expert reviews
in molecular medicine

Influenza reverse genetics: dissecting immunity and pathogenesis

Siying Ye[1,2,*], Justin G. Evans[1,2] and John Stambas[1,2]

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, on-going 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

[1]School of Medicine, Deakin University, Waurn Ponds, VIC 3217, Australia

[2]AAHL CSIRO Deakin Collaborative (ACDC) Biosecurity laboratory, Australian Animal Health Laboratory, CSIRO, East Geelong, VIC 3219, Australia

*Corresponding author: Siying Ye, AAHL CSIRO Deakin Collaborative (ACDC) Biosecurity Laboratory, Australian Animal Health Laboratory (AAHL), CSIRO, 5 Portarlington Road, East Geelong, VIC 3219, Australia. E-mail: siying.ye@deakin.edu.au

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

(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

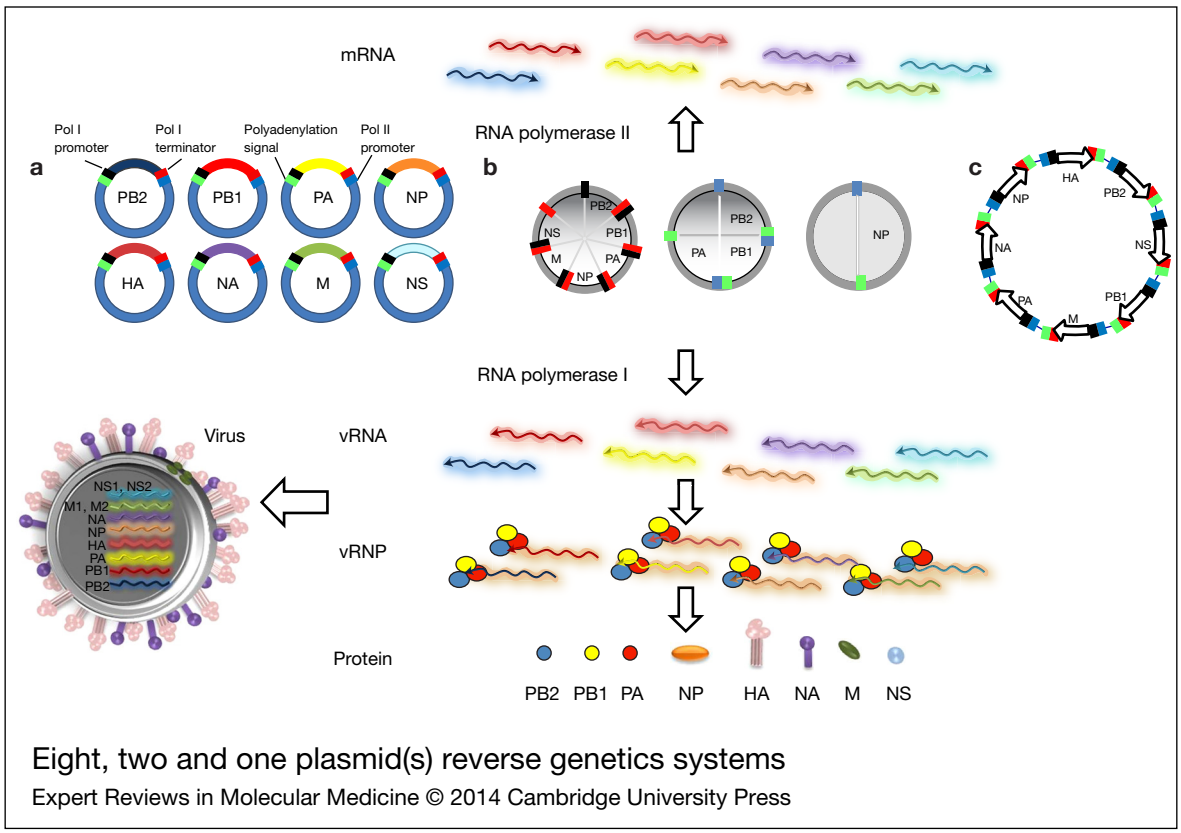
Reverse genetics is the process of generating live viruses entirely from cloned cDNA (Ref. 10). This approach has simplified the manipulation of the virus genome, allowing not only the introduction of point mutations, but also the generation of reassortant viruses through the replacement of virus gene 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

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press



Eight, two and one plasmid(s) reverse genetics systems

Expert Reviews in Molecular Medicine © 2014 Cambridge University Press

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 promotor–teminater 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,

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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-α2,3-galactose (SAα2,3-Gal), which is abundant on avian intestinal epithelia (Ref. 26). Human-adapted influenza viruses bind to SAα2,6-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 SAα2,6-Gal receptor to avian SAα2,3-Gal receptor (Ref. 33). Other studies have shown that for H1 viruses, E190D and D225 G substitutions facilitate preferential binding to the human SAα2,6-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

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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).

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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–Q_7–R_6–R_5–R_4–K_3–K_2–R_1) 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 SAα2,6Gal when compared with avian SAα2,3Gal (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 SAα2,6Gal 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

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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 immundominant 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^bNP_{366} and D^bPA_{224}, 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^bNP_{366}-specific response is clearly immunodominant (increased by at least 14-fold in the spleen) when compared with the D^bPA_{224} 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^bNP_{366} and

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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 NP_{366} and PA_{224} 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-OVA_{257}) 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-OVA_{257}-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^bOVA_{257} (Ref. 105). Co-dominance of the D^bNP_{366} and K^bOVA_{257} $CD8^+$ 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^bOVA_{257} $CD8^+$ 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, $A_1S_2N_3E_4N_5M_6E_7T_8M_9$, have been used to study this further (Ref. 106). Analysis of IFN-γ production through intracellular cytokine staining demonstrated that M_6 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 NP_{366} and NP_{366}M6A 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

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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 neutraliszing 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 (Aβ42) was used to confer protection against Aβ42-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).

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

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 adamantine 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, A30 T, 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 R292 K 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, E119 V, 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 E119 V oseltamivir-resistant H3N2 influenza A virus recovered from an immunocompromised patient can be partially restored with the addition of a I222 V mutation using reverse genetics

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

(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.

Acknowledgment and financial support

This work is supported by Alfred Deakin Postdoctoral Research Fellowship, Deakin University (S.Y.).

Conflicts of interest

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

References

1 WHO (2009) Influenza Seasonal Fact Sheet, No. 211. WHO Media Centre
2 Mao, L., Yang, Y. and Qiu, Y. (2012) Annual economic impacts of seasonal influenza on US

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

counties: spatial heterogeneity and patterns. International Journal of Health Geographics 11, 16
3 Webster, R.G. et al. (1992) Evolution and ecology of influenza A viruses. Microbiological Review 56, 152-179
4 Jagger, B.W. et al. (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337, 199-204
5 Bruns, K. et al. (2007) Structural characterization and oligomerization of PB1-F2, a proapoptotic influenza A virus protein. Journal of Biological Chemistry 282, 353-363
6 Portela, A. and Digard, P. (2002) The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. Journal of General Virology 83(Pt 4), 723-734
7 Martin, K. and Helenius, A. (1991) Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell 67, 117-130
8 Takeda, M. et al. (2002) Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture. Journal of Virology 76, 1391-1399
9 Garcia-Sastre, A. et al. (1998) Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252, 324-330
10 Neumann, G., Whitt, M.A. and Kawaoka, Y. (2002) A decade after the generation of a negative-sense RNA virus from cloned cDNA – what have we learned? Journal of General Virology 83(Pt 11), 2635-2662
11 Webby, R.J. et al. (2004) Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 363, 1099-1103
12 Li, S. et al. (1993) Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. Proceedings of the National Academy of Sciences USA 90, 5214-5218
13 Sexton, A. et al. (2009) Evaluation of recombinant influenza virus-simian immunodeficiency virus vaccines in macaques. Journal of Virology 83, 7619-7628
14 Taniguchi, T., Palmieri, M. and Weissmann, C. (1978) QB DNA-containing hybrid plasmids giving rise to QB phage formation in the bacterial host. Nature 274, 223-228
15 Palese, P. et al. (1996) Negative-strand RNA viruses: genetic engineering and applications. Proceedings of the National Academy of Sciences USA 93, 11354-11358
16 Parvin, J.D. et al. (1989) Promoter analysis of influenza virus RNA polymerase. Journal of Virology 63, 5142-5152
17 Luytjes, W. et al. (1989) Amplification, expression, and packaging of foreign gene by influenza virus. Cell 59, 1107-1113
18 Schnell, M.J., Mebatsion, T. and Conzelmann, K.K. (1994) Infectious rabies viruses from cloned cDNA. EMBO Journal 13, 4195-4203
19 Bridgen, A. and Elliott, R.M. (1996) Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proceedings of the National Academy of Sciences USA 93, 15400-15404
20 Neumann, G. et al. (1999) Generation of influenza A viruses entirely from cloned cDNAs. Proceedings of the National Academy of Sciences USA 96, 9345-9350
21 Zobel, A., Neumann, G. and Hobom, G. (1993) RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Research 21, 3607-3614
22 Hoffmann, E. et al. (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proceedings of the National Academy of Sciences USA 97, 6108-6113
23 Neumann, G. et al. (2005) An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proceedings of the National Academy of Sciences USA 102, 16825-16829
24 Zhang, X. et al. (2009) A one-plasmid system to generate influenza virus in cultured chicken cells for potential use in influenza vaccine. Journal of Virology 83, 9296-9303
25 Skehel, J.J. and Wiley, D.C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review in Biochemistry 69, 531-569
26 Connor, R.J. et al. (1994) Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205, 17-23
27 Glaser, L. et al. (2007) Effective replication of human influenza viruses in mice lacking a major alpha2,6 sialyltransferase. Virus Research 126, 9-18
28 Oshansky, C.M. et al. (2011) Avian influenza viruses infect primary human bronchial epithelial cells unconstrained by sialic acid alpha2,3 residues. PLoS One 6, e21183. doi:10.1371/journal.pone.0021183
29 Wilson, I.A. and Cox, N.J. (1990) Structural basis of immune recognition of influenza virus hemagglutinin. Annual Review of Immunology 8, 737-771
30 Schweiger, B., Zadow, I. and Heckler, R. (2002) Antigenic drift and variability of influenza viruses.

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

Medical Microbiology and Immunology 191, 133-138
31 Colman, P.M. et al. (1989) Three-dimensional structures of influenza virus neuraminidase-antibody complexes. Philosophical Transactions of the Royal Soceity of London B – Biological Sciences 323, 511-518
32 Reid, A.H. et al. (1999) Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proceedings of the National Academy of Sciences USA 96, 1651-1656
33 Matrosovich, M. et al. (2000) Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. Journal of Virology 74, 8502-8512
34 Korsman, K. (2006) Vaccines. Influenza Report. Flying Publisher, Paris, France
35 Fleming, D.M. (2010) Influenza and influenza vaccines – the need for further developments. European Infectious Disease 4(Suppl. 1), 2-6
36 Cox, R.J., Brokstad, K.A. and Ogra, P. (2004) Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scandinavian Journal of Immunology 59, 1-15
37 Govaert, T.M. et al. (1994) The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. Journal of American Medical Association 272, 1661-1665
38 Corrigan, E.M. and Clancy, R.L. (1999) Is there a role for a mucosal influenza vaccine in the elderly? Drugs Aging 15, 169-181
39 MedImmune Vaccines (2003) Influenza virus vaccine live intranasal – MedImmune vaccines: CAIV-T, influenza vaccine live intranasal. In Drugs R & D (2003/09/04 edn), pp. 312-319
40 Fiore, A.E., Bridges, C.B. and Cox, N.J. (2009) Seasonal influenza vaccines. Current Topics in Microbiology and Immunology 333, 43-82
41 Carter, N.J. and Curran, M.P. (2011) Live attenuated influenza vaccine (FluMist(R); Fluenz): a review of its use in the prevention of seasonal influenza in children and adults. Drugs 71, 1591-1622
42 Doherty, P.C. and Kelso, A. (2008) Toward a broadly protective influenza vaccine. Journal of Clinical Investigation 118, 3273-3275
43 Du, L., Zhou, Y. and Jiang, S. (2010) Research and development of universal influenza vaccines. Microbes and Infection 12, 280-286
44 Lee, L.Y. et al. (2008) Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. Journal of Clinical Investigation 118, 3478-3490
45 Thomas, P.G. et al. (2006) Cell-mediated protection in influenza infection. Emerging Infectious Diseases 12, 48-54
46 Goodman, A.G. et al. (2011) A human multi-epitope recombinant vaccinia virus as a universal T cell vaccine candidate against influenza virus. PLoS One 6, e25938. doi:10.1371/journal.pone.0025938
47 Booy, R. et al. (2006) Pandemic vaccines: promises and pitfalls. Medical Journal of Australia 185(10 Suppl), S62-S65
48 Subbarao, K. et al. (2003) Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 305, 192-200
49 Hatta, M. et al. (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293, 1840-1842
50 Watanabe, T. et al. (2009) Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. Proceedings of the National Academy of Sciences USA 106, 588-592
51 Taubenberger, J.K. et al. (2005) Characterization of the 1918 influenza virus polymerase genes. Nature 437, 889-893
52 Subbarao, E.K., London, W. and Murphy, B.R. (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal of Virology 67, 1761-1764
53 Gabriel, G. et al. (2005) The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proceedings of the National Academy of Sciences USA 102, 18590-18595
54 Shinya, K. et al. (2004) Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine. Journal of Virology 78, 3083-3088
55 Li, Z. et al. (2005) Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. Journal of Virology 79, 12058-12064
56 de Jong, M.D. et al. (2006) Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nature Medicine 12, 1203-1207
57 Detjen, B.M. et al. (1987) The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. Journal of Virology 61, 16-22
58 Pappas, C. et al. (2008) Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

virus. Proceedings of the National Academy of Sciences USA 105, 3064-3069
59 Chen, W. et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nature Medicine 7, 1306-1312
60 Mazur, I. et al. (2008) The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cellular Microbiology 10, 1140-1152
61 McAuley, J.L. et al. (2007) Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host and Microbe 2, 240-249
62 Conenello, G.M. et al. (2007) A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathogens 3, 1414-1421
63 de la Luna, S. et al. (1995) Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. Journal of Virology 69, 2427-2433
64 Ehrhardt, C. et al. (2007) Influenza A virus NS1 protein activates the PI3 K/Akt pathway to mediate antiapoptotic signaling responses. Journal of Virology 81, 3058-3067
65 Donelan, N.R., Basler, C.F. and Garcia-Sastre, A. (2003) A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. Journal of Virology 77, 13257-13266
66 Guo, Z. et al. (2007) NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. American Journal of Respiratory Cell and Molecular Biology 36, 263-269
67 Mibayashi, M. et al. (2007) Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. Journal of Virology 81, 514-524
68 Opitz, B. et al. (2007) IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cellular Microbiology 9, 930-938
69 Taubenberger, J.K. (2005) The virulence of the 1918 pandemic influenza virus: unraveling the enigma. Archives of Virology, Supplement (19), 101-115
70 Egorov, A. et al. (1998) Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. Journal of Virology 72, 6437-6441
71 Guan, Y. et al. (2002) Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proceedings of the National Academy of Sciences USA 99, 8950-8955
72 Seo, S.H., Hoffmann, E. and Webster, R.G. (2002) Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nature Medicine 8, 950-954
73 Obenauer, J.C. et al. (2006) Large-scale sequence analysis of avian influenza isolates. Science 311, 1576-1580
74 Sheng, M. and Sala, C. (2001) PDZ domains and the organization of supramolecular complexes. Annual Review in Neuroscience 24, 1-29
75 Jackson, D. et al. (2008) A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proceedings of the National Academy of Sciences USA 105, 4381-4386
76 Wang, W. et al. (1999) RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA 5, 195-205
77 Min, J.Y. et al. (2007) A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology 363, 236-243
78 Hale, B.G. et al. (2008) The multifunctional NS1 protein of influenza A viruses. Journal of General Virology 89(Pt 10), 2359-2376
79 Ayllon, J. et al. (2012) Contribution of NS1 effector domain dimerization to Influenza A virus replication and virulence. Journal of Virology 86, 13095-13098
80 Bottcher, E. et al. (2006) Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. Journal of Virology 80, 9896-9898
81 Horimoto, T. and Kawaoka, Y. (1994) Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. Journal of Virology 68, 3120-3128
82 Wang, M. et al. (2012) Residue Y161 of influenza virus hemagglutinin is involved in viral recognition of sialylated complexes from different hosts. Journal of Virology 86, 4455-4462
83 Glaser, L. et al. (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. Journal of Virology 79, 11533-11536
84 van Doremalen, N. et al. (2011) A single amino acid in the HA of pH1N1 2009 influenza virus affects cell tropism in human airway epithelium, but not transmission in ferrets. PLoS One 6, e25755. doi:10.1371/journal.pone.0025755

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

85 de Vries, R.P. et al. (2011) Only two residues are responsible for the dramatic difference in receptor binding between swine and new pandemic H1 hemagglutinin. Journal of Biological Chemistry 286, 5868-5875
86 Schmolke, M. and Garcia-Sastre, A. (2010) Evasion of innate and adaptive immune responses by influenza A virus. Cellular Microbiology 12, 873-880
87 Takeuchi, O. and Akira, S. (2010) Pattern recognition receptors and inflammation. Cell 140, 805-820
88 Le Goffic, R. et al. (2007) Cutting Edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. Journal of Immunology 178, 3368-3372
89 Goodbourn, S., Didcock, L. and Randall, R.E. (2000) Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. Journal of General Virology 81(Pt 10), 2341-2364
90 Lopez, C.B. et al. (2003) Type I interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells induced by negative-strand RNA viruses. Journal of Infectious Diseases 187, 1126-1136
91 Fernandez-Sesma, A. et al. (2006) Influenza virus evades innate and adaptive immunity via the NS1 protein. Journal of Virology 80, 6295-6304
92 Haye, K. et al. (2009) The NS1 protein of a human influenza virus inhibits type I interferon production and the induction of antiviral responses in primary human dendritic and respiratory epithelial cells. Journal of Virology 83, 6849-6862
93 Davis, M.M. et al. (1998) Ligand recognition by alpha beta T cell receptors. Annual Review of Immunology 16, 523-544
94 Boon, A.C. et al. (2002) Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. Journal of Virology 76, 2567-2572
95 Goulder, P.J. et al. (2001) Evolution and transmission of stable CTL escape mutations in HIV infection. Nature 412, 334-338
96 Goulder, P.J. et al. (1997) Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nature Medicine 3, 212-217
97 Yewdell, J.W. and Bennink, J.R. (1999) Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annual Review of Immunology 17, 51-88
98 Townsend, A.R. et al. (1986) The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 44, 959-968
99 Belz, G.T. et al. (2000) A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. Journal of Virology 74, 3486-3493
100 Sigal, L.J. and Wylie, D.E. (1996) Role of non-anchor residues of Db-restricted peptides in class I binding and TCR triggering. Molecular Immunology 33, 1323-1333
101 Price, G.E. et al. (2000) Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. Journal of Experimental Medicine 191, 1853-1867
102 Webby, R.J. et al. (2003) Protection and compensation in the influenza virus-specific CD8+ T cell response. Proceedings of the National Academy of Sciences USA100, 7235-7240
103 Andreansky, S.S. et al. (2005) Consequences of immunodominant epitope deletion for minor influenza virus-specific CD8 + -T-cell responses. Journal of Virology 79, 4329-4339
104 La Gruta, N.L. et al. (2006) A virus-specific CD8+ T cell immunodominance hierarchy determined by antigen dose and precursor frequencies. Proceedings of the National Academy of Sciences USA 103, 994-999
105 Jenkins, M.R. et al. (2006) Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting. Journal of Immunology 177, 2917-2925
106 Kedzierska, K. et al. (2008) Complete modification of TCR specificity and repertoire selection does not perturb a CD8+ T cell immunodominance hierarchy. Proceedings of the National Academy of Sciences USA 105, 19408-19413
107 Florido, M. et al. (2013) Influenza A virus infection impairs mycobacteria-specific T cell responses and mycobacterial clearance in the lung during pulmonary coinfection. Journal of Immunology 191, 302-311
108 Jegaskanda, S. et al. (2012) Comparison of influenza and SIV specific CD8 T cell responses in macaques. PLoS One 7, e32431. doi:10.1371/journal.pone.0032431
109 Wojtasiak, M. et al. (2004) Persistent expression of CD94/NKG2 receptors by virus-specific CD8 T cells is initiated by TCR-mediated signals. International Immunology 16, 1333-1341

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

110 Reece, J.C. et al. (2010) Timing of immune escape linked to success or failure of vaccination. PLoS One 5, e12774. doi:10.1371/journal.pone.0012774
111 Gerhard, W. et al. (1997) Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunological Reviews 159, 95-103
112 Sealy, R. et al. (2003) Antibody response to influenza infection of mice: different patterns for glycoprotein and nucleocapsid antigens. Immunology 108, 431-439
113 Laursen, N.S. and Wilson, I.A. (2013) Broadly neutralizing antibodies against influenza viruses. Antiviral Res 98, 476-483
114 Kang, S.M., Song, J.M. and Compans, R.W. (2011) Novel vaccines against influenza viruses. Virus Research 162, 31-38
115 Job, E.R. et al. (2013) Addition of glycosylation to influenza A virus hemagglutinin modulates antibody-mediated recognition of H1N1 2009 pandemic viruses. Journal of Immunology 190, 2169-2177
116 Davtyan, H. et al. (2011) The immunological potency and therapeutic potential of a prototype dual vaccine against influenza and Alzheimer's disease. Journal of Translational Medicine 9, 127
117 Thomas, P.G. et al. (2006) An unexpected antibody response to an engineered influenza virus modifies CD8+ T cell responses. Proceedings of the National Academy of Sciences USA 103, 2764-2769
118 Snyder, M.H. et al. (1985) Attenuation of wild-type human influenza A virus by acquisition of the PA polymerase and matrix protein genes of influenza A/Ann Arbor/6/60 cold-adapted donor virus. Journal of Clinical Microbiology 22, 719-725
119 Maassab, H.F. and DeBorde, D.C. (1985) Development and characterization of cold-adapted viruses for use as live virus vaccines. Vaccine 3, 355-369
120 Snyder, M.H. et al. (1988) Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. Journal of Virology 62, 488-495
121 Donabedian, A.M. et al. (1988) A mutation in the PA protein gene of cold-adapted B/Ann Arbor/1/66 influenza virus associated with reversion of temperature sensitivity and attenuated virulence. Virology 163, 444-451
122 Restifo, N.P. et al. (1998) Transfectant influenza A viruses are effective recombinant immunogens in the treatment of experimental cancer. Virology 249, 89-97
123 Zheng, H., Palese, P. and Garcia-Sastre, A. (2000) Antitumor properties of influenza virus vectors. Cancer Research 60, 6972-6976
124 Strobel, I. et al. (2000) Efficient expression of the tumor-associated antigen MAGE-3 in human dendritic cells, using an avian influenza virus vector. Human Gene Therapy 11, 2207-2218
125 Li, S. et al. (1993) Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. Journal of Virology 67, 6659-6666
126 Ferko, B. et al. (1998) Chimeric influenza virus replicating predominantly in the murine upper respiratory tract induces local immune responses against human immunodeficiency virus type 1 in the genital tract. Journal of Infectious Diseases 178, 1359-1368
127 Muster, T. et al. (1995) Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. Journal of Virology 69, 6678-6686
128 Gonzalez-Aseguinolaza, G. et al. (2003) Induction of protective immunity against malaria by priming-boosting immunization with recombinant cold-adapted influenza and modified vaccinia Ankara viruses expressing a CD8 + -T-cell epitope derived from the circumsporozoite protein of Plasmodium yoelii. Journal of Virology 77, 11859-11866
129 Gilleland, H.E., Jr. et al. (1997) Chimeric influenza viruses incorporating epitopes of outer membrane protein F as a vaccine against pulmonary infection with Pseudomonas aeruginosa. Behring Institute of Mitteilungen 98, 291-301
130 Staczek, J. et al. (1998) A chimeric influenza virus expressing an epitope of outer membrane protein F of *Pseudomonas aeruginosa* affords protection against challenge with *P. aeruginosa* in a murine model of chronic pulmonary infection. Infection and Immunity 66, 3990-3994
131 McCutchan, F.E. et al. (2000) Diversity of envelope glycoprotein from human immunodeficiency virus type 1 of recent seroconverters in Thailand. AIDS Research and Human Retroviruses 16, 801-805
132 Korber, B. et al. (2001) Evolutionary and immunological implications of contemporary HIV-1 variation. British Medical Bulletin 58, 19-42
133 Perrin, H. et al. (2010) New approaches to design HIV-1 T-cell vaccines. Current Opinion in HIV and AIDS 5, 368-376
134 Rerks-Ngarm, S. et al. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. New England Journal of Medicine 361, 2209-2220

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

135 Tang, X. and Chen, Z. (2010) The development of an AIDS mucosal vaccine. Viruses 2, 283-297
136 Gherardi, M.M. et al. (2003) Prime-boost immunization schedules based on influenza virus and vaccinia virus vectors potentiate cellular immune responses against human immunodeficiency virus Env protein systemically and in the genitorectal draining lymph nodes. Journal of Virology 77, 7048-7057
137 Nakaya, Y. et al. (2004) Induction of cellular immune responses to simian immunodeficiency virus gag by two recombinant negative-strand RNA virus vectors. Journal of Virology 78, 9366-9375
138 Goulder, P.J. and Watkins, D.I. (2004) HIV and SIV CTL escape: implications for vaccine design. Nature Reviews Immunology 4, 630-640
139 Pielak, R.M., Schnell, J.R. and Chou, J.J. (2009) Mechanism of drug inhibition and drug resistance of influenza A M2 channel. Proceedings of the National Academy of Sciences USA 106, 7379-7384
140 Itzstein, J.C.D.a.M.v. (2001) Anti-influenza virus drug design: sialidase inhibitors. Australian Journal of Chemistry 54, 663-670
141 Hayden, F.G. and de Jong, M.D. (2011) Emerging influenza antiviral resistance threats. Journal of Infectious Diseases 203, 6-10
142 Whitley, R.J. et al. (2013) Global assessment of resistance to neuraminidase inhibitors, 2008–2011: the Influenza Resistance Information Study (IRIS). Clinical Infectious Disease 56, 1197-1205
143 Yen, H.L. et al. (2013) Resistance to neuraminidase inhibitors conferred by an R292 K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. MBio 4, e00396-13. doi: 10.1128/mBio.00396-13
144 Abed, Y., Goyette, N. and Boivin, G. (2005) Generation and characterization of recombinant influenza A (H1N1) viruses harboring amantadine resistance mutations. Antimicrobial Agents and Chemotherapy 49, 556-559
145 Abed, Y., Baz, M. and Boivin, G. (2006) Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. Antiviral Therapy 11, 971-976
146 Pizzorno, A. et al. (2011) Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors. Journal of Infectious Diseases 203, 25-31
147 Nelson, M.I. et al. (2009) The origin and global emergence of adamantane resistant A/H3N2 influenza viruses. Virology 388, 270-278
148 Gubareva, L.V. et al. (2001) Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. Journal of Infectious Diseases 183, 523-531
149 Wang, M.Z., Tai, C.Y. and Mendel, D.B. (2002) Mechanism by which mutations at his274 alter sensitivity of influenza a virus n1 neuraminidase to oseltamivir carboxylate and zanamivir. Antimicrobial Agents and Chemotherapy 46, 3809-3816
150 Gubareva, L.V. et al. (2010) Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. Antiviral Therapy 15, 1151-1159
151 Gubareva, L.V. et al. (1996) Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. Journal of Virology 70, 1818-1827
152 Simon, P. et al. (2011) The I222 V neuraminidase mutation has a compensatory role in replication of an oseltamivir-resistant influenza virus A/H3N2 E119 V mutant. Journal of Clinical Microbiology 49, 715-717
153 Manicassamy, B. et al. (2010) Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proceedings of the National Academy of Sciences USA 107, 11531-11536
154 Kittel, C. et al. (2004) Rescue of influenza virus expressing GFP from the NS1 reading frame. Virology 324, 67-73
155 Ku, A.S. and Chan, L.T. (1999) The first case of H5N1 avian influenza infection in a human with complications of adult respiratory distress syndrome and Reye's syndrome. Journal of Paediatrics and Child Health 35, 207-209
156 Fouchier, R.A., Garcia-Sastre, A. and Kawaoka, Y. (2012) Pause on avian flu transmission studies. Nature 481, 443
157 Herfst, S. et al. (2012) Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336, 1534-1541
158 Imai, M. et al. (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486, 420-428
159 Song, M.S. et al. (2013) Establishment of Vero cell RNA polymerase I-driven reverse genetics for Influenza A virus and its application for pandemic (H1N1) 2009 influenza virus vaccine production. Journal of General Virology 94(Pt 6), 1230-1235
160 Hamamoto, I. et al. (2013) High yield production of influenza virus in Madin Darby canine kidney

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press

(MDCK) cells with stable knockdown of IRF7. PLoS One 8, e59892. doi: 10.1371/journal.pone.0059892
161 Fouchier, R.A. et al. (2012) Pause on avian flu transmission research. Science 335, 400-401
162 Fouchier, R.A. et al. (2013) Transmission studies resume for avian flu. Science 339, 520-521
163 Gao, R. et al. (2013) Human infection with a novel avian-origin influenza A (H7N9) virus. New England Journal of Medicine 368, 1888-1897
164 Watanabe, T. et al. (2013) Characterization of H7N9 influenza A viruses isolated from humans. Nature 501, 551-555
165 Tong, S. et al. (2012) A distinct lineage of influenza A virus from bats. Proceedings of the National Academy of Sciences USA 109, 4269-4274
166 Garcia-Sastre, A. (2012) The neuraminidase of bat influenza viruses is not a neuraminidase. Proceedings of the National Academy of Sciences USA 109, 18635-18636
167 Schmid, S., Zony, L.C. and Tenoever, B.R. (2013) A versatile RNA vector for delivery of coding and non-coding RNAs. Journal of Virology 88, 2333-2336

Features associated with this article

Figure
Figure 1. Eight, two and one plasmid(s) reverse genetics systems.

Citation details for this article

Siying Ye, Justin G. Evans and John Stambas (2014) Influenza reverse genetics: dissecting immunity and pathogenesis. Expert Rev. Mol. Med. Vol. 16, e2, February 2014, doi:10.1017/erm.2014.4

Accession information: doi:10.1017/erm.2014.4; Vol. 16; e2; February 2014
© Cambridge University Press 2014

https://doi.org/10.1017/erm.2014.4 Published online by Cambridge University Press