Opinion

Advances in Influenza Virus Research: A Personal Perspective

Kanta Subbarao

1 WHO Collaborating Centre for Reference and Research on Influenza, The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC 3000, Australia; kanta.subbarao@influenzacentre.org; Tel.: +61-03-9342-9300

2 Department of Microbiology and Immunology, The University of Melbourne, The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC 3000, Australia; kanta.subbarao@unimelb.edu.au

Received: 30 November 2018; Accepted: 17 December 2018; Published: 18 December 2018

Abstract: Technical advances in the last decade have made it possible to investigate influenza virus infection from the cellular and subcellular level to intact animals and humans. As a result, we have gained a new understanding of the virus and disease.

Keywords: influenza; technical advances

1. Introduction

Influenza viruses and the disease that bears the same name have fascinated researchers for decades. While some researchers are interested in the clinical illness, disease severity and its complications, others focus on virus biology, specific viral proteins, and virus-host interactions whereas some immunologists have used influenza viruses and proteins as model antigens in basic research. Technical advances in the last decade, many within the last few years, have made it possible to investigate influenza virus infection from the cellular and subcellular level to intact animals and humans. As a result, we have gained a new understanding of the virus and disease; some dogma has been questioned or even overturned and, in some instances, investigators have re-discovered what was previously known. I will present my own perspective on advances that have occurred during my career, with the caveat that this is not a comprehensive or thorough assessment of progress in the field as a whole.

2. Ten Remarkable Advances

1. The technique of reverse genetics, the generation of an infectious influenza virus starting from the sequence of the virion RNA, is a very powerful technique that has changed our ability to study the biology of influenza viruses, down to the level of individual amino acid residues in specific viral proteins and has certainly changed how vaccines are or can be made. The technology evolved over a few years from the ability to rescue (or recover) one cloned viral gene segment using a helper virus to supply the remaining seven gene segments [1], to plasmid-based reverse genetics that did not require a helper virus but required co-transfection of 12 to 17 plasmids [2,3], and then to an optimized system of using eight bi-directional plasmids that can be transcribed to produce viral and messenger RNA [4]. Reverse genetics is now widely applied in all aspects of influenza research (see below).

2. The 1918 influenza pandemic was the largest single infectious disease event of the 20th century, causing an estimated 50 million deaths worldwide. For decades, it was assumed that the reason that the 1918 pandemic was so severe that it was caused by a particularly virulent influenza A virus. The painstaking work to recover and sequence short overlapping RNA fragments that covered the entire influenza virus genome from paraffin-embedded formalin-fixed tissue blocks from soldiers who had died during the 1918 pandemic, confirmed by recovery of similar sequence data from the body of...
a fatal case of influenza that was buried in the permafrost in Alaska, revealed that the 1918 influenza A virus did not contain molecular signatures associated with highly virulent influenza viruses, such as a highly cleavable hemagglutinin protein [5,6]. The 1918 influenza virus was reconstructed by reverse genetics techniques and studied in animal models including mice, ferrets and non-human primates [7–10]. These studies revealed that the virus elicits an exaggerated and aberrant inflammatory response that is not controlled by the innate immune system [7,8]. Bacterial co-infection was also a major factor in the mortality associated with the 1918 influenza pandemic [11]. Secondary bacterial infection following influenza and its contribution to severe disease is an active topic of research.

3. For many years it was believed that animal influenza viruses were restricted in their ability to directly infect humans. This was based on the fact that (i) the 1957 and 1968 pandemic viruses were reassortant viruses that derived two or three gene segments from an avian influenza virus and the remaining gene segments from a previously circulating human influenza virus [12,13] and (ii) avian influenza A viruses administered to humans during experimental infection studies failed to infect or cause severe illness [14]. Several events over the last 20 years have changed this perception: first, the reconstruction of the genome of the 1918 virus indicates that the virus was a wholly avian influenza virus [15] and second, direct infection of humans by avian influenza viruses of different subtypes including H5, H6, H7, H9 and H10 have been identified, associated with illness ranging from mild (H9) to severe sporadic infections (H6 and H10) and severe illness in hundreds of sporadic cases (H5, H7) [16–21].

4. The identification of influenza infections at the animal human interface since 1997, caused by many different influenza A subtypes, has led to closer collaboration and communication between human and animal public health researchers, surveillance in wild birds and poultry and greater recognition of the ‘One Health’ vision [22].

5. As a result of advances and access to molecular methods and international sharing of genetic sequence data, the sequences of H7N9 avian influenza viruses that caused human and poultry infections in China have been available for analysis by researchers around the world.

6. Advances in antiviral drug development: Amantadine and its derivative Rimantadine are ion-channel inhibitors that were available for use for prophylaxis or treatment of seasonal human influenza till the early 2000s, when resistance to these drugs emerged and spread globally [23]. Fortunately, the neuraminidase inhibitor class of drugs, that were generated by rational computer-aided design [24] remain effective and are now the mainstay for treatment. In 2018, nearly 20 years after the licensure of the neuraminidase inhibitors, a polymerase inhibitor has been licensed for treatment of influenza in Japan and the USA [25].

7. Immortalization of B cells and characterization of the antibody repertoire using phage display libraries [26–28] following H5N1 infections and more widely, following the 2009 H1N1 pandemic [29,30], facilitated the identification of a highly conserved epitope in the HA stem. A similar murine antibody was first described in 1993 [31]; the conserved HA stem epitope was re-discovered using the equivalent human antibodies [32]. The HA stem is one of the most promising targets for the development of a universal influenza vaccine.

8. With the application of molecular techniques [33] and B cell probes [34], a much more detailed understanding of the antibody response to the haemagglutinin and neuraminidase [35] of influenza viruses is emerging, that includes mapping of epitope specificity and characterization of the role of glycans in shielding epitopes [36–38].

9. Following a long gap after the first report of the crystal structure of the influenza A (H3) HA in 1981 [39], there has been an explosion of structural information in the last decade of different HA subtypes, many in association with well-characterized antibodies [32,40–42]. In conjunction with advances in protein engineering, this knowledge will enable rational design of vaccines [43,44]. The conventional inactivated influenza vaccine may be replaced in the future by a vaccine containing a computationally designed and optimized or antigenically advanced HAs [45,46], presented as nucleic acid [47–49] or protein.
Some of the most remarkable advances in our understanding of the biology of influenza viruses, the pathophysiology of infection at the level of the single cell as well as intact animals and our understanding of virus-host interactions have resulted from the application of advances in imaging techniques. It is possible to track and image infection in single cells and in intact animals [50,51], to study how gene segments interact as they are transported through the cytoplasm and assemble into daughter virions [52], to determine how many gene segments are present in a virion that can cause a productive infection [53] and to study reassortment and packaging of influenza virus gene segments [54]. Labelled viruses have been used to identify which cells are infected in an intact animal following intranasal administration of live virus, to explore whether viral replication in vivo is uniform [55] and what happens to bystander cells, and which cells survive infection in vivo [56,57] and what they can tell us [58].

Despite the many advances, several challenges remain. The controversies regarding dual-use research of concern (DURC) and the risks and benefits of conducting and sharing information gleaned from such research have been polarizing. The resulting regulations pose an ongoing challenge for individual investigators, institutions, journal editors and funding bodies. Although the prospects of improved and/or novel influenza vaccines are good, equal access to vaccines and antiviral drugs for the global population remains a challenge.

Many big questions about influenza are yet to be answered. Why is influenza a seasonal disease in temperate climates? What makes some influenza viruses more virulent than others? Why do some influenza viruses induce a cytokine storm? What makes some influenza viruses transmissible in people while others are not? Will it be possible to forecast influenza evolution? What is the mechanistic basis for the concept of immunologic imprinting [59]? Will a universal influenza vaccine become a reality and will it be an adjunct to or replace conventional influenza vaccines? I am confident that the advances in molecular and quantitative virology, immunology, evolutionary biology and ecology, transmission and environmental control, modelling and novel therapeutics will answer many of these questions. With the tools that are available and a very talented group of young scientists who have entered the field, the future of influenza research is bright!

**Funding:** This work at the Melbourne WHO Collaborating Centre for Reference and Research on Influenza was supported by the Australian Government Department of Health.

**Conflicts of Interest:** The author declares no conflict of interest.

**References**

1. Luytjes, W.; Krystal, M.; Enami, M.; Parvin, J.D.; Palese, P. Amplification, expression and packaging of a foreign gene by influenza virus. *Cell* **1989**, *59*, 1107–1113. [CrossRef]
2. Fodor, E.; Devenish, L.; Engelhardt, O.G.; Palese, P.; Brownlee, G.G.; Garcia-Sastre, A. Rescue of influenza A virus from recombinant DNA. *J. Virol.* **1999**, *73*, 9679–9682. [PubMed]
3. Neumann, G.; Watanabe, T.; Ito, H.; Watanabe, S.; Goto, H.; Gao, P.; Hughes, M.; Perez, D.R.; Donis, R.; Hoffmann, E.; et al. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9345–9350. [CrossRef] [PubMed]
4. Hoffmann, E.; Neumann, G.; Kawaoka, Y.; Hobom, G.; Webster, R.G. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6108–6113. [CrossRef] [PubMed]
5. Reid, A.H.; Fanning, T.G.; Hultin, J.V.; Taubenberger, J.K. Origin and evolution of the 1918 “Spanish” influenza virus hemagglutinin gene. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1651–1656. [CrossRef] [PubMed]
6. Taubenberger, J.K.; Reid, A.H.; Kraft, A.E.; Bijwaard, K.E.; Fanning, T.G. Initial genetic characterization of the 1918 “Spanish” influenza virus. *Science* **1997**, *275*, 1793–1796. [CrossRef] [PubMed]
7. Kash, J.C.; Tumpey, T.M.; Proll, S.C.; Carter, V.; Perwitasari, O.; Thomas, M.J.; Basler, C.F.; Palese, P.; Taubenberger, J.K.; Garcia-Sastre, A.; et al. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **2006**, *443*, 578–581. [CrossRef] [PubMed]
8. Kobasa, D.; Jones, S.M.; Shinya, K.; Kash, J.C.; Copps, J.; Ebihara, H.; Hatta, Y.; Kim, J.H.; Halfmann, P.; Hatta, M.; et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature 2007*, *445*, 319–323. [CrossRef] [PubMed]

9. Tumpey, T.M.; Garcia-Sastre, A.; Taubenberger, J.K.; Palese, P.; Swayne, D.E.; Pantin-Jackwood, M.J.; Schultz-Cherry, S.; Solorzano, A.; Van Rooijen, N.; Katz, J.M.; et al. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: Functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J. Virol. 2005*, *79*, 14933–14944. [CrossRef] [PubMed]

10. Watanabe, T.; Kawoaka, Y. Pathogenesis of the 1918 pandemic influenza virus. *PLoS Pathog.* 2011, *7*, e1001218. [CrossRef] [PubMed]

11. Morens, D.M.; Taubenberger, J.K.; Fauci, A.S. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. *J. Infect. Dis. 2008*, *198*, 962–970. [CrossRef] [PubMed]

12. Kawoaka, Y.; Krauss, S.; Webster, R.G. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol. 1989*, *63*, 4603–4608. [PubMed]

13. Schafer, J.R.; Kawoaka, Y.; Bean, W.J.; Suss, J.; Senne, D.; Webster, R.G. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology 1993*, *194*, 781–788. [CrossRef] [PubMed]

14. Beare, A.S.; Webster, R.G. Replication of avian influenza viruses in humans. *Arch. Virol. 1991*, *119*, 37–42. [CrossRef] [PubMed]

15. Taubenberger, J.K.; Reid, A.H.; Janczewski, T.A.; Fanning, T.G. Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. *Philos. Trans. R. Soc. Lond. B Biol. Sci. 2001*, *356*, 1829–1839. [PubMed]

16. Chen, H.; Yuan, H.; Gao, R.; Zhang, J.; Wang, D.; Xiong, Y.; Fan, G.; Yang, F.; Li, X.; Zhou, J.; et al. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: A descriptive study. *Lancet 2014*, *383*, 714–721. [CrossRef]

17. Harfoot, R.; Webby, R.J. H5 influenza, a global update. *J. Microbiol. Soc. Lond. B Biol. Sci. 2001*, *356*, 1829–1839. [PubMed]

18. Peiris, M.; Yuen, K.Y.; Leung, C.W.; Chan, K.H.; Ip, P.L.; Orr, W.K.; Shortridge, K.F. Human infection with influenza H9N2. *Lancet 1999*, *354*, 916–917. [CrossRef]

19. Shi, W.; Shi, Y.; Wu, Y.; Liu, D.; Gao, G.F. Origin and molecular characterization of the human-infecting H6N1 influenza virus in Taiwan. *Protein Cell 2013*, *4*, 846–853. [CrossRef] [PubMed]

20. Subbarao, K.; Klimov, A.; Katz, J.; Regnery, H.; Lim, W.; Hall, H.; Perdue, M.; Swayne, D.; Bender, C.; Huang, J.; et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science 1999*, *279*, 393–396. [CrossRef] [PubMed]

21. Wang, X.; Jiang, H.; Wu, P.; Uyeki, T.M.; Feng, L.; Lai, S.; Wang, L.; Huo, X.; Xu, K.; Chen, E.; et al. Epidemiology of avian influenza A H7N9 virus in human beings across five epidemics in mainland China, 2013-17: An epidemiological study of laboratory-confirmed case series. *Lancet Infect. Dis. 2017*, *17*, 822–832. [CrossRef]

22. Anderson, T.; Capua, I.; Dauphin, G.; Donis, R.; Fouchier, R.; Mumford, E.; Peiris, M.; Swayne, D.; Thierrmann, A. FAO-OIE-WHO Joint Technical Consultation on Avian Influenza at the Human-Animal Interface. *Influenza Respir. Viruses 2010*, *4* (Suppl. 1), 1–29.

23. Bright, R.A.; Shay, D.K.; Shu, B.; Cox, N.J.; Klimov, A.I. adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. *JAMA 2006*, *295*, 891–894. [CrossRef] [PubMed]

24. von Itzstein, M.; Wu, W.Y.; Kok, G.B.; Pegg, M.S.; Dyason, J.C.; Jin, B.; Van Phan, T.; Smythe, M.L.; White, H.F.; Oliver, S.W. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature 1993*, *363*, 418–423. [CrossRef] [PubMed]

25. Hayden, F.G.; Sugaya, N.; Hirotsub, N.; Lee, N.; de Jong, M.D.; Hurt, A.C.; Ishida, T.; Sekino, H.; Yamada, K.; Portsmouth, S.; et al. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. *N. Engl. J. Med. 2018*, *379*, 913–923. [CrossRef] [PubMed]

26. Kashyap, A.K.; Steel, J.; Oner, A.F.; Dillon, M.A.; Swale, R.E.; Wall, K.M.; Perry, K.J.; Faynboym, A.; Ilhan, M.; Horowitz, M.; et al. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc. Natl. Acad. Sci. USA 2008*, *105*, 5986–5991. [CrossRef] [PubMed]
27. Simmons, C.P.; Bernasconi, N.L.; Suguitan, A.L.; Mills, K.; Ward, J.M.; Chau, N.V.; Hien, T.T.; Sallusto, F.; Ha do, Q.; Farrar, J.; et al. Prophylactic and therapeutic efficacy of human monoclonal antibodies against H5N1 influenza. *PLoS Med.* 2007, 4, e178. [CrossRef] [PubMed]

28. Throsby, M.; van den Brink, E.; Jongeneelen, M.; Poon, L.L.; Alard, P.; Cornelissen, L.; Bakker, A.; Cox, F.; van Deventer, E.; Guan, Y.; et al. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS ONE* 2008, 3, e3942. [CrossRef] [PubMed]

29. Sui, J.; Hwang, W.C.; Perez, S.; Wei, G.; Aird, D.; Chen, L.M.; Santelli, E.; Stec, B.; Cadwell, G.; Ali, M.; et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 2009, 16, 265–273. [CrossRef] [PubMed]

30. Wrammert, J.; Koutsonanos, D.; Li, G.M.; Edupuganti, S.; Sui, J.; Morrissy, M.; McCausland, M.; Skountzou, I.; Hornig, M.; Lipkin, W.I.; et al. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J. Exp. Med.* 2011, 208, 181–193. [CrossRef] [PubMed]

31. Okuno, Y.; Isegawa, Y.; Sasao, F.; Ueda, S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* 1993, 67, 2552–2558. [PubMed]

32. Ekiert, D.C.; Bhabha, G.; Elsliger, M.A.; Friesen, R.H.; Jongeneelen, M.; Throsby, M.; Goudsmit, J.; Wilson, I.A. Antibody recognition of a highly conserved influenza virus epitope. *Science* 2009, 324, 246–251. [CrossRef] [PubMed]

33. Lee, J.; Boutz, D.R.; Chromikova, V.; Joyce, M.G.; Vollmers, C.; Leung, K.; Horton, A.P.; DeKosky, B.J.; Lee, C.H.; Lavinder, J.J.; et al. Molecular-level analysis of the serum antibody repertoire in young adults before and after seasonal influenza vaccination. *Nat. Med.* 2016, 22, 1456–1464. [CrossRef] [PubMed]

34. Whittle, J.R.; Wheatley, A.K.; Wu, L.; Lingwood, D.; Kanekiyo, M.; Ma, S.S.; Narpala, S.R.; Yassine, H.M.; Frank, G.M.; Yewdell, J.W.; et al. Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. *J. Virol.* 2014, 88, 4047–4057. [CrossRef] [PubMed]

35. Chen, Y.Q.; Wohlbold, T.J.; Zheng, N.Y.; Huang, M.; Huang, Y.; Neu, K.E.; Lee, J.; Wan, H.; Rojas, K.T.; Kirkpatrick, E.; et al. Influenza Infection in Humans Induces Broadly Cross-Reactive and Protective Neuraminidase-Reactive Antibodies. *Cell* 2018, 173, 417–429. [CrossRef] [PubMed]

36. Huang, K.Y.; Rijal, P.; Schimanski, L.; Powell, T.J.; Lin, T.Y.; McCauley, J.W.; Daniels, R.S.; Townsend, A.R. Focused antibody response to influenza linked to antigenic drift. *J. Clin. Investig.* 2015, 125, 2631–2645. [CrossRef] [PubMed]

37. Linderman, S.L.; Chambers, B.S.; Zost, S.J.; Parkhouse, K.; Li, Y.; Herrmann, C.; Ellebedy, A.H.; Carter, D.M.; Andrews, S.F.; Zheng, N.Y.; et al. Potential antigenic explanation for atypical H1N1 infections among middle-aged adults during the 2013–2014 influenza season. *Proc. Natl. Acad. Sci. USA* 2014, 111, 15798–15803. [CrossRef] [PubMed]

38. Zost, S.J.; Parkhouse, K.; Guma, M.E.; Kim, K.; Diaz Perez, S.; Wilson, P.C.; Treanor, J.J.; Sant, A.J.; Cobey, S.; Hensley, S.E. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proc. Natl. Acad. Sci. USA* 2017, 114, 12578–12583. [CrossRef] [PubMed]

39. Wiley, D.C.; Wilson, I.A.; Skehel, J.J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 1981, 289, 373–378. [CrossRef] [PubMed]

40. Bangaru, S.; Zhang, H.; Gilchuk, I.M.; Voss, T.G.; Irving, R.P.; Gilchuk, P.; Matta, P.; Zhu, X.; Lang, S.; Nieuwma, T.; et al. A multifunctional human monoclonal neutralizing antibody that targets a unique conserved epitope on influenza HA. *Nat. Commun.* 2018, 9, 2669. [CrossRef] [PubMed]

41. Stevens, J.; Corper, A.L.; Basler, C.F.; Taubenberger, J.K.; Palese, P.; Wilson, I.A. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 2004, 303, 1866–1870. [CrossRef] [PubMed]

42. Xiong, X.; Corti, D.; Liu, J.; Pinna, D.; Foglierini, M.; Calder, I.J.; Martin, S.R.; Lin, Y.P.; Walker, P.A.; Collins, P.J.; et al. Structures of complexes formed by H5 influenza hemagglutinin with a potent broadly neutralizing human monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 2015, 112, 9430–9435. [CrossRef] [PubMed]
43. Crowe, J.E., Jr. Principles of Broad and Potent Antiviral Human Antibodies: Insights for Vaccine Design. Cell. Host Microbe 2017, 22, 193–206. [CrossRef] [PubMed]

44. Laursen, N.S.; Friesen, R.H.E.; Zhu, X.; Jongeneelen, M.; Blokland, S.; Vermond, J.; van Eijgen, A.; Tang, C.; van Diepen, H.; Obmolova, G.; et al. Universal protection against influenza infection by a multidomain antibody to influenza hemagglutinin. Science 2018, 362, 598–602. [CrossRef] [PubMed]

45. Wong, T.M.; Allen, J.D.; Bebin-Blackwell, A.G.; Carter, D.M.; Alefantis, T.; DiNapoli, J.; Kleanthous, H.; Ross, T.M. Computationally Optimized Broadly Reactive Hemagglutinin Elicits Hemagglutination Inhibition Antibodies against a Panel of H3N2 Influenza Virus Cocirculating Variants. J. Virol. 2017, 91, e01581-17. [CrossRef] [PubMed]

46. Li, C.; Hatta, M.; Burke, D.F.; Ping, J.; Zhang, Y.; Ozawa, M.; Taft, A.S.; Das, S.C.; Hanson, A.P.; Song, J.; et al. Selection of antigenically advanced variants of seasonal influenza viruses. Nat. Microbiol. 2016, 1, 16058. [CrossRef] [PubMed]

47. Bahl, K.; Senn, J.J.; Yuzhakov, O.; Bulychev, A.; Brito, L.A.; Hassett, K.J.; Laska, M.E.; Smith, M.; Almarsson, O.; Thompson, J.; et al. Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses. Mol. Ther. 2017, 25, 1316–1327. [CrossRef] [PubMed]

48. Magini, D.; Giovanni, C.; Mangiavacchi, S.; Maccari, S.; Cecchi, R.; Ulmer, J.B.; De Gregorio, E.; Geall, A.J.; Brazzoli, M.; Bertholet, S. Self-Amplifying mRNA Vaccines Expressing Multiple Conserved Influenza Antigens Confer Protection against Homologous and Heterosubtypic Viral Challenge. PLoS ONE 2016, 11, e0161193. [CrossRef] [PubMed]

49. Pardi, N.; Parkhouse, K.; Kirkpatrick, E.; McMahon, M.; Zost, S.J.; Mui, B.L.; Tam, Y.K.; Kariko, K.; Barbosa, C.J.; Madden, T.D.; et al. Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies. Nat. Commun. 2018, 9, 3361. [CrossRef] [PubMed]

50. Brandes, M.; Klauschen, F.; Kuchen, S.; Germain, R.N. A systems analysis identifies a feedforward inflammatory circuit leading to lethal influenza infection. Cell 2013, 154, 197–212. [CrossRef] [PubMed]

51. Manicassamy, B.; Manicassamy, S.; Belicha-Villanueva, A.; Pisanelli, G.; Pulendran, B.; Garcia-Sastre, A. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc. Natl. Acad. Sci. USA 2010, 107, 11531–11536. [CrossRef] [PubMed]

52. Lakdawala, S.S.; Wu, Y.; Wawrzusin, P.; Kabat, J.; Broadbent, A.J.; Lamirande, E.W.; Fodor, E.; Altan-Bonnet, N.; Shroff, H.; Subbarao, K. Influenza a virus assembly intermediates fuse in the cytoplasm.PLoS Pathog. 2014, 10, e1003971. [CrossRef] [PubMed]

53. Noda, T.; Murakami, S.; Nakatsu, S.; Imai, H.; Muramoto, Y.; Shindo, K.; Sagara, H.; Kawaoya, Y. Importance of the 1+7 configuration of ribonucleoprotein complexes for influenza A virus genome packaging. Nat. Commun. 2018, 9, 54. [CrossRef] [PubMed]

54. Brooke, C.B.; Ince, W.L.; Wei, J.; Bennink, J.R.; Yewdell, J.W. Influenza A virus nucleoprotein selectively decreases neuraminidase gene-segment packaging while enhancing viral fitness and transmissibility. Proc. Natl. Acad. Sci. USA 2014, 111, 16854–16859. [CrossRef] [PubMed]

55. Sjaastad, L.E.; Fay, E.J.; Fiege, J.K.; Macchietto, M.G.; Stone, I.A.; Markman, M.W.; Shen, S.; Langlois, R.A. Distinct antiviral signatures revealed by the magnitude and round of influenza virus replication in vivo. Proc. Natl. Acad. Sci. USA 2017, 115, 9610–9615. [CrossRef] [PubMed]

56. Hamilton, J.R.; Sachs, D.; Lim, J.K.; Langlois, R.A.; Palese, P.; Heaton, N.S. Club cells surviving influenza A virus infection induce temporary nonspecific antiviral immunity. Proc. Natl. Acad. Sci. USA 2016, 113, 3861–3866. [CrossRef] [PubMed]

57. Heaton, N.S.; Langlois, R.A.; Sachs, D.; Lim, J.K.; Palese, P.; tenOever, B.R. Long-term survival of influenza virus infected club cells drives immunopathology. J. Exp. Med. 2014, 211, 1707–1714. [CrossRef] [PubMed]

58. Fukuyama, S.; Katsura, H.; Zhao, D.; Ozawa, M.; Ando, T.; Shoemaker, J.E.; Ishikawa, I.; Yamada, S.; Neumann, G.; Watanabe, S.; et al. Multi-spectral fluorescent reporter influenza viruses (Color-flu) as powerful tools for in vivo studies. Nat. Commun. 2015, 6, 6600. [CrossRef] [PubMed]

59. Gostic, K.M.; Ambrose, M.; Worobey, M.; Lloyd-Smith, J.O. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. Science 2016, 354, 722–726. [CrossRef] [PubMed]