7 General Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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