zoonotic diseases

MDPI

Review

The Value of a Comparative Approach with Equine Vaccine Development for the Development of Human Influenza DNA Vaccines

Ahmed F. Abdelkhalek [1,2] and Janet M. Daly [2,*]

[1] Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt; ahmed.mahmoud2@nottingham.ac.uk
[2] One Virology-Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Loughborough LE12 5RD, UK
* Correspondence: janet.daly@nottingham.ac.uk

Simple Summary: DNA vaccines give promising results in mice, the most routinely used laboratory animal, but the results often cannot be translated in human clinical trials. The same is true in the horse, the immune system of which shares more similarities with humans than that of the mouse. Hence, testing of human influenza DNA vaccine concepts in horses, which are naturally infected by influenza A viruses, could be more predictive of what would be seen in humans than testing in mice. In this review, we outline reasons for suggesting that by taking a comparative approach, the horse may provide more insight than the mouse model for developing human DNA vaccines against influenza.

Abstract: A comparative medicine approach, whereby similarities and differences in biology between human and veterinary species are used to enhance understanding for the benefit of both, is highly relevant to the development of viral vaccines. Human and equine influenza share many similarities in pathogenesis and immune responses. The DNA vaccine approach offers potential advantages for responding rapidly and effectively to outbreaks or pandemics in both humans and animals, especially in under-resourced regions. The European and American vaccine regulatory authorities require demonstration of vaccine efficacy in animal models. However, mice, the most widely used model, are not naturally infected with influenza viruses, resulting in different pathobiology. Additionally, mice as a model for DNA vaccine testing appear to overestimate the humoral immune response compared to other mammalian species. In this review, we propose that testing of DNA vaccines against influenza type A viruses (and other shared pathogens) in the horse can provide valuable knowledge for the development of human DNA vaccines.

Keywords: DNA vaccines; animal model; equine; influenza virus; immune system

Citation: Abdelkhalek, A.F.; Daly, J.M. The Value of a Comparative Approach with Equine Vaccine Development for the Development of Human Influenza DNA Vaccines. *Zoonotic Dis.* **2024**, *4*, 245–258. https://doi.org/10.3390/zoonoticdis4040021

Academic Editor: Stephen K. Wikel

Received: 31 October 2023
Revised: 30 September 2024
Accepted: 11 October 2024
Published: 14 October 2024

1. Introduction

Zoonotic diseases comprise 60% of human infectious diseases, and 75% of newly emerging diseases [1]. Influenza A virus (IAV), an emerging and re-emerging pathogen, continues to cause human infections and mortality, despite the availability of traditional inactivated virus vaccines for several decades. For instance, highly pathogenic avian influenza (HPAI) of the H5N1 subtype caused 868 zoonotic infections, and 457 deaths, a case fatality rate (CFR) of 53%, from January 2003 to January 2023 [2]. However, these zoonotic influenza strains do not transmit efficiently between humans [3]. Nonetheless, even after becoming established in humans, seasonal human influenza continues to cause three to five million severe infections and nearly 500,000 deaths annually worldwide [4]. Influenza A viruses also circulate in equids causing an acute respiratory tract infection [5].

Influenza type A is classified in the genus *Alphainfluenzavirus*, which is one of nine genera in the segmented negative sense single-stranded virus family *Orthomyxoviridae* [6]. Influenza A virus classification into subtypes (e.g., the HPAI H5N1 subtype as mentioned above) is determined by the antigenic relationship between haemagglutinin (HA) and neuraminidase (NA) proteins using the traditional serological tests haemagglutination inhibition (HI) and neuraminidase inhibition (NI), respectively, and genome sequencing recently. There are 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11) [7]. Like other RNA viruses, the high replication rate and lack of a proof-reading mechanism results in the rapid accumulation of mutations. When mutations accumulate in the HA and NA proteins, and result in proteins that are no longer recognized by antibodies to previous infection or vaccination, it is called antigenic drift. Furthermore, the emergence of new viruses after reassortment may cause human pandemics; reassortment (mixing of different gene segments) is called antigenic shift when it involves HA and/or NA [8]. In humans, there are currently two circulating IAV subtypes, H1N1and H3N2, which cause seasonal epidemics [9]. In equids, there is currently only the H3N8 subtype circulating, which outcompeted the H7N7 subtype [10]. The H3N8 subtype diverged into American and Eurasian lineages in the 1980s [11]. Furthermore, the American lineage is subdivided into Florida, South America and Kentucky [12]. Of these sub-lineages, the Florida sub-lineage is currently circulating with two clades. There is a continuous effort to control IAV in veterinary species and humans by vaccination, and vaccination of competition horses is mandatory under many jurisdictions such as the Fédération Équestre Internationale.

In the era of gene-based vaccines, DNA offers many merits over the traditional inactivated influenza vaccine candidate. The DNA vaccine approach has lower production costs, higher stability (does not need to be stored or maintained in a cold chain), safety in pregnant and immunocompromised individuals and is effective in the presence of maternal antibodies. The ease of design means that DNA vaccines can be tailored rapidly to respond to outbreaks [13]. However, few DNA vaccines have progressed beyond the clinical phase of development. The COVID-19 pandemic stimulated renewed interest in the potential of DNA vaccines, particularly for low- and middle-income countries. Indeed, a DNA vaccine encoding the SARS-CoV-2 spike protein received emergency use authorization in India in 2021. On the other hand, 16 years earlier in 2005, the first DNA vaccine to be licensed was for use in horses against West Nile virus, although the vaccine, which was developed by Fort Dodge, was discontinued by Pfizer.

Regulatory authorities require evidence of vaccine efficacy in an animal model for granting vaccine licenses [14]. However, mice, although most widely used, are not the best model for DNA vaccines for several reasons that we will explain here.

2. Influenza A Virus Immunity in Humans and Equids

The innate immune response plays a crucial role in the rapid immunological response that is essential to limit viral infection. Subsequently, the role of the adaptive immune response appears in viral eradication and developing immunological memory for protection from further infection.

2.1. Innate Immunity

Innate immune responses, which start by sensing risk and invoking adaptive immunity, are the initial line of defence against infection. Influenza A viruses have complementarity between the 5′ and 3′ ends of the viral RNA segments, resulting in short stretches of double-stranded RNA (dsRNA) [15]. This pathogen-associated molecular pattern (PAMP) is detected by toll-like receptor 3 (TLR3), which is a pattern recognition receptor (PRR) [16]. The dsRNA recognition leads to activation of the intracellular signalling pathway of the TIR domain-containing adaptor protein TRIF (Figure 1), which leads to the induction of interferon inducible genes, such as interferon beta (IFN-ß), interferon gamma inducible protein (IP-1) or CXCL10, and the 'regulated on activation normal T cell expressed and secreted' (RANTES) protein or CCL5. Interferon ß is a type-I interferon that has antiviral

activity and stimulates the other two chemotactic factors, CXCL10 and CCL5, that attract leukocytes to infected tissue [17].

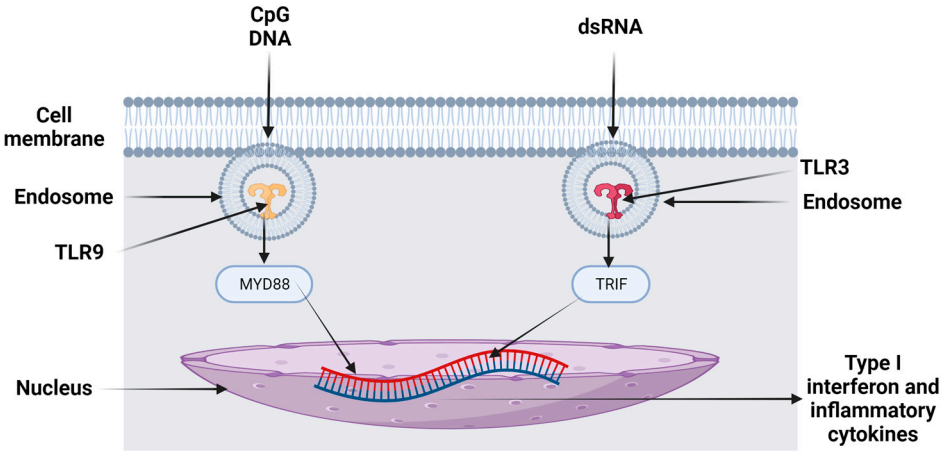

Figure 1. The type-I interferon and other inflammatory cytokines are stimulated by endosomal pattern recognition receptors (PRRs) and associated adaptor molecules. Toll-like receptor (TLR) 3 and its ligand, double-stranded RNA (dsRNA), stimulates the production of antiviral type-I interferons via TIR domain-containing adaptor protein (TRIF). Another toll-like receptor, TLR-9, stimulates production of the same molecules as TLR-3, but their ligand is unmethylated CpG that can be found in the plasmid backbone of DNA vaccines and the adapter protein is myeloid differentiation primary response 88 protein (MyD88). Adapted from [18]. Created with BioRender.com.

The target cells for IAV are the epithelial lining in the respiratory airways, which produce the previously mentioned chemotactic agents leading to the recruitment of effector cells of innate immunity. After this, innate effector cells such as neutrophils, macrophages, dendritic cells (DCs) and natural killer (NK) cells begin to migrate. Natural killer cells have two large groups of receptor families, killer cell immunoglobulin-like receptors (KIRs) and killer cell lectin-like receptors (KLRs) [19]. These receptors have been found to be linked to severe influenza infections in children [20]. The cytotoxic activity of NK cells is related to natural killer protein receptors NKp44 and NKp46, and their interaction with influenza HA causes lysis of IAV-infected cells [21]. Equine NKp46 protein has only 65% identity (the murine protein only has 58% identity) with the human ortholog, which is insufficient for cross-reaction and required the development of a specific equine reagent [22].

Lung macrophages play a key role in controlling viral spread. Influenza-infected cells are phagocytosed by activated macrophages, which prevents viral propagation and controls the subsequent adaptive immune response [23]. Alveolar and interstitial macrophages are the two primary types of lung macrophages that are constitutively expressed in healthy mice and humans [24]. However, horses additionally have a peculiar type of macrophage known as pulmonary intravascular macrophage (PIM) [25]. It is thought these cells may explain why the horse is particularly susceptible to endotoxemia [26]. Although PIMs are usually absent in mice and healthy humans, they can be induced in mice and human patients with liver failure [27]. Liver injury is one of the extra pulmonary pathogenic effects of influenza infection that has been observed in humans [28].

During IAV infection, DCs, one of the professional antigen-presenting cells (APCs), mediate between the innate and adaptive immune responses. Conventional DCs (cDCs) migrate from the lungs to the lymph nodes after IAV infection [29]. In DCs, the engulfed antigens of IAV are processed before being presented on major histocompatibility (MHC class I and class II) molecules to stimulate cellular and humoral adaptive immune responses [30]. Monocyte DCs are one of the numerous varieties of DCs that develop during inflammation; in equine species, clusters of differentiation (CD83) and c-type lectin (CD206) receptors are expressed on both mature and immature monocyte dendritic cells [31]. However, in murine and human species, whereas CD206 is abundantly expressed on immature

DCs, CD83 expression is highly expressed on the surface of mature DCs [32]. Immature dendritic cells are less capable than mature dendritic cells of stimulating naive T cells [31]. Hence, attempting to improve DNA vaccine delivery by targeting CD83 or CD206 (as has been proposed, e.g., [33]) may have different effects in horses than in mice or humans.

2.2. Adaptive Immune Response in Equids and Humans

Activated DCs, in turn, stimulate activation of $CD4^+$ T cells to differentiate them into type 1 helper (Th1) or Th2 cells, which can be differentiated by the cytokines they produce [34,35]. The Th1 response is characterized by the production of interferon gamma (IFN-γ) and interleukin (IL)-2 and stimulation of cell-mediated immunity, which is represented by $CD8^+$ cytotoxic T-lymphocytes (CTLs) [36]. The CTLs migrate to the site of infection and clear viral-infected cells [37] either directly by perforins and granzymes that lyse the infected cells, or indirectly by producing cytokines that cause the development of death receptors and, eventually, apoptosis [38]. The development of memory CTLs has a role in the rapid response to subsequent infections by the same or different influenza strains. The CTLs are directed mainly to conserved internal proteins of IAV and are critical in heterosubtypic protection [39].

The humoral immune response is stimulated by Th2-type cytokines (IL-4, IL-5, IL10 and IL-13), which is characterized by B-cell differentiation into plasma cells that produce neutralizing and non-neutralizing antibodies against IAV [40]. The life span and the speed of response of memory B cells are the two key determinants of the efficacy of vaccines. All mammals produce five classes of antibody (IgG, IgM, IgA, IgD and IgE) with IgA having the main role in preventing the airborne transmission of a virus [41], whereas IgG is the dominant class of antibodies in the protection against IAV pathogenesis. The horse has a higher number of IgG constant region genes than either humans or mice, encoding seven IgG subclasses (IgG1–7), whereas mice express five subclasses (IgG1, IgG2a, IgG2b, IgG2c and IgG3) and humans express four (IgG1–4, from highest to lowest abundance). In human influenza infection, IgG1 and IgG3 are the main effectors of protection (reviewed by [42]). It has been proposed that for effective equine vaccine-induced immunity, the subclasses IgG1, IgG3, IgG4 and IgG7 are required [43]. Activated B cells are essential for heterosubtypic protection, either through antibody-dependent cell cytotoxicity (ADCC) or non-neutralizing antibodies that promote CD8+ T cell proliferation [40,44].

In summary, the primary function of CTLs is the rapid clearance of virally infected cells, whereas the humoral immune response neutralising antibody mechanisms are largely responsible for preventing initial infection. Strong induction of both these arms of the adaptive immune response by vaccines is desirable.

3. Currently Available Commercial Influenza Vaccines Have Similar Problems in Equids and Humans

Both humans and horses can benefit from vaccination as a means of protection against IAV [45–47]. The currently available influenza vaccines can be broadly categorised into inactivated virus (typically produced in eggs), 'modified live' (i.e., attenuated virus vaccines), and viral vectored (e.g., canarypox vectored vaccines in equine species) or subunit (split viral vaccines in human species) [48,49]. The pros and cons of these are briefly discussed in this section.

The first available vaccine for human influenza was produced in the 1940s, and for equine influenza, in the 1960s [50,51]. The virus was grown in embryonated hens' eggs, inactivated chemically, and then mixed with adjuvants in the final product. Because of this drawn-out process, the vaccine produced may be ineffective against the circulating strain, as demonstrated by the human influenza H3N2 2014–2015 outbreak [52]. Another prominent drawback of egg-based vaccines is the acquisition of mutations that are acquired by propagating virus during serial passages in eggs, mainly in human but also equine IAV [53,54]. These mutations may affect the antibodies that bind haemagglutinin (HA), leading to a weak immunogenic response [55]. In addition to the previously mentioned

concerns, the dependence on egg supply that becomes particularly demanding during outbreaks can result in a shortage in vaccine supply.

The best immunity can be stimulated by a vaccination that mimics a natural infection, especially if it is administered via the same route by which the virus initiates infection. The fundamental benefit of attenuated vaccines is that, in addition to inducing a strong humoral immune response, they can also produce cell-mediated immunity and mucosal immunity, both of which are limited or absent from inactivated virus immune responses. The primary concerns of attenuated influenza vaccines in both humans and equids are the potential for reversion to virulence when administered in the target host and reassortment with field strains. However, this has not been observed in clinical use in either species [56,57].

A canarypox virus vector that expresses HA as a transgene is commercially available as an equine influenza vaccine. It was successfully exploited in the 2007 Australian EIV outbreak as a vaccine that enables differentiation of infected from vaccinated animals (DIVA) [58]. It also provides an immediate, long-lasting immunological response and stimulates a cell-mediated immune response [59,60]. Despite numerous studies of vectored vaccines for human influenza, none of them have progressed beyond the preclinical stage yet [61,62]. The disadvantage of vaccines that contain only one or two of the surface glycoproteins such as the viral vectored or 'split' vaccines is that they lack T-cell responses against the more conserved viral proteins.

Although a range of vaccines are available for both equine and human use, they cannot always prevent the subclinical infection in either species; furthermore, some of them can cause adverse post-vaccinal reactions [11,63]. There are also issues with vaccine effectiveness, length of protection, the time of vaccine manufacturing and the cost of production. As a result, there is room for further developing IAV vaccine platforms.

4. Influenza Gene-Based Vaccines as Promising Candidates

As a swift reaction to influenza pandemics is required, traditional whole inactivated influenza vaccines cultivated in eggs, which at their best were only accessible after 6 months, had little value; this was evident in the 2009 influenza pandemic [64]. Gene-based vaccines offer greater potential than conventional inactivated or subunit recombinant proteins because they are easier to synthesise and can be rapidly produced from genes of the most immunogenic protein of a pathogen. Furthermore, the gene-based vaccine approach depends on the expression of certain proteins in the target host's cells; therefore, it mimics the process by which the body produces protection following a natural infection (Figure 2).

However, delivering gene-based vaccines to the desired cells is a challenge for both DNA and messenger RNA (mRNA) with DNA having a nuclear barrier in addition to a cellular barrier (Figure 3). It is estimated that 1 in 10,000 RNA molecules can escape the endosome to the cytoplasm, and just 1% of naked DNA can be successfully delivered to the nucleus [65,66].

The major obstacle in the early development of mRNA vaccines was the instability (in the vial and in vivo) of mRNA. A preclinical mRNA rabies vaccine, however, had a two-year shelf life in the temperature range of −80 °C to +70 °C [67]. However, in clinical trials, a local and significant (but not fatal) systemic adverse event was recorded [68]. This highlights the inherent inflammatory response of RNA; for example, mRNA can stimulate the innate immune response via several PRRs such as TLR3 and the TIR domain-containing adapter-inducing interferon-β (TRIF) pathway, or TLR7 and myeloid differentiation primary response 88 (MyD88) [65]. These pathways can induce type-I interferons that can stall the translation and affect the final expression of the encoded protein. For example, this was observed in an HIV mRNA vaccine trial, in which there was a decrease in the net immunity of the vaccine [69]. As a result, the effectiveness of subsequent doses of mRNA vaccines following exposure to an inappropriate environment from the initial dose is questionable. Ex vivo delivery using dendritic cells [70], which is impractical for mass immunization, and cationic lipids that carry amine, which shield the negatively charged RNA from the negatively charged cellular membrane, are two methods to deliver the

mRNA molecules with the least amount of nuclease degradation [71]. The lipid encapsulated mRNA approach was used in the approved COVID-19 vaccines of Pfizer and Moderna [72,73]. Synthesized nucleosides are another technique for improving stability; they were employed in the development of a multivalent influenza vaccine that protects against all subtypes of influenza A and influenza B [74]. However, clinical toxicity studies in humans conducted using mRNA containing synthetic nucleosides suggest the need for caution due to potential toxicity [75,76].

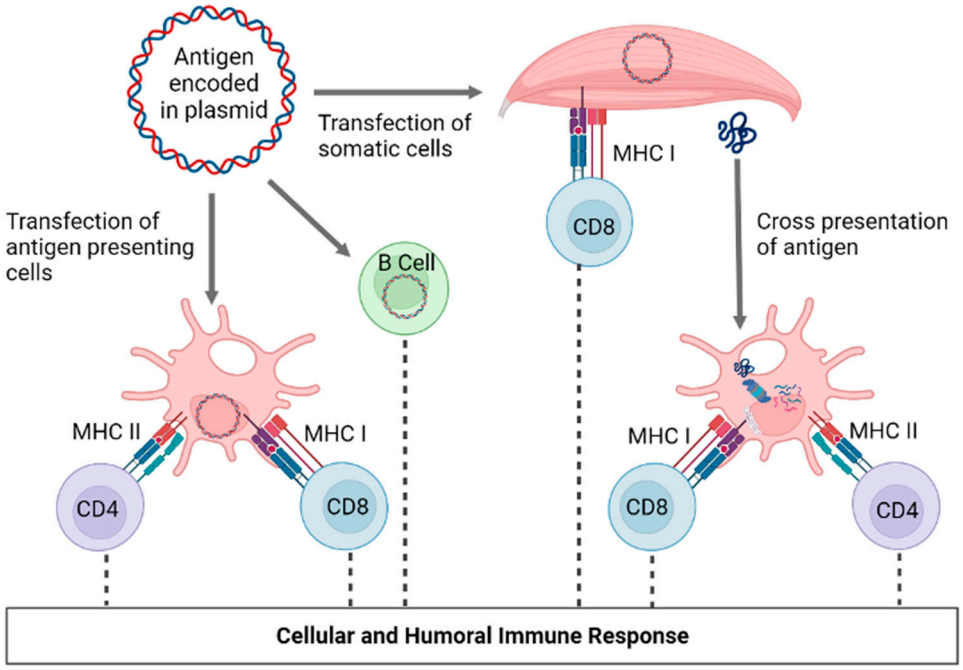

Figure 2. Mechanism of antigen presentation and immune response in DNA vaccines.

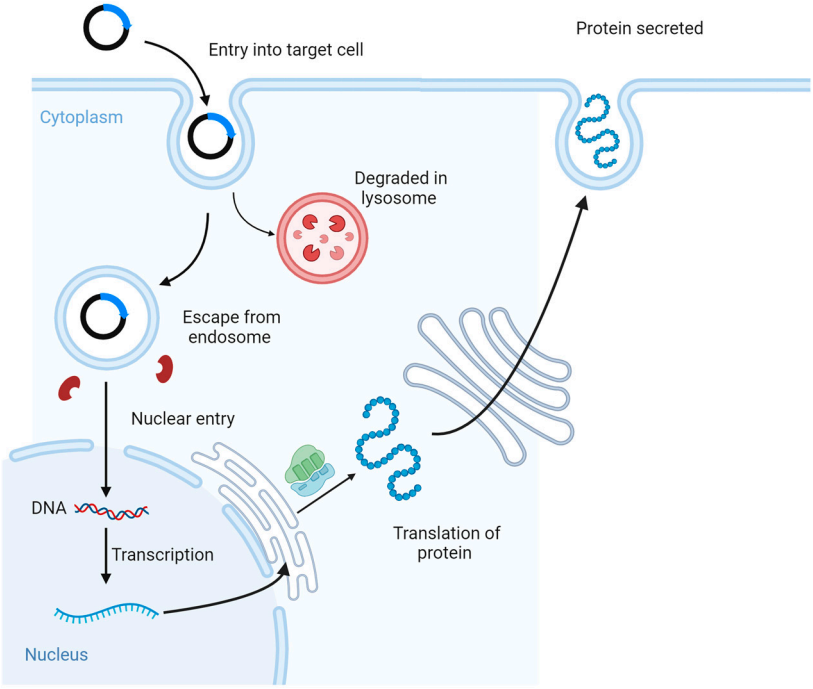

Figure 3. Pathway and barriers encountered after immunisation with a plasmid DNA vaccine.

In contrast to mRNA, plasmid DNA offers more templates to produce mRNA molecules that will be translated into proteins. Additionally, DNA vaccines are more stable than mRNA vaccines. There are some concerns around DNA vaccines, such as the potential for the integration of plasmid in the chromosome of the host or autoimmunity to the host DNA, although neither of these were observed in the clinical studies of DNA vaccines [77]. For mRNA vaccines, the possibility of integration is not entirely impossible, notably in HIV-positive humans or in healthy people with human internal retro viruses in their genomes that supply the replicative machinery that is necessary for integration [78]. In terms of the price of in vitro transcription and formulation, mRNA vaccines are more expensive than DNA vaccines. However, as mentioned above, delivery of the nucleic acid to the desired cells and cellular compartments is a particular challenge for DNA vaccines. Although nanograms of protein are created after transfection of cells with plasmid DNA in vitro, after immunisation, much of the plasmid DNA is lost by being taken up by inefficient non-immune cells like myocytes or keratinocytes [79]. The DNA vaccine pathway after injection is shown in Figure 4. When the naked plasmid is injected intramuscularly, then the transduced myocytes act as a reservoir for the antigen, which is taken up and processed by the antigen-presenting cells (APCs). Then, the activated DCs stimulate the cellular and humoral immune responses through MHC class I and class II [80].

Obstacles in the delivery of DNA vaccines have been at least partially overcome by using a biolistic delivery device ('gene gun') that 'fires' gold particles coated with plasmid DNA using high-pressure gas or an electroporation device. Neither of these approaches lends itself to mass immunisation. Nonetheless, the first licensed gene-based vaccine was a DNA vaccine against West Nile virus (WNV) in horses [81] and a WNV DNA vaccine produced a neutralising antibody in humans [82]. Additionally, the first two Zika vaccines that entered clinical trials were DNA vaccines [83]. Hence, the DNA approach is a current feasible option for influenza vaccines in both horses and people.

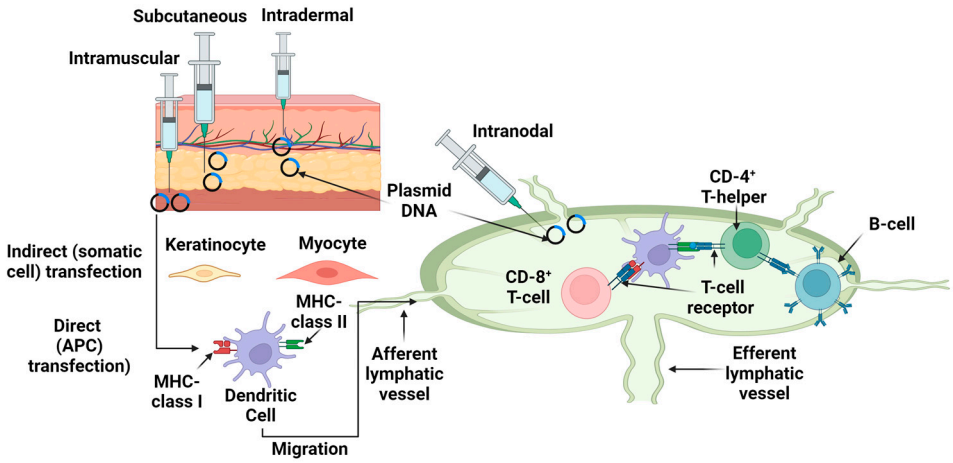

Figure 4. Different parenteral injections of DNA vaccines and the pathway of the injected plasmid until effective stimulation of immune response. After vaccine injection, plasmids either transfect the myocytes or keratinocytes according to the route of injection, or the plasmids can be engulfed by the resident antigen-presenting cells (APCs), such as dendritic cells (DCs). The later engulfment by DCs is called direct transfection, while the previous transfection of myocytes or keratinocytes is called indirect transfection. Finally, the activated mature DCs migrate either directly by intranodal injection or after migration from the injection sites activates the $CD8^+$ and $CD4^+$ T cells through major histocompatibility (MHC) class I or II, respectively. Adapted from [84]. Created with BioRender.com.

5. The Case for a Comparative Approach to Development of Influenza DNA Vaccines

Influenza A virus has been the most studied pathogen as a model for DNA vaccines since the 1990s. For example, Ulmer, Donnelly [85] injected naked plasmid encoding nucleoprotein (NP), which protected mice against a heterologous influenza challenge. Similar

success was achieved in another trial, in which different routes of plasmid administration were used to reduce the plasmid concentration, and the vaccine achieved protection against influenza in mice [86]. Moreover, a single immunisation with DNA vaccines can result in a quick and sustained immune response in mice [87]. Laboratory mice are an inbred species and therefore have genetic consistency, and there are abundant available immunological reagents; therefore, they are the most widely used model in preclinical influenza vaccine testing [88]. However, the mouse is not naturally infected with the influenza virus, and laboratory infection by most human influenza strains needs previous adaptation [89]. Moreover, the clinical manifestations of influenza infection in humans differ greatly from those in mice; the disease manifests in mice as a lower respiratory tract disease, whereas influenza is an upper respiratory infection in humans and horses.

Despite the protection afforded by DNA influenza vaccines in mice, this success was not observed in humans or other large animals such as horses [90,91]. It was initially thought this was simply a scale-up problem, but it has been suggested that differences in immune response might be the cause. It is noteworthy that the repertoire of human CTL is larger than mice, which have a defective response of class-I alleles for IAV [92]. Furthermore, the broad-spectrum subtype-specific, or heterosubtypic-specific protection observed in mice, is rarely observed in humans [93]. Additionally, serum amyloid P (SAP), which is a DNA binding protein, acts as a weak negative regulator for both adaptive and innate immune responses in mice, in contrast to the strong effect of human SAP [85].

Finally, the number, structure and distribution of TLRs, which as previously mentioned play key roles as innate immune sensors, are different between humans and mice. There are 13 TLRs in mice but the gene for TLR10 is not expressed; in humans, TLRs 11, 12 and 13 are not represented. Very recently, 12 TLRs were identified in members of the family *Equidae* [94]. The murine and human TLR-9, the receptor for unmethylated cytosine phosphate guanosine (CpG) oligonucleotide (ODN) motifs that are found in bacterial plasmids and widely used as adjuvants [95], share only 75% amino acid homology. Furthermore, the expression profile of TLR-9 in humans is on plasmacytoid dendritic cells and B cells [64], whereas the expression in mice is on macrophage and myeloid-derived dendritic cells. This led Kayraklioglu, Horuluoglu [96] to suggest that there will be an exaggerated immune response to CpG in mice. The innate immune response is more similar in equine and human species. For example, some of the pattern recognition receptors (PRRs), such as TLR9, the receptor of bacterial CpG, are expressed in many cell types of both equine and human lungs [66,97]. The amino acid identity of the equine TLR9 amino acid sequence compared to that of humans is 84%, and the same expression pattern of TLR9 was observed in leukocytes of both equine and human species [98]. The main differences and similarities between the immune systems of horses, mice and humans are summarised in Table 1.

A key advantage of the equine model for vaccine efficacy studies is that experimental infection models that recapitulate natural infection have been developed from initial intranasal installation of a virus to exposure to a nebulised aerosol in a room, culminating in the use of an individual face mask such as Flexineb®, which is marketed for delivering inhaled medication to horses [99].

The IgA antibodies are one of the key players in the prevention of influenza infection at the site of entry in both humans and equine species, as mentioned above. It has a role in cross-protection. The best route to attain the protective level for respiratory infection such as IAV is thought to be the mucosal route, to generate secretory IgA as well as systemic IgG antibodies [100]. However, protection was achieved in the absence of an IgA response in ponies [57].

In both equine and human species, the focus of influenza DNA vaccine development is on the HA glycoprotein, and the NA glycoprotein is not considered or included in most of the DNA vaccine trials [90,91]. The antigenic drift of the HA glycoprotein is accompanied by changes in the NA glycoprotein to maintain the balance between the mutations occurring in both glycoproteins, which aid in the effective viral replication and fitness [101]. Furthermore, NA is very important in viral release and has an important role

in the influenza viral entry, the later effects are inhibited, when the anti-NA antibodies are produced and reduced viral shedding and disease signs after challenging humans [102]. This partial immunity is observed when NA is used alone and is not accompanied with HA [103]. Thus, it is necessary to determine target epitopes of NA in both equine and human influenza viruses to aid in the production of more effective vaccines.

Table 1. Summary of key difference of equine, murine and human immune systems.

	Human	Mice	Equids
Clinical disease	Mainly upper respiratory infection	Virus usually must be adapted and typically results in weight loss and death	Same as human—enables study of transmission
Toll-like receptor 3		About 50% protein identity	About 86% protein identity
	Among immune cells, only myeloid DCs, macrophages and mast cells express TLR3	Higher expression on alveolar macrophages	Expressed on perivascular alveolar macrophages
Toll-like receptor 9		75% protein identity	84% protein identity
	Expressed on monocytes and lymphocytes of human species	Expressed on macrophage and myeloid-derived dendritic cells	Expression like human (on granulocytes, monocytes and lymphocytes)
Pulmonary intravascular macrophages (PIMs)	Absent in healthy humans but can be induced in patients with hepatopulmonary diseases	Can be induced in ligated bile duct mice	Constitutively present
Killer cell immunoglobulin-like receptors	Present	Absent	Present
CD83 and DEC206	Immature DCs highly express DEC 206, mature DCs express CD83	Like humans	Expression of CD83 and DEC206 is on both mature and immature dendritic cells
IgG isotypes	IgG1, IgG2, IgG3, IgG4	IgG1, IgG2a, IgG2b, IgG3	IgG1–IgG7

There have been trials to avoid the continuously changing glycoproteins (HA and NA) to produce a broader immunity by using a matrix ectodomain sequence of M2 or mucosal IgA antibodies [104,105]. However, an M2 approach failed; instead of protection, it enhanced the pathogenesis of the swine influenza when the vaccine was tested in pigs [106]. This emphasises the need for a balanced immune response to vaccination in terms of antigens targeted and humoral and cellular immunity. Not only is the pig a natural host of IAV, but pigs can also be infected with human isolates; therefore, pig has also been proposed as an undervalued model for human IAV infection studies [107].

Equids have been increasingly proposed as a model for various human diseases, including virus vaccine development [108,109], and they have similar pathogenesis and immunity to influenza infection. There are, however, several limitations to using equids solely as a model species for the development of human influenza vaccines including cost, difficulty in obtaining unexposed animals, more limited specific immunological reagents and ethical objections. Some of these limitations similarly apply to the use of pigs as a model organism. On the other hand, horses are often highly valued (either economically as competition animals or as companion animals) and are long-lived, and research to develop improved influenza vaccines for use in horses is ongoing. Thus, studies in equids are justified for the development of both equine and human vaccines.

6. Future Directions

As described above, there is further work to be carried out to optimize the antigen(s) presented by DNA vaccines, which could potentially include expression of the matrix M1

protein with HA and NA, which can then self-assemble into virus-like particles (VLPs). Expression of a single viral protein suffers from the same disadvantage as recombinant protein vaccines, which are poor inducers of immunity without a strong adjuvant, whereas by displaying viral antigens similarly to virus, VLPs are more potent. The plasmid pVAX1 was designed specifically for DNA vaccine development, avoiding extraneous elements and including a kanamycin rather than ampicillin resistance gene. Nonetheless, there are disadvantages to using bacteria to amplify plasmid DNA and alternative platforms to produce DNA for immunization are being explored, for example, enzymatically produced Doggybone™ DNA as reviewed by [110]. Immunization with DNA is also typically performed using a device such as a 'gene gun', which fires gold particles coated with plasmid DNA, to target the skin as intradermal delivery appears to induce a stronger immune response than intramuscular delivery in mice [110]. Due to their small size, mice are not suitable for testing some delivery devices, such as biojectors, which use compressed gas to force the DNA into the dermis. Here, large animal models such as the horse and pig can again afford some advantage over the use of the mouse model. On the other hand, the use of such devices may not lend themselves to mass immunization in the event of a human influenza pandemic, and formulations to improve traditional intramuscular injection using a needle and syringe are under development [111].

7. Conclusions

DNA vaccines against influenza viruses show promising results in small animal models, but the results in large including horses and humans are often not at the expected level. It appears that mice do not represent the immune response in the target host results, and this may, at least in part, be due to the difference in distribution of some critical receptors on antigen-presenting cells. However, the availability of reagents and a genetically consistent inbred host makes mice the most widely accepted model in testing vaccine efficacy including DNA vaccines. There are challenges to using equine species purely as a model for DNA vaccine development. Nonetheless, research to develop an equine influenza DNA vaccine is ongoing, potentially providing valuable information to inform human DNA vaccine development in a comparative approach. Finally, although progress is being made, there is still scope for research to enhance the delivery and immunogenicity of DNA vaccines.

Author Contributions: Conceptualization, A.F.A. and J.M.D.; writing—original draft preparation, A.F.A.; writing—review and editing, A.F.A.; visualization, AM.; supervision, J.M.D. All authors have read and agreed to the published version of the manuscript.

Funding: A.F.A. is funded by an Egyptian Ministry of Higher Education scholarship.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Salyer, S.J.; Silver, R.; Simone, K.; Barton Behravesh, C. Prioritizing Zoonoses for Global Health Capacity Building-Themes from One Health Zoonotic Disease Workshops in 7 Countries, 2014–2016. *Emerg. Infect. Dis.* **2017**, *23*, S55–S64. [CrossRef] [PubMed]
2. WHO. Cumulative Number of Confirmed Human Cases for Avian Influenza A(H5N1) Reported to WHO, 2003–2023, 5 January 2023. Available online: https://www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a(h5n1)-reported-to-who-2003-2022-5-jan-2023 (accessed on 12 October 2024).
3. Yen, H.L.; Lipatov, A.S.; Ilyushina, N.A.; Govorkova, E.A.; Franks, J.; Yilmaz, N.; Douglas, A.; Hay, A.; Krauss, S.; Rehg, J.E.; et al. Inefficient transmission of H5N1 influenza viruses in a ferret contact model. *J. Virol.* **2007**, *81*, 6890–6898. [CrossRef] [PubMed]
4. Paget, J.; Spreeuwenberg, P.; Charu, V.; Taylor, R.J.; Iuliano, A.D.; Bresee, J.; Simonsen, L.; Viboud, C. Global mortality associated with seasonal influenza epidemics: New burden estimates and predictors from the GLaMOR Project. *J. Glob. Health* **2019**, *9*, 020421. [CrossRef]
5. Cullinane, A.; Newton, J.R. Equine influenza—A global perspective. *Vet. Microbiol.* **2013**, *167*, 205–214. [CrossRef]
6. Walker, P.J.; Siddell, S.G.; Lefkowitz, E.J.; Mushegian, A.R.; Adriaenssens, E.M.; Dempsey, D.M.; Dutilh, B.E.; Harrach, B.; Harrison, R.L.; Hendrickson, R.C.; et al. Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). *Arch. Virol.* **2020**, *165*, 2737–2748. [CrossRef]

7. Pleschka, S. Overview of Influenza Viruses. In *Swine Influenza*; Richt, J.A., Webby, R.J., Eds.; Springer: Berlin/Heidelberg, Germany, 2013; pp. 1–20.
8. Fuller, T.L.; Gilbert, M.; Martin, V.; Cappelle, J.; Hosseini, P.; Njabo, K.Y.; Abdel Aziz, S.; Xiao, X.; Daszak, P.; Smith, T.B. Predicting hotspots for influenza virus reassortment. *Emerg. Infect. Dis.* **2013**, *19*, 581–588. [CrossRef]
9. Petrova, V.N.; Russell, C.A. The evolution of seasonal influenza viruses. *Nat. Rev. Microbiol.* **2018**, *16*, 47–60. [CrossRef] [PubMed]
10. Murcia, P.R.; Wood, J.L.N.; Holmes, E.C. Genome-Scale Evolution and Phylodynamics of Equine H3N8 Influenza A Virus. *J. Virol.* **2011**, *85*, 5312–5322. [CrossRef]
11. Daly, J.M.; Newton, J.R.; Mumford, J.A. Current perspectives on control of equine influenza. *Vet. Res.* **2004**, *35*, 411–423. [CrossRef]
12. Lai, A.C.K.; Chambers, T.M.; Holland, J.R.E.; Morley, P.S.; Haines, D.M.; Townsend, H.G.G.; Barrandeguy, M. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. *Arch. Virol.* **2001**, *146*, 1063–1074. [CrossRef]
13. Wong, S.S.; Webby, R.J. Traditional and new influenza vaccines. *Clin. Microbiol. Rev.* **2013**, *26*, 476–492. [CrossRef] [PubMed]
14. Thangavel, R.R.; Bouvier, N.M. Animal models for influenza virus pathogenesis, transmission, and immunology. *J. Immunol. Methods* **2014**, *410*, 60–79. [CrossRef] [PubMed]
15. Pflug, A.; Guilligay, D.; Reich, S.; Cusack, S. Structure of influenza A polymerase bound to the viral RNA promoter. *Nature* **2014**, *516*, 355–360. [CrossRef]
16. Guillot, L.; Le Goffic, R.; Bloch, S.; Escriou, N.; Akira, S.; Chignard, M.; Si-Tahar, M. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* **2005**, *280*, 5571–5580. [CrossRef] [PubMed]
17. Figueiredo, M.D.; Vandenplas, M.L.; Hurley, D.J.; Moore, J.N. Differential induction of MyD88- and TRIF-dependent pathways in equine monocytes by Toll-like receptor agonists. *Vet. Immunol. Immunopathol.* **2009**, *127*, 125–134. [CrossRef]
18. Kawai, T.; Akira, S. The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. *Nat. Immunol.* **2010**, *11*, 373–384. [CrossRef]
19. Brown, M.G.; Dokun, A.O.; Heusel, J.W.; Smith, H.R.C.; Beckman, D.L.; Blattenberger, E.A.; Dubbelde, C.E.; Stone, L.R.; Scalzo, A.A.; Yokoyama, W.M. Vital Involvement of a Natural Killer Cell Activation Receptor in Resistance to Viral Infection. *Science* **2001**, *292*, 934–937. [CrossRef]
20. Aranda-Romo, S.; Garcia-Sepulveda, C.A.; Comas-García, A.; Lovato-Salas, F.; Salgado-Bustamante, M.; Gómez-Gómez, A.; Noyola, D.E. Killer-cell immunoglobulin-like receptors (KIR) in severe A (H1N1) 2009 influenza infections. *Immunogenetics* **2012**, *64*, 653–662. [CrossRef]
21. Mendelson, M.; Tekoah, Y.; Zilka, A.; Gershoni-Yahalom, O.; Gazit, R.; Achdout, H.; Bovin, N.V.; Meningher, T.; Mandelboim, M.; Mandelboim, O.; et al. NKp46 O-glycan sequences that are involved in the interaction with hemagglutinin type 1 of influenza virus. *J. Virol.* **2010**, *84*, 3789–3797. [CrossRef]
22. Noronha, L.E.; Harman, R.M.; Wagner, B.; Antczak, D.F. Generation and characterization of monoclonal antibodies to equine NKp46. *Vet. Immunol. Immunopathol.* **2012**, *147*, 60–68. [CrossRef]
23. Tumpey, T.M.; García-Sastre, A.; Taubenberger, J.K.; Palese, P.; Swayne, D.E.; Pantin-Jackwood, M.J.; Schultz-Cherry, S.; Solórzano, 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]
24. Gibbings, S.L.; Thomas, S.M.; Atif, S.M.; McCubbrey, A.L.; Desch, A.N.; Danhorn, T.; Leach, S.M.; Bratton, D.L.; Henson, P.M.; Janssen, W.J.; et al. Three Unique Interstitial Macrophages in the Murine Lung at Steady State. *Am. J. Respir. Cell Mol. Biol.* **2017**, *57*, 66–76. [CrossRef] [PubMed]
25. Karagianni, A.E.; Lisowski, Z.M.; Hume, D.A.; Scott Pirie, R. The equine mononuclear phagocyte system: The relevance of the horse as a model for understanding human innate immunity. *Equine Vet. J.* **2021**, *53*, 231–249. [CrossRef] [PubMed]
26. Aharonson-Raz, K.; Singh, B. Pulmonary intravascular macrophages and endotoxin-induced pulmonary pathophysiology in horses. *Can. J. Vet. Res.* **2010**, *74*, 45–49.
27. Brain, J.D.; Molina, R.M.; DeCamp, M.M.; Warner, A.E. Pulmonary intravascular macrophages: Their contribution to the mononuclear phagocyte system in 13 species. *Am. J. Physiol.* **1999**, *276*, L146–L154. [CrossRef]
28. Sellers, S.A.; Hagan, R.S.; Hayden, F.G.; Fischer, W.A., 2nd. The hidden burden of influenza: A review of the extra-pulmonary complications of influenza infection. *Influenza Other Respir. Viruses* **2017**, *11*, 372–393. [CrossRef]
29. Holt, P.G.; Strickland, D.H.; Wikström, M.E.; Jahnsen, F.L. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* **2008**, *8*, 142–152. [CrossRef] [PubMed]
30. GeurtsvanKessel, C.H.; Bergen, I.M.; Muskens, F.; Boon, L.; Hoogsteden, H.C.; Osterhaus, A.D.M.E.; Rimmelzwaan, G.F.; Lambrecht, B.N. Both Conventional and Interferon Killer Dendritic Cells Have Antigen-Presenting Capacity during Influenza Virus Infection. *PLoS ONE* **2009**, *4*, e7187. [CrossRef]
31. Moyo, N.A.; Marchi, E.; Steinbach, F. Differentiation and activation of equine monocyte-derived dendritic cells are not correlated with CD206 or CD83 expression. *Immunology* **2013**, *139*, 472–483. [CrossRef]
32. Mellman, I.; Turley, S.J.; Steinman, R.M. Antigen processing for amateurs and professionals. *Trends Cell Biol.* **1998**, *8*, 231–237. [CrossRef]
33. Apostolopoulos, V.; Thalhammer, T.; Tzakos, A.G.; Stojanovska, L. Targeting antigens to dendritic cell receptors for vaccine development. *J. Drug Deliv.* **2013**, *2013*, 869718. [CrossRef] [PubMed]

34. Schmitt, C.; Cogné, M.; Agrapart, M.; Ballet, J.J. Major histocompatibility complex restriction of tetanus toxoid-specific human T lymphocyte clones. *Eur. J. Immunol.* **1984**, *14*, 1131–1136. [CrossRef] [PubMed]
35. Shu, U.; Kiniwa, M.; Wu, C.Y.; Maliszewski, C.; Vezzio, N.; Hakimi, J.; Gately, M.; Delespesse, G. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur. J. Immunol.* **1995**, *25*, 1125–1128. [CrossRef] [PubMed]
36. Romagnani, S. Th1/Th2 cells. *Inflamm. Bowel Dis.* **1999**, *5*, 285–294. [CrossRef] [PubMed]
37. Ho, A.W.; Prabhu, N.; Betts, R.J.; Ge, M.Q.; Dai, X.; Hutchinson, P.E.; Lew, F.C.; Wong, K.L.; Hanson, B.J.; Macary, P.A.; et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. *J. Immunol.* **2011**, *187*, 6011–6021. [CrossRef]
38. Allie, S.R.; Randall, T.D. Pulmonary immunity to viruses. *Clin. Sci.* **2017**, *131*, 1737–1762. [CrossRef]
39. McGee, M.C.; Huang, W. Evolutionary conservation and positive selection of influenza A nucleoprotein CTL epitopes for universal vaccination. *J. Med. Virol.* **2022**, *94*, 2578–2587. [CrossRef]
40. Rangel-Moreno, J.; Carragher, D.M.; Misra, R.S.; Kusser, K.; Hartson, L.; Moquin, A.; Lund, F.E.; Randall, T.D. B cells promote resistance to heterosubtypic strains of influenza via multiple mechanisms. *J. Immunol.* **2008**, *180*, 454–463. [CrossRef]
41. Seibert, C.W.; Rahmat, S.; Krause, J.C.; Eggink, D.; Albrecht, R.A.; Goff, P.H.; Krammer, F.; Duty, J.A.; Bouvier, N.M.; García-Sastre, A.; et al. Recombinant IgA is sufficient to prevent influenza virus transmission in guinea pigs. *J. Virol.* **2013**, *87*, 7793–7804. [CrossRef]
42. Krammer, F. The human antibody response to influenza A virus infection and vaccination. *Nat. Rev. Immunol.* **2019**, *19*, 383–397. [CrossRef]
43. Lewis, M.J.; Wagner, B.; Woof, J.M. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. *Mol. Immunol.* **2008**, *45*, 818–827. [CrossRef] [PubMed]
44. Jegaskanda, S.; Vandenberg, K.; Laurie, K.L.; Loh, L.; Kramski, M.; Winnall, W.R.; Kedzierska, K.; Rockman, S.; Kent, S.J. Cross-Reactive Influenza-Specific Antibody-Dependent Cellular Cytotoxicity in Intravenous Immunoglobulin as a Potential Therapeutic Against Emerging Influenza Viruses. *J. Infect. Dis.* **2014**, *210*, 1811–1822. [CrossRef] [PubMed]
45. Gasparini, R.; Amicizia, D.; Lai, P.L.; Panatto, D. Influenza vaccination: From epidemiological aspects and advances in research to dissent and vaccination policies. *J. Prev. Med. Hyg.* **2016**, *57*, E1–E4. [PubMed]
46. Perkins, N.R.; Webster, W.R.; Wright, T.; Denney, I.; Links, I. Vaccination program in the response to the 2007 equine influenza outbreak in Australia. *Aust. Vet. J.* **2011**, *89* (Suppl. S1), 126–134. [CrossRef] [PubMed]
47. Paillot, R. A Systematic Review of Recent Advances in Equine Influenza Vaccination. *Vaccines* **2014**, *2*, 797–831. [CrossRef]
48. Grohskopf, L.A.; Alyanak, E.; Ferdinands, J.M.; Broder, K.R.; Blanton, L.H.; Talbot, H.K.; Fry, A.M. Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices, United States, 2021–2022 Influenza Season. *MMWR Recomm. Rep.* **2021**, *70*, 1–28. [CrossRef]
49. Pavulraj, S.; Bergmann, T.; Trombetta, C.M.; Marchi, S.; Montomoli, E.; Alami, S.S.E.; Ragni-Alunni, R.; Osterrieder, N.; Azab, W. Immunogenicity of Calvenza-03 EIV/EHV(®) Vaccine in Horses: Comparative In Vivo Study. *Vaccines* **2021**, *9*, 166. [CrossRef]
50. Fontaine, M.; Fontaine, M.P. Recent progress in influenza virus research and outlook for the control of equine influenza. In *Equine Infectious Diseases: 3rd International Conference, Paris 1972*; Bryans, J.T., Gerber, H., Eds.; S. Karger AG: Berlin, Germany, 1974; pp. 487–502.
51. Barberis, I.; Myles, P.; Ault, S.K.; Bragazzi, N.L.; Martini, M. History and evolution of influenza control through vaccination: From the first monovalent vaccine to universal vaccines. *J. Prev. Med. Hyg.* **2016**, *57*, E115–E120.
52. Chambers, B.S.; Parkhouse, K.; Ross, T.M.; Alby, K.; Hensley, S.E. Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014–2015 Influenza Season. *Cell Rep.* **2015**, *12*, 1–6. [CrossRef]
53. Said, A.W.; Kodani, M.; Usui, T.; Fujimoto, Y.; Ito, T.; Yamaguchi, T. Molecular changes associated with adaptation of equine influenza H3N8 virus in embryonated chicken eggs. *J. Vet. Med. Sci.* **2011**, *73*, 545–548. [CrossRef]
54. Zost, S.J.; Parkhouse, K.; Gumina, 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]
55. Gaglani, M.; Pruszynski, J.; Murthy, K.; Clipper, L.; Robertson, A.; Reis, M.; Chung, J.R.; Piedra, P.A.; Avadhanula, V.; Nowalk, M.P.; et al. Influenza Vaccine Effectiveness Against 2009 Pandemic Influenza A(H1N1) Virus Differed by Vaccine Type During 2013–2014 in the United States. *J. Infect. Dis.* **2016**, *213*, 1546–1556. [CrossRef]
56. Bandell, A.; Ambrose, C.S.; Maniaci, J.; Wojtczak, H. Safety of live attenuated influenza vaccine (LAIV) in children and adults with asthma: A systematic literature review and narrative synthesis. *Expert. Rev. Vaccines* **2021**, *20*, 717–728. [CrossRef] [PubMed]
57. Lunn, D.P.; Hussey, S.; Sebing, R.; Rushlow, K.E.; Radecki, S.V.; Whitaker-Dowling, P.; Youngner, J.S.; Chambers, T.M.; Holland, R.E., Jr.; Horohov, D.W.; et al. Safety, efficacy, and immunogenicity of a modified-live equine influenza virus vaccine in ponies after induction of exercise-induced immunosuppression. *J. Am. Vet. Med. Assoc.* **2001**, *218*, 900–906. [CrossRef]
58. Kannegieter, N.J.; Frogley, A.; Crispe, E.; Kirkland, P.D. Clinical outcomes and virology of equine influenza in a naïve population and in horses infected soon after receiving one dose of vaccine. *Aust. Vet. J.* **2011**, *89* (Suppl. S1), 139–142. [CrossRef]
59. Minke, J.M.; El-Hage, C.M.; Tazawa, P.; Homer, D.; Lemaitre, L.; Cozette, V.; Gilkerson, J.R.; Kirkland, P.D. Evaluation of the response to an accelerated immunisation schedule using a canarypox-vectored equine influenza vaccine, shortened interdose intervals and vaccination of young foals. *Aust. Vet. J.* **2011**, *89* (Suppl. S1), 137–139. [CrossRef]

60. Paillot, R.; Kydd, J.H.; Sindle, T.; Hannant, D.; Edlund Toulemonde, C.; Audonnet, J.C.; Audonnet, J.C.; Minke, J.M.; Daly, J.M. Antibody and IFN-gamma responses induced by a recombinant canarypox vaccine and challenge infection with equine influenza virus. *Vet. Immunol. Immunopathol.* **2006**, *112*, 225–233. [CrossRef] [PubMed]
61. Kreijtz, J.H.; Suezer, Y.; de Mutsert, G.; van den Brand, J.M.; van Amerongen, G.; Schnierle, B.S.; Kuiken, T.; Fouchier, R.A.; Löwer, J.; Osterhaus, A.D.; et al. Preclinical evaluation of a modified vaccinia virus Ankara (MVA)-based vaccine against influenza A/H5N1 viruses. *Vaccine* **2009**, *27*, 6296–6299. [CrossRef]
62. Van Kampen, K.R.; Shi, Z.; Gao, P.; Zhang, J.; Foster, K.W.; Chen, D.T.; Marks, D.; Elmets, C.A.; Tang, D.C. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine* **2005**, *23*, 1029–1036. [CrossRef]
63. Becker, T.; Elbahesh, H.; Reperant, L.A.; Rimmelzwaan, G.F.; Osterhaus, A.D.M.E. Influenza Vaccines: Successes and Continuing Challenges. *J. Infect. Dis.* **2021**, *224* (Suppl. S4), S405–S419. [CrossRef]
64. Krammer, F.; Palese, P. Advances in the development of influenza virus vaccines. *Nat Rev Drug Discov.* **2015**, *14*, 167–182. [CrossRef] [PubMed]
65. Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-based therapeutics—Developing a new class of drugs. *Nat. Rev. Drug Discov.* **2014**, *13*, 759–780. [CrossRef] [PubMed]
66. Babiuk, L.A.; Pontarollo, R.; Babiuk, S.; Loehr, B.; van Drunen Littel-van den Hurk, S. Induction of immune responses by DNA vaccines in large animals. *Vaccine.* **2003**, *21*, 649–658. [CrossRef] [PubMed]
67. Stitz, L.; Vogel, A.; Schnee, M.; Voss, D.; Rauch, S.; Mutzke, T.; Ketterer, T.; Kramps, T.; Petsch, B. A thermostable messenger RNA based vaccine against rabies. *PLoS Neglect. Trop. Dis.* **2017**, *11*, e0006108. [CrossRef] [PubMed]
68. Alberer, M.; Gnad-Vogt, U.; Hong, H.S.; Mehr, K.T.; Backert, L.; Finak, G.; Gottardo, R.; Bica, M.A.; Garofano, A.; Koch, S.D.; et al. Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: An open-label, non-randomised, prospective, first-in-human phase 1 clinical trial. *Lancet* **2017**, *390*, 1511–1520. [CrossRef]
69. Pollard, C.; Rejman, J.; De Haes, W.; Verrier, B.; Van Gulck, E.; Naessens, T.; De Smedt, S.; Bogaert, P.; Grooten, J.; Vanham, G.; et al. Type I IFN counteracts the induction of antigen-specific immune responses by lipid-based delivery of mRNA vaccines. *Mol. Ther.* **2013**, *21*, 251–259. [CrossRef]
70. Kowalski, P.S.; Rudra, A.; Miao, L.; Anderson, D.G. Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery. *Mol. Ther.* **2019**, *27*, 710–728. [CrossRef]
71. Blanco, E.; Shen, H.; Ferrari, M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat. Biotechnol.* **2015**, *33*, 941–951. [CrossRef]
72. Baden, L.R.; El Sahly, H.M.; Essink, B.; Kotloff, K.; Frey, S.; Novak, R.; Diemert, D.; Spector, S.A.; Rouphael, N.; Creech, C.B.; et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N. Engl. J. Med.* **2021**, *384*, 403–416. [CrossRef]
73. Polack, F.P.; Thomas, S.J.; Kitchin, N.; Absalon, J.; Gurtman, A.; Lockhart, S.; Perez, J.L.; Pérez Marc, G.; Moreira, E.D.; Zerbini, C.; et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **2020**, *383*, 2603–2615. [CrossRef]
74. Arevalo, C.P.; Bolton, M.J.; Le Sage, V.; Ye, N.; Furey, C.; Muramatsu, H.; Alameh, M.G.; Pardi, N.; Drapeau, E.M.; Parkhouse, K.; et al. A multivalent nucleoside-modified mRNA vaccine against all known influenza virus subtypes. *Science* **2022**, *378*, 899–904. [CrossRef] [PubMed]
75. Feng, J.Y.; Johnson, A.A.; Johnson, K.A.; Anderson, K.S. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. *J. Biol. Chem.* **2001**, *276*, 23832–23837. [CrossRef]
76. Moyle, G. Toxicity of antiretroviral nucleoside and nucleotide analogues: Is mitochondrial toxicity the only mechanism? *Drug Saf.* **2000**, *23*, 467–481. [CrossRef] [PubMed]
77. Kutzler, M.A.; Weiner, D.B. DNA vaccines: Ready for prime time? *Nat. Rev. Genet.* **2008**, *9*, 776–788. [CrossRef]
78. Douville, R.N.; Nath, A. Human endogenous retroviruses and the nervous system. *Handb. Clin. Neurol.* **2014**, *123*, 465–485.
79. Boyle, J.S.; Brady, J.L.; Lew, A.M. Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature* **1998**, *392*, 408–411. [CrossRef] [PubMed]
80. Corr, M.; Lee, D.J.; Carson, D.A.; Tighe, H. Gene vaccination with naked plasmid DNA: Mechanism of CTL priming. *J. Exp. Med.* **1996**, *184*, 1555–1560. [CrossRef]
81. Meeusen, E.N.T.; Walker, J.; Peters, A.; Pastoret, P.-P.; Jungersen, G. Current Status of Veterinary Vaccines. *Clin. Microbiol. Rev.* **2007**, *20*, 489–510. [CrossRef] [PubMed]
82. Ledgerwood, J.E.; Pierson, T.C.; Hubka, S.A.; Desai, N.; Rucker, S.; Gordon, I.J.; Enama, M.E.; Nelson, S.; Nason, M.; Gu, W.; et al. A West Nile virus DNA vaccine utilizing a modified promoter induces neutralizing antibody in younger and older healthy adults in a phase I clinical trial. *J. Infect. Dis.* **2011**, *203*, 1396–1404. [CrossRef]
83. Gaudinski, M.R.; Houser, K.V.; Morabito, K.M.; Hu, Z.; Yamshchikov, G.; Rothwell, R.S.; Berkowitz, N.; Mendoza, F.; Saunders, J.G.; Novik, L.; et al. Safety, tolerability, and immunogenicity of two Zika virus DNA vaccine candidates in healthy adults: Randomised, open-label, phase 1 clinical trials. *Lancet* **2018**, *391*, 552–562. [CrossRef]
84. Hobernik, D.; Bros, M. DNA Vaccines-How Far from Clinical Use? *Int. J. Mol. Sci.* **2018**, *19*, 3605. [CrossRef] [PubMed]
85. Ulmer, J.B.; Donnelly, J.J.; Parker, S.E.; Rhodes, G.H.; Felgner, P.L.; Dwarki, V.J.; Gromkowski, S.H.; Deck, R.R.; DeWitt, C.M.; Friedman, A. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **1993**, *259*, 1745–1749. [CrossRef] [PubMed]
86. Fynan, E.F.; Webster, R.G.; Fuller, D.H.; Haynes, J.R.; Santoro, J.C.; Robinson, H.L. DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11478–11482. [CrossRef] [PubMed]

87. Andersen, T.K.; Bodin, J.; Oftung, F.; Bogen, B.; Mjaaland, S.; Grodeland, G. Pandemic Preparedness Against Influenza: DNA Vaccine for Rapid Relief. *Front. Immunol.* **2021**, *12*, 747032. [CrossRef]
88. van der Laan, J.W.; Herberts, C.; Lambkin-Williams, R.; Boyers, A.; Mann, A.J.; Oxford, J. Animal models in influenza vaccine testing. *Expert. Rev. Vaccines* **2008**, *7*, 783–793. [CrossRef]
89. Hirst, G.K. Studies on the Mechanism of Adaptation of Influenza Virus to Mice. *J. Exp. Med.* **1947**, *86*, 357–366. [CrossRef]
90. Lee, L.Y.Y.; Izzard, L.; Hurt, A.C. A Review of DNA Vaccines Against Influenza. *Front. Immunol.* **2018**, *9*, 1568. [CrossRef]
91. Olsen, C.W. DNA vaccination against influenza viruses: A review with emphasis on equine and swine influenza. *Vet. Microbiol.* **2000**, *74*, 149–164. [CrossRef] [PubMed]
92. Bennink, J.R.; Yewdell, J.W. Murine cytotoxic T lymphocyte recognition of individual influenza virus proteins. High frequency of nonresponder MHC class I alleles. *J. Exp. Med.* **1988**, *168*, 1935–1939. [CrossRef]
93. Tamura, S.; Tanimoto, T.; Kurata, T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. *Jpn. J. Infect. Dis.* **2005**, *58*, 195–207. [CrossRef]
94. Stejskalova, K.; Janova, E.; Splichalova, P.; Futas, J.; Oppelt, J.; Vodicka, R.; Horin, P. Twelve toll-like receptor (TLR) genes in the family Equidae—Comparative genomics, selection and evolution. *Vet. Res. Commun.* **2023**, *48*, 725–741. [CrossRef] [PubMed]
95. Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; et al. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745. [CrossRef] [PubMed]
96. Kayraklioglu, N.; Horuluoglu, B.; Klinman, D.M. CpG Oligonucleotides as Vaccine Adjuvants. In *DNA Vaccines: Methods and Protocols*; Sousa, Â., Ed.; Springer: New York, NY, USA, 2021; pp. 51–85.
97. Schneberger, D.; Caldwell, S.; Suri, S.S.; Singh, B. Expression of toll-like receptor 9 in horse lungs. *Anat. Rec.* **2009**, *292*, 1068–1077. [CrossRef]
98. Zhang, Y.W.; Davis, E.G.; Blecha, F.; Wilkerson, M.J. Molecular cloning and characterization of equine Toll-like receptor 9. *Vet. Immunol. Immunopathol.* **2008**, *124*, 209–219. [CrossRef]
99. Garrett, D.; Montesso, F.; Fougerolle, S.; Lopez-Alvarez, M.R.; Birand, I.; De Bock, M.; Huang, C.M.; Legrand, L.; Pronost, S.; Paillot, R. Refinement of the equine influenza model in the natural host: A meta-analysis to determine the benefits of individual nebulisation for experimental infection and vaccine evaluation in the face of decreased strain pathogenicity. *Vet. Microbiol.* **2017**, *211*, 150–159. [CrossRef] [PubMed]
100. Zaman, M.; Chandrudu, S.; Toth, I. Strategies for intranasal delivery of vaccines. *Drug Deliv. Transl. Res.* **2013**, *3*, 100–109. [CrossRef]
101. McAuley, J.L.; Gilbertson, B.P.; Trifkovic, S.; Brown, L.E.; McKimm-Breschkin, J.L. Influenza Virus Neuraminidase Structure and Functions. *Front. Microbiol.* **2019**, *10*, 39. [CrossRef]
102. Couch, R.B.; Kasel, J.A.; Gerin, J.L.; Schulman, J.L.; Kilbourne, E.D. Induction of Partial Immunity to Influenza by a Neuraminidase-specific Vaccine. *J. Infect. Dis.* **1974**, *129*, 411–420. [CrossRef] [PubMed]
103. Johansson, B.E.; Moran, T.M.; Kilbourne, E.D. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6869–6873. [CrossRef]
104. Okuya, K.; Yoshida, R.; Manzoor, R.; Saito, S.; Suzuki, T.; Sasaki, M.; Saito, T.; Kida, Y.; Mori-Kajihara, A.; Kondoh, T.; et al. Potential Role of Nonneutralizing IgA Antibodies in Cross-Protective Immunity against Influenza A Viruses of Multiple Hemagglutinin Subtypes. *J. Virol.* **2020**, *94*, e00408-20. [CrossRef]
105. Park, K.S.; Seo, Y.B.; Lee, J.Y.; Im, S.J.; Seo, S.H.; Song, M.S.; Choi, Y.K.; Sung, Y.C. Complete protection against a H5N2 avian influenza virus by a DNA vaccine expressing a fusion protein of H1N1 HA and M2e. *Vaccine* **2011**, *29*, 5481–5487. [CrossRef] [PubMed]
106. Heinen, P.P.; Rijsewijk, F.A.; de Boer-Luijtze, E.A.; Bianchi, A.T.J. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. *J. Gen. Virol.* **2002**, *83 Pt 8*, 1851–1859. [CrossRef] [PubMed]
107. Rajao, D.S.; Vincent, A.L. Swine as a Model for Influenza A Virus Infection and Immunity. *ILAR J.* **2015**, *56*, 44–52. [CrossRef] [PubMed]
108. Naveed, A.; Eertink, L.G.; Wang, D.; Li, F. Lessons Learned from West Nile Virus Infection:Vaccinations in Equines and Their Implications for One Health Approaches. *Viruses* **2024**, *16*, 781. [CrossRef] [PubMed]
109. Badenhorst, M.; Saalmüller, A.; Daly, J.M.; Ertl, R.; Stadler, M.; Puff, C.; de le Roi, M.; Baumgärtner, W.; Engelmann, M.; Brandner, S.; et al. An Equine Model for Vaccination against a Hepacivirus: Insights into Host Responses to E2 Recombinant Protein Vaccination and Subsequent Equine Hepacivirus Inoculation. *Viruses* **2022**, *14*, 1401. [CrossRef]
110. Shafaati, M.; Saidijam, M.; Soleimani, M.; Hazrati, F.; Mirzaei, R.; Amirheidari, B.; Tanzadehpanah, H.; Karampoor, S.; Kazemi, S.; Yavari, B.; et al. A brief review on DNA vaccines in the era of COVID-19. *Future Virol.* **2021**, *17*, 49–66. [CrossRef]
111. Nguyen, T.N.; Lai, D.C.; Sillman, S.; Petro-Turnquist, E.; Weaver, E.A.; Vu, H.L.X. Lipid nanoparticle-encapsulated DNA vaccine confers protection against swine and human-origin H1N1 influenza viruses. *mSphere* **2024**, *9*, e00283-24. [CrossRef]