shown similar results, with mice lacking Ifitm3 showing a sustained heightened influenza virus infection compared with wild type controls (Bailey *et al.* 2012; Everitt *et al.* 2012). Interestingly, results from patients possessing the rs12252-CC SNP, which may truncate IFITM3 in humans (Everitt *et al.* 2012), indicate that they also show heightened viral replication kinetics during influenza virus infection (Zhang *et al.* 2013b).

This study shows for the first time that not only is this form of vaccine potentially safe for use in this subset of patients, but that it is also effective in protecting against infection in the *Ifitm3*$^{-/-}$ mouse model. The study demonstrated that animals lacking this critical viral restriction factor are largely unaffected by the administration of the attenuated virus and are capable of mounting a typical adaptive immune response that clears a high dose of A/(H1N1)pdm09 influenza virus.

In Chapter 4, I noted that some of the key contributing factors to the morbidity and mortality of *Ifitm3*$^{-/-}$ mice when challenged with influenza A virus were the heightened viral burden, pathological damage and immune dysregulation. All of these factors were countered by the administration of LAIV prior to virus challenge. One of the most striking alterations associated with vaccination was the amelioration of the gross pathological damage on the surface and within the lungs. This is most likely due to the adaptive immune recall response being fast enough to allow vaccinated mice to efficiently reduce the viral load (Figure 5.3).

The lower viral load resulted in a significant reduction in neutrophil and macrophage infiltration, which were both observed to be higher with A/X-31 infection, previously. While neutrophils and

macrophages can aid in the clearance of influenza virus, they can also contribute to acute immunopathology and airway damage when in sufficient quantities (Narasaraju *et al.* 2011; Damjanovic *et al.* 2012). Indeed, it appears as though both macrophages and neutrophils may be one of the primary causes of the excessive lung damage seen in mice infected with H5N1 and 1918 'Spanish' H1N1 influenza (Maines *et al.* 2008; Perrone *et al.* 2008). Therefore, such a marked decrease in their numbers in the lungs of vaccinated mice may be having a beneficial impact on the gross pathology (Figures 5.6 and 5.7).

Further to this, the inflammatory cytokine dysregulation observed in unvaccinated *Ifitm3*$^{-/-}$ mice was altered by vaccination, with levels of MCP-1, G-CSF, IL-6 and TNFα, all being significantly lower (Figure 5.9). Overall, the same trend was observed here as with sub-lethal doses of A/X-31 influenza (Figure 4.19a), with unvaccinated *Ifitm3*$^{-/-}$ mice displaying an exaggerated cytokine cascade compared to unvaccinated wild type controls. Strikingly, vaccination lowered the levels of these cytokines in *Ifitm3*$^{-/-}$ mice beyond those of unvaccinated wild type mice to a level that was similar to vaccinated wild type mice. The reduction in these cytokines has been shown previously upon LAIV immunisation and challenge (Lanthier *et al.* 2011), and is important in the context of the *Ifitm3*$^{-/-}$ mice as such molecules recruit and maintain populations of monocytes, macrophages and neutrophils in the lung, as well as trigger the acute phase inflammatory response in infected tissues (Damjanovic *et al.* 2011; Hermesh *et al.* 2012); all of which are seemingly exacerbated in unvaccinated *Ifitm3*$^{-/-}$ mice. Although these classes of molecules have been demonstrated to be crucial in promoting recovery from influenza virus infection (Dessing *et al.* 2007; Dienz *et al.* 2012; Hermesh *et al.* 2012), the reduction in quantity may be a significant contributor to the survival of *Ifitm3*$^{-/-}$ mice.

This study provides the first robust evidence that Ifitm3-deficient animals possess an adequately functioning adaptive immune response, with the loss of Ifitm3 not impacting upon cellular or humoral immunity. Results showed that *Ifitm3*$^{-/-}$ mice produce a strong antibody response following vaccination in their lungs and systemically. Interestingly, the gross, non-antigen specific antibody quantities were higher in all immunoglobulin subclasses, and in some cases significantly so; specifically IgA, IgG2a and IgG2b in the lungs (Figure 5.4). Such elevated titres

may therefore account for the reduction in peak viral titres at day three post-infection in the vaccinated *Ifitm3*$^{-/-}$ mice, relative to vaccinated wild type controls (Figure 5.3).

Similarly, vaccination induced a robust cellular response to infection in vaccinated *Ifitm3*$^{-/-}$ mice; with a highly significant ($p < 0.001$) increase in the presence of CD8+ T-cells in the lungs over the course of infection, relative to unvaccinated mice (Figure 5.8). IFITM3 expression is crucial for the survival of resident memory CD8+ T-cells in the lung tissue during influenza infection (Wakim *et al.* 2013). It is interesting to note that on day five post-infection there is a small reduction in the number of CD8+ T-cells in vaccinated *Ifitm3*$^{-/-}$ mice relative to vaccinated wild type controls. Although this reduction was non-significant, it is plausible that this loss of CD8+ T-cells was due to influenza infection; reducing the number of live cells.

It is noteworthy that *Ifitm3*$^{-/-}$ mice lost a small, but significant (~5%) amount of body weight during challenge when immunised with either FluMist or delNS1 influenza virus (Figure 5.2). Observation of the same trend in independent trials would suggest that this response is not an anomaly. Although it is ultimately important to note that all vaccinated mice survived the challenge, it is interesting to speculate why this weight loss was observed. In addition to the previously described reduction in CD8+ T-cells on day five post-infection, neutralising titres of antibodies in *Ifitm3*$^{-/-}$ mice were lower than wild type littermates, which may both have contributed to weight loss. Furthermore, variations in G-CSF, which was significantly higher in vaccinated *Ifitm3*$^{-/-}$ mice than vaccinated wild type mice (Figure 5.9), and other cytokines that were not assayed here, may have resulted in the minor, transient weight loss. However, as mentioned at the outset, such results do not alter the conclusion that vaccination is safe and efficacious in *Ifitm3*$^{-/-}$ mice and by extension should protect humans carrying the rs12252-CC allele.

Increasingly, the field is showing that underlying genetic determinants may confer resistance or susceptibility to infection by a pathogen through the use of large scale knockdown techniques (Brass *et al.* 2008; Krishnan *et al.* 2008; Brass *et al.* 2009) or by conducting genome-wide association studies (Bellamy *et al.* 2000; Timmann *et al.* 2012). Not all genetic polymorphisms confer the carrier with an obvious, identifiable phenotype unless certain environmental

conditions are met, such as infection with a particular virus. Such mutations represent a novel challenge for medicine and for the pharmaceutical industry to address. Although the current study suggests the rs12252-C polymorphism in IFITM3 should not present complications for LAIV use, it demonstrates the need for broader-based clinical trials to incorporate genetic polymorphisms that may be present within and between populations and account for therapy-associated adverse events.

5.3.2 Discussion: effect of amphotericin B on IFITM3 function

The collaborative work on the actions of amphotericin B/AmBisome and its interactions with IFITM3 are important considering the widespread use of the drug in the clinic to prevent fungal infections in patients. The study has shown that AmphoB is capable of reversing the antiviral actions of IFITM3 and it promotes influenza virus infection both *in vitro* and *in vivo*.

As discussed, AmBisome exerts its antifungal actions through incorporation into the fungal cell membrane, thereby forming pores through which protons and cations can travel, which may in turn alter the fluidity of the membrane (Younsi *et al.* 2000). It is therefore plausible that the incorporation of these pores into the host cell membrane may disrupt the actions of IFITM3 either through physically preventing IFITM3-IFITM3 interaction and aggregation, which has been shown to be necessary for antiviral action (Yount *et al.* 2010), or through altering the biomechanical properties of the membrane (Amini-Bavil-Olyaee *et al.* 2013; John *et al.* 2013) by increasing fluidity thus permitting fusion of viral and cellular membranes.

Previously, in Chapter 3, I discussed the shortcomings of *in vitro* work with respect to using RNAi knockdowns to reveal antiviral functions, as *in vitro* models are not always representative of what may happen *in vivo*. Similarly, the same shortcomings could have been true with the present study. However, the *in vivo* work strongly supports what was observed *in vitro* with wild type mice showing profound morbidity and mortality upon repeat exposure to the otherwise harmless AmBisome. This may mimic the situation in humans, who would be continuously administered with the drug when treated. In particular, it is important to note that wild type mice exposed to two doses of AmBisome recovered from the infection with no mortality. This therefore suggests that cessation of treatment could prevent the terminal decline that was

observed with the three dose regimen. The finding that the rs12252-C SNP in *IFITM3* is medically relevant suggests that IFITM3 is a functioning antiviral protein in human health (Everitt *et al.* 2012; Zhang *et al.* 2013b). This would also suggest that the effects of AmBisome *in vitro* and in murine *in vivo* models would be true for humans; thus making the current study particularly noteworthy and indicating that AmBisome should be contraindicated to influenza.

The histological analysis of the lungs on day four post-infection further supports the idea that AmBisome inhibits the actions of Ifitm3, as the lungs of untreated wild type mice are strikingly different from all other lung sections. They show minimal cellular infiltrate and inflammation, as is typical with A/X-31 influenza infections (Figure 4.11); particularly at such an early time point post-infection. However, AmBisome-treated wild type lungs showed severe inflammation and cellular infiltrate; mimicking the observations seen in *Ifitm3*$^{-/-}$ mice (Figure 5.17). Similarly, the viral load in the lungs on day three post-infection in treated wild type mice was as high as that seen in *Ifitm3*$^{-/-}$ mice and was dissimilar to untreated wild type littermates, which had begun to clear the infection.

It is interesting to note that *Ifitm3*$^{-/-}$ mice were also adversely affected by AmBisome; losing weight at a more rapid rate than untreated *Ifitm3*$^{-/-}$ mice (Figure 5.16). This effect could be due to AmBisome interfering with the actions of either Ifitm1 or Ifitm2 during the course of infection. As discussed previously, the Ifitm family show a degree of redundancy in their actions; despite the fact that some members are more capable of restricting certain viruses better than others. Ifitm1 and Ifitm2 are both capable of restricting influenza virus (Brass *et al.* 2009); therefore the more rapid weight loss could be attributed to the additive effect of inhibition of their function.

This study has shown how IFITM3 functionality may impact on human health, but in a different manner to that that has been previously discussed here. The administration of the commonly-used drug amphotericin B may be exposing patients to a greater risk of viral infection through its apparent bypassing of IFITM3. Although the current study has shown the impact of AmphoB on influenza virus infection, it is important to consider the broad range of viruses that can be restricted by this family of proteins. Therefore, the fact that AmphoB is routinely given to immunocompromised hosts that may be harbouring chronic HIV-1 or HCV infections presents a

particular risk. Studies have indicated that IFITM proteins may restrict these viruses (Lu *et al.* 2011; Yao *et al.* 2011); therefore AmphoB treatment may not only increase the chances of developing a new infection, but be accelerating the rate of viral replication of an existing infection through antagonism of a crucial antiviral protein.