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).

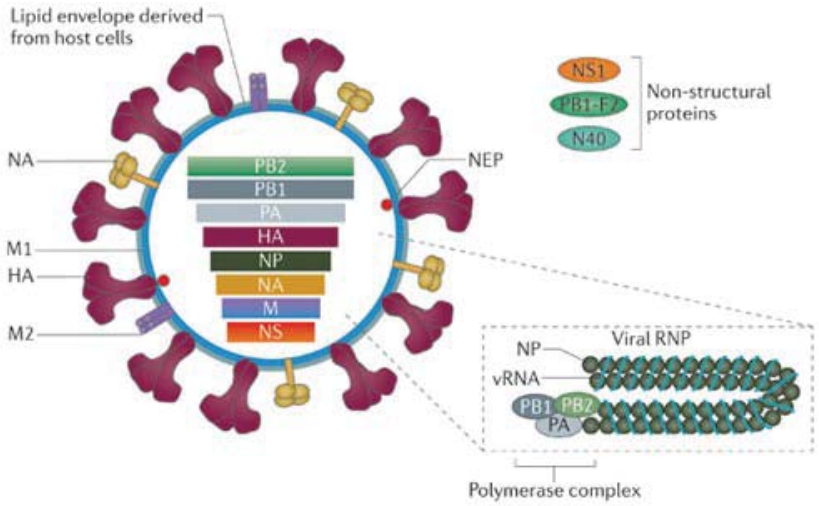

Figure 1.1: Schematic of an influenza A virus. The virion encapsulates a total of eight vRNP's that encode at least 11 proteins. These are: PB1, PB2 and PA form the components of the RNA polymerase complex (PB1 also encodes N40 and PB1-F2, whilst PA also encodes several variants of itself (Chen *et al.* 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)

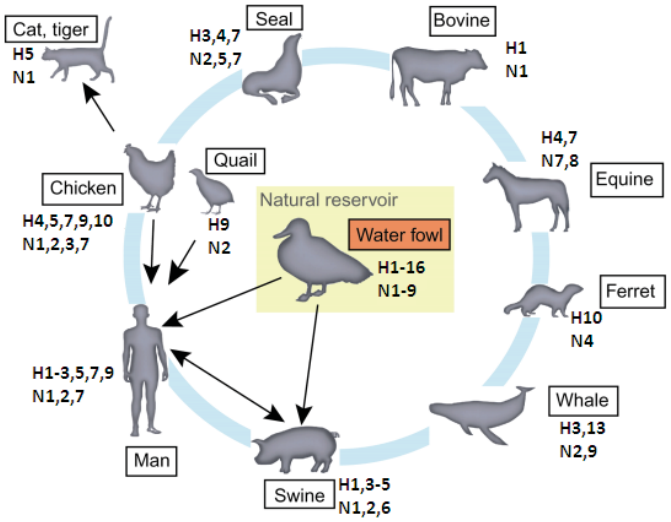

Figure 1.2: Host range of influenza viruses. The different influenza HA and NA subtypes are capable of infecting a range of animal species. Transmission events are shown by the arrows. The newly discovered H17 hemagglutinin is not shown on the diagram, but is currently thought to primarily target bats. 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.

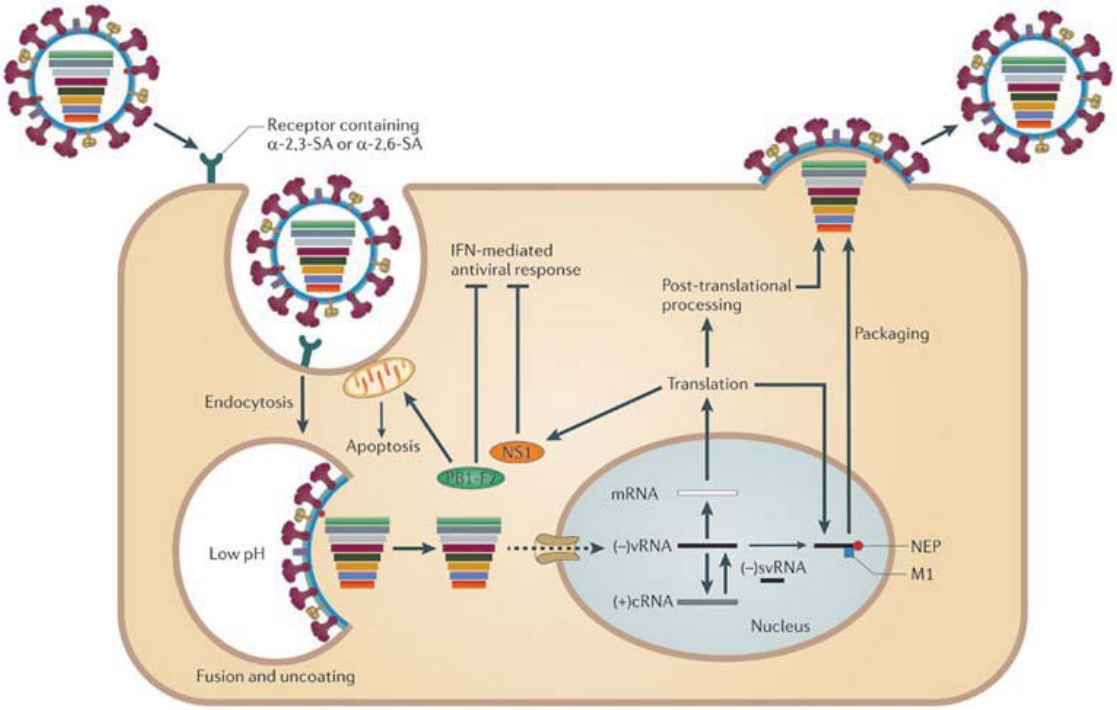

Figure 1.3: Schematic to illustrate the influenza replication cycle. The key components of the replication cycle are discussed in the text. However, it is pertinent to address the roles of NS1 and PB1-F2, which are responsible for antagonising the host immune response. NS1 is primarily an interferon-antagonist, 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.

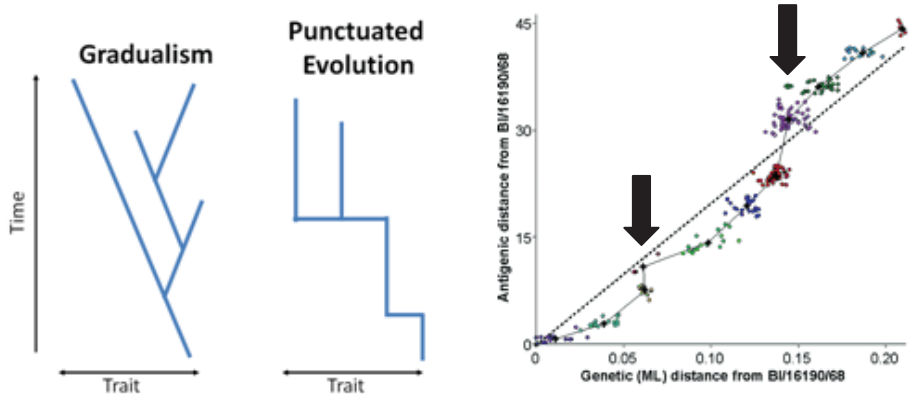

Figure 1.4: Models of evolutionary change and the evolution of H3N2 influenza. The schematics show how the theories of gradualism and punctuate evolution operate: gradual vs. rapid phenotypic changes. The right hand panel shows the correlation between genetic and antigenic evolution of H3N2, where the 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.

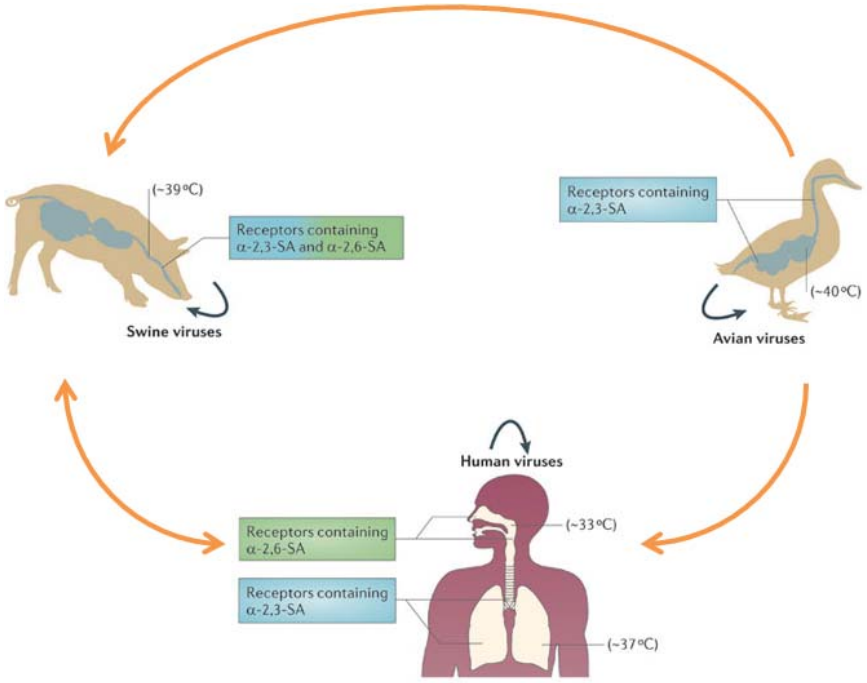

Figure 1.5: The anatomical distribution of α2,3-galactose (α-2,3-SA) and α2,6-galactose (α-2,6-SA) linked sialic acid receptors in three key species infected by influenza and the direction of inter-species viral transmission. Temperatures of the epithelial surfaces within the various species are indicated 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^{o}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).

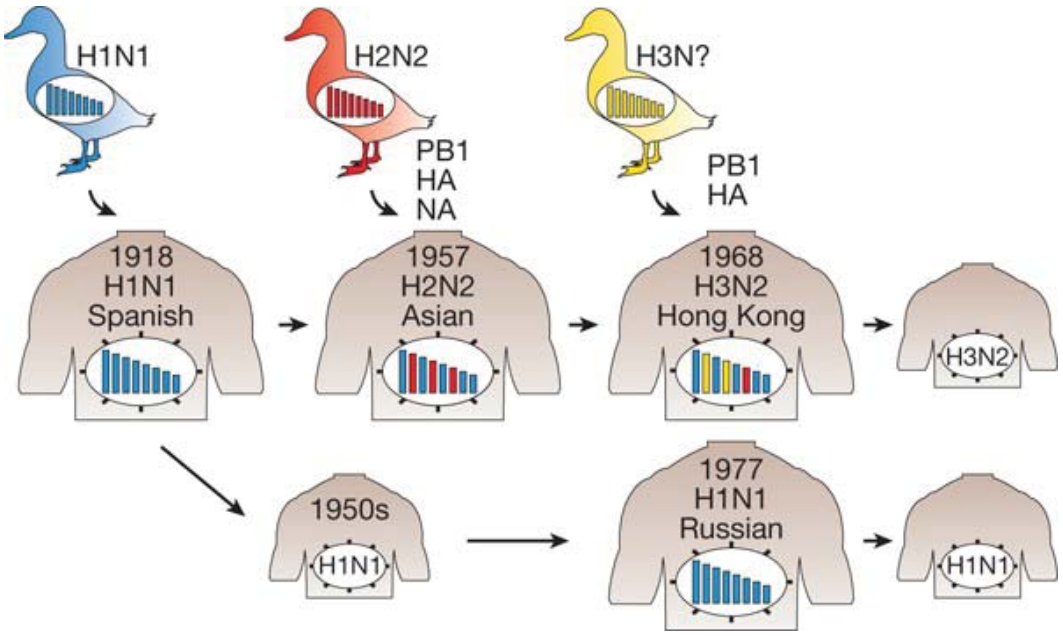

Figure 1.6: The role of the 1918 'Spanish' influenza virus in the pandemics of the 20th Century. The original 'Spanish' influenza has undergone several genetic reassortments with wild avian influenza viruses to yield the pandemics of 1957 and 1968. The original H1N1 virus was largely absent for the majority 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.

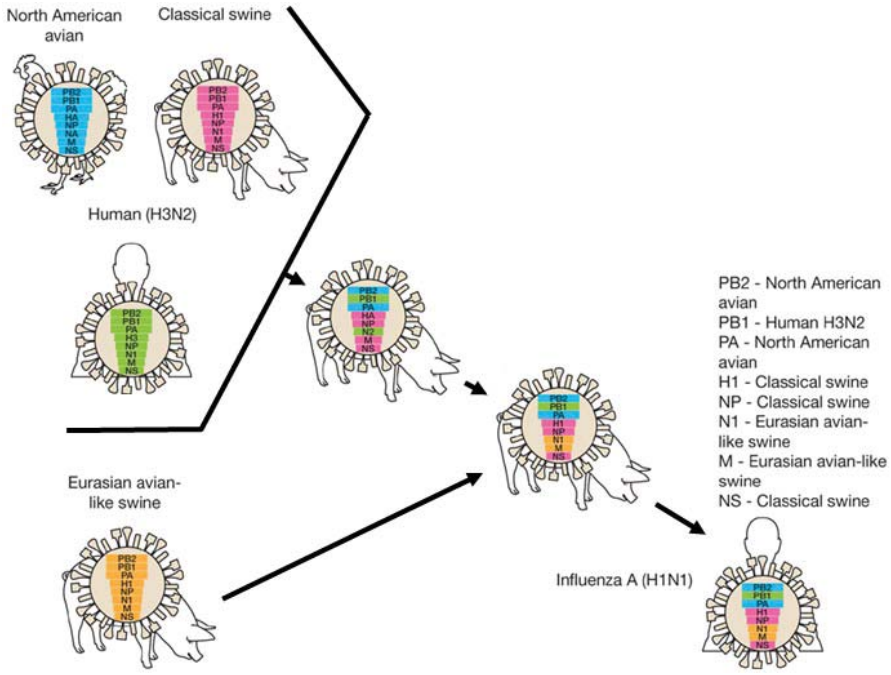

Figure 1.7: Schematic to show the genetic reassortments that led to the development of the 2009 H1N1 strain of influenza. The resulting virus contained genes from four different lineages: a quadruple reassortment, owing to the recombination of a pre-existing triple reassortant virus with the Eurasian 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).

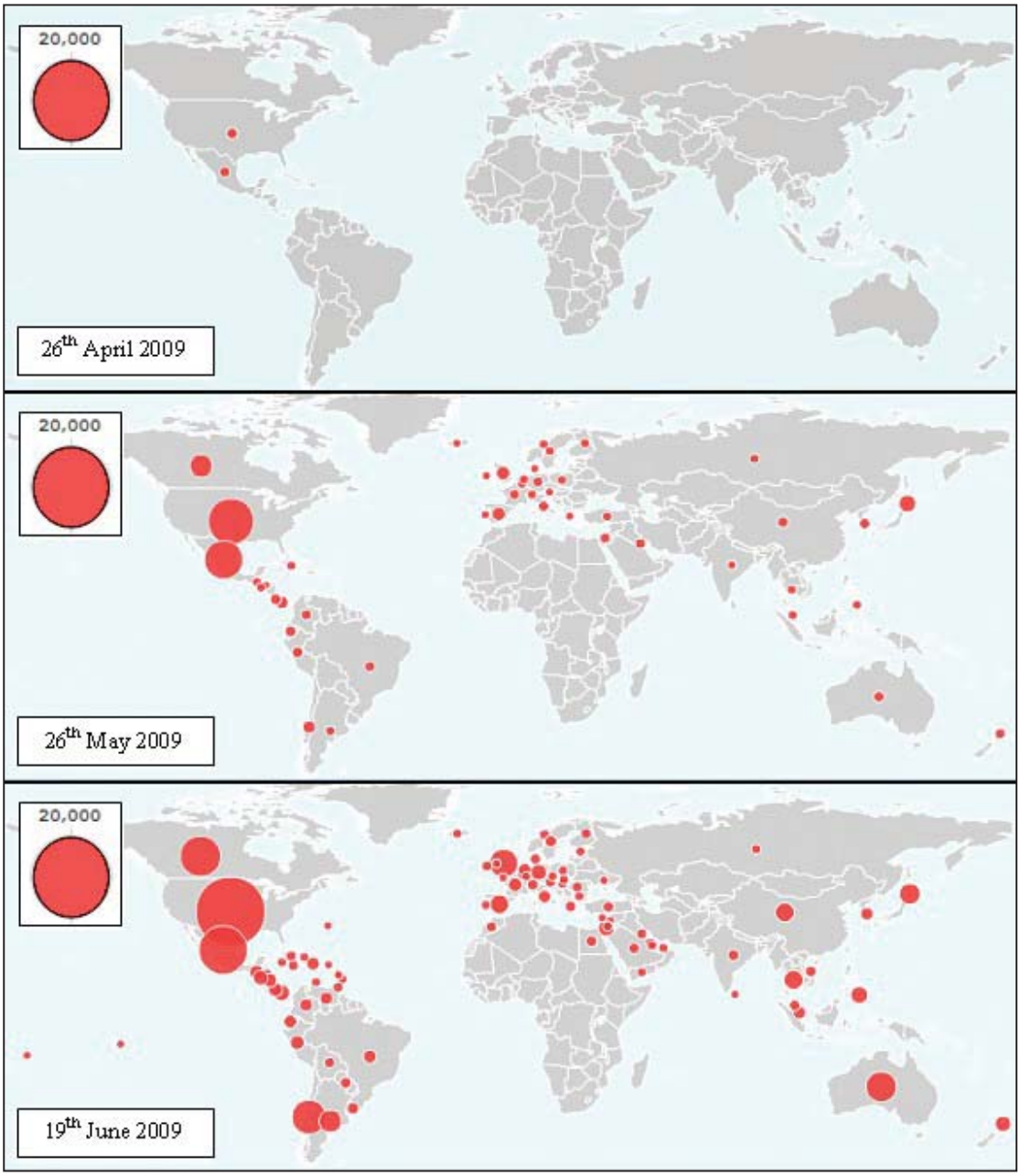

Figure 1.8: Geographical spread of the A(H1N1)pdm09 virus over the course of the first two months of global transmission. All data points are based on reported and clinically confirmed cases according to the WHO. The notable absence of cases in Africa may be due to gross underreporting by these countries (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.

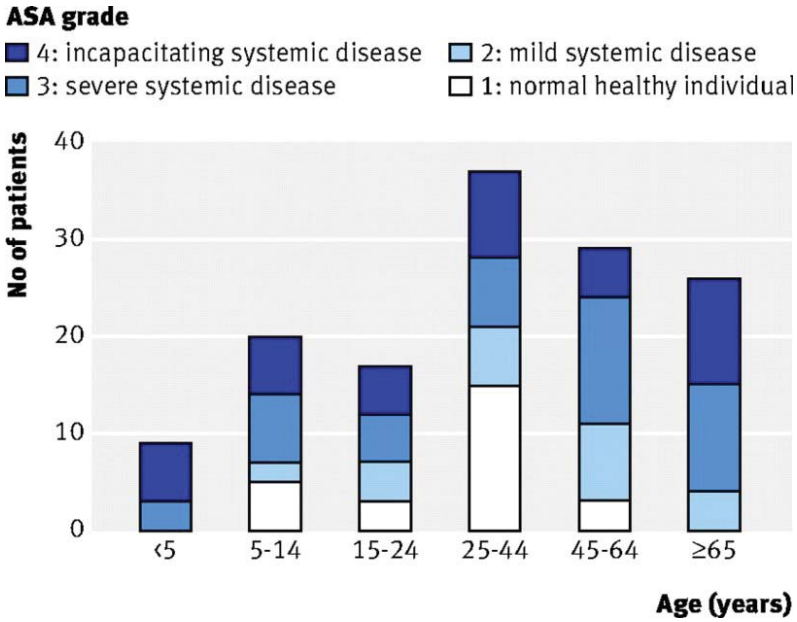

Figure 1.9: Age and pre-illness health of patients that died of pandemic influenza infection in 2009 in England. Bar colours co-ordinate with the co-morbidity severity of the patients. (Liam *et al.* 2009)

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.

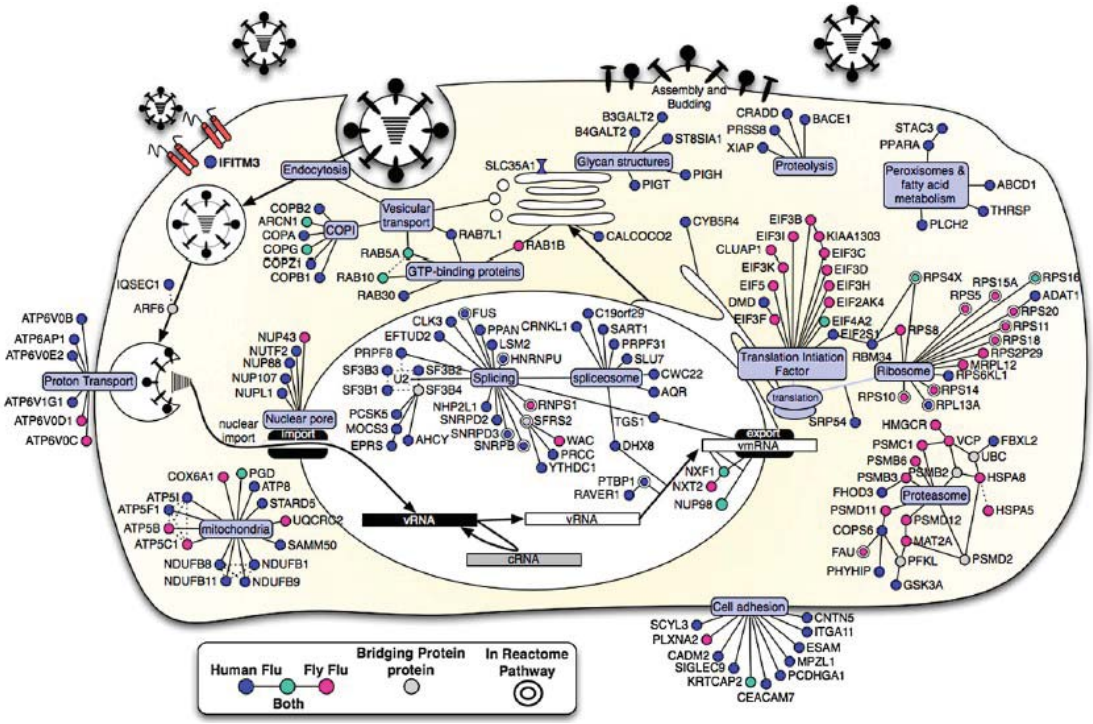

Figure 1.10: Host factors involved in influenza A virus replication as deduced from human and fly RNAi screens. Proteins shown to have an interaction with influenza from fly (pink) (Hao *et al.* 2008a) and human (blue) screens (Brass *et al.* 2009), as well as those that have occurred in both (green) 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.

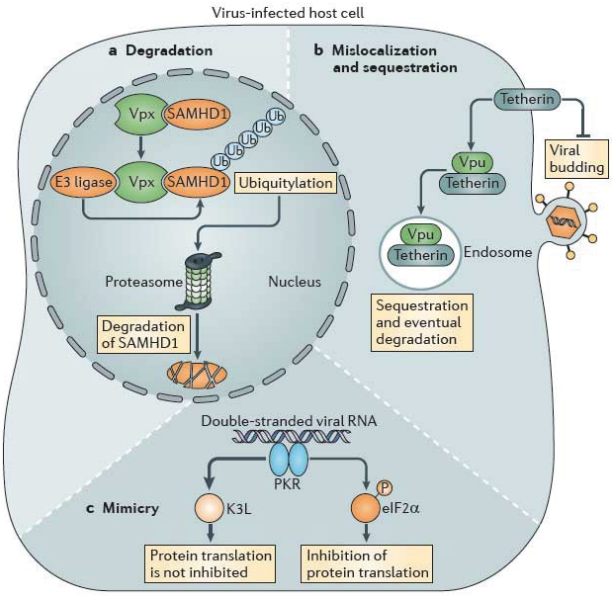

Figure 1.11: Broad mechanisms of viral antagonism of the host's innate immune response. The schematic depicts three of the key ways in which viral proteins are capable of antagonising the host's defences in order to continue proliferation. a) degradation: where the viral proteins signal for the destruction 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).

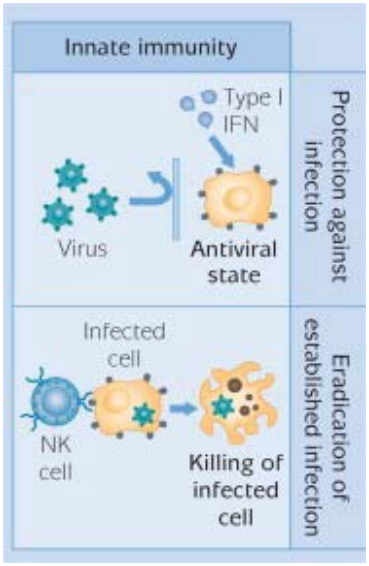

Figure 1.12: The stages of the innate immune response to viral infection. The innate immune system initially relies on intrinsic antiviral responses within the cell to protect against viral replication. Should these fail, or the virus subvert them, then the cell is brought into an antiviral state, primarily 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.

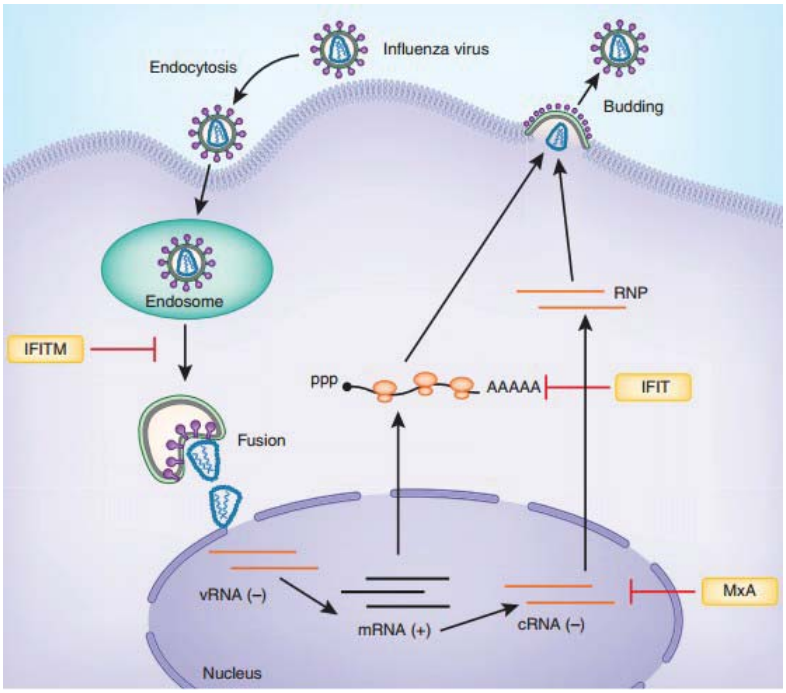

Figure 1.13: Intrinsic antiviral restriction factors that inhibit influenza virus. The schematic illustrates the three major identified families / proteins that intrinsically protect cells against influenza virus: IFITM, IFIT and MxA (Mx1 in mice). The actions of these families are discussed further 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).

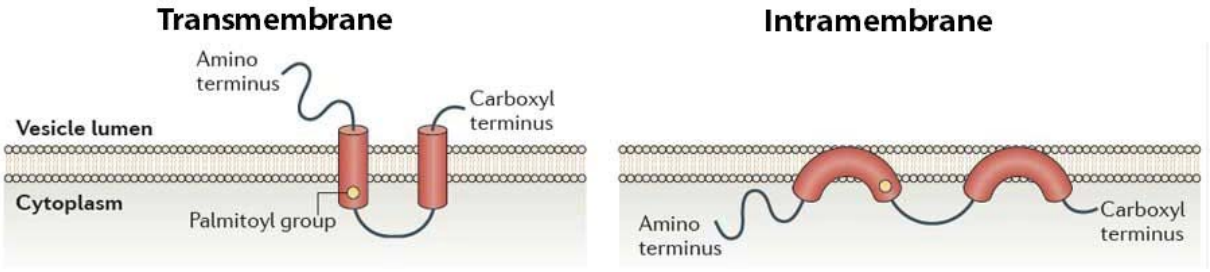

Figure 1.14: Topologies suggested for the IFITM family of proteins. The "transmembrane" model was what was initially predicted to be the structure of the IFITM family, with two anti-parallel transmembrane domains and the N- and C- termini facing into the ER lumen / endosome / extracellular space. However, 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.

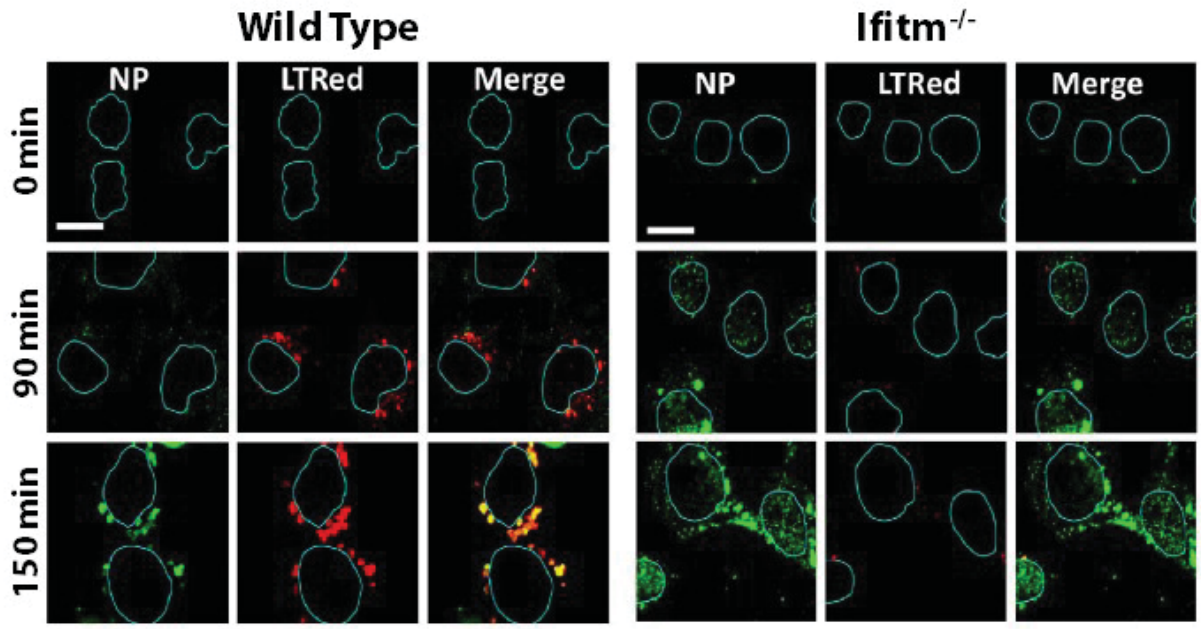

Figure 1.15: The Ifitm proteins are necessary for restricting influenza virus in the late endosomes and preventing vRNP entry into the nucleus. The images show the advance of H1N1 influenza (NP) into the nuclei (blue circles) of IFNγ-treated murine embryonic fibroblasts (MEFs) that either have (Wild 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.

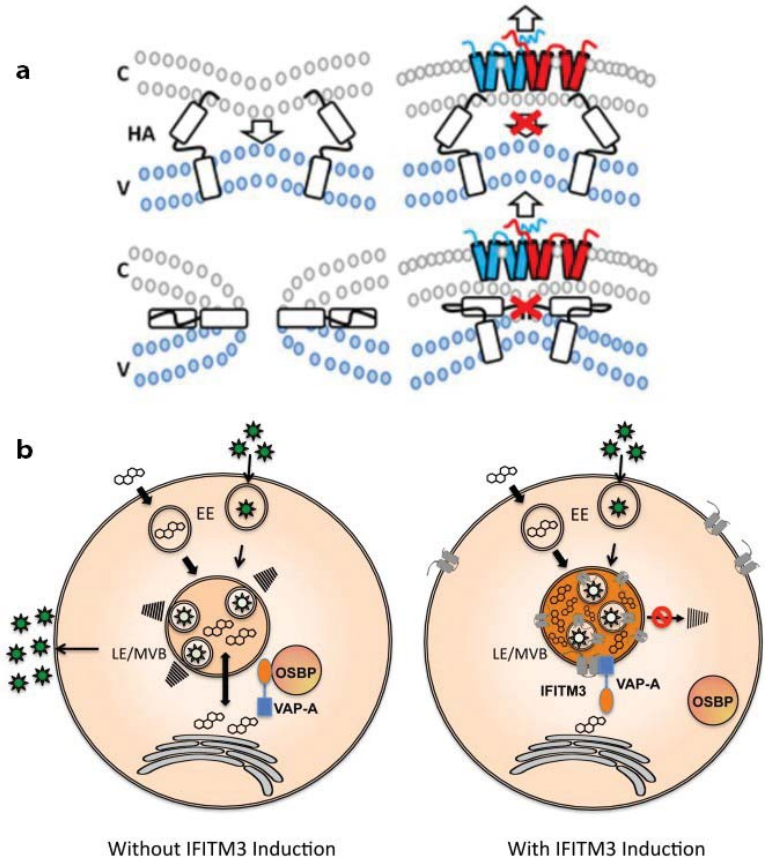

Figure 1.16: Currently suggested models of IFITM restriction. a, schematic illustrating how the IFITM proteins may be mechanically strengthening the cell's endosomal membrane (C), which prevents viral membrane (V) fusion. The left hand panels show "regular" fusion in the absence of IFITM expression, 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).

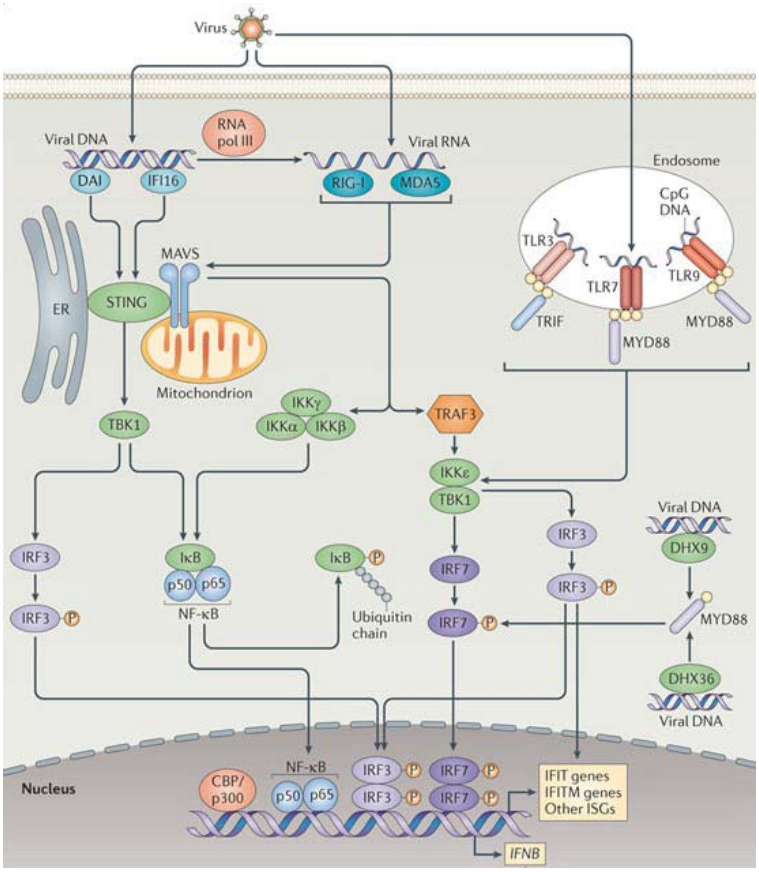

Figure 1.17: Modes of detection of incoming virus and their subsequent signalling pathways. The schematic shows a generalised signalling pathway for viruses. As mentioned in the body of text, the key influenza receptors are RIG-I, TLR3 and TLR7; therefore the subsequent signalling pathways stemming from 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).

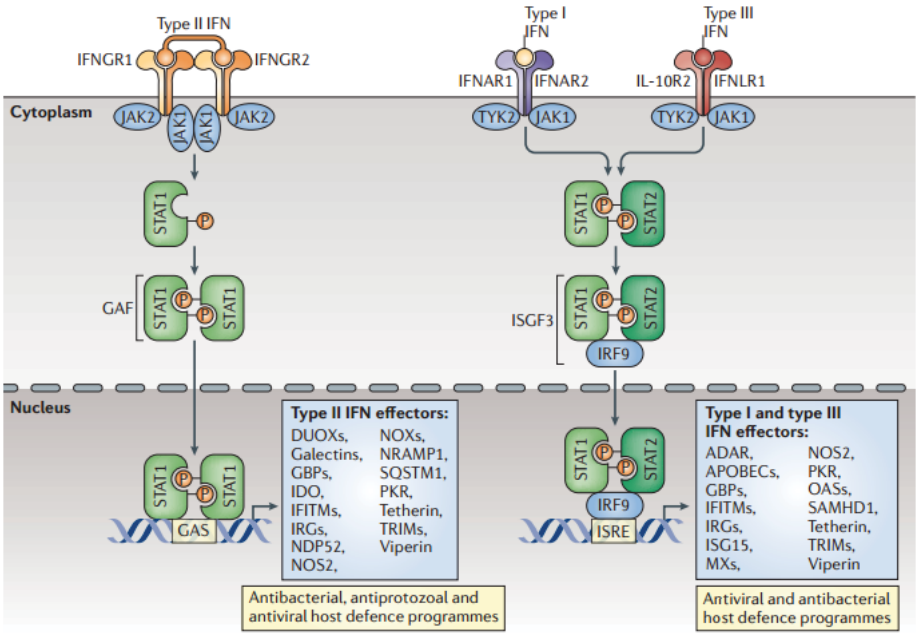

Figure 1.18: Signalling pathways of type I, II and III interferons. The three classes of IFN bind to their own independent receptor molecules on the cell surface. Type I IFN binds to a heterodimer of IFNα receptor 1 (IFNAR1) and IFNAR2, type II binds to a tetramer of two IFNγ receptor 1 (IFNGR1) chains 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.

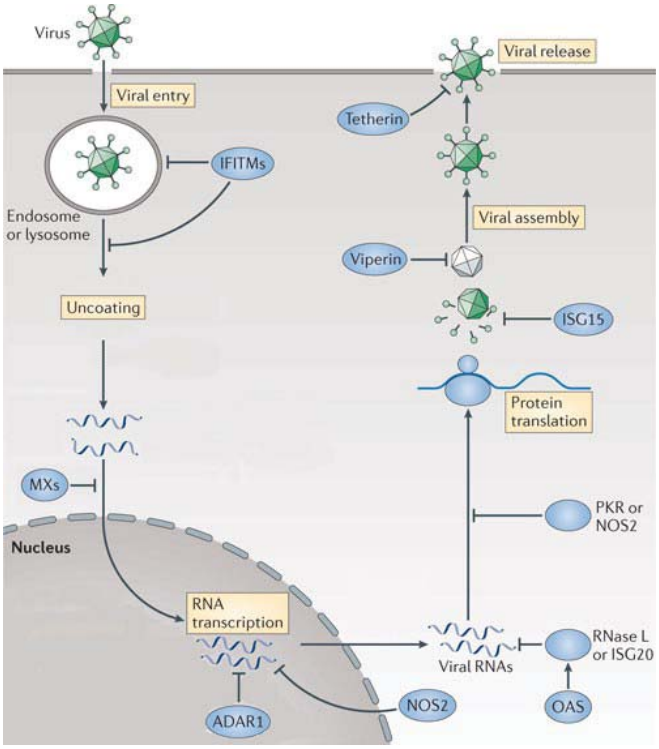

Figure 1.19: Some of the interferon-induced proteins thought to be capable of restricting influenza virus. The schematic illustrates the generalised life cycle of an infecting virion, which will act as a surrogate for influenza virus. The cell uses many autonomous techniques to prevent further viral 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).

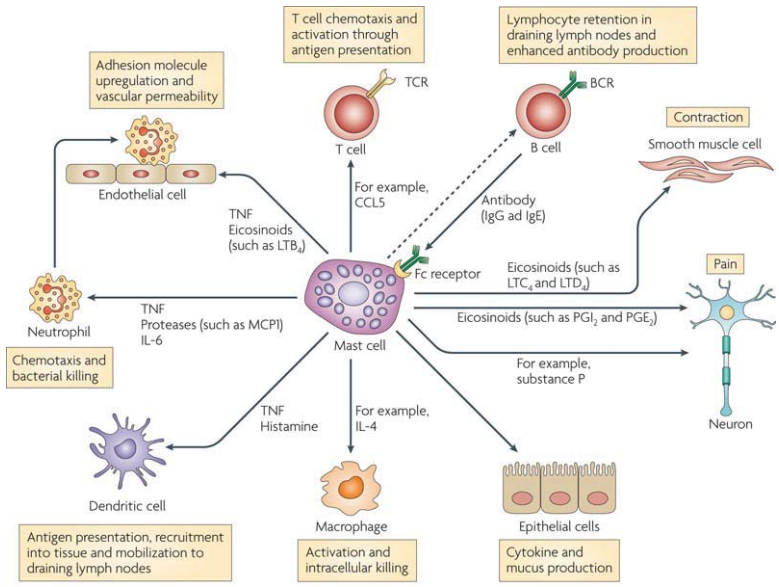

Figure 1.20: The role of mast cells in host defence. The schematic illustrates how mast cells communicate and moderate the actions of various cell populations, with the aim of modulating host immunity. Orange boxes show the functional consequences of the signalling and chemical signals / cytokines are 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).

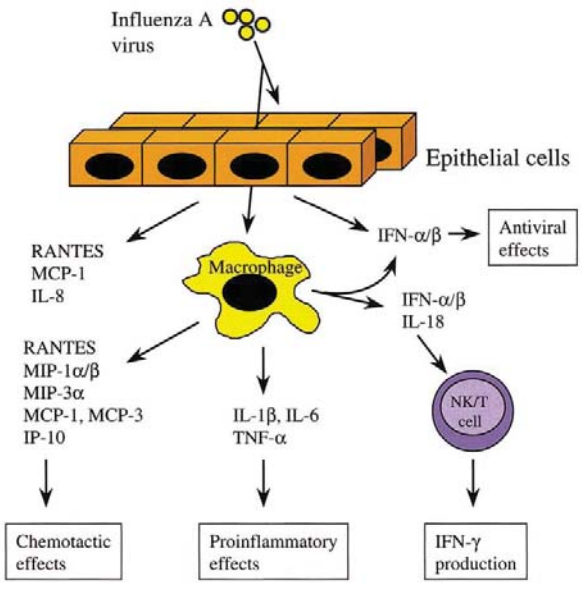

Figure 1.21: Cytokines produced by influenza-infected macrophages and their downstream effects. The production of RANTES, MCP-1 and IL-8 by infected epithelial cells also acts as a chemoattractant for monocyte populations, which mature into macrophages in the lung tissue. (From (Julkunen *et al.* 2001))

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).

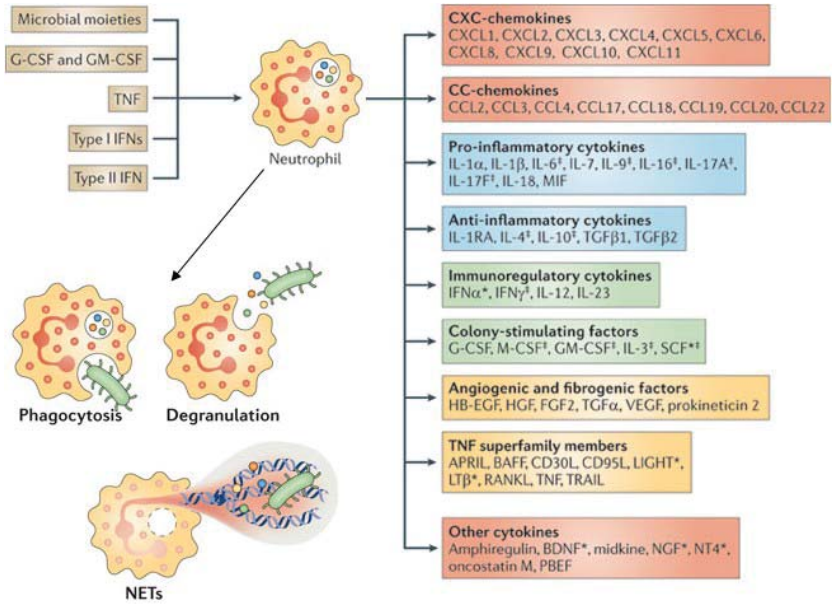

Figure 1.22: Killing mechanisms and signalling molecules generated by neutrophils during the innate immune response. The schematic illustrates the scope of immunomodulation possible by neutrophils. Phagocytosis: the engulfment of pathogens or apoptotic cells by the neutrophil, which are disposed of by 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.

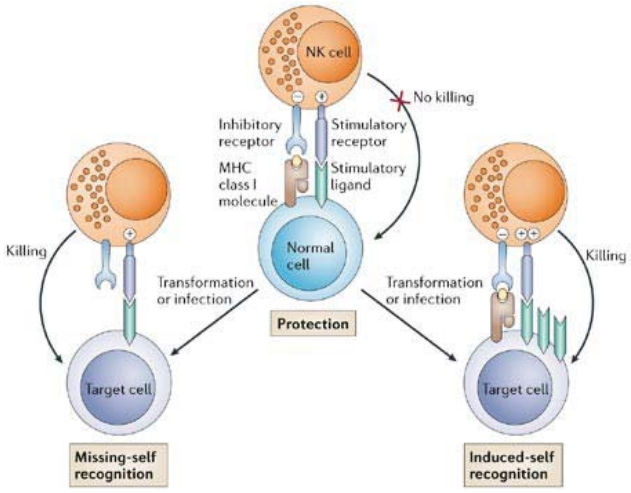

Figure 1.23: Natural killer cell control of activation state. NK cells possess inhibitory and stimulatory receptors on their cell surface wherein the ratio of inhibition to stimulatory signals stemming from the target cell dictates NK cell activation. When MHC-I receptors are withdrawn from the cell 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.

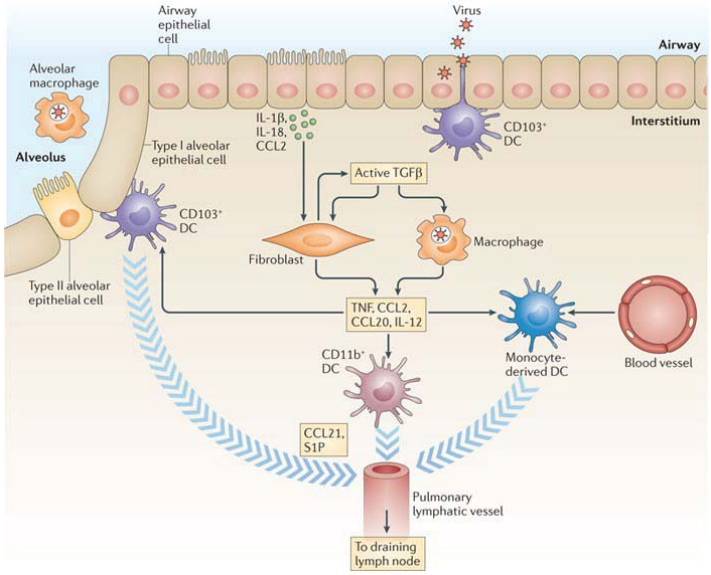

Figure 1.24: The innate immune response of DC populations at the respiratory surface. As described in section 1.4.1.2. the epithelial cells form the primary target for influenza virus and subsequently release cytokine and chemokine signals such as CCL2 (MCP-1), IL-1β and IL-18 into the surrounding cells, 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.

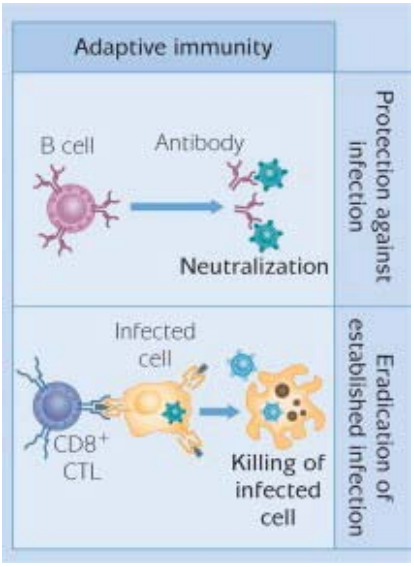

Figure 1.25: The functions of the adaptive immune response to influenza infection. After priming by DC populations, the adaptive immune response is triggered to counteract the infecting virus. This response has two purposes, with the first of which being protection against the virus; neutralising it 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.

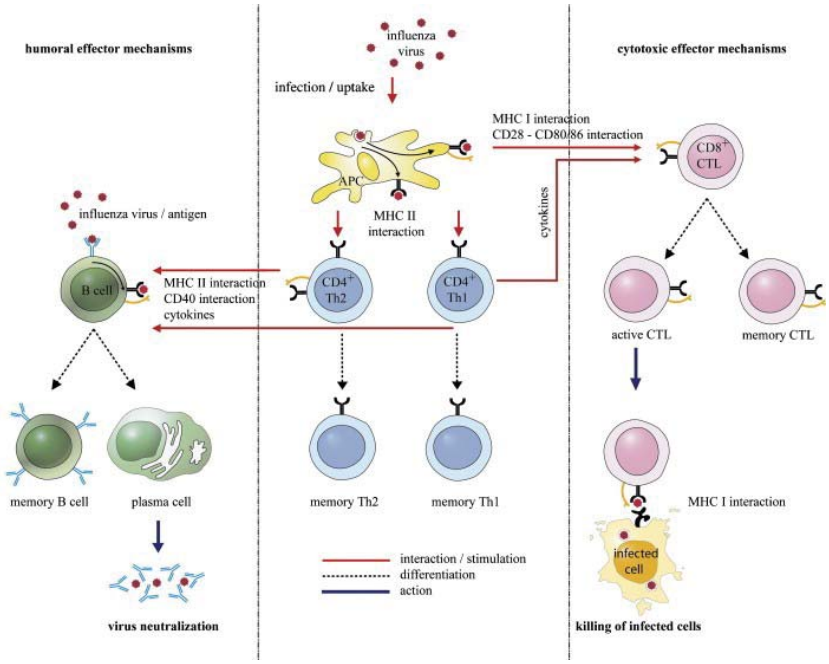

Figure 1.26: Generation of the adaptive immune response to influenza. The schematic illustrates the interactions between APCs and effector cells that lead to the development of the adaptive immune response. The roles of MHC-I and MHC-II complexes in the activation of the repertoire, along with the interactions 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.

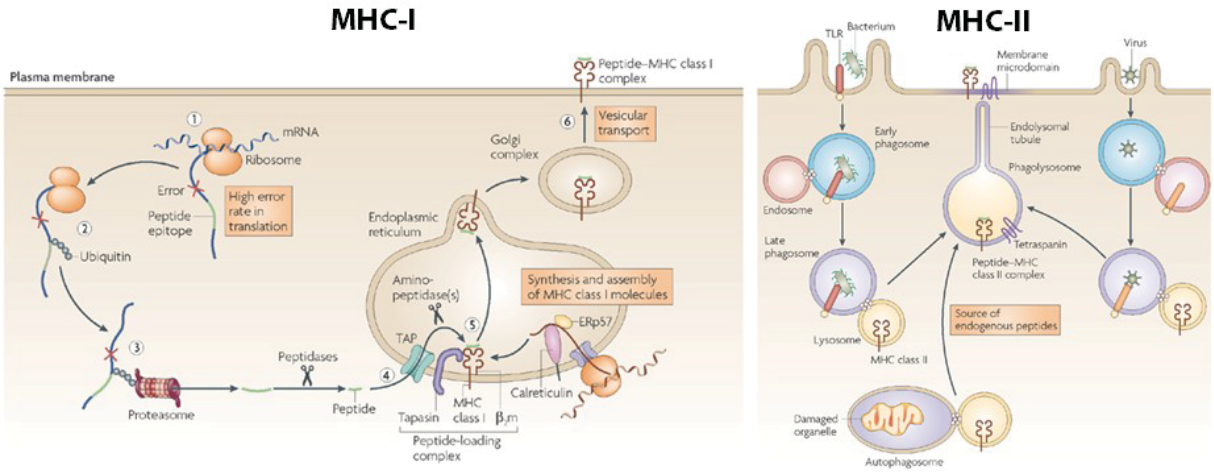

Figure 1.27: Mechanisms underpinning antigen presentation via MHC-I and MHC-II. The six stages of antigen presentation via the MHC-I pathway: 1) acquisition of error-laden protein antigens, 2) misfolded proteins are ubiquitinated for degradation, 3) proteasome degradation, 4) peptides transported to 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.

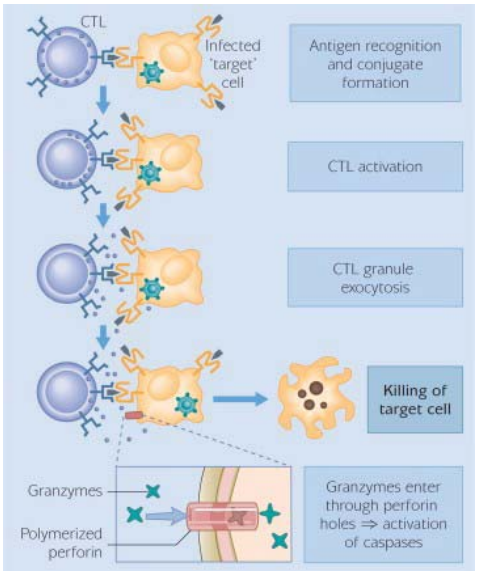

Figure 1.28: Killing mechanism of cytotoxic T-lymphocytes. Interaction via the MHC-I complex allows the CTL to detect an infected target cell. This subsequently leads to the CTL targeting the infected cell and releasing granzymes and perforin, which enter the cell and degrade the DNA and disrupt the 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).

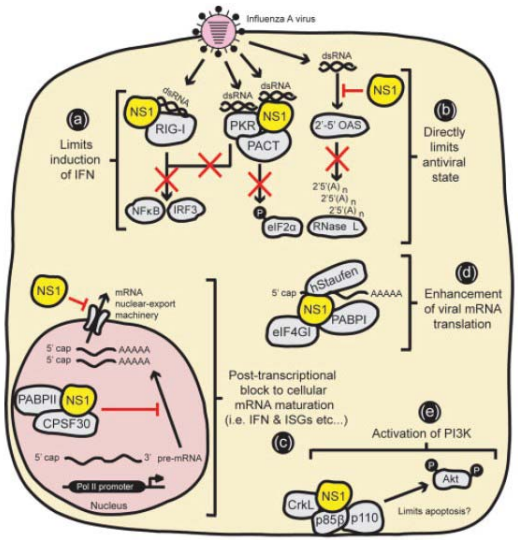

Figure 1.29: The multi-functional role of influenza's NS1 protein in antagonising the cell-autonomous innate immune response. a) pre-transcriptional block of IFN induction, b) PKR and OAS inhibition, c) block of processing and nuclear export of cellular mRNA, d) enhancement of vRNA translation, e) activation 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).

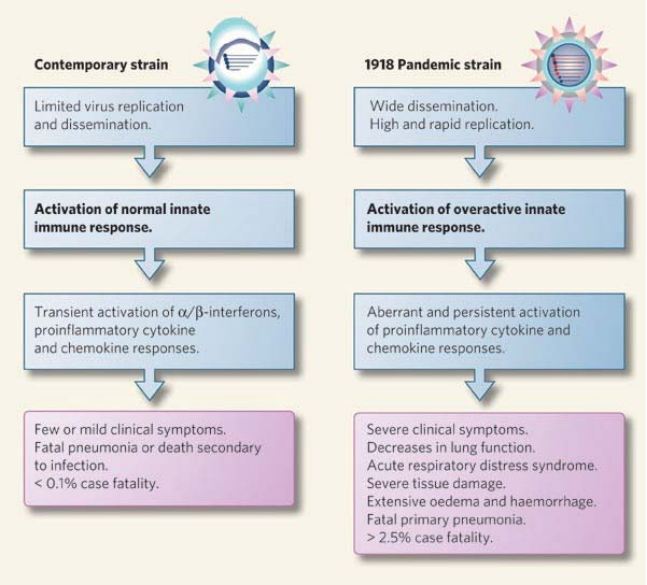

Figure 1.30: Differences in the host response elicited by low and high pathogenicity viruses. Contemporary influenza viruses are used to typify "low pathogenicity" infections, which usually show limited spread, mild symptoms and are cleared by the host. The 1918 'Spanish' virus is used to illustrate 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.

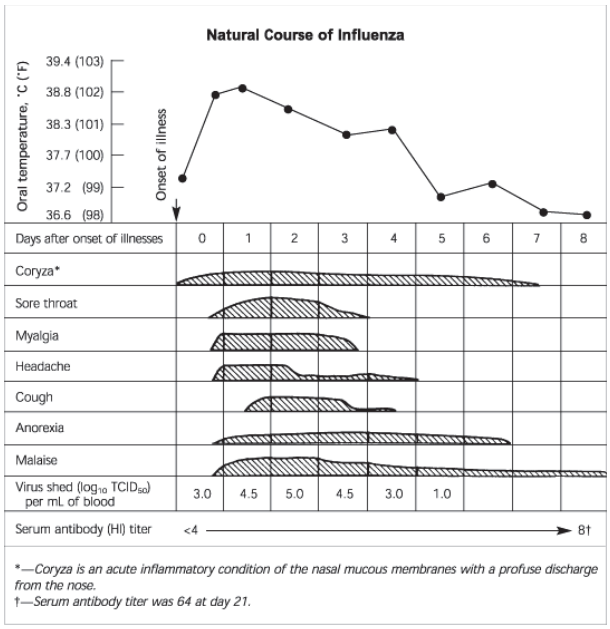

Figure 1.31: Clinical symptoms and disease progression associated with uncomplicated influenza virus infection. Results demonstrate the extent of morbidity from a healthy 28 year old male. (From (Montalto 2003))

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 × $\log_{10}$ 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.

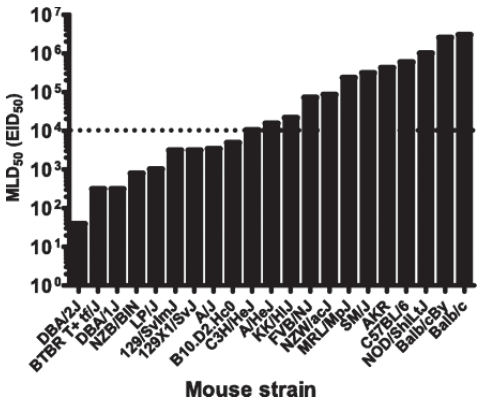

Figure 1.32: Susceptibility of 21 inbred mouse strains to highly pathogenic H5N1 influenza A virus. The 50% mouse lethal dose (MLD_{50}) of A/Hong Kong/213/03 is shown for all inbred strains. The dotted line indicates the median dose required for lethality. (From (Boon *et al.* 2011))

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].

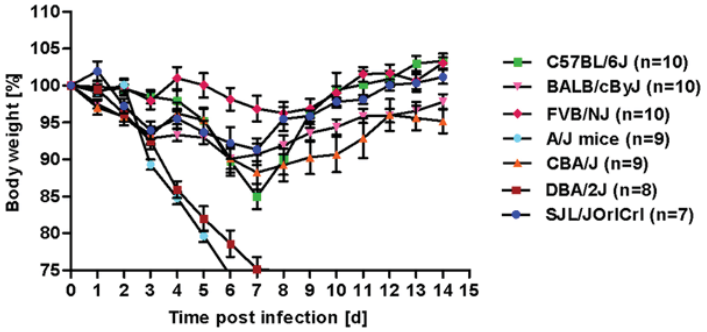

Figure 1.33: Weight loss induced by influenza A virus infection in an array of inbred strains of mice. All mice were infected with 3000 FFU (fluorescence forming units) of PR/8 H1N1 influenza and monitored for weight loss. Note the differences in susceptibility between inbred strains, but notice that 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.

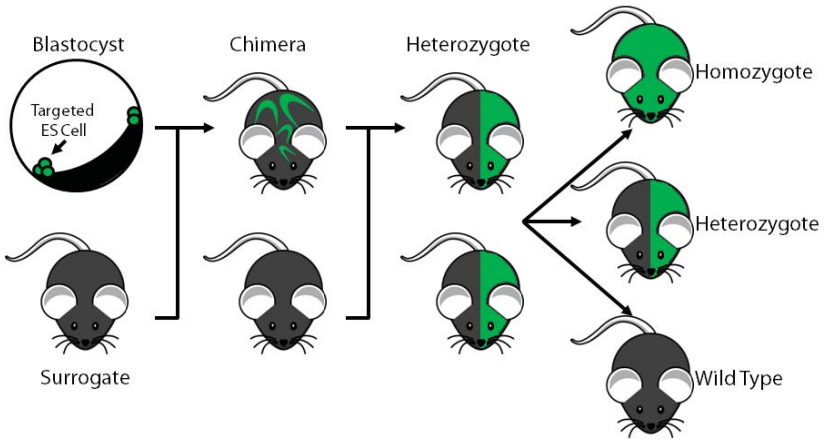

Figure 1.34: Schematic of the generation of knockout mice. Targeted embryonic stem cells (ES cells) carrying the gene deletion are inserted into blastocysts, which are then brought to term by surrogate wild type mice. These chimeras are crossed onto wild type backgrounds until a pairing is found to carry 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.