

Influenza reverse genetics: dissecting immunity and pathogenesis

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Reverse genetics systems allow artificial generation of non-segmented and segmented negative-sense RNA viruses, like influenza viruses, entirely from cloned cDNA. Since the introduction of reverse genetics systems over a decade ago, the ability to generate ‘designer’ influenza viruses in the laboratory has advanced both basic and applied research, providing a powerful tool to investigate and characterise host-pathogen interactions and advance the development of novel therapeutic strategies. The list of applications for reverse genetics has expanded vastly in recent years. In this review, we discuss the development and implications of this technique, including the recent controversy surrounding the generation of a transmissible H5N1 influenza virus. We will focus on research involving the identification of viral protein function, development of live-attenuated influenza virus vaccines, host-pathogen interactions, immunity and the generation of recombinant influenza virus vaccine vectors for the prevention and treatment of infectious diseases and cancer.

Influenza A viruses

Influenza A virus outbreaks are an annual, ongoing public health concern that place a great social and economic burden on society. Influenza virus infection is currently the leading cause of combined morbidity and mortality in the world, causing 250 000–500 000 deaths worldwide per year (Ref. 1). In the event of a pandemic influenza outbreak, these numbers can rise exponentially. A recent study indicated that the total economic costs associated with annual influenza epidemics were \$29 billion in the USA alone (Ref. 2). In light of this, rapid and cost-effective production of effective vaccines

targeting seasonal influenza or emerging influenza virus outbreaks is essential. Moreover, development of effective vaccines and disease prevention depend on profound knowledge of the virus itself. Reverse genetics has been exploited extensively to dissect the roles of influenza virus gene segments in disease pathogenicity, host-pathogen interactions and to develop new vaccine strategies.

Influenza A belongs to the Orthomyxoviridae family of RNA viruses. The influenza A genome is divided into eight separate segments of single-stranded negative-sense RNA, which express at least 11 functional proteins: polymerase basic

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(PB) 2, PB1, PB1-F2, nucleoprotein (NP), haemagglutinin (HA), neuraminidase (NA), matrix (M) 1, M2, polymerase acidic (PA), non-structural protein (NS) 1, and nuclear export protein (NEP; previously known as NS2) (Ref. 3), although a 12th has recently been described and is currently being characterised (Ref. 4). PB2, PB1 and PA form part of the RNA-dependent RNA polymerase complex, required for the synthesis of viral mRNA and viral replication (Ref. 3). PB1-F2 is thought to enhance virus-induced apoptosis and is translated from an alternative open reading frame overlapping the PB1 open reading frame (Ref. 5). HA is a surface glycoprotein responsible for attachment to host cells via surface sialic acid (SAs) receptors, and NA cleaves cell-surface SAs, releasing newly packaged virus from host cells. Individual viral RNAs (vRNAs) are bound by NP, which then interacts with the viral polymerase to form the viral ribonucleoprotein (vRNP) complex triggering the initiation of viral RNA transcription and replication (Ref. 6). The matrix structural protein M1 is required for vRNP export to the cytosol (Ref. 7) and the ion channel protein M2 is required for virus entry and the production of infectious viral particles (Refs 3, 8). NS1 and NEP proteins are encoded by gene segment 8 again through alternative splicing. The major role of NS1 protein is the suppression of host innate type I interferon (IFN) responses (Ref. 9). NEP is an adaptor protein that mediates the export of viral RNPs from the cell nucleus for virus assembly (Ref. 3). Reverse genetics has been used to incorporate changes in the majority of the aforementioned gene segments to address questions about protein function and pathogenicity, the findings of which will be discussed further in this review.

Introduction to reverse genetics systems

Reverse genetics is the process of generating live viruses entirely from cloned cDNA (Ref. 10). This approach has simplified the manipulation of the virus genome, allowing not only the introduction of point mutations, but also the generation of reassortant viruses through the replacement of virus gene segments from different influenza virus strains or subtypes. This has enabled the delineation of protein function and the contribution each protein has made to virulence and pathogenicity, as well as the generation and development of influenza

virus vaccines (Ref. 11). Furthermore, reverse genetics has also been utilised to develop influenza virus-based gene delivery systems (Refs 12, 13).

Reverse genetics systems were first established in 1978 with the generation of the non-segmented positive-stranded RNA virus coliphage, Q_β (Ref. 14). This was achieved through the conversion of its viral RNA genome into double-stranded cDNA using reverse transcription. The cDNA was then cloned into a plasmid for transfection into *Escherichia coli* to produce phage Q_β (Ref. 14). These early studies laid the foundation for the generation of more complex virus systems containing segmented negative-sense genomes (e.g. influenza virus) (Ref. 10). Progress towards the rescue of segmented negative-sense RNA viruses was slow and technically difficult. In contrast to the non-segmented positive-stranded RNA viruses, the genome of the negative-stranded RNA viruses is non-infectious. The two major factors contributing to these difficulties were: (i) that initial viral RNA transcription and replication relied on the presence of endogenous viral RNA polymerase, and (ii) that viral genomic RNA required NP to form a RNP complex (Ref. 15). The first functional influenza RNP complex was generated *in vitro* in 1989 in the presence of purified NP and polymerase proteins (Ref. 16). Additional systems were established in subsequent years, based on a similar technique, to generate a recombinant influenza A virus that expressed a foreign gene replacing the NS gene segment (Ref. 17). Progress towards the generation of negative-stranded RNA viruses entirely from cloned cDNA gained momentum with the production of a single negative-stranded Rabies virus in 1994 (Ref. 18), followed by generation and rescue of a segmented (three RNA segments) negative-stranded Bunyamwera bunyavirus in 1996 (Ref. 19).

It was not until 1999 that an influenza virus was generated entirely from cloned cDNA using reverse genetics (Ref. 20). To generate and rescue this viable influenza virus, a total of 12 plasmids were initially required; eight plasmids encoding the eight vRNAs (PB2, PB1, PA, NP, HA, NA, M and NS) (Ref. 21) in combination with four plasmids that facilitated the production of polymerase complex proteins, PB2, PB1, PA and NP (Ref. 20). In the year 2000, a more efficient reverse genetics system was developed using

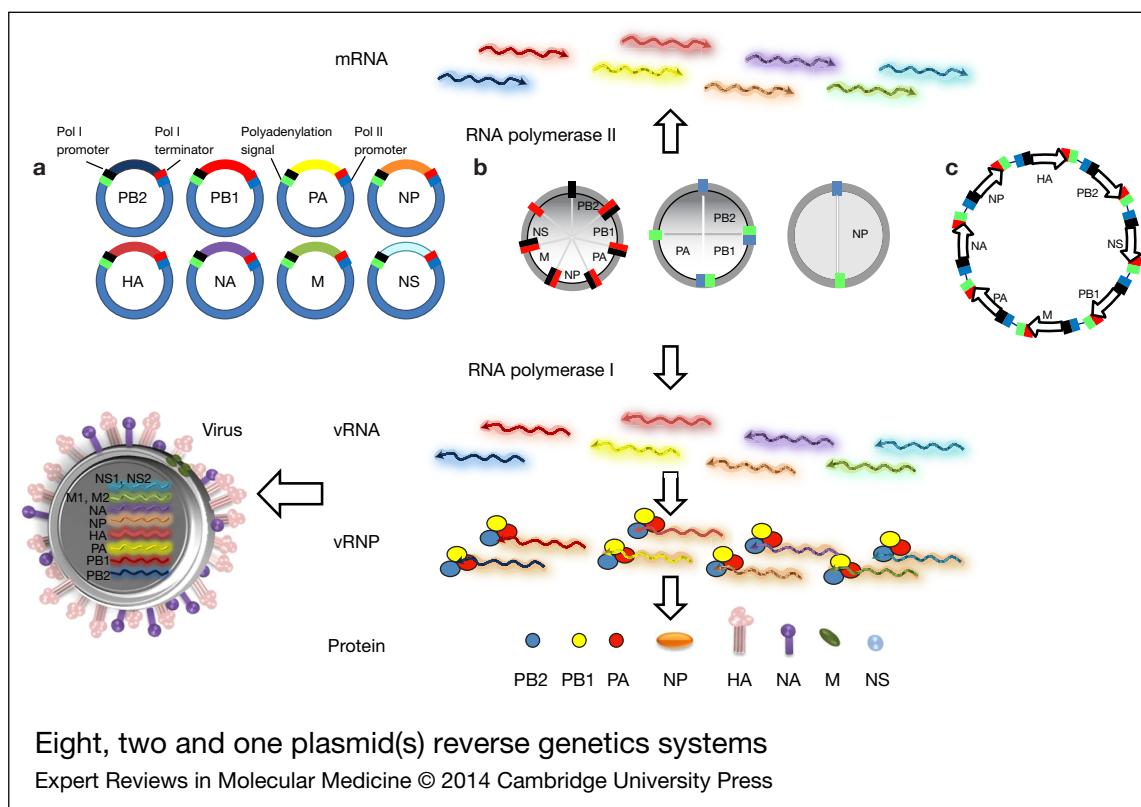


Figure 1. Eight, two and one plasmid(s) reverse genetics systems. (a) Eight plasmids reverse genetics system (Ref. 22). In each of the expression plasmid, the cDNA encoding one of the eight influenza viral segments was inserted between a human pol I promoter and a murine pol I terminator. This pol I cassette is then flanked by the pol II promoter of the human cytomegalovirus and the polyadenylation signal of the gene encoding bovine growth hormone. This pol I-pol II system will result in the transcription of the eight vRNAs through the pol I promoter using cellular pol I, and the transcription of viral mRNAs through pol II. vRNAs will then be translated into viral proteins. The core of the virus contains the vRNPs that is wrapped up around NP and joined with the viral RNA polymerase complex (PB1, PB2 and PA). Infectious influenza virus will be formed following assembly of vRNPs and proteins HA, NA, M1, M2 and NS. (b) Three plasmids reverse genetics system (Ref. 23). One plasmid contains cDNAs for all eight viral segments, each flanked by pol I promoter-terminator pair; the other plasmid contain three RNA pol II transcription cassettes containing cDNA encoding PB2, PB1 and PA for the transcription of the three mRNAs; the third plasmid contains a pol II transcription unit of NP. (c) One plasmid reverse genetics system (Ref. 24). Expression plasmids were generated using a similar pol I-pol II system as (a). All eight cDNA cassettes for the eight viral segments were combined into one expression plasmid. Each cDNA cassette consists of the cDNA flanked by the pol I-pol II system.

only eight plasmids (Ref. 22) (Fig. 1a). In this modified system, viral cDNA was inserted between a human polymerase I (pol I) promoter and a mouse pol I terminator, driving the synthesis of negative-sense vRNAs (Ref. 22). The pol I transcription cassette was flanked by a human pol II promoter and a bovine polyadenylation signal from which mRNAs were transcribed and translated into viral proteins (Ref. 22). This enabled negative-sense

vRNAs and positive-sense mRNAs encoding viral proteins to be synthesised from the same cDNA template (Ref. 22). This technological breakthrough simplified and streamlined the rescue of influenza viruses and made reverse genetics a mainstream procedure in many influenza laboratories.

The reduction in the number of plasmids required for virus rescue increased the transfection efficiency of mammalian cells,

resulting in higher virus yields (Refs 22, 23). To this end, a three-plasmid system has been developed to further improve efficiency and yield (Ref. 23) (Fig. 1b). Briefly, each of the influenza virus cDNAs were flanked by the human pol I promoter and the mouse pol I terminator to form a RNA pol I transcription cassette. The eight transcription cassettes were then cloned into one plasmid. The other two plasmids encoded the viral polymerases PB2, PB1 and PA or NP, respectively, with the cDNA flanked by a pol II promoter and a polyadenylation sequence (Ref. 23). The use of this three-plasmid system resulted in higher transfection efficiency and virus yields in both Vero and HEK293 T cells when compared with other pre-existing reverse genetics systems (Ref. 23).

More recently, a one-plasmid reverse genetics system was developed (Ref. 24) (Fig. 1c). cDNAs encoding PB2, PB1, PA and NP were first inserted between a pol I promoter–terminator and a pol II promoter–terminator. The pol II promoter was omitted from the cassette containing HA, NA, M or NS. All eight cassettes were then ligated to form a single plasmid. Virus yields generated using the one-plasmid system were comparable with the eight-plasmid system in chicken embryonic fibroblasts (CEFs), and were improved substantially with co-culture of CEFs and Madin–Darby canine kidney (MDCK) cells (Ref. 24). One of the key features of this system is the use of unique restriction sites between cassettes, allowing replacement of viral genes with circulating seasonal influenza viral genes for vaccine production (Ref. 24). Moreover, it provides a useful research tool for the study of virulence determinants through the generation of recombinant viruses. Despite these incremental improvements in technology, the eight-plasmid system remains the workhorse in the field.

Current applications for influenza virus reverse genetics systems

Use of influenza reverse genetics to study host range

Influenza viruses infect host cells by binding to SA moieties via HA, followed by receptor-mediated endocytosis and fusion of the viral and endosomal membranes (for review, see (Ref. 25)). Avian influenza viruses bind to cell-surface receptors whose sugars terminate in SA- α 2,3-galactose (SA- α 2,3-Gal), which is abundant on avian intestinal epithelia (Ref. 26). Human-

adapted influenza viruses bind to SA- α 2,6-Gal-linked receptors expressed for the most part on the surface of airway epithelia (Ref. 26). It should also be noted that there is emerging evidence to suggest that avian influenza viruses can infect host cells in the absence of canonical sugar moieties (Refs 27, 28). The phrase ‘antigenic drift’ describes the accumulation of mutations (predominantly in HA and to a lesser extent in NA), resulting in the emergence of antigenic variants of influenza virus and is responsible for our annual seasonal outbreaks (Refs 29, 30). The antigenic sites where changes occur have been mapped to the globular head of HA in an area surrounding the receptor-binding pocket (Ref. 29) or the substrate-binding site of NA (Ref. 31). Mutations at the receptor-binding site (RBS) can influence the host range of influenza viruses. It is thought that the avian origin 1918 pandemic virus acquired its ability to infect humans through mutations in HA (Ref. 32). Use of reverse genetics to analyse human and avian host range (for H2 and H3 viruses) has demonstrated that Q226L and G228S substitutions change the binding preference for these viruses from human SA- α 2,6-Gal receptor to avian SA- α 2,3-Gal receptor (Ref. 33). Other studies have shown that for H1 viruses, E190D and D225 G substitutions facilitate preferential binding to the human SA- α 2,6-Gal receptors (Ref. 33).

Use of reverse genetics to produce inactivated and live influenza vaccines

Vaccines (live and inactivated) are currently available to control influenza virus infection and provide between 80 and 100% protection in children and adults under the age of 65 (Refs 34, 35). There are currently two vaccine formulations available to protect humans against seasonal influenza; inactivated vaccines and live-attenuated influenza vaccines (LAIV) (Ref. 36). Both vaccines are generated using reverse genetics systems, incorporating the HA and NA gene segments from circulating strains in combination with six backbone gene segments to produce a product using egg-based technology (Ref. 36). There are some limitations with these vaccines, including poor efficacy in the elderly (Ref. 37). In addition, as the inactivated vaccine is administrated intramuscularly, it does not induce a strong mucosal immune response of the upper

respiratory tract as seen during natural infection (Ref. 38). Reverse genetics has been used to generate an effective commercially available LAIV (FluMist®) (Ref. 39) that is delivered as a nasal spray and has been licensed for use in the USA since 2003 (Ref. 40) and in the European Union since 2011 as Fluenz™ (Ref. 41).

Use of reverse genetics to develop universal influenza vaccines

The 2009 pandemic caused by the swine-origin influenza virus (S-OIV) H1N1 and the on-going pandemic threat associated with the highly pathogenic avian influenza (HPAI) H5N1 subtype highlight the urgent need for the development of a universal influenza vaccine with a capacity to induce cross-protective cellular immunity (Ref. 42). Current research has focused on conserved sequences in structural proteins, such as the extracellular domain of the matrix protein 2 (M2e) and NP (Ref. 43). A recent study analysing memory CD4⁺ and CD8⁺ T cells from individuals in the UK and Vietnam demonstrated cross-reactive immune responses targeting internal proteins, M1 and NP, following A/Vietnam/CL26/2005 (H5N1) and A/New York/232/2004 (H3N2) infection (Ref. 44). Moreover, mice primed with two viruses, A/Puerto Rico/8/1934 H1N1 (PR8) and A/Hong Kong/X31 H3N2 (X31), showed significantly improved protection (90% in primed mice *versus* 10% in unprimed mice) following challenge with HPAI A/Vietnam/1203/2004 (H5N1) that was attributed to the cross-protective cellular immunity (Ref. 45). Other studies have shown that reverse genetics can be used to generate recombinant A/WSN/33 (WSN) H1N1 viruses expressing previously defined human influenza T cell epitopes (M1, NS1, NP, PB1 and PA epitopes) in the NP (NPmix) or together with conserved sequences in the N-terminus of NA and C-terminus of HA from H5N1 virus (NA-HA) (Ref. 46). Vaccinated BALB/c mice primed with the two corresponding recombinant NP DNA constructs showed increased influenza virus-specific CD4⁺ and CD8⁺ T cell responses in splenocytes stimulated with NP peptide (TYQRTRALV), which clearly correlated with decreased viral load in the lungs (Ref. 46).

Despite these advances, a major limitation of current influenza vaccination strategies is the long lead-time (up to 6 months) required to produce vaccine (Ref. 47) and the possibility of

vaccine shortages in the event of a pandemic (Ref. 47). In view of these limitations, a rapid, cost-effective and consistent production pipeline is essential. Established reverse genetics systems, along with well-defined egg-based technology, and emerging *in vitro* approved cell lines for seasonal vaccine production are the foundation of future pandemic preparedness. The effectiveness of this reverse genetics technology was demonstrated by the production of a safe, high-yielding human influenza vaccine in Vero cells in response to HPAI H5N1 virus in a period of 4 weeks in 2003 (Ref. 11). The polybasic amino acids from the H5N1 HA cleavage site were removed to attenuate the virus and a reassortant vaccine virus strain was rescued using a modified HA (H5) and NA (N1) from A/Hong Kong/491/97 H5N1 virus, along with the standard backbone internal gene segments from PR8 H1N1 virus (Ref. 48).

Use of reverse genetics to elucidate determinants of viral pathogenicity

Effective prevention and control of influenza viruses depends on a detailed understanding of the virus at the molecular level. The on-going advances in our understanding of molecular mechanisms driving influenza pathogenicity have been made possible with the establishment and refinement of reverse genetics technology (Refs 49, 50). Below we highlight and discuss critical examples noted in the literature and present.

The PB2 protein

The viral RNP complex consisting of PB2, PB1, PA and NP plays a key role in adaptation and virulence of avian influenza viruses (Refs 50, 51). The PB2 protein is responsible for the initiation of viral mRNA transcription and RNA polymerase activity within infected cells (Ref. 3). Several amino acids within PB2, such as 701 and 627, have been implicated in mammalian host range adaptation (Refs 52, 53) and virulence (Refs 49, 54). A single mutation in the PB2 subunit at position 701 from aspartic acid (D) (consensus residue in avian viruses) to asparagine (N) (D701N) in avian H5N1 and H7N1 subtypes enable the viruses to infect mice (Refs 53, 55) and humans (Ref. 56). In addition, the PB2 D701N has been found in human isolates of HPAI H5N1 virus, as well as in avian-like swine virus (Ref. 53).

Another amino acid residue in the PB2 segment that has come under intense scrutiny is amino acid 627. In general, avian influenza viruses usually encode a glutamic acid (E) at position 627 (E627) in PB2, whereas human viruses possess a lysine (K627). Sequence analyses and comparisons between two well-studied H5N1 human isolates, A/Hong Kong/483/97 (HK483-lethal in mice) and A/Hong Kong/486/97 (HK486-non-lethal in mice), uncovered eight amino acid differences, including K in HK483 *versus* E in HK486 at position 627 (Ref. 49). HK483 influenza viruses with a K627E substitution in PB2 (generated with reverse genetics) displayed attenuated virulence in mice (Ref. 49). In contrast, HK486 influenza viruses with an E627 K substitution in PB2 became increasingly adapted to mouse cell lines when compared with wild-type virus (Ref. 54) and were lethal in mice (Refs 49, 54).

The PB1-F2 protein

Segment 2 of the influenza genome encodes two proteins, PB1 and PB1-F2, from different open reading frames. PB1, as part of the polymerase complex, is involved in viral mRNA synthesis and elongation (Ref. 57). Use of reverse genetics to generate single 1:7 gene reassortants between the 1918 Spanish pandemic strain and a seasonal human H1N1 A/Texas/36/91 (1918 PB1:Tx/91) demonstrated that PB1 was a major virulence determinant in the 1918 virus by increasing replication efficiency in human airway cells and lungs of mice (Ref. 58).

PB1-F2 can act as a pro-apoptotic protein sensitising cells to apoptosis (Ref. 59) and regulate viral replication through its interaction with PB1, influencing polymerase activity (Ref. 60). The PB1-F2 sequence of the PR8 strain was compared with that of the 1918 pandemic virus and the seven amino acids differences between the two were incorporated into the PR8 PB1-F2 open reading frame (R33H, G40D, R60Q, N66S, I68 T, L69P, F71S, END88W) to generate a 1918-like PB1-F2 using reverse genetics (Ref. 61). The mutant virus, PR8-PB1-F2 (1918), demonstrated accelerated viral replication kinetics *in vivo* and resulted in higher mortality, increased incidence of pulmonary inflammation, secondary bacterial infection and mortality in mice when compared with the wild-type PR8 virus (Ref. 61).

Sequence alignment of PB1-F2 from human isolates of the Hong Kong H5N1 outbreak (A/

Hong Kong/483/1997) and the 1918 H1N1 pandemic virus revealed the presence of a serine (S) at position 66 in both the Hong Kong 1997 H5N1 virus and the 1918 H1N1 virus. Low pathogenic avian influenza (LPAI)/intermediate H5N1 viruses contain an N in the same position (Ref. 62). Two recombinant WSN viruses containing an S or an N at position 66 were generated using reverse genetics to determine the impact on virulence (Ref. 62). The WSN N66S virus demonstrated increased virulence in infected mice as indicated by increased weight loss, higher virus titres in the lungs and delayed virus clearance. In contrast, a reconstructed 1918 virus with the S66N mutation showed significant attenuation (Ref. 62).

The NS1 protein

The NS1 protein is a recognised virulence determinant and has been shown to be involved in numerous processes associated with replication and host immunity. Firstly, it has been shown to increase NP and M1 protein expression through the stimulation of viral mRNA translation (Ref. 63). In addition, NS1 has been shown to activate the phosphatidylinositol-3 kinase (PI3 K)/Akt pathway to prevent virus-induced apoptosis in cells (Ref. 64). The best characterised function of NS1 is its suppression of host antiviral immune responses through its actions as a type I IFN antagonist, preventing important IFN-mediated antiviral responses. It interrupts retinoic acid-inducible gene I (RIG-I)-mediated signalling in response to viral RNA (Refs 65, 66, 67, 68). It has been proposed that the type I IFN antagonistic action of NS1 contributed to the virulence of the 1918 'Spanish Flu' (Ref. 69). C-terminal truncations and single amino acid substitutions of the NS gene segment generated through reverse genetics have been used to identify regions that affect virulence (Refs 9, 70). Deletion of the NS1 gene segment at position 80–84 (Ref. 71) and a D to E substitution at position 92 (D92E) (Ref. 72) were shown to be associated with increased virulence and resistance to antiviral IFNs.

Further in-depth analysis of the NS1 protein identified a four-amino acid peptide sequence (predominantly ESEV in avian, and RSKV in human) that forms a PDZ domain ligand (PL) (Ref. 73). PDZ domains are highly conserved protein–protein interaction modules that bind to PLs in target proteins (Ref. 74) and are involved

in trafficking and assembly of signalling complexes (Ref. 74). The avian NS1 protein containing the ESEV motif bound to a wide range of human PDZ domains, whereas human NS1 protein containing the RSKV motif showed little binding (Ref. 73). Recombinant WSN viruses encoding different PL motifs, including those from the 1918 H1N1 virus, HPAI H5N1 virus, and seasonal human H3N2 strains, were generated using reverse genetics. Recombinant viruses containing PL motifs from HPAI H5N1 and 1918 H1N1 viruses showed increased virulence in mice, while deletion of the PL motif resulted in decreased virulence (Ref. 75).

The NS1 protein consists of an N-terminal dsRNA-binding domain (residues 1–73) that is important in RIG-I inhibition and a C-terminal domain (residues 74–230) that coordinates interactions with host proteins and stabilises the RNA-binding domain and an NS1 tail. NS1 exists as a homodimer, which is essential for dsRNA binding (Ref. 76). Binding of NS1 to dsRNA blocks the dsRNA-activated antiviral protein kinase, PKR, dampening the host antiviral response (Ref. 77). A tryptophan at position 187 (W187) is critical for this process (Ref. 78). A recombinant PR8 virus with a single mutation of tryptophan at position 187 to arginine (rPR8 NS1–W187R) was generated using reverse genetics to disrupt dimerisation (Ref. 79). rPR8 NS1–W187R showed decreased dsRNA-binding ability that correlated with poor inhibition of the IFN- β promoter. Moreover, mice infected with rPR8 NS1–W187R displayed improved survival rates with lower virus titres in the lung compared with controls.

The HA protein

The ability of influenza virus to bind and infect cells to gain access to cell machinery requires cleavage of the HA protein (HA0) by cellular proteases to form two subunits: HA1 and HA2 (Ref. 29). The availability and location of these cellular proteases influence the site of virus replication. For example, influenza virus replication in mammals is generally restricted to the respiratory tract because this is where serine proteases that cleave HA are produced (Ref. 80). However, the HA of HPAI H5 and H7 viruses can be cleaved by ubiquitously expressed proteases due to the presence of the multiple basic amino acid motif (R–X–K/R–R) at the HA1–HA2 cleavage site (Ref. 81). Reverse

genetics has been utilised to generate mutant viruses with altered HA cleavage sites to correlate HA cleavability with virulence (Ref. 81). Results suggest that the length of the cleavage site (P_8 –Q₇–R₆–R₅–R₄–K₃–K₂–R₁) as well as the presence of basic amino acid (K) at positions 2 and 3 is important for optimal cleavage (Ref. 81).

The HA RBS of influenza virus is located on each HA monomer as part of a trimeric HA globular head (Ref. 25). A key amino acid on the surface of the HA1, tyrosine (Y) 161, has been shown to be essential for HA receptor recognition and virus entry (Ref. 82). An Y161A mutation introduced into A/Hong Kong/213/03 (H5N1) virus using reverse genetics resulted in a change of host tropism from avian species to mammals. In addition, *in vitro* infection studies with this H5N1 Y161A recombinant virus showed impaired growth in MDCK cells (Ref. 82). Detailed analyses of two 1918 pandemic strains have identified a single amino acid difference at position 225 of HA (Ref. 83). The strain with a D at position 225 showed higher binding affinity to human SA α 2,6Gal when compared with avian SA α 2,3Gal (Ref. 83). Moreover, replacement of D to E using reverse genetics (as per most LPAI avian viruses) at position 190 (D190E) reduced binding affinity for the human SA α 2,6Gal receptor (Ref. 83). Another key residue identified within the H1 HA RBS is residue 227 (Refs 84, 85). While human H1N1 viruses, including the 2009 pandemic H1N1 virus strain, encode E227, swine or S-OIV generally contain an alanine (A) in this position (Ref. 84). Introduction of an E227A mutation or expression of swine HA in the 2009 H1N1 virus via reverse genetics altered cell tropism and resulted in poorer fitness in primary human airway epithelial cells (Ref. 84).

Utilising reverse genetics to dissect influenza-specific immunity

The innate immune response against influenza virus infection is characterised by the infiltration of neutrophils and monocytes into the lung. The release of pro-inflammatory cytokines and chemokines by alveolar macrophages, lung epithelial cells and dendritic cells (DC) leads to the induction of adaptive immunity characterised by the generation of influenza-specific neutralising antibodies by B cells and the activation and expansion of virus-specific CD4 $^{+}$ and CD8 $^{+}$ T cells that assist in clearing

virus (Ref. 86). Reverse genetics can be used as a valuable tool to study how influenza virus manipulates host cellular and immune environments to ensure optimal propagation and prolonged survival.

Innate immunity

Toll-like receptors (TLRs) expressed by DCs, natural killer (NK) cells and macrophages play a critical role in regulating innate immune responses following influenza virus infection (Refs 87, 88). TLR3, 7, 8 and 9 expressed in the endosomal membranes recognise nucleic acid and viral components upon infection. This triggers the activation of intracellular signalling pathways and leads to the induction of antiviral cytokine production (e.g. type I IFNs) by virus-infected cells (Refs 87, 89, 90). Type I IFNs bind to cell-surface receptors, activating the JAK-STAT, TLR and RIG-I-like receptor (RLR) signalling pathways, resulting in the augmentation of IFN-stimulated/responsive genes and induction of pro-inflammatory responses (Refs 89, 90). Importantly, IFNs induce maturation of DCs, contributing to the augmentation of the antiviral state, including activation of T cell immunity (Ref. 90). As a consequence, viruses have developed various mechanisms to evade protective type I IFN defence mechanisms. The influenza NS1 protein plays a major role in evading host immune responses as mentioned earlier in this review (Refs 91, 92). Influenza virus reverse genetics systems have been used to carefully dissect these host-pathogen interactions. *In vitro* infection of human peripheral blood DCs with a recombinant PR8 virus incorporating an NS1 gene deletion resulted in suboptimal maturation, activation and migration of mature myeloid DCs. This correlated with reduced cytokine gene expression and anti-viral function (including T-cell activation) compared with wild-type virus as a result of weak stimulatory signals (Ref. 91). Similar results were also observed with a seasonal influenza virus A/Tx/91 and its A/Tx/91 NS1-deletion counterparts (Ref. 92). Furthermore, the NS1 C-terminus has been shown to interrupt RIG-I signalling by inhibiting IFN regulator factor 3 (IRF3) phosphorylation and activation, preventing transcription of type I IFNs and other IFN-induced genes (Ref. 92). When reverse genetics was used to delete the NS1 C-terminal region involved in this interaction, the newly

generated recombinant virus replicated poorly in primary human bronchial epithelial cells (Ref. 92).

Influenza-specific CD8⁺ T cell immunity

CD8⁺ T cell responses are stimulated by the presentation of viral peptides bound to major histocompatibility complex (MHC) class I glycoproteins to T cell receptors (TCR) on the T cell surface (Ref. 93). The design and development of a cross-reactive universal CD8⁺ T cell-based influenza virus vaccine targeting the highly conserved internal proteins can only be achieved if our underlying knowledge of influenza-specific CD8⁺ T cell-mediated immunity is clearly defined. Within this context, consideration must also be given to the application of immune pressure in order to avoid the emergence of mutations in the virus that could lead to CTL immune escape (Refs 94, 95, 96). Importantly, the magnitude of CD8⁺ T cell responses of different specificities following infection is not uniform. The way in which these specificities are ordered is referred to as an immunodominance hierarchy (Ref. 97). Understanding the significance of immunodominance hierarchies is critical for improving our understanding of cellular immunity and informing future vaccine design and development. In the C57BL/6J (B6, H2^b) mouse model of infection, two immunodominant influenza A virus-specific CD8⁺ T cell epitopes, nucleoprotein (NP_{366–374}) and acid polymerase (PA_{224–233}), are presented via the H2D^b MHC class I molecule (D^bNP₃₆₆ and D^bPA₂₂₄, respectively) (Refs 98, 99). The asparagine anchor residue at position 5 in these well-defined epitopes is responsible for high affinity binding to H2D^b (Refs 100, 101). Analyses of primary infection in studies conducted by the Doherty group have demonstrated co-dominance between the two specificities. However, following secondary infection, the D^bNP₃₆₆-specific response is clearly immunodominant (increased by at least 14-fold in the spleen) when compared with the D^bPA₂₂₄ response (Ref. 99). To understand the significance of this immunodominance hierarchy and its underlying mechanisms, recombinant viruses were generated using reverse genetics to substitute the N at position 5 for both epitopes to glutamine (Q), thereby abrogating presentation and ensuring loss of specificity (Refs 102, 103, 104). This loss of specificity for D^bNP₃₆₆ and

D^bPA_{224} allowed compensatory expansion of the normally subdominant K^bPB1_{703} CD8 $^{+}$ T cell population after secondary challenge (Ref. 102). Further detailed analyses revealed additional compensatory expansion of other minor epitopes (K^bNS2_{114} and the K^bM_{1128}) (Ref. 103). These experiments clearly demonstrated the plasticity of subdominant CD8 $^{+}$ T cell populations and gave us important insights into their ability to contribute to immunity when required in different/unique circumstances. The role of protective antibody also became more prominent following secondary infection when recombinant viruses expressing both NP₃₆₆ and PA₂₂₄ N–Q mutations were used to infect mice (Ref. 102). The Doherty group has also used reverse genetics to equilibrate expression of D^bNP_{366} and D^bPA_{224} epitopes to examine the role of antigen presentation in establishing immunodominance hierarchies by inserting the aforementioned epitopes within the NA stalk (NA^{NP} and NA^{PA}, respectively). Endogenous expression was disabled using N–Q mutations (Ref. 104) and results suggested that the development of CD8 $^{+}$ T cell responses to a certain MHC class I/epitope complex following primary infection was determined not only by the number of naïve CD8 $^{+}$ T cell precursors available, but also by the type of antigen-presenting cells (APC) expressing the epitope (Ref. 104). The recall response in these experiments was influenced by the number of CD8 $^{+}$ memory T cells present following primary infection and the level of epitope presentation (Ref. 104).

The significance of T cell immunodominance was further investigated through the introduction of the exogenous OVA_{257–264} immunodominant peptide (SIINFEKL; presented by H2K b , K b -OVA₂₅₇) into the NA stalk of PR8 and X31 influenza viruses (PR8-OVA and HK-OVA) using reverse genetics (Ref. 105). The presence of a normally immunodominant exogenous antigen did not extensively alter normal endogenous influenza-specific responses upon infection. The magnitude of the K b -OVA₂₅₇-specific CD8 $^{+}$ T cell response was equivalent to that of the D^bNP_{366} -specific CD8 $^{+}$ T cell population after primary infection (Ref. 105). Two other specificities, D^bPA_{224} and K^bPB1_{703} , were slightly reduced after primary and secondary infections. However, the magnitude of overall CD8 $^{+}$ T cell response was similar in the presence or absence of K b OVA₂₅₇ (Ref. 105). Co-dominance of the

D^bNP_{366} and K b OVA₂₅₇ CD8 $^{+}$ T cell responses was only disrupted when mice were administered virus intraperitoneally, resulting in abortive infection. As a result, the D^bNP_{366} CD8 $^{+}$ T cell response was observed to be immunodominant over the exogenous K b OVA₂₅₇ CD8 $^{+}$ T cell population in a process influenced by viral protein abundance (Ref. 105).

Another factor thought to play a key role in immunodominance is engagement of the MHC-peptide complex with the TCR (Ref. 106). Numerous recombinant X31 viruses containing single amino acid substitutions along the NP_{366–374} peptide, A₁S₂N₃E₄N₅M₆E₇T₈M₉, have been used to study this further (Ref. 106). Analysis of IFN- γ production through intracellular cytokine staining demonstrated that M₆ is critical for TCR recognition of D^bNP_{366} . The methionine to alanine substitution (M6A) was shown not to affect overall virus fitness or NP peptide expression and presentation in mutant viruses. As a result, the D^bNPM6A CD8 $^{+}$ T cell population detected following infection of mice was immunodominant (just as the D^bNP_{366} CD8 $^{+}$ T cell population in wild-type virus) and showed little cross-reactivity, but did display weaker TCR avidity (Ref. 106). This was validated when the crystal structure for both interactions was solved, showing similar binding patterns for NP₃₆₆ and NP₃₆₆M6A to the H2-D b antigen-binding cleft. The lack of CD8 $^{+}$ T cell cross-reactivity was attributed to altered interactions at the binding cleft as a result of the M6A mutation (Ref. 106). These studies have highlighted the fact that pre-immunisation against mutant influenza viruses may in fact elicit new CD8 $^{+}$ T cell memory populations to assist in protection against emerging mutant strains.

The majority of studies dissecting influenza-specific CD8 $^{+}$ T cell immunity using reverse genetics have directly or indirectly involved members of the Doherty laboratory. Other research groups have also taken advantage of this system to generate influenza viruses expressing exogenous T cell epitopes of interests to study pathogen-specific responses and pathogen-pathogen interactions. A recent study using a recombinant PR8 virus expressing the CD8 T cell epitope of *Mycobacterium tuberculosis*, MPT64, demonstrated that co-infection with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) resulted in exacerbated disease and

delayed mycobacterial clearance in lungs influenced perhaps by lower MHC Class I and II expression on DCs and impaired activation of the pathogen-specific CD4 and CD8 T cells (Ref. 107). Further examples include the use of recombinant X31 and PR8 influenza A viruses expressing multiple SIV CD8 T cell epitopes to induce simian immunodeficiency virus (SIV)- and influenza-specific responses in non-human primates or the use of a recombinant WSN influenza virus expressing the gB CD8 T cell epitope of HSV-1 to characterise the expression of CD94/NKG2 receptors on the virus-specific CD8 T cells (Refs 108, 109, 110).

Influenza-specific B cell immunity

Influenza-specific B cell responses are critical for protection following primary and secondary influenza virus infections (Ref. 111). Influenza-specific antibodies are rapidly induced against several influenza viral proteins (Ref. 112). Antibody responses targeting the HA glycoprotein are often stronger and have significant virus neutralising capacity (Ref. 113). In addition to the aforementioned vaccine strategies that aim to elicit influenza virus-specific CD8 T cell responses, cross-reactive neutralising antibodies that can target diverse viral clades are being developed as an alternative treatment option (Ref. 113). Current research on cross-reactive neutralising antibodies has focused on identifying antibodies that target conserved HA stalk domains (Refs 113, 114). Importantly, reverse genetics has been used to alter glycosylation sites on the surface of the HA. Work by Job *et al.* has identified a glycosylation site at Asn136 on the HA of the 2009 Pandemic influenza A (H1N1) virus that was associated with resistance to neutralising antibodies and higher virus titres in mice (Ref. 115). Although reverse genetics has not been extensively used to study influenza-specific B cell immunity or the development of neutralising antibodies, a recombinant WSN influenza virus expressing a immunodominant B cell epitope of the β -amyloid peptide (A β 42) was used to confer protection against A β 42-mediated neurotoxicity, a neuropathological feature of Alzheimer's Disease (Ref. 116). Moreover, novel cross-reactive antibody responses have been generated following insertion of the OVA_{323–339} CD4 T cell epitope (OT-II) into the HA using influenza reverse genetics (Ref. 117). These

studies demonstrate the potential value of using reverse genetics to dissect B cell immunity and antibody production and its use should be investigated further in the future.

Influenza A viruses as vectors for vaccine development

Advances in technology, such as reverse genetics, have enabled the genetic modification of negative-strand RNA viruses to express T cell epitopes and genes. The availability of live-attenuated influenza virus strains, such as influenza A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 cold-adapted viruses, for seasonal influenza vaccination (Refs 118, 119, 120, 121) also provides an established safety profile for future use in humans. Furthermore, reverse genetic techniques have been optimised over the last decade, allowing production of high-titre recombinant viruses (Refs 20, 23, 24).

Influenza viruses have been used as vectors to treat cancer (Refs 122, 123, 124) and infectious diseases, such as AIDS (Refs 125, 126, 127), malaria (Refs 12, 128) and *pseudomonas aeruginosa* (Refs 129, 130). Influenza A virus vectors have been engineered to express a β -galactosidase (β -gal) CD8 $^+$ T cell epitope in the HA or NA protein for the recognition of β -gal expressing tumours (Ref. 122). Mice vaccinated with the recombinant influenza virus expressing the β -gal CD8 $^+$ T cell epitope (MINIGAL, NAGAL and BHAGAL) stimulated specific cytotoxic CD8 $^+$ T cells that targeted tumour cells expressing β -gal, and reduced lung metastases and increased survival was observed in vaccinated mice with established experimental tumours (Refs 122, 123). Regression (>6 months) was also observed in mice with lower initial tumour burdens (Ref. 123).

Development of a safe and effective HIV vaccine remains one of the scientific community's great challenges. High genetic diversity (Refs 131, 132) and a lack of knowledge regarding immune correlates have impeded progress (Ref. 133). A recently completed Phase III trial (RV144) conducted in Thailand tested a combination prime-double boost vaccination regimen using a live recombinant canarypox vector and gp120 protein for boosting (Ref. 134). The trial demonstrated 31% efficacy, suggesting for the first time that a preventive HIV vaccine was achievable using a live virus-based delivery system (Ref. 134).

It is evident that cellular immunity, especially virus-specific CD8⁺ T cells are involved in the control of HIV (Ref. 133). Influenza viruses replicate in respiratory organs, but are capable of inducing mucosal immune responses at distal mucosa, including the urogenital where exposure to HIV occurs (Refs 126, 127). In addition, vaccination usually produces long-lasting, virus-specific humoral responses, such as secretory IgA, which is the major humoral defence mechanism at mucosal surfaces (Ref. 135). Recombinant influenza viruses have been used in mice to stimulate HIV envelope-specific cellular and humoral immunity at mucosal sites (Refs 136, 137). In recent work, recombinant H1N1 or H3N2 influenza virus vectors expressing SIV CD8⁺ T cell epitopes in the NA stalk have been evaluated for their ability to stimulate mucosal T cell immunity and efficacy in a rhesus macaque model (Ref. 13). These viruses were generated using reverse genetics and elicited SIV-specific and influenza specific CD8⁺ T cell responses in animals following a prime-boost protocol via the respiratory tract. Vaccination of SIV-exposed macaques resulted in a significant expansion of SIV-specific CD8⁺ T cell in the peripheral blood (approximately 14%). Despite the fact that SIV challenge of influenza virus-SIV vaccinated resulted in a strong recall of SIV-Gag specific CD8⁺ T cell responses, SIV viraemia and CD4⁺ T cell counts were not altered compared with controls and the vaccination regimen was thought to have contributed to the early establishment of CTL escape mutants. Use of influenza virus vectors expressing multiple HIV antigens to elicit broader HIV-specific CD8⁺ T cell responses may be required to minimise the impact of CTL escape (Ref. 138).

Utilising reverse genetics to dissect the mechanisms of influenza virus drug resistance

Current antiviral drugs that are FDA approved for clinical use against influenza in the USA can be classified into two categories: (i) viral M2 ion channel blockers (the adamantane drugs, amantadine and rimantadine) and (ii) the NA inhibitors (NAIs) (zanamivir (Relenza[®]) and oseltamivir (Tamiflu[®]) (<http://www.cdc.gov>)). The former target the transmembrane domain of the M2 protein and are effective against influenza A viruses (Ref. 139), whereas the latter

bind to the NA catalytic site and are effective for both influenza A and B viruses due to the high degree of conservation of the residues (Ref. 140). Influenza viral RNA polymerases have poor proof-reading ability that results in high mutation rates during replication. Therefore, antiviral resistance can happen spontaneously or under selection pressure following antiviral therapy (Ref. 141). As such, widespread resistance to adamantanes and/or oseltamivir has been documented in circulating influenza A H3N2 and seasonal and pandemic H1N1 subtypes (Ref. 142). In addition, the novel H7N9 virus that recently caused an outbreak in China has been found to contain mutations that can confer resistance to both classes of antiviral agents (Ref. 143). These events highlight the urgent need to acquire a better understanding of viral resistance patterns and the characteristics of the resistant strains. Reverse genetics systems have been extensively used to generate or recapitulate mutations identified in a clinical setting (Refs 144, 145, 146). The molecular mechanism for amantadine-resistance is relatively straightforward and has been characterised as a single mutation at S31N in the transmembrane domain of the M2 gene (Refs 144, 147). Other M2 mutations (L26F, V27A, A30T, G34E) that have been associated with resistance to amantadine were also validated using recombinant WSN H1N1 viruses generated with reverse genetics (Ref. 144). NAI resistance is far more complex. NA mutations are found to be subtype-specific and be induced *in vitro* using different NAIs or observed clinically following treatment of infected individuals (Refs 148, 149). The H274Y mutation conferring resistance is frequently described for the H1N1 subtype (Refs 148, 149, 150), while R292K is commonly observed in the N2 and N9 subtypes (Refs 142, 151). Unlike the M2 mutations that maintain viral fitness and infectivity, NA mutations, such as H274Y, E119G, E119V, N294S, of the N1 and N2 subtypes show lower levels of viral fitness and virulence as demonstrated *in vitro* in cell culture or in mice infected with reverse genetics-generated recombinant WSN viruses (Refs 145, 146). Moreover, subsequent studies have shown that the impaired viral fitness of an E119V oseltamivir-resistant H3N2 influenza A virus recovered from an immunocompromised patient can be partially restored with the addition of a I222V mutation using reverse genetics

(Ref. 152). The compensatory effects of the I222 V mutation were further validated through the generation of a recombinant pandemic H1N1 virus (A/Québec/144147/09), demonstrating that the I222 V mutation can also restore the fitness of the H274Y mutant and promote its resistance to both oseltamivir and peramivir (Ref. 146). It is important to note that a single I222 V mutation alone only confers minor resistance to oseltamivir (Ref. 146). Knowledge of antiviral drug-resistant phenotypes is important for monitoring the emergence and spread of resistance and forms an integral part of pandemic planning.

Future perspectives

The ability to generate and manipulate live recombinant influenza viruses using reverse genetics systems continues to provide insights into the mechanisms involved in virus infection on host immunity. One example is a recent study in which a GFP reporter gene was expressed from the NS segment of PR8 (NS1-GFP virus) that has enabled localisation and replication studies of the virus *in vivo* (Ref. 153). The recombinant NS1-GFP influenza virus is pathogenic in mice and capable of replicating, although less virulent than unmanipulated wild-type PR8. This valuable tool can now be used to study movement of virus through tissue following infection (Refs 54, 153, 154) and cell populations infected by GFP-tagged viruses can be isolated by flow cytometry.

Since the first human infection of HPAI H5N1 influenza virus in 1997 (Ref. 155), 648 laboratory confirmed human cases have been identified with a mortality rate of nearly 60% (http://www.who.int/influenza/human_animal_interface). To date, zoonotic transmission of HPAI H5N1 has been largely associated with close contact with infected poultry. Given the high mutation rates of influenza viruses, it remains a significant concern that HPAI H5N1 viruses could acquire the ability to transmit between humans. In recent years, scientists have been focusing on identifying factors that influence the transmissibility of HPAI H5N1 viruses. Two independent groups recently generated H5N1 mutant viruses using reverse genetics that were readily transmitted via airborne particles in ferrets (Ref. 156), and identified key amino acid residues that facilitate transmission of H5N1 in mammals (Refs 157, 158). Knowledge of

these residues is important for surveillance and implementation of pandemic prevention strategies.

Some of the major issues facing clinicians and scientists in the influenza field include (but are not limited to); emergence of novel epidemic or pandemic virus strains; virus resistance to multiple anti-viral drugs; improvement of vaccine production (including use of enhanced cell lines that improve rescue of viruses or inhibit antiviral immunity) (Refs 159, 160); development of universal vaccines (T cell or antibody-based); improvement of vaccine efficacy in the elderly; and finally, ethical concerns involving the use of reverse genetics to generate potentially pandemic viruses with so called 'dual use' potential (Refs 161, 162).

Reverse genetics has played a critical role in improving our understanding of influenza virus pathogenesis and immunity and will continue to do so in the future. This was clearly evidenced recently through the rapid identification and characterisation of the circulating H7N9 virus subtype in China (Refs 163, 164) and will certainly help answer questions arising from the identification of novel virus strains such as the H17N10 virus isolated from bats in Guatemala (Refs 165, 166). New and exciting technological advances in influenza reverse genetics, such as the expression of miRNAs, offers scientists opportunities to regulate virus replication and host immune responses and has implications in areas such as vaccine production (Ref. 167). In conclusion, our ability to reconstruct and manipulate the influenza virus genome using reverse genetics and the knowledge gained will undoubtedly lead to improved therapeutics and vaccines in the future.

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Conflicts of interest

None of the authors have any financial interests in the subject matter of this review.

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