2 Materials and Methods

2.1 Materials

2.1.1 Media

Table 2.1: Media compositions used in the study.

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

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

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

2.1.2 Cell lines

Table 2.2: Cell lines used during the study.

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

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

2.1.3 Viruses

Table 2.3: Viruses used during the study.

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

2.1.4 Oligonucleotide primers

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

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

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

2.1.5 Antibodies for flow cytometry

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

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

2.1.6 Silencing RNAs (siRNA)

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

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

2.1.7 Mice

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

2.2 Methods

2.2.1 Animal methods

2.2.1.1 Mouse infection

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

2.2.1.2 Titration of virus in mice

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

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

2.2.1.3 Mouse vaccination

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

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

2.2.1.3 Anti-fungal treatment

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

2.2.1.4 Bone marrow transfer

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

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

2.2.2 Tissue processing

2.2.2.1 Division of the respiratory system

The respiratory tract was cut at the point of bifurcation between the oesophagus and trachea and removed from the chest cavity. Lungs were either segregated by lobe for individual purposes (Figure 2.1), or the entire respiratory tract (trachea and all lobes) were immersed in 4% formaldehyde for immunohistochemistry (section 2.2.6).

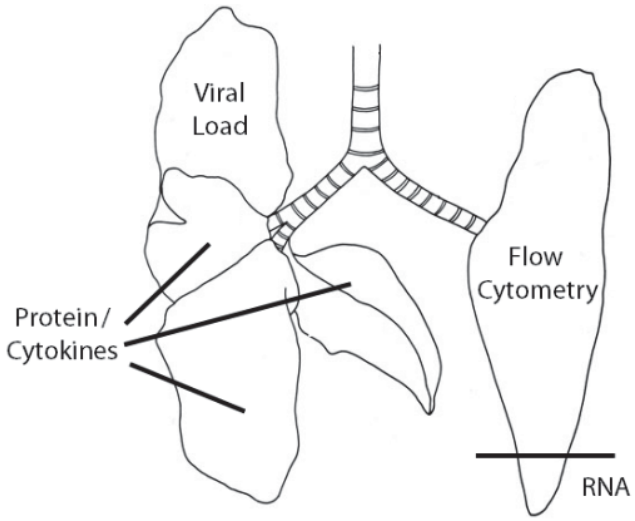

Figure 2.1: Uses of murine lung tissue in the study. Lungs were either carefully dissected and used for the listed purposes, or the entire respiratory system was excised for pathological analysis.

2.2.2.2 Flow cytometry preparation

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

2.2.2.3 Viral load preparation

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

2.2.2.4 RNA extraction preparation

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

2.2.2.5 Protein extraction preparation

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

2.2.3 Replicating virus quantification

2.2.3.1 Plaque assay

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

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

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

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

Where: d = dilution factor where plaques were counted (10^{-1}-10^{-10})
V = volume of diluted virus added to the well (0.2ml)

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

2.2.3.2 Tissue culture infective dose ($TCID_{50}$)

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

2.2.4 Molecular methods

2.2.4.1 Nucleic acid extraction

2.2.4.1.1 DNA extraction

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

2.2.4.1.2 RNA extraction

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

2.2.4.2 Polymerase chain reaction (PCR)

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

i. 15 minutes at 95^{o}C.
ii. 30 cycles of 30 seconds at 94^{o}C, 90 seconds at 60^{o}C, 90 seconds at 72^{o}C.
iii. 10 minutes at 72^{o}C

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

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

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

i. 15 minutes at 95°C.
ii. 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C.

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

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

i. 2 minutes at 50°C.
ii. 10 minutes at 95°C
iii. 40 cycles of 15 seconds at 95°C, 1 minute at 60°C.

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

2.2.4.4 Agarose gel electrophoresis

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

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

2.2.5 Cellular methods

2.2.5.1 Flow cytometry

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

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

2.2.5.2 Murine embryonic fibroblasts (MEFs)

2.2.5.2.1 Generation

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

2.2.5.2.2 Transfection and transduction

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

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

2.2.5.3 RNA interference (RNAi)

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

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

2.2.5.4 *In vitro* infection assays

2.2.5.4.1 RNAi studies

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

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

2.2.5.4.2 LCL infections

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

2.2.5.4.3 MEF infections

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

2.2.6 Tissue analysis

2.2.6.1 Peripheral leukocytes

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

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

2.2.6.2 Histology

2.2.6.2.1 Pathology scoring

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

2.2.6.2.2 Protein immunohistochemistry

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

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

2.2.6.2.3 RNA immunohistochemistry

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

2.2.7 Protein analysis

2.2.7.1 ELISA

2.2.7.1.1 Cytokine ELISA

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

2.2.7.1.2 Anti-influenza antibody ELISA

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

2.2.7.2 Luminex

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

antibody profiles of the mice. Analysis was conducted on a Luminex FlexMAP3D, using the following mouse antibody bead kits: Millipore's Cytokine/Chemokine 17-plex (G-CSF, GM-CSF, IFNγ, IL-10, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IP-10, KC-like, MCP-1, MIP-1α, RANTES, and TNFα), Millipore's Cytokine/Chemokine 4-plex (G-CSF, MCP-1, IL-6, TNFα) Millipore's Immunoglobulin Isotyping Kit (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM) and Invitogen's Inflammatory Cytokine Panel (GM-CSF, IL-1β, IL-6, and TNFα). All assays were conducted according to the manufacturer's instructions, with >100 bead events being counted per cytokine, per assay. Results were analysed and quality control checked using Masterplex QT 2010 and Masterplex Readerfit 2010 (MiraiBio).

2.2.7.3 Western blot

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

2.2.7.4 Microneutralisation assay

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

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

2.3 Statistical analyses

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