Investigating the Role of Interferon-Inducible Transmembrane 3 (IFITM3) in Infection.

By

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Abstract

My thesis focuses on the interaction between interferon-inducible transmembrane 3 (IFITM3) and influenza viruses. IFITM3 confers cells *in vitro* with resistance to multiple pathogenic viruses, including influenza, dengue and West Nile virus amongst others (Brass *et al.* 2009; Huang *et al.* 2011). Although the current mechanism of restriction is unknown, it is thought that aggregation of IFITM3 within the late endosomes prevents the membrane fusion necessary for the release of viral nucleic acids and proteins into the cells' cytoplasm (Feeley *et al.* 2011; John *et al.* 2013).

My thesis aims to further understanding of IFITM3 through the use of a knockout mouse model with an ablation of the *Ifitm3* allele (*Ifitm3*$^{-/-}$). Challenge of the mouse with sub-lethal doses of influenza A virus showed that the loss of Ifitm3 resulted in heightened susceptibility to the virus, which resulted in accelerated weight loss, fulminant viral pneumonia, a persistent viral burden and ultimately death. These phenotypic effects are more commonly associated with infections using highly pathogenic 1918 'Spanish' influenza and avian H5N1 influenza viruses.

These findings were taken further by analysing the prevalence of single nucleotide polymorphisms (SNPs) in the *IFITM3* locus of humans hospitalised during the 2009 H1N1 pandemic. Through international collaboration, SNP rs12252-C, which is thought to be associated with sub-optimal IFITM functioning, was identified as being over-represented in these patients. Typically, 0.3% of the European Caucasian population are homozygous for the rs12252-C allele; however, the study showed that in patients hospitalised with influenza virus this proportion increased to 5.7%: a significant enrichment.

Furthering this observation, the thesis also investigates the effects and interactions of IFITM3 on medically-relevant treatments. Primarily, studies were employed to test the safety and efficacy of live attenuated influenza virus vaccines in *Ifitm3*$^{-/-}$ mice to assess the potential for vaccine-associated morbidity in individuals possessing sub-optimally functioning IFITM3, and if protection is elicited against subsequent infection. This showed the vaccine was safe in these mice, and induced a normal, robust immune response that protected mice from a lethal challenge with pandemic H1N1 influenza virus. Furthermore, the mouse model was employed to assess the

effects of AmBisome, a commonly used antifungal agent, on Ifitm3 function, as it had been shown to cause a bypass of IFITM3-based restriction *in vitro*. The wild type mice treated with AmBisome prior to, and during, influenza virus infection show weight loss and morbidity similar to *Ifitm3$^{-/-}$* mice; suggesting that AmBisome may heighten viral susceptibility in patients treated with this drug.

The thesis concludes with a meta-analysis investigating the *in vivo* effects of Ifitm3 in restricting a range of bacterial, viral and protozoan pathogens. This demonstrates the specificity of Ifitm3 for restricting only specific viral pathogens, despite the fact that a variety of pathogens utilise the endosomal pathway for entry into cells.

In conclusion, the thesis furthers our knowledge of IFITM3 by showing for the first time its *in vivo* effects on viral restriction and the criticality of IFITM3 in preventing the morbidity and mortality associated with influenza viruses.

Acknowledgements

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

Dr. S. Clare from the Wellcome Trust Sanger Institute assisted the author in all live animal work and conducted all inoculations, immunisations and animal procedures during the course of experiments. Dr. A.L. Brass gave guidance and practical help with *in vitro* MEF transductions and infections, immunofluorescence imaging of *in vitro* influenza infections, and in RNA immunohistochemistry of tissue sections whilst I was part of his lab at the Ragon Institute, USA. Dr. D. Goulding performed the GMA-embedded protein immunohistochemistry.

All other protocols relating to influenza challenge, including *in vitro* and *ex vivo* work, were conducted by the author.

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Abbreviations

aDC	Alveolar dendritic cell
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
CFU	Colony forming unit
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbant assay
g	Force of gravity
GenISIS	Genetics of *Influenza* Susceptibility in Scotland
GMA	Glycol methacrylate
HA	Hemagglutinin
HCV	Hepatitis C virus
HP	High pathogenicity
HTS	High throughput screening
IFN	Interferon
Ig	Immunoglobulin
i.v.	Intravenous
IL	Interleukin
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
ISG	Interferon-stimulated gene
i.v.	Intravenous
kDa	Kilo Dalton
LAIV	Live attenuated influenza vaccine
LCL	Lymphoblastoid cell line
LD	Lethal dose
LP	Low pathogenicity
MEF	Murine embryonic fibroblast

MHC	Major histocompatability complex
MOSAIC	Mechanisms of Severe Acute Influenza Consortium
NA	Neuraminidase
NK	Natural killer
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PRR	Pathogen recognition receptor
QIV	Quadrivalent influenza vaccine
QTL	Quantitative trait loci
RNAi	RNA interference
RSV	Respiratory syncytial virus
SA	Sialic acid
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TCID	Tissue culture infective dose
TIV	Trivalent influenza vaccine
TLR	Toll-like receptor
pDC	Plasmacytoid dendritic cell
VRDF	Viral replication dependence factor
VRF	Viral restriction factor
WHO	World Health Organisation
WTSI	Wellcome Trust Sanger Institute

1 Introduction

1.1 Influenza virus

"When we think of the major threats to our national security, the first to come to mind are nuclear proliferation, rogue states and global terrorism. But another kind of threat lurks beyond our shores, one from nature, not humans – a flu pandemic."

- Barack Obama (2005)

1.1.1 General features

Influenza virus is a respiratory pathogen that belongs to the *Orthomyxoviridae* family of viruses. This family of single-stranded RNA viruses comprises three distinct, but related types: A, B and C. The most common form of influenza virus is influenza A, which is capable of infecting a range of animal species, including humans, birds, pigs and horses. The second most common is influenza B, which is restricted to infections in humans and seals, whilst the rarest type, influenza C solely affects humans and pigs.

The influenza A genome consists of eight RNA segments that are encapsulated by the virus' nucleoproteins to produce vRNPs (Figure 1.1). The total genome size of influenza A is 13,000 nucleotides (nt), with the segments demarked 1-8 based on their relative size, with each encoding for a minimum of one viral protein (Figure 1.1 & Table 1.1). Influenza B similarly consists of eight RNA segments, but differs in the number and form of proteins that the RNA encodes, whilst influenza C viruses encode only seven segments (Palese and Shaw 2007).

One of the key determinants of the virus' ability to infect cells resides in the composition of the glycoproteins on its surface; the two most abundant of which are hemagglutinin (HA) and neuraminidase (NA). These glycoproteins can be further subdivided based on their antigenic subtype. Currently, 17 forms of HA exist in the wild giving rise to H1-H17 alongside nine forms of NA; thus providing N1-N9 (Tong *et al.* 2012). Each HA and NA subtype exhibits differing host specificity, with some solely infecting a single species, whilst others are capable of infecting multiple hosts (Figure 1.2).
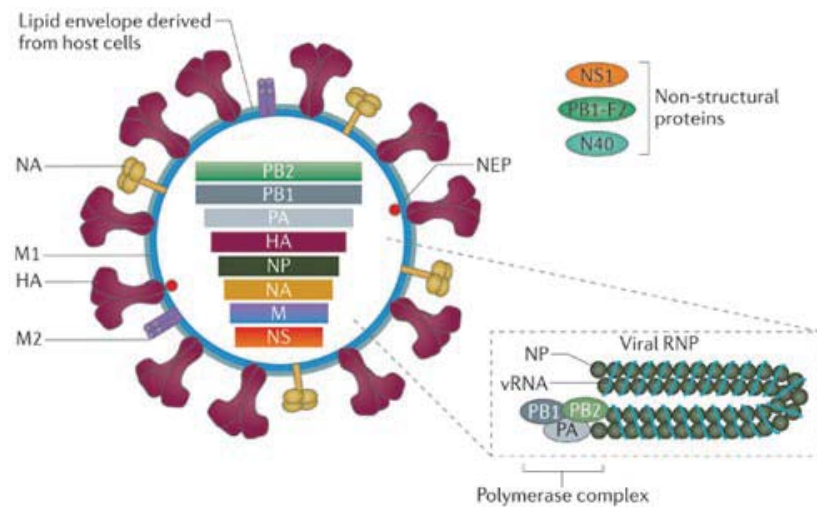
Figure 1.1: Schematic of an influenza A virus. The virion encapsulates a total of eight vRNP's that encode at least 11 proteins. These are: PB1, PB2 and PA form the components of the RNA polymerase complex (PB1 also encodes N40 and PB1-F2, whilst PA also encodes several variants of itself (Chen *et al.* 2001b; Wise *et al.* 2009; Jagger *et al.* 2012; Muramoto *et al.* 2013)); HA, the attachment protein hemagglutinin; NA, neuraminidase, the enzyme used to cleave the virus from the cell; NP, nucleoprotein, M, the segment which encodes both M1 (matrix protein) and M2 (ion channel); and NS, which encodes the interferon-antagonist NS1 (non-structural protein 1) and nuclear export protein (NEP), which is translated into NS1 and NEP (nuclear export protein). (Medina and Garcia-Sastre 2011)

Table 1.1: Influenza A gene products and their functions.

Segment number	vRNA segment length (nt)	Gene product	Polypeptide length (aa)	Function
1	2341	PB2	759	Polymerase component, RNA cap recognition
2	2341	PB1	757	Polymerase component, endonuclease activity
		PB1-F2	87	Pro-apoptotic protein
		PB1-N40	717	Unknown function
3	2233	PA*	716	Polymerase component, protease
4	1778	HA	566	Surface binding glycoprotein, major antigen
5	1565	NP	498	RNA binding, synthesis and nuclear import
6	1413	NA	454	Cleavage of virus from the cell surface
7	1027	M1	252	Viral matrix protein
		M2	97	Ion channel activity
8	890	NS1	230	Interferon antagonist
		NS2	121	RNP nuclear export

*: Various splice forms of PA are encoded in segment 3.

Adapted from (Palese and Shaw 2007)
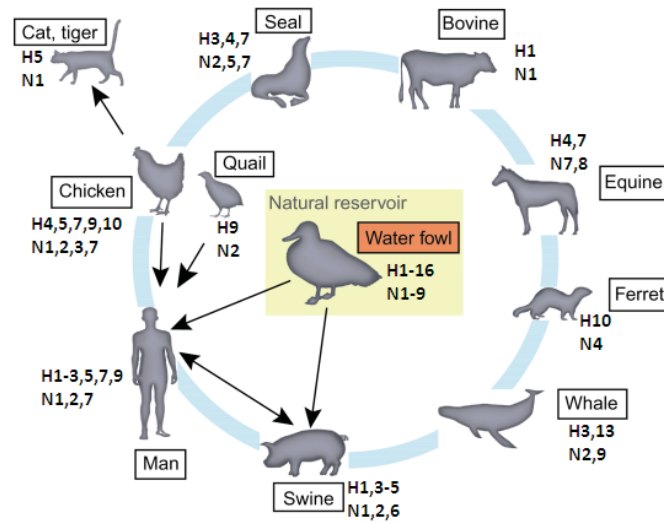
Figure 1.2: Host range of influenza viruses. The different influenza HA and NA subtypes are capable of infecting a range of animal species. Transmission events are shown by the arrows. The newly discovered H17 hemagglutinin is not shown on the diagram, but is currently thought to primarily target bats. Redrawn and updated from (Suzuki 2005).

1.1.2 The influenza replication cycle

Influenza primarily targets and replicates in host epithelial surfaces, although it is capable of entering a broad variety of cell types, including immune cells (Chen *et al.* 2001b). As shown in Figure 1.3, the virus enters the cell through the binding of the HA protein to sialic acids on the cell surface, before being endocytosed. The endosomal vesicle is subsequently acidified, which consequently triggers the M2 ion channel to acidify the interior of the virus; thus enabling unpackaging and infection. Through further conformational changes triggered by the acidity, the HA protein fuses with the surface of the endosomal membrane to release the viral ribonucleoprotein (vRNP) complexes into the cytoplasm, which subsequently allows the vRNA to enter the nucleus to replicate and be translated, producing numerous progeny viruses. The viral components are trafficked to the surface of the cell, where they are packaged and released by NA, which cleaves the attachment between the sialic acid and HA proteins.
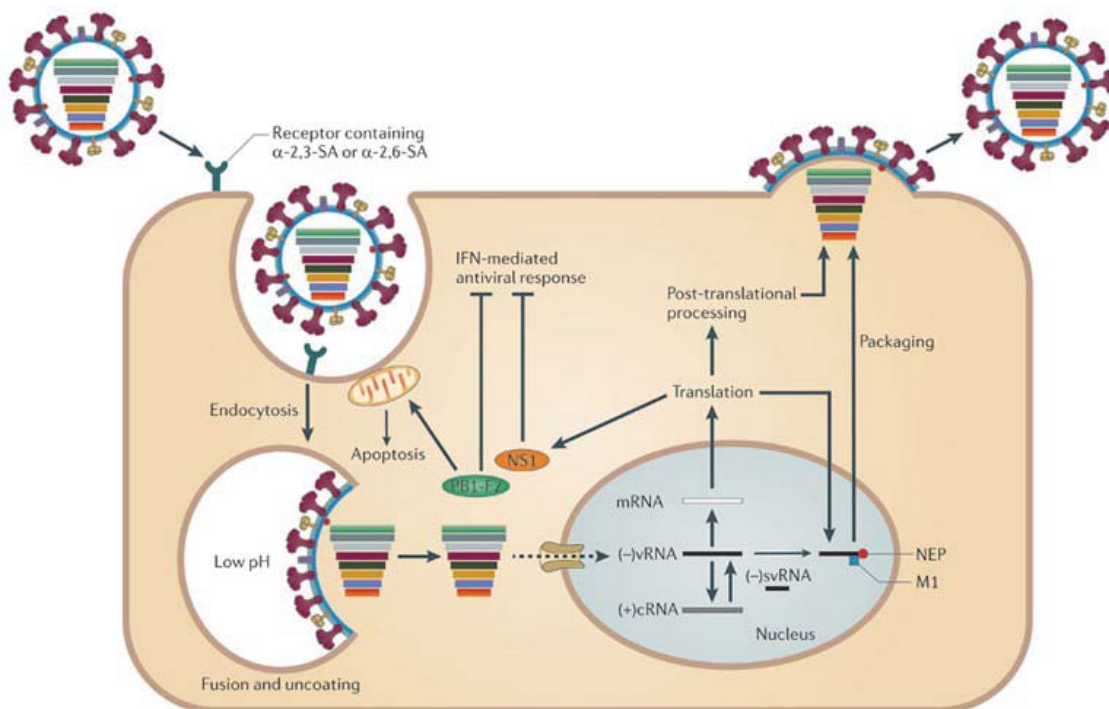
Figure 1.3: Schematic to illustrate the influenza replication cycle. The key components of the replication cycle are discussed in the text. However, it is pertinent to address the roles of NS1 and PB1-F2, which are responsible for antagonising the host immune response. NS1 is primarily an interferon-antagonist, which is important as interferon is the first signal released by the cell in order to commence the innate immune response. PB1-F2 can also act as an interferon-antagonist, but also triggers apoptosis. It is thought that this ability is primarily used when influenza infects immune cells, in order to hinder the host's cellular antiviral response. (Medina and Garcia-Sastre 2011)

Ultimately, the viral replication cycle can cause cellular damage; leading to the rupture of the cellular membrane. Such cellular bursts can release large numbers of live virus into the surrounding space, leading to a highly concentrated infection in a localised area. The pattern is then repeated with the surrounding cells being exposed to viruses at a high multiplicity of infection (MOI), wherein multiple viruses infect the cell simultaneously.

Although the schematic in Figure 1.3 illustrates a single viral particle infecting a cell, it is not untypical for multiple viruses to infect the cell simultaneously. This is given a further layer of complexity when several antigenically distinct influenza subtypes infect the cell simultaneously. In dual or multiple virus infected cells, genome packaging does not discriminate between the distinct infecting genome segments, resulting in the packaging of eight vRNA segments,

regardless of origin. This phenomenon is known as "reassortment" and can lead to entirely novel viruses emerging from the cell, much in the same way that sexual reproduction produces progeny that are recombinants of the two parents. Such reassortant viruses are an important evolutionary process for influenza virus leading to new variant combinations previously unseen by the host immune system.

1.1.3   Influenza mutation & variation

Influenza replication by the viral RNA polymerase complex is a relatively inefficient process, which in turn leads in the introduction of spontaneous mutations in the genome. Influenza A viruses typically mutate at a mean rate of 2.3×10^{-5} sequence changes per nucleotide per cell infection ($\mu_{s/n/c}$), whilst influenza B viruses change at the slower rate of $1.7 \times 10^{-6} \mu_{s/n/c}$ (Sanjuan *et al.* 2010). Such mutation rates can be further subdivided owing to the observation that HA subtypes also differ amongst one another, with H3 evolving more rapidly than H1 viruses (Ferguson *et al.* 2003). These mutations create an enormous diversity of influenza antigenic variation, even within HA and NA subtypes; thus making influenza virus variants able to evade the host's immune system over time, leading to new seasonal epidemics. In contrast, rhinoviruses that can cause the common cold mutate at a rate of c. $6.9 \times 10^{-5} \mu_{s/n/c}$, whilst the rapidly-changing hepatitis C virus (HCV) mutates at a rate of c. $1.2 \times 10^{-4} \mu_{s/n/c}$ (Sanjuan *et al.* 2010).

Monitoring of the mutations within the influenza genome can provide valuable insights into the phylodynamics and geographical spread of the viruses over the course of their seasonal epidemics. Analysis of archived viral strains can also reveal important data, such as detailing how the influenza genome has evolved between epidemics to evade the host immune responses (Smith *et al.* 2004; Ghedin *et al.* 2005; Koelle *et al.* 2006). This becomes even more valuable as a tool for predicting the viruses' future antigenicity and spread. Such monitoring and prediction is used extensively by the World Health Organisation (WHO) and vaccine industry to predict the new viral strains to be included in the upcoming vaccine season. Furthermore, it can also prove useful in addressing concerns about antiviral resistance spreading within the global influenza virus population (Bloom *et al.* 2010).

Indeed, the monitoring of mutations should not be limited to samples of human origin; owing to the diversity of hosts that influenza A virus can infect. The monitoring of pigs and birds for novel mutations and subsequent calculations regarding future spread of these viruses can be equally, if not more important, than monitoring the spread in humans due to the risk of zoonotic events and future epidemic events (Campitelli *et al.* 2006; Li *et al.* 2010; Vijaykrishna *et al.* 2011).

1.1.3.1 Antigenic drift

Of the eight influenza genes, HA, NA, M2 and PB1 exhibit the highest mutation rate, whilst NP and M1 are the most conserved (Ducatez *et al.* 2007). Such a high level of mutation in the HA and NA genes leads to the phenomenon of "antigenic drift" wherein the HA and NA gradually accumulate mutations to a degree that the host immune system can no longer recognise the pathogen; therefore rendering previous neutralising cross-reactive antibodies ineffective.

Although there are correlations between the underlying mutation rate within the viral genome and the "shifting" of antigenicity, it should be noted that the latter displays a greater degree of punctuated evolution than would be expected from genetic analyses alone (Smith *et al.* 2004). Research has shown that certain mutations may result in a dramatic antigenic shift, whilst others are largely ineffectual in altering the overall antigenicity. Figure 1.4 shows how the evolutionary theories of gradualism and punctuated evolution differ from one another, with the former favouring a steady change over time, whilst the latter favours stasis followed by rapid evolutionary change. Although influenza overall evolves at a gradual rate, the more rapid, punctuated changes in antigenicity are also visible.

Antigenic drift is one of the primary reasons that novel influenza vaccines are manufactured and released on an annual basis. However, other factors are also key considerations in these decisions, such as the emergence of newly circulating strains.
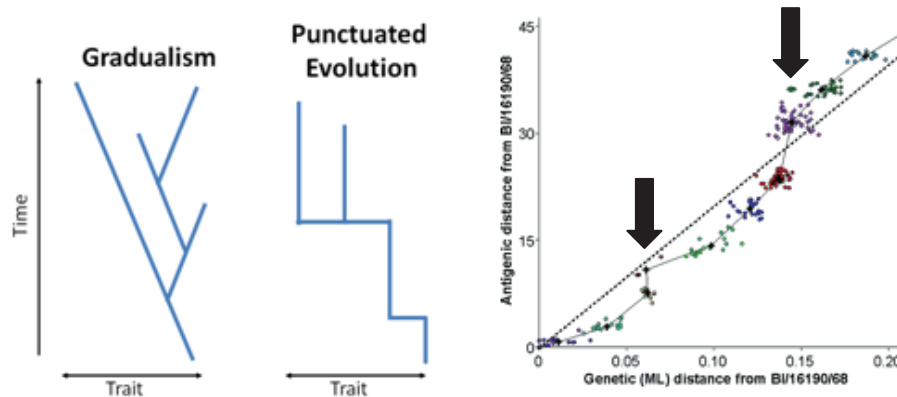
Figure 1.4: Models of evolutionary change and the evolution of H3N2 influenza. The schematics show how the theories of gradualism and punctuate evolution operate: gradual vs. rapid phenotypic changes. The right hand panel shows the correlation between genetic and antigenic evolution of H3N2, where the solid line connects the clusters of samples and the dashed line denotes a linear fit with a forced zero-intercept. Note how the change is overall linear, but with sporadic rapid antigenic evolution, indicated by arrows. H3N2 influenza data from Smith *et al*. (2004).

1.1.3.2 Antigenic shift

A less common, but more dramatic form of antigenic change is known as "antigenic shift". This occurs when a divergent or novel HA or NA antigen enters the current circulating viral population; thus hosts are exposed to a pathogen to which they have had no prior exposure, and therefore have little or no cross protective adaptive immunity.

Antigenic shifts are primarily driven by the introduction of previously non-human-adapted HA or NA into the circulating viral population. Typically, such "novel" surface gylcoproteins arise from either avian or porcine lineages. Whilst instances of illness-inducing bird-to-human transmission events are rare, they are currently the primary driver of H5 and H9 infections and represent a threat to humans (Lin *et al.* 2000; Abdel-Ghafar *et al.* 2008). However, perhaps a bigger zoonotic threat could come from pigs, which essentially act as "mixing vessels" for both mammalian and avian strains of influenza (Ito *et al.* 1998; Salomon and Webster 2009). This ability is a result of pigs possessing two forms of sialic acids (the binding partner of HA) in their tracheas (Figure 1.5). Avian-adapted influenza typically binds to sialic acid α2,3-galactose (α-2,3-SA) linked receptors, whereas mammalian strains bind to sialic acid α2,6-galactose (α-2,6-SA) linked receptors (Medina and Garcia-Sastre 2011). The co-located sialic acids of the pig therefore mean that they can become co-infected with multiple strains of virus from entirely

different lineages. Should these viruses reassort within the pig, then an antigenically shifted virus is produced, which may subsequently accumulate more mutations and thus become human-adapted, but contain major non-human lineage antigens.

The overall effect of such antigenic shifts is that the host has little protective immunity. The ongoing bird-to-human transmission of avian H5N1 influenza has resulted in a profile of lethality, which would be expected from a novel virus in humans. However, H5N1 has also been shown to cause asymptomatic infections in some patients (Palese and Wang 2012). This would suggest that other non-viral factors are having an effect on the susceptibility of the host to a lethal infection. In the absence of adaptive immune control an individual's genetic predisposition to viral infection may also be a key factor. Regardless, it is primarily the phenomenon of antigenic shift that creates global pandemics that cause large scale morbidity and mortality.
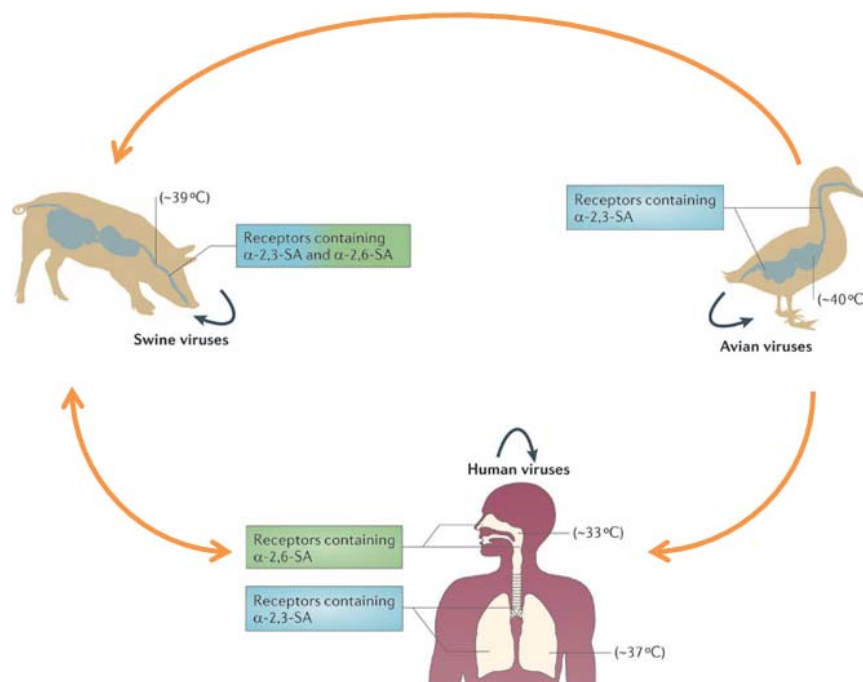
Figure 1.5: The anatomical distribution of α2,3-galactose (α-2,3-SA) and α2,6-galactose (α-2,6-SA) linked sialic acid receptors in three key species infected by influenza and the direction of inter-species viral transmission. Temperatures of the epithelial surfaces within the various species are indicated in the diagram. Particularly of note is the fact that humans possess a temperature gradient across their respiratory surfaces; a factor that is important for the use of live attenuated influenza vaccines, which is replication incompetent at temperatures above 33°C (Medina and Garcia-Sastre 2011)

1.1.3.3 Virus adaptation mutations

Influenza viruses accumulate mutations as they adapt to new hosts, particularly those that affect their transmissibility and pathogenicity (Taubenberger and Kash 2010). In particular, much attention has been paid to mutations arising within the HA and vRNP-encoding genes, as evidence has amassed supporting the role of specific mutations in adaptation. As discussed previously, HA is the influenza surface protein responsible for binding to sialic acids on the surface of cells that permits viral entry. Therefore individual mutations in *HA* that can switch binding preferences from avian α-2,3-SA to α-2,6-SA should more successfully attach in the human upper respiratory tract. Indeed, mutations at residue 225 of the HA of 1918 and 2009 H1N1 pandemic viruses can result in dual affinity for both α-2,3-SA and α-2,6-SA binding (Zhang *et al.* 2013a), although the discovery of α-2,6-SA-adapted avian viruses that show poor human infectivity complicates the role of HA-sialic acid binding as a driver of human adaptation (Taubenberger and Kash 2010). However, HA mutations that increase pH stability within the endosomes have also been implicated in improving virus fitness in humans (Shelton *et al.* 2013).

Similarly, mutations in the *PB2* gene, in particular at site 627, have been implicated in improving the virulence and replication efficiency of the virus (Hatta *et al.* 2001; Shinya *et al.* 2004). However, other studies have downplayed the importance of mutations at this site, as the introduction of the supposedly higher virulence E627K mutation into 2009 H1N1 pandemic viruses failed to increase infectivity in cells and mice (Jagger *et al.* 2010).

Recently, efforts have been made to explore the basis for avian H5N1 mammal-to-mammal transmission using ferret models as surrogates for humans (Herfst *et al.* 2012; Imai *et al.* 2012). Serial passage between ferrets resulted in the ability of the virus to gain airborne transmissibility with as few as five mutations (four in HA, one in PB2). Such studies demonstrate that few mutations need to be introduced for viruses to become adapted to their host and potentially gain the ability to transmit and cause pathogenicity. Therefore, spontaneous mutations and reassortment between viruses introduces a wealth of variation into the influenza genome, which can result in pandemic viruses.

1.2   Twentieth century influenza pandemics

"Spanish influenza killed more people in a year than the Black Death of the Middle Ages killed in a century. It killed more people in 24 weeks than AIDS has killed in 24 years."

- John Barry (2005)

To be successful, a pathogen must be able to survive, replicate, and spread from host to host. Owing to its propensity for accruing genetic mutations and its ability to reassort with phenotypically distinct subtypes, influenza remains a globally relevant pathogen. It causes seasonal epidemics in countries in the temperate regions and establishes itself throughout the year in more tropical climes (Viboud *et al.* 2006). Although we regularly generate vaccines against the circulating viruses, antigenic drift results in the need to update the vaccine on an annual basis. However, antigenic shift results in influenza viruses with pandemic potential.

WHO recognises six phases of pandemic alert to denote the severity of a new influenza outbreak (Table 1.2). Briefly, the more the virus transmits between humans across global territories, the higher the alert status. Although pandemic outbreaks of influenza are infrequent, they do occur once every 10-50 years (Potter 2001). This is primarily driven by the generation of novel zoonotic viruses.

Although influenza is thought to have existed for thousands of years, based on historical accounts of disease symptoms, the virus was only isolated in 1933 (Smith *et al.* 1933). Incidences of outbreaks or pandemics prior to the start of the twentieth century can be approximated from written accounts, but cannot be verified (Potter 2001). However, several pandemics have now been experienced in the "modern" era; thus informing us of how the virus spreads, the impact it has, and the ways in which we can prepare for future events.

Table 1.2: The six phases of pandemic alert.

Phase	Description
1	No viruses circulating amongst animal populations are reported to cross species barriers to infect humans.
2	An animal-borne influenza virus circulating amongst wild or domesticated animals has caused infection in a human host.
3	An animal or human-animal reassortment has caused sporadic pockets of outbreaks in different geographical areas within the same nation. No human to human transmission has been recorded at this stage.
4	Verified transmission of a virus between humans, causing "community-level outbreaks" within a single country. A pandemic can still be prevented at this stage.
5	Recorded human to human transmission of the virus in at least two different countries in one WHO region. Six regions exist: Africa, Europe, Eastern Mediterranean, Americas, South-East Asia and Western Pacific. A pandemic is thought to be imminent at this point.
6	The pandemic phase. The criteria are as those established in phase 5, except that now transmission is recorded in more than one WHO region to form "community-level outbreaks" on a global scale.

(Source: WHO)

1.2.1 1918 'Spanish' influenza

The most devastating example of an influenza pandemic is perhaps that of 1918-20; the so-called 'Spanish' influenza (subtype H1N1). It is estimated that in total, 30% of the entire global population contracted the disease (Xu *et al.* 2008), resulting in between 30-50 million deaths (Mills *et al.* 2004; Murray *et al.* 2006). Such a figure is even more remarkable when considering the fact that international transportation was still in its infancy and was not something as freely available as it is in the present day. However, many questions remain as to why this pandemic was as serious as reported (Morens and Taubenberger 2012).

Although one may at first associate the death rate with the conditions of the time: poorer sanitation, less developed healthcare systems and no vaccination regime, recent studies have revealed great insights into the virus itself. Sequencing and regeneration of the recovered virus has shown that Spanish H1N1 exhibits remarkable pathogenesis in non-human and human tissues (Tumpey *et al.* 2005a). Indeed, viruses just containing the surface HA and NA proteins of the 1918 virus are sufficient to generate a lethal phenotype in mice (Kobasa *et al.* 2004; Pappas *et al.* 2008). However, the mortality witnessed during the pandemic may also be due to extensive

immunopathological damage (Kobasa *et al.* 2007; Perrone *et al.* 2008) or to the well-documented secondary bacterial pneumonias that occurred following the initial influenza infection (Brundage and Shanks 2008).

1.2.2 Influenza pandemics 1957-1977

After 1920, the 'Spanish' influenza virus seemingly disappeared as the case numbers fell. At this point the virus had retreated back into animal hosts, which appear to act as reservoirs for these viruses in inter-pandemic periods. However, its signature has remained throughout the majority of the 20th Century, either through the antibodies it generated in surviving individuals, or through the genetic material it transferred to subsequent pandemic viruses via genetic reassortment (Figure 1.6).
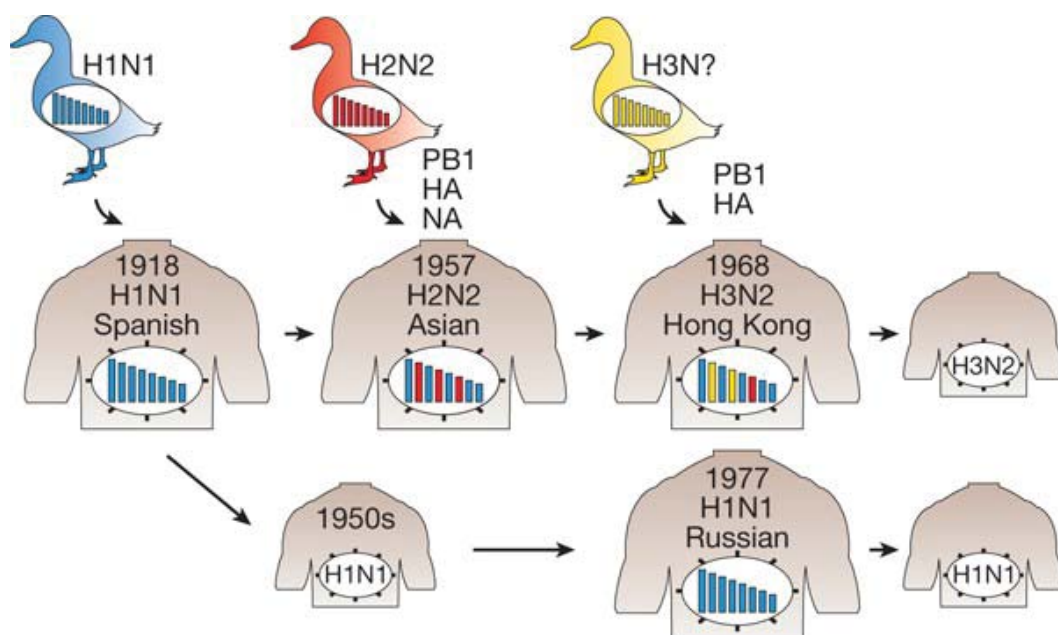
Figure 1.6: The role of the 1918 'Spanish' influenza virus in the pandemics of the 20th Century. The original 'Spanish' influenza has undergone several genetic reassortments with wild avian influenza viruses to yield the pandemics of 1957 and 1968. The original H1N1 virus was largely absent for the majority of the 20th Century, but re-emerged in the Soviet Union / Northern China in 1977 from unknown origins. Both the H3N2 and H1N1 viruses are still in circulation, as of 2013. However, the "Russian" strain of H1N1 has been supplanted by the swine-origin pandemic H1N1 since 2009. (Neumann *et al.* 2009)

1.2.2.1 1957: the 'Asian Influenza' pandemic

Genetic reassortment was the key driver behind the emergence of a novel H2N2 strain in Asia during 1957. As shown in Figure 1.4, the novel virus emerged as a result of reassortment between the 1918 'Spanish' strain of H1N1 influenza and an avian H2N2 virus. The resulting progeny contained HA, NA and PB1 genes from H2N2 and all other segments from the H1N1 virus (Kawaoka *et al.* 1989).

Although the death rate of the 1957 pandemic was far lower than its forbearer (Oxford 2000), this pandemic was important for two reasons: 1) it was the first pandemic of the "modern" era, where influenza was a known causative agent; and 2) it presented the first occasion to trial influenza vaccines. Knowledge gained since the first isolation of the influenza virus in 1933 allowed the detection of the virus and determination that it was antigenically distinct from the 1918 strain (Kilbourne 2006). Additionally, unlike the 1918 pandemic, it showed that influenza virus alone could be remarkably pathogenic and induce severe pneumonia in the lungs, without the requirement for a secondary bacterial infection (Kilbourne 2006); thus highlighting how capable this pathogen was of not only transmitting, but of causing severe morbidity and mortality without obvious co-infection. Although the vaccination efforts were largely seen as a failure (Killingray and Phillips 2003), owing to sub-optimal doses and not enough vaccine being manufactured, they marked the first widespread trial (Kilbourne 2006).

1.2.2.2 1968: the 'Hong Kong Influenza' pandemic

The circulating H2N2 strain of influenza was soon supplanted with the recombination of a novel H3 avian influenza antigen into the human virus; thus generating the H3N2 pandemic virus. Although this represented an introduction of a previously-unseen HA antigen, the virus retained the same NA that was present during the 1957 pandemic, thus providing those that were previously infected with a degree of cross-protection. Indeed, H2N2 vaccine was shown to significantly increase immunity to the novel virus (Kilbourne 2006).

However, issues remained regarding the vaccination regime. Whilst the 1957 pandemic had highlighted the shortcomings in administering an effective dose of the vaccine, the 1968

pandemic revealed the failings in getting vaccine to those most in need of it, with large corporations purchasing the majority of the stock to protect worker productivity (Davis 2006).

1.2.2.3 1976/77: The 'Fort Dix' virus and 'Russian' influenza pseudo-pandemics

1976 saw the emergence of an influenza strain that was thought to carry pandemic potential, which subsequently failed to materialise. The Fort Dix virus is particularly noteworthy as it represents the first instance of widespread distribution of vaccine against the emerging swine-borne H1N1 virus. However, this 'pseudo-pandemic' is more notorious for the vaccine's side effects, which is thought to have led to over 500 people developing Guillian-Barre syndrome in the USA (Schonberger *et al.* 1979).

A year later, H1N1 was reported as arising from the Soviet Union. Strikingly, the virus was related to the strain that had disappeared in the 1950's but seemingly lacked any evidence of antigenic drift, which would have been expected. The reason for the re-emergence of the virus is unknown, although some postulate it was accidentally released from a research institute in China (Nakajima *et al.* 1978; Palese 2004).

1.2.3   The threat of an avian influenza pandemic

"In April 1997 Hong Kong issued a set of postage stamps celebrating the migratory birds that flock each winter to the city's marshes. One of the birds depicted on a new stamp is a handsome, medium-sized duck called the falcated teal. Amongst the flu subtypes identified in a Hong Kong teal is H5N1. That might well make the falcated teal the duck of the apocalypse."

- (Davis 2006)

In 1997 reports arose of a novel, lethal strain of influenza in Hong Kong. Analysis of its genome revealed it to be an H5N1 isolate that was closely related to a strain that had been circulating in poultry (Claas *et al.* 1998). Subsequently, large scale slaughtering of poultry was enacted throughout the affected areas in order to destroy any infected birds that could potentially transmit the virus to humans. However, the H5N1 virus had re-emerged in Asia by 2004, with migrating wild duck populations acting as a reservoir for the virus (Li *et al.* 2004). Although the virus so far remains incapable of widespread human-to-human transmission, its virulence in animal

models is remarkably high (Dybing *et al.* 2000; Cameron *et al.* 2008; Perrone *et al.* 2008). Recently, reports have been published demonstrating that animal-to-animal transmission is possible in the ferret model after serial intra-nasal passage (Herfst *et al.* 2012; Imai *et al.* 2012). The results revealed that airborne transmission was possible when the wild type virus accumulated five amino acid alterations. Although this clearly shows that the virus is theoretically capable of widespread transmission, it has yet to happen, with only limited accounts of reported human-to-human transmission (Ungchusak *et al.* 2005). Similarly, there is no consensus on the case fatality rate of the virus, with estimates varying between 1% and 60%, due to under-reporting of non-serious events and over-reporting of the fatal cases (Palese and Wang 2012).

It now appears as though the H5N1 strain of avian influenza may not be the sole pandemic threat arising from birds. In 2013, fatalities were reported in China resulting from an infection with H7N9 – another antigenic combination that is previously unseen in humans (Parry 2013). The main difference with this virus is that it induces very low pathogenicity in its avian hosts, unlike H5N1 (Bertran *et al.* 2012). This will therefore hamper efforts to diagnose and cull flocks that are infected with the virus.

This recent addition of another novel antigen into the human population creates another layer of complexity, as there is now another viral subtype that could undergo genetic reassortment with a regular seasonal strain of influenza. Such a reassortant could potentially lead to a virulent, highly transmissible virus, as originally feared with the 2009 H1N1 pandemic.

1.3   The 2009 H1N1 pandemic

"Influenza viruses are the ultimate moving target. Their behaviour is notoriously unpredictable.
The behaviour of pandemics is as unpredictable as the viruses that cause them. No one can say
how the present situation will evolve."

- Margaret Chan, Director-General of WHO (2009)

Since the late 1990's, much attention had been paid to the emerging avian influenza threat from Asia (Section 1.2.3), as it was perceived that the H5 subtype of viruses would yield the next true influenza pandemic. However, on 24th April 2009, a growing number of infections were reported in Mexico to the WHO, from a novel influenza virus with the subtype H1N1.

1.3.1   Origins

Geographically, the pandemic form of H1N1 (A(H1N1)pdm09) originated in Mexico before spreading into the neighbouring USA. However, its genomic origins are much more complex and highlight the need for the monitoring of pigs as well as birds as a source of pandemic viruses. As shown in Figure 1.7, the pandemic virus was a product of multiple reassortments over time to generate a hybrid of four differing influenza genomes (Butler 2009; Neumann *et al.* 2009). Genomic analysis has shown that the virus that eventually caused the pandemic was a quadruple reassortent containing elements of human, avian and porcine influenza viruses, along with a so-called "avian-like swine" virus. As described earlier in section 1.1.3.2, the key to this virus' zoonosis was the pigs' ability to act as a 'mixing vessel' for all of these viruses, which led to the ultimate transmission event into humans.
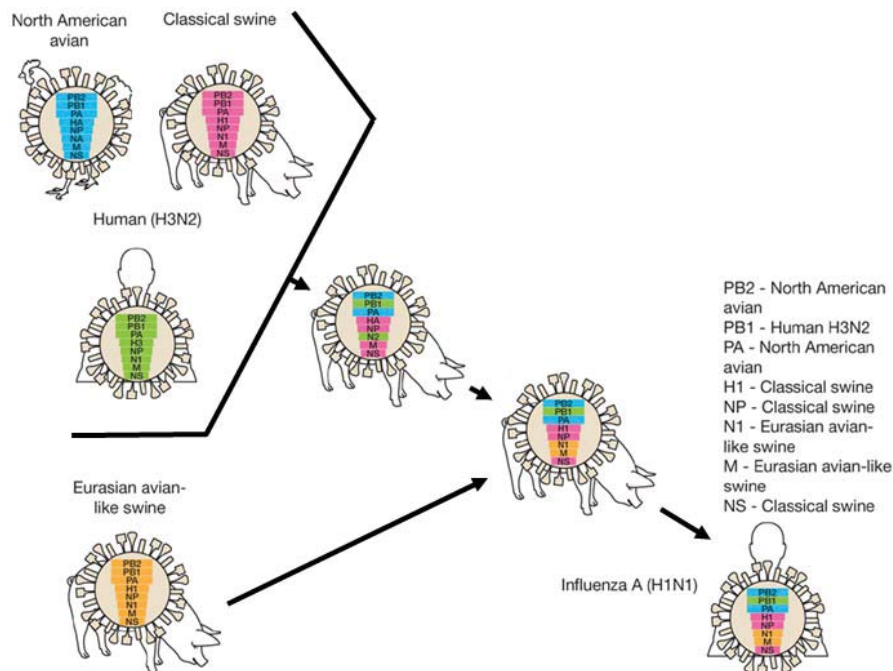
Figure 1.7: Schematic to show the genetic reassortments that led to the development of the 2009 H1N1 strain of influenza. The resulting virus contained genes from four different lineages: a quadruple reassortment, owing to the recombination of a pre-existing triple reassortant virus with the Eurasian avian-like swine influenza virus. (Neumann *et al.* 2009)

1.3.2 Epidemiology

The first outbreak of an influenza-like illness was reported to the WHO on 12th April 2009 in the Veracruz area of Mexico. Over the subsequent nine weeks, the virus spread internationally (Figure 1.8), causing the pandemic phase to be raised to 4, 5 and finally 6 on the 27th April, 29th April and 11th June respectively. Within three months of identification, the virus had spread to all WHO regions.

The rapid spread of the virus is attributable to both the genetics of the virus and the trappings of 21st Century international transport. In the early stages of the pandemic, the R_0 (basic reproduction number of the virus) was estimated to be between 1.2 - 3.2 (Fraser *et al.* 2009; Yang *et al.* 2009; Boelle *et al.* 2011), although this has subsequently been revised towards the lower end of the scale (Boelle *et al.* 2011; Kenah *et al.* 2011).
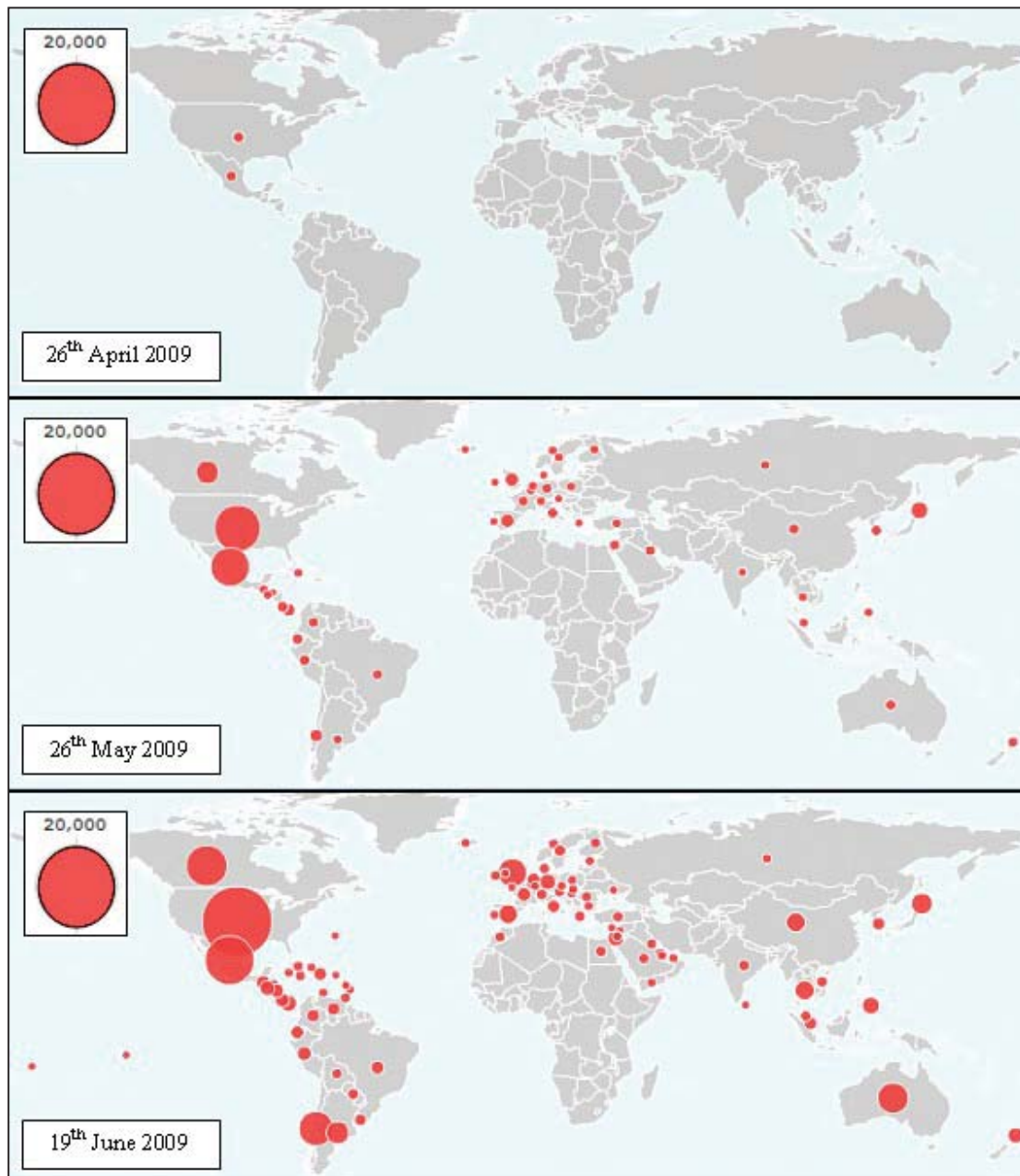
Figure 1.8: Geographical spread of the A(H1N1)pdm09 virus over the course of the first two months of global transmission. All data points are based on reported and clinically confirmed cases according to the WHO. The notable absence of cases in Africa may be due to gross underreporting by these countries (WHO website).

1.3.3 Morbidity & mortality profile

Despite initial fears that this new virus would cause mortality not seen on a scale since 1918, the pandemic proved overall to be no more virulent than seasonal influenza, albeit with an atypically

affected age profile. Estimates of the total mortality stemming from the virus range greatly; partly owing to the different reporting regimes of each WHO territory. For instance, Africa and Southeast Asia both show conspicuously low death totals (168 and 1,992, respectively) (WHO 2010), despite the fact that 38% of the global population lives in these areas. The WHO lists the global death toll for the first year of the pandemic to be 18,499 (WHO 2010), whilst mathematical modelling that takes into account the underreporting of cases in certain regions puts the figure at >280,000 deaths in the first year (Dawood *et al.* 2012). In contrast, seasonal influenza viruses typically result in an annual death toll of between 250,000 and 500,000 individuals globally (WHO 2003); highlighting the mild pathogenicity of the A(H1N1)pdm09 virus.

However, it is not the number of recorded deaths that is most remarkable about the pandemic, but the population demographic that were adversely affected. Typically, those at most at risk from influenza-related death are the over-65 year olds and very young children. Counter to this, the 2009 pandemic resulted in over 90% of the reported deaths occurring in individuals <65 years old (Bautista *et al.* 2010). Even more strikingly, 25-50% of these deaths were in individuals displaying no known co-morbidities such as chronic lung or cardiovascular diseases (Bautista *et al.* 2010). Figure 1.9 illustrates the atypical mortality profile of the individuals infected, with a significant minority of patients being noted as "previously healthy" (Liam *et al.* 2009).

Such findings would at first suggest viral drift and mutation as a cause for the increased severity of illness in these patients. Although there has been some evidence of mutations within the HA protein of some of the severe cases of illness (Kilander *et al.* 2010; Chan *et al.* 2011; Rykkvin *et al.* 2013), which have been linked with the ability of this virus to induce pneumonia, overall the virus associated with severe disease was largely identical to that found in patients that showed mild symptoms. Such findings would suggest that other, previously undetermined risk factors could be a cause of disease severity. As the adaptive response is largely absent during the 2009 H1N1 pandemic, these risks could therefore be located within host defences against the virus, either through an exaggerated, or sub-optimally functioning innate immune response.
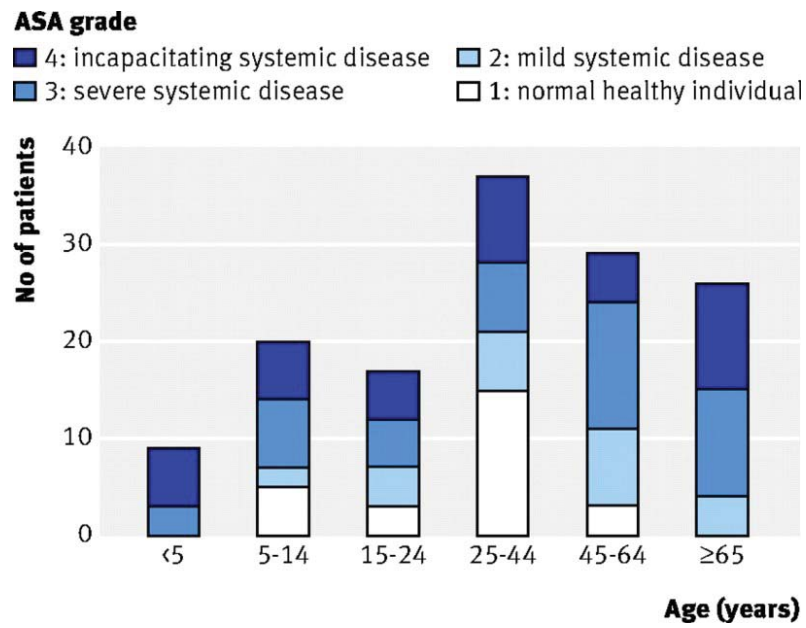
Figure 1.9: Age and pre-illness health of patients that died of pandemic influenza infection in 2009 in England. Bar colours co-ordinate with the co-morbidity severity of the patients. (Liam *et al.* 2009)

1.4   Host-Virus Interactions

The interplay between the host and infecting virus determines both the recovery of the former and successful replication of the latter. As viruses are obligate intracellular parasites of the host, they necessitate the use of host cell components in order to replicate; thus resulting in a high degree of interaction between the two organisms. However, both these conflicting selective pressures drive the development of countermeasures against one another to ensure their respective fitness advantage.

Figure 1.10 shows the approximate extent of host-virus interaction that occurs during a single influenza replication cycle within the cell. The host proteins shown in Figure 1.10 have been elucidated through the use of RNA interference (RNAi) screens to systematically knockdown the translation of individual host genes at the cellular level. Subsequently, the cells are then infected with influenza virus and assayed to determine the extent of viral replication over time (Brass *et al.* 2009; Shapira *et al.* 2009; Karlas *et al.* 2010). Not only have such studies have been critical in understanding the host proteins that facilitate viral replication, but also in indentifying those that restrict viral replication within individual cells: the so-called intrinsic and innate immune defences (discussed further in section 1.4.1).

One of the key protein families identified by these screens was the interferon-inducible transmembrane (IFITM) family of proteins (Brass *et al.* 2009), which have been shown to be capable of restricting multiple pathogenic viruses including flaviviruses, filoviruses and SARS-Coronavirus, amongst others (Brass *et al.* 2009; Huang *et al.* 2011) (see Section 1.4.1 for further discussion of the IFITM proteins). Such RNAi screen information can then be used in knockout animal models to determine whether there is an effect at the organism level, which may therefore inform future human disease therapies. For instance, these studies have shown that the ATPase and COPI complexes are both indispensible for influenza replication (Brass *et al.* 2009; Karlas *et al.* 2010); thus making them potential targets for drugs to reduce their expression.
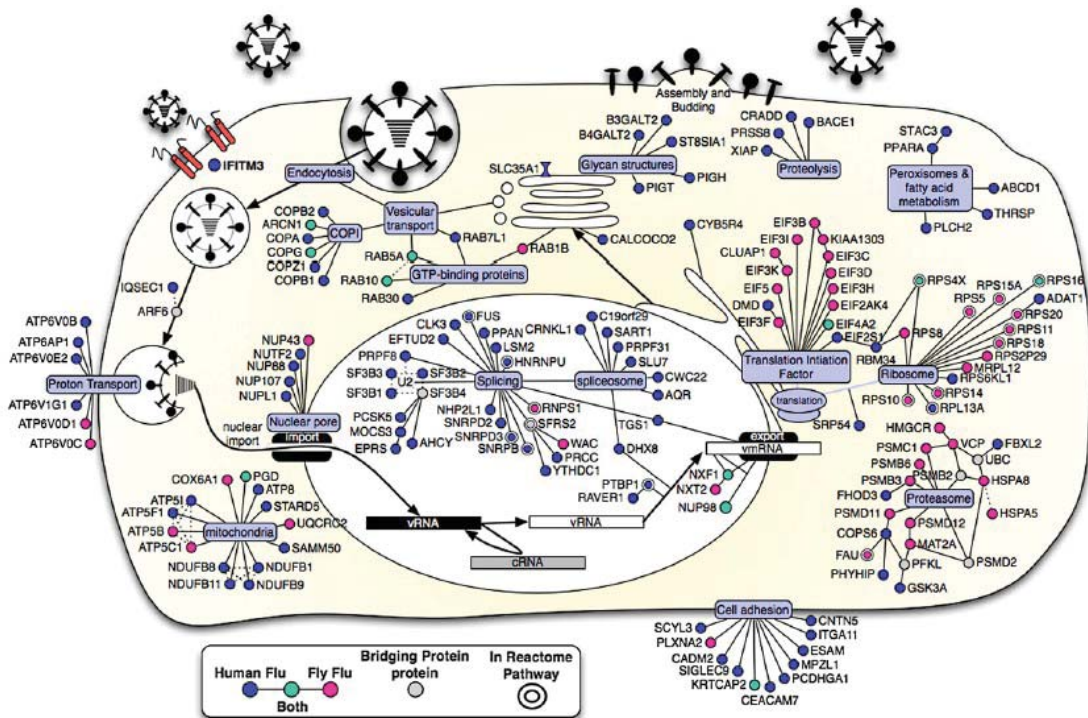
Figure 1.10: Host factors involved in influenza A virus replication as deduced from human and fly RNAi screens. Proteins shown to have an interaction with influenza from fly (pink) (Hao *et al.* 2008a) and human (blue) screens (Brass *et al.* 2009), as well as those that have occurred in both (green) are shown. Bridging proteins that were not detected in the screens, but are putatively thought to have an important function are also shown (grey). Double circles indicate the protein is present in the Reactome influenza A virus infection pathway (Vastrik *et al.* 2007). Solid lines between genes indicate the presence of an interaction, whilst dotted lines indicate an inferred interaction based on the literature. (Brass *et al.* 2009)

Further to the ability of the host to restrict the invading pathogens, viruses also possess their own suite of countermeasures designed to counteract the host immune repertoire. Broadly, these factors can be divided into three key areas (Figure 1.11), wherein the virus: 1) degrades, 2) sequesters, or 3) mimics the host's defence proteins as a dominant negative regulator. The competition between the virus and the host results in both evolving and counter-evolving in order to gain an advantage over its opponent is an ongoing process and has occurred across deep evolutionary time (Duggal and Emerman 2012). Specific examples of the methods employed by influenza viruses are discussed in section 1.4.1.5.
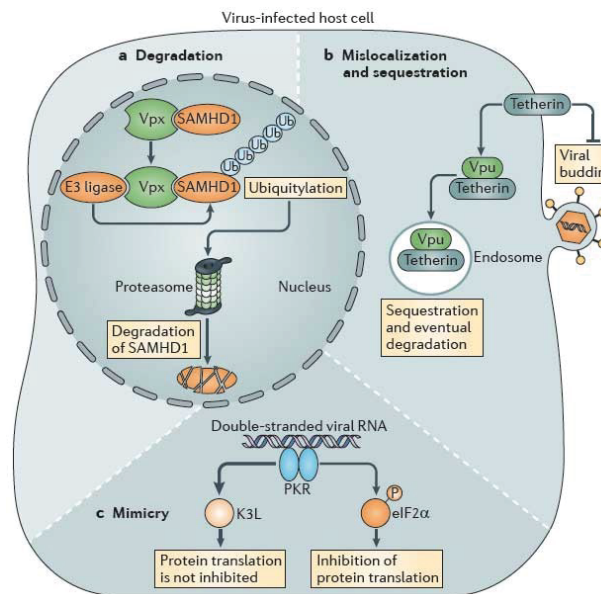
Figure 1.11: Broad mechanisms of viral antagonism of the host's innate immune response. The schematic depicts three of the key ways in which viral proteins are capable of antagonising the host's defences in order to continue proliferation. a) degradation: where the viral proteins signal for the destruction of the host's antiviral proteins. In this instance, it is illustrated by Vpx of HIV-1 signalling for the destruction of SAMHD1 by simultaneously binding to the host's E3 ligase, which subsequently results in the ubiquitylation of SAMHD1. The cell therefore processes the SAMHD1 protein for degradation as a result of this added signal (Laguette *et al.* 2011). b) mislocalisation and sequestration: where the virus forces the location of the host protein away from where it would serve its primary antiviral function. Tetherin is a potent antiviral restriction factor that binds budding HIV-1 virions to the cell membrane of the infected cell; preventing escape. However, the virus' Vpu protein can also bind tetherin and direct it into the endosomes, where it cannot achieve its antiviral function (Kueck and Neil 2012). c) mimicry: where the virus produces a protein with high similarity to that of the host's. This is illustrated here by K3L, which is encoded by poxviruses. K3L shows high structural similarity with eIF2α, which would typically down-regulate protein translation to halt the propagation of virus. K3L therefore competes with eIF2α for PKR; resulting in uninhibited translation (Dar and Sicheri 2002). (From (Duggal and Emerman 2012))

1.4.1 The innate response to influenza virus

The innate immune system is the first line of defence against pathogens that have successfully penetrated the barriers to infection, such as skin and mucus, and have reached a suitable site for infection. The response that is triggered upon cellular exposure to a pathogen such as influenza is non-specific and multifaceted; relying on cells recognising, restricting and eradicating the virus, whilst simultaneously signalling to other cells to trigger an antiviral state. Broadly, innate immune responses can be classified as those that either result in protection of the cell via

intrinsic and cell-autonomous mechanisms, or those that require the recruitment of specialised immune cells to the site of infection to aid in clearance (Figure 1.12).
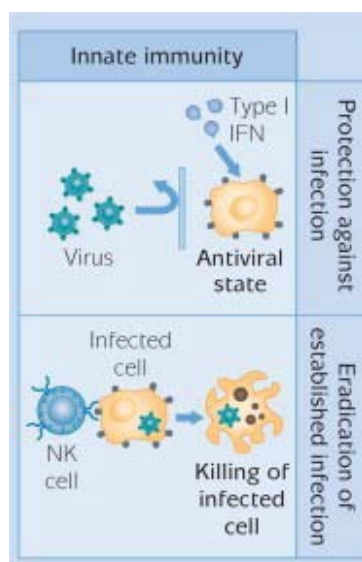
Figure 1.12: The stages of the innate immune response to viral infection. The innate immune system initially relies on intrinsic antiviral responses within the cell to protect against viral replication. Should these fail, or the virus subvert them, then the cell is brought into an antiviral state, primarily through the actions of type I interferons that are released upon detection of viral replication within the cells (top panel). The second "arm" of the innate immune response relies on the recruitment of innate responder cell types to the site of the established infection, through the release of signalling cytokines and chemokines by infected cells. In the schematic, this is illustrated by the arrival of an "NK cell", which subsequently detects which cell is infected before killing the cell to prevent further replication. Both "arms" of the innate system interact and work together in order to halt the spread of the virus. Further immune responses are classified as the "adaptive immune response" and are discussed in Section 1.4.2. From (Saunders 2003)

1.4.1.1 Intrinsic antiviral responses

Intrinsic antiviral responses are defined as those that are latently resident within cells to detect and restrict viruses prior to the triggering of interferon production and the consequent cascade of interferon-stimulated genes (ISGs); although it should be noted that these intrinsic effectors can also be up-regulated by interferon too. Such intrinsic antiviral responses therefore represent the first line of defence against incoming viruses in the cell. A list of the currently recognised intrinsic antiviral effectors, the viruses they restrict and their mode of action can be seen in Table 1.3.

Table 1.3: Intrinsic antiviral factors.

Name	Target viruses	Key role(s)	Key reference
IFITM family	Influenza, Dengue, West Nile, Ebola, SARS-Coronavirus	Block cytosolic entry	(Feeley *et al.* 2011)
IFIT family	Influenza	Recognise 5-triphosphate and the lack of 2-O-methylation in vRNA and inhibit translation	(Daffis *et al.* 2010)
Mx	Influenza, other RNA viruses	Block transcription	(Tumpey *et al.* 2007)
APOBEC3G	HIV-1, SIV, MLV, hepatitis B	Edit C to U in HIV DNA; inhibit reverse transcription and integration	(Mangeat *et al.* 2003)
TRIM5a	HIV-1, MLV	Block uncoating of incoming virions; promote innate immune signalling	(Pertel *et al.* 2011)
Tetherin	HIV-1, MLV, Ebola, KSHV	Block release of enveloped viruses	(Neil *et al.* 2008)
SAMHD1	HIV-1	Inhibit replication in myeloid cells	(Laguette *et al.* 2011)
TREX1	HIV-1	Remove cytosolic non-productive reverse-transcribed DNA; inhibit innate immune responses to HIV-1	(Yan *et al.* 2010)
RNase L	Many RNA viruses	Cleave single-stranded RNA in U-rich sequences; activate antiviral innate immunity	(Chakrabarti *et al.* 2011)
PKR	Many RNA viruses	Inhibit virus translation by protein phosphorylation; promote innate immune signalling	(Pindel and Sadler 2011)
cGAS	DNA viruses	Senses cytosolic DNA and activates the Type I IFN pathway via STING	(Sun *et al.* 2013)

Adapted from (Yan and Chen 2012)

As shown in Table 1.3, three specific anti-influenza protein families have currently been identified as intrinsic restriction factors: IFITM, IFIT and MX. Their roles in the influenza replication cycle are shown in Figure 1.13 and are subsequently discussed within this sub-section.
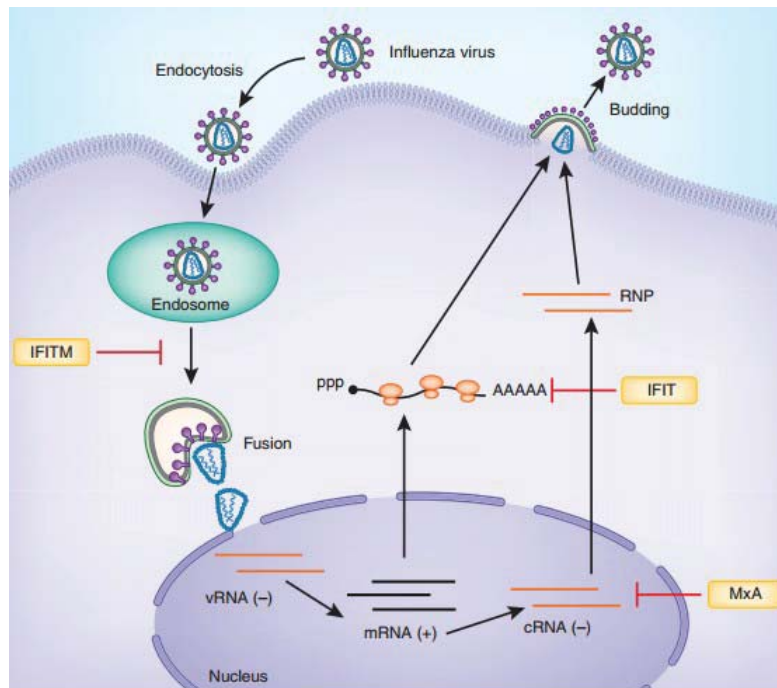
Figure 1.13: Intrinsic antiviral restriction factors that inhibit influenza virus. The schematic illustrates the three major identified families / proteins that intrinsically protect cells against influenza virus: IFITM, IFIT and MxA (Mx1 in mice). The actions of these families are discussed further in the text. (Yan and Chen 2012)

1.4.1.1.1 The IFITM family

The interferon-induced transmembrane (IFITM) family of proteins were first identified in 1984 as key responders following exposure of human cell lines to interferon treatment (Friedman *et al.* 1984). The IFITM family (previously known as 1-8, MIL or Fragilis) in humans consists of IFITM1, IFITM2, IFITM3 and IFITM5, whilst in mice the family is made up of orthologous Ifitm1, Ifitm2, Ifitm3, Ifitm5, Ifitm6 and Ifitm7 (Siegrist *et al.* 2011), however only IFITM1-3 and their murine orthologs have been shown to display significant antiviral effects and will form the basis of this section.

Initially, it was thought that these small 14-17kDa proteins all had a similar topology, consisting of a dual-pass transmembrane arrangement in the cellular membranes, with their longer N- and shorter C- termini facing the extracellular space (when on the cell surface) or the lumen (when on endosomal vesicles). However, it is now thought that the proteins display an intramembrane topology (Figure 1.14), owing to their patterns of palmitoylation and ubiquitination, which are otherwise incompatible with a transmembrane structure (Yount *et al.* 2012).
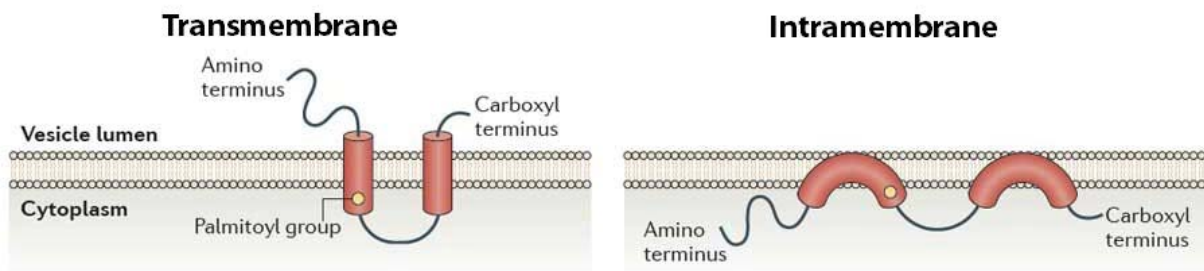
Figure 1.14: Topologies suggested for the IFITM family of proteins. The "transmembrane" model was what was initially predicted to be the structure of the IFITM family, with two anti-parallel transmembrane domains and the N- and C- termini facing into the ER lumen / endosome / extracellular space. However, subsequent analysis has shown such an arrangement to be less likely, owing to the post-translational modification profile of IFITM3. The alternative "intramembrane" topology has the transmembrane sections arranged in an intramembrane ordering, with the N- and C- termini facing in the opposite direction of that previously: into the cytosol. The yellow dots indicate the area of the palmitoylation sites that are crucial for the antiviral action of the protein (Adapted from (Diamond and Farzan 2013))

In addition to their anti-influenza restriction, the IFITM family have also been implicated in development, cancer and cellular proliferation (Tanaka *et al.* 2005; Li *et al.* 2011; Siegrist *et al.* 2011). Furthermore, the proteins have been shown to restrict a broad range of viruses (Brass *et al.* 2009; Weidner *et al.* 2010; Yount *et al.* 2010; Huang *et al.* 2011; Schoggins *et al.* 2011; Anafu *et al.* 2013; Mudhasani *et al.* 2013). Initially it was thought that the IFITM family could only restrict enveloped viruses, as they were the only viruses blocked during *in vitro* studies, although studies on reovirus infection have also revealed a restriction role for IFITM3 (Anafu *et al.* 2013). Reovirus, although non-enveloped, does utilise the endosomal pathway during viral entry, which is consistent with hypotheses on how the IFITM family restricts viral replication.

Although there are questions over the structure and position of the IFITM proteins within the cellular membranes (Diamond and Farzan 2013) and the number of viruses restricted by the family continues to expand, debate remains about how the IFITM proteins achieve their antiviral role in the cell. Currently, it is thought that the most potent antiviral family member, IFITM3, associates with the endosomal pathway and achieves restriction in the late endosomes (Figure 1.15), which is largely agreed upon. However, the exact mechanism of restriction remains unknown.
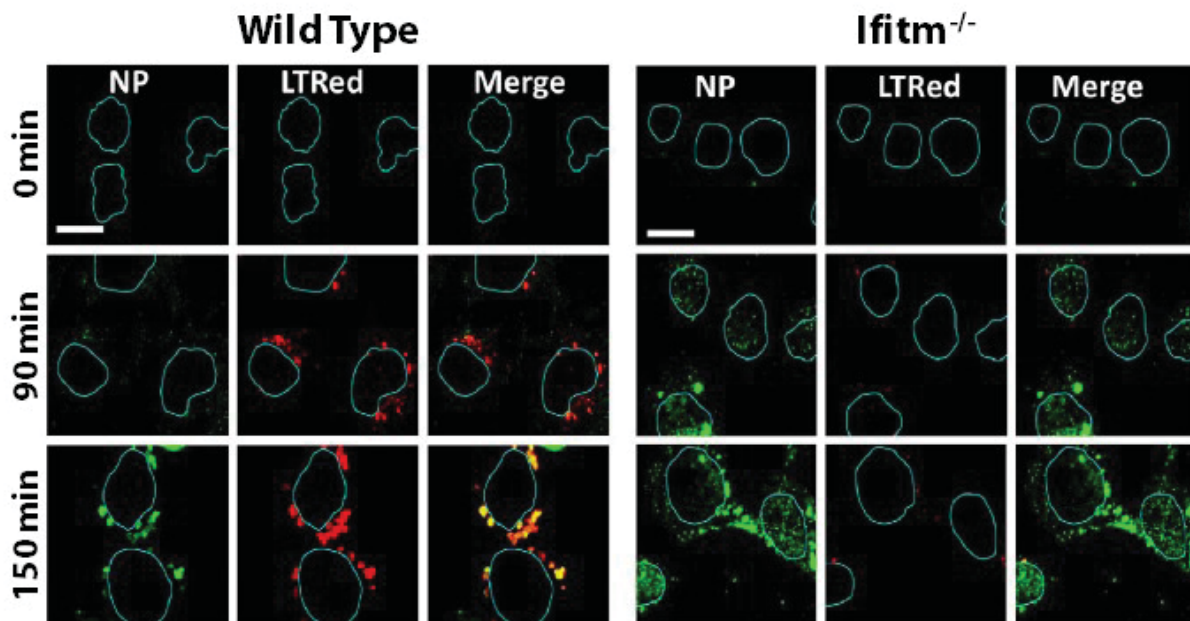
Figure 1.15: The Ifitm proteins are necessary for restricting influenza virus in the late endosomes and preventing vRNP entry into the nucleus. The images show the advance of H1N1 influenza (NP) into the nuclei (blue circles) of IFNγ-treated murine embryonic fibroblasts (MEFs) that either have (Wild Type) or lack (Ifitm$^{-/-}$) all Ifitm proteins. Of note is the fact that the Ifitm$^{-/-}$ cells show viral NP within the nuclei over the course of infection – something that is not seen in Wild Type cells. Furthermore, the inclusion of lysotracker red (LTRed) shows that the endosomes aggregated around the nucleus at 150min post-infection co-localise with the influenza NP signal in Wild Type cells; thus lending support to the hypothesis that the IFITM family of proteins restricts incoming virus in the late endosome pathway and prevents cytosolic release. (From (Feeley *et al.* 2011))

In light of the recent evidence stemming from the new theories regarding IFITM protein intramembrane topology (Figure 1.13), research has shown that IFITM3 may be playing a role in structurally reinforcing the plasma membrane; thus preventing the fusion of the viral and cellular membranes by raising the energy required by the viral fusion proteins (HA for influenza) to merge the membranes, which would account for the aggregation of virus within the endosomes (John *et al.* 2013; Li *et al.* 2013). Furthermore, another role for the IFITM proteins has been suggested, relating to their association with vesicle-membrane-protein-associated protein A (VAPA), which is involved in intracellular cholesterol homeostasis (Amini-Bavil-Olyaee *et al.* 2013). These newly suggested models of how the IFITM proteins impact viral release from the late endosomes are seen in Figure 1.16.
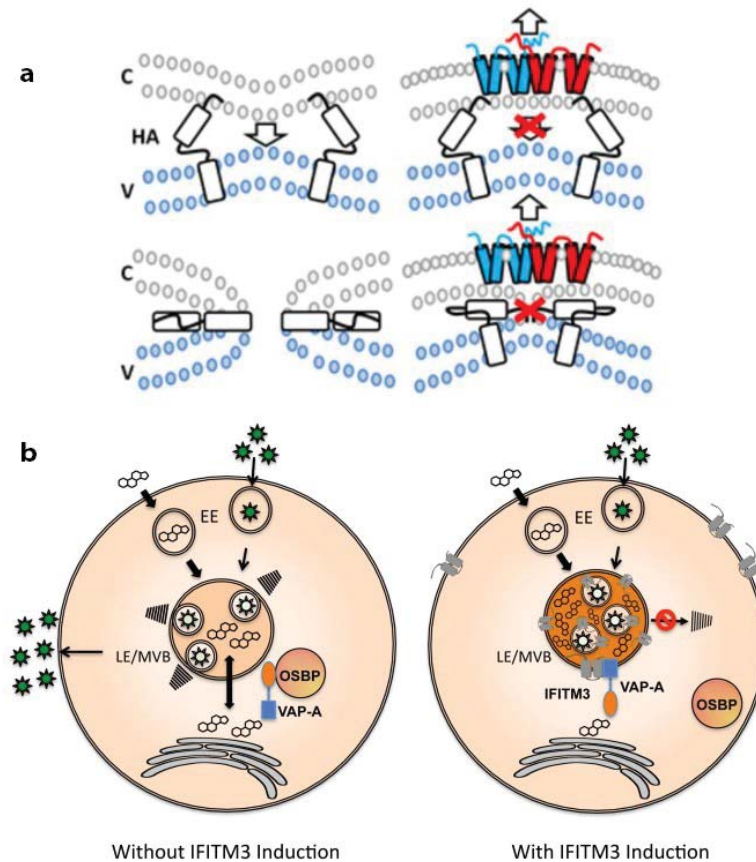
Figure 1.16: Currently suggested models of IFITM restriction. a, schematic illustrating how the IFITM proteins may be mechanically strengthening the cell's endosomal membrane (C), which prevents viral membrane (V) fusion. The left hand panels show "regular" fusion in the absence of IFITM expression, whilst the right hand panels demonstrate how IFITM proteins accumulate (blue and red show two molecules aggregating) and change the intermolecular properties of the membrane, which also compresses the lipid bilayer. It is argued that this increases the rigidity of the endosomal membrane, which cannot be overcome by influenza's HA protein. **b**, the schematic illustrates how without the presence of IFITM3, the virus and cholesterol (chemical structure) enter through the early endosomal (EE) pathway and aggregate in the late endosome / multi-vesicular body (LE/MVB) and cholesterol can reach regular homeostasis levels with the cytosol. However, in the presence of IFITM3 expression, VAP-A becomes associated with IFITM3; thus forming a block to cholesterol homeostasis, which leads to an aggregation of cholesterol within the late endosome. It is suggested that the accumulation of cholesterol prevents the fusion of the membranes and release of vRNP. (From (Amini-Bavil-Olyaee *et al.* 2013; John *et al.* 2013))

Although much of the focus has been placed on IFITM3, as it is seen as the most potent antiviral member of the family (Brass *et al.* 2009), not all IFITMs function at the same point of viral infection and associate with the late endosomes. Indeed, it appears as though IFITM1 may be more associated with the early endosomes and cell surface, whilst IFITM2 and IFITM3 are more

closed aligned to the late endosomes (John *et al.* 2013). The differences in their expression pattern may account for how IFITM1 and IFITM3 show differing degrees of restriction of certain viruses, with IFITM3 playing a larger role in influenza restriction, whilst IFITM1 appears to restrict filoviruses and hepatitis C virus (HCV) more successfully (Huang *et al.* 2011; Wilkins *et al.* 2013).

Despite difficulties in elucidating the mechanisms of action of the IFITM proteins, they nevertheless represent a family of critical intrinsic viral restriction factors and may be one of the first lines of defence against incoming viral pathogens. Furthermore, the fact that the family is also interferon-inducible means that they serve a dual role as a key innate immune effector and ISG.

1.4.1.1.2 MxA / Mx1

Orthomyxovirus resistance gene 1 (*Mx1*) was one of the first intrinsic anti-influenza restriction factors to be discovered in mice. Like IFITM3, Mx1 and its human homologue MxA are both intrinsically expressed, but can also be up-regulated by the actions of interferon (the actions of interferon and its upregulation of the cell to an antiviral state are considered in section 1.4.1.2). Although both Mx1 and MxA share an evolutionary history, they differ in their sub-cellular localisation; with Mx1 acting in a nuclear role, whilst MxA is cytoplasmic (Pavlovic *et al.* 1992).

The most striking evidence for a role for the Mx family in influenza restriction comes from murine mouse lines. Many inbred mouse lines lack a functional copy of Mx1, which is believed to be due to a founder effect of the colonies routinely used for *in vivo* studies (Haller *et al.* 2010). Infection of mice with non-functional copies of *Mx1* leads to a rapid and lethal infection. However, this can be overcome by the restoration of the Mx1 gene, which in turn confers complete protection to the mouse (Arnheiter *et al.* 1990). Strikingly, the restoration of the *Mx* allele in mice also confers them with protection against the highly lethal 1918 Spanish influenza and avian H5N1 viruses (Tumpey *et al.* 2007); thus demonstrating the remarkable protective ability of a single protein.

The exact mechanism underpinning the restrictive capacity of the Mx family is still the subject of debate. Currently, it is suggested that the nuclear murine Mx1 blocks primary viral transcription, whilst human cytoplasmic MxA acts to prevent secondary transcription and viral replication (Yan and Chen 2012). However, evidence regarding a physical interaction between MxA and the influenza virus nucleocapsid may provide some clues as to how it retards the spread of virus (Turan *et al.* 2004). Indeed, it has been shown that mutations within the 1918 and 2009 H1N1 pandemic influenza viruses' nucleoprotein (NP) complex results in them being able to overcome restriction by MxA in human cell lines (Manz *et al.* 2013). Further to this, the introduction of the mutated NP into a previously Mx-restricted H5N1 virus resulted in a gain of MxA resistance. A single MxA protein may recognise viral proteins through such interactions and can then signal for the recruitment of multiple MxA proteins, which form copolymers to immobilise and mis-sort the virus (Haller *et al.* 2007). Taken together, the current body of data suggests that the MxA protein is highly important in resistance against zoonotic influenza viruses and those viruses carrying mutations with resistance to MxA may successfully transmit into humans more easily.

1.4.1.1.3   The IFIT family

The interferon-induced proteins with tetratricopeptide repeats (IFIT) family consists of four members in humans: IFIT1, IFIT2, IFIT3 and IFIT5, and three members in mice: Ifit1, Ifit2 and Ifit3. Like the other intrinsic antiviral proteins described here, the IFIT proteins can also be up-regulated by the actions of interferon, but they also act as pattern recognition receptors (PRRs), like the Mx family (and potentially the IFITM family) (Diamond and Farzan 2013).

Research into the IFIT family of proteins has revealed the family to be multi-functional; restricting viral replication in a number of ways. Several studies have indicated that the family, in particular IFIT1, can act as a cytoplasmic sensor for uncapped 5'-triphosphorylated or non-2'-*O*-methylated RNA that is released from RNA viruses upon infection of the cell; a distinct 'non-self' signal that is detected by the host (Daffis *et al.* 2010; Pichlmair *et al.* 2011). Upon detection of the vRNA in the cell, IFIT1 recruits IFIT2 and IFIT3 to form a trimer and binds to the vRNA in order to sequester it from further replication. Although this complex has been shown to restrict the virus, the fate of the IFIT-vRNA complex is unknown; thus the exact disposal route is still debated (Yan and Chen 2012; Diamond and Farzan 2013).

Further to their role in detection and sequestration of vRNA, the IFIT family have also been implicated in binding to human papillomavirus proteins, as well as other host proteins, such as eIF3 to prevent translation of vDNA (Hui *et al.* 2003; Saikia *et al.* 2010). Interestingly, the IFIT family has also been purported to have an immunomodulatory role. Although contentious, some studies have shown that the IFIT family can reduce expression of many ISGs, inflammatory chemokines and interferon signals and therefore reduce the extent of immunopathology caused by the cellular response to viral infection (Berchtold *et al.* 2008; Li *et al.* 2009; Diamond and Farzan 2013).

1.4.1.2 Cell-autonomous responses

Although the intrinsic antiviral mechanisms listed in sub-section 1.4.1.1 are effective in sequestering and immobilising invading virus, they are all capable of being up-regulated by the actions of interferon signals. Cell-autonomous responses to viral infection typically rely on the actions of interferon to create a feedback loop in order to induce the expression of numerous ISGs to combat the established infection and prime surrounding cells in a paracrine manner for the potential burst of progeny viruses from the infected cell.

However, prior to the transcription and translation of interferon and the subsequent ISG cascade, the cell must first activate its innate immune repertoire through detection of the vRNA. Although various host-viral binding interactions have been discussed previously, they do not directly prime the cell to activate all of its antiviral defences. The detection of influenza's vRNA is primarily mediated by a number of receptors that are resident in the cytoplasm and are embedded within endosomal and mitochondrial membranes. The purpose of these PRRs is to act as sensors for non-host RNA and to commence a signalling cascade. These receptors can take numerous forms in mammals: toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs) and C-type lectin receptors (Yan and Chen 2012). As shown in Figure 1.17, the primary receptors involved in the detection of influenza are the RLR RIG-I (Kato *et al.* 2006), which identifies cytoplasmic vRNA, and the TLRs TLR3 and TLR7, which monitor the endosomal compartments for single stranded RNA (ssRNA) that may be accidentally released from damaged virions undergoing acidification as part of the fusion process (Crozat and Beutler 2004;

Lund *et al.* 2004; Le Goffic *et al.* 2007). These pathways operate in a redundant fashion, wherein the abrogation of either the RLR or TLR pathway can be compensated for by the other processes that still generate interferon responses to control influenza replication in murine lungs (Koyama *et al.* 2007).
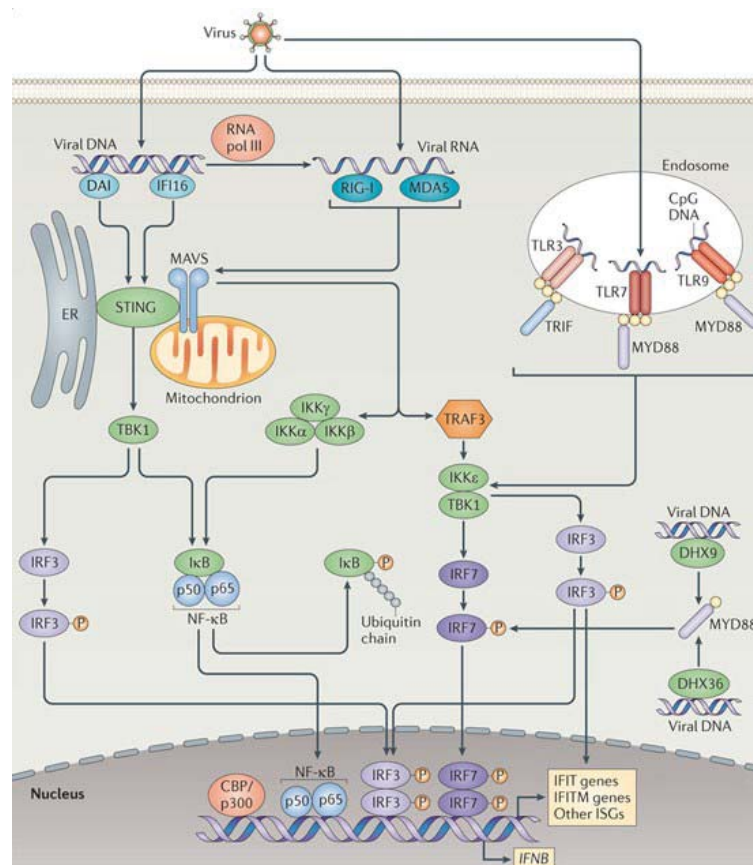
Figure 1.17: Modes of detection of incoming virus and their subsequent signalling pathways. The schematic shows a generalised signalling pathway for viruses. As mentioned in the body of text, the key influenza receptors are RIG-I, TLR3 and TLR7; therefore the subsequent signalling pathways stemming from these proteins are most relevant to the innate immune response to influenza virus. The release of vRNA by influenza viruses is recognised by RIG-I in the cytosol and TLR3 and TLR7 in the endosomes. RIG-I interacts with mitochondrial antiviral signalling protein (MAVS; also known as IPS-1), which recruits TNFR-associated factor 3 (TRAF3), TBK1 and the IκK complex, which results in the activation and nuclear translocation of IRF3 and NF-κB. Meanwhile, the TLRs interact with TRIF and MyD88, which activates IRF3 or IRF7. The binding of NF-κB, IRF3 and IRF7 to the IFN and ISG promoters leads to the transcription of the interferon and other ISGs. (From (Diamond and Farzan 2013))

The primary function of the PRR pathways is sensing incoming virus to trigger the transcription of the key antiviral signalling molecule: interferon, as can be seen in Figure 1.17. However, it

should also be noted that certain genes, such as the IFIT family can be transcribed in an interferon-independent manner through the actions of IRF3 (Grandvaux *et al.* 2002).

Interferons (IFN) are crucial in readying the organism to combat the incoming infection as it is capable of altering the immune state of the infected cell in an autocrine manner, as well as systemically readying neighbouring cells, and indeed the body, in a paracrine manner. IFNs are broadly classified into three "types": I, II and III. In humans, type I IFNs consist of the 13 IFNα members and IFNβ, ε, κ, and ω, type II are IFNγ and type III are the newly studied, but little understood, IFNλ class (Platanias 2005; Sadler and Williams 2008).

Briefly, Type I IFNs are normally the first to be produced following virus infection via the PRR pathways shown in Figure 1.17. In influenza infections of mammals, the respiratory epithelium is the primary target of the virus and as such these will be the first cells to produce type I IFN. However, various other cell types reside in the airways, including plasmacytoid dendritic cells (pDCs) and alveolar macrophages, which may also become infected (these leukocytes and others are discussed in sub-section 1.4.1.3). Research has shown that these cells produce higher amounts of Type I IFN and may therefore be responsible for the paracrine signalling in the lungs (Takeuchi and Akira 2009). Type II IFNs are distinct and highly dissimilar to type I IFNs, but also regulate the production of ISGs. This class of IFN is largely produced by activated T-cells and natural killer cells and as such play a larger role in the adaptive response than the innate response to infection (discussed further in section 1.4.2). The final class of IFNs, the type III IFNλ, represent an emerging field in immunology, owing to their recent discovery (Kotenko *et al.* 2003). Like the type I IFNs, they are produced by epithelial surfaces and also seemingly regulate a similar set of ISGs (Sommereyns *et al.* 2008). They have been implicated in clearance of hepatitis C virus (Ge *et al.* 2009), as well as aiding in the restriction of influenza virus infection, they have yet to be shown as crucial, unlike type I IFNs (Mordstein *et al.* 2008; Jewell *et al.* 2010; Mordstein *et al.* 2010).

Once released from the stimulated cell, the IFN molecules then bind to their respective receptors at the cell surface where they can trigger their respective JAK/STAT pathways (Figure 1.18). These signalling pathways ultimately stimulate the binding of the STAT complex to the

interferon stimulated response elements (ISREs), which in turn up-regulates the generation of hundreds of interferon-stimulated genes geared to combat infection (Haller *et al.* 2006; Rusinova *et al.* 2013).
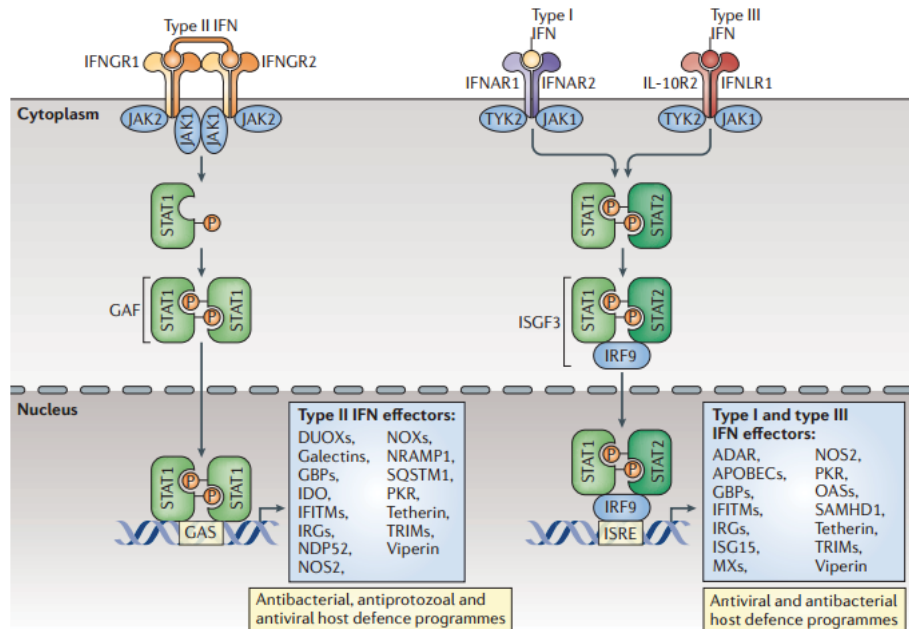
Figure 1.18: Signalling pathways of type I, II and III interferons. The three classes of IFN bind to their own independent receptor molecules on the cell surface. Type I IFN binds to a heterodimer of IFNα receptor 1 (IFNAR1) and IFNAR2, type II binds to a tetramer of two IFNγ receptor 1 (IFNGR1) chains and two IFNGR2 chains, and type III binds to the interleukin-10 receptor 2 (IL-10R2) / IFNλ receptor 1 (IFNLR1) complex. Both type I and III IFNs largely use the same pathway to stimulate ISGs, wherein the receptors' pre-associated tyrosine kinase (TYK) and janus kinase (JAK), result in phosphorylation upon receptor-binding. The phosphorylated signal is carried to the signal transducers and activators of transcription (STAT) complex, which subsequently binds IRF9 to form the IFN-stimulated gene factor 3 (ISGF3). Type II IFN signalling is largely similar, but relies on a STAT1 homodimer to form the IFNγ activation factor (GAF), which binds to the DNA instead of ISGF3. The result of this transduction is the transcription of an array of IFN effector molecules; some of which are displayed. (From (MacMicking 2012)).

A final consequence of the PRRs detecting virus, generating IFN and the signal being transduced by the JAK/STAT pathway is the production of an array of ISGs. Although hundreds of these proteins can be generated following an infection, not all are capable of restricting influenza virus. The cell detects the presence of viral components and therefore produces a general response to account for a broad-cross spectrum of potential pathogenic infections. As previously discussed in sub-section 1.4.1.1, the intrinsically expressed IFITM, IFIT and MX proteins are all further

induced by the actions of IFN, which can all potentially restrict influenza viruses. However, the vast majority of the genes transcribed as a result of IFN stimulation have unknown functions. Some of those with known functions against influenza are depicted in Figure 1.19.
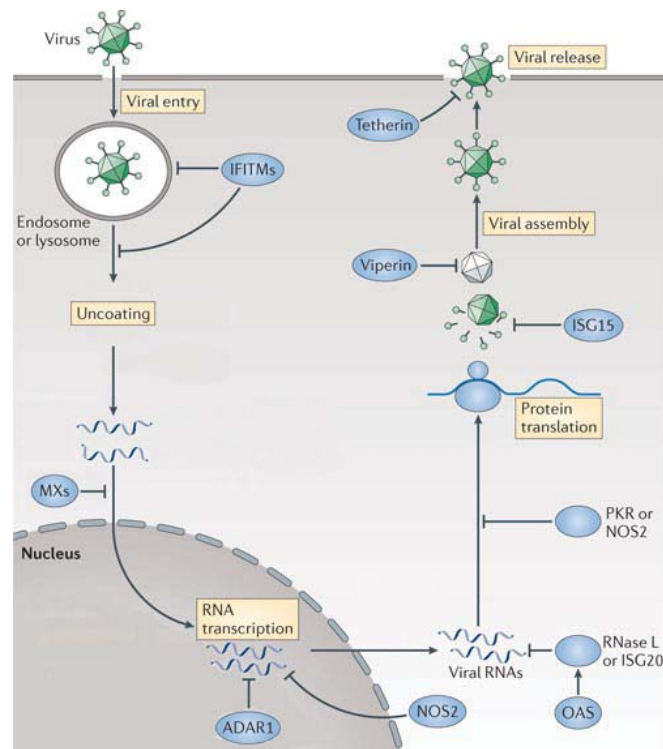
Figure 1.19: Some of the interferon-induced proteins thought to be capable of restricting influenza virus. The schematic illustrates the generalised life cycle of an infecting virion, which will act as a surrogate for influenza virus. The cell uses many autonomous techniques to prevent further viral replication. As discussed previously, the IFITM and MX family of proteins block replication at early stages of the viral life cycle. ADAR1 (adenosine deaminase, RNA-specific 1), NOS2 (nitric oxide synthase 2), OASs (2'-5' oligoadenylate synthases), RNase L, ISG20 and PKR (RNA-dependent protein kinase) inhibit RNA transcription at various stages, whereas ISG15, viperin and tetherin prevent post-translation assembly of the final virions. (Modified from (MacMicking 2012))

1.4.1.2.1   ISGs against influenza: pre-translation

As shown in Figure 1.19, ISGs are generated in such a way as to prevent viral replication at multiple stages and therefore mitigate the chances of viral escape mutants arising (discussed in section 1.4.1.5). Although many hundreds of ISGs are encoded to prevent viral replication, sub-sections 1.4.1.2.1 and 1.4.1.2.2 will primarily focus on those shown in Figure 1.19 to aid clarity and comprehension.

The actions of the IFITM and MX families of ISGs have been discussed and will therefore not be covered in this section. However, it is important to highlight that they continue to play a crucial role post-interferon stimulation. Indeed, administration of type I or II IFN to cell lines stimulates a significant induction of the IFITM family, which shows a far greater degree of influenza restriction than unstimulated cells (Feeley *et al.* 2011).

Within the nucleus, the ISGs ADAR1 and NOS2 contribute to inhibiting viral replication (the Mx proteins can also contribute at this stage, as discussed previously). Briefly, ADAR1 is thought to be responsible for hypermutation of A to G in exposed vRNA within the nucleus (Suspene *et al.* 2011); thus introducing nonsense mutations into the viral genome to prevent successful replication. Increasingly, evidence indicates that the p150 isoform (the interferon-stimulated form) contributes to influenza restriction (Suspene *et al.* 2011; Ward *et al.* 2011). NOS2 utilises a different approach to halting viral replication, as it generates nitric oxide (NO) as a reactive species with the intention of destabilising viral proteases (Karupiah *et al.* 1998; Saura *et al.* 1999). Although some evidence shows NOS2 to be important in clearing certain viral infections, such as MCMV (Noda *et al.* 2001), NOS2 also contributes to immunopathology associated with influenza, as it can also adversely affect uninfected cells; causing non-specific damage to the adjacent infected tissues (Jayasekera *et al.* 2006). This highlights a potential problem in IFN triggering a generalised innate immune response: certain ISG protein functions may be cell toxic.

Following nuclear export, the vRNA can then be antagonised by OAS/RNase L, ISG20 and PKR. OAS recognises the replication intermediate of influenza, dsRNA, which triggers its activation, which subsequently activates the latent RNase L (MacMicking 2012). RNase L is then free to cleave ssRNA stemming from the virus in the cytoplasm, as well as also cleaving certain host mRNA signals, which in turn feedback to RIG-I and MDA5 to further stimulate the IFN pathway (Boo and Yang 2010). ISG20 is also an RNase with specificity for ssRNA that contributes to anti-influenza virus activity. Indeed, over-expression of ISG20 alone, without IFN-stimulation, can greatly restrict influenza viruses, as well as VSV and EMCV (Espert *et al.* 2003). PKR on the other hand utilises an entirely different mode of action to combat infection, as not only can it restrict virus replication through phosphorylation of eukaryotic initiation factor 2α

(eIFα), which in turn slows translation (Boo and Yang 2010), but it can also trigger cell death by upregulating several pro-apoptotic genes (Gil and Esteban 2000). Furthermore, like RNase L, PKR can stimulate the autocrine production of IFN by signalling to TRAF and subsequently NFκB. Indeed, the ablation of PKR expression in knockout mice revealed it to be crucial in reducing influenza viral burden; highlighting its crucial role as an ISG and in the autocrine signalling pathway (Balachandran *et al.* 2000).

1.4.1.2.2   ISGs against influenza: post-translation

The host immune response also generates a number of ISGs to prevent viral assembly and budding from the cells surface. Three such proteins are ISG15, viperin and tetherin, which will be discussed in this sub-section.

ISG15, much like PKR, is a multifaceted protein that plays several roles in the innate antiviral response. Although it has been established that ISG15 is involved in the antiviral repertoire, owing to the fact that when it is ablated in knockout mice they show modestly increased susceptibility to influenza A and B viruses, as well as various herpes viruses (Lenschow *et al.* 2007), an extensive understanding of its mechanism is lacking. It has been observed that ISG15 facilitates so-called ISGylation wherein it is conjugated onto various host and viral proteins (Skaug and Chen 2010). The binding to host proteins (such as Mx, RIG-I and RNase L) could boost their protective effect in the cell, whereas binding to viral proteins, such as NS1 of influenza virus has been shown to result in a "loss of function" effect; greatly reducing viral infectivity (Zhao *et al.* 2010). Additionally, ISG15 can inhibit degradation of IRF3, which viruses seek to down-regulate in order to retard the IFN-signalling cascade (Sadler and Williams 2008; Boo and Yang 2010). Therefore, although the exact mechanism of ISG15 is yet to be elucidated, much like the IFITM family of proteins, it clearly plays an important role in the antiviral response. Furthermore, evidence of viral countermeasures, in the form of the NS1 protein of influenza B virus actively binding and sequestering ISG15 (Zhao *et al.* 2013), act to support the importance of ISG15 as an antiviral molecule.

In the final stages of viral assembly, both viperin and tetherin may play a role in restricting the export of influenza virus into the extracellular space. Viperin, like many ISGs, shows broad

neutralising ability against a variety of viral pathogens. However, with influenza viruses it is purported to target a route specific to the manner in which influenza buds from the cell surface. During escape from the cell, influenza preferentially associates with lipid rafts, which are rich in cholesterols and glycosphingolipids. These areas may act as microdomains where the viral HA and NA surface proteins aggregate (Nayak *et al.* 2004). Viperin disturbs these lipid rafts; fragmenting them in the process, which in turn affects the ability of influenza to successfully bud from the surface (Wang *et al.* 2007). However, the only evidence of a role for viperin in restriction of influenza virus has come from *in vitro* assays. Studies on viperin knockout mice have shown no obvious effect following challenge with influenza viruses (Sen Tan *et al.* 2012).

Tetherin is also associated with preventing viral budding, but works in a distinctly different manner to viperin. Much of our understanding regarding this ISG comes from work on HIV-1 (Neil *et al.* 2008; Perez-Caballero *et al.* 2009), which has shown that tetherin physically participates in anchoring budding virions to the cell surface before they are then re-endocytosed and degraded. However, evidence regarding participation of tetherin in restricting influenza virus has proved to be more contentious than that of HIV-1. Studies have indicated that tetherin can modulate release of influenza virions from the surface of the cell, which can be cleaved by certain NA subtypes (Yondola *et al.* 2011). In spite of this, assays involving wild type viruses, as opposed to virus-like particles (VLPs) or pseudoviruses, have shown no restrictive capacity for tetherin (Watanabe *et al.* 2011). It has consequently been argued that influenza may possess multiple tetherin countermeasures, just as HIV-1 possesses Vpu (Mangeat *et al.* 2012). Ultimately it appears as though evidence regarding the role of tetherin in restriction of influenza viruses is circumstantial at best.

1.4.1.3 Leukocyte responses to influenza

The epithelial cells that are normally the target of influenza virus are capable of mounting their own autonomous innate immune response, as previously discussed. However, another crucial component of the innate immune response to infection is mediated by non-epithelial cells: the leukocytes. Their functions can include detecting and signalling the presence of virus to other cellular populations, destroying infected cells through direct cellular interaction and the secretion of chemical signals, and priming T-cell populations for the adaptive immune response. The

cellular immune response to pathogens is characterised by its rapid onset and non-specific nature; it is not targeting a single pathogen, much like the cell autonomous response produces a broad cascade of antiviral proteins.

1.4.1.3.1 Mast cells

Mast cells are a resident, sentinel population of leukocytes present throughout the body and particularly at mucosal surfaces, such as the nasal cavity and lungs. These cells have been implicated in the control of allergic diseases, such as asthma, but have also increasingly been shown to aid in the innate immune response to bacteria, parasites and viruses (Abraham and St John 2010). The primary function of mast cells during viral infection is in the production of various cytokine signals, which in turn influence a multitude of innate and adaptive immune cells (Figure 1.20).
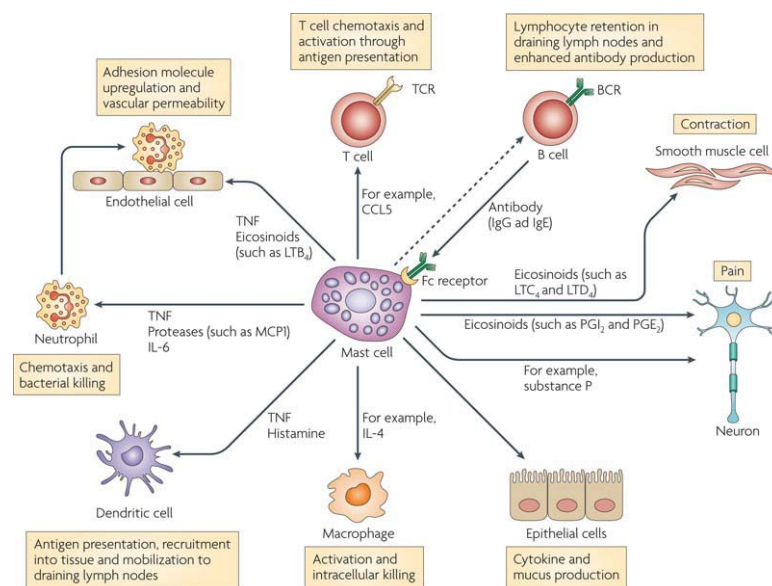
Figure 1.20: The role of mast cells in host defence. The schematic illustrates how mast cells communicate and moderate the actions of various cell populations, with the aim of modulating host immunity. Orange boxes show the functional consequences of the signalling and chemical signals / cytokines are shown to indicate how mast cells achieve their mediating actions. (From (Abraham and St John 2010))

Mast cells are now emerging as a key component of the innate immune response to influenza virus infection (Graham *et al.* 2013). These cells can become infected with influenza, detect vRNA through RIG-I signalling, and commence the proinflammatory process. Interestingly, like many other leukocytes that contribute to the innate response to influenza, this proinflammatory

response elicited by mast cells can also cause severe immunopathology and widespread apoptosis within the lungs through the release of IFNγ; thus exacerbating the severity of disease (Hu *et al.* 2012). This study showed that mast cells may contribute to the severe pathology associated with avian H5N1 influenza infection, as depletion of the cell population ameliorated the symptoms associated with the virus in mice. Furthermore, Hu and colleagues showed how depletion of mast cells within the lungs also improved the efficacy of the antiviral drug oseltamivir; again implicating the cells in the severity of disease.

1.4.1.3.2   Macrophages

A further immune cell population that is responsible for promoting the development of a proinflammatory environment within the lungs are the macrophages. Also, much like mast cells, macrophages are mediators of the innate immune response through the cascade of cytokines and chemokines that they release upon infection with influenza virus (Figure 1.21). However, unlike mast cells, macrophages are phagocytes and are therefore able to engulf pathogens and apoptotic cells to control the spread of disease (Fujimoto *et al.* 2000). Additionally, they also possess the ability to dampen the immune response at the site of infection through their CD200R antigen, which prevents excessive inflammation and therefore morbidity (Snelgrove *et al.* 2008).
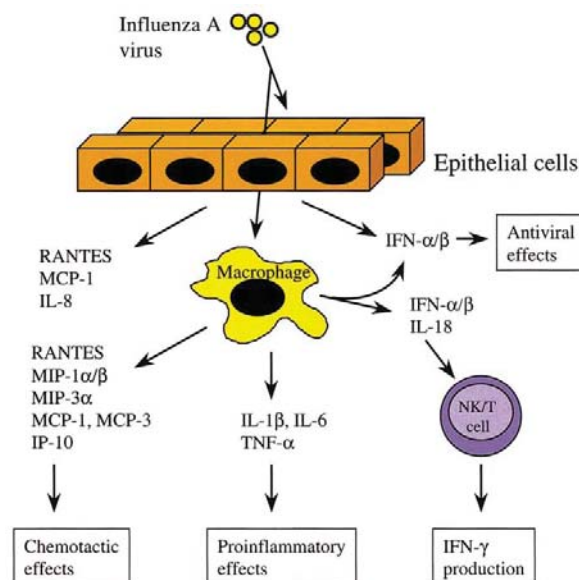
Figure 1.21: Cytokines produced by influenza-infected macrophages and their downstream effects. The production of RANTES, MCP-1 and IL-8 by infected epithelial cells also acts as a chemoattractant for monocyte populations, which mature into macrophages in the lung tissue. (From (Julkunen *et al.* 2001))

Macrophages have evolved several properties that compliment their environmental niche, with specialised populations located in tissues around the body in addition to those deriving from infiltrating monocytes. The lungs possess their own sub-population of macrophages: alveolar macrophages (AMs), which act as sentinels for detecting infection. Notably, macrophages, much like the epithelial cells, can also act as targets for influenza virus infection (Wang *et al.* 2009). However, AMs and bone-marrow derived macrophages differ in their permissibility to influenza infection; whilst general macrophages can become infected with human and avian lineages of influenza, AMs are susceptible to H5N1 infection, but not human H1N1 or H3N2 subtypes (Yu *et al.* 2011; Wang *et al.* 2012). The capacity of macrophages to be infected by influenza viruses, as well as engulf infected cells, has important consequences in their role as antigen presenting cells (APCs), which will be discussed in section 1.4.2. Interestingly, infection with influenza viruses also promotes the ability of macrophages to phagocytose other infected cells (Hoeve *et al.* 2012).

Although macrophages are crucial in ameliorating influenza virus infection, with evidence showing that depletion of these cells within the lungs results in lethal infections in mice and pigs (Tumpey *et al.* 2005b; Kim *et al.* 2008), their proinflammatory response can in itself cause excessive morbidity, as seen with H5N1 infections (Cheung *et al.* 2002). The release of IL-6 and TNFα in particular results in the recruitment of monocytes into the lung, which differentiate into exudate "inflammatory" macrophages, in turn increasing the scale of inflammation in the lungs (McGill *et al.* 2009). Indeed, macrophages have also been recorded as causing excessive damage to the airway epithelial cells, resulting in alveolar leakage; thus making the viral infection increasingly lethal (Herold *et al.* 2008). Therefore, much like other innate immune cells, they play roles in the recovery from, and pathogenesis of, influenza virus (Damjanovic *et al.* 2012).

1.4.1.3.3 Neutrophils

Neutrophils are another class of innate immune cells involved in the acute response to influenza virus infection in the lungs. Indeed, a large proportion of mammalian neutrophils are concentrated within the lung vasculature, although as yet the reasoning for this is unknown (Kolaczkowska and Kubes 2013). However, it is the immune cascade generated by the sentinel mast cells and macrophages (Figures 1.20 and 1.21) within the lungs that signals the

extravasation of neutrophils into the tissue. Once present at the site of infection and activated by the presence of pathogens or chemokines released by infected cells, neutrophils can employ a number of mechanisms to either directly remove the pathogen or attract other immune cell populations through the release of a cascade of cytokines and chemokines (Figure 1.22).
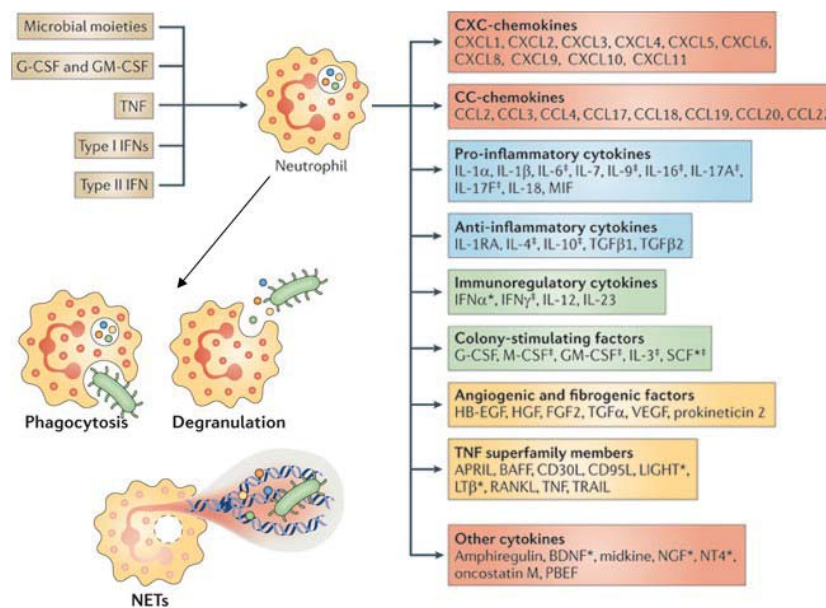
Figure 1.22: Killing mechanisms and signalling molecules generated by neutrophils during the innate immune response. The schematic illustrates the scope of immunomodulation possible by neutrophils. Phagocytosis: the engulfment of pathogens or apoptotic cells by the neutrophil, which are disposed of by reactive oxygen species or antimicrobial agents. Degranulation: the neutophil transports their secretory vesicles to the cell surface and deliver proteases and proinflammatory cytokines into the extracellular space. NETs (Neutrophil Extracellular Traps): these are primarily used to immobilise pathogens and consist of a mixture of the neutrophil's DNA, histones and enzymes. *: only mRNA evidence for production, ‡: data is controversial for human neutrophils. (Redrawn from (Mantovani *et al.* 2011; Kolaczkowska and Kubes 2013))

As with the other immune cell populations, neutrophils are regarding as having polar effects on host morbidity and clearance of virus (Damjanovic *et al.* 2012). Whilst some studies have revealed that neutrophils are one of the leading causes of acute lung injury (Grommes and Soehnlein 2011; Narasaraju *et al.* 2011), others have conversely shown them to be indispensible for viral clearance. This has been clearly shown in mice infected with sub-lethal doses of low pathogenicity X-31 influenza, wherein the mice succumb to infection when neutropenia was induced (Tate *et al.* 2009; Tate *et al.* 2011). This would again suggest that the body must attain a

balance of neutrophil numbers, much like it must with macrophages: too great an infiltration causes excessive cellular damage, while too few leads to uncontrolled viral replication.

1.4.1.3.4 Natural killer cells

Natural killer (NK) cells represent an important arm of the innate immune response to viral infection; they possess the ability to directly lyse and kill infected cells through a balance of stimulatory and inhibitory signals generated by potential target cells (Figure 1.23). Under healthy conditions, cells present major histocompatibility complex (MHC) class I complexes on their surface, which are detected by NK cells; thus they are recognised as "self" and are not killed. However, cells may lose their MHC-I molecules, notably during periods of infection. Importantly, influenza viruses do not appear to stimulate the removal of MHC-I from the cell surface (Achdout *et al.* 2008). Instead, it appears as though influenza virus manipulates the MHC-I complex and repositions it in lipid rafts on the cell surface. This positioning increases the strength of the inhibitory signal sent to NK cells, which increases resistance to NK-mediated attack.
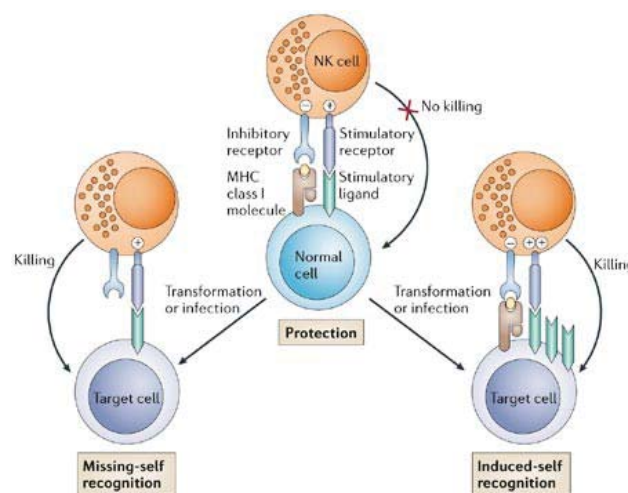
Figure 1.23: Natural killer cell control of activation state. NK cells possess inhibitory and stimulatory receptors on their cell surface wherein the ratio of inhibition to stimulatory signals stemming from the target cell dictates NK cell activation. When MHC-I receptors are withdrawn from the cell surface, typically by viral manipulation, the stimulatory signals result in cell killing (missing-self recognition). It should be noted that MHC-I molecules are the primary mode of inhibition, although other non-MHC ligands can also inhibit NK cell activity. Conversely, an over-proliferation of stimulatory signals on the cell surface, which indicate the presence of infection, will also result in cellular killing (induced-self recognition). (From (Raulet and Vance 2006))

However, despite this manipulation of the MHC-I complex, NK cells are capable of detecting and destroying virus infected cells. This detection of influenza infected cells is primarily driven by an interaction by the NK cells' NKp44 or NKp46 surface receptor and viral HA, which is present on the surface of infected cells (Mandelboim *et al.* 2001; Ho *et al.* 2008). The importance of NK cell populations and in particular their NKp46-HA interactions has been shown in mice with a deletion of the receptor, wherein influenza infection became lethal in mice lacking NKp46 (Gazit *et al.* 2006). Similarly, individuals that develop severe A(H1N1)pdm09 infections have a reduced CD8 T-cell and NK cell count (Fox *et al.* 2012), implicating them as crucial to resistance. However, it is again apparent that the scale of response by this immune cell population plays a role in the pathogenesis of disease, as NK cells have been shown to be detrimental to the host when challenged with high doses of influenza infection (Zhou *et al.* 2013). Importantly, such immunopathology was not observed with low-to-medium doses of inoculating virus.

1.4.1.3.5   Dendritic cells

Dendritic cells (DCs) form the bridging component between the innate and adaptive immune response. Their broad distribution throughout the body and respiratory tissues means that they act as sentinels capable of sensing incoming pathogens and priming the innate immune response; however they also act as one of the primary APCs, along with macrophages, which primes the adaptive response to infection (McGill *et al.* 2009). APC migration, presentation and B and T-cell interaction are discussed in section 1.4.2.1.

DCs, like macrophages, are a heterogeneous population of cells that serve different functions during infection (Hao *et al.* 2008b). Resident in the lungs are the alveolar DCs (aDCs), which are positioned at the epithelial surface to detect incoming pathogens and the interstitial DCs (iDCs), which are the major producers of inflammatory cytokines (McGill *et al.* 2009; Braciale *et al.* 2012). In addition to the resident populations, both plasmacytoid DCs (pDCs) and inflammatory monocyte-derived DCs migrate into the lung tissue following pulmonary infection (McGill *et al.* 2009). The spatial distribution and pre-adaptive immunity response of respiratory DCs is shown in Figure 1.24.
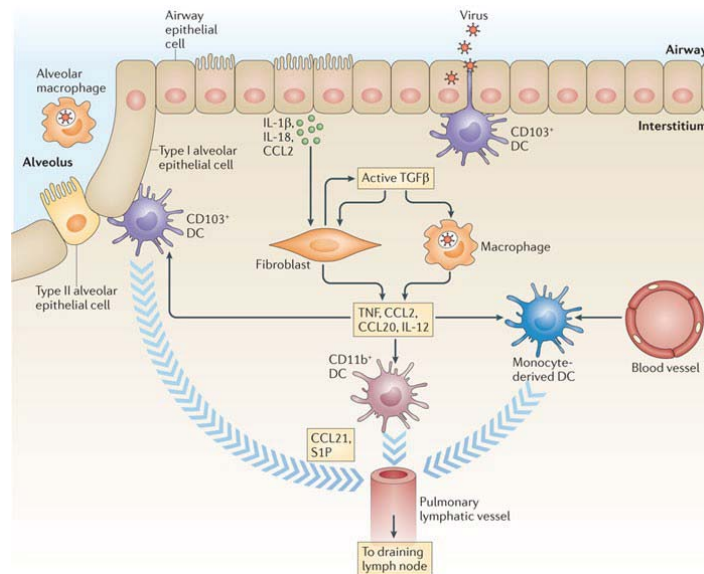
Figure 1.24: The innate immune response of DC populations at the respiratory surface. As described in section 1.4.1.2. the epithelial cells form the primary target for influenza virus and subsequently release cytokine and chemokine signals such as CCL2 (MCP-1), IL-1β and IL-18 into the surrounding cells, such as fibroblasts, who in turn product an active form of TGFβ, which up-regulates the production of inflammatory cytokines by macrophages and other localised immune cells. Such signals result in the maturation of CD103+ aDCs and CD11b+ iDCs, as well as signalling for the extravasation of other DCs into the lung. Antigen acquisition by these cells and those at the epithelial surface result in migration from the lungs in the lymph nodes in order to prime the adaptive immune response. (From (Braciale *et al.* 2012))

Importantly, DCs are capable of becoming infected with influenza virus, but viral replication is aborted within the cells (Bender *et al.* 1998). The result is that the DCs accumulate internalised viral proteins, which they are able to present to the adaptive T-cell populations. One of the most important contributors to the innate immune system is the pDC, as it is one of the most strongly activated cell types during influenza virus infection; leading to the production of large amounts of interferon and proinflammatory cytokines (Summerfield and McCullough 2009). However, this importance is debated, as mice that are depleted for pDC populations have been shown to clear virus as effectively as control mice when infected with influenza (GeurtsvanKessel *et al.* 2008; Wolf *et al.* 2009). However, mice used in these studies were deficient in the intrinsic and induced antiviral Mx proteins, which may not make the results analogous to those seen in humans. It would follow that should Mx have been present, the large amount of IFN released by the pDCs would have up-regulated Mx1, which would have bolstered the mouse's immune state and may have made them resist the virus more effectively than those lacking pDCs.

Regardless of the perceived necessity of certain DC sub-populations, the DC population of the lung is indispensible in clearing influenza virus. Although they are critical in boosting the innate immune response through the generation of IFN, cytokines and chemokines, they also perform a direct role in the adaptive immune response. Their priming of the specific anti-influenza response is now discussed in section 1.4.2.

1.4.2   The adaptive response to influenza virus

The adaptive immune response differs from the innate insofar that it is a specific response to the invading pathogen, which is designed to clear virus from the body, as opposed to primarily slowing its progress. Therefore, when influenza viruses are detected by the innate PRRs, the adjoining adaptive immune response is specific for influenza antigens and directly seeks out virions or cells displaying viral HA or NA on their surface or MHC complexes. As shown in Figure 1.25, the adaptive immune response can largely be classified as having two effects: protection against infection and eradication of established infection.
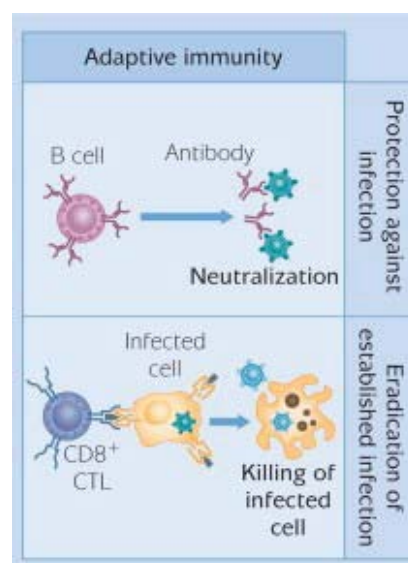
Figure 1.25: The functions of the adaptive immune response to influenza infection. After priming by DC populations, the adaptive immune response is triggered to counteract the infecting virus. This response has two purposes, with the first of which being protection against the virus; neutralising it prior to its entry into cells. This is primarily mediated by B-cells and their generation of antibodies, which bind directly to the HA surface antigens. The second wing of the adaptive immune response is designed to eradicate the already established virus, which is primarily driven by cytotoxic T lymphocytes (CTLs), which detect viral antigen displayed on the surface of infected cells before killing them. From (Saunders 2003)

The effector cells that mediate these processes are two forms of lymphocytes: B-cells and T-cells (Figure 1.26), which are discussed in this section. Crucially, the adaptive response also maintains a memory-based component wherein certain B-cell and T-cell populations retain their specificity for the strain of infecting virus and are retained within the body. Therefore, should the organism become re-infected with the same strain of pathogen, the body can mount a much more rapid response to it; resulting in lower morbidity. It is this memory component that forms the basis of influenza vaccinology, which is discussed in section 1.4.5.
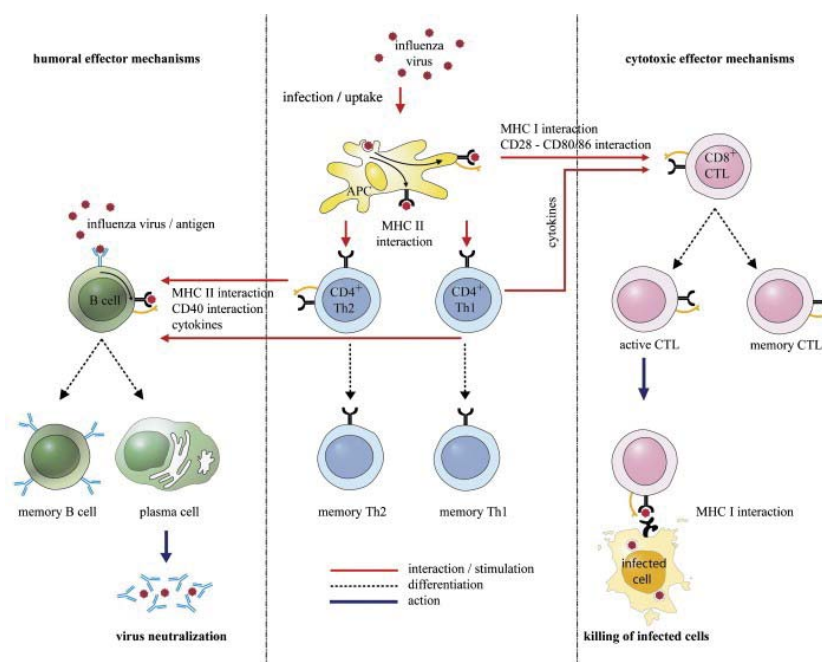
Figure 1.26: Generation of the adaptive immune response to influenza. The schematic illustrates the interactions between APCs and effector cells that lead to the development of the adaptive immune response. The roles of MHC-I and MHC-II complexes in the activation of the repertoire, along with the interactions that lead to the development of humoral and cytotoxic / cell-mediated immunity are discussed in subsequent sub-sections. (From (Holvast *et al.* 2007))

1.4.2.1 MHC and antigen presentation

Although DCs, macrophages and epithelial cells play a key role in the detection of influenza virus and the induction of the interferon signalling cascade, they play a further role as professional APCs; a lynchpin function in the successful resolution of influenza virus infection. Of these cell types (in addition to B-cells), DCs are considered to be the most important APC

(McGill *et al.* 2009). The critical molecules involved in such priming of the adaptive immune response are MHC-I and MHC-II. Both of these complexes are used by DCs to begin the first stages of the adaptive response after internalisation of antigen (via phagocytosis of infected cells, or direct influenza infection) and migration to the draining lymph nodes: priming the CD4 (via MHC-II) and CD8 (via MHC-I) T-cell responses (Holvast *et al.* 2007).

The MHC complexes display "grooves" in their extracellular-facing structures, where antigenic peptides can be loaded into and presented. Typically, MHC-I complexes display antigens that are endogenously derived; therefore signalling that a cell is "self" and should not become the target of attack by innate immune cells such as NK cells (discussed in sub-section 1.4.1.3.4). However, APCs can undergo "cross-presentation" of antigens derived from influenza virus that is replicating in the cytosol (Vyas *et al.* 2008; Ballesteros-Tato *et al.* 2010). Some of the molecular mechanisms involved in this are shown in Figure 1.27. As previously mentioned and shown in Figure 1.26, MHC-I presentation of antigen is critical in activating and focusing the CD8 T-cell response towards influenza virus.
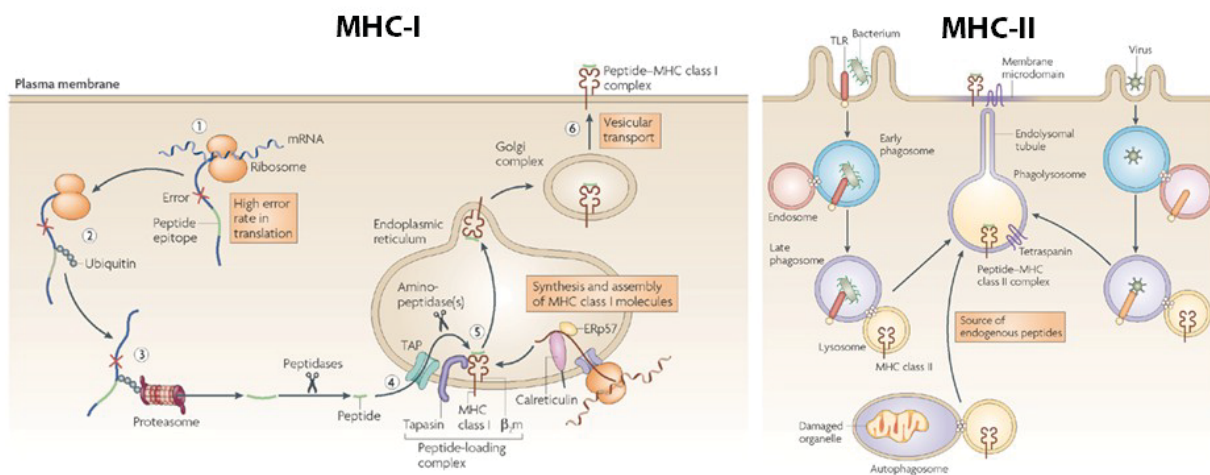
Figure 1.27: Mechanisms underpinning antigen presentation via MHC-I and MHC-II. The six stages of antigen presentation via the MHC-I pathway: 1) acquisition of error-laden protein antigens, 2) misfolded proteins are ubiquitinated for degradation, 3) proteasome degradation, 4) peptides transported to the ER via the transporter associated with antigen processing (TAP) complex, 5) peptide loading onto the MHC-I molecules, 6) transport to the surface via the Golgi. This pathway is also largely followed, but not shown, for MHC-II loading, but differs in the use of lysosomes and phagosomes to acquire the peptide fragments from the exogenous virus. Loaded MHC-II molecules are transported to the surface via endolysosomal tubules. (From (Vyas *et al.* 2008))

MHC-II molecules differ from MHC-I molecules insofar that they are typically used to express exogenously derived antigens on their surface, which are typically acquired by APCs phagocytosing pathogens and infected or damaged cells (Figure 1.27). However, MHC-II molecules are also capable of displaying endogenously generated viral antigens for display (Vyas *et al.* 2008; Eisenlohr *et al.* 2011). Both the endogenous and exogenous peptides are subsequently used to activate CD4+ T-cells, which can then be used to prime the humoral and cell-mediated wings of the adaptive immune response.

1.4.2.2 B-cell response (humoral immunity)

The priming of CD4 Th2 cells subsequently results in the activation and priming of B-cell populations to influenza virus. The key role of B-cells in the adaptive immune response to infection is the generation of a spectrum of antibodies that bind and neutralise the invading pathogen. As seen in Figure 1.26, the stimulated B-cell will develop down one of two pathways: it will either become a resident memory cell, which enables the body to mount a more rapid response should it encounter the same antigenic stimulus, or it will become a plasmablast, which is capable of generating neutralising antibodies.

Broadly, two waves of antibody responses are produced by the plasmablasts in response to viral activation. Chronologically, the first "wave" of antibody production has a weighting towards IgM, the "natural antibody" (Dörner and Radbruch 2007), which although polyvalent and showing low specificity for viral antigens, has been shown to be crucial in aiding the clearance of influenza virus in mice (Kopf *et al.* 2002). The second "wave", which is retained at the mucosal surface and within the serum following influenza infection, primarily consists of IgA and IgG classes of antibody. This second wave is more typically useful in restricting the onset of a re-infection (and in vaccination), as these classes are typically produced after much of the viral burden has been resolved (Lambrecht and Hammad 2012).

Briefly, IgA is largely localised to the mucosa following influenza infection and is secreted into the airways of the nose, trachea and lungs, with a skew towards the upper respiratory tract (Tamura and Kurata 2004). It is a highly potent neutralising antibody that can prevent influenza virus from even attaching to sialic acids; therefore never triggering an innate inflammatory

response (van Riet *et al.* 2012). Conversely, IgG primarily acts in the lower respiratory tract as a secondary defence should the virus evade IgA and infect the lung tissues, at which point IgG-secreting plasmablasts extravasate from the pulmonary blood vessels to lower the extent of viral shedding by replicating viruses (Renegar *et al.* 2004). Both IgA and IgG are crucial in protection of the host from repeat infection by influenza virus and also form the basis of influenza vaccinology, which is discussed in section 1.4.5.

1.4.2.3 Cytotoxic T-cell response (cell-mediated immunity)

Whilst B-cell mediated humoral immune responses are vital in preventing repeat infection, they do not normally serve a central role in clearance of the initial infection. However, CD8+ cytotoxic T-lymphocytes (CTLs) are thought to be crucial to host recovery from these initial infections (Doherty *et al.* 1997; Schmolke and García-Sastre 2010).

After priming by APCs via MHC-I recognition (Figure 1.26), the CD8+ T-cell population can either become a memory cell or can activate and commence killing of infected cells through the use of degrading granzymes (Figure 1.28). Such cytotoxic activity by the CTLs not only correlates with viral clearance, but it may also contribute to the immunopathology associated with severe influenza infections when leukocyte recruitment is dysregulated (Damjanovic *et al.* 2012). However, counter to this, evidence has also shown that CD8+ effector cells also secrete high levels of anti-inflammatory cytokines such as IL-10 (Sun *et al.* 2009). This would suggest that CD8+ T-cells are equally capable of reducing inflammation as they are of generating it.

As with memory B-cells and CD4+ T-cells, the development of memory populations of CTLs is crucial in protecting against re-infection. Like diverse antibody cascades, CTLs can also contribute to heterosubtypic immunity wherein the cells / antibodies recognise antigens from different influenza subtypes (Nguyen *et al.* 1999; Nguyen *et al.* 2007). Not only are such responses useful when considering the route of vaccine administration (intranasal vs. intramuscular, discussed in section 1.4.5), but are important at times of a pandemic when a novel zoonotic subtype is in transmission, as heterosubtypic immunity may provide some protection.
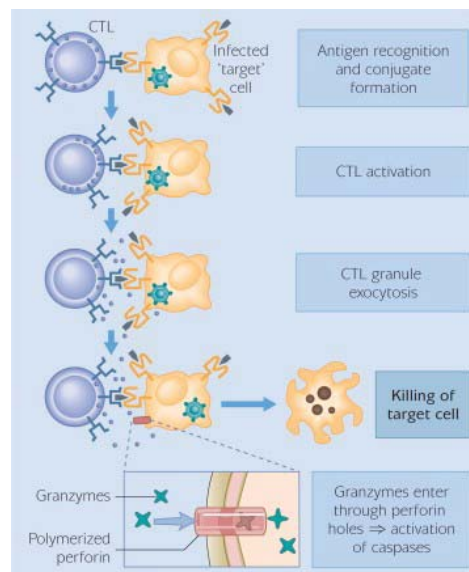
Figure 1.28: Killing mechanism of cytotoxic T-lymphocytes. Interaction via the MHC-I complex allows the CTL to detect an infected target cell. This subsequently leads to the CTL targeting the infected cell and releasing granzymes and perforin, which enter the cell and degrade the DNA and disrupt the mitochondria; causing irreparable damage to the cell. (From (Abbas and Lichtman 2004))

1.4.3   Viral antagonism of the immune response

The host intrinsic, innate and adaptive immune repertoires present a series of formidable barriers to infection for influenza viruses. However, as briefly discussed previously, influenza possesses a number of counter-measures designed to antagonise the immune system and evade clearance before replication and shedding. Primarily, our understanding of these mechanisms relates to the ability of the virus to impede the IFN-signalling pathway that is crucial to the development of the innate immune response; although viruses that directly attack specific immune cell populations to impede the cellular responses to infection have also been observed.

One of the most well-characterised, but still yet to be fully elucidated mechanisms of antagonism by influenza virus stems from the viral NS1 component (Hale *et al.* 2008). As can be seen in Figure 1.29, NS1 is capable of interacting with the host's cells at various points in order to repress the innate immune response by either binding to crucial host proteins, or through up-regulating certain host processes to the benefit of the virus. Indeed, in mice it has been observed that in the first 48h of infection with the PR/8 strain of influenza virus, the host does not mount a robust immune response. It is thought that this so-called "stealth phase" is a result of the

inhibitory mechanisms of NS1; thus allowing relatively unhindered viral replication (Schmolke and García-Sastre 2010). Strikingly, the NS1 component has also been linked to the increased virulence of the 1918 'Spanish' influenza virus. Transferral of the NS1 gene into a laboratory-strain of influenza greatly increased the immunosuppressive abilities of the virus, with lower levels of ISG induction (Geiss *et al.* 2002).
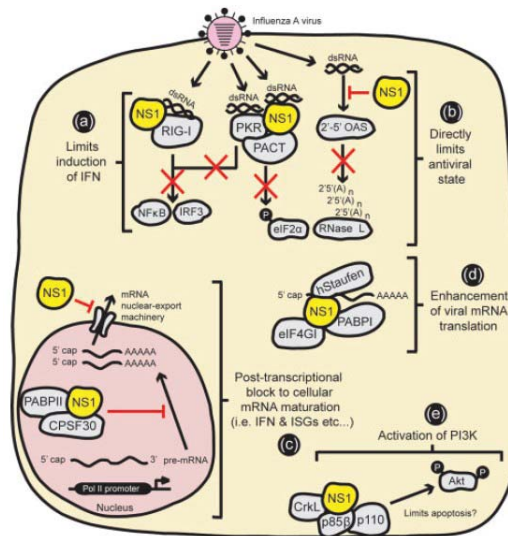
Figure 1.29: The multi-functional role of influenza's NS1 protein in antagonising the cell-autonomous innate immune response. a) pre-transcriptional block of IFN induction, b) PKR and OAS inhibition, c) block of processing and nuclear export of cellular mRNA, d) enhancement of vRNA translation, e) activation of PI3K, which is involved in anti-apoptosis, cell growth and cytokine production. (From (Hale *et al.* 2008))

NS1 is not the only viral protein to antagonise the host immune response. Several polymerase subunits, including PB1-F2 and PB2, also impact on viral pathogenesis through host-virus interactions within the cell (Garcia-Sastre 2011). PB1-F2 exerts its function through the induction of apoptosis within the infected cell, particularly infected CD8 T-cells and alveolar macrophages. This destruction of vital immune cells permits the virus to persist for longer within the host and heighten the pathogenesis of the infection (Zamarin *et al.* 2006). PB2, like PB1-F2, targets the cell's mitochondria, but differs insofar that it has been reported to inhibit the generation of IFNβ; thus suppressing the ISG cascade (Graef *et al.* 2010). Recently, a further polymerase subunit, known as PA-X, has been discovered. Although work as to its function is ongoing, it appears as though it interacts with host mRNA to dampen the host immune response, although PA-X deficient viruses are more pathogenic in mice (Jagger *et al.* 2012). This may be

advantageous as it may delay the arrival of immune cells at the site of infection. Further to the polymerase subunits, viral NP has been shown to correlate with sensitivity to the Mx protein response. Viruses containing A(H1N1)pdm09's NP could kill $Mx1^{+/+}$ mice, whilst avian H5N1 NP are sensitive to Mx (Zimmermann *et al.* 2011; Manz *et al.* 2013). Although influenza viruses only express a small number of effector proteins, it is clear that they possess multiple roles, with some directly involved in host cell antagonism. However, it should be noted that not all influenza subtypes and strains possess the entire repertoire of antagonistic elements (McAuley *et al.* 2010). The presence of different mutations and proteins in different strains of influenza, results in viral evolution and differentiation. Such variations may therefore account for why certain strains are more pathogenic than others, and can develop pandemic potential (McAuley *et al.* 2010; Manz *et al.* 2013).

Further to the suppressive abilities of individual viral proteins, such as NS1, influenza viruses also display a number of techniques where they actively evade certain immune cells, or even target, infect and destroy cell populations in order to prevent viral clearance by the immune system. This is particularly evident with NK Cells, which can be infected and killed by influenza viruses triggering cellular apoptosis (Mao *et al.* 2009). Furthermore, viruses can relocate the MHC-I-HA complex to lipid rafts to prevent NK Cell detection (Achdout *et al.* 2008), or even overwhelm the NK Cells by loading the cell surface with HA, which interestingly produces an inhibitory signal that prevents cell-mediated killing (Mao *et al.* 2010).

1.4.4 Pathogenesis of influenza

Influenza viruses vary greatly in their genetic and antigenic compositions. Similarly, the effects of these viruses on their hosts varies greatly too. Sometimes these pathogenic effects are attributable to the virus itself, which may be configured in a way that results in an infection that causes severe damage to the host (Figure 1.30).
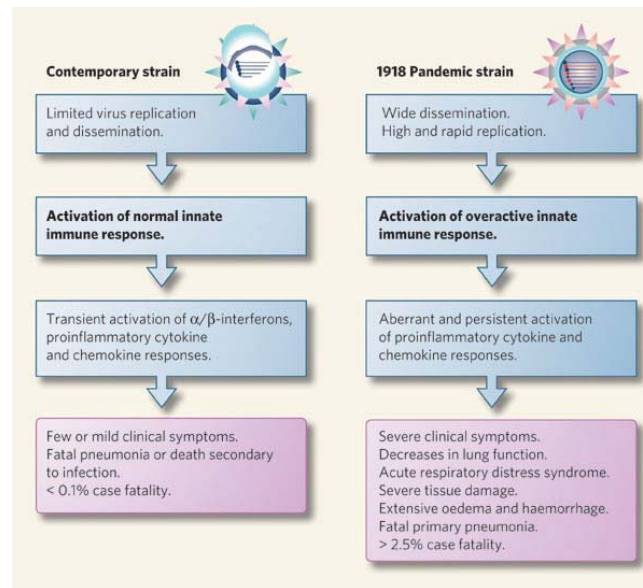
Figure 1.30: Differences in the host response elicited by low and high pathogenicity viruses. Contemporary influenza viruses are used to typify "low pathogenicity" infections, which usually show limited spread, mild symptoms and are cleared by the host. The 1918 'Spanish' virus is used to illustrate the effects of "high pathogenicity" viruses, where viral spread is much more rapid and dispersed in the host, which leads to an aberrant immune response and severe pathological damage, which may result in death. Blue: observations in animal models; purple: clinical observations in humans. (From (Loo and Gale 2007))

However, there is not always a direct correlation between perceived virulence of the virus and the extent of the morbidity and mortality that it causes, as evidenced by the A(H1N1)pdm09 virus hospitalising some previously-healthy individuals, whilst most required no intervention. It is thought that in these instances certain host responses or underlying genetic predispositions may increase host sensitivity to severe viral infections, just as the polymorphic CCR5 receptor can influence potential susceptibility to HIV-1 (Huang *et al.* 1996). Humans can be diagnosed as being "at risk" of severe influenza viral infection if they are of a certain age (<2 years old, >65 years old), have underlying medical conditions (asthma, pregnancy, chronic diseases etc.) (Taubenberger and Morens 2008), or if they have a genetic predisposition to infection (an area currently under studied). This section however focuses on the clinical pathogenesis of disease, regardless of the viral serotype or potential risk factors.

1.4.4.1 Mild clinical symptoms of influenza virus infection in humans

Typically, contemporary strains of circulating human influenza virus cause mild symptoms in those that are infected. However, there can be a spectrum of disease pathology across humans infected with the same viral subtype, in part due to the previously mentioned underlying risk factors, which can consequently make an infection more severe in some, whilst others are asymptomatic.

The symptoms and time course of an uncomplicated influenza infection are shown in Figure 1.31 and stem mainly from the initial viral replication and subsequent release of inflammatory cytokines (Kuiken and Taubenberger 2008). Although much of the histopathological evidence regarding the spread of the virus in these mild infections is weak due to the lack of autopsy material (Taubenberger and Morens 2008), it has been established that the virus primarily infects the upper respiratory tract, from the nasal mucosa to the bronchi, leading to tracheobronchitis (van Riel *et al.* 2007; Kuiken and Taubenberger 2008; Damjanovic *et al.* 2012). However, as shown in Figure 1.31, the virus is soon cleared and the pathological damage is resolved by the host's immune and repair systems.
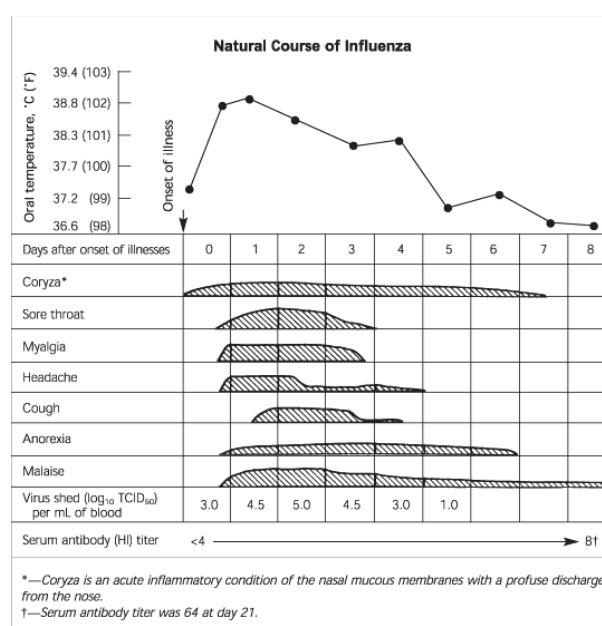
Figure 1.31: Clinical symptoms and disease progression associated with uncomplicated influenza virus infection. Results demonstrate the extent of morbidity from a healthy 28 year old male. (From (Montalto 2003))

1.4.4.2 Severe clinical symptoms of influenza virus infection in humans

Influenza viruses also have the ability to cause severe infections in people due to the pathogenic potential of the virus itself, an exacerbated host immune response, or through a combination of the two (Figure 1.30). Certain viral serotypes are intrinsically more capable of causing more severe symptoms in patients, which subsequently requires the need for medical intervention and hospitalisation (Guarner and Falcon-Escobedo 2009). Avian H5N1 influenza viruses, which have a purportedly high case fatality rate, show a much more diffuse pattern of binding in the respiratory system than contemporary human strains. These viruses can penetrate deep into the alveoli of the lungs and cause alveolar damage (van Riel *et al.* 2007; Kuiken and Taubenberger 2008); something that is not witnessed with "low pathogenicity" viruses.

As discussed previously, the impact of an influenza virus infection can be exacerbated, and the pathological damage increased, by the host immune response. Such aberrant responses are not typically observed in low pathogenicity virus infections; therefore the genetics of the invading influenza virus or underlying host genetic polymorphisms conferring viral susceptibility may be culpable. This has been supported by evidence from fatalities from the 2009 pandemic wherein the virus did not replicate to higher levels, nor did it have an obviously higher mutation rate, in these patients relative to the circulating virus that caused mild illness (Peiris *et al.* 2010). In these cases, and those stemming from high pathogenicity H5N1 infections, cytokine dysregulation, hypercytokinemia or "cytokine storms" have been attributed to varying degrees as causing much of the respiratory damage (Chan *et al.* 2005; de Jong *et al.* 2006; Salomon *et al.* 2007). In these infections, proinflammatory cytokines, such as TNFα and IL-6, are at far higher concentrations than in a low pathogenicity virus infection, leading to the infiltration of macrophages and neutrophils into the airways, causing congestion and further exacerbation of inflammatory signalling, and leading to acute lung injury (Cheung *et al.* 2002; Horimoto and Kawaoka 2005). Indeed, this dysregulation in H5N1 infections may be a result of the viral NS1 being able to resist the antiviral effects of interferon and TNF; leading to the body generating larger, cell toxic responses (Seo *et al.* 2002). However, it should be noted that evidence regarding influenza's ability to acquire resistance to interferon and cytokines such as TNF has recently been disputed in experiments attempting to replicate the work of Seo *et al.* (Ngunjiri *et al.* 2012). This suggests that the virulence of H5N1 may not be due to escape from interferon, as first thought.

The ultimate effect of atypical virus dissemination, cytokine dysregulation and excessive cellular infiltrate is acute lung damage and primary viral pneumonia, which has been seen in patients infected with high pathogenicity H5N1 and H7N9 (Taubenberger and Morens 2008; Peiris *et al.* 2010; Gao *et al.* 2013), and more rarely in infections with the 2009 strain of H1N1 and seasonal viruses (Mauad *et al.* 2010; Peiris *et al.* 2010). The viral pneumonia can manifest as widespread oedema, haemorrhaging, necrosis and hyperplasia amongst other symptoms. This, along with acute respiratory distress syndrome, is one of the leading causes of influenza-related death (Taubenberger and Morens 2008; Louie *et al.* 2009); although systemic viremia and secondary bacterial pneumonia can also contribute greatly to the overall pathology associated with severe influenza infections (Kuiken and Taubenberger 2008).

1.4.5   Influenza vaccinology

One of the most effective therapies used to protect against influenza virus infections are vaccines (Nichol 2003). Vaccines are a critical way of protecting ourselves and various domesticated animal species from contracting influenza virus, which is especially important during a pandemic when individuals may encounter a novel zoonotic virus (Ferguson *et al.* 2006). Although the technology used to generate these vaccines is beyond the scope of this discussion, it is suffice to say that the advances in this area from the current industry standard of propagation in chicken eggs through the use of cell culture bioreactors, recombinant proteins and entirely novel forms of vaccine such as those based on DNA and nanoparticles will only improve our pandemic preparedness (Cox and Hollister 2009; Lambert and Fauci 2010; Kanekiyo *et al.* 2013). This section details two of the major routes of administration of the current generation of vaccines: intramuscular trivalent inactivated vaccine, and intranasal live attenuated vaccine, and evaluates their relative effectiveness.

1.4.5.1 Intramuscular, inactivated vaccine

The traditional mode and route of vaccine administration is intramuscular (IM) injection of inactivated virus, which accounts for over 90% of vaccines administered (Osterholm *et al.* 2012). The trivalent inactivated vaccine (TIV) consists of three strains of influenza virus that aim to antigenically match those viruses circulating in the current season: typically H1N1, H3N2 and an influenza B strain. However, the quadrivalent influenza vaccine (QIV) has recently been

approved and will potentially supersede the TIV with its inclusion of a second influenza B strain (Lee *et al.* 2012).

Inactivated vaccines are produced and formulated in several different ways: whole inactivated, split virion and subunit. "Whole inactivated" contains the entire killed-virus, "split-virion" is surfactant treated and contains all envelope proteins and "subunit" only contains HA and NA proteins (although typically just HA). All vary in the degree of host immunogenic response they induce, but also have differing side-effects, with the subunit eliciting the weakest response, but also having the least side effects (Geeraedts *et al.* 2008). Regardless of mode of vaccine used, the aim is to deliver an antigenic stimulus into host tissues, which is consequently processed by APCs and an adaptive immune response mounted. The typical immunoglobulin profile generated by IM vaccination is biased towards an IgG and IgM profile in the serum, owing to the fact the virus was delivered into a non-respiratory tissue (Chen *et al.* 2001a; Tamura and Kurata 2004). IM vaccines therefore generate poor mucosal IgA antibodies, which as detailed earlier, are important in preventing influenza from binding to the respiratory epithelium (sub-section 1.4.2.2).

1.4.5.2 Live attenuated vaccines

Live attenuated influenza vaccines (LAIVs) differ from the traditional IM influenza vaccines insofar that they: 1) are administered intranasally and 2) are replication-competent. LAIVs rely on the vaccine to mimic a natural influenza infection without causing morbidity to the patient. This is achieved through the use of an attenuated form of virus that restricts its infective capacity; in currently available commercial LAIVs this is achieved through cold-adaptation, wherein the virus can solely replicate in the cooler nasal cavity, as opposed to in the $37^{o}C$ lower respiratory tract (Figure 1.5) (Maassab and Bryant 1999).

The fact that LAIVs mimic natural infections results in a similar adaptive immune response to that described in 1.4.2; namely the production of mucosal IgA and systemic IgG antibodies in addition to the generation of a site-directed CTL response and the production of long-lived CTL memory cells (Cox *et al.* 2004; Powell *et al.* 2007). Importantly, these vaccines limit the inflammatory cascade following infection post-immunisation (Lanthier *et al.* 2011). These

responses are important as they are triggered at the primary site of infection: the respiratory tract. In particular, the generation of CTLs has important implications as they also mean that treatment with LAIVs confers the patient with heterosubtypic immunity, which is not seen with traditional IM vaccines (Tamura and Kurata 2004). However, the IgG response elicited by intranasal vaccines has been reported to be inferior to those administered IM (Beyer *et al.* 2002).

Currently, studies debate which form of influenza vaccine is superior (Cox *et al.* 2004) with some observations being biased by publications involving individuals working for pharmaceutical companies that could financially benefit from positive findings (Jefferson *et al.* 2010). Independent meta-analyses of published studies indicate that the two different routes of immunisation result in similarly efficacious protection against influenza-related illness (Beyer *et al.* 2002). However, it is noted that the LAIVs are more efficient at protecting children <7 years old, but are only moderately-protective in the elderly (Cox *et al.* 2004; Osterholm *et al.* 2012).

1.5   Mouse models of influenza virus infection

One of the primary ways of studying influenza viruses, the host antiviral immune response, and testing the feasibility of vaccine approaches is through the use of model organisms. Although influenza is restricted in the wild to animals such as birds, pigs and horses, the feasibility of routinely using these organisms for laboratory study is low. Various surrogate animals are therefore routinely used in the study of influenza; each with their own advantages and disadvantages.

The most common models used to analyse influenza pathogenesis are mice, ferrets and non-human primates (O'Donnell and Subbarao 2011). Mice have the advantage of being cheap and having well-understood and genetically alterable genomes, but cannot transmit virus and are not natural hosts. Ferrets are natural reservoirs of virus and can transmit virus via aerosol, but are more expensive and genetic knockout animals are not available. Finally, non-human primates are the most anatomically and genetically similar to humans and therefore have great relevancy, but are extremely expensive and have many ethical issues regarding their *in vivo* use. This section will focus on the use of the mouse model, as the availability of genetic knockout mice is a huge asset in understanding the host-virus interactions that occur during influenza infection.

1.5.1   The influence of mouse background

Laboratory mice (*Mus musculus*) are not a homogeneous population and therefore display various unique phenotypes. At present, over 450 strains of inbred mice exist, each with their own unique set of phenotypic traits (Beck *et al.* 2000). Such traits vary greatly from increased tendency to alcohol dependency to propensity to generating cancers, but also include susceptibility to pathogens, including influenza. In Figure 1.32, 21 of the predominant inbred mouse strains are shown and their mouse lethal dose (MLD) for influenza virus is recorded. One can see that this dose varies by approximately $5 \times \log_{10}$ across the strains; highlighting how disparate the susceptibility of these mouse populations is. It is not just the susceptibility to lethal infection that varies between strains, as the extent of the pathological damage, viral burden and cytokine response also vary between strains when challenged with the same infectious dose of influenza (Srivastava *et al.* 2009). Comparison of the transcriptome of certain mouse strains following infection with influenza A virus has revealed that these mice have differing gene expressions and has identified several candidates genes as causative (Boon *et al.* 2009). It is thought that such observations may explain why humans show such differing responses to influenza virus.
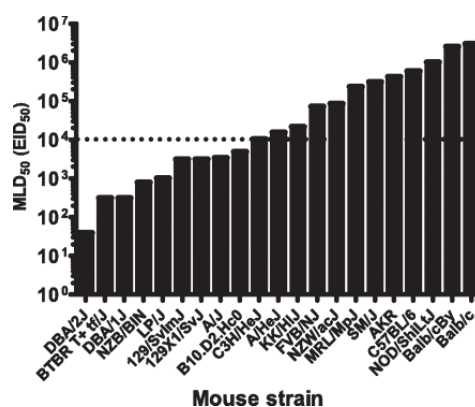
Figure 1.32: Susceptibility of 21 inbred mouse strains to highly pathogenic H5N1 influenza A virus. The 50% mouse lethal dose (MLD$_{50}$) of A/Hong Kong/213/03 is shown for all inbred strains. The dotted line indicates the median dose required for lethality. (From (Boon *et al.* 2011))

Although some of these differences are due to single gene mutations (e.g. in *Mx1*), which are relatively simple to identify and quantify, a large proportion are due to polygenic traits arising from multiple quantitative trait loci (QTL) throughout the murine genome, which mimics the

situation seen in an outbred population such as humans. Although studies, such as that described previously, have shown that differences between inbred lines can be caused by differing gene expression levels (Boon *et al.* 2009), a more comprehensive picture is needed to include single nucleotide polymorphism (SNP) data.

Recently there has been a move to ensure that mice are bred with the intent of mimicking the complex situation seen in humans, which has led to the founding of the Collaborative Cross (CC) (Churchill 2004; Collaborative Cross 2012). The aim of this project is to generate blends of inbred mice that carry QTL influencing phenotypic traits that reflect human phenotypes. Although ongoing, the initial generation of the "pre-CC" murine lines has resulted in the production of a cohort of mice with 40 million SNPs across their genomes (Collaborative Cross 2012). In particular relation to influenza, several studies have now started to provide results based on the pre-CC murine models. One such study focused on the "extreme" responders to influenza virus infection: those that lost <5% of their body weight by day 4 (low responders) and those that lost >15% by the same time point (high responders) (Bottomly *et al.* 2012). This study successfully identified 21 eQTLs implicated as being causative in the host response to influenza; many of which would have been overlooked using standard, defined inbred lines, but became apparent through the use of the crossing procedure of the CC. The utility of this approach has been further shown in a study that demonstrated that a striking 9.7% of the total variation in weight loss (see sub-section 1.5.2 for the importance of weight loss in murine pathology) was attributable to a single QTL containing 69 genes and 10 non-coding RNAs (Ferris *et al.* 2013). Although these are based on pre-CC mouse populations, they demonstrate the potential utility of the vast CC library in identifying critical QTLs and SNPs that may be relatable to human disease.

The addition of the CC to the already established inbred lines and knockout lines of mice will only further our understanding of the complexities underpinning influenza pathogenesis in humans. By the same token, the establishment of such a broad array of murine lines also establishes the fact that considerable variation can exist within mice and that their backgrounds can greatly influence disease severity.

1.5.2 A "typical" phenotypic response

Regardless of mouse background, certain phenotypic traits are common amongst mice when infected with influenza virus, although the extent of the phenotypic change is dependent upon both mouse and virus strain used in the experiment (sub-sections 1.5.1 and 1.5.3, respectively). The most commonly used measure of morbidity following influenza infection in mice is weight. When infected with influenza, mice will typically show an initial loss of body weight (the extent of which is strain and virus specific), which typically persists until 7 days post-infection after which point the mice will regain their weight (Figure 1.33). The "typical" phenotypic responses described here are primarily concerned with mouse-adapted non-lethal influenza viruses such as PR/8 or X-31, which is an engineered hybrid of human A/Hong Kong/1/68 [H3N2] and mouse-adapted A/Puerto Rico/8/33 [H1N1].
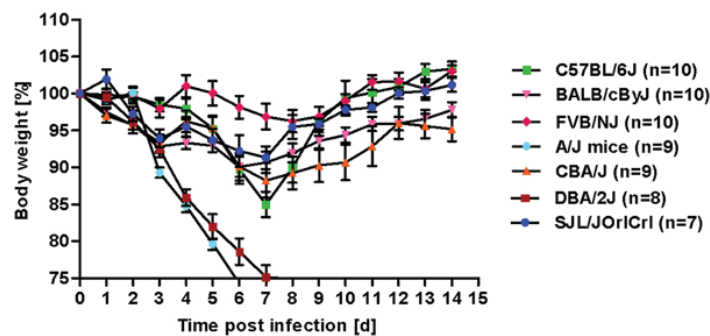
Figure 1.33: Weight loss induced by influenza A virus infection in an array of inbred strains of mice. All mice were infected with 3000 FFU (fluorescence forming units) of PR/8 H1N1 influenza and monitored for weight loss. Note the differences in susceptibility between inbred strains, but notice that the majority follow the same weight loss profile over the duration of the challenge. (From (Srivastava *et al.* 2009))

The weight loss profile of mice differs from the associated viral load and the local and systemic immune response. Typically, influenza virus reaches peak titres between 2-3 days post-infection before declining to a level that it is undetectable by day 10 post-infection (Flynn *et al.* 1999). The weight loss profile correlates most strongly with the extent of the cellular infiltrate and immune response by the host (Flynn *et al.* 1999), and not the viral burden, which is similar to the situation seen in humans wherein the malaise and morbidity is predominantly caused by the host immune response upon infection with seasonal influenza viruses. However, unlike humans, mice

to not exhibit fever and instead display hypothermia. Additionally, the temporary pathological damaged caused by the virus is typically located in the lower respiratory tract of mice, as opposed to the upper tract, as is seen in humans (Bouvier and Lowen 2010).

1.5.3   The influence of influenza strain on murine pathology

Deviations from the "typical" phenotypic response to influenza virus infection are seen when infecting with higher pathogenicity (HP) strains of virus (Bouvier and Lowen 2010). Just as the mouse genetic background can influence susceptibility to a lethal infection (Figure 1.33), the viral strain can also influence the morbidity and mortality profile of the mice in a similar way. As seen in Table 1.4, viruses vary greatly in their lethality, with the avian H5N1 strains of virus capable of killing mice with as few as 13 PFU of virus, whilst the mildly pathogenic human pandemic H1N1/09 strain of influenza can require $>10^6$ PFU for the mice to succumb to infection.

Table 1.4: Susceptibility of BALB/c mice to different strains of influenza virus.

Viral Strain	50% lethal dose (LD_{50})
PR/8 [H1N1]	10^2 PFU
WSN/33 [H1N1]	$10^2 - 10^{3.3}$ PFU
X-31 [H3N2]	10^6 PFU
1918 Spanish influenza [H1N1]	$10^{3.5}$ PFU
A/California/04/2009 [H1N1]	$10^{4.7} - >10^6$ PFU
A/Vietnam/1203/2004 [H5N1]	$10^{1.3}$ PFU
A/Hong Kong/483/1997 [H5N1]	$10^{1.6} - 10^{2.4}$ EID_{50}

PFU: plaque forming units; EID_{50}: 50% egg infectious dose. Adapted from (Bouvier and Lowen 2010)

However, it should also be noted that there can also be variation in the lethality of viruses amongst those within a certain strain (Belser *et al.* 2010). For instance, two different isolates of A(H1N1)pdm09 from the Netherlands and California, which are nearly identical at the sequence level, exhibit differing lethality in C57BL/6 mice despite being administered at the same dose, with the Netherlands strain proving to be more virulent than that from California (Manicassamy *et al.* 2010).

In addition to the lethality of the virus, there is variation in the symptoms and course of disease caused by the virus upon infection; similar to the situation in humans. Severe infections of mice can be observed prior to lethality, as the mouse typically exhibits piloerection, fur ruffling, lethargy and anorexia. In the respiratory tract, the HP strains of virus, such as avian H5N1, do not exhibit the same profile of viral replication described in section 1.5.3, as the virus reaches high titres at 2-3 days post-infection but persists at this level until death (Perrone *et al.* 2008); showing no characteristic decline. The persistent presence of the virus in the respiratory tract is not the only deviation from the "typical" phenotypic response, as extensive cellular infiltration, necrosis, oedema, cytokine dysregulation and lymphopenia have also been recorded in challenges with HP strains of influenza in mice (Dybing *et al.* 2000; Tumpey *et al.* 2000; Kobasa *et al.* 2004; Perrone *et al.* 2008). Furthermore, the virus can disseminate through the mouse and spread to multiple organs, including the heart and brain (Maines *et al.* 2005; Bouvier and Lowen 2010). All of these factors highlight the broad spectrum of phenotypes that can arise during influenza virus infection in mice.

1.5.4   The use of knockout mouse models for studying the host immune response

One of the final variables that can be altered to examine the extent of host-virus interactions in mice is gene expression, through the use of knockout mice (Figure 1.34). Although not all knockouts are successful, owing to difficulty in targeting an allele or prenatal lethality caused by the gene loss, the knockout mouse models provide an invaluable way to elucidate the role of a gene in a mammalian organism. This approach has distinct advantages over cell-based RNAi screening approaches, as a knockout mouse will show the potential adverse-effects of deleting a gene that may not be apparent from solely relying on cell lines.
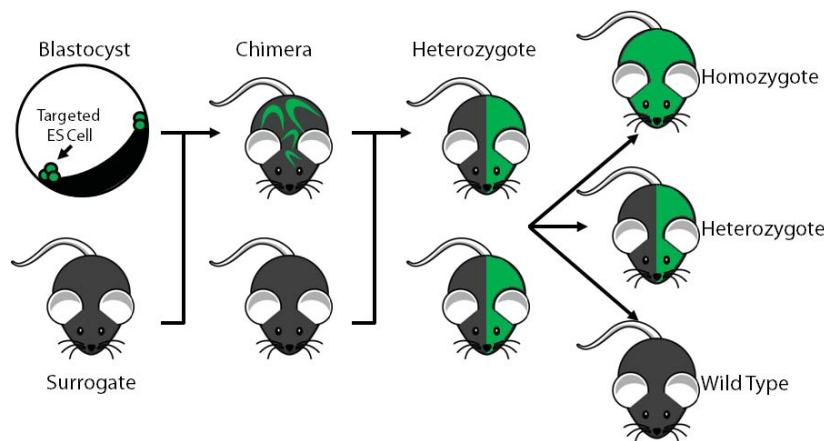
Figure 1.34: Schematic of the generation of knockout mice. Targeted embryonic stem cells (ES cells) carrying the gene deletion are inserted into blastocysts, which are then brought to term by surrogate wild type mice. These chimeras are crossed onto wild type backgrounds until a pairing is found to carry the gene deletion in the germ line, which generates a heterozygote. The production of pure homozygote knockout mouse lines then requires heterozygote x heterozygote pairings.

Large scale knockout programmes, such as the Mouse Genome Project (MGP; http://www.sanger.ac.uk/mouseportal), have been established to systematically ablate individual genes in the mouse genome and record their resultant phenotype through an array of screening techniques, which look for differences in physiology, behaviour and immunity, amongst others. In relation to immunity, mouse models have been used to study the functions and criticality of immune cell populations (Gazit *et al.* 2006; Snelgrove *et al.* 2008), but have also been used to test for host-pathogen interactions. The removal of certain genes has revealed roles in the restriction of a variety of pathogens including bacteria, viruses and parasites (Riopel *et al.* 2001; Kurt-Jones *et al.* 2004; Zheng *et al.* 2008; Kum *et al.* 2011; Longley *et al.* 2011).

In relation to influenza virus infection, knockout mice have yielded insights into the host-virus interactions that occur over the course of infection. Numerous genes have been shown to be involved in restricting influenza in both the innate and adaptive arms of the immune system (Bergmann *et al.* 2000; Gazit *et al.* 2006; Koerner *et al.* 2007; Lenschow *et al.* 2007). Furthermore, knockout mice have revealed how ablating the expression of certain proteins, such as TLR3, IL-15 and IL-17 can conversely reduce the damage and mortality associated with influenza (Le Goffic *et al.* 2006; Crowe *et al.* 2009; Nakamura *et al.* 2010). Therefore this model

organism can reveal genes and proteins that prevent, as well as contribute to, the overall pathogenesis of influenza.

In mice, microarray analysis has shown approximately 495 genes have significantly (>2-fold) altered gene expression in the lungs during influenza virus infection (Ding *et al.* 2008). Thus, any dysregulated gene may be an interesting target for investigation of a role for that gene in antiviral immunity. An alternative approach through which to identify which knockout mouse should be generated and tested with influenza is to use RNAi screens (Brass *et al.* 2009; Shapira *et al.* 2009; Karlas *et al.* 2010) as a proof-of-principle that the gene of interest may have a phenotypic effect. Alternatively, one could use SNPs purported to be involved in influenza resistance or susceptibility from human studies to inform which mice should be prioritised for generation.

Knockout mice are therefore a valuable resource for identifying drug targets and uncovering the effects of genetic mutations that may pervade in the human population. Although some have questioned the utility of pure knockout mouse lines as representing the situation in humans, where genetic polymorphisms are more abundant than gene ablations (Ferris *et al.* 2013), the targeted approach of knockout mice allows for a deepening of our knowledge of mammalian genetics; especially in relation to genes affecting immune function and pathogen resistance.

1.6   Hypothesis

Interferon-inducible transmembrane 3 (IFITM3) has been shown to be a potent antiviral molecule *in vitro*, with the capacity to restrict multiple pathogenic viruses including influenza, West Nile and dengue viruses. At the commencement of my studies in 2010, little was known about the actions of IFITM3, except that it exhibited a transmembrane topology and was dispensable for embryonic development (Lange *et al.* 2008). Brass *et al.* (2009) provided the first evidence for the IFITM family controlling viral infection, but all work was conducted *in vitro*. Therefore, I hypothesise that Ifitm3 will be critical in restricting influenza virus in an *in vivo* mouse model, and that the removal of this gene will result in heightened pathological damage and an increased viral burden; ultimately leading to mortality.

1.7   Thesis aims

The aims of this thesis are to move beyond *in vitro* studies and characterise the *in vivo* effects resulting from a loss of Ifitm3, using a knockout mouse model that was generated on-site at the Wellcome Trust Sanger Institute (WTSI). The primary aim of the work described in the thesis is to use influenza A viruses to determine the Ifitm3 knockout mouse's response to viral infection and fully characterise the resultant phenotype. I hope to gain an understanding of the role of Ifitm3 at the respiratory surfaces and how its loss affects the local and systemic response to influenza. Should there be a dramatic phenotype, then one could infer that similar results would be seen using other pathogens that have been shown to be restricted by IFITM3 *in vitro*. Furthermore, should there be a phenotype, I would be interested to determine whether humans carry any polymorphisms in their *IFITM3* alleles, as this gene could potentially be a biomarker for viral susceptibility.

A second aim of my thesis is to determine vaccine efficacy in the *Ifitm3*^{-/-} mice, using commercially available LAIV. As previously stated, influenza viruses can replicate more efficiently in IFITM3 deficient cells, therefore it is pertinent to address the safety and efficacy of LAIVs, as they potentially represent the most hazardous form of vaccine in organisms lacking IFITM3.

The final aim of my work is to explore the effects of a loss of *Ifitm3* in mice using a range of pathogenic micro-organisms. The current body of literature suggests that *IFITM3* is up-regulated following challenge with non-viral pathogens, but proof has yet to be provided as to whether IFITM3 is genuinely involved in the restriction of these pathogens, or whether it is an artefact generated by an increase in interferon levels in the infected individual.

2 Materials and Methods

2.1 Materials

2.1.1 Media

Table 2.1: Media compositions used in the study.

Name	Components	Manufacturer
Complete DMEM (cDMEM)	Dulbecco's Modified Essential Medium	Invitrogen
	10% fetal calf serum (FCS)	Sigma
	Penicillin / streptomycin	Invitrogen
	2mM L-glutamine	Invitrogen
Serum free medium (SFM)	Dulbecco's Modified Essential Medium	Invitrogen
	Penicillin / streptomycin	Invitrogen
	2mM L-glutamine	Invitrogen
Overlay medium	Avicel medium (see below)	AMC Corporation
	SFM	Invitrogen
	0.2% BSA	Sigma
	2µg/ml TPCK-trypsin	Worthington Biochemical Corporation

Complete F-12K (cF12-K) consists of the same components as cDMEM, but with the replacement of DMEM by Ham's F-12K cell culture medium (Invitrogen). All other media, including RPMI 1640 and Leibovitz's L-15 were also supplied by Invitrogen.

Avicel medium (Matrosovich *et al.* 2006) was made by dispersal of 2.5g of Avicel powder (AMC Corporation) in 100ml of distilled water and stirred for one hour at room temperature. The homogeneous solution was autoclaved at $121^{o}C$ for 20 minutes to sterilise and it was allowed to cool to room temperature before use.

2.1.2 Cell lines

Table 2.2: Cell lines used during the study.

Name	Source	Description	Culture Media
MDCK	ATCC (CCL-34)	Canine kidney	cDMEM
MDCK SIAT-1	ECACC (05071502)	Canine kidney, sialic acid over expression	cDMEM
A549	ATCC (CCL-185)	Human lung epithelium carcinoma	cF-12K
U-2 OS	ECACC (92022711)	Human bone osteosarcoma	cDMEM
LA-4	ATCC (CCL-196)	Mouse lung epithelium adenoma	cF-12K

ATCC: American Type Culture Collection; ECACC: European Collection of Cell Cultures

2.1.3 Viruses

Table 2.3: Viruses used during the study.

Name	Subtype	Notes	Source
A/X-31	H3N2	Mouse-adapted	Prof. T. Hussell, Imperial College
A/Puerto Rico 8/33	H1N1	Reverse-genetics engineered delNS1: strain of PR/8 carrying a deletion of the viral NS1 gene.	Prof. P. Digard, Cambridge
A/WSN/33	H1N1	Lab-adapted	ATCC
A/England/195/09	A(H1N1)pdm09	Human isolate	Prof. P. Digard, Cambridge
A/California/07/09	A(H1N1)pdm09	Human isolate	NIMR, England
B/Bangladesh/3333/2007	B	Human isolate	NIMR, England

2.1.4 Oligonucleotide primers

Table 2.4: Custom-designed primers used during the study.

Target	Primer direction	Primer sequence (5'- 3')
Influenza matrix 1 (*M1*)	F	TGAGTCTTCTAACCGAGGTC
	R	GGTCTTGTCTTTAGCCATTCC
Mouse *β-actin*	F	CTAAGGCCAACCGTGAAAAG
	R	ACCAGAGGCATACAGGGACA
Mouse *Gapdh*	F	TTGGGCTACACTGAGGACCAG
	R	GATAGGGCCTCTCTTGCTCAG
Mouse *Ifitm3*	F	GTTATCACCATTGTTAGTGTCATC
	R	AATGAGTGTTACACCTGCGTG
Wild type *Ifitm3*^EGFP / *Ifitm3*^+/+ allele	F	GACTGCATAGCCACCGAAGATATTCC
	R	CCCATCTCAGCCACCTCATATTCTTCC
Knockout *Ifitm3*^EGFP / *Ifitm3*^-/- allele	F	GCAGAAGAACGGCATCAAGGTG
	R	CCCATCTCAGCCACCTCATATTCTTCC

All other primers were pre-designed and TaqMan-conjugated with non-disclosed sequences (Applied Biosystems).

2.1.5   Antibodies for flow cytometry

Table 2.5: Antibodies used for flow cytometry during the study.

Target	Conjugation	Host	Isotype	Source
CD4	PerCP-Cy5.5	rat	$IgG_{2a,k}$	BD Biosciences
CD8a	PE-Cy7	rat	$IgG_{2a,k}$	BD Biosciences
CD69	APC	hamster	$IgG_{1,A3}$	eBioscience
NKp46	BD-V450	rat	$IgG_{2a,k}$	BD Biosciences
CD11b	PerCp-Cy5.5	rat	$IgG_{2a,k}$	BD Biosciences
CD11c	PE-Cy7	rat	$IgG_{2a,k}$	BD Biosciences
F4/80	APC	hamster	$IgG_{1,A3}$	AbD Serotec
Ly6g	BD-V450	rat	$IgG_{2a,k}$	BD Biosciences
MHCII	PE	rat	$IgG_{2a,k}$	BD Biosciences
B220	PerCp	rat	$IgG_{2a,k}$	BD Biosciences
CD19	PE-Cy7	rat	$IgG_{2a,k}$	BD Biosciences
Influenza NP	FITC	mouse	IgG_1	Abcam

2.1.6   Silencing RNAs (siRNA)

Table 2.6: List of Ambion-validated siRNAs used in the study.

Target	Ambion product number	
Arcn1	s1541	s1542
Calcoco2	s19994	s19995
Copg	s22430	s22431
Gapdh	4390849	-
Ido1	s7425	s7426
Ifitm3	s195033	s195034
Tm9sf4	s18882	s18883
Sms	s13173	s13174

2.1.7   Mice

All knockout mice were generated at the WTSI as part of the Mouse Genetics Programme, except for the *Ifitm3*^{-/-} mice. The *Ifitm3*^{-/-} mice and their littermate controls were generated as a faculty project by Dr. David Adams, as described previously (Lange *et al.* 2008). Briefly, the knockout mice had their *Ifitm3* expression ablated by the introduction of an EGFP coding region into exon 1. Genetic testing (Charles River, UK) showed all mice to be >95.5% C57BL/6.

2.2 Methods

2.2.1 Animal methods

2.2.1.1 Mouse infection

Background matched wild type (WT) and *Ifitm3*$^{-/-}$ mice were challenged with influenza at an age of 8-10 weeks. Groups of >5 isofluorane-anaesthetised mice were intranasally inoculated with influenza virus diluted in sterile PBS (Sigma-Aldrich); totalling 50µl. Mice were monitored daily and sacrificed by cervical dislocation at pre-determined time points, or when their weight loss exceeded 25% of their original weight. All animal husbandry and killing are in accordance with UK Home Office guidelines, UK Animals Scientific Procedures Act 1986 under the project license PPL80/2099 or PPL80/2596.

2.2.1.2 Titration of virus in mice

Mice were infected with serial 10× dilutions of X-31, PR/8 and England/195 influenza viruses in order to determine viral infectivity *in vivo*. Doses were subsequently refined to give non-lethal doses; thereby resulting in 10-15% weight loss by day seven post-infection in WT C57BL/6 mice. If a virus used at a near-neat dilution could not elicit sufficient weight loss the virus was subsequently mouse-adapted. This was accomplished by infecting mice with a high dose of virus, as described in sub-section 2.2.2. Mice were killed day three post-infection and their lungs were excised. All lung tissue was homogenised in a rotor-stator homogenizer in 1ml DMEM. The homogenate was centrifuged at 596×g for five minutes and the supernatant was used to reinfect a new cohort of mice. This process was repeated until the virus developed sufficient pathogenicity in mice.

The final influenza doses used in the study were as follows: X-31, 1×10^4 PFU and England/195, 200 PFU. Both of these doses resulted in non-lethal infections in C57BL/6 mice, and a peak weight loss of ~15% by day 7 post-infection. All feasibly accurate PR/8 doses (>50 PFU) resulted in mortality in our mice. For lethal challenge experiments following vaccination, a 10× lethal dose (LD$_{50}$) was used to determine vaccine efficacy.

2.2.1.3   Mouse vaccination

Mice were anaesthetized and inoculated intranasally with 20μl (1/10th human dose) of FluMist (2012-2013 formulation; MedImmune), which was followed by a vaccine boost three weeks later. Animals were challenged with 10× LD$_{50}$ of England/195 (2000 PFU) six weeks after the initial immunization. Mice were also given a higher dose of FluMist (50μl: 1/5th human dose) to test their tolerance to the vaccine and were monitored and weighed for 10 days after their initial dose. Additionally, FluMist vaccine was substituted for an attenuated strain of PR/8 lacking a functional NS1 gene (delNS1) for certain experiments. This virus was administered intranasally at the same time intervals as described above and mice were subsequently challenged with 5000 PFU of PR/8.

Mice were also vaccinated with Fluvirin (Novartis). The experimental conditions were the same as those for FluMist, but the vaccine (50μl of vaccine diluted 1:1 in sterile PBS) was injected twice, three weeks apart, intramuscularly into the hind leg.

2.2.1.3   Anti-fungal treatment

AmBisome (Gilead) was resuspended in sterile water (Sigma), as per the manufacturer's instructions, and subsequently diluted in 5% dextrose solution (Sigma) to provide a dose of 3mg/kg (milligrams of AmBisome per kilogram of mouse body weight). 200μl of the diluted drug was delivered into mice intravenously two hours before infection and at days 2 and 4 post-infection. Mice were monitored daily for signs of illness and were sacrificed using the guidelines in sub-section 2.2.1.1.

2.2.1.4   Bone marrow transfer

Bone marrow was flushed with Hank's buffered salt solution (HBSS; Sigma) from WT and *Ifitm3*$^{-/-}$ mice's hind femurs using a 21-gauge needle. Bone marrows from multiple mice were pooled by genotype and red blood cells were lysed by the addition of 10ml ACK cell lysis buffer (Invitrogen). Treated cells were subsequently centrifuged at 400×g for 10 minutes, the supernatant removed and the pellet washed twice with HBSS. WT and *Ifitm3*$^{-/-}$ bone marrow were resuspended at a concentration of 5×10^6 cells/ml. 200ml of WT or *Ifitm3*$^{-/-}$ bone marrow was immediately injected intravenously into the tails of irradiated (2 × 4.5Gy doses) recipient

WT or *Ifitm3*^{-/-} mice. All mice were then left to recover for six weeks until challenge and were kept on drinking water containing clindamycin (250mg/L) for the first two weeks post-bone marrow transfer.

2.2.2   Tissue processing

2.2.2.1   Division of the respiratory system

The respiratory tract was cut at the point of bifurcation between the oesophagus and trachea and removed from the chest cavity. Lungs were either segregated by lobe for individual purposes (Figure 2.1), or the entire respiratory tract (trachea and all lobes) were immersed in 4% formaldehyde for immunohistochemistry (section 2.2.6).
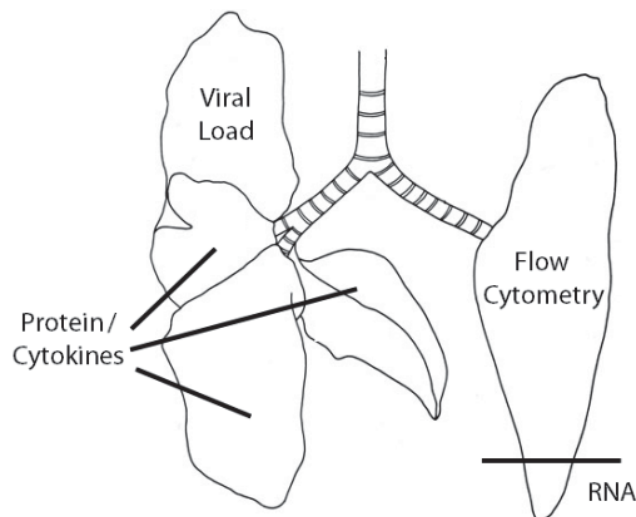
Figure 2.1: Uses of murine lung tissue in the study. Lungs were either carefully dissected and used for the listed purposes, or the entire respiratory system was excised for pathological analysis.

2.2.2.2   Flow cytometry preparation

Tissues that were to be used for flow cytometry were excised and immediately submersed in 5ml of ice cold RPMI 1640 medium. These tissues were immediately processed by first passing them through 100μm sterile filters (BD Biosciences). The cell homogenate was centrifuged at 500×g for 5 minutes, the pellet was lysed with ACK lysis buffer, re-centrifuged, and cleaned twice more with sterile PBS. The white blood cell pellet was then ready for analysis by flow cytometry, which is detailed further in section 2.2.5.

2.2.2.3   Viral load preparation

Tissues that were to be used for quantification of viral load by plaque assay or $TCID_{50}$ (section 2.2.4) were extracted and immediately snap frozen in liquid nitrogen, before being stored at -$70^{o}C$ until use. Tissue was thawed on ice, weighed, and submerged in 5% weight / volume (w/v) of Leibovitz L-15 media containing antibiotic-antimycotic. The tissue was then processed in a rotor-stator homogeniser and centrifuged at 546×g, $4^{o}C$ for 5 minutes. Supernatant was either used immediately for viral load quantification or was snap frozen in liquid nitrogen. The maximum number of freeze-thaw cycles was limited to two to ensure minimal loss of viral titre.

2.2.2.4   RNA extraction preparation

Tissues that were to have their RNA extracted were exised and submerged in room temperature RNAlater (Invitrogen). The tissues were incubated at $4^{o}C$ overnight and were subsequently stored at -$20^{o}C$ until use. The processing of these samples is discussed further in section 2.2.4.1.

2.2.2.5   Protein extraction preparation

Tissues were excised from the mouse and immediately snap-frozen in liquid nitrogen before being stored at -$70^{o}C$ until use. Tissues were thawed on ice, weighed, and homogenised in 5% w/v of Tissue Protein Extraction Reagent (Thermo Scientific) containing "cOmplete Protease Inhibitor" (Roche). Homogenate was centrifuged at 546×g for 5 minutes and the supernatant was either snap-frozen and stored, or used immediately for assays.

2.2.3   Replicating virus quantification

2.2.3.1   Plaque assay

MDCK cells were grown in either T-75 or T-150 flasks in cDMEM under standard incubator conditions ($37^{o}C$, 5% CO_2). Cells were passaged every 2-3 days by incubation with 0.05% trypsin-EDTA (Invitrogen) to remove cells from the tissue culture flask. Plaque assays were conducted as described previously (Matrosovich *et al.* 2006). Briefly, cells were seeded into 12-well tissue culture treated plates (BD Biosciences) at 2×10^5 cell/well in cDMEM and left overnight until they formed a confluent monolayer. Media was aspirated from the wells, with care not to disturb the monolayer and the cells were washed twice with sterile PBS. Samples containing virus were diluted 1:10 in SFM and serially diluted 10-fold in SFM to create a

dilution series between $10^{-1} - 10^{-9}$. 200μl of each of the dilution series was transferred into a well of the 12-well plate and were left to incubate at 37°C for 1-2 hours with agitation every 15 minutes.

After incubation, 1ml of overlay medium was added to each well and incubated, undisturbed for 72 hours at 37°C. Plates were then removed, the overlay medium aspirated and cells incubated at room temperature with 5% formal saline for 30 minutes, at which point the formal saline was removed and the cells were covered with 5% toluidine blue (Sigma) and left for 15-30 minutes to stain. Plaques were counted in the well of the highest dilution with >10 plaques present and the PFU/ml was determined using the following calculation:

$$Plaque\ forming\ units\ per\ ml = \frac{\#\ plaques}{d\ \times V}$$

Where: d = dilution factor where plaques were counted (10^{-1}-10^{-10})

V = volume of diluted virus added to the well (0.2ml)

All plaque assays were carried out in duplicate to ensure accuracy of the final concentration of virus.

2.2.3.2   Tissue culture infective dose (TCID$_{50}$)

The England/195 strain of influenza yielded no plaques and was therefore quantified by TCID$_{50}$. Approximately 3×10^{4} MDCKs were seeded into the 96-well flat bottom plates (BD Biosciences) and allowed to settle overnight to reach confluency. Samples were diluted 1:10 in SFM and serially diluted 10-fold across the length of the 96-well plate, in quadruplicate, and incubated for 1-2 hours at 37°C. Samples were flicked onto an absorbent pad and each well was covered with SFM supplemented with 2μg/ml of TPCK-trypsin before incubation at 37°C for 72 hours. The finished plates were fixed with 5% formal saline and stained with toluidine blue, as in sub-section 2.2.3.1. The endpoint was determined as the greatest dilution showing signs of cytopathic effect (CPE) as observable by microscopy. Final TCID$_{50}$ values were then calculated by the Reed & Muench method.

2.2.4   Molecular methods

2.2.4.1   Nucleic acid extraction

2.2.4.1.1   DNA extraction

Cells or tissues were first digested by incubation with proteinase K (Qiagen) at 56°C until the sample was fully lysed. The samples were then processed through a column purification series as part of the DNeasy Blood & Tissue Kit (Qiagen); following the manufacturer's instructions. Final DNA elution concentrations were quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.2.4.1.2   RNA extraction

Cells and tissues were lysed using the lysis buffer provided in the RNeasy Plus Mini Kit (Qiagen). Cells were homogenised by passing the lysate through QIAshredder disruption columns (Qiagen), whilst tissues were homogenised using a rotor-stator homogeniser. The samples were subsequently processed using the manufacturer's instructions. Final RNA elution concentrations were quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.2.4.2   Polymerase chain reaction (PCR)

Amplification of DNA extracts was achieved by using HotStarTaq DNA Polymerase, as part of the Multiplex PCR Kit (Qiagen) on a DNA engine DIAD thermal cycler (MJ Research), using the primers listed in Table 2.4 and following the standard manufacturer's protocol. Each reaction utilised ~100ng of gDNA and was performed using the following programme:

    i.   15 minutes at 95°C.

    ii.   30 cycles of 30 seconds at 94°C, 90 seconds at 60°C, 90 seconds at 72°C.

    iii.   10 minutes at 72°C

2.2.4.3   Real time quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription of RNA to cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions, with each reaction containing 50ng of RNA. RT-qPCR was then either conducted with SYBR green dye (Qiagen) or TaqMan-probed primers (Applied Biosystems) in MicroAmp Optical 96-well Reaction Plates

(Applied Biosystems). The primers used for the SYBR green protocols are listed in Table 2.4 and were diluted to 0.5µM per reaction, or were alternatively supplied by Takara as part of their "Transgene Detection Primer Set", designed to amplify eGFP DNA. The cycling conditions used for RT-qPCR were as follows:

i.   15 minutes at 95$^{\text{o}}$C.
ii.   40 cycles of 15 seconds at 94$^{\text{o}}$C, 30 seconds at 55$^{\text{o}}$C, 30 seconds at 72$^{\text{o}}$C.

Negative controls containing no primers, no DNA/cDNA, or both were included with each run and melt curves were also conducted to detect non-specific amplification in each well.

Duplex TaqMan assays were conducted using 100ng DNA/cDNA, the TaqMan Gene Expression Master Mix (Applied Biosystems) and validated primer sets for: *Actb*, *Arcn1*, *Calcoco2*, *Copg*, *Gapdh*, *Ido1*, *Ifitm3*, *Tm9sf4* and *Sms* (Applied Biosystems). All reactions were conducted according to the manufacturer's instructions, with the following cycling conditions:

i.   2 minutes at 50$^{\text{o}}$C.
ii.   10 minutes at 95$^{\text{o}}$C
iii.   40 cycles of 15 seconds at 95$^{\text{o}}$C, 1 minute at 60$^{\text{o}}$C.

All RT-qPCR reactions were run in triplicate on a StepOne Plus PCR cycler (Applied Biosystems) and used either GAPDH or β-actin primers as endogenous housekeeping genes for calculation of the comparative C_T ($\Delta\Delta C_T$) values for RNA expression analyses. All data were analysed with StepOne software v2.1 (Applied Biosystems).

2.2.4.4   Agarose gel electrophoresis

DNA products were run on 0.5-1.5% agarose gels, which were made by boiling agarose (Sigma) in TAE buffer before being allowed to cool and set with the addition of ethidium bromide. Wells were loaded with 2µl DNA product, 2µl tracking dye (Thermo Scientific) and 8µl distilled water and run at a constant voltage of between 80-100V along with either Hyperladder III or

Hyperladder IV (Fisher Scientific) depending on expected band size. The DNA was visualised by UV illumination.

2.2.5   Cellular methods

2.2.5.1   Flow cytometry

Single cell suspensions were prepared from tissues (section 2.2.2.2) or cell lines. Live cells were counted by hemocytometer and assessed for viability by Trypan blue exclusion. Cells were subsequently diluted so that each sample contained the same number of cells and were added to round-bottom 96-well plates (BD Falcon). The plate was centrifuged at 546 × g, 4°C for 5 minutes and resuspended in FACS buffer (PBS, 1% BSA, 0.05% sodium azide) and centrifuged again. Murine samples were incubated with Mouse Fc Block (BD Biosciences) for 10 minutes at room temperature. Antibody-dye mixtures were subsequently added to all wells and incubated at 4oC for 2 hours. Cells were centrifuged and washed twice with FACS buffer and were analysed immediately on a FACSAria II (BD Biosciences) using FACSDiva (BD Biosciences).

Cells were characterised as follows: T-lymphocytes CD4^{+} or CD8^{+}, T-lymphocytes (activated) CD4^{+}CD69^{+} or CD8^{+}CD69^{+}, neutrophils CD11b^{hi}CD11c^{-}Ly6g^{+}, dendritic cells CD11c^{+}CD11b^{lo}Ly6g^{lo} MHC class II high, macrophages CD11b^{+}CD11c^{+}F4/80^{hi}, natural killer cells NKp46^{+}CD4^{-}CD8^{-}, B-cells CD19^{+}B220^{+}.

2.2.5.2   Murine embryonic fibroblasts (MEFs)

2.2.5.2.1   Generation

Pregnant female WT and *Ifitm3*$^{-/-}$ mice were killed on day 13 of gestation. Embryos were removed from the uterus, washed and dissected to remove the brain and red tissue. The tissue was then minced with a scalpel and homogenised with trypsin-EDTA for 15 minutes in a shaking incubator at 37°C. Cells were centrifuged and resuspended in cDMEM and incubated overnight in petri-dishes. Cells that had adhered to the plate and grown overnight were MEFs, which were subsequently frozen in 90% FCS / 10% DMSO or used for experiments.

2.2.5.2.2   Transfection and transduction

Retroviral vectors were constructed by transfecting plasmids into 293T cells. Briefly, this was accomplished using a combination of *Ifitm3* cDNA-containing pQXCIP plasmid (Clontech; supplied by Dr. Abraham L. Brass), VSV-G and Gag/Pol plasmids, which were mixed at a 5:1:1 ratio and delivered into 293T cells. The resultant pseudotyped retroviruses were removed in the supernatant approximately every 6 hours after 48 hours of infection. The retroviruses were either frozen at -70°C for storage, or used immediately.

MEFs were transduced with VSV-G pseudotyped retroviruses expressing either the empty vector control (pQXCIP, Clontech), or one expressing *Ifitm3*. Successfully transduced cells were isolated by 2μg/ml puromycin selection, and transductions were confirmed by Western blot.

2.2.5.3   RNA interference (RNAi)

A549 or U-2 OS cells were subjected to forward transfection with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Pre-designed and validated siRNAs (Ambion) used are listed in Table 2.6. Additional, custom-designed siRNAs to influenza NP (5'-AAGCAGGGUAGAUAAUCACUU-3', 5'-GUGAUUAUCUACCCUGCUUUU-3', GCAGGGUAGAUAAUCACUCUU-3', 5'-GAGUGAUUAUCUACCCUGCUU-3') were used as a positive control and a scrambled siRNA (Ambion) used a negative control.

Briefly, cells were seeded into tissue-culture treated 24-well plates (BD Falcon) at 4×10^4 cells / well in antibiotic-free cDMEM (U-2 OS) or cF-12K (A549) medium and were left to adhere overnight at 37°C. The following day, the RNAi duplex-Lipofectamine RNAiMAX (15pmol of each siRNA, 1μl Lipofectamine RNAiMAX, 99μl Opti-MEM I Medium (Invitrogen)) was added to each well in triplicate, and incubated at 37°C for 48 hours after which they were analysed or infected with influenza.

2.2.5.4   *In vitro* infection assays
2.2.5.4.1   RNAi studies

After transfection, cells were infected at an MOI of 0.1-0.5 PFU/cell with WSN/33 influenza virus and incubated for a further 18 hours at 37°C, after which they were fixed and permeabilised

with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells were subsequently stained with Hoescht 33342 (Invitrogen) and anti-influenza NP (Abcam) and analysed by flow cytometry for infectivity.

2.2.5.4.2   LCL infections

Lymphoblastoid cell lines (LCLs) were grown in RPMI-1640 containing 10% FCS, 2mM L-glutamine, 1mM sodium pyruvate, 1 x MEM non-essential amino acids solution, and 20mM HEPES (Invitrogen). Cells were either treated with recombinant human IFN-α2 (PBL Interferon Source at 100 units/ml or PBS for 16h. The LCL cells were then counted, resuspended, and plated on a round-bottom 96-well plate. The cells were then challenged with WSN/33 influenza A virus at an MOI of 0.1 PFU/cell. After 18h, the cells were washed twice with PBS, and fixed / permeabilised using BD Cytofix/Cytoperm (BD Biosciences), following the manufacturer's instructions. Cells were stained with FITC-conjugated anti-influenza A NP antibody (Abcam) and analysed by flow cytometry using a FACSAria II (BD Biosciences).

2.2.5.4.3   MEF infections

Cells were challenged with either A/X-31 or PR/8 at an MOI of 0.4 PFU/cell. For PR/8 infections, after 12h the media was removed and the cells were then fixed with 4% formalin and stained with purified anti-HA monoclonal antibody (Wistar Institute). For A/X-31 experiments, cells were processed similarly, but additionally were permeabilised, followed by staining for NP expression (Millipore). Both sets of experiments were completed using an Alexa Fluor 488-conjugated goat anti-mouse secondary (Invitrogen). The cells were imaged on an automated Image Express Micro microscope (Molecular Devices), and images were analysed using the Metamorph Cell Scoring software program (Molecular Devices).

2.2.6   Tissue analysis

2.2.6.1   Peripheral leukocytes

Mice (*n*=3 per genotype per day) were bled on days 0, 1, 2, 3, 4 and 6 by tail vein puncture. Leukocyte counts were determined by haemocytometer, whilst blood cell differential counts were calculated by counting from duplicate blood smears stained with Wright-Giemsa stain

(Sigma-Aldrich). At least 100 leukocytes were counted per smear. All blood analyses were conducted in a blinded fashion.

2.2.6.2   Histology

2.2.6.2.1   Pathology scoring

Gross lung pathology was compared by removal of the entire respiratory system from the chest cavity and being immediately photographed. Tissues were also embedded in paraffin following a >48 hour emersion in 4% formaldehyde. 5-μm sections of paraffin-embedded tissue were stained with hematoxylin and eosin (Sigma-Aldrich) and were examined and scored twice, once by a pathologist under blinded conditions. The TUNEL assay for apoptosis was conducted using the TACS XL DAB *In Situ* Apoptosis Detection Kit (R&D Systems), using the standard manufacturer's protocol.

2.2.6.2.2   Protein immunohistochemistry

For visualisation of viral spread, lungs were excised at days 1, 3 and 6 post-infection and were embedded in glycol methacrylate (GMA). 2-μm sections were blocked with 0.1% sodium azide and 30% hydrogen peroxide followed by a second block of RPMI 1640 (Invitrogen) containing 10% FCS (Sigma-Aldrich) and 1% BSA (Invitrogen). Viral antigen was stained using M149 polyclonal antibody to influenza A, B (Takara) and visualised with a secondary goat anti-rabbit antibody conjugated to AP (Dako). Sections were counterstained with hematoxylin (Sigma-Aldrich).

Paraffin-embedded lungs were stained for Ifitm1 and Ifitm3 protein expression with either anti-Ifitm1 or anti-Ifitm3 antibody (Abcam). Sections were also stained for DNA with Hoechst 33342 (Sigma). Alternatively, various tissues, including lung, were processed for light microscopy using a Benchmark XT automatic stainer (Ventana), using primary anti-Ifitm3 (Abcam) and secondary anti-rabbit (Jackson ImmunoResearch) antibodies with the Omnimap Rabbit Kit (Ventana). The stainer was run using the standard protocol, which includes deparaffinisation, blocking, primary and secondary antibody incubations and development of DAB staining.

2.2.6.2.3   RNA immunohistochemistry

Viral RNA was visualised in 5-µm paraffin-embedded sections using the QuantiGene viewRNA kit (Affymetrix), following the manufacturer's instructions. Briefly, sections were rehydrated and incubated with Proteinase K. They were subsequently incubated with a viewRNA probe set designed against the negative stranded vRNA encoding the *NP* gene of A/X-31 (Affymetrix) or the positive stranded *NP* mRNA. The AP signal was amplified before incubation with labelled probes and counterstaining with Hoescht 33342 (Invitrogen).

2.2.7   Protein analysis

2.2.7.1   ELISA

2.2.7.1.1   Cytokine ELISA

ELISAs for cytokine detection were conducted using either homogenised lung tissue, or mouse sera isolated from blood following either cardiac puncture or tail bleed. All tests were conducted using pre-designed, pre-validated kits and were conducted according to the manufacturer's instructions in all cases. ELISAs for MCP-1, IL-6, G-CSF, TNFα were supplied by R&D Systems, whilst the ELISA for OPN was from Abcam. Samples were analysed and checked using Masterplex Readerfit 2010 (MiraiBio).

2.2.7.1.2   Anti-influenza antibody ELISA

Flat-bottom non-tissue culture treated 96-well plates (Nunc) were coated with recombinant hemagglutinin (rHA) based on the sequence of England/195/09 H1N1 influenza virus (supplied by Prof. A.R.M. Townsend). Mouse sera were heat-inactivated at 56°C for two hours and diluted 1:20 in DMEM and serial 1:2 dilutions were made across the plate, which was incubated at room temperature for two hours. Plates were washed and antibody bound with HRP-conjugated anti-mouse antibody (Dako) for one hour before being developed with PM Blue substrate (Roche), and read at 450 nm. Titres were expressed as the last dilution to give >50% of the plateau positive signal.

2.2.7.2   Luminex

Lung homogenates and sera were collected at specified time points over the course of the experiments. At least 4 mice of each genotype were used to assess the chemokine / cytokine /

antibody profiles of the mice. Analysis was conducted on a Luminex FlexMAP3D, using the following mouse antibody bead kits: Millipore's Cytokine/Chemokine 17-plex (G-CSF, GM-CSF, IFNγ, IL-10, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IP-10, KC-like, MCP-1, MIP-1α, RANTES, and TNFα), Millipore's Cytokine/Chemokine 4-plex (G-CSF, MCP-1, IL-6, TNFα) Millipore's Immunoglobulin Isotyping Kit (IgA, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$, IgM) and Invitogen's Inflammatory Cytokine Panel (GM-CSF, IL-1β, IL-6, and TNFα). All assays were conducted according to the manufacturer's instructions, with >100 bead events being counted per cytokine, per assay. Results were analysed and quality control checked using Masterplex QT 2010 and Masterplex Readerfit 2010 (MiraiBio).

2.2.7.3 Western blot

Total protein was quantified by BCA assay (Thermo Scientific) and was normalised before being heated to 95$^{\text{o}}$C for 5 minutes with loading buffer. Samples were allowed to cool, and loaded into wells of pre-cast gels (Bio-Rad), with Magic Mark standards (Invitrogen) and run at 150V for one hour. Separated proteins were transferred to 0.45μm nitrocellulose membranes (Bio-Rad) for one hour at 100V, and incubated overnight in PBS-T (PBS, 0.05% Tween-20) containing 5% non-fat dried milk (Marvel). Immunoblots were conducted with the following primary antibodies: mouse Ifitm2 (Santa Cruz Biotechnology), Ifitm3 (Abcam), β-actin (Abcam), and Osteopontin (Abcam). After washing with PBST, all membranes were exposed to species-appropriate HRP-conjugated secondary antibodies (Dako) for one hour, washed, and incubated with ECL Plus Western Blotting Detection Reagents (GE Healthcare), according to the manufacturer's instructions.

2.2.7.4 Microneutralisation assay

Mouse blood was obtained by cardiac puncture at defined time points and was centrifuged at 1000 × g for 10 minutes to separate the cells from the sera. Sera were heat inactivated at 56$^{\text{o}}$C for 30 minutes and diluted in DMEM containing penicillin-streptomycin and 0.1% BSA to give a final concentration of 1:20. Sera were serially diluted 1:2 across 96-well plates. Sera were then mixed with 4 HAU of England/195/09 influenza and incubated for 2 hours at 37$^{\text{o}}$C. 3 x 10^4 MDCK-SIAT1 cells were then added to each well of the plate and incubated overnight. Monolayers were subsequently fixed and permeabilised before detection of influenza infection

was conducted using human anti-NP IgG1 monoclonal antibody (produced by Prof. Alain Townsend) and HRP-labelled secondary anti-human Ig (Dako). Titers were defined as the final dilution of serum that caused >50% reduction in NP expression.

2.3 Statistical analyses

All experiments that could be analysed were subjected to a two-way Student's t-test, or two-way ANOVA. A p value of <0.05 was considered to be statistically significant in all cases. Construction of "Best Fit" regressions for ELISA and Luminex assays were conducted using Masterplex 2010 software (MiraiBio), which selected for the best weighting and parameters to construct a line of best fit. All other testing and graphing was done using GraphPad Prism 5 (GraphPad Software).

3   Screening for host factors involved in the restriction of influenza virus, using *in vitro* assays and knockout mouse models.

3.1   Introduction

The use of knockdown technology has proven to be an invaluable tool in investigating the actions of innumerable host proteins in development, homeostasis and immunity (Mohr *et al.* 2010). It also provides a crucial first step in identifying putative targets whose expression can be ablated in a model organism, which can be more insightful and revealing than studying cell lines in isolation.

RNA interference (RNAi) is a eukaryotic cell technique based on a system that exists in mammalian cells to regulate gene expression and RNAi has been utilised to reduce the expression of specific genes in cells and organisms (Kim and Rossi 2008). The premise of the technique is that specifically designed short interfering RNA molecules (siRNAs) of about 21-22bp in length bind to a specific site on mRNA, which ultimately stimulates the cell to enzymatically degrade the mRNA; thus preventing translation (Fire *et al.* 1998).

The advantages of *in vitro* RNAi technologies are that it is very quick and economical to gather large amounts of information about the actions of potentially every gene in a targeted genome under a particular physiological condition, such as differentiation, tumorigenesis, or pathogen infection (Kamath *et al.* 2003; Boutros *et al.* 2004; Westbrook *et al.* 2005; Brass *et al.* 2009). The use of these high-throughput screening (HTS) techniques at the organismal level in "simple" model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, removes the need for gene trapping and mutagenesis, which are exploited in other more "complex" model organisms. Indeed, it is now possible to target genes in a tissue specific manner in *D. melanogaster* (Dietzl *et al.* 2007); thus further expanding the utility of RNAi technology in understanding gene function. Furthermore, this again demonstrates the relative simplicity of these model organisms, compared with mice, which would require the time-consuming generation of lines with tissue-specific Cre-drivers that have to then be crossed with an appropriate transgenic mouse with *LoxP* sites (Gu *et al.* 1994).

Recently, a number of studies have been published that utilise RNAi technology to analyse the host-virus interactions that occur at the cellular level, in both insect (Hao *et al.* 2008a) and mammalian systems (Brass *et al.* 2009; Shapira *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010). These studies have identified several candidate virus replication dependence factors (VRDFs): host proteins that are exploited by the virus during replication, and viral restriction factors (VRFs): host proteins that prevent viral replication (Wash *et al.* 2012). Although the methodologies employed by such studies vary by cell type, screening technique and viral subtype, there is a degree of overlap, with some host factors being identified in several of the studies. Such cross-study confirmation would suggest that these host proteins should be more thoroughly investigated at the cellular and organismal level.

Whilst it may be useful to analyse the roles of these genes in *C. elegans* and *D. melanogaster*, such findings may not be directly applicable to humans, although some cross-over between insect and human screens is evident (Figure 1.10). In order to explore the relevance of these genes to human health, it is appropriate, in addition to RNAi, to employ knockout mouse models, as they are amenable to genetic manipulation and serve as an appropriate organism for influenza virus challenges (discussed in section 1.5).

Although there was a degree of consensus amongst the influenza RNAi studies, it is accepted that false positive results can be reported through screen noise, experimental duration and analysis techniques (Mohr *et al.* 2010). Therefore, the aim of this set of studies was to first validate a number of the key targets identified by performing my own small scale RNAi studies, and simultaneously screen some of the currently available knockout mouse lines that have been generated at the WTSI, as part of the Mouse Genetics Programme. These particular lines have been selected as the literature, either through HTS RNAi screening or individual experiments, would suggest a phenotypic effect upon exposure to influenza virus.

3.1.2   Targets for validation of antiviral function
3.1.2.1   ARCN1
Archain 1 (*ARCN1 / COPD*) is the δ portion of seven-subunit coat protein I (COPI) coatomer complex (Kirchhausen 2000). The primary function of the COPI complex concerns intracellular

trafficking of vesicles between the ER and Golgi: a cell function that is utilised by influenza virus during its replication cycle. The *ARCN1* gene was identified as being involved in the influenza virus replication cycle in three of the currently available HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010). These findings would suggest that ARCN1 is a VRDF and that its knockdown restricts influenza virus infection.

3.1.2.2 CALCOCO2

Calcium-binding and coiled-coil domain-containing 2 (*CALCOCO2 / NDP52*) is a dimeric, multimeric, cytoplasmic and nucleus-associated protein (Sternsdorf *et al.* 1997), which is thought to primarily be either a) a negative regulator of secretion (Morriswood *et al.* 2007), or b) acting as a receptor for ubiquitin-tagged proteins within endosomes. The detection of ubiquitin by CALCOCO2 is thought to result in autophagy by the cell (Thurston *et al.* 2009). It is capable of restricting bacterial infection in cells through the recruitment of various other host proteins to commence autophagy (Thurston *et al.* 2009; Thurston *et al.* 2012; Watson *et al.* 2012). The *CALCOCO2* gene was identified as being involved in the influenza replication cycle by two of the currently published HTS studies (Brass *et al.* 2009; Shapira *et al.* 2009), where it is suggested that CALCOCO2 is a VRDF, wherein its knockdown restricts influenza virus infection.

3.1.2.3 COPG

Coatomer subunit gamma (COPG) forms the γ subunit of the COPI complex, which ARCN1 is also associated with (COPG and ARCN1 functions are discussed in sub-section 3.1.2.1). Similarly, COPG was identified in three of the currently published HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010) and is thought to act as a VRDF.

3.1.2.4 IDO1

Indoleamine 2,3-dioxygenase 1 (*IDO1 / INDO*) is an enzyme involved in tryptophan catabolism and is thought to have an important immunomodulatory role in the host through its influence on T-cell apoptosis (Munn *et al.* 1999) and may also form a crucial component of the innate immune system of the cell, owing to its interactions with STAT1, IFNγ and TNFα (Chon *et al.* 1996; Adams *et al.* 2004). IDO1 has thus far been implicated in the control of multiple pathogenic viruses, including vaccinia virus, West Nile virus, murine leukaemia virus (MLV)

and hepatitis B virus (Terajima and Leporati 2005; Hoshi *et al.* 2010; Mao *et al.* 2011; Munoz-Erazo *et al.* 2012), as well as being linked with the development of a number of cancers (Uyttenhove *et al.* 2003; Liu *et al.* 2010b; Bonanno *et al.* 2012). Although IDO1 was not revealed as a candidate by the HTS studies, it has been included in the study owing to its known role in the life-cycles of a broad range of viruses, much like IFITM3 (Brass *et al.* 2009), and evidence indicating it may have a role in influenza virus infection through its role in inflammation (van der Sluijs *et al.* 2006).

3.1.2.5 SMS

Spermine synthase (*SMS* / *SPS*) is an enzyme that converts spermidine and S-adenosylmethionine into spermine. Spermine, a polyamine, has multiple roles in cells; influencing cell growth, differentiation and cell death (Wallace *et al.* 2003). As with *IDO1*, *SMS* was not a hit in the HTS studies, but has been included owing to a potential role of spermine in the inactivation of multiple viruses, including influenza, West Nile and vaccinia viruses (Bachrach 2007).

3.1.2.6 TM9SF4

Transmembrane 9 superfamily protein member 4 (*TM9SF4*) is a nonaspanin molecule, associated with the endosomal membrane. Although the exact function of the TM9SF family is unknown, it is becoming apparent that these proteins show strong evolutionary conservation amongst animal species (Pruvot *et al.* 2010). TM9SF4 plays a role in cell adhesion, as well as in macrophage engulfment of pathogens, with knockout *D. melanogaster* showing a depleted ability to engulf Gram-negative bacteria (Bergeret *et al.* 2008). Interestingly, TM9SF4 has also been linked with tumour cannibalism in humans, wherein it is seen to directly interact with RAB5A in the endosomal membrane; its silencing was shown to directly influence the acidification of the endosomal compartment (Lozupone *et al.* 2009). Although TM9SF4 has not been shown to be a target in the influenza HTS studies, it is a VRDF in West Nile virus HTS studies (Krishnan *et al.* 2008). Furthermore, its role in endosomal acidification, which is exploited by influenza virions, and its putative role in phagocytosis potentially suggest it may have a role in influenza virus replication.

3.2   Results

3.2.1   The impact of gene knockdown on susceptibility to influenza virus infection in human cell lines

To investigate the effects of the loss of translation directed by mRNA from the genes listed in sub-section 3.1.2 on influenza virus infection, RNAi studies were conducted using two human cell lines: U2-OS and A549 cells. Although A549 cells are the more relevant cell line, owing to being derived from lung epithelia, they also lack the expression of IFITM3 (data not shown), a crucial antiviral restriction factor. Therefore, U2-OS cells were also used to investigate the effects of the gene knockdown. Cells were transfected in duplex with siRNAs (Table 2.6) and subsequently infected with WSN/33 influenza virus.

As shown in Figure 3.1, the siRNAs had varying effects on influenza infectivity. The screening revealed that the greatest effects were elicited from the silencing of *ARCN1* (A549, $p < 0.001$; U2-OS, $p < 0.001$) and *COPG* (A549, $p < 0.001$; U2-OS, $p = 0.003$) in both cell lines, which significantly reduced influenza virus replication over the 18 hours of infection. This was similar to the effect observed when cells were treated with siRNAs specific to the influenza virus' *NP* (A549, $p < 0.001$; U2-OS, $p < 0.001$), which are intended to impede influenza replication and act as a positive control for reduction of virus replication. Interestingly, a mild, but significant reduction in infectivity in U2-OS cells was also observed when *SMS* was silenced ($p = 0.003$), but this effect could not be seen in A549 cells.

Conversely, the reduction in expression of IDO1 resulted in a small, but significant increase in viral replication in both cell lines (A549, $p = 0.012$; U2-OS, $p = 0.011$). However, no statistically significant alterations in viral infectivity was observed with siRNAs designed against *CALCOCO2* or *TM9SF4*.
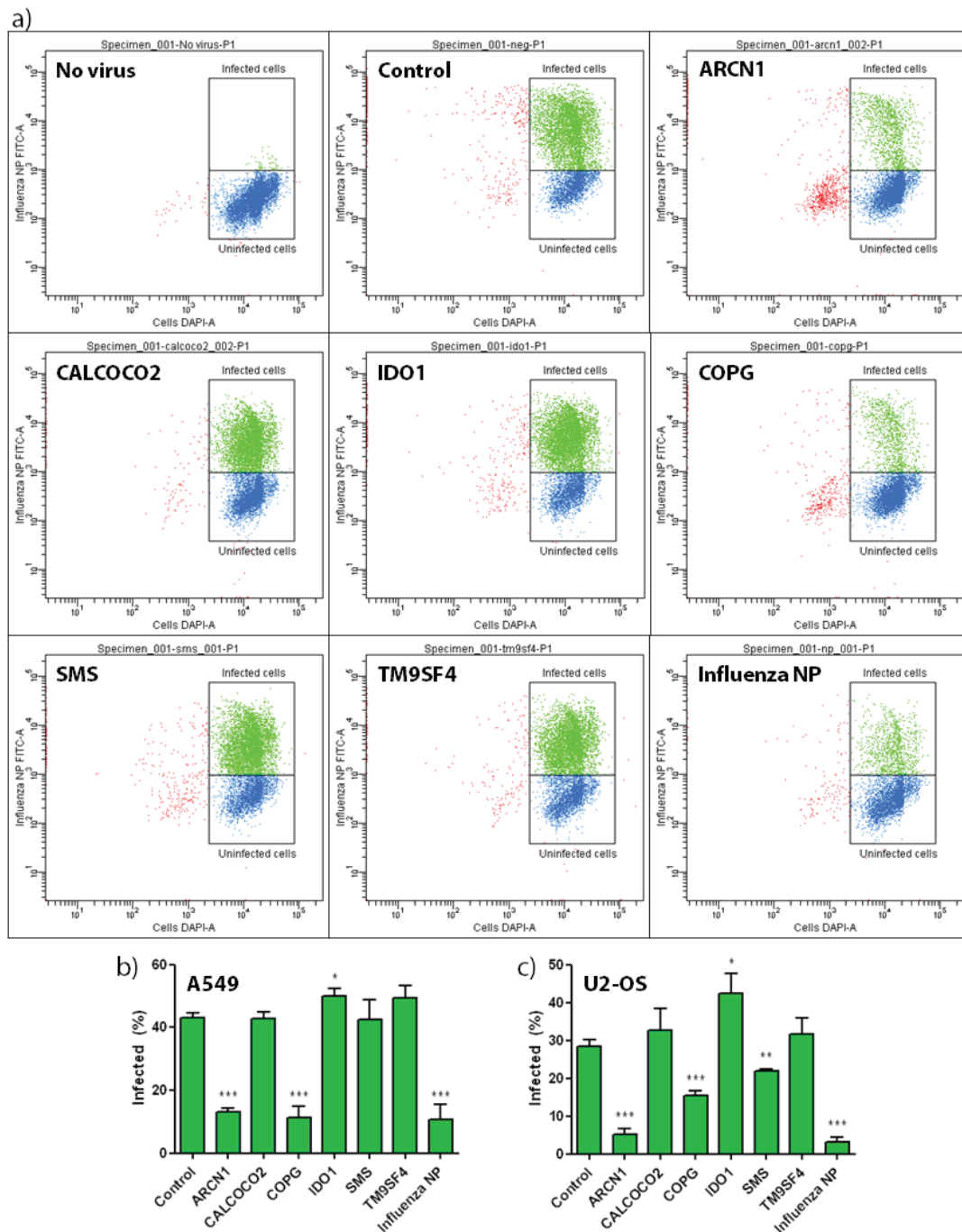
Figure 3.1: The impact of gene knockdown on influenza infection in A549 and U2-OS cell lines. Human cell lines were transfected in duplex with the indicated siRNAs for 48 hours, after which they were infected with WSN/33 influenza virus (MOI = 0.1 PFU/cell) for 18 hours before being assayed for infectivity by flow cytometry. Representative A549 flow cytometry profiles (**a**) for each of the siRNAs tested in the study are shown. Cells were double stained with Hoescht (cell nuclei) and FITC (influenza NP). Mean percentage of A549 (**b**) and U2-OS (**c**) cells staining positive for influenza virus are also shown. Results show means from >3 biological repeats ± S.D. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Gene expression and related knockdowns were confirmed by RT-qPCR analysis 48 hours after transfection, which showed that on average the targeted genes were reduced to 14% the expression of control cells receiving scrambled siRNAs (Table 3.1).

Table 3.1: Percentage expression of targeted genes in A549 cells following siRNA knockdown.

	siRNA target gene					
	Arcn1	Calcoco2	Copg	Ido1	Tm9sf4	Sms
Percentage of "normal" expression:	4.2	14.8	24.3	21.2	5.5	13.9

All percentages are relative to cells transfected with control scrambled siRNAs.

3.2.2   The impact of gene knockout on susceptibility to influenza virus infection in mouse knockout lines.

Several of the candidate genes listed in section 3.2.1 have been knocked out in C57BL/6 mice as part of the WTSI's Mouse Genome Project. To test the effects of the loss of genes *in vivo* during influenza virus infection, mice were intra-nasally challenged with 10^4 PFU of A/X-31 influenza: a sub-lethal dose, and monitored for 10 days for signs and symptoms of disease. Some groups of mice were also re-challenged 3 weeks later to test for any defects in their humoral and cellular adaptive immune response.

The mouse lines available for infection over the course of these studies were those with mutations of *Arcn1*, *Calcoco2*, *Copg*, *Ido1*, *Sms* and *Tm9sf4*. However, difficulties in breeding meant that not all lines could be challenged with influenza virus: *Sms* knockout mice were homozygotic lethal in females, which led to an insufficiently large colony; *Ido1* knockout mice bred poorly, which also led to an insufficiently large colony; and *Arcn1* knockout mice were shown to be incorrectly targeted at the gene level, with reportedly *Arcn1*$^{-/-}$ mice possessing an intact *Arcn1* allele.

Homozygous mice (n < 5 per genotype) with ablations of *Calcoco2* and *Tm9sf4* were challenged with influenza, but showed no statistical difference in weight loss profiles (Figure 3.2a,b), indicating there was no effect of these mutations. *Copg*$^{-/-}$ mice were homozygotic lethal, so *Copg*$^{+/-}$ mice were challenged to test for heterozygotic effects. Similarly, no statistically

significant phenotype was detectable in these mice (Figure 3.2c). All mice also showed no loss of weight loss of morbidity when re-challenged with A/X-31 influenza.
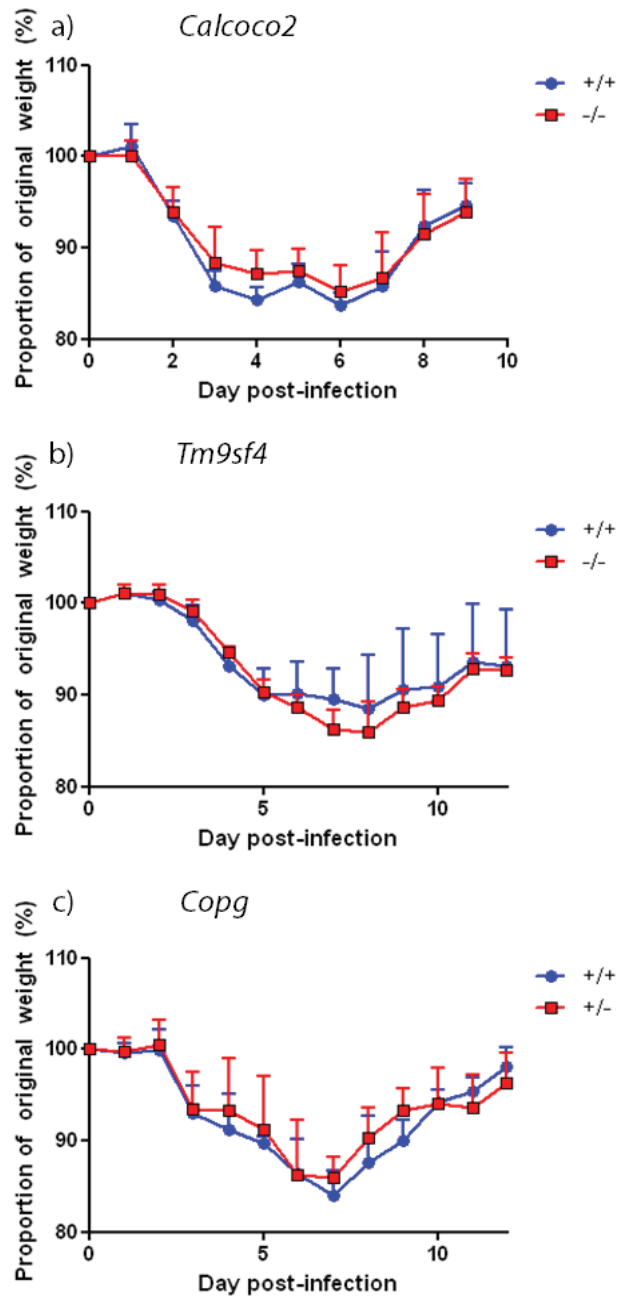
Figure 3.2: Weight loss profiles of knockout mice screened for susceptibility to influenza virus infection. Mice with specific deletions in their a) *Calcoco2*, b) *Tm9sf4* and c) *Copg* alleles were infected intra-nasally with 10^4 PFU of A/X-31 influenza virus, weighed daily and monitored for signs of morbidity. Results show means from n > 5 mice per genotype, ± S.D.

3.3   Discussion

In this study, I explored the effects of a small set of gene knockdowns in both human cell lines and mice. The body of literature generated through HTS studies has suggested that numerous host proteins inhibit or promote viral replication *in vitro*. The aim of this pilot study was to validate these screens in order to ascertain whether they may yield a phenotype in knockout mouse lines.

The strongest phenotypic effects seen in the study were from ARCN1 and COPG, which form two portions of the COPI complex, which is involved in retrograde transport of vesicles between the ER and Golgi apparatus. Highly significant p-values ($p < 0.001$) were attained for siRNA-mediated knockdowns in both A549 and U2-OS cells. These findings ratify those reported in previous HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010), where they produce a significant reduction in influenza virus infectivity. Since the current study was conducted, Sun and colleagues (2012) investigated the specific role of the COPI complex in relation to influenza infectivity. They too showed that siRNA-mediated depletion of ARCN1 resulted in a significant reduction in influenza virus replication; concluding that the COPI complex was involved in indirectly influencing vesicle trafficking in the late endosomal stages. Furthermore, they also inhibited the COPI complex via pharmaceutical intervention, which uncovered a role in potentially assisting in viral membrane formation prior to budding. Although my findings are consistent with those published in the literature, I also observed significant cell death as a result of the siRNAs directed against ARCN1 and COPG. Such cytotoxicity was not observed in any other *in vitro* knockdowns, which would suggest that these genes are intrinsic to cellular viability. Interestingly, this was not reported in previous studies looking at depletion of the COPI complex by siRNA (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010; Sun *et al.* 2012), but was observed in all independent repeats by both myself and colleagues at the WTSI. However, it is possible that such differences are due to internal practices and experimental setup, and may not be as a result of the knockdown.

However, the attempts to generate mice with a gene ablation in the COPI complex would suggest that these genes are essential for survival. *Copg*$^{-/-}$ mice proved to be homozygotic lethal; therefore impeding the study as I could only monitor heterozygous mice, which yielded no

significant obvious deviations from the normal progression of influenza virus infection. In order to investigate the effects of complete ablation of *Copg* under influenza challenge, I sought to acquire and cross the *LoxP*-containing *Copg$^{+/-}$* mice with those possessing a CCSP-driven Cre allele (Bertin *et al.* 2005). CCSP (Clara cell secretory protein) is specifically expressed in the respiratory tissues, and as such would result in the excision of the remaining *LoxP*-flanked *Copg* allele. However, we entered difficulties in acquiring the Materials Transfer Agreement (MTA); thus making the transgenic generation infeasible. The WTSI will now attempt to generate this mouse on-site for future lung-specific gene deletions.

The impact of the loss of *IDO1* in my screen was also notable. The knockdown of *IDO1 in vitro* resulted in a small, but significant ($p < 0.05$) increase in viral infection in both A549 and U2-OS cells, which would suggest IDO1 is an antiviral molecule. Previously, HTS results have shown that knockdown of IDO1 results in increased infectivity with West Nile virus and chikungunya virus *in vitro* (Schoggins *et al.* 2011), but this presents the first evidence that IDO1 may impact upon influenza virus. Although the *Ido1$^{-/-}$* mouse line is viable and available at the WTSI, sufficient numbers of mice were unavailable to perform a robust series of experiments. However, the current RNAi evidence indicates that this line should be pursued as a priority, as it may yield a positive phenotype. This is especially true when considering the potential role of IDO1 in moderating T-cell responses (Munn *et al.* 1999; Larrea *et al.* 2007) and in the stabilisation of mRNA coding for pro-inflammatory cytokines such as IL-6 (van Wissen *et al.* 2002). It is anticipated that the *Ido1$^{-/-}$* mouse will show increased pathological damage and heightened viral replication when challenged, but confirmatory data is awaited.

Of the knockout animals challenged in this study, *Calcoco2$^{-/-}$* mice represent the only viable homozygotes to be represented in multiple HTS studies for influenza infectivity. However, I was unable to replicate the results found in previous studies, with the knockdown of *CALCOCO2* in cell lines resulting in non-significant differences compared to control cells. Similarly, the mice challenged with influenza virus showed a marginal trend towards protection from the virus, but these differences were also non-significant. Interestingly, sequence alignment of the human (AAH15893) and mouse (NP_001257949) proteins using ClustalW2 (Larkin *et al.* 2007) revealed only 39% similarity at the amino acid level. It may therefore not be surprising to note

that the published effects of CALCOCO2 knockdown in cell lines are not replicated in mice, owing to divergent evolution.

This study highlights several key issues that must be considered with both *in vitro* assays and the use of knockout mouse lines. Perhaps the most evident problem is that *Copg*$^{-/-}$ mice have a lethal phenotype. HTS studies, and this RNAi work, would suggest that the ablation or knockdown of the COPI complex would result in reduced influenza virus infection. One could therefore surmise that this complex may be a viable target for antiviral interventions. However, the observation that these cells exhibited increased rates of cell death and that *Copg*$^{-/-}$ mice were non-viable (one would presume the same to be true for the *Arcn1*$^{-/-}$ mice should they have been generated appropriately), would suggest this may not be the case. Whilst RNAi screens are useful for understanding cellular dynamics and viral replication, as well as focusing attention onto certain genes, they may also focus research onto areas that have no translational potential to human therapies.

The second, more general problem, stems from the use of knockout mouse lines to mimic the situation seen in humans. The discrepancies that were observed between the effects of the loss of *Calcoco2* in mice and the knockdown of *CALCOCO2* in HTS studies with human cells may be a result of divergence between the orthologs, which may have resulted in altered functionality. Previous comparisons of the genetic and amino acid sequences of human and mouse orthologs have revealed approximately 80-85% sequence similarity (Batzoglou *et al.* 2000; Chinwalla *et al.* 2002). Therefore, the poor degree of sequence homology here (39%) would indicate that the *Calcoco2*$^{-/-}$ mouse may not be an accurate model for the study of the effect of silencing the human gene *in vivo*. Such extensive sequence divergence should therefore be considered when comparing across species barriers.

In conclusion, HTS studies are an important tool in rapidly identifying genes that may be involved in cellular processes, and in particular those involved in influenza infection cycles. Whilst the current study encountered some discrepancies with the published literature, they illustrate how it is possible to translate *in vitro* into *in vivo* studies through the use of knockout animal models, such as mice. Ultimately, the goal of such progression would be to take a further

step and utilise the information ascertained from cell lines and mice through to human studies. Such translational research could inform potentially important diagnostic and therapeutic tools.

4   IFITM3 restricts the morbidity and mortality associated with influenza.

4.1   Introduction

The IFITM family of proteins represents an important intrinsic and innate block to viral infection. Initially identified as playing a role in development and germ cell homing (Tanaka *et al.* 2004; Tanaka *et al.* 2005), IFITM3 has subsequently been shown to block an increasing number of viruses *in vitro*, which currently includes influenza A and B viruses (IAV and IBV), flaviviruses such as West Nile virus (WNV) and dengue virus, and Ebola virus, amongst others (Brass *et al.* 2009; Jiang *et al.* 2010; Weidner *et al.* 2010; Feeley *et al.* 2011; Huang *et al.* 2011; Schoggins *et al.* 2011; Anafu *et al.* 2013; Mudhasani *et al.* 2013; Wilkins *et al.* 2013).

As described briefly in section 1.4.1, the IFITM family consists of multiple members across various species. Interestingly, it appears as though the family has also diverged so that individual members are more capable of restricting certain viruses than others, with IFITM1 shown to have higher restrictive capacity against the filoviruses (Huang *et al.* 2011), whilst IFITM3 has been shown to be more capable of restricting influenza viruses (Brass *et al.* 2009). IFITM3 is thought to be associated with late endosomal membranes, where it effectively blocks the release of viruses into the cytosol (Weidner *et al.* 2010; Feeley *et al.* 2011). However, as discussed in section 1.4.1, the exact biochemical function of IFITM3 has yet to be elucidated, although it does appear to be playing a role in membrane fusion, potentially through moderation of cholesterol homeostasis (Figure 1.16) (Amini-Bavil-Olyaee *et al.* 2013; John *et al.* 2013). Additionally, a reported association with vacuolar ATPase complex suggests a role in mediation of endosomal pH and clathrin-mediated phagocytosis (Wee *et al.* 2012).

Analysis of the amino acid structure of IFITM3 and its post-translational modifications through palmitoylation and ubiquitination has aided in furthering our understanding of its mode of action (Yount *et al.* 2012; John *et al.* 2013). Systematic, non-biased alanine scanning of IFITM3 has shown that the majority of the anti-influenza restrictive capacity is encoded in the protein's N-terminal residues (Figure 3.1) (John *et al.* 2013). Further to this, research has shown that the key IFITM3 domain determining its ability to restrict viruses resides within the N-terminal 20 amino

acids (Weidner *et al.* 2010). Together, these studies would suggest that the tyrosine residue at position 20 (Y20) is a functionally critical amino acid.
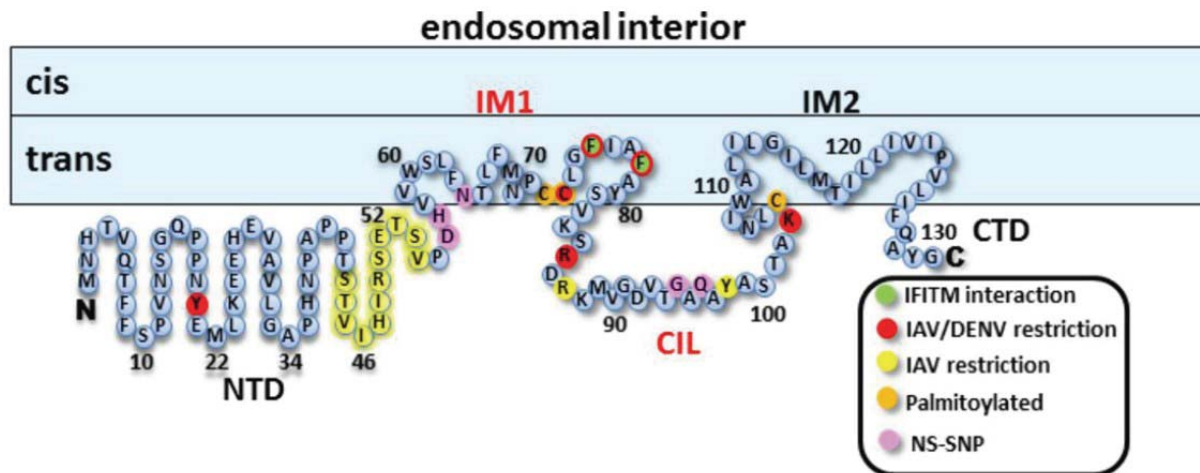
Figure 4.1: Analysis of each IFITM3 amino acid's influence on antiviral restriction of influenza and dengue viruses. Alanine-scanning mutagenesis was used to examine the individual amino acid's function on IFITM3-mediated restriction. The sites that most influenced the properties of IFITM3 are shown and explained in the legend, with NS-SNP indicating SNPs that occur in both IFITM2 and IFITM3. NTD: N-terminal domain; IM1: intra-membrane region 1; CIL: conserved intracellular loop; IM2: intra-membrane region 2; CTD: C-terminal domain. IM1 and CIL are shown in red to indicate that they comprise the two components of the CD225 domain. From (John *et al.* 2013)

The IFITM family has been implicated in multiple processes in addition to the immune system, such as primordial germ cell homing and cancer (Tanaka *et al.* 2005; Andreu *et al.* 2006). To investigate the effects of the IFITM family on primordial germ cell homing and embryonic development, Lange and colleagues (2008) generated a knockout mouse with an ablation of the entire *Ifitm* family locus to create an *Ifitm*[del] mouse. Surprisingly, the mouse developed normally and was phenotypically similar to wild type littermates. In addition to this, they generated a targeted knockout of Ifitm3 to examine whether there were any gradient-dependent effects stemming from the loss of a single family member; this mouse line also showed no obvious phenotypic effect (Lange *et al.* 2008). The Ifitm3 knockout mouse (*Ifitm3*^{-/-}) was generated through the insertion of an EGFP locus into exon 1 of the coding sequence; thus generating EGFP instead of Ifitm3 upon stimulation (Figure 4.2). This mouse was used for the duration of the study to test for other phenotypic effects induced by pathogens.
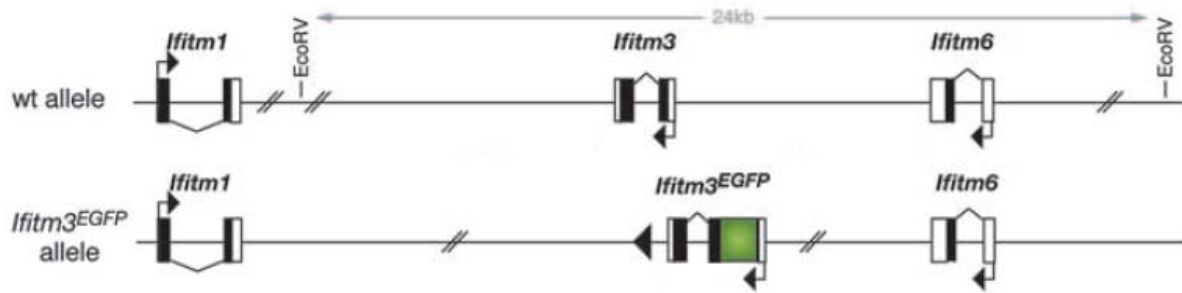
Figure 4.2: Schematic of the targeted ablation of the *Ifitm3* locus in *Ifitm3*$^{-/-}$ mice. The insertion of EGFP into exon 1 of the *Ifitm3* locus generated an Ifitm3-null mutant mouse. The *Ifitm3*^{EGFP} mouse will be referred to *Ifitm3*$^{-/-}$ for the remainder of the discourse. From (Lange *et al.* 2008)

Although humans do not carry an ablation of their *IFITM3* allele, multiple SNPs are reported across the length of the coding transcript (Figure 4.3). Currently, 13 non-synonymous, 13 synonymous, one in-frame stop and one splice site acceptor-altering SNPs have been reported in the *IFITM3* sequence, which could putatively have a dramatic effect on the activity or the expression pattern of the protein.
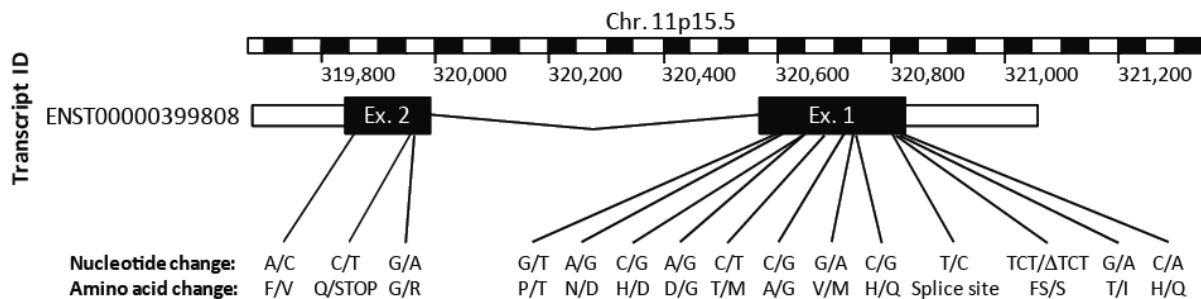
Figure 4.3: Single nucleotide polymorphisms of the *IFITM3* exons. All reported non-synonymous SNPs recorded in Ensembl are listed; noting the nucleotide change and its subsequent effects on the amino acid sequence.

The aim of the current study was to characterise the *Ifitm3*$^{-/-}$ mouse in terms of its susceptibility to influenza infection and for the first time, assess the role of Ifitm3 under virus challenge *in vivo*. Furthermore, a subset of individuals that were hospitalised during the H1N1 pandemic in 2009-2011, and had DNA samples taken as part of the Mechanisms of Severe Acute Influenza Consortium (MOSAIC) and Genetics of Influenza Susceptibility in Scotland (GenISIS) consortia, were analysed. In collaboration with Sarah Smith, we sought to sequence their IFITM3

loci and look for any discrepancies in the prevalence of SNPs within their alleles. Ultimately, the aim of the study was to move from cell lines to a model organism and translate those findings to humans.

4.2   Results

4.2.1   The impact of the loss of Ifitm3 on susceptibility to influenza virus infection in cell lines

To investigate the impact of the loss of Ifitm3 in mouse cells, RNAi studies were conducted in a murine alveolar epithelium cell lines (LA-4). Cells were transfected in duplex with either a scrambled siRNA or one specific to Ifitm3 and were subsequently infected with WSN/33 influenza virus at an MOI of 0.1 PFU/cell for 18 hours.

As shown in Figure 4.4, the targeted knockdown of Ifitm3 in the epithelial cell line resulted in significant increase in the levels of viral infection, with 12.6% of cells becoming infected, as opposed to 4.2% of cells when scrambled siRNA was used (p = 0.001).
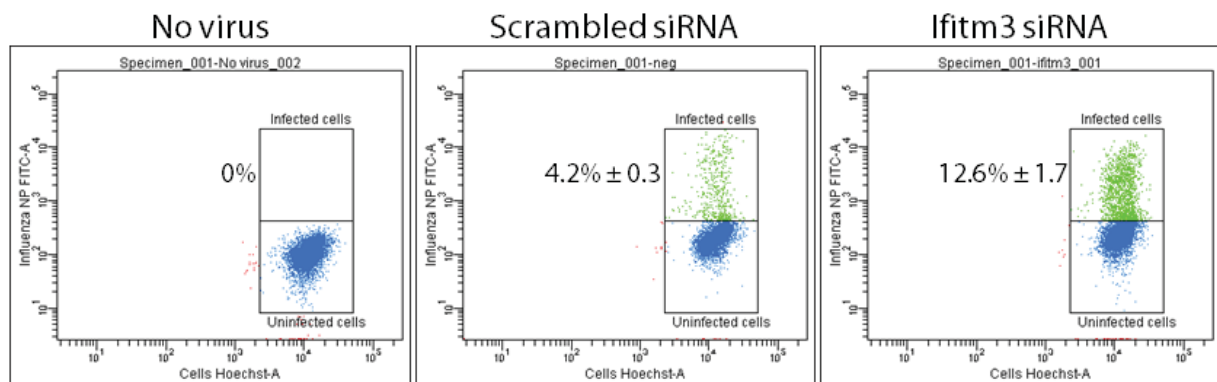
Figure 4.4: The impact of Ifitm3 knockdown in murine LA-4 cells. LA-4 cells were treated with either scrambled siRNA or siRNA specific to Ifitm3 and incubated for 48 hours. Cells were subsequently infected with WSN/33 influenza at an MOI of 0.1 PFU/cell for 18 hours and analysed for the expression of influenza NP (FITC) by flow cytometry. Figures indicate the mean ± SD of three biological replicates.

MEFs were also generated from wild type and *Ifitm3*$^{-/-}$ mice. Additionally, knock-in MEF lines were also created by the introduction of plasmids containing full length *Ifitm3* coding sequences into *Ifitm3*$^{-/-}$ cells to restore wild type expression. Cells were treated with IFNα or IFNγ for 24

hours and were challenged with either A/X-31 or PR/8 influenza at an MOI of 0.4 PFU/cell for 12 hours before being assayed for relative influenza protein expression.

As shown in Figure 4.5a, *Ifitm3*$^{-/-}$ MEFs were significantly more susceptible to influenza virus infection (p < 0.0001), regardless of whether they were pre-treated with IFN or not. The reintroduction of *Ifitm3* into *Ifitm3*$^{-/-}$ cells was also shown to return infectivity levels to comparable levels as the wild type cells (Figure 4.5b). The successful reintroduction of Ifitm3 was confirmed by Western Blot (Figure 4.5c).
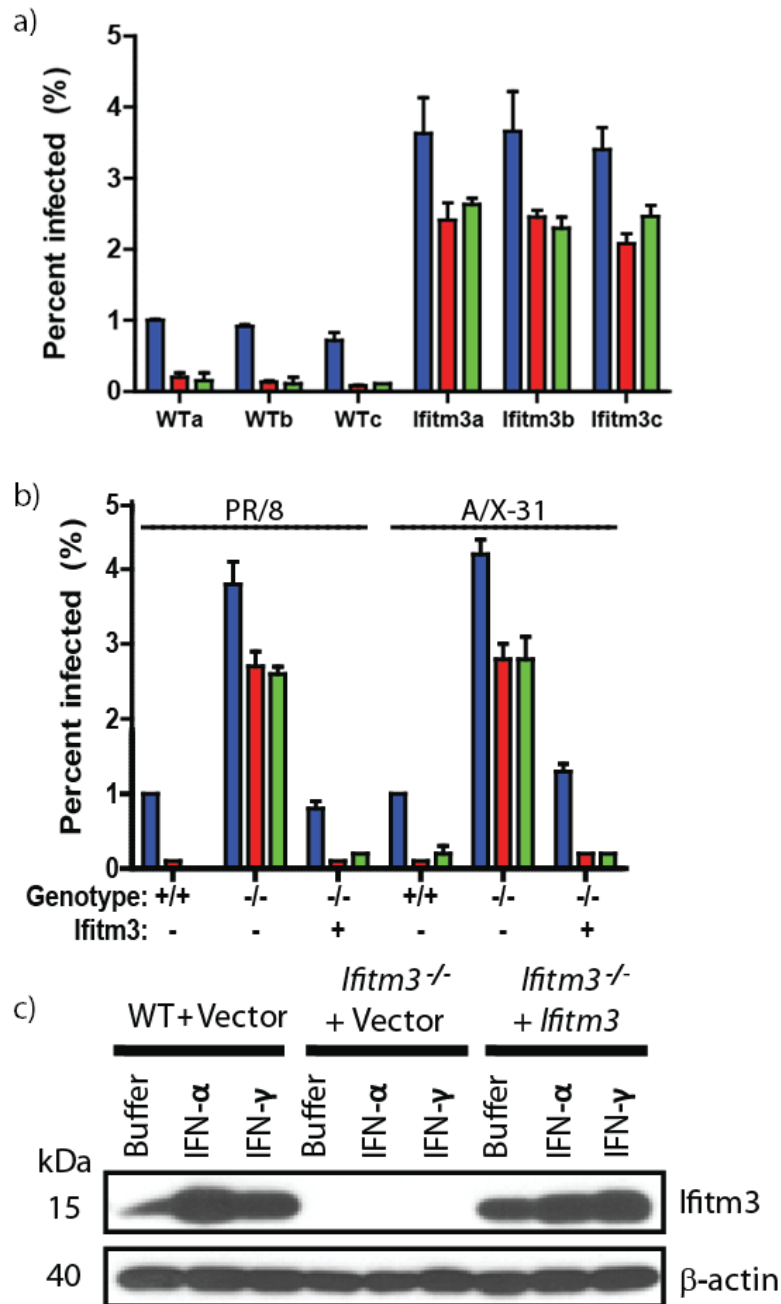
Figure 4.5: Infection levels of murine embryonic fibroblasts (MEFs) with and without the presence of Ifitm3 after influenza A challenge. MEFs from three different embryos from wt and *Ifitm3⁻/⁻* mice, denoted a-c, were challenged with PR/8 influenza virus following IFN treatment (a). Three lines of MEFs were generated: +/+, which are from wt mice, -/-, which are from *Ifitm3⁻/⁻* mice, and -/- with the reintroduction of Ifitm3 expression (b); "Ifitm3: -" indicates no plasmid was present, "Ifitm3: +" indicates presence of Ifitm3 plasmid. Similarly, these MEFs were challenged with either X-31 or PR/8 influenza virus. Transduction of *Ifitm3* into *Ifitm3⁻/⁻* was confirmed by Western blot (c). Results show means ± SD (n < 3). Blue bars: treated with buffer (control); red bars: IFNα treated; green bars IFNγ treated.

4.2.2   Confirmation of mouse genotype

Prior to further experimentation, mouse genotype and levels of Ifitm3 expression in the respiratory tissues were confirmed in wild type and *Ifitm3*⁻/⁻ mice. PCR analysis of the locus revealed that the mice were assigned to breeding colonies correctly, with wild type mice possessing the full length *Ifitm3* coding sequence, whilst *Ifitm3*⁻/⁻ mice possessed the *Ifitm3*⁻/⁻ allele and insertion of EGFP, in accordance with the original publication (Figure 4.6a) (Lange *et al.* 2008). This genotype was confirmed by Western blot to assess for expression of Ifitm3 (Figure 4.6b).
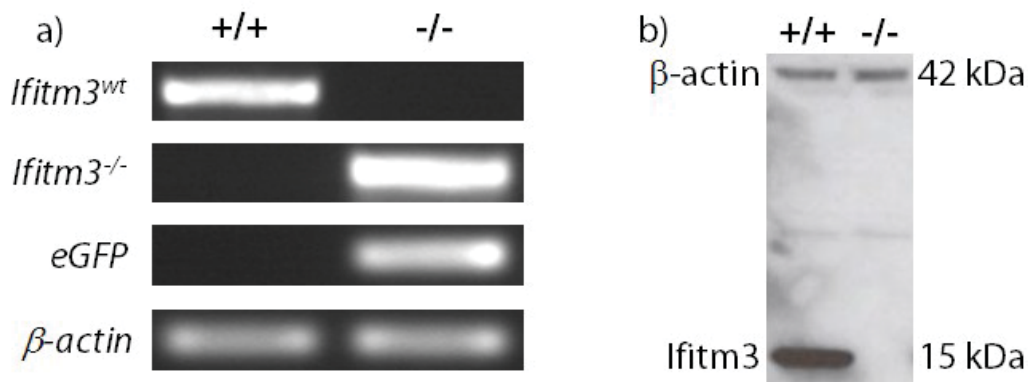
Figure 4.6: Confirmation of the loss of *Ifitm3* expression in *Ifitm3*⁻/⁻ mice. DNA was extracted from lung tissue and analysed by PCR for the presence of full length (*Ifitm3*ʷᵗ) and ablated (*Ifitm3*⁻/⁻) sequences, as well as the presence of *eGFP* which is inserted in the *Ifitm3* sequence in *Ifitm3*⁻/⁻ mice (a). Protein was also extracted from lung homogenate and assayed by Western blot for the presence or absence of Ifitm3 (b). In both cases, β-actin was used as an endogenous loading control.

4.2.3   Influenza challenge of *Ifitm3*⁻/⁻ mice

Mice were infected intra-nasally with 50µl of sterile PBS containing either A/X-31 (10^4 PFU), A/England/195/09 (200 PFU), wild type or delNS1 A/PR/8/33 ($50\text{-}10^3$ PFU) and monitored over the course of infection for clinical symptoms associated with severe illness (weight loss, piloerection, reduced motility etc.). Individuals were either culled at pre-determined time points or allowed to progress to monitor the overall weight loss profile. Mice that lost >25% of their original body weight were killed in accordance with UK Home Office regulations.

4.2.3.1   **Weight loss and survival**

All pathogenic viruses (X-31, England/195, PR/8) showed a similar statistically significant trend when comparing wild type to *Ifitm3*$^{-/-}$ mice, wherein *Ifitm3*$^{-/-}$ mice showed an accelerated weight loss and increased morbidity when compared to their wild type littermate controls (Figure 4.7). PR/8 was found to be highly pathogenic at the lowest accurately achievable dose (50 PFU in 50µl) and as such it was not possible to titrate the virus down any further in order to achieve wild type mouse survival. However, a statistically significant trend was still evident on days 4-6 post-infection, with the *Ifitm3*$^{-/-}$ showing significantly greater weight loss.

Infection with PR/8 delNS1 virus was conducted to assess whether *Ifitm3*$^{-/-}$ mice were IFN-competent and were capable of mounting an IFN response (Garcia-Sastre *et al.* 1998). The loss of the host-antagonist protein, NS1, results in an attenuated infection wherein the host mounts an unopposed IFN response to eliminate the virus. Infection with this virus resulted in no weight loss or morbidity differences between the wild type and *Ifitm3*$^{-/-}$ mice.
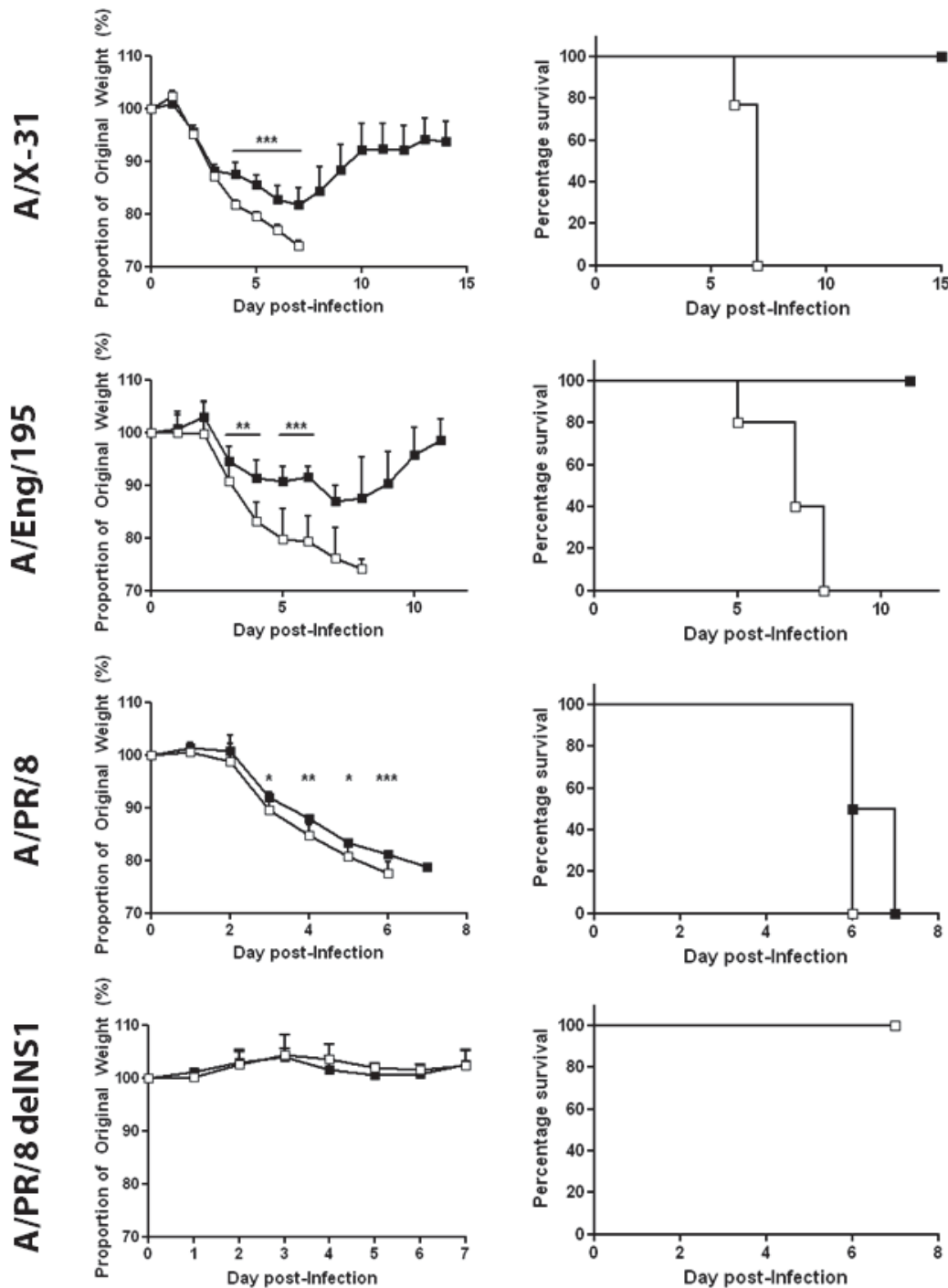
Figure 4.7: Weight loss and survival profiles of wild type and *Ifitm3*^{-/-} mice infected with various influenza A subtypes. Mice were dosed as follows: A/X-31 (H3N2), 10^4 PFU; A/Eng/195 (A(H1N1)pdm09), 200 PFU; PR/8 (H1N1), 50 PFU; PR/8 delNS1 (H1N1), 10^3 PFU and monitored for the indicated time period, assessing daily for weight loss and morbidity. Mice that surpassed 25% weight loss were killed. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. (n > 5) Statistical significance was assessed by ANOVA (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

4.2.3.2   Viral burden and distribution

Mice infected with X-31 influenza virus were assessed for viral load within their lungs via plaque assay and systemically for signs of viremia by qPCR. As shown in Figure 4.8, peak viral load at day 2 post-infection showed no significant difference between wild type and *Ifitm3*$^{-/-}$ mice. However, virus persisted within the *Ifitm3*$^{-/-}$ lungs to give a 10-fold higher burden at day six post-infection (p = 0.0001). These differences were confirmed by qPCR for influenza NP RNA on days one and six post-infection; showing the same trend. Analysis of the blood, spleen, brain and heart revealed no signs of viral RNA in organs outside of the respiratory system.
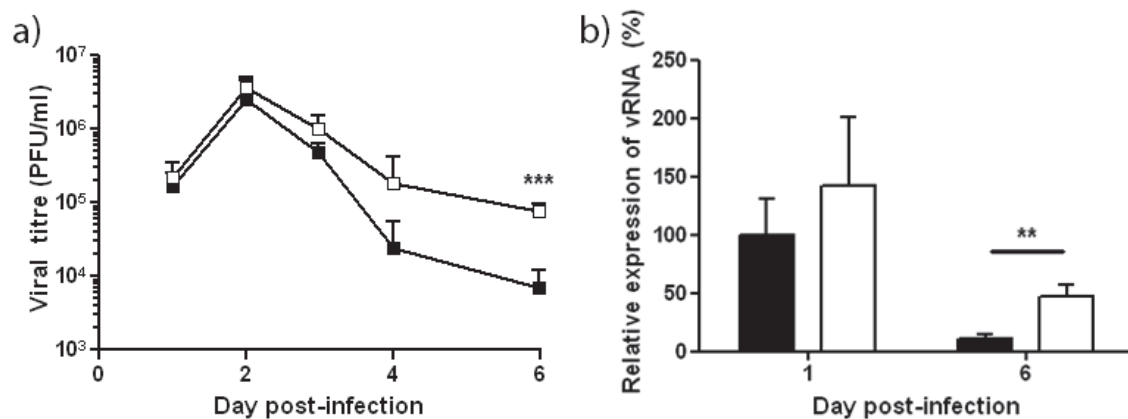
Figure 4.8: Lung viral burden over the course of influenza A virus infection. Results show the viral replication kinetics of X-31 influenza virus, as assessed by plaque assay (a). Results were verified by qPCR (b), wherein levels of NP expression were normalised to levels observed in wild type mice on day one post-infection. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 4). Statistical significance was assessed by Student's *t*-test (**: p < 0.01, ***: p < 0.001).

The distribution of virus within the lungs was analysed by immunohistochemistry (IHC) for both viral proteins (Figure 4.9a) and viral RNA (Figure 4.9b). RNA visualisation confirmed a much higher amount of vRNA within the lungs at day 6 post-infection in *Ifitm3*$^{-/-}$ mice; supporting the viral load quantification. Interestingly, protein IHC indicated *Ifitm3*$^{-/-}$ mice displaying more viral antigen deeper in the lung tissue than wild type littermates, where virus was restricted to large airways.
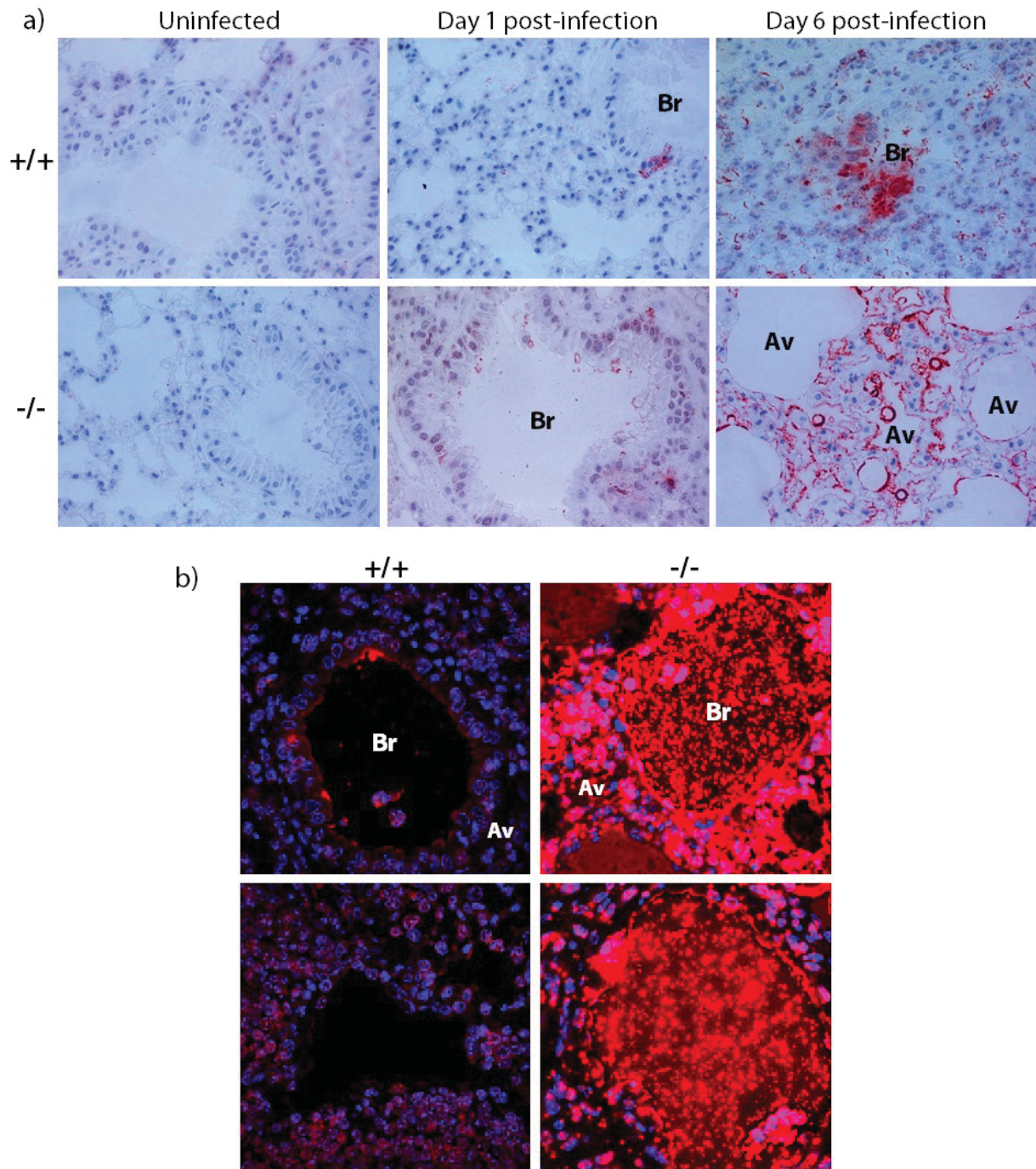
Figure 4.9: Viral antigen distribution through the lungs over the course of infection. Protein immunohistochemistry (a) over the course of infection showed the spread of virus in *Ifitm3⁻ᐟ⁻* lungs into the terminal bronchi and alveoli by day six post-infection, which was absent in wild type mice. At earlier time points there were no differences in virus distribution. Viral RNA immunohistochemistry (b) showed a greater abundance of vRNA on day 6 post-infection in *Ifitm3⁻ᐟ⁻* lungs, compared to wild type littermates (red: virus, blue: cell nuclei, Av: alveolus, Br: bronchiole). All images were taken at 20× magnification.

4.2.3.3   Pathology

Organs were removed over the course of infection and were assessed for pathological damage by a variety of means. Freshly excised *Ifitm3*⁻/⁻ lungs showed signs of extensive damage, with multiple large lesions present on their surface at day 6 post-infection (Figure 4.10). Sectioning of *Ifitm3*⁻/⁻ lungs revealed fulminant viral pneumonia, with severe inflammation, gross cellular infiltrate, oedema, red blood cell extravasation and hemorrhagic pleural effusion (Figure 4.10). Wild type mice showed moderate inflammation, with less extensive infiltration and oedema.
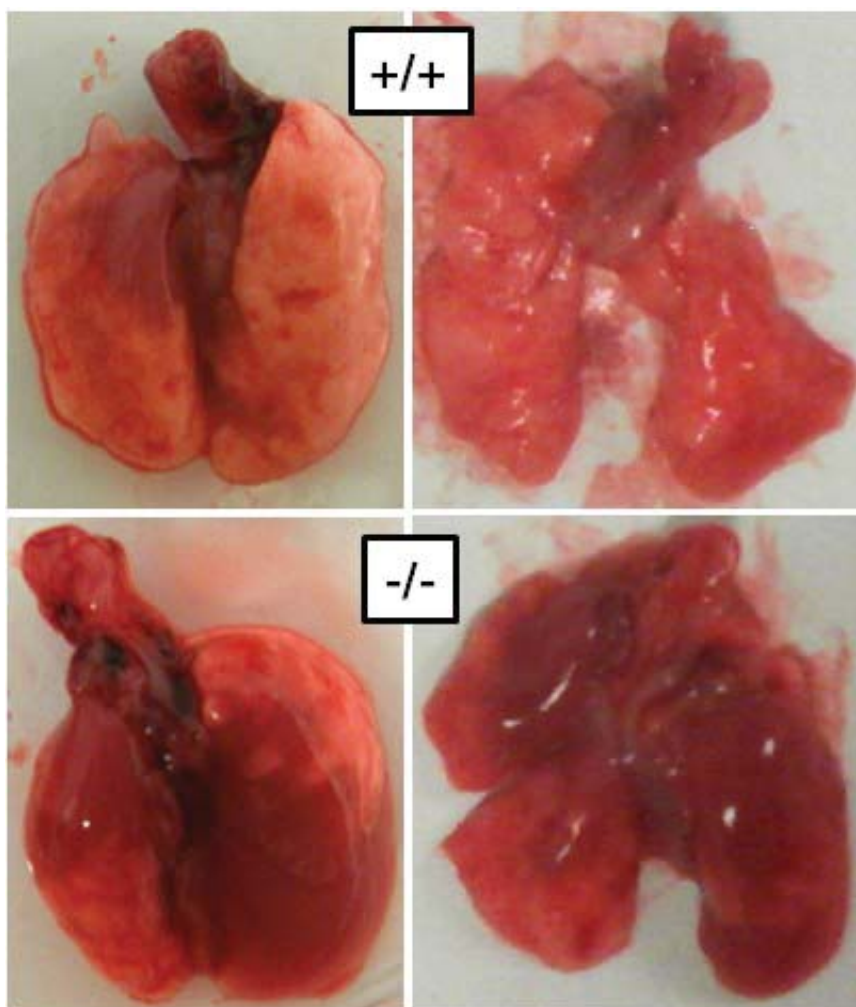
Figure 4.10: Gross lung pathology of mice following influenza A virus challenge. Mice were infected with X-31 influenza virus and pathological damaged assessed at day 6 post-infection. Gross pathology showed more extensive damage and several large lesions on the pleural surface of *Ifitm3*⁻/⁻ lungs. Photos show distal views of the lungs with lobes as they are in-situ (left) and splayed (right) to reveal the extent of damage.
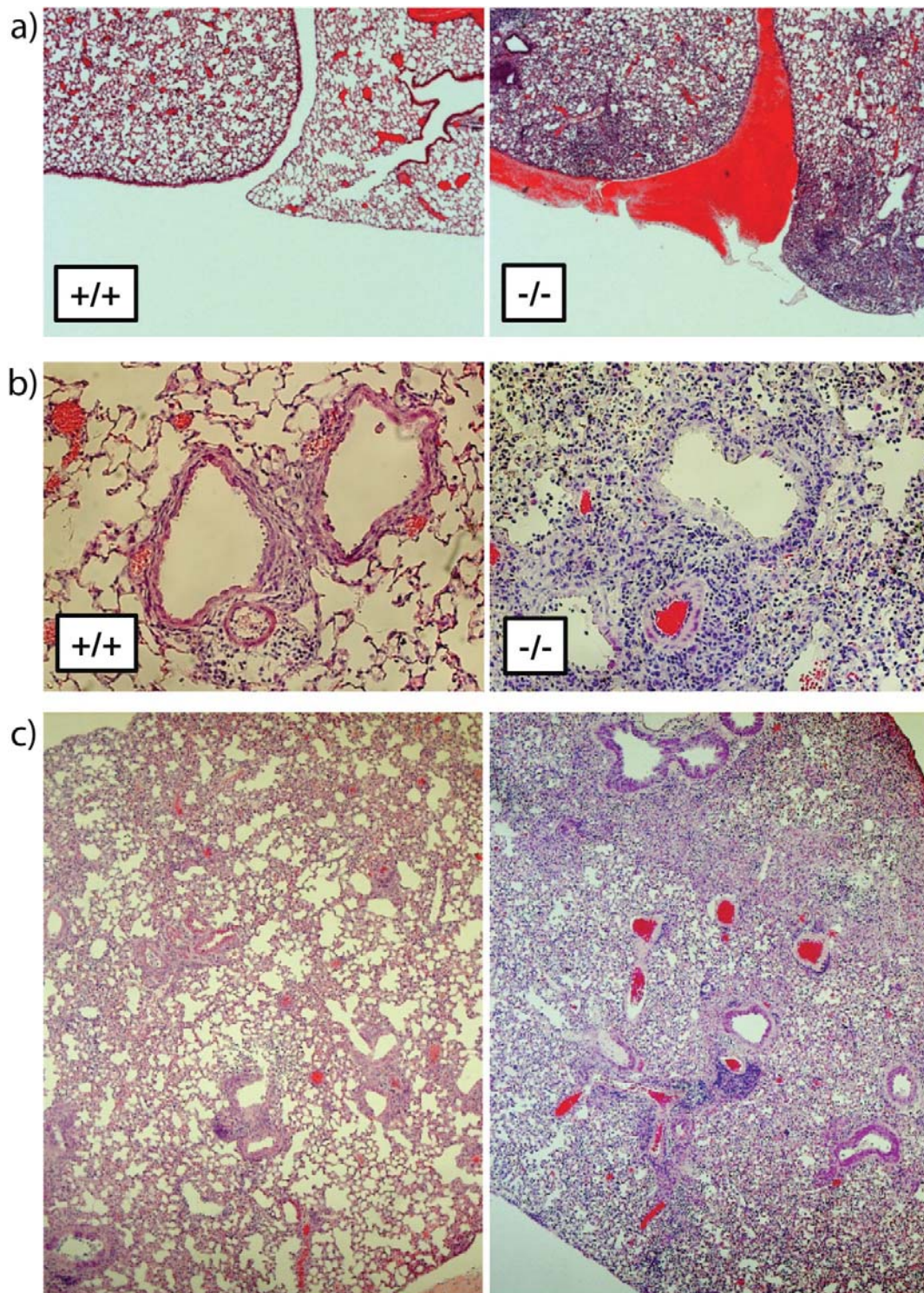
Figure 4.11: Lung sections of mice following influenza A virus challenge. Mice were infected with X-31 influenza virus and pathological damaged assessed at day six post-infection. Hemorrhagic pleural effusion (a), oedema, cell debris and cellular infiltrate (b, c) were also more pronounced in *Ifitm3*$^{-/-}$ lungs. Original magnification for (a) and (c), 5×, and for (b), 20×.

Furthermore, pathology was indirectly assessed by measuring lung weight. Excised respiratory systems were immediately cleaned and weighed to determine the extent of cellular infiltrate and water content present within the infected lungs on day six post-infection (Figure 4.12a). This revealed that *Ifitm3*$^{-/-}$ lungs were significantly heavier at the peak of morbidity, when compared to wild type littermates (p = 0.0007). Lungs were also removed and dried for seven days at 50°C to investigate the amount of water present in the lungs at the same time point (Figure 4.12b). This showed that *Ifitm3*$^{-/-}$ mice had significantly more water in their lungs (p = 0.02); supporting the pathologically observed pneumonia.
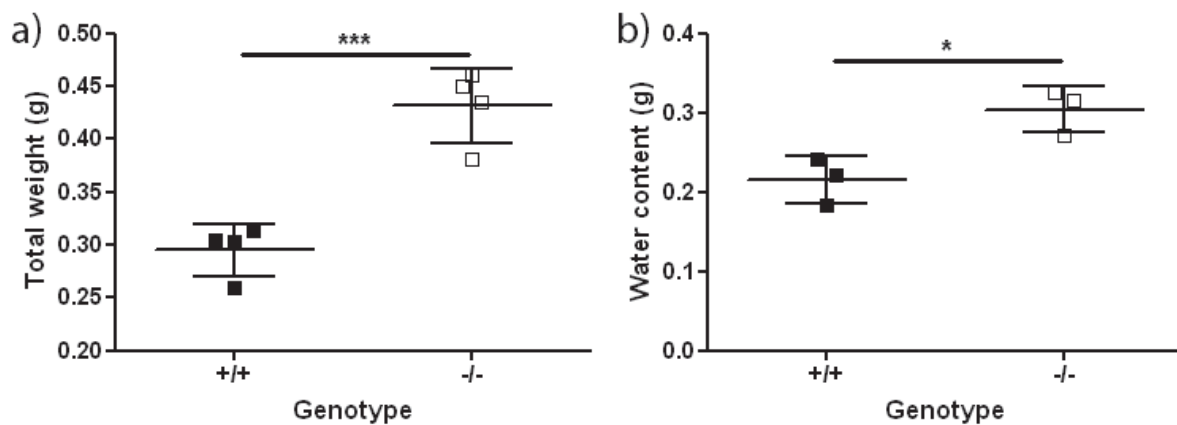
Figure 4.12: Total weight and water content of mouse lungs excised at day six post-influenza infection. Freshly removed lower respiratory systems (n = 4) trachea and lungs) were weighed to determine the extent of cellular and water infiltrate (a). Separately, lungs (n = 3) were dried for seven days and water content was calculated (b). ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. Statistical significance was assessed by Student's *t*-test (*: p < 0.05, ***: p < 0.001).

Lungs were also assessed for necrotic and apoptotic damage using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Figure 4.13). This revealed extensive and widespread cell death across the breadth of the sectioned lobe in *Ifitm3*$^{-/-}$ mice. However, instances of such damage were isolated and limited in wild type littermates.
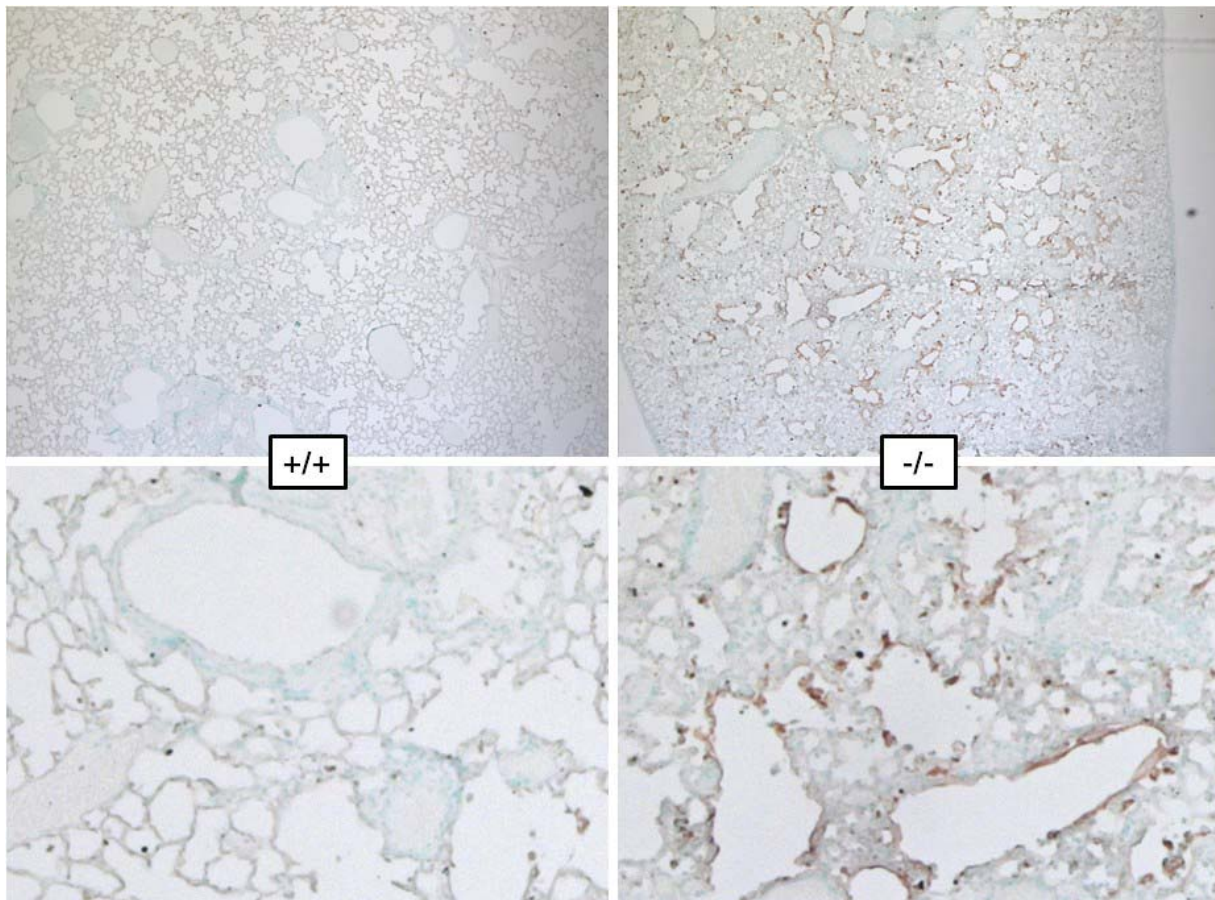
Figure 4.13: TUNEL assay for cell death in influenza-infected lungs. Lung sections from mice six days post-infection with influenza A were assayed for cell death. The assay revealed widespread and extensive damage across the entire love in *Ifitm3*$^{-/-}$ mice, but instances of such damage were limited and isolated in wild type control mice. Brown staining indicates the presence of DNA fragmentation, which is associated with apoptosis and necrosis. All cells have been counterstained with methyl green to aid visibility and contrast. Original magnification 5×.

4.2.3.3   Ifitm3 and osteopontin expression during infection

In order to monitor Ifitm3 expression over the course of infection, lungs were homogenised at set time points in order to assess whether expression was temporally regulated during viral infection. Western blot analysis qualitatively revealed that Ifitm3 expression increased over the course of infection up until day six post-infection in wild type mice (Figure 4.14a). Further to this, levels of osteopontin (Opn) were qualified by Western blot (Figure 4.14a) and quantified by ELISA (Figure 4.14b) and qPCR (Figure 4.14c). This was conducted owing to recent discoveries that Ifitm3 directly binds *Opn* mRNA and as such prevents its translation (El-Tanani *et al.* 2010). The ELISA and qPCR for osteopontin expression indicate there to be significantly more protein

present at day six post-infection (p = 0.01), and a trend towards elevated RNA levels (p = 0.07), at the time when Ifitm3 expression is typically highest. Interestingly, constitutive expression of Opn was also higher in uninfected animals.

Ifitm3 expression was also qualitatively examined *in vivo* through the use of IHC. Staining showed an up-regulation of Ifitm3 levels in infected lungs, compared to uninfected animals (Figure 4.15). Counterstaining for Ifitm1 also revealed that the ablation of Ifitm3 had no downstream effect on other Ifitm family members; thus confirming the specificity of the knockout.
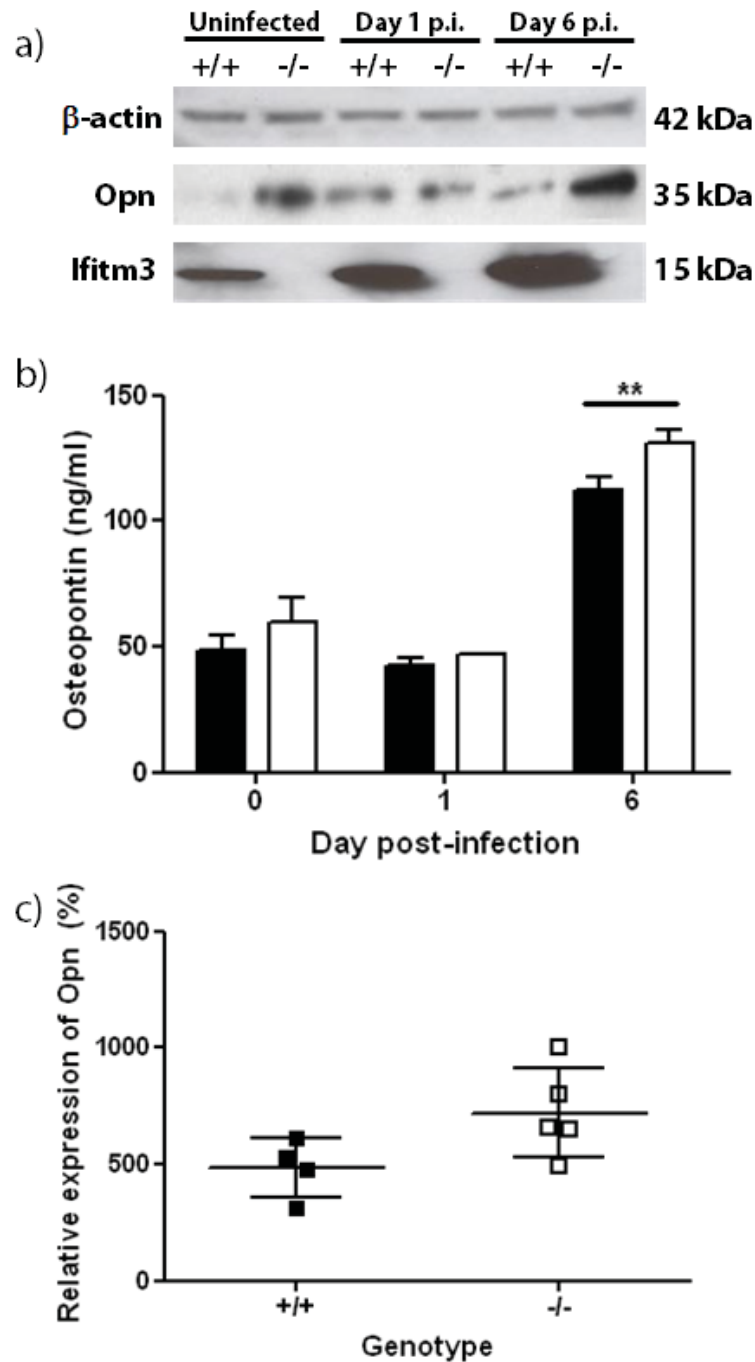
Figure 4.14: Expression levels of Ifitm3 and osteopontin over the course of infection. Western blot analysis showed Ifitm3 expression to increase over the duration of infection (a); similarly, osteopontin levels reached their highest levels in *Ifitm3*^{-/-} mice at day six post-infection. This was confirmed by ELISA (b), which showed levels of Opn to be significantly higher than those observed in wild type mice. The trend was also apparent at the RNA level on day six post-infection (c), where expression was normalised to uninfected animals' *Opn* levels. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. (n > 4). Statistical significance was assessed by Student's *t*-test (**: p < 0.01).
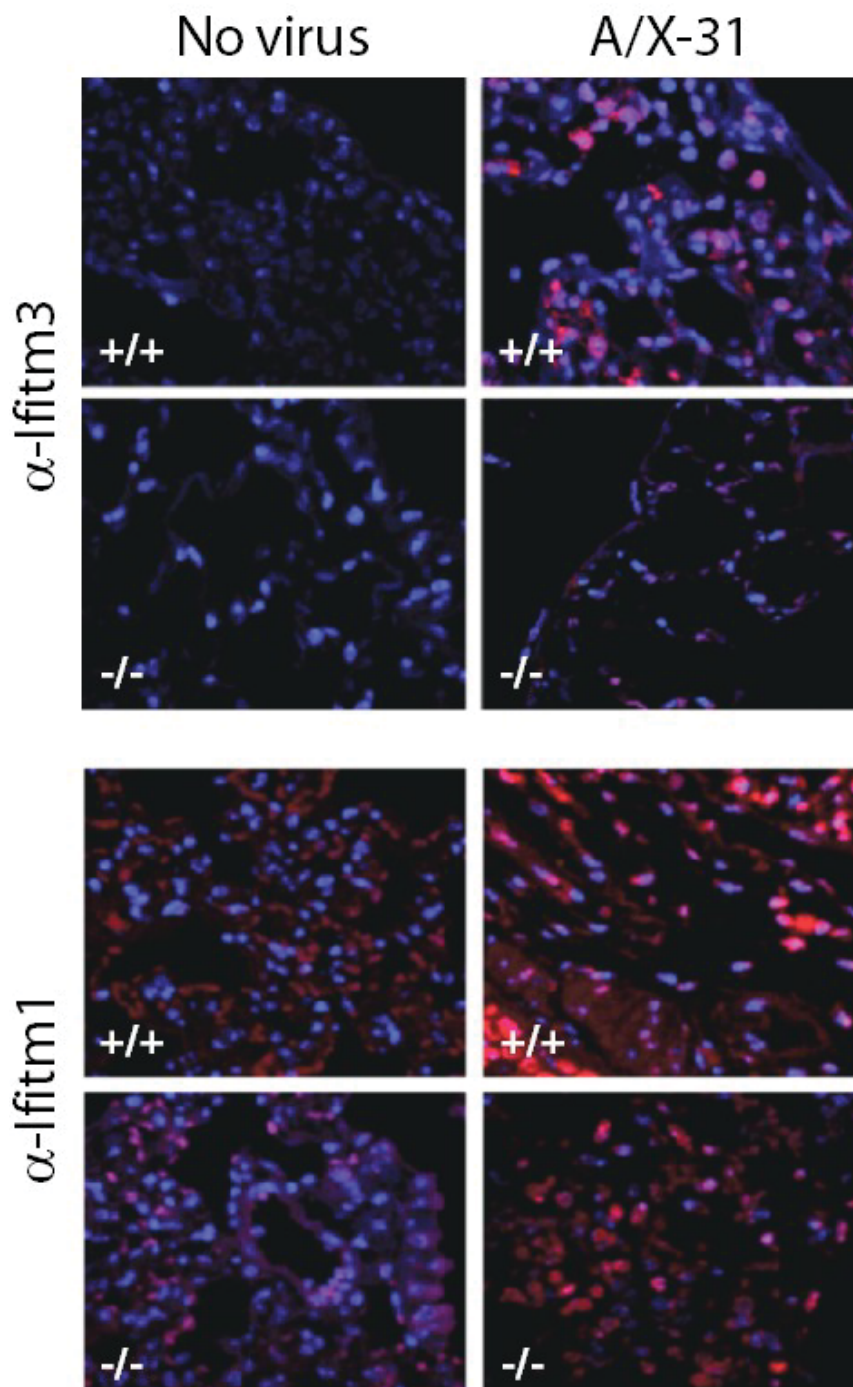
Figure 4.15: Expression of Ifitm1 and Ifitm3 in mouse lungs with or without influenza infection. Lung sections from wild type and *Ifitm3⁻ᐟ* mice at two days post-A/X-31 infection were stained to assess the expression of Ifitm3 and Ifitm1 (both red). Tissue was counterstained for DNA (blue). Viral infection is shown to up-regulate both Ifitm1 and Ifitm3 in lungs, but the loss of Ifitm3 does not influence Ifitm1 expression.

4.2.3.3   Immunology

4.2.3.3.1   Cellular response: respiratory system

The leukocyte response to viral infection is crucial to defence against influenza virus infection (discussed in section 1.4.1.3). To examine the cellular response, lungs were excised, homogenised and cells stained for a variety of cell types over the course of infection. Total cell numbers in the bronchoalveolar lavage (BAL) fluid and lung tissue were calculated to quantify the extent of cellular infiltrate seen in pathology sections (Figure 4.11). Counts showed there to be significantly more leukocytes present in the BAL of *Ifitm3*$^{-/-}$ mice, six days post-infection (p = 0.001) (Figure 4.16a). Similarly, there was a trend for larger leukocyte numbers in total in the lungs at the same point of infection (p = 0.06) (Figure 4.16b).
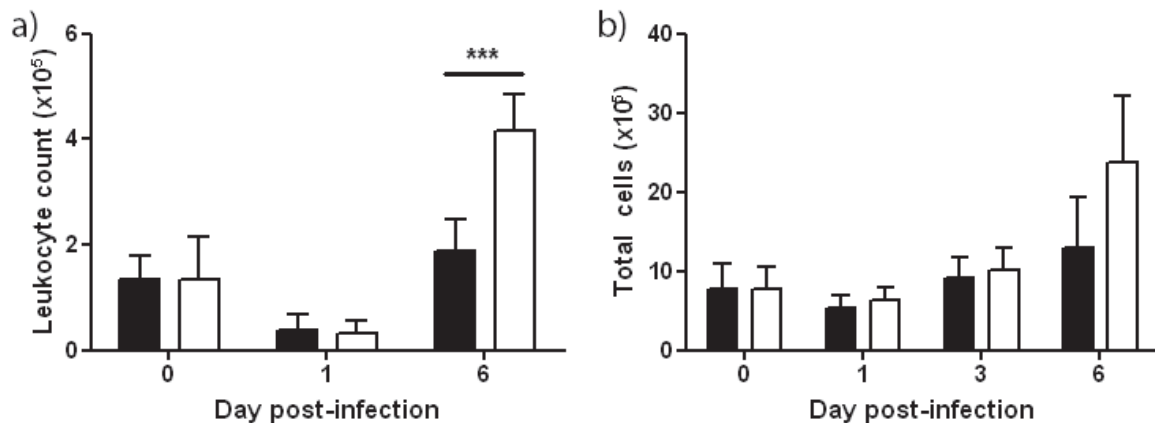
Figure 4.16: Respiratory system cell counts during influenza infection. Live cells were counted in either BAL (a) or total homogenised lung (b) at the indicated time points following challenge with A/X-31 influenza virus. ■: wild type, □: *Ifitm3*$^{-/-}$. Results show means ± S.D. (n > 4). Statistical significance was assessed by Student's *t*-test (***: p < 0.001).

Leukocytes were further characterised by flow cytometry to qualify the contribution of various cell subtypes in the immune response to influenza virus in *Ifitm3*$^{-/-}$ mice. Analysis showed that during the early infection stage (days 1-3) there were minor differences in the major immune cell populations between the genotypes of mice, with elevated numbers of neutrophils (p = 0.05) and NK cells (p = 0.02) on days one and three, respectively. However, at day six post-infection there was significant evidence of lymphopenia, with reductions in CD4 T-cell (p = 0.004), CD8 T-cell (p = 0.02) and NK cell (p = 0.0001) populations in the lungs of *Ifitm3*$^{-/-}$ mice, which was

accompanied by significantly higher numbers of neutrophils ($p = 0.007$) compared to wild type mice (Figure 4.17).
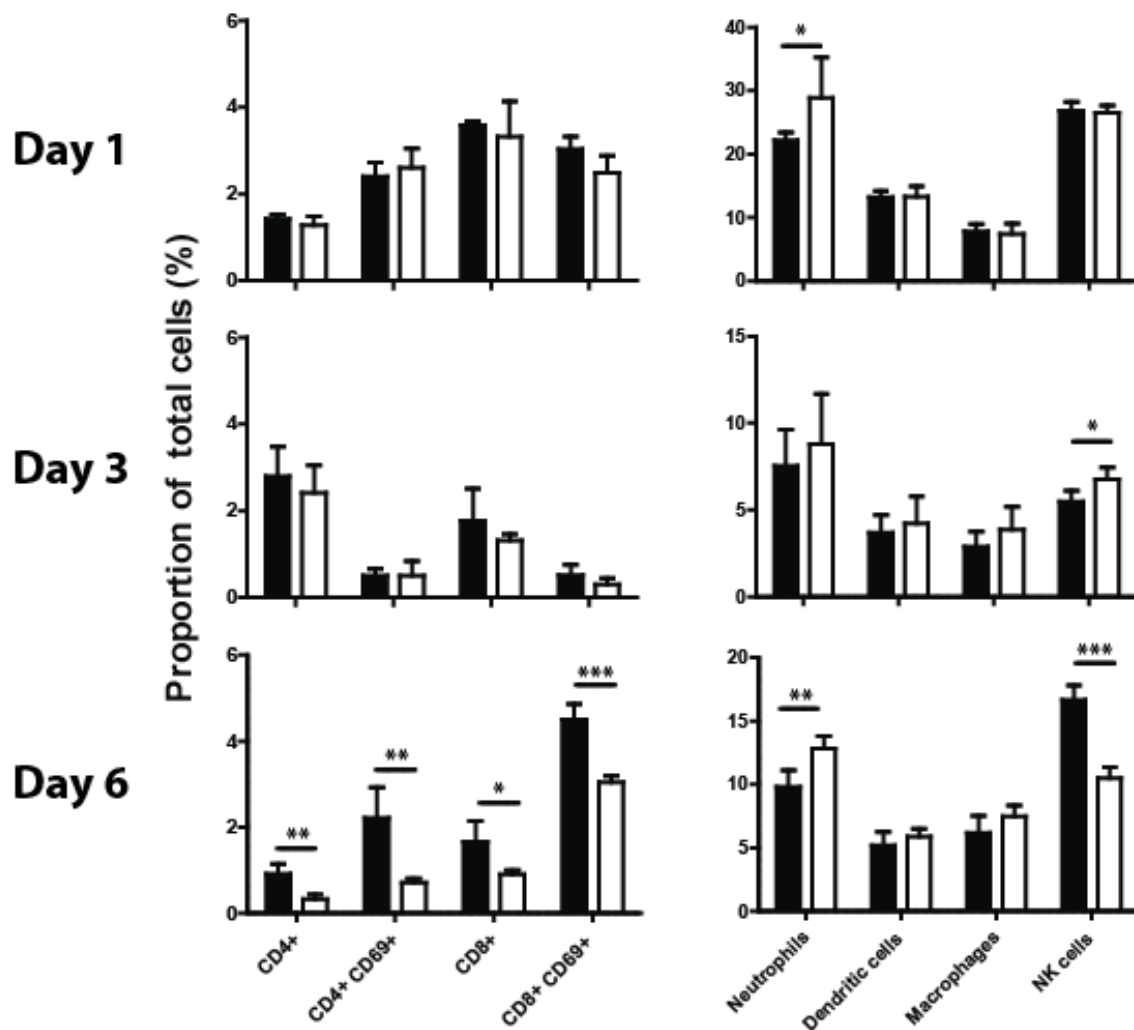
Figure 4.17: Immune cell populations over the course of influenza virus infection. Lungs were excised, homogenised and analysed by flow cytometry to quantify the contribution of various cellular subtypes at the indicated times post-infection. Widespread significant differences were observed on day six post-infection, wherein lymphopenia and an excess of neutrophils are seen. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. (n > 4). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). .

4.2.3.3.2 Cellular response: systemic

In order to quantify the systemic immune response to infection, mice were either killed by cardiac puncture and total leukocyte counts were calculated (Figure 4.18a), or mice were bled by

tail vein puncture and blood smears taken to analyse blood differential cell counts and quantify the levels of leukocytes, polymorphonuclear (PMN) cells and monocytes during infection (Figure 4.18b). The results of the total leukocyte count revealed that *Ifitm3*^{-/-} mice were largely unresponsive and failed to show the early peak in leukocyte numbers on day two post-infection. Similarly, *Ifitm3*^{-/-} mice showed a significantly lower number of leukocytes in circulation on day six post-infection when compared with wild type littermates (p = 0.005). Significant lymphopenia was also observed on day two post-infection (p = 0.04), with a reduction in the number of circulating lymphocytes.
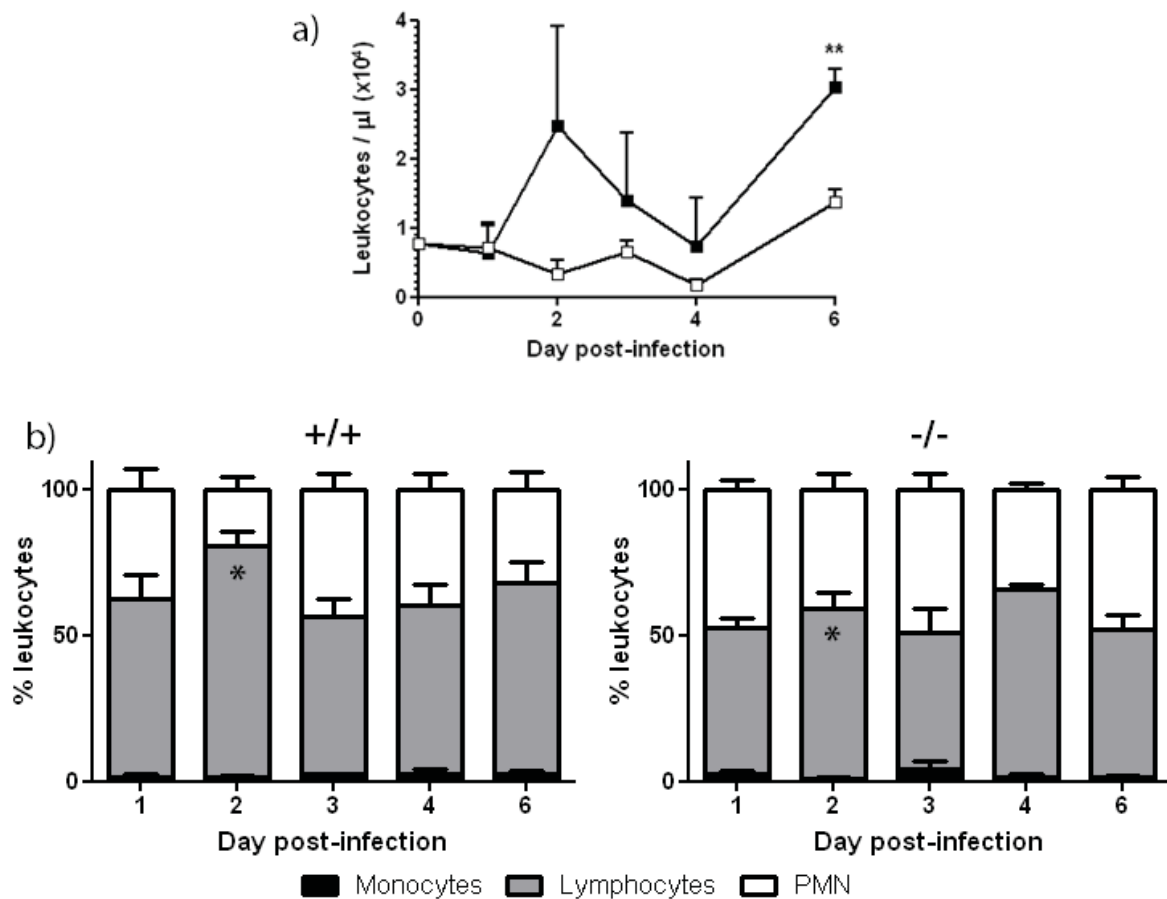
Figure 4.18: Systemic leukocyte responses to influenza virus infection. Total leukocyte counts (a) were taken by cardiac puncture of >3 mice per genotype at the indicated time points post-infection. Blood differentials (b) were calculated in a blinded fashion by assessing leukocyte populations on blood smears collected by tail vein puncture from >4 mice per genotype. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. Statistical significance was assessed by Student's *t*-test (*: p < 0.05, **: p < 0.01).

4.2.3.3.3   Cytokine response

Cytokines, along with immune cell populations, are one of the key mediators of the immune response to invading pathogens. Additionally, they can also be responsible for the immunopathology associated with severe disease, as discussed in section 1.4.4. *Ifitm3*^{-/-} mice differed in their cytokine cascades when compared with wild type mice, generally showing a more exaggerated response. Some of the most important deviations were observed with the pro-inflammatory cytokines IL-6, TNFα, G-CSF and MCP-1 (Figure 4.19a); all of which were significantly up-regulated over the course of infection. A further 11 inflammatory and anti-inflammatory cytokines were also assessed by bead-based Luminex assay over the course of infection (Figure 4.19b), which showed a similarly exaggerated trend.
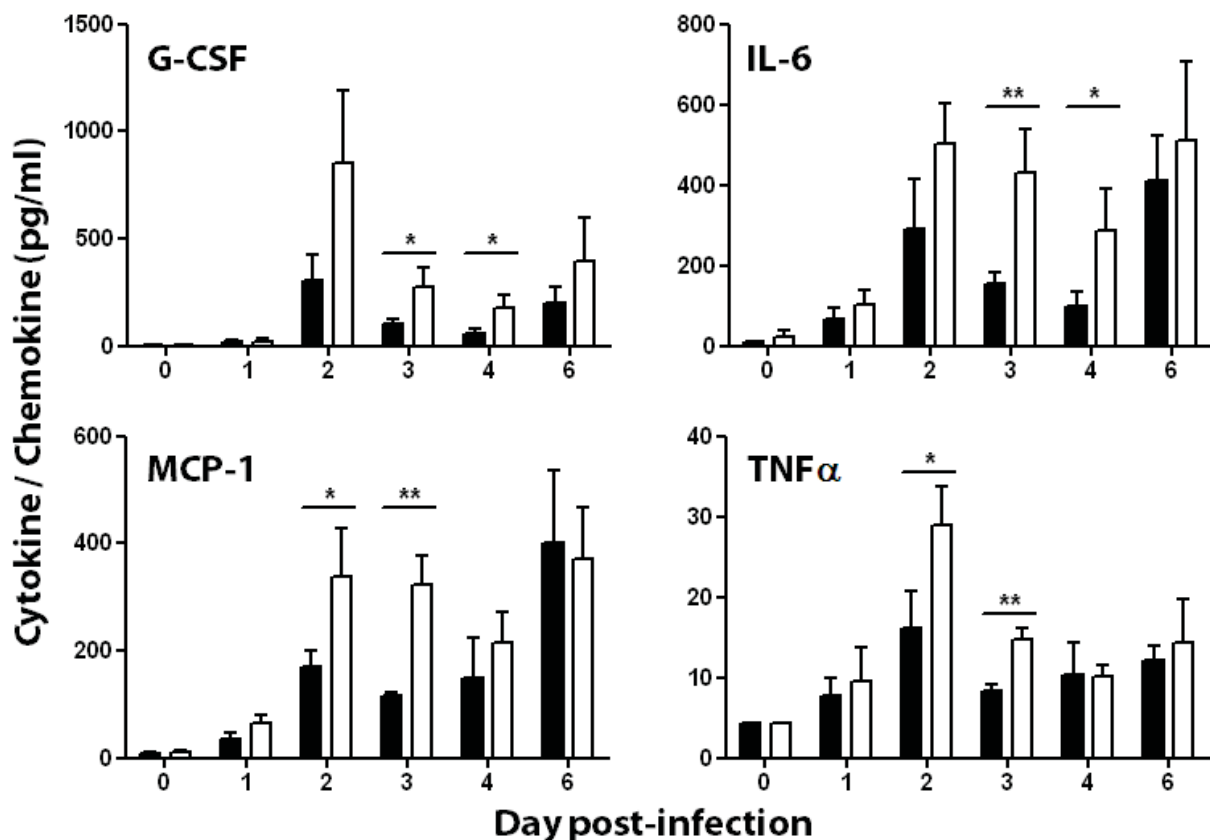
Figure 4.19a: Cytokine responses in the lungs of mice infected with influenza virus. Concentrations of a panel of cytokines present in the lungs over the course of A/X-31 infection were measured by Luminex assay. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. Statistical significance was assessed by Student's *t*-test (*: p < 0.05, **: p < 0.01).
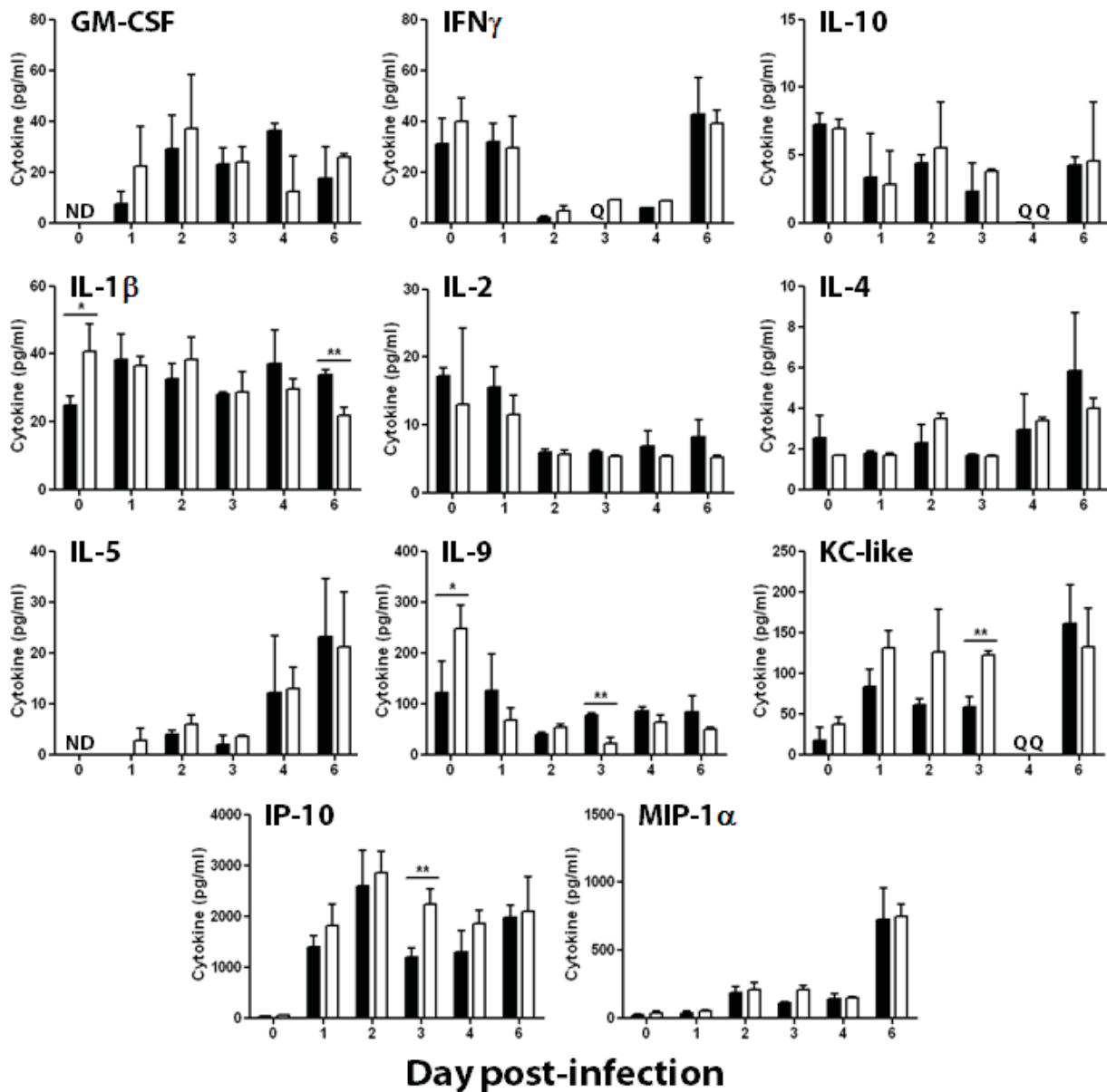
Figure 4.19b: Cytokine responses in the lungs of mice infected with influenza virus. Concentrations of a panel of cytokines present in the lungs over the course of A/X-31 infection were measured by Luminex assay. Q = <50 beads detected, therefore recorded as quality control failure, ND = not detected. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. Statistical significance was assessed by Student's *t*-test (*: p < 0.05, **: p < 0.01).

4.2.3.3.4   Adoptive bone marrow transfer

To evaluate the relative contribution of the immune system against influenza in *Ifitm3*^{-/-} mice, adoptive bone marrow transfer was conducted. Both wild type and *Ifitm3*^{-/-} mice were irradiated and bone marrow was transferred between animals to create chimeras (henceforth termed wt[BM-

^{Ifitm3}: wild type mice with *Ifitm3*^{-/-} bone marrow, and Ifitm3^{BM-wt}): *Ifitm3*^{-/-} mice with wild type bone marrow). Mice that survived for 10 days post-transfer were deemed to have been successfully repopulated with immune progenitor cells and were infected with A/X-31 influenza after eight weeks and recorded for phenotypic differences (Figure 4.20).
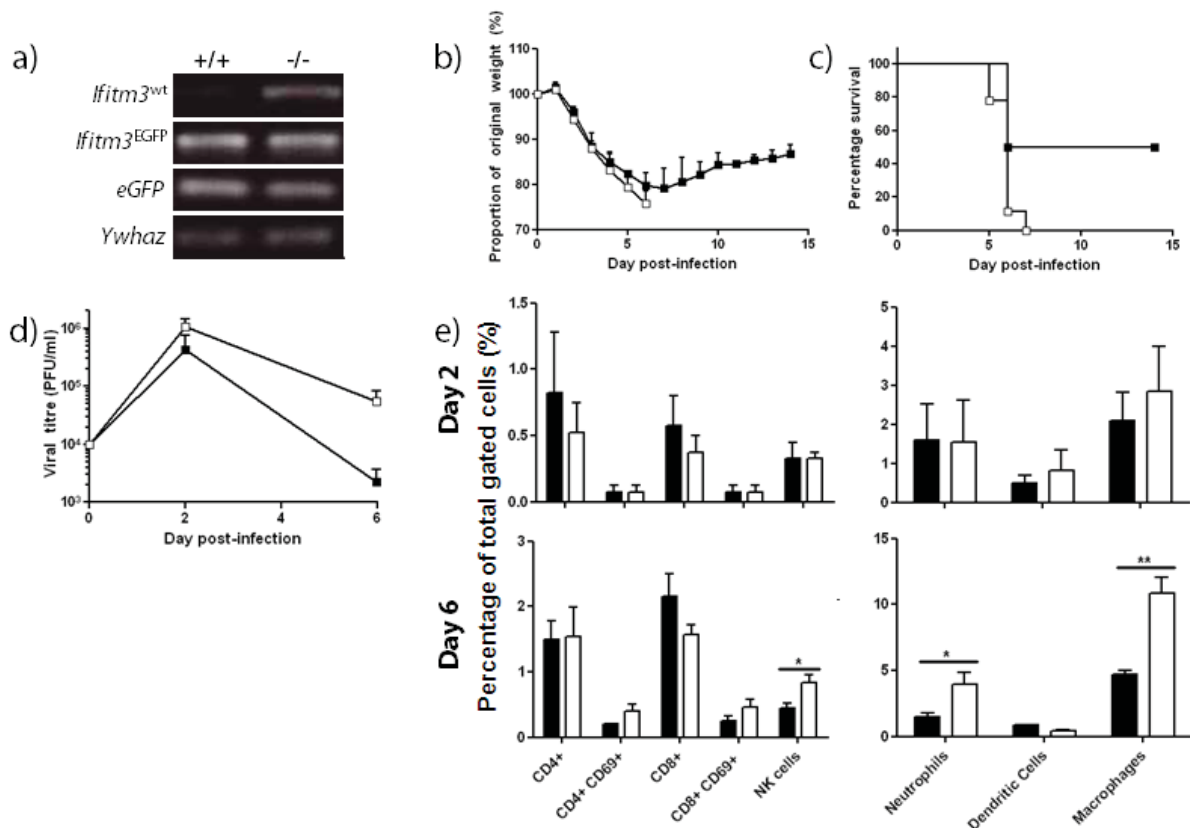
Figure 4.20: Influenza challenge of chimeric mice. Wild type and *Ifitm3*^{-/-} mice were irradiated and bone marrow was transferred between genotypes to create chimeras. Spleens were excised from animals and analysed by PCR for the presence of the wild type *Ifitm3* allele (*Ifitm3*^{wt}), *Ifitm3*^{-/-} allele (*Ifitm3*^{EGFP}) and *eGFP*, with *Ywhaz* included as a reference loading gene (a). Mice were infected with 10^4 PFU of A/X-31 and weight loss and survival recorded for 14 days post-infection (b,c). Lungs were removed on days two and six post-infection to quantify viral load (d) and resident immune cell populations (e). ■: wt^{BM-Ifitm3}, □: Ifitm3^{BM-wt}. Results show means ± S.D. (n > 3). Statistical significance was assessed by Student's *t*-test (*: $p < 0.05$, **: $p < 0.01$).

To verify the chimeras, spleens were removed and PCR analysis conducted for wild type and *Ifitm3*^{EGFP} allele presence in both sets of mice. This confirmed that both sets of mice had become successfully reconstituted with the donor's bone marrow (Figure 4.20a). qPCR analysis of these samples revealed that wt^{BM-Ifitm3} mice contained 28× more Ifitm3^{EGFP} DNA than knockout mice,

whilst Ifitm3$^{\text{BM-wt}}$ mice contained 10× more *Ifitm3*$^{\text{wt}}$ DNA than wild type mice in their spleens (data not shown).

Infection of the chimeric mice with influenza virus resulted in >20% weight loss in both genotypes of mice (Figure 4.20b), with 100% mortality in the Ifitm3$^{\text{BM-wt}}$ group, and 50% mortality in the wt$^{\text{BM-Ifitm3}}$ group (Figure 4.20c). Viral kinetics in both sets of mice were the same as those observed in non-chimeric challenges (Figure 4.7), with Ifitm3$^{\text{BM-wt}}$ mice showing slower resolution of viral infection, with a >10-fold higher viral burden on day six post-infection (Figure 4.20d). Significant differences were observed on day six post-infection, with Ifitm3$^{\text{BM-wt}}$ mice having significantly more NK cells (p = 0.03), neutrophils (p = 0.04) and macrophages (p = 0.006) present in their lung tissue.

4.2.4   Collaborative work on human IFITM3 genetics[1]

To assess the IFITM3 allelic diversity in humans, samples were collected from patients hospitalised with confirmed influenza virus infections during the 2009-2010 H1N1 pandemic. These patients were all Caucasians with no known co-morbidities. A total of 53 DNA samples were collected in association with the *MOSAIC* and *GenISIS consortia* from England and Scotland, which were then subsequently sequenced. These were aligned to the human *IFITM3* encoding reference sequence (Acc. No.: NC_000011.9) and Phred values compared.

Significant deviations in some of the sequenced samples from the human reference sequence occurred at DNA position 320772, which encodes SNP rs12252, wherein a majority T is mutated to a minority C. In total, we found 46 TT, 4 TC, and 3 CC individuals (Figure 4.21a). In collaboration with others at the WTSI and Roslin Institute in Edinburgh, we found the genotypes associated with rs12252 differed significantly from ethnically matched Europeans in the 1000 Genomes sequence data and from genotypes imputed against the June 2011 release of the 1000 Genomes phased haplotypes from the UK, Netherlands and Germany (Table 4.1). Patients' genotypes also depart from Hardy Weinberg equilibrium (*p*=0.003), showing an excess of C

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[1]Collaboration was sought to complete the human genetics component of the IFITM3 work. Sarah E. Smith sequenced the patients' *IFITM3* genes and scored Phred values; Aarno Palotie and Verneri Anttila performed 1000 Genomes analyses and imputation of SNPs; Chris Tyler-Smith performed evolutionary analyses on the *IFITM3* allele; and J. Kenneth Baillie performed principle component analyses.

alleles in this population (Figure 4.21a). Principal components analysis of over 100K autosomal SNPs showed no evidence of a hidden population structure differences between WTCCC controls and a subset of the hospitalised individuals from this study (Figure 4.21b). Further collaboration with Chris Tyler-Smith's group at the WTSI showed evidence for positive selection on the *IFITM3* locus in human populations acting over the last tens of thousands of years in Africa (Figure 4.21c).
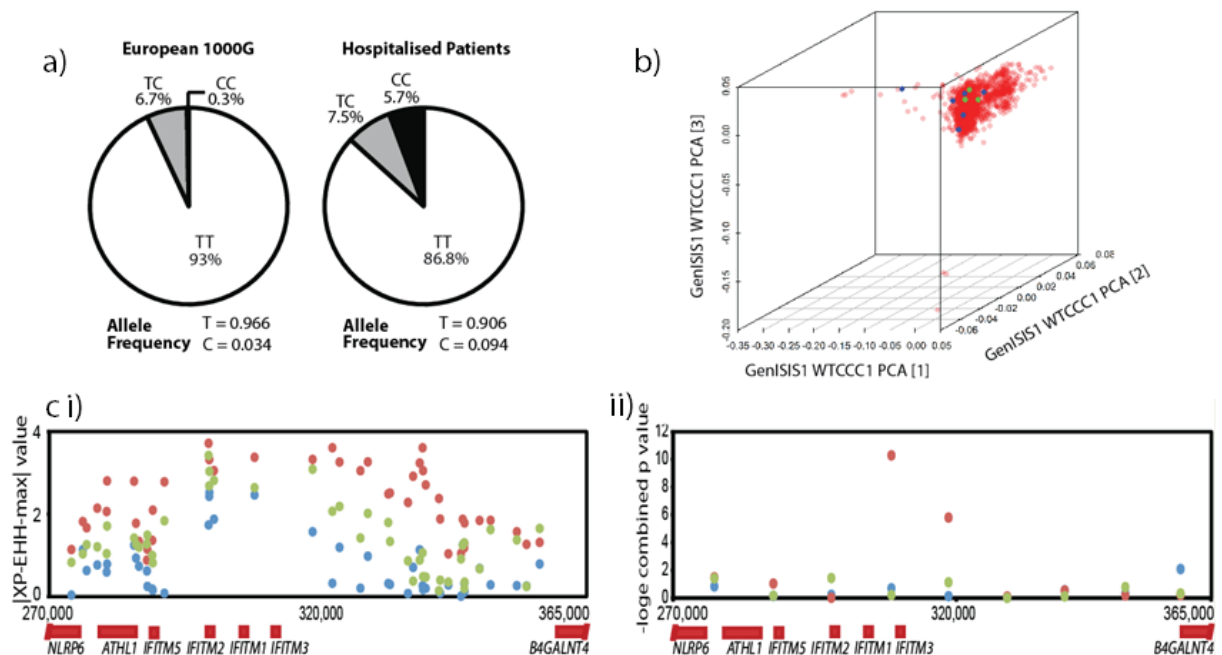
Figure 4.21: Single nucleotide polymorphisms of the human *IFITM3* gene and the prevalence of SNP rs12252. Sequencing of patients hospitalised with influenza virus during the 2009-2010 influenza pandemic showed an overrepresentation of individuals with the minority C allele at SNP rs12252. Principle component analyses (b) were conducted to check for clustering of a selection of TT (blue circles) and TC / CC (green circles) samples against 1499 controls from the WTCCC 1958 Birth Cohort (red circles). Positive selection analysis were conducted using a haplotype-based test (|XP-EHH-max|, (ci) where data points above 2.7 in the YRI (red), 3.9 in the CEU (blue) and 5.0 in the CHB+JPT (green) are in the top 1% of values and using a combination of three allele frequency spectrum-based test statistics, namely Tajima's D, Fay and Wu's H and Nielsen *et al.*'s CLR (cii), on 10 kb windows along chromosome 11 encompassing the *IFITM3* locus. Evidence for positive selection is seen only in the YRI.

Table 4.1: Allele and genotype distribution derived from multiple global populations of the 1000 Genomes Project and patients hospitalised with influenza for SNP rs12252 of IFITM3.

Population	Allele Frequency		Genotype			Total Samples	HW[1]	P-value[5]
	C	T	CC	CT	TT			
YRI[2]	0.093	0.907	1	9	49	59	0.40	-
CHB/JPT[2]	0.3	0.7	9	18	33	61	0.03	-
CEU/FIN/GBR/IBI/TSI[2]	0.036	0.964	1	24	335	360	0.37	-
Hospitalised patients[3]	0.094	0.906	3	4	46	53	0.003	-
WTCCC1[4]	0.028	0.972	-	-	-	2938	0.73	6.46×10^{-5}
Netherlands[4]	0.026	0.974	-	-	-	8892	0.67	1.11×10^{-5}
Germany[4]	0.029	0.971	-	-	-	6253	0.82	6.93×10^{-5}

[1] Probability that observed genotype frequencies depart from Hardy-Weinberg equilibrium.
[2] Allele and genotype frequencies obtained from 1000 Genomes sequence data, (YRI, African ancestry, CHB/JPT, Chinese and Japanese ancestry, CEU/FIN/GBR/IBS/TSI, European ancestry).
[3] Allele and genotype frequencies determined in this study.
[4] Allele frequencies determined in this study imputed against the June 2011 release of 1000 Genomes phased haplotypes.
[5] P-value for additive model association analysis of hospitalised patients vs. the population samples, using SNPTEST v2.1.1.

4.2.5   Restrictive capacity of truncated and rs12252-C containing IFITM3

The rs12252-C SNP is purported to act as a splice site acceptor site, which in turn would truncate the full length IFITM3 protein at its N-terminal by 21 amino acids (NΔ21). In collaboration with Abraham L. Brass, Harvard University, plasmids encoding either the full length or NΔ21 DNA sequence were transduced into A549 cells to examine the effect of the loss of the terminal 21 amino acids at the N-terminal. Cells were confirmed to be stably expressing either of the constructs by Western blot (Figure 4.22a) and were subsequently challenged with four strains of influenza virus (A/WSN/33, A/California/07/2009, A/Uruguay/716/2007 and B/Brisbane/60/2008). Viral NP expression was quantified 12 hours post-infection (Figure 4.22b). The *in vitro* assays showed that NΔ21 IFITM3 confers minimal restrictive capacity, whilst full length IFITM3 was capable of significantly restricting all strains of influenza A and B tested.

a) **IFITM3**   MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP
       NΔ21   MLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP
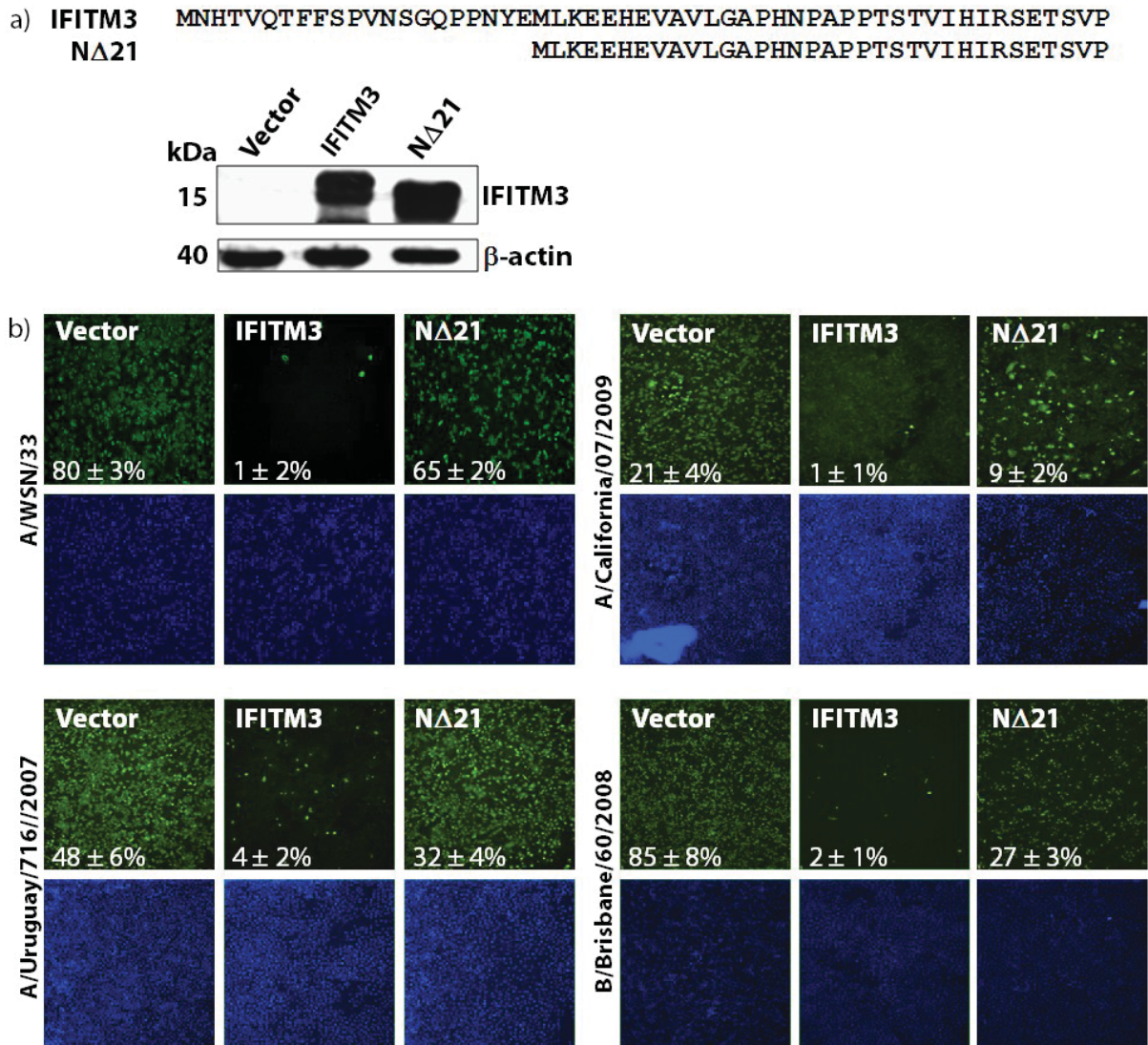
Figure 4.22: Impact of IFITM3 NΔ21 truncation on restriction of influenza A and B viruses. A549 cells were transduced with plasmids to express either empty vector control (Vector), full-length IFITM3 (IFITM3), or an N-terminally truncated IFITM3 (NΔ21) protein. Expression was confirmed by Western blot (a). Cells were subsequently infected with the indicated strains of influenza virus (b) and were assayed after 12 hours for influenza NP expression. Green: influenza NP expressing cells, blue: DAPI staining of cells. Results show mean level of infection ± S.D. (n = 3).

To assess the function of the rs12252-C SNP, human lymphoblastoid cell lines (LCLs) were sequenced to identify those containing the rs12252-T and rs12252-C alleles. These were subsequently infected with WSN/33 influenza A virus to determine viral susceptibility. As shown in Figure 4.23, the presence of CC resulted in increased susceptibility to influenza virus

infection in both an unstimulated and IFN-induced environment. Furthermore, Western blot analysis showed that IFITM3 expression was lower in rs12252-C containing LCLs in the latent state. However, IFN stimulation resulted in a qualitatively similar level to that of the rs12252-T containing LCLs.
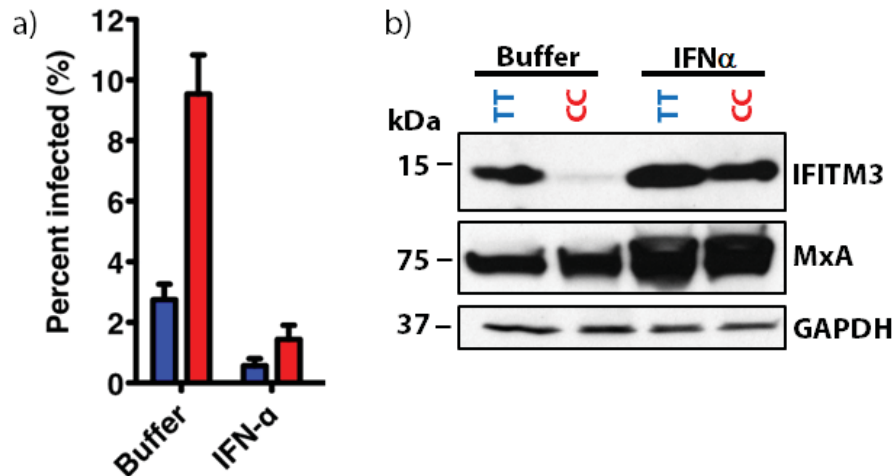
Figure 4.23: Viral replication and IFITM3 expression in rs12252-TT and rs12252-CC containing human cells. LCLs homozygous for either the majority T (blue) or minority C (red) alleles were challenged with WSN/33 influenza with or without IFN stimulation (a). Western blots for these LCLs were also conducted (b) and probed for IFITM3 expression, MxA for assessment of ISG expression and GAPDH as a housekeeping loading control. Results show means ± S.D. (n = 3).

4.3   Discussion

This study showed that Ifitm3 is a crucial antiviral restriction factor necessary for resistance to influenza virus in mice. The loss of Ifitm3 results in a persistent viral infection in the lungs, and the onset of fulminant viral pneumonia when mice are challenged with a low pathogenicity strain of influenza virus, which subsequently results in heightened morbidity and mortality. Furthermore, these studies showed for the first time that influenza resistance and susceptibility may have a heritable component in humans, with the enrichment of SNP rs12252 in a cohort of hospitalised patients suggesting that defects in IFITM3 may result in a more severe disease phenotype. This SNP is thought to act as a splice site acceptor, which truncates IFITM3 at its N-terminus by 21 amino acids (NΔ21). This study shows that this mutation greatly reduces the antiviral activity of IFITM3.

Typically, low pathogenicity (LP) influenza viruses, such as A/X-31 and A/Eng/195 cause mild weight loss and are largely asymptomatic in wild type mice, at the doses used here (Mozdzanowska *et al.* 2000; Guo *et al.* 2011; Vlahos *et al.* 2011). They do not normally cause extensive viral replication throughout the lungs, or cause the cytokine dysregulation and death typically seen after infection with highly-pathogenic (HP) viral strains (Belser *et al.* 2010). However, *Ifitm3*$^{-/-}$ mice became moribund and showed severe signs of clinical symptoms by day six post-infection, as a result of delayed viral clearance, extensive respiratory tissue damage and fulminant viral pneumonia, which subsequently resulted in death. It therefore appears as though the removal of a functional Ifitm3 protein enables a typically LP influenza virus to elicit symptoms more commonly associated with HP infections.

Indeed, experiments using the HP 1918 'Spanish' influenza strain and avian H5N1 strains have resulted in severe pathological damage and death in a range of animals, including mice and primates (Tumpey *et al.* 2005a; Kobasa *et al.* 2007; Maines *et al.* 2008). Further to the extensive pathological damage caused by these viruses, several other symptoms seen in the current study would also more commonly be associated with HP influenza infection. One of the notable features seen in the study was the depletion of NK, CD4+ and CD8+ lymphocytes both within the lungs and systemically. T-cells are seen as crucial in the clearance of influenza virus from the body (Schmolke and García-Sastre 2010; Zhang and Bevan 2011); therefore making their absence counterintuitive. However, lymphopenia has been noted in HP (but not LP) influenza infections in both animals and humans (Tumpey *et al.* 2000; Maines *et al.* 2008; Perrone *et al.* 2008; Belser *et al.* 2010); although the reasoning as to why this depletion occurs is currently unknown.

In addition to the leukopenia and lymphopenia, two other traits associated with HP infections are dysregulated cytokine production and excessive cellular infiltrate into the lungs during infection; both of which were seen in the current study. The observed exaggerated pro-inflammatory responses in the lungs of *Ifitm3*$^{-/-}$ mice are particularly noteworthy, with higher levels of TNFα, IL-6, G-CSF and MCP-1 showing the most marked changes. This is indicative of the extent of viral spread within the lungs, as TNFα and IL-6 are released from cells upon infection (Julkunen *et al.* 2000). Consistent with the pathological damage, these changes are comparable in level to

those observed in non-H5N1 HP influenza infections (Belser *et al.* 2010). A novel observation was the elevated levels of osteopontin (Opn) in the lungs following influenza infection. Opn, which can act as a neutrophil chemoattractant (Nishimichi *et al.* 2011), is up-regulated in severe acute respiratory syndrome coronavirus (SARS-CoV) infections (Smits *et al.* 2011) and has been shown to directly but inversely interact with IFITM3 (El-Tanani *et al.* 2010). Thus, it is interesting that elevated expression of Opn correlates with an increase in cellular infiltrate, especially neutrophils, in the airways on day six post-infection (Figure 4.14). Such heightened levels may be contributing to neutrophil accumulation in the lungs. Neutrophil chemotaxis, together with elevated proinflammatory cytokine secretion, has previously been reported as one of the primary causes of acute lung injury (Yum *et al.* 2001; Grommes and Soehnlein 2011; Narasaraju *et al.* 2011). Similarly, the observed elevated levels of Opn in uninfected mice may serve to promote more rapid neutrophil chemotaxis, which could be contributing to the overall immunopathology.

Interestingly, the adoptive bone marrow transfer experiment did little to negate the effects of the loss of Ifitm3 in *Ifitm3*$^{-/-}$ mice, with a broadly similar phenotype occurring as that seen earlier in the study with non-chimeric mice. This would suggest that possessing immune cells with functioning copies of *Ifitm3* is not sufficient to rescue the *Ifitm3*$^{-/-}$ animal; it would appear as though the altered viral kinetics in the lungs is perhaps the primary cause of the overall phenotype. Further to this, infection with the delNS1 strain of PR/8 influenza virus would also indicate that the phenotype was not the result of impeded IFN production as such attenuated viruses are pathogenic to IFN-deficient mice (Garcia-Sastre *et al.* 1998). Taken cumulatively, these data would suggest the murine phenotype is a result of elevated and sustained viral replication in the respiratory tract and subsequent immune dysregulation; therein causing severe pathological damage.

Since publication of this work (Everitt *et al.* 2012), another group has independently verified our findings by showing the increased susceptibility of mice carrying the *Ifitm3*-null allele (Bailey *et al.* 2012). They also show that heterozygotic *Ifitm3*$^{+/-}$ mice display an intermediate phenotype, and that mice carrying a cumulative deletion of *Ifitm1*, *Ifitm2* and *Ifitm3* are phenotypically

indistinguishable from *Ifitm3^{-/-}* mice; supporting the premise that Ifitm3 is the crucial member of the Ifitm family that controls influenza virus infection *in vivo*.

The prevalence of the rs12252-C SNP in the sampled cohort of hospitalised patients is particularly interesting, as it suggests a heritable trait that can account for viral susceptibility; just as the CCR5-Δ32 mutation can aid resistance to HIV (Dean *et al.* 1996; Samson *et al.* 1996). As discussed previously, the 2009 H1N1 virus was less virulent than was first anticipated; resulting in far fewer mortalities than would be predicted from a novel zoonotic virus. What was remarkable was the prevalence of severe illness and death in individuals that had no known co-morbidities that were not classed as traditionally "at risk" (Donaldson *et al.* 2009). The discovery of the increased susceptibility of the rs12252-CC containing cells and loss of restrictive capacity of NΔ21 IFITM3-expressing cells suggests an important role for fully functional IFITM3 in humans. It is the elucidation of host resistance and susceptibility factors, such as the rs12252-C SNP in IFITM3 that may aid in prediction of disease severity. Although there are clearly many more host factors that may be contributing to the overall illness stemming from influenza virus, our findings provide the first evidence of such a phenomenon.

Recently, these findings have been independently verified in a Chinese cohort of patients, where the prevalence of the rs12252-C SNP is far higher than in European Caucasian populations (Zhang *et al.* 2013b). The study found a significant overrepresentation of the minority SNP in patients hospitalised with severe influenza infection, as well as higher viral loads and levels of MCP-1 in these patients, which are features recorded in our study's *in vivo* murine work (Figures 4.8 and 4.19a). Although the rs12252-CC genotype is rare in Europeans (0.5%), it is far more frequent in Chinese (25%) and Japanese (44%) populations. This data therefore highlights the importance of the SNP, as potentially large numbers of people could possess this genotype; therefore making them more susceptible to the range of viruses that IFITM3 can restrict.

Taken together, this study shows how the loss of a single immune effector, Ifitm3, can transform a potentially mild influenza virus infection into one with remarkable severity. The enrichment of the rs12252 C-allele in those hospitalised with influenza infections, together with the decreased IFITM3 levels and the increased infection of the rs12252-CC cells *in vitro*, suggests that IFITM3

also plays a pivotal role in defence against human influenza virus infections. This innate resistance factor is all the more important during encounters with a novel pandemic virus, when host acquired immune defences are less effective. Indeed, IFITM3-compromised individuals, and in turn populations with a higher percentage of such individuals, may be more vulnerable to the initial establishment and spread of a virus against which they lack adaptive immunity, which would suggest novel vaccination practices should be evaluated to include such groups. In light of its ability to curtail the replication of a broad range of pathogenic viruses *in vitro*, these *in vivo* results suggest that IFITM3 may also shape the clinical course of additional viral infections in favour of the host.

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).
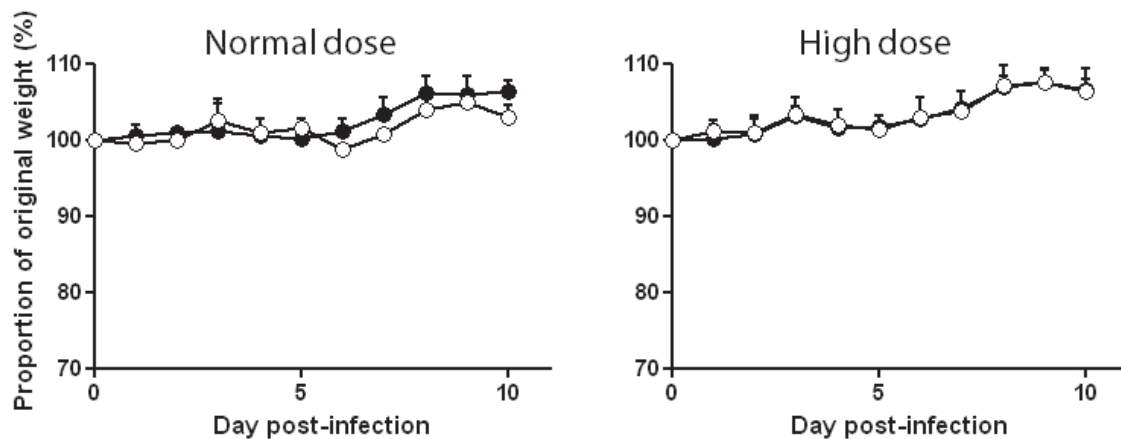
Figure 5.1: Tolerance of the live attenuated influenza vaccine, FluMist, in wild type and *Ifitm3*$^{-/-}$ mice. Mice were either administered intra-nasally with 1/10th human dose (normal) or 1/5th human dose (high) of FluMist and were weighed for 10 days post-vaccination. ●: vaccinated wild type, ○: 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.
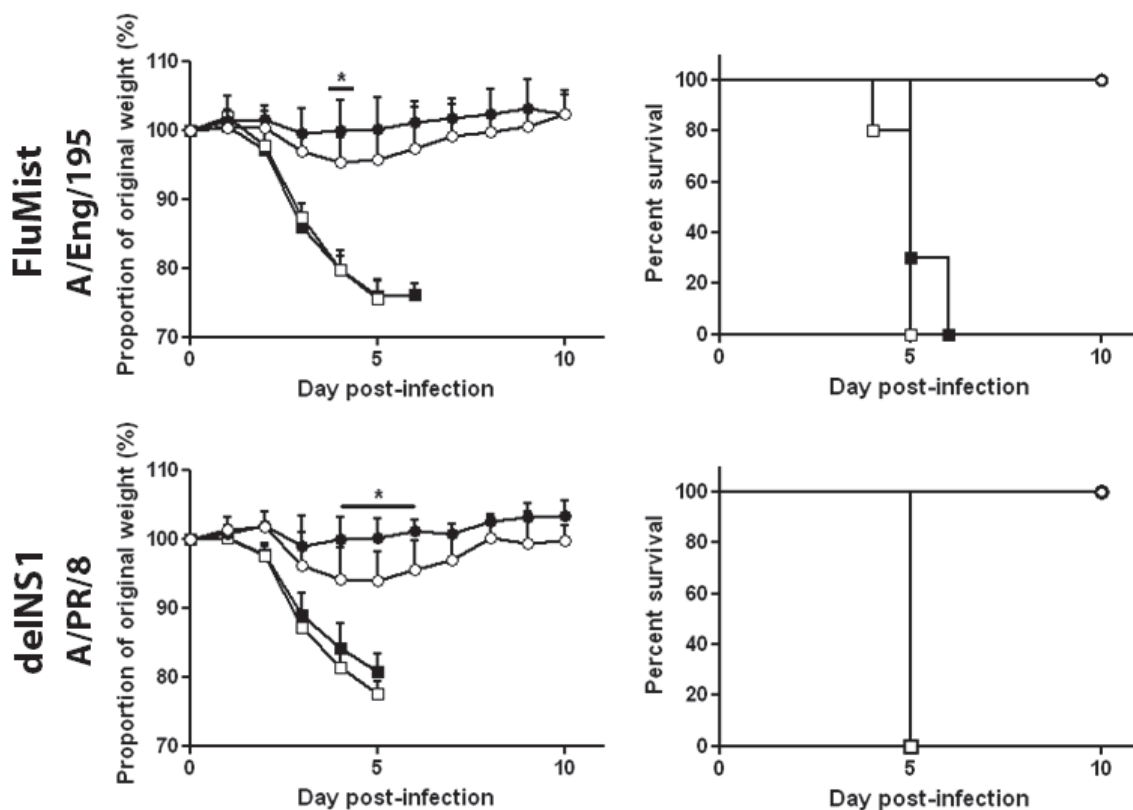
Figure 5.2: Efficacy of live attenuated influenza vaccines in terms of weight loss and survival of wild type and *Ifitm3*$^{-/-}$ mice. Mice were primed and boosted with either FluMist or PR/8 delNS1 influenza virus before challenge with 10 lethal dose of virulent virus. Animals were weighed daily and 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 $10\times$ 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).
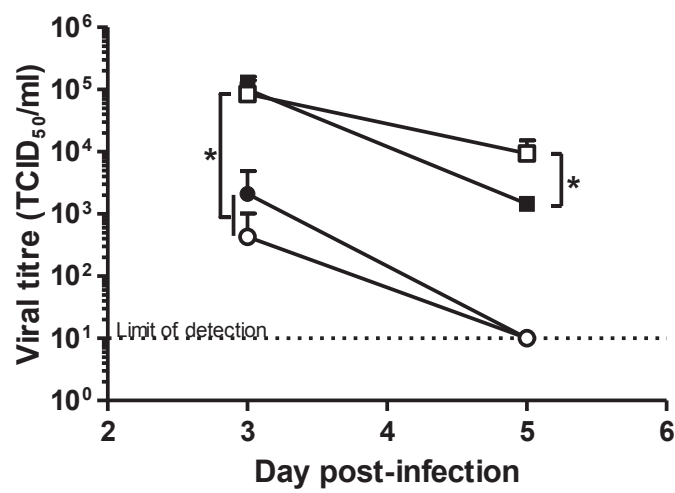
Figure 5.3: Effect of FluMist vaccination on influenza A viral kinetics in wild type and *Ifitm3*$^{-/-}$ mice. Results show the viral replication kinetics of Eng/195 influenza virus, as assessed by $TCID_{50}$ assay, in vaccinated and unvaccinated mice. ■: wild type, □: *Ifitm3*$^{-/-}$, ●: vaccinated 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.
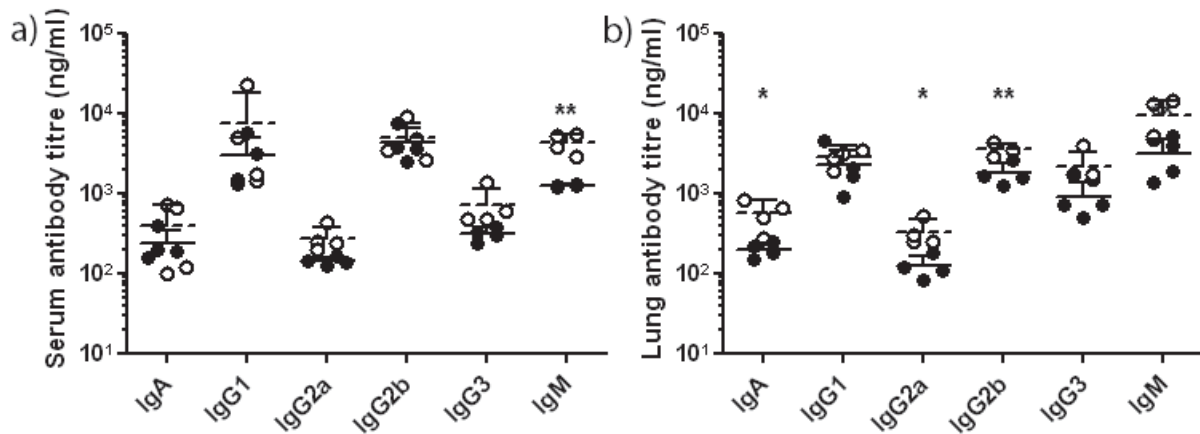
Figure 5.4: Immunoglobulin profile of the blood and lungs of wild type and *Ifitm3*^{-/-} mice following immunisation with FluMist vaccine. Antibodies in the sera (a) and lung homogenate (b) of immunised, but uninfected, mice were quantified by bead-based array. ●: vaccinated wild type, ○: vaccinated *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.
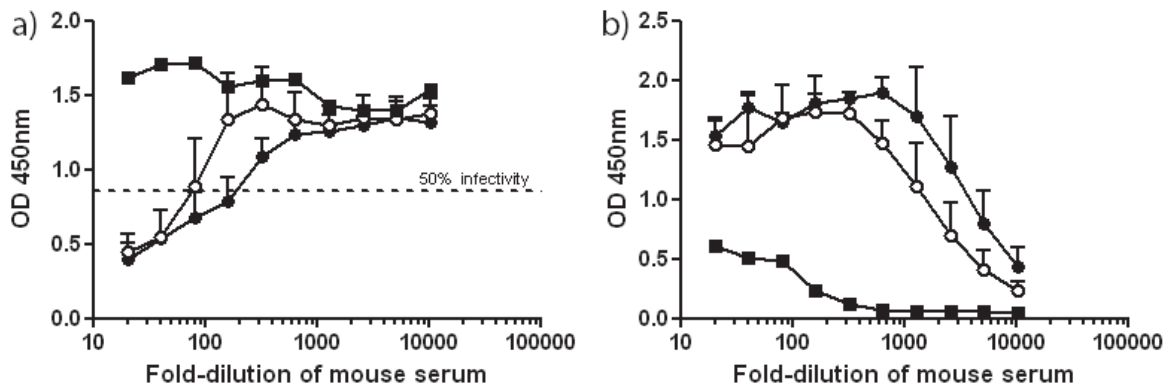
Figure 5.5: Influenza-binding capacity of wild type and *Ifitm3*$^{-/-}$ antibodies following FluMist immunisation. The neutralising capacity of sera antibodies were determined by microneutralisation (MN) assay (a), and influenza-binding capacity was measured by ELISA (b). MN titres were determined to 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.
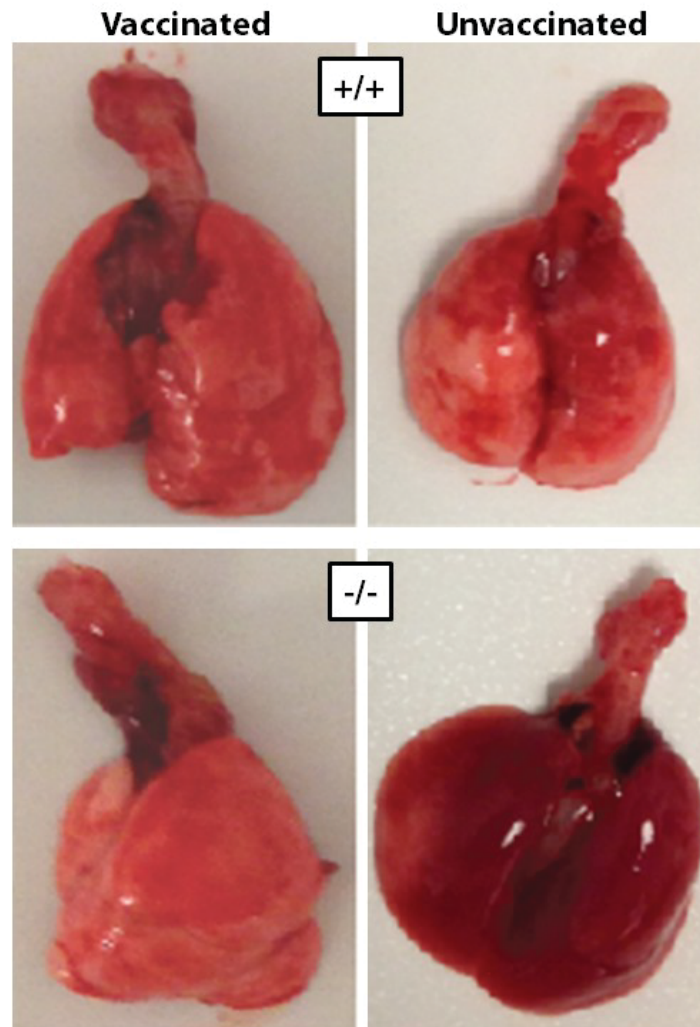
Figure 5.6: Effect of vaccination on pathological damage to the respiratory system following a lethal influenza A challenge. Vaccinated and unvaccinated wild type (+/+) and *Ifitm3*$^{-/-}$ (-/-) mice were challenged with a 10× lethal dose of Eng/195 influenza virus and their lungs were excised on day 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.
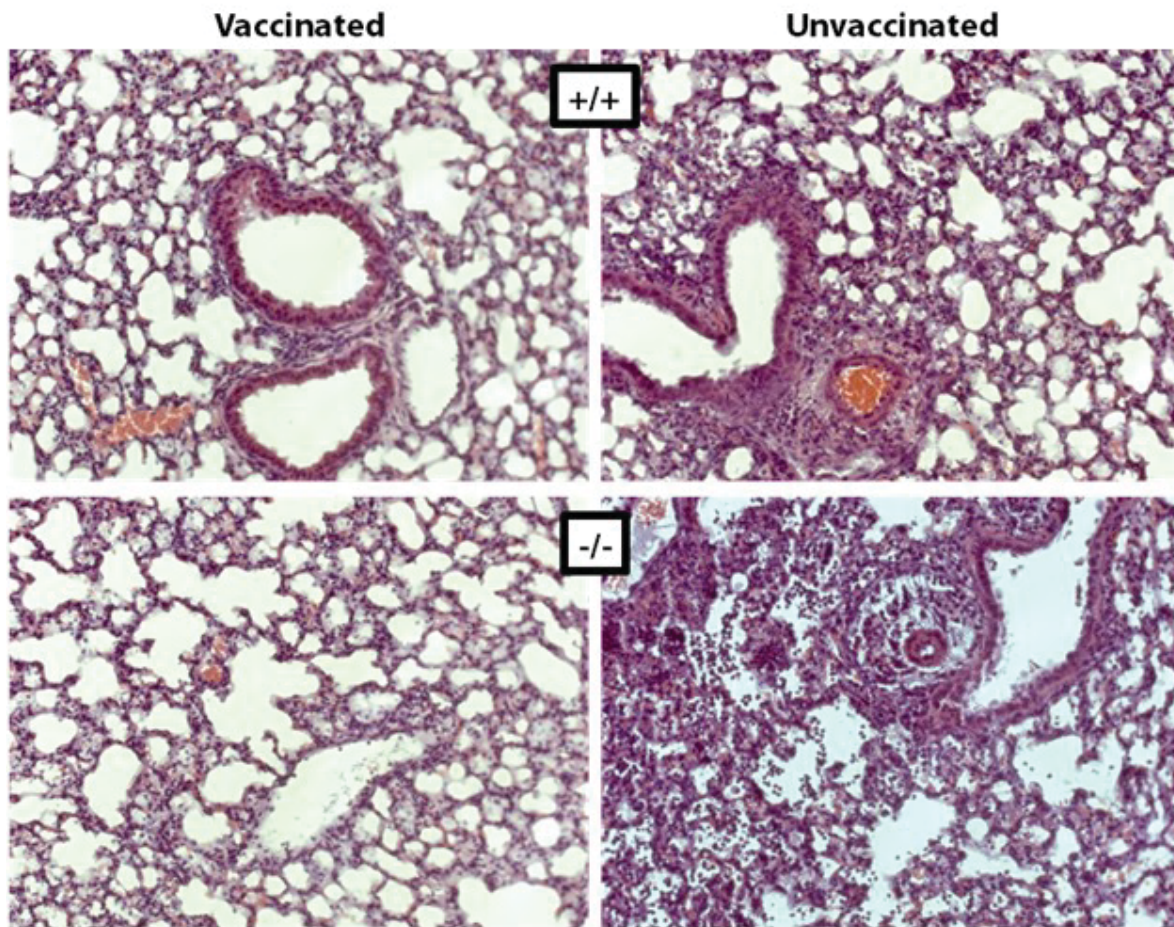
Figure 5.7: Histological impact of vaccination in wild type and *Ifitm3*-/- mice lungs following a lethal influenza A infection. Images show the extent of inflammation and damage in murine lungs at day five post-infection with a 10× lethal dose of Eng/195 influenza. Original magnification 20×.

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.
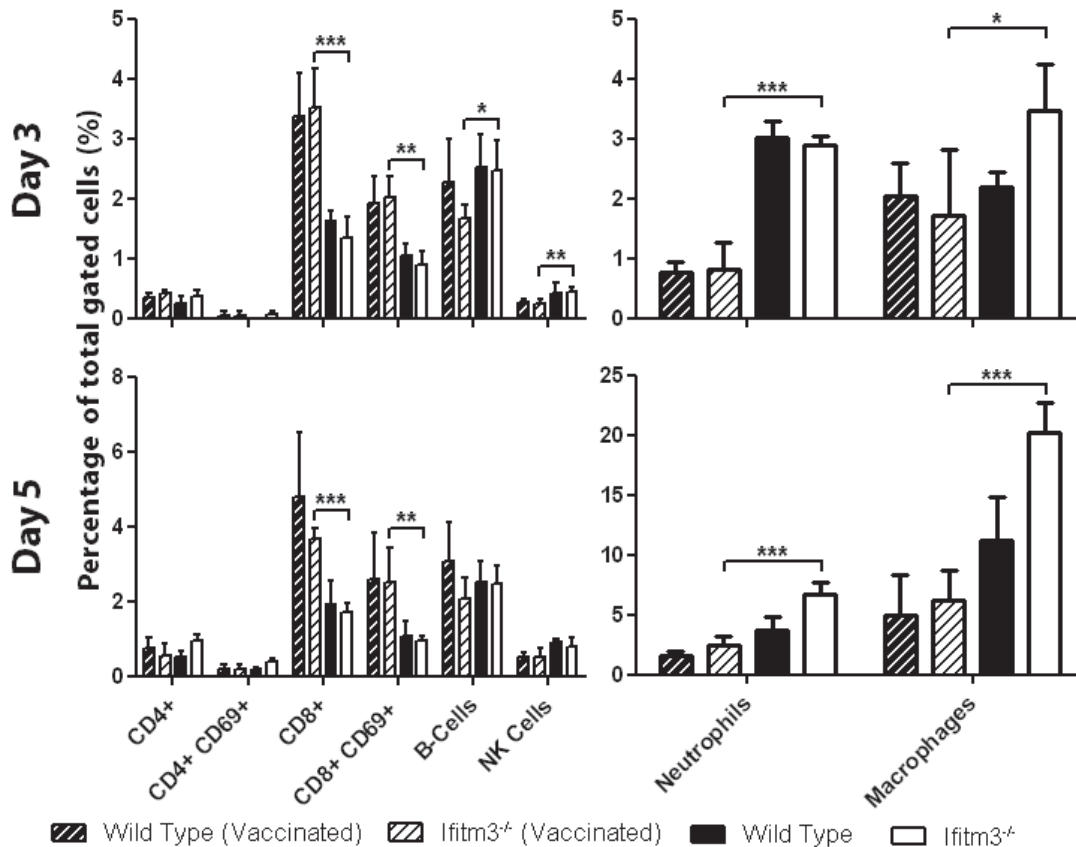
Figure 5.8: Impact of vaccination on immune cell populations within the lungs of wild type and *Ifitm3*$^{-/-}$ mice following influenza A infection. Leukocytes were isolated from total lung homogenates and analysed by flow cytometry to quantify the effects of vaccination. Legend is shown in the Figure. 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.
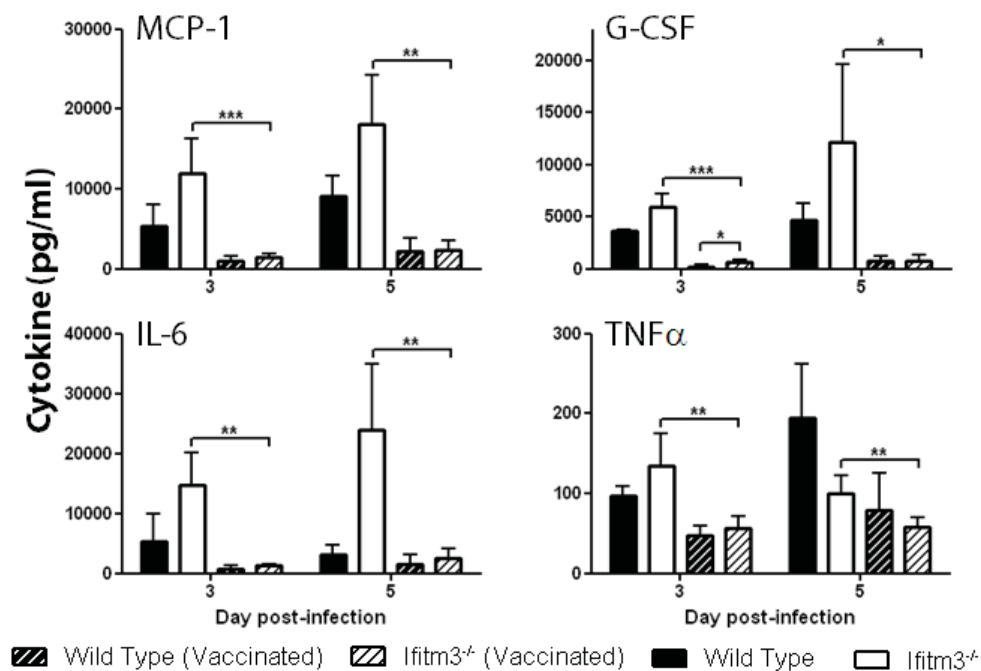
Figure 5.9: Effect of vaccination on inflammatory cytokine production in wild type and *Ifitm3*^{−/−} mice following influenza A infection. Lung homogenate was analysed for the levels of MCP-1, G-CSF, IL-6 and TNFa; all of which were significantly up-regulated in non-vaccine-based challenges (section 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: $1/10$[th] 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.
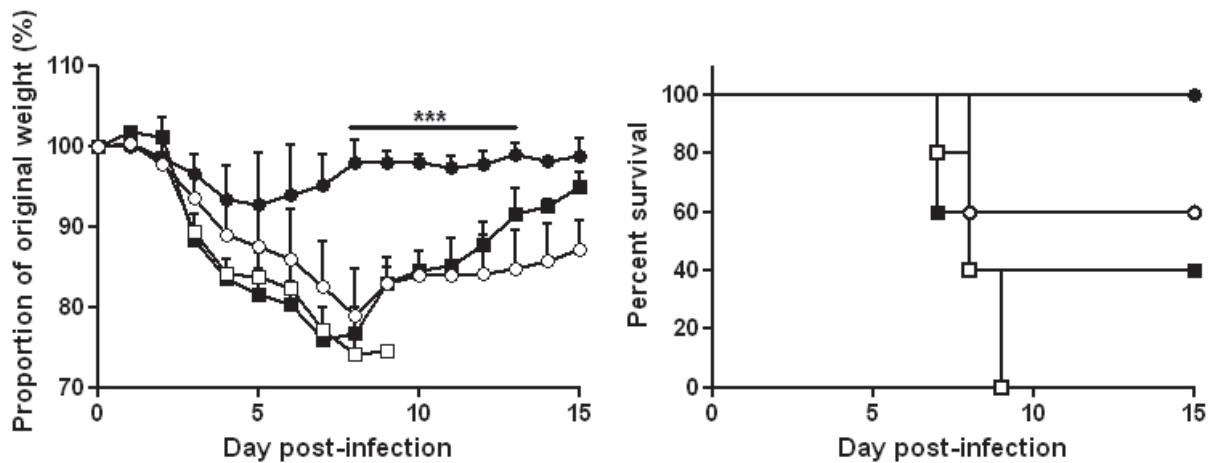
Figure 5.10: Effect of intra-muscular influenza vaccination on the weight loss and survival of wild type and ***Ifitm3*-/-** **mice following influenza A infection.** Mice were weighed daily and monitored for signs of severe illness; those exceeding 25% weight loss were killed in accordance with Home 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.
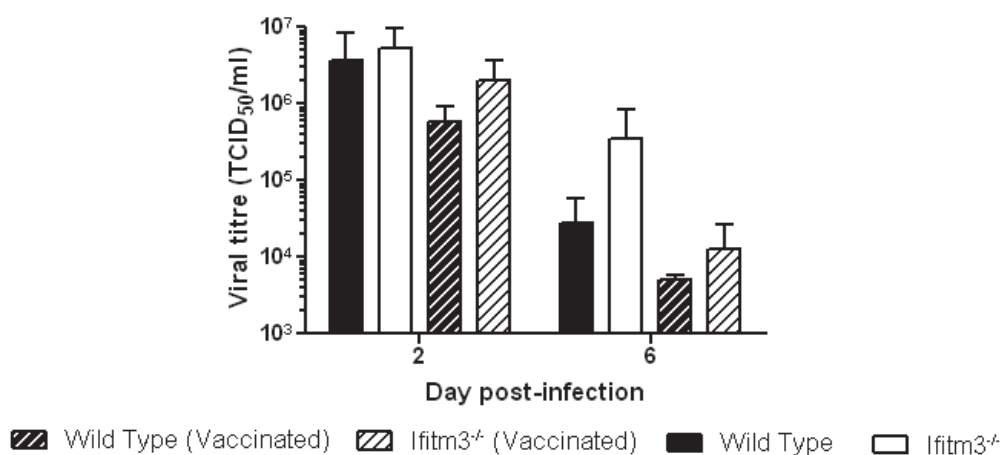
Figure 5.11: Effect of intra-muscular vaccination on viral load in the lungs of wild type and ***Ifitm3*-/-** **mice following influenza A infection.** Lungs were excised and homogenised on days two and six post-infection to quantify viral load by $TCID_{50}$ assay. Legend is shown in the Figure. Results 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.
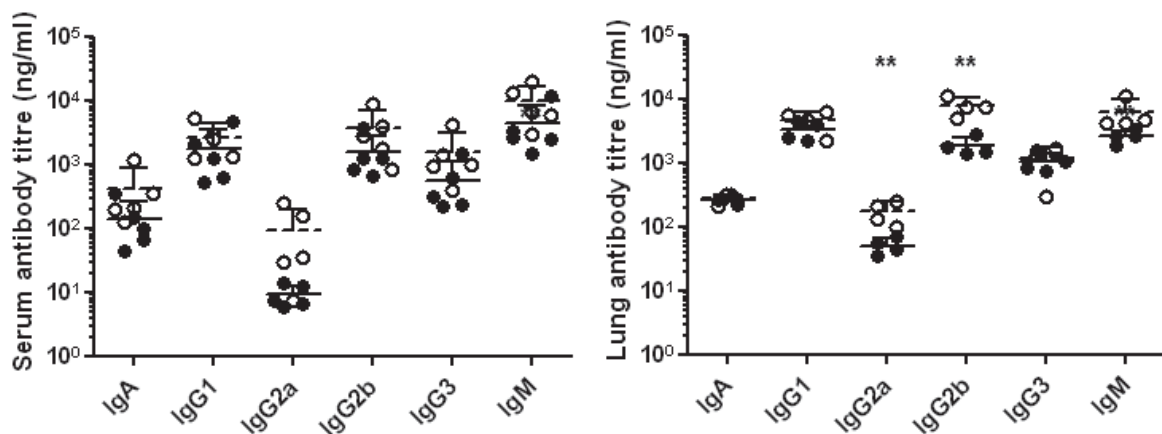
Figure 5.12: Immunoglobulin profile of the blood and lungs of wild type and *Ifitm3*^{-/-} mice following immunisation with Fluvirin intra-muscular vaccine. Antibodies in the sera (a) and lung homogenate (b) of immunised, but uninfected, mice were quantified by bead-based array. ●: vaccinated wild type, ○: 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.
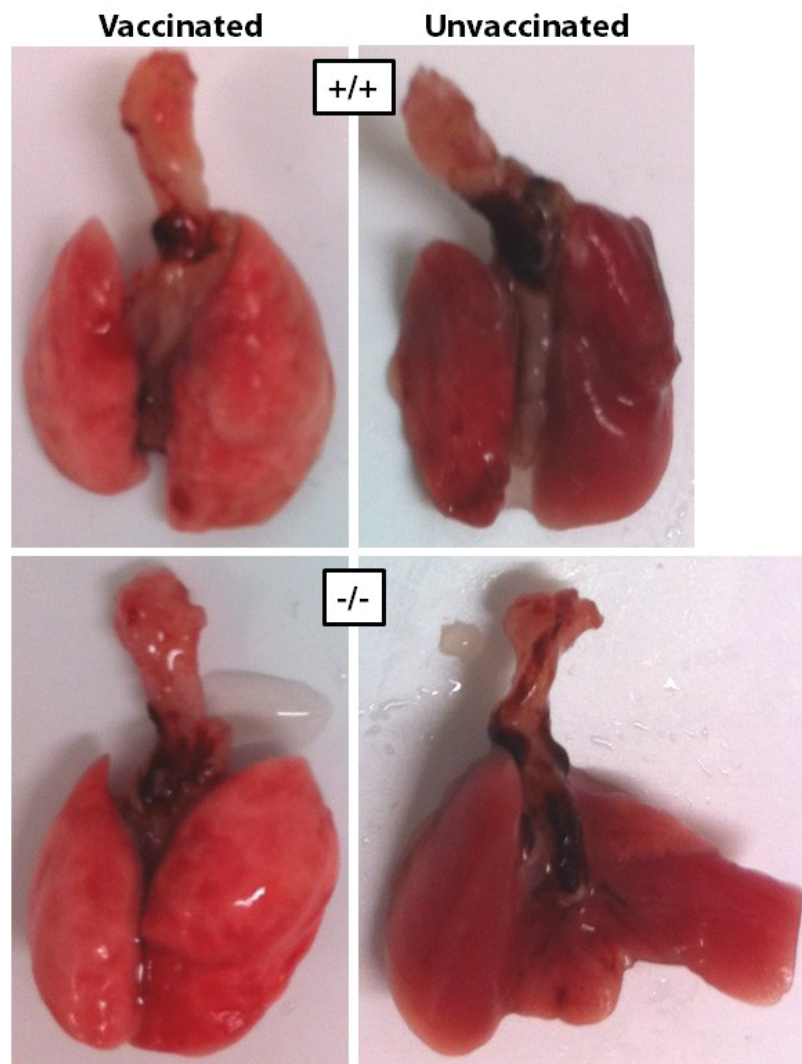
Figure 5.13: Impact of intra-muscular vaccination on pathological damage caused by a lethal challenge with influenza A virus. Vaccinated and unvaccinated wild type (+/+) and *Ifitm3*$^{-/-}$ (-/-) mice were challenged with a 10× lethal dose of Eng/195 influenza virus and their lungs were excised on day 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.
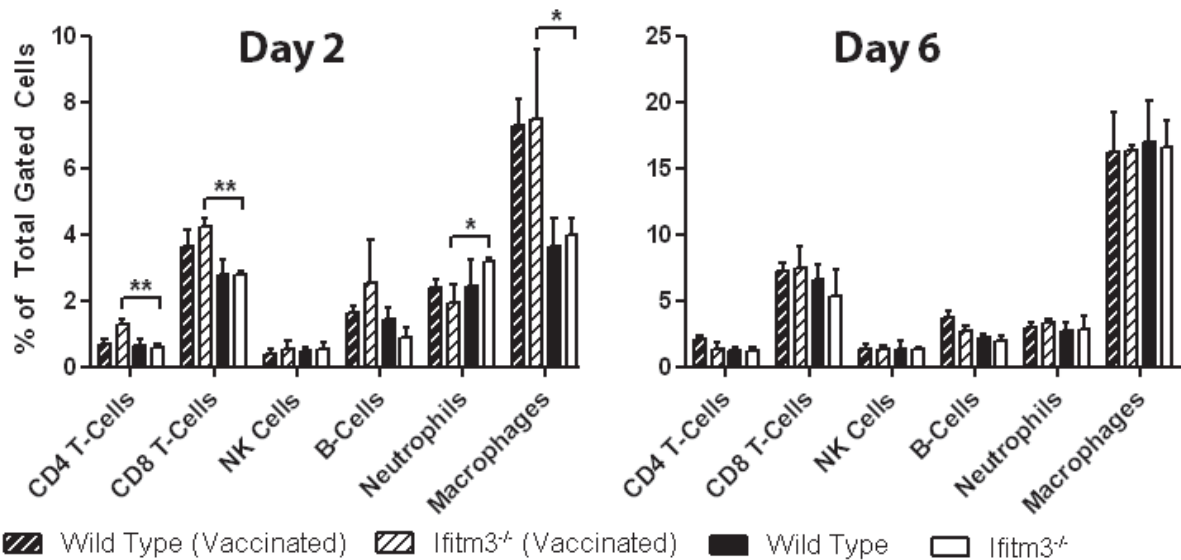
Figure 5.14: Effect of intra-muscular vaccination on leukocyte populations in the lungs of wild type and *Ifitm3*^{-/-} mice following influenza A infection. Lungs were removed and cell populations analysed by flow cytometry on days two and six post-infection. Legend is shown in the Figure. Results show 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).
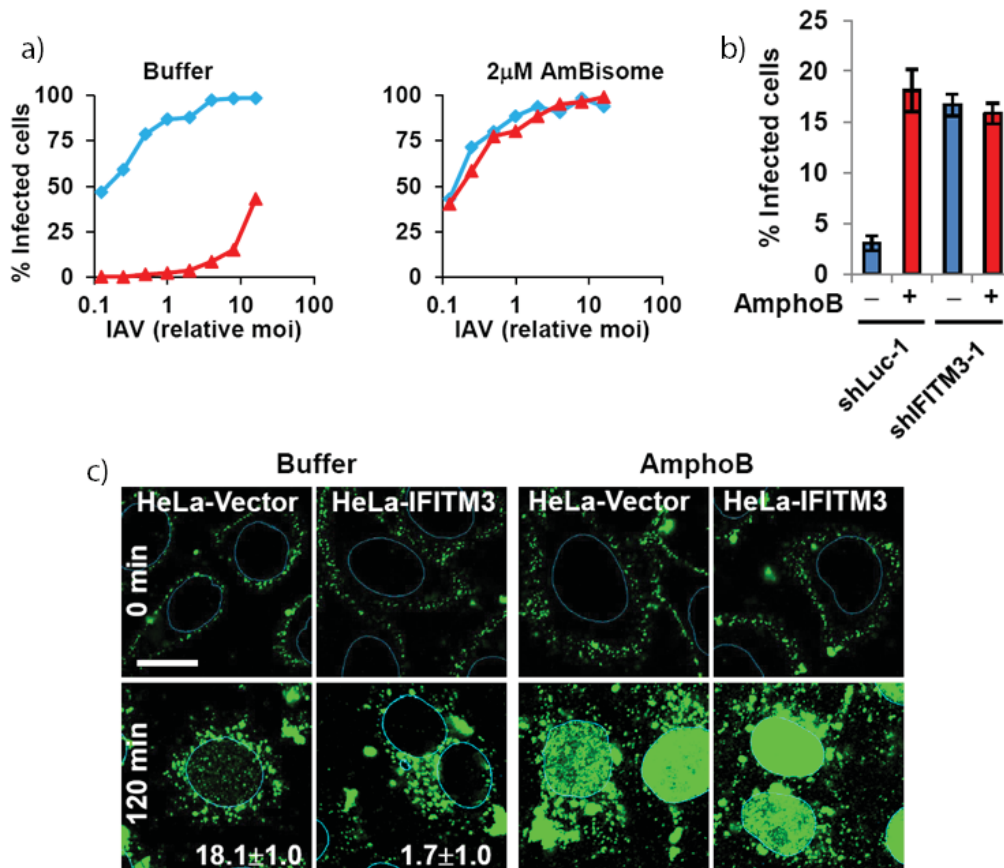
Figure 5.15: *In vitro* **effects of amphotericin B on IFITM3-mediated restriction of influenza virus.** (a) A549 cells expressing no (blue) or full-length IFITM3 (red) were incubated in the absence or presence of 2μM AmBisome and infected at the indicated range of MOIs with WSN/33 influenza. (b) HeLa 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).
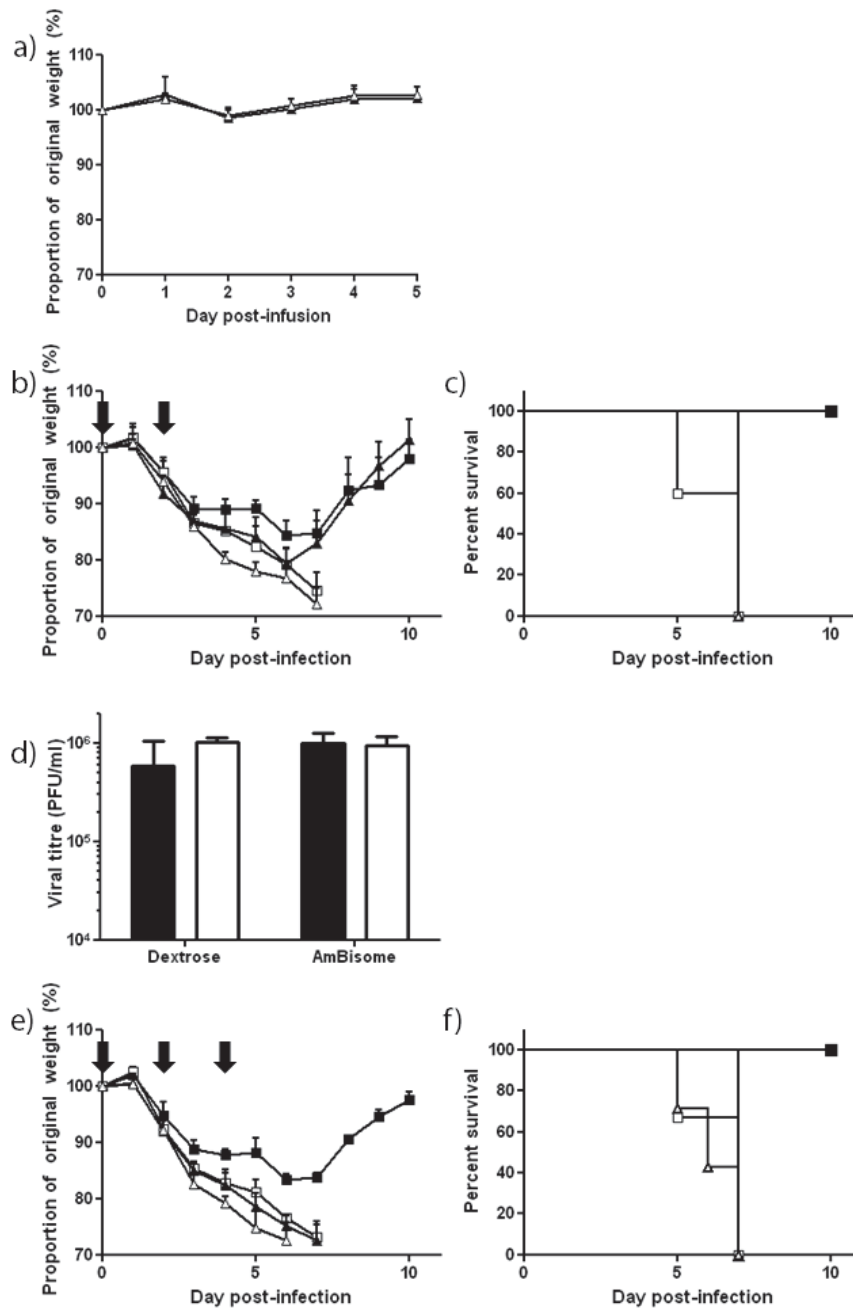
Figure 5.16: Effects of AmBisome on wild type and *Ifitm3*^{-/-} mice during influenza A infection. Mice were injected intravenously with either 5% dextrose or 3mg/kg AmBisome in 5% dextrose and monitored for signs of weight loss or morbidity (a). With AmBisome yielding no adverse effects, mice were injected with two or three doses of AmBisome at the indicated time points (arrows) during challenge with X-31 influneza 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.
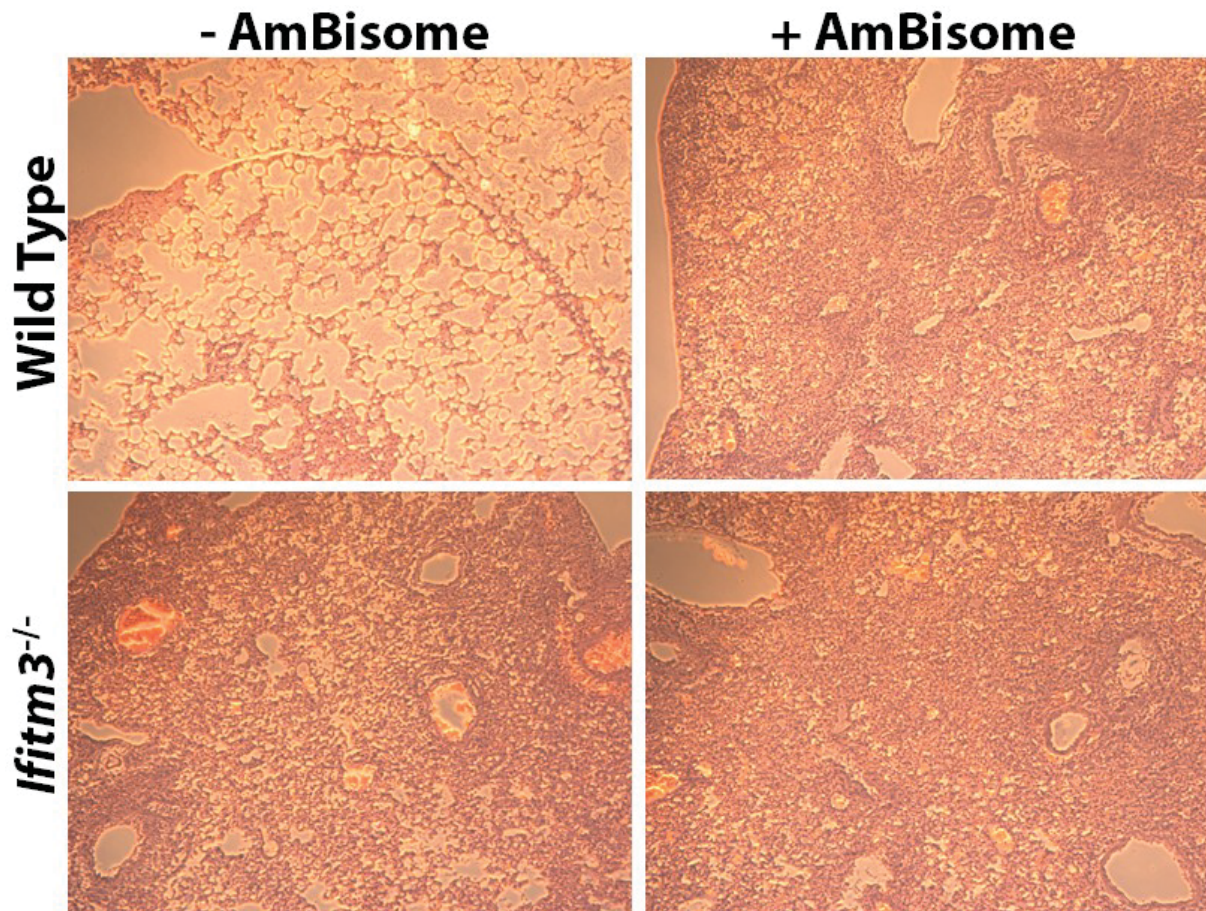
Figure 5.17: Effect of AmBisome on lung histology during influenza virus infection. Lungs were excised on day four post-infection with A/X-31 influenza. Mice had either received two infusions of dextrose (- AmBisome) or 3mg/kg AmBisome (+ AmBisome) on the day of infection and two days post-infection. 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.

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*.

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[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.
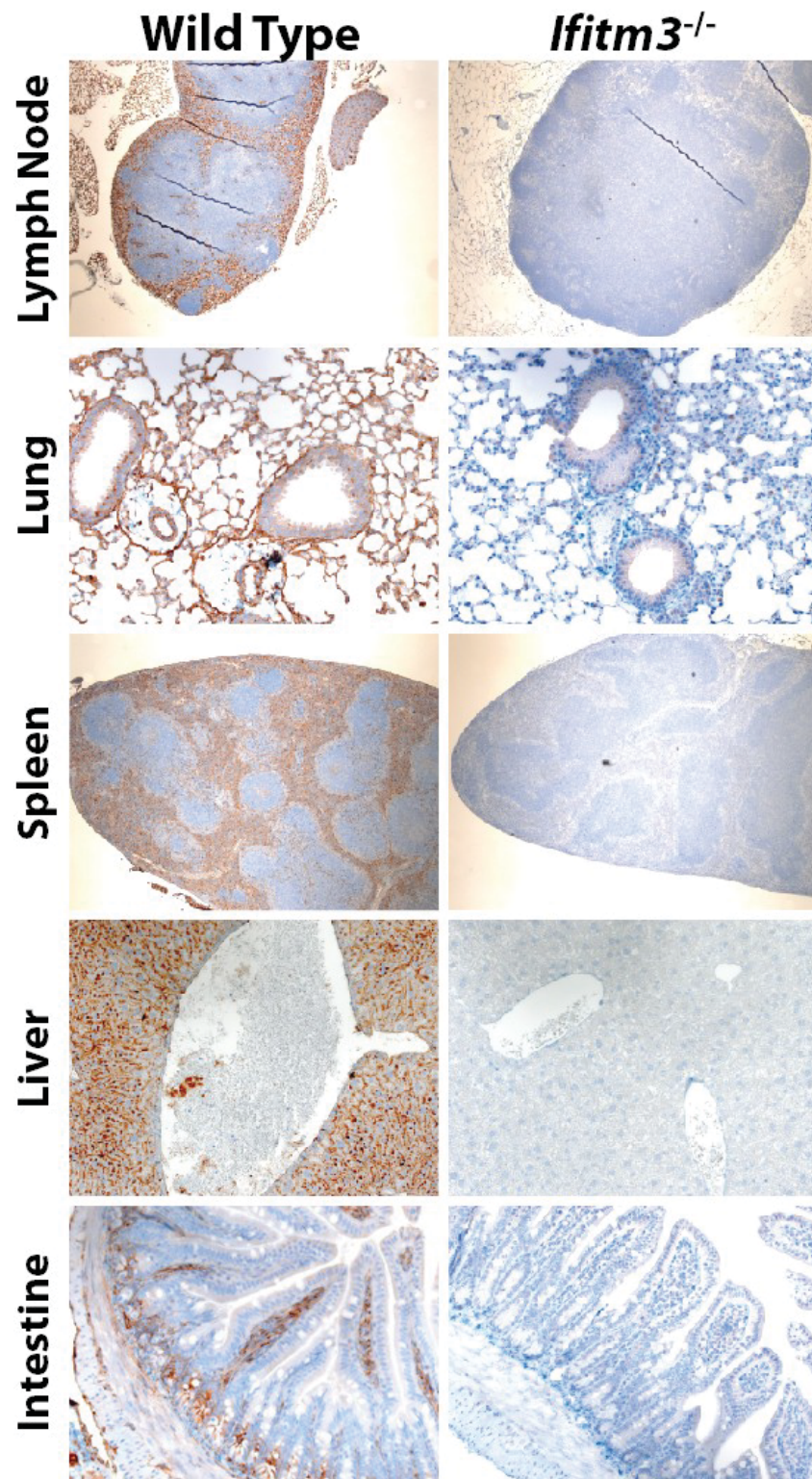
Figure 6.1: Expression of Ifitm3 at the predominant sites of pathogen infection. Paraffin-embedded sections from wild type and *Ifitm3-/-* mice were cut and stained for expression of Ifitm3 (brown), and counterstained with hematoxylin (blue). Original magnification of lymph node and spleen 10×; lung 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.
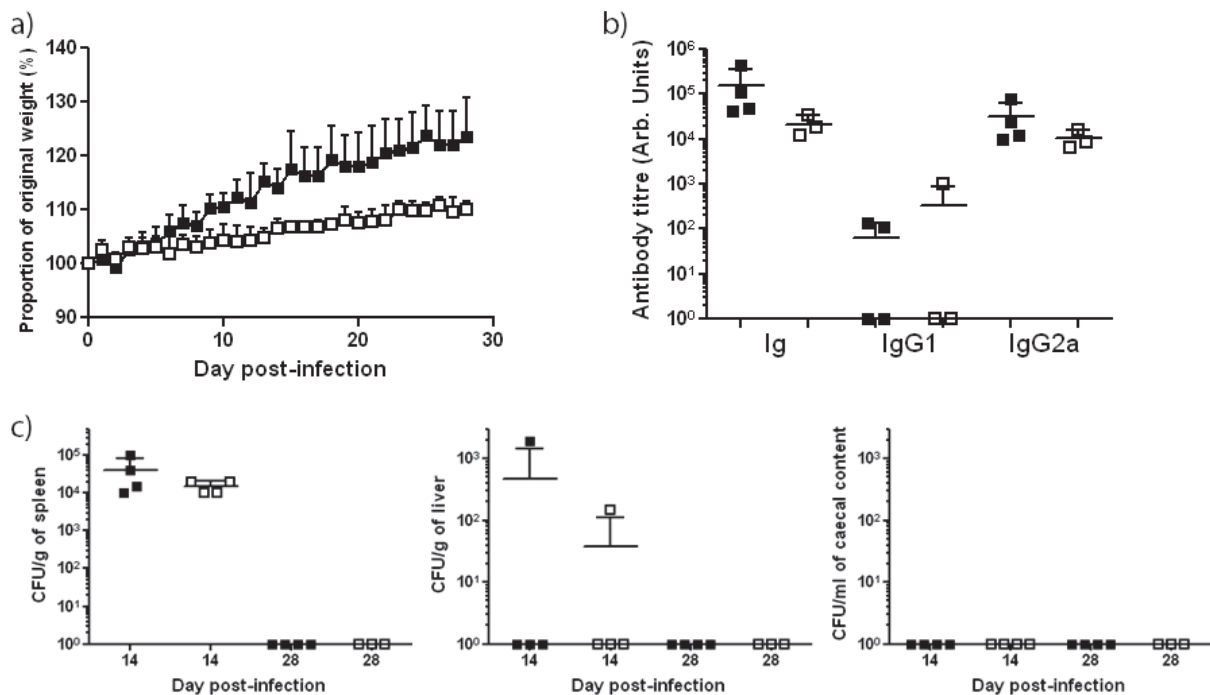
Figure 6.2: *S.* Typhimurium challenge of wild type and *Ifitm3*^{-/-} mice. Mice were intravenously injected with *S.* Typhimurium and observed for weight loss for 28 days post-infection (a). Mice were killed on day 28 post-infection to assess neutralising antibody titre against *S.* Typhimurium (b). 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 faeceal 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).
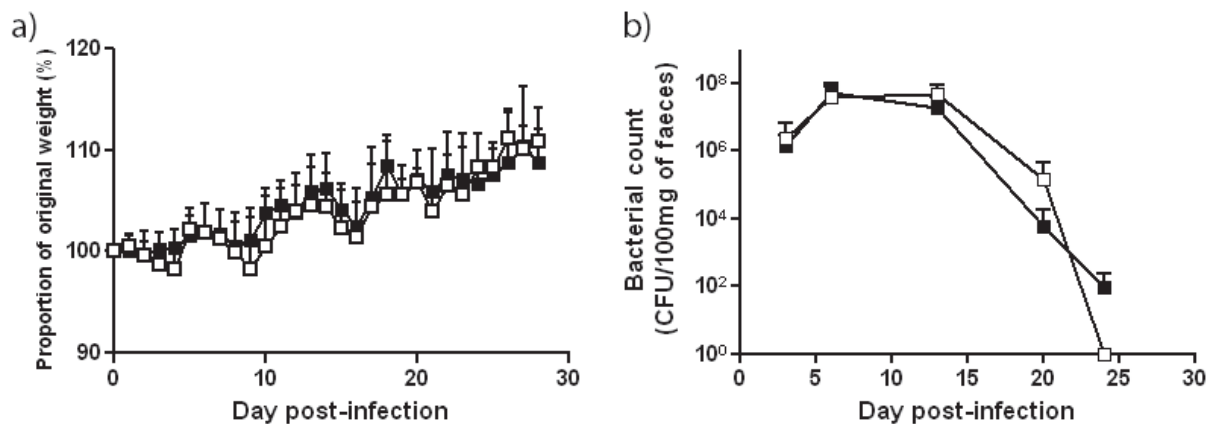
Figure 6.3: Weight loss and bacterial shedding of wild type and *Ifitm3*^{-/-} mice infected with *C. rodentium*. Mice were orally infected with *C. rodentium* and weighed daily to monitor morbidity (a). Faecal samples were taken over the course of infection (b), and were homogenised, diluted and plated 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.
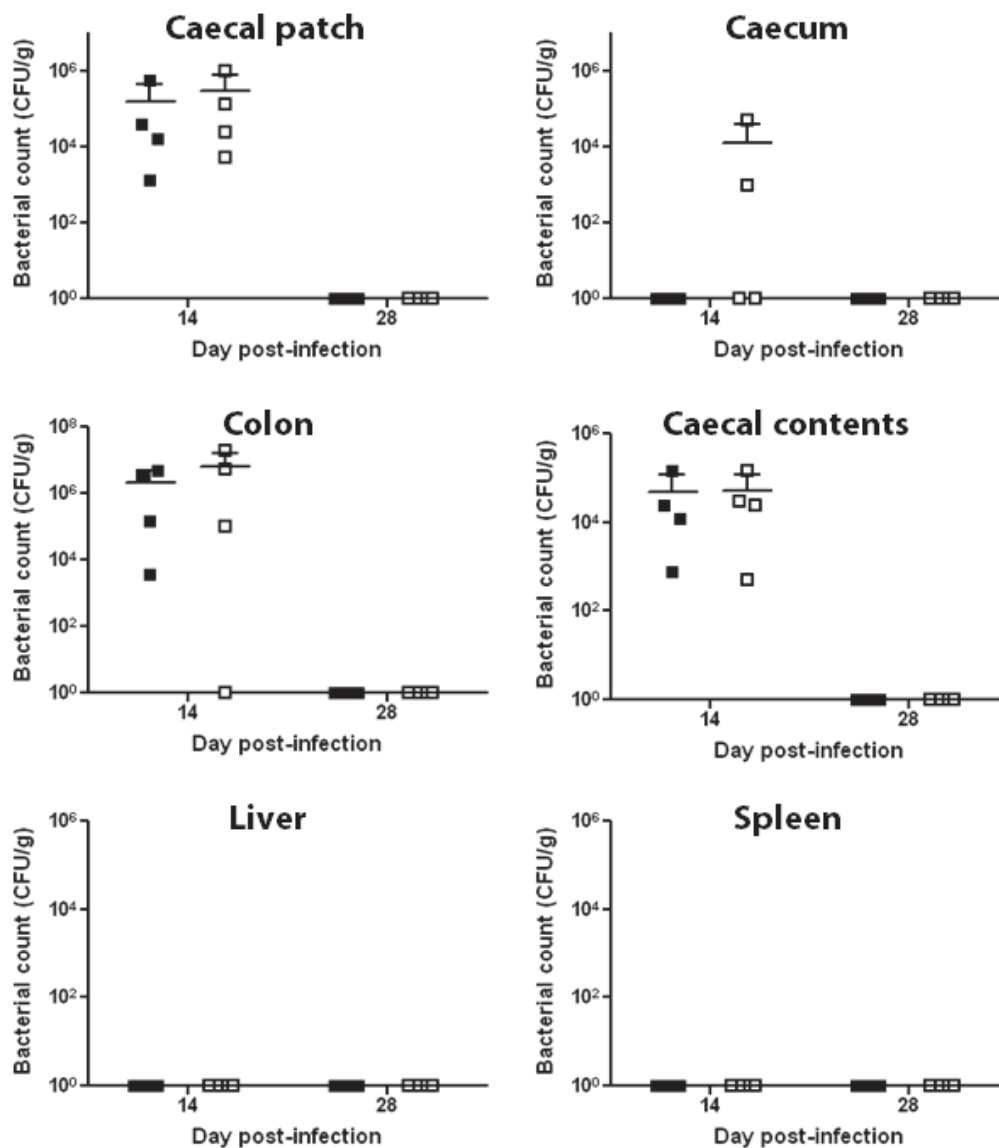
Figure 6.4: Bacterial counts of wild type and *Ifitm3*^{-/-} mice infected with *C. rodentium* over the course of infection. Mice were killed on days 14 and 28 post-infection and the indicated organs were excised, homogenised and plated to count the number of colony forming units of *C. rodentium*. ■: 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.
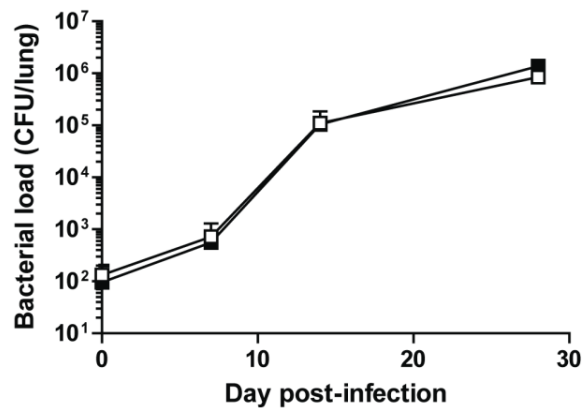
Figure 6.5: Bacterial growth kinetics of *M. tuberculosis* in the lungs of wild type and *Ifitm3*^{-/-} mice. Mice were killed over the course of infection with H37Rv *M. tuberculosis* to determine the bacterial load within their lungs. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. (n > 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).
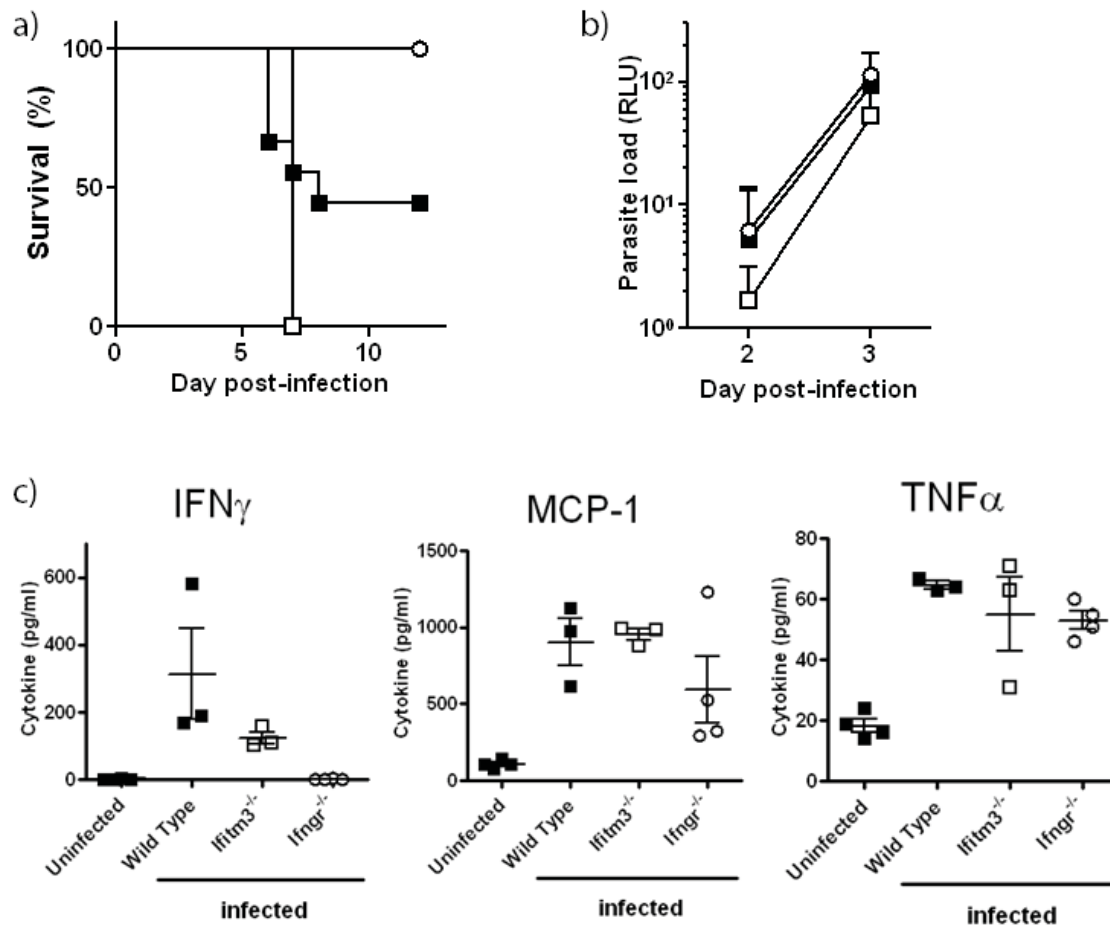
Figure 6.6: Malarial challenge of wild type and *Ifitm3*^{-/-} mice with *P. berghei* ANKA. Mice were intravenously injected with red blood cells containing *P. berghei* ANKA and were monitored for survival for 12 days post-infection (a). Parasite burden was measured by luminescence readings from blood 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).
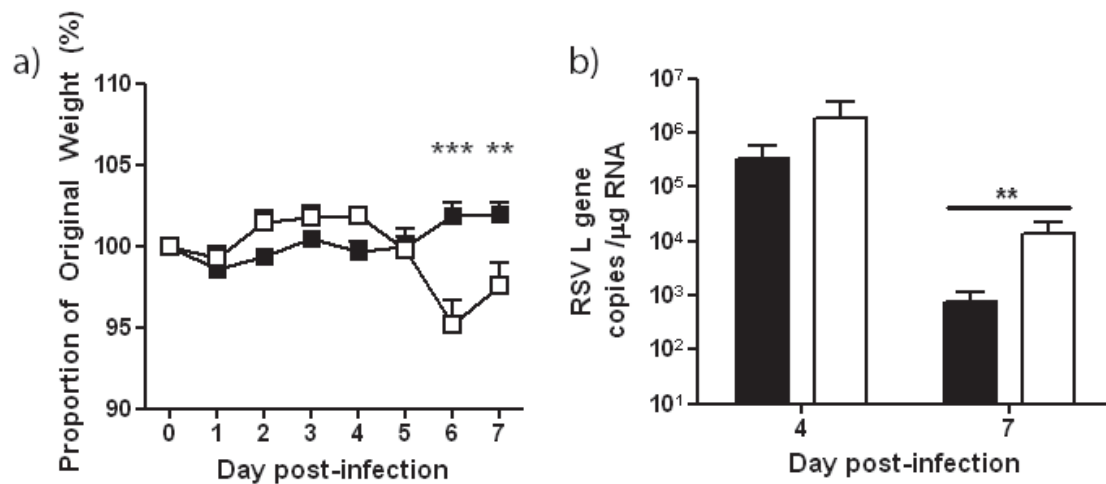
Figure 6.7: Weight loss and viral load associated with RSV infection of wild type and *Ifitm3*^{-/-} mice. Mice were intranasally infected with RSV-A and weighed for seven days post-infection (a). Cohorts of mice were killed on days four and seven post-infection and viral titres calculated by RT-qPCR (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.
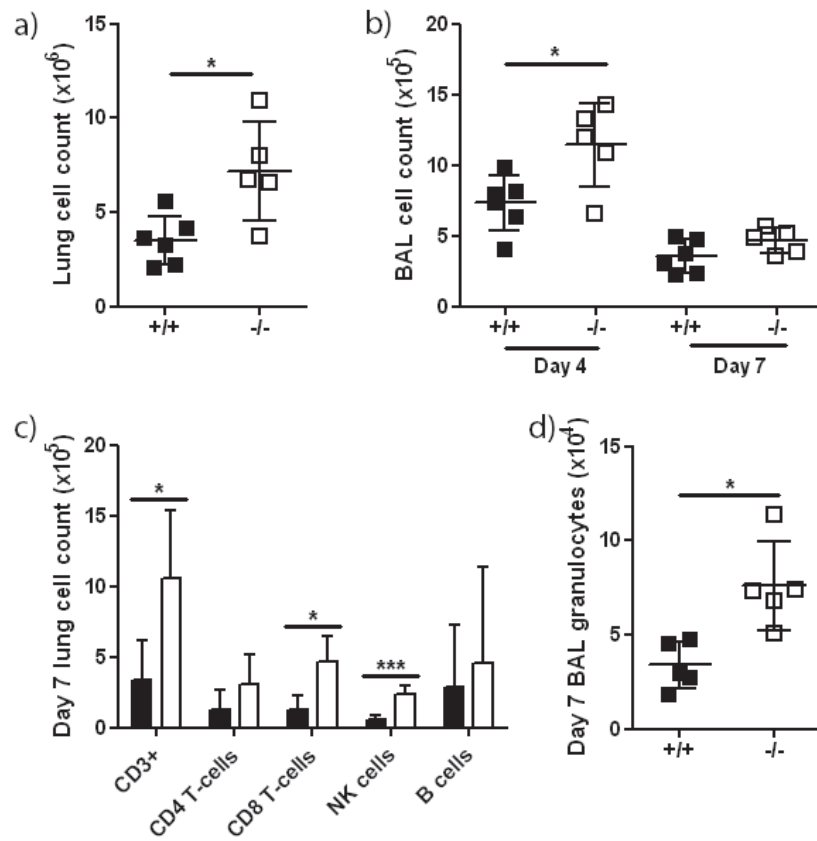
Figure 6.8: Cellular response of wild type and *Ifitm3*^{-/-} mice to RSV infection. Total viable cell counts were calculated from the lungs on day seven post-infection (a) and from broncho-alveolar lavage (BAL) on days four and seven post-infection (b). On day seven, cellular sub-populations were identified 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).
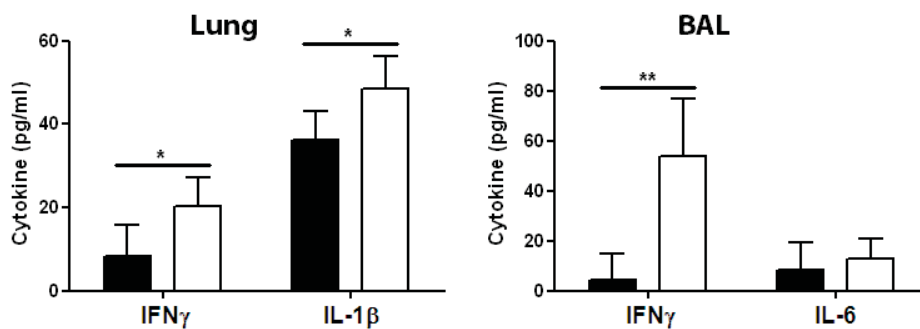
Figure 6.9: Inflammatory cytokines in the lungs and BAL on day seven post-infection in RSV-infected wild type and *Ifitm3*^{-/-} mice. Levels of IFNγ, IL-1β and IL-6 were quantified by ELISA on day seven post-infection. ■: wild type, □: *Ifitm3*^{-/-}. Results show means ± S.D. (n > 5). Statistical significance 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.

7   General Discussion

The intrinsic immune response is critical in preventing pathogens from establishing an infection within the host species. Should a micro-organism subvert these intrinsic barriers, the complex array of innate and adaptive immune responses are crucial in controlling and eradicating invasive organisms from the host in order to prevent the onset of severe morbidity and mortality. Although pathogens have evolved to subvert and antagonise these immune responses, resulting in sustained microbial survival, replication and induction of pathogenesis, the immune response can itself be to the detriment of the host, should they become dysregulated. This can in itself exacerbate the disease through immunopathological damage and its associated morbidity.

Influenza viruses vary greatly in their pathogenic potential, with infections ranging from asymptomatic to lethal. Indeed, a single isolate of influenza virus is capable of creating a spectrum of disease, both within and between the host species that it infects. The zoonotic ability for influenza viruses to cross species boundaries, particularly between avian and mammalian hosts, is the reason for the infrequent, but regular occurrence of pandemic strains of the virus in humans. During a pandemic, the immune system is likely exposed to a virus that it has not previously encountered; therefore the humoral and cell mediated T-cell responses are largely ineffectual, rendering the host particularly susceptible to infection by the virus and potentially developing a severe illness. A current example of a strain that is causing particular concern is the avian-borne H7N9 virus. Whilst it has yet to show evidence of sustained human-to-human transmission, which is a pre-requisite for a successful pandemic virus, it has shown a 28% case-fatality rate in humans (Morens *et al.* 2013). Whilst avian strains, such as H5N1, have been known to lead to lethal infections of humans, H7N9 is largely asymptomatic in its avian hosts, unlike H5N1. This will make eradication of the pathogen particularly challenging.

The detection of a novel swine-origin strain of H1N1 influenza in the early summer of 2009 in Mexico caused particular concern and the virus progressed to cause the first pandemic of the 21st Century. Although the virus was zoonotic in origin, and was a quadruple reassortant of avian, swine and human influenza viruses, it failed to induce the widespread excess of mortality that was feared at the time of detection. However, what was notable about the pandemic was the

atypical epidemiology of the virus, which caused deaths in groups not traditionally regarded as being "at risk" (Liam *et al.* 2009; Bautista *et al.* 2010). Analysis of the virus over the course of the pandemic revealed that mutations to enhance its virulence were not the reason for the atypical morbidity profile. This would suggest that other, undetermined host factors may have contributed to the overall impact of the pandemic.

This study has added to the current body of knowledge by furthering our understanding of the role of host genetics in relation to the morbidity and mortality associated with influenza virus. Previously, proteins of the MX and IFIT families have been characterised *in vivo* and have been shown to have crucial antiviral roles in the restriction of pathogenic viruses, particularly influenza (Tumpey *et al.* 2007; Pichlmair *et al.* 2011). In recent years, a novel family of proteins, the IFITMs, has been identified as playing a role in restricting multiple pathogenic viruses *in vitro* (Brass *et al.* 2009; Jiang *et al.* 2010; Weidner *et al.* 2010; Huang *et al.* 2011; Schoggins *et al.* 2011; Anafu *et al.* 2013; Mudhasani *et al.* 2013; Wilkins *et al.* 2013). Although *in vitro* assays are useful in generating hypotheses and analysing protein function (Brass *et al.* 2009; Shapira *et al.* 2009; Karlas *et al.* 2010), they do not always reveal the impact of the protein *in vivo*.

Indeed, the programme of work described here attempts to analyse the effects of a subset of genes predicted by RNAi screens *in vivo* revealed that certain genes identified as being involved in susceptibility and resistance to influenza virus could not be knocked out without causing lethality (Chapter 3). Furthermore, problems were highlighted with using mice to model the effect of human genes *in vivo*. This was exemplified in experiments involving the infection of *Calcoco2*$^{-/-}$ mice with influenza virus, which yielded no phenotype, despite *in vitro* evidence (Brass *et al.* 2009; Shapira *et al.* 2009). Subsequent sequence analysis revealed that human and mouse genes were highly divergent; therefore perhaps compromising the model. Although *in vitro* and animal models are useful in modelling the phenotypic effects that may be observed in humans, several caveats in their usage exist and have been highlighted in this study.

This study was successful in replicating the results obtained *in vitro* with the IFITM family of proteins in a model organism (Chapter 4). For the first time, it was shown that the loss of Ifitm3,

the most potent anti-influenza protein of the family (Brass *et al.* 2009), resulted in the onset of fulminant viral pneumonia, acute pathological damage and ultimately death in the *Ifitm3*$^{-/-}$ mouse. These mice exhibited a prolonged, elevated viral infection in their respiratory system, with accompanying immunological effects more closely mirroring infections with highly pathogenic influenza viruses such as H5N1 and 1918 'Spanish' H1N1 influenza viruses, than the low pathogenicity X-31 influenza virus used in the challenge. This would support the idea of an evolutionary arms race between the host and virus. Just as the loss of the viral anti-host defence NS1 protein improves the effectiveness of the immune system (Garcia-Sastre *et al.* 1998), the loss of a host protein such as Ifitm3 enhances the pathogenesis and replicative abilities of the virus. Indeed, it would be interesting to further the current findings by analysing the relative contributions of the intrinsic immune defence families identified in section 1.4.1.1: MX, IFIT and IFITM. As is the case with many studies utilising inbred mouse lines, the *Mx1* gene is already ablated (Tumpey *et al.* 2007) due to a mutation that occurred when the lines were initially derived; thus effectively making knockout mice "dual knockouts" for *Mx1* and the target gene. Therefore, it would be compelling to generate *Ifit* and *Ifitm* knockout mice on an *Mx1*$^{+/+}$ C57BL/6 mouse line. The relative contributions of these antiviral families to resistance to influenza viruses could then be determined absolutely by infecting in parallel.

The findings obtained from the murine model were taken further by analysing the effects of human IFITM3 *in vivo*. Through international collaboration, a SNP in *IFITM3* was identified as being overrepresented in a cohort of patients that were hospitalised with confirmed influenza virus infection during the 2009 H1N1 pandemic. The minority IFITM3 genotype, rs12252-CC, has a prevalence of 0.3% in European Caucasian populations, but this was significantly enriched, with 5.7% of the sequenced patients possessing the rs12252-CC genotype. This therefore suggested that influenza susceptibility and the risk of developing a severe virus infection may have a heritable component in humans.

Here, murine and human analyses were subsequently independently ratified in separate publications, which showed the increased pathogenesis of influenza virus in *Ifitm3*$^{-/-}$ mice (Bailey *et al.* 2012), and the replication of the role of the rs12252 SNP in the severity of influenza infections in humans (Zhang *et al.* 2013b). Indeed, the discovery of the abundance of

the rs12252-CC genotype in Chinese and Japanese populations (25% and 44%, respectively) prompted investigation of whether individuals with sub-optimally functioning IFITM3 protein could be protected from potentially contracting a severe influenza infection by vaccination (Chapter 5).

In the study, LAIVs were chosen as they potentially represent the greatest risk to health in an individual whose immune system cannot fully control viral infection, owing to the fact that the vaccine relies on live but attenuated virus to provide immunity. Using the *Ifitm3*^{-/-} mouse model, the study showed for the first time that mice lacking a crucial antiviral restriction factor could tolerate LAIV and mount an adequate adaptive immune response when challenged with a lethal dose of pandemic H1N1 influenza virus. This was typified by a lower peak viral burden, significantly reduced pathological damage and reduction in immune infiltrate. This preclinical model would suggest that individuals with the rs12252-CC genotype, who are genetically "at risk" of infection, can be protected by vaccination.

Although not within the scope of the current study, one outstanding concern relating to the use of LAIV is the possibility of genome reassortment should the patient become co-infected with a wild type strain of influenza virus (Hai *et al.* 2011). If a patient has a sub-optimally functioning version of IFITM3, they may have more attenuated virus present for a prolonged period within the upper respiratory tract. This would therefore expand the timeframe in which co-infection could occur, which may result in a novel reassortant virus. Current evidence indicates that any such events would be very rare and would produce highly weakened strains, should they occur (Kiseleva *et al.* 2012). Nevertheless, the implications of the loss of IFITM3 expression do increase such a risk and merit investigation.

The role of Ifitm3 in pathogen restriction *in vivo* was further investigated by collaborating nationally to challenge the *Ifitm3*^{-/-} mice with a range of bacteria, protozoa and viruses (Chapter 6). The study further defined Ifitm3 as a potent antiviral protein. Despite reports of Ifitm3 being involved in the immune response to diseases such as TB and malaria (Sharma *et al.* 2011; Shen *et al.* 2013), no significant phenotypic effects were recorded when mice were challenged with these pathogens. This highlights an issue with the interpretation of data from RNA expression

based assays in relation to ISGs. IFN is generated upon infection with multiple pathogens, which leads to the initial up-regulation of a broad cascade of ISGs in a non-specific manner. Therefore, the presence of *Ifitm3* mRNA may be a hardwired response to infection in general, and not to the restriction of a particular invading pathogen.

However, it was shown that Ifitm3 was capable of restricting RSV: a virus that does not enter via the late endosomal pathway, which is regarded as the spatial site of Ifitm3 restriction (Feeley *et al.* 2011). Although this would at first seem counterintuitive to the proposed models of Ifitm3 function in the endosomal pathway, it could be suggested that this is evidence of the overlapping function of the divergent Ifitm proteins (Diamond and Farzan 2013). It was suggested that although a mild, but significant degree of restriction of RSV was seen in *Ifitm3*$^{-/-}$ mice, one would hypothesise that Ifitm1 would be the most potent restrictor of RSV, based on the fact that it is the predominant Ifitm family member on the plasma membrane (John *et al.* 2013), which is where RSV fuses with the cell. This could be investigated *in vitro*, and ultimately through the generation of an *Ifitm1*$^{-/-}$ mouse. This would be a valuable knockout mouse, as arguably Ifitm1 and Ifitm3 are proving to be the most crucial members of the family; functioning at spatially different sites and restricting different viruses *in vitro* (Huang *et al.* 2011; Diamond and Farzan 2013; Wilkins *et al.* 2013).

Presently, only influenza virus and RSV have been shown to yield a phenotype in *Ifitm3*$^{-/-}$ mice. It would be pertinent to challenge these mice with other reportedly restricted pathogens that can be modelled in mice, such as SARS-Coronavirus, West Nile virus and dengue virus (Wang *et al.* 2004; Roberts *et al.* 2007; Yauch and Shresta 2008). Should *Ifitm3*$^{-/-}$ mice be shown to have a highly susceptible phenotype, then they could be used as a preclinical model for severe infections to test novel antiviral drugs and vaccines, similar to how it could be used in influenza research in the future.

Although much is yet to be ascertained regarding the functional role of IFITM3, and indeed its structure, it is certainly a potent antiviral molecule. Increasingly, the field is uncovering evidence of IFITM orthologs in a variety of species including other mammals, reptiles, birds and fish, which are capable of restricting influenza and other viruses (Huang *et al.* 2011; Hickford *et al.*

2012; Huang *et al.* 2013; Zhu *et al.* 2013). The presence of this family across a divergent range of species highlights its evolutionary importance in host defence against viruses. The body of work discussed here has further defined the role of this family in the restriction of pathogens *in vivo* and will hopefully contribute to research that has medical and translational significance to human health in the future.

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