Enhancing protective efficacy of avian influenza vaccines through targeted delivery of protective antigens to chicken immune cells



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Declarations

I declare that the work presented in this thesis entitled “Enhancing protective efficacy of avian influenza vaccines through targeted delivery of protective antigens to chicken immune cells” is entirely my work, except in those instances where the contribution of others has been acknowledged. Any materials quoted, referenced, or paraphrased from other sources has been clearly indicated and its origins cited. Some of the work presented in Chapter 5 (analysis of vaccine immunogenicity in H9HA-specific maternally derived antibodies (MDA) positive chickens) was done at MSD Animal Health, The Netherlands. Rick Meeuws (MSD Animal Health) carried out blood sampling for the entire duration of the MDA study, and the serum samples were shipped to the Pirbright institute where I carried out all the analysis. For the MDA animal study, Nobilis® Influenza H9N2 + ND and Nobilis® H9N2+ND P vaccines (MSD Animal Health) were also included for comparison. However, due to the commercial confidentiality, data for the commercial vaccines could not be presented in this thesis. The recombinant Herpesvirus of Turkey vector vaccines were generated with the assistance of Dr. Pengxiang Chang (The Pirbright institute).

I hereby confirm that this thesis has not been submitted for any other qualification or degree at this university or any other institution.

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Thesis Abstract

Avian influenza viruses (AIVs) are highly contagious viruses that are widespread in birds. Wild aquatic birds (e.g., waterfowls, gulls, and shorebirds) are their natural hosts. However, they can be easily transmitted between different bird species and infect gallinaceous poultry including chickens, quails, and turkeys, resulting in severe economic losses. AIVs also pose a significant zoonotic and pandemic threats. Most of the commercially available avian influenza vaccines for poultry are inactivated whole virus vaccines that have several drawbacks including sub-optimal immunogenicity, need for multiple dose administration and interference from maternally derived antibodies (MDAs). Here, the immunogenicity and protective efficacy of recombinant subunit poultry AIV vaccine were enhanced by selective delivery of antigens to the professional antigen presenting cells (APCs) like dendritic cells and macrophages. This led us to the development of targeted antigen delivery vaccines whereby the haemagglutinin (HA) protein of H9N2 AIV was fused to single chain fragment variable antibodies (scFv) specific to chicken Dec205, CD11c and CD83 receptors. Out of the three targeted antigens tested, CD83 targeting induced higher serum haemagglutinin inhibition and virus neutralising antibodies compared to the untargeted H9HA and inactivated H9N2 vaccine. Furthermore, chickens vaccinated with CD83 targeted H9HA showed a reduced virus shedding post challenge compared to the untargeted H9HA, as well as a reduced interference from H9HA-specific MDAs. Overall, CD83 targeted H9HA was able to generate antibodies that were cross-reactive against different heterologous H9N2 viruses. Following on, the recombinant Herpesvirus of Turkey (rHVT) was also assessed for its use as a viral vector for *in vivo* expression of

secreted and native membrane bound forms of H9HA antigen. The antibody titres generated by rHVT expressing native membrane bound H9HA was higher than any of the secreted forms.

Overall, these results will assist in developing the next generation vaccines for preventing animal and human infectious diseases.

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List of abbreviations

AIV	Avian influenza virus
APC	Antigen presenting cells
BCA	Bicinchoninic acid
BS3	Bis(sulfosuccinimidyl)suberate
CD83	Cluster of Differentiation 83
CD11c	Cluster of Differentiation 11c
cDNA	Complementary DNA
CDRs	Complementarity determining regions
CMI	Cell mediated immunity
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
CTLD	C-type lectin domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DC	Dendritic cells
Dec205	Dendritic cell receptor for Endocytosis-205
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
EID_{50}	Egg infectious dose 50

ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immune absorbent spot
FITC	Fluorescein isothiocyanate
F protein	Fusion protein
HA	Haemagglutinin
HA assay	Haemagglutination assay
HDR	Homology directed repair
HI	Haemagglutinin inhibition
HPAI	High pathogenicity avian influenza
HRP	Horseradish peroxidase
HVT	Herpes virus of Turkey
IAV	Influenza A virus
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
IFN	Interferon
Ig	Immunoglobulin
ILTV	Infectious laryngotracheitis virus
kb	Kilobase
kDa	Kilodalton
LPAI	Low pathogenicity avian influenza

LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MDA	Maternally Derived antibodies
MDCK	Madin-Darby Canine Kidney cells
MDV	Marek disease virus
MHC	Major Histocompatibility Complex
MOI	Multiplicity of infection
MNT	Micro-neutralisation
mRNA	Messenger RNA
NA	Neuraminidase
NDV	Newcastle disease virus
NHEJ	Non-homologous end joining
OIE	World Health Organisation for Animal Health
PAMP	Pathogen Associated Molecular Patterns
PCR	Polymerase chain reaction
PE	Phycoerythin
PFU	Plaque forming unit
PRR	Pattern Recognition Receptor
PRV	Pseudorabies virus
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RBC	Red blood cells
RNA	Ribonucleic acid
SA	Sialic acid
scFv	Single chain fragment variable
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPF	Specified pathogen free
TADV	Targeted antibody delivery vaccine
$TCID_{50}$	Tissue culture infectious dose 50
TPCK	Tosyl phenylalanyl chloromethyl ketone
TLR	Toll like receptor
VP2	Viral protein 2
vRNA	Viral RNA

Chapter 1: General introduction

1.1. Influenza viruses

Influenza viruses are members of the *Orthomyxoviridae* family and are divided into four genera, *Influenza* A, B, C and D viruses[1]. *Influenza A* viruses *(*IAVs*)* are the only viruses from this family to have caused pandemic infections[2]. They can infect different animal species, including birds, pigs, horses, seals, and dogs, and are responsible for causing most human seasonal infections. Aquatic birds of the *Anseriformes* and C*haradriiforme*s orders are the primary natural reservoir for most IAVs however, these viruses have also been isolated from bats indicating that they have multiple reservoir hosts[1]. The nomenclature for influenza A subtypes is based on the combination of the viral glycoproteins, haemagglutinin (HA) and neuraminidase (NA) e.g., H1N1 or H9N2. To date, 16 HA and 9 NA subtypes have been identified circulating within the aquatic bird reservoir. However, recently two further HA and NA subtypes have been identified in bats (H17 and H18, N10 and N11)[3,4]. *Influenza B* viruses are only found in humans and seals, and they cause seasonal influenza infection in humans hence, they are constituents of human seasonal influenza vaccines[5]. *Influenza C* viruses have been found in humans and typically cause mild or asymptomatic respiratory disease, primarily in children[6]. *Influenza D viruses*, which infect cattle and swine, have only recently been characterised and are more closely related to *Influenza C* viruses[7]. From this point only IAVs will be discussed.

1.1.1. Influenza virus genome

The influenza A virus genome is about 13.4 kb in size and comprises of 8 negative sense, single stranded viral RNA (vRNA) segments. These 8 gene segments code for 10 core proteins as well as various accessory proteins[1] (**Table 1**). The 8 segments are numbered in order of decreasing length. The key proteins translated from each gene segment include Polymerase Basic protein 2 (PB2, segment 1), Polymerase Basic protein 1 (PB1, segment 2), Polymerase Acidic protein (PA, segment 3), Haemagglutinin (HA, segment 4), Nucleoprotein (NP, segment 5), Neuraminidase (NA, segment 6), Matrix protein 1 (M1, segment 7) and Non-Structural protein 1 (NS1, segment 8). However, various mechanisms like alternative splicing, frameshifting and alternative translation initiation events allows the expression of two or more proteins from the same gene segment, increasing the coding capacity of the genome[8–10]. For example, segment 7 and 8 both code for splice products; M1 and matrix protein 2 (M2), and NS1 and Nuclear Export protein (NEP), respectively. Each vRNA segment forms a helical hairpin in shape coated by NP and is bound by the heterotrimeric RNA polymerase complex (PB1, PB2 and PA)[11]. Each segment is flanked on its 3- and 5- prime ends by a conserved semi-complementary set of untranslated regions (UTRs) which serves as a promoter for vRNA replication and transcription. These regions also contain mRNA polyadenylation signals and some signals for virus assembly[12,13].

Table 1: **The genomic segments of influenza A viruses and their encoded proteins (modified from Bouvier and Palese[1])**

Segment	Approximate segment length in nucleotides	Encoded proteins	Protein function	Refs
1	~2341	PB2	Polymerase subunit, mRNA cap recognition	14
2	~2341	PB1	Polymerase subunit, RNA elongation, endonuclease activity	15
		PB1-F2	Accessory protein, Pro-apoptotic activity	16
3	~2233	PA	Polymerase subunit, protease activity	17
		PA-X	Accessory protein, host cell shut off	18
4	~1778	HA	Surface glycoprotein, receptor binding and fusion	19
5	~1565	NP	RNA binding protein, nuclear import regulation	12
6	~1413	NA	Surface glycoprotein, sialidase activity, virus release	20
7	~1027	M1	Matrix protein, vRNP interaction, RNA nuclear export regulation, viral budding	21
		M2	Ion channel, virus uncoating and assembly	22
8	~890	NS1	Interferon antagonist	23
		NEP/NS2	Nuclear export of RNA	24

PB1/PB2: Polymerase Basic protein 1 and 2, **PA**: Polymerase Acidic protein, **HA**: Haemagglutinin, **NP**: Nucleoprotein, **NA**: Neuraminidase, **M1/M2**: Matrix protein 1 and 2, **NS1/NS2**: Non-Structural protein 1 and 2, **NEP**: Nuclear Export protein **vRNP**: Viral ribonucleoprotein

1.1.2. Influenza virion structure

Influenza virions are pleomorphic with spherical and filamentous morphologies[25]. Whilst most virions are spherical in shape (100 nm diameter), some filamentous virions are also observed (~20 μm length and 100 nm diameter), particularly in clinical isolates[26]. The exact role of filamentous virion formation still remains unclear however, it is thought that filamentous morphology might facilitate virus spread *in vivo*[27]*.* A diagrammatic representation of influenza virion is shown in **Figure 1.1.** The influenza virion is an enveloped virus whose membrane is derived from the host cell membrane. It contains three virally encoded transmembrane proteins: HA, NA and M2. The most abundant surface glycoprotein is HA followed by NA, usually present in a ratio of 4:1[1]. Both HA and NA proteins protrude from the virion, acting as antigenic determinants, while M2 protein, which functions as a proton channel, transverses the lipid membrane[22]. The M1 protein is the most abundant protein within the virion, and it forms a matrix beneath the viral membrane providing structural integrity to the virion[28]. At the centre of the virion are the 8 gene segments packaged as ribonucleoproteins (RNPs), along with NEP and other cellular factors which vary depending on the originating host species[28,29].

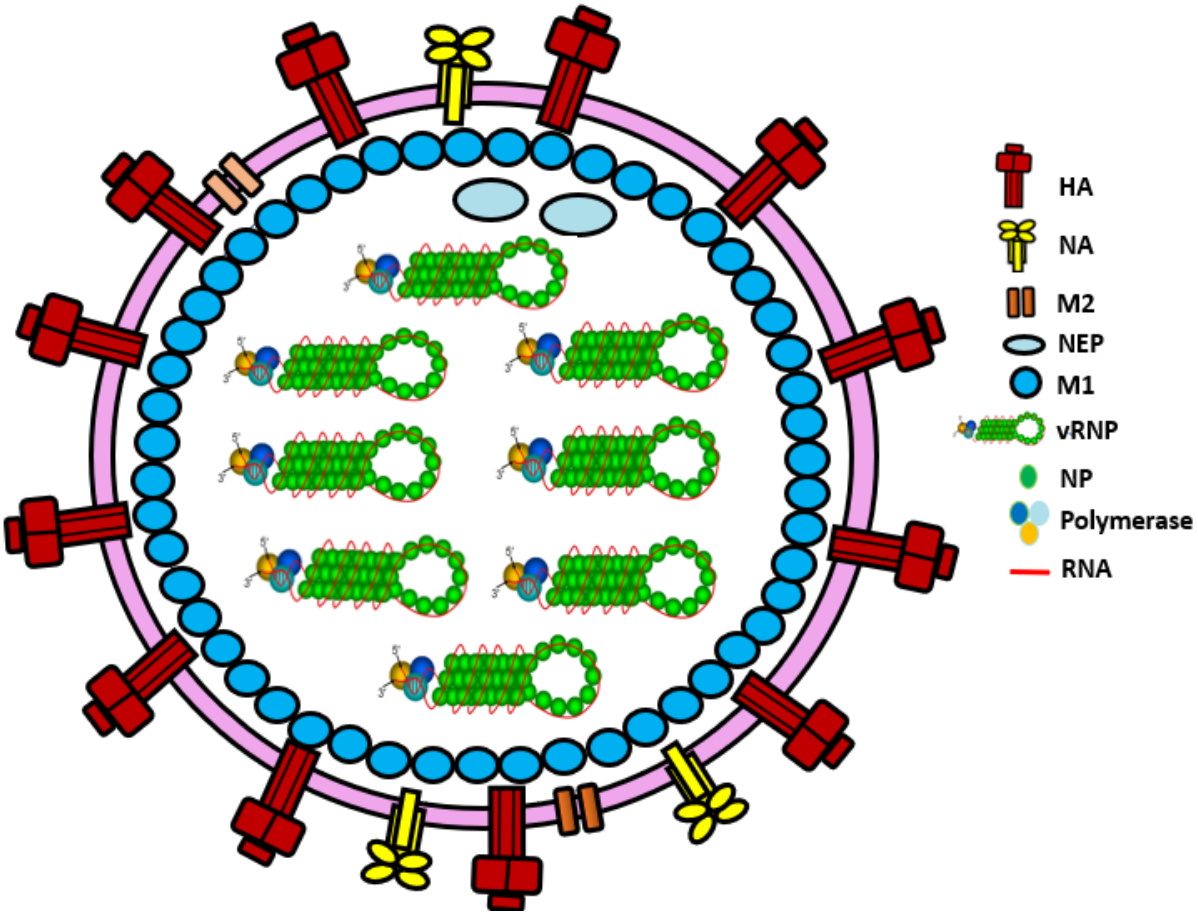


Figure 1.1. Structure of influenza virion. Image inspired from *http://www.virology.ws/2009/04/30/structureof-influenza-virus/*. **HA**: Haemagglutinin **NA**: Neuraminidase **M1/M2**: Matrix protein 1 and 2 **NEP**: Nuclear export protein **vRNP**: Viral ribonucleoprotein **NP**: Nucleoprotein

The influenza virus proteins are described in detail below:

1.1.2.1. Haemagglutinin

Haemagglutinin is the most abundant surface protein making up around 80% of influenza virus surface glycoprotein[30]. Due to its abundance and key role in virus life cycle, it is the main target for the neutralising antibody response. Therefore, it forms a major constituent of recombinant influenza vaccines. It is homotrimer in structure and facilitates receptor binding and membrane fusion activity[31]. Each HA monomer comprises of a head domain and a stalk domain (**Figure 1.2**). The head

domain contains receptor binding sites (RBSs) whereas the stalk domain contains membrane fusion activity[31]. HA is synthesised as an inactive precursor, HA0 whereby three copies of HA0 assemble into a non-covalent trimer[32]. The HA0 is cleaved by host proteases into HA1 and HA2. HA1 contains the entire globular head domain and a part of the stalk domain, whilst HA2 contains the remaining stalk, the fusion peptide, a transmembrane domain (TM) and a short cytoplasmic domain[30]. The RBSs in the head domain of HA have binding specificity for terminal sialic acid (SA). There are two major SA species distributed differentially across hosts, with avian influenza viruses (AIV) preferentially binding SA attached to galactose via an alpha (2,3) linkage (α2,3-SA) and human influenza viruses preferentially recognising SA attached to galactose via an alpha (2,6) linkage (α2,6-SA)[33]. Studies have shown that the switch in SA species preference is the molecular basis for adaptation of influenza viruses from one host to another[34,35].

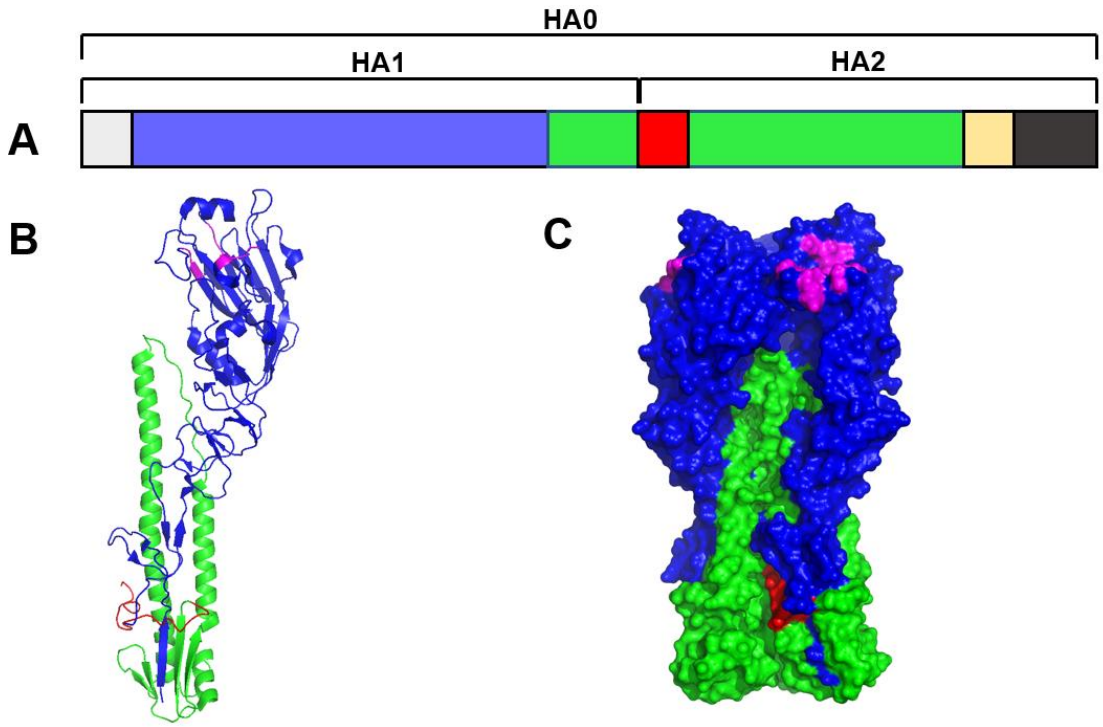


Figure 1.2. Structure of haemagglutinin. **A)** Primary structure of HA: signal sequence (grey), head domain (blue), stalk domain (green), fusion peptide (red), transmembrane domain (yellow) and cytoplasmic domain (black). **B)** Monomeric HA: head domain (blue),

stalk domain (green), fusion peptide (red) and receptor binding sites (magenta). **C)** Trimeric HA: head domain (blue), stalk domain (green), fusion peptide (red) and receptor binding sites (magenta). PDB ID: 1JSD[36]. Structures made using Pymol[37].

1.1.2.2. Neuraminidase

Neuraminidase is the second most abundant influenza surface proteins representing around 20% of the total viral surface glycoprotein[38]. It is homo-tetramer in structure (**Figure 1.3**). Each NA monomer consists of globular head domain facilitating sialidase activity, and stalk and transmembrane domain which functions to anchor the NA to the viral membrane[38]. The main function of NA is to catalyse the cleavage of terminal SA from glycan chains[39]. Therefore, NA has a role in cleaving airway mucins thus, it ensures that the virus reaches and infects their target cells. Furthermore, it also facilitates the cleavage of cellular SA from virion HA, allowing the release of progeny virus from the infected cells. The NA activity also prevents the aggregation of progeny virions and promotes efficient budding[39,40].

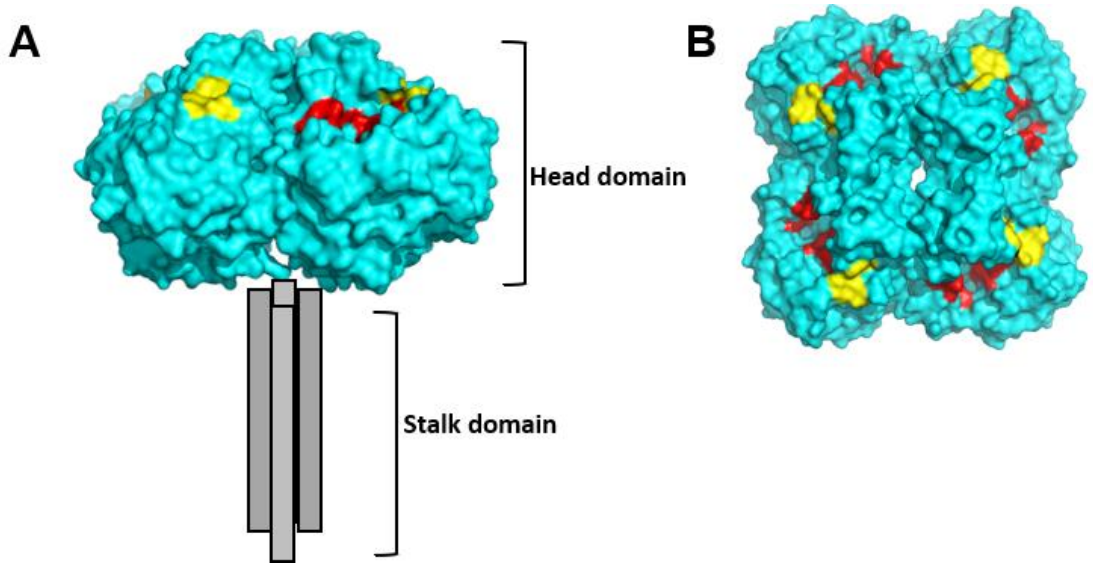


Figure 1.3. Structure of neuraminidase (N2). **A)** Tetrameric NA shown from the side: catalytic sites (red) and secondary sialic acid binding sites (yellow). Proposed schematic of stalk domain shown with grey bars as only the head domain of NA has been successfully

crystallised. **B)** Tetrameric NA shown from above: catalytic sites (red) and secondary sialic acid binding sites (yellow). PDB ID: 4H52[41]. Structures made using Pymol[37].

1.1.2.3. Polymerase and nucleoprotein

The influenza virus RNA-dependent RNA polymerase comprises of three subunits: PB1, PB2 and PA[42]. It is involved in the synthesis of viral messenger RNA (mRNA) and replication of viral genome segments. PB1 is the core of viral polymerase and houses the RNA-binding domain for vRNA and complementary RNA (cRNA). It catalyses the polymerisation of nucleotide during mRNA synthesis and vRNA replication[43]. PB2 is responsible for binding to host pre-mRNA transcripts (particularly small non-coding RNAs) containing a 5' cap (methylated nucleotide). In a process called 'cap-snatching' the endonuclease domain of PA cleaves this 5' capped host pre-mRNA transcripts to initiate viral mRNA synthesis[11,17]. The 5' and 3' ends of RNA are complementary and their binding to PB1 facilitates the formation of panhandle structure, which acts as a promoter for vRNA transcription[12]. PB1 elongates the nascent viral mRNA in the 3' to 5' direction until poly-uridine residues at the 5' end of vRNA is encountered. This leads to polyadenylation of mRNA and transcription termination[44]. All the polymerase components contain nuclear localisation signals (NLS) allowing them to enter the nucleus[43,44].

NP is an RNA binding protein which coats both vRNA and cRNA. It has a net positive charge allowing it to bind to the negatively charged ribose-phosphate backbone of vRNA in a non-specific manner[45].

1.1.2.4. Matrix protein

M1 and M2 matrix proteins are encoded from the same gene segment (segment 7) of influenza genome through alternate splicing. Hence, they share 9 amino acids at the N-terminus[21]. M1 is the most abundant protein within the influenza virion and are multifunctional. M1 proteins form a matrix beneath the lipid membrane and interact with viral RNP complexes via protein-protein interactions. It has a key role in virus assembly and budding[39]. The assembly of virion begins with coalescence of HA and NA proteins in lipid rafts at the apical membrane. M1 then binds to the cytoplasmic tails of HA and NA which enables them to be included into the budding virion[21,39].

On the other hand, M2 is a tetrameric membrane spanning proton (H^+) channel that pumps protons from the cellular endosome into the virion interior, lowering the pH. This facilitates the disassembly of the M1 matrix layer and uncoating of the virus[46]. M2 has been one of the major targets of anti-influenza drugs like amantadine and rimantadine. These antiviral drugs act by inhibiting the proton pumping activity of M2 thus, preventing the release of viral ribonucleoproteins (vRNPs) to the cytoplasm[47].

1.1.2.5. NS1 and NEP

NS1 and NEP are both encoded from segment 8 of influenza genome through alternative splicing and share 10 amino acids at the N-terminus[48]. The NS1 protein has a major role as an antagonist of the host innate immune response. It antagonises interferon alpha/beta (IFN-α/β) dependent responses, limits the production of IFN-β and inhibits the activity of protein kinase R (PKR) and 2′-5′-

oligoadenylate synthetase (OAS)[49,50]. Structurally it forms homodimer with each monomer comprising of two structural domains, a N-terminal RNA binding domain and a C-terminal effector domain. It can target over 50 viral and host proteins[48,51,52].

Moreover, NEP protein is involved in the export of nascent vRNPs from the nucleus to the cytoplasm. The current model of RNP export involves protein-protein interactions between RNP and M1, which in turn binds to the C-terminal of NEP. The N-terminal of cognate NEP contains a nuclear export signal (NES) which facilitates the nuclear export of the RNP-M1-NEP complex[24,51]. Furthermore, NEP also regulates the switch between viral transcription and viral replication and facilitates budding of nascent virions[48].

1.1.2.6. Accessory proteins

There are several additional proteins that are encoded by the 8 genome segments of IAVs, in addition to the 10 core proteins. Various mechanisms like alternative splicing, alternative translation initiation events and ribosomal frameshifting are involved in the synthesis of these accessory proteins. Some of these accessory proteins include PB1-F2, PB1-N40, PA-X, PA-N155, PA-N182, M42 and NS3. The function of many of these accessory proteins is unknown however, PB1-F2 and PA-X have been well characterised[53]. PB1-F2 is produced from an alternative reading frame from the mRNA of segment 2. During translation, some ribosomes miss the PB1 start codon and instead scan further down the mRNA reaching the stronger Kozak sequence of PB1-F2[53]. PB1-F2 exists as either a ‘full-length’ protein (≥87/90 amino acid) or a ‘truncated’ protein (≤79 amino acid)[16]. In chickens, full-length PB1-F2 has been associated with increasing viral shedding times and lengthening the transmission period for infection of a new host[54]. PB1-

F2 has also been associated with pro-apoptotic activity as they can interact with pro-apoptotic factors such as voltage-dependent anion-selective channel 1 (VDAC-1)[55].

PA-X is an accessory protein encoded by segment 3 of influenza genome and is a result of ribosomal frameshifting event[56]. It contains the N-terminal endonuclease domain of PA. PA-X is involved in degrading host mRNAs leading to host shutdown. This is thought to dampen the host antiviral response and free cellular translational machinery to focus on viral protein synthesis[57].

1.1.3. Influenza virus life cycle and vaccine targets

Unlike other non-retroviral RNA viruses, IAVs undergo replication in the host nucleus rather than the cytoplasm[58]. IAVs accomplish this by utilising a combination of viral and cellular machineries to co-ordinate the transport of their proteins and vRNA gene segments in and out of the host nucleus.

1.1.3.1. Virus life cycle

The first step in influenza virus life cycle is attachment to the host cell SA moieties via HA protein (**Figure 1.4**). This is followed by endocytosis of virus into an early endosome by either clathrin-mediated endocytosis or micropinocytosis[58]. The acidic pH of the early endosome (pH ~5.0) induces a conformational change in HA which triggers the fusion peptide to insert itself into the endosome membrane, facilitating the fusion of viral and host endosome membranes[31]. Furthermore, the acidification of endosome activates M2 proton channels which pump protons from the endosome to the virus interior depolymerising M1 viral core and releasing vRNP

to the cytoplasm[46]. The free vRNP then enters the nucleus by interacting with nuclear import machinery, making use of NLS on NP[43]. Once inside the nucleus, vRNP starts to synthesise viral mRNA in a primer-dependent process requiring the 'cap-snatching' activity of PB2 and PA. These caps are cleaved from host RNAs and transferred to the polymerase site for viral mRNA transcription initiation[59]. The nascent viral mRNA is then either directly exported to the cytoplasm for translation by cellular ribosomes, or undergoes splicing using host splicing machinery in the nucleus. The newly synthesised NP and polymerase proteins are imported back to the nucleus from the cytoplasm for use in further viral replication and transcription[60]. At some stage, probably due to the accumulation of NEP, the viral polymerase switches from transcription of viral mRNA to replication of the viral genome[24]. Unlike transcription, vRNA replication does not require a primer. Initially, positive sense RNA complementary to the negative sense vRNA is formed. This cRNA is packaged into complementary viral ribonucleoprotein which acts as a template for the formation of new vRNA. The newly formed vRNA are encapsidated by free NP and polymerase subunits within the nucleus to form vRNPs. The vRNPs form a nuclear assembly complex with chromosomal maintenance 1 (CRM1), NEP and M1 for nuclear export[61]. In the cytoplasm, viral mRNAs are translated into viral proteins by the host translation machinery. HA and NA glycoproteins are shuttled to the host cell membrane via the Golgi apparatus where they associate with lipid raft domains[39]. HA and NA are thought to facilitate the recruitment of other viral proteins and vRNPs[62,63]. Furthermore, M1 is also thought to play an important role during virus assembly and budding since it coalesces HA and NA together in the host cell membrane. In addition, M1 can also recruit M2 and vRNPs to the budding virus[39]. M1 and M2 can mediate membrane scission process. Once membrane

scission has occurred, the virion still remains attached to the cell surface due to HA-SA interactions. NA is responsible for the cleavage of HA-SA linkage allowing the virion to be released from the host cell[40].

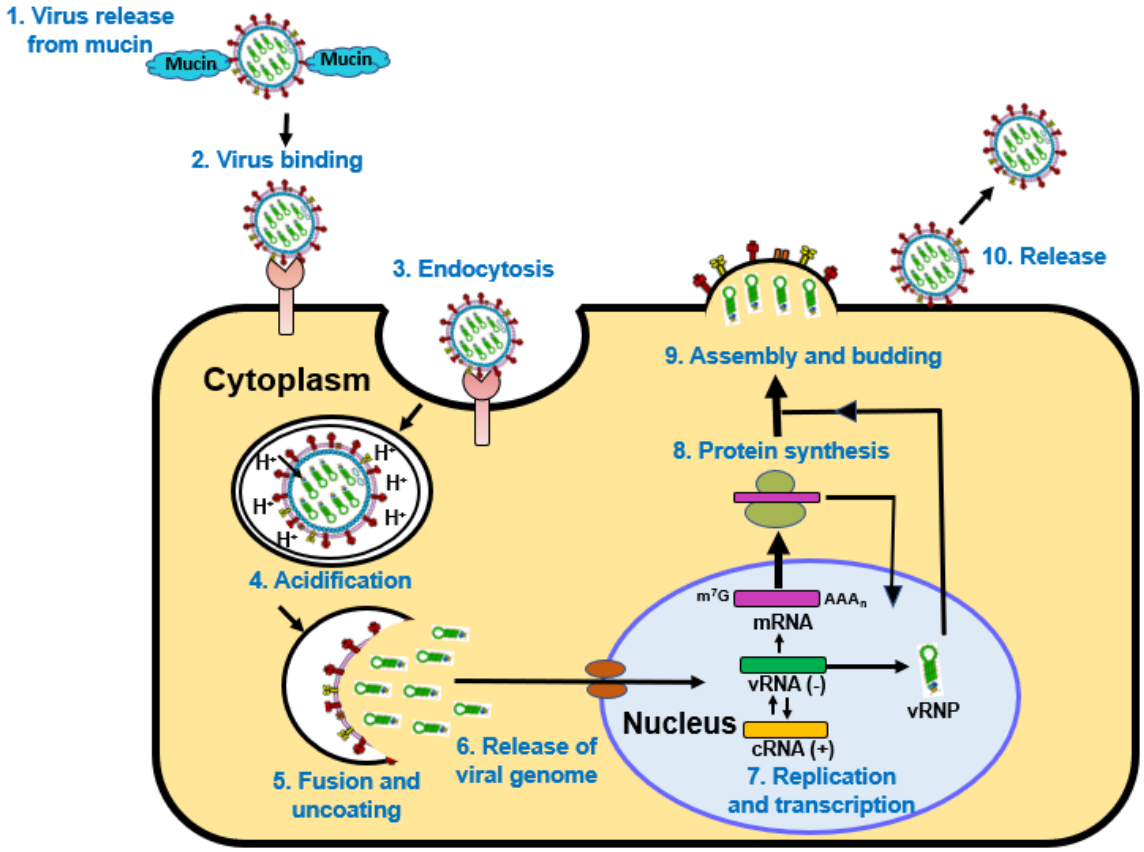


Figure 1.4. The replication cycle of influenza virus. The NA activity frees incoming virions from decoy receptors on mucins. The binding of virus HA protein to the SA receptor on the host cell facilitates receptor mediated endocytosis. Low pH in the endosome mediates fusion of the viral and host membranes releasing vRNP into the cytoplasm, which is then imported into the nucleus. The vRNA is transcribed into mRNA and the newly formed viral mRNA is exported to the cytoplasm for protein synthesis. Some of the proteins like polymerase and nucleoproteins are transported back to the nucleus for aiding vRNA transcription and replication. Newly synthesised copies of the vRNA assemble with viral proteins to form vRNP. Assembly and budding of new virions take place at the cell surface. Finally, the NA activity releases the newly formed virion from the host cell surface. **vRNP**: Viral ribonucleoprotein **SA**: Sialic acid

1.1.3.2. Key vaccine targets

Some of the influenza virus surface proteins like HA, NA and M2 exhibit important roles during the virus replication cycle. Thus, they are good targets for vaccine development (**Figure 1.5**). Influenza HA protein is the primary immunogen during infection and vaccination therefore, it is the main neutralising target for the antibody response against influenza viruses[64]. The HA protein is made up of two structurally and functionally distinct domains: the variable globular head domain and the conserved stalk domain[65]. Typically, antibodies against the head domain are induced at much higher levels than those against the stalk[66]. One of the mechanisms proposed for immunodominance of the globular head domain of HA over the stalk domain include poor accessibility of the HA stalk epitopes due to steric shielding by the HA globular head[66]. Another mechanism explains that the HA stalk-reactive B cells are poly-reactive. The poly-reactivity of the HA stalk antibodies can increase the chances of dynamic interactions with less accessible HA stalk epitopes. However, this property of poly-reactivity is likely to further reduce the number of B cells in an already restricted repertoire that can bind the HA stalk, indicating that the poly-reactive B cells have a selective disadvantage during B cell development[66,67]. Moreover, antibodies against the HA head domain can inhibit receptor binding activity of HA hence, they are referred to as neutralising antibodies (nAbs)[31]. The antigenic drift process accumulates mutations on the HA head domain allowing the virus to escape these nAbs[68]. This is the main reason why conventional human influenza vaccine seed strains have to be updated every year in order to protect against the newly circulating strains. Recently, there has been a great interest in developing ‘universal’ influenza vaccines focussing on antibodies that target the stalk region of HA[69]. Unlike the head domain, the stalk domain is

much more conserved across different HA subtypes[69]. Hence, these stalk-specific antibodies can overcome the problem of antigenic drift and protect against multiple antigenically diverse virus subtypes[65]. IAVs can be categorised into two phylogenetic groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) and group 2 (H3, H4, H7, H10, H14 and H15)[66]. The breadth of stalk-reactive antibodies is mostly restricted to one haemagglutinin group (group 1 or group 2). Group 1 constructs based on the H1 stalk domain have been successfully tested in mice and ferrets, and protect them from heterologous (H1N1) and heterosubtypic (H5N1 and H6N1) challenge, but not from challenge with group 2 viruses (H3N2)[70,71]. Similarly, group 2 constructs based on the H3 stalk domain have shown to protect mice against various H3N2 viruses and against heterosubtypic challenge virus such as H7N1 and H7N9[72,73]. In some cases, HA stalk-specific monoclonal antibodies have been isolated from humans that bind across both group 1 and group 2 virus groups increasing the breadth of cross-reactivity[74]. The HA stalk-specific antibodies provide host protection by mediating antibody dependent cell-mediated cytotoxicity (ADCC) or complement cytotoxicity[75]. Although HA stalk-specific antibodies are more cross-reactive than HA head-specific antibodies, they are produced in low levels after infection or vaccination with conventional influenza vaccines compared to those against the immunodominant globular head domain[66]. In recent years, various strategies including sequential vaccination with chimeric HAs containing different heads but the same stalk region, and ‘headless’ HA proteins consisting only the HA stalk domain have been implemented to boost the production of stalk-reactive antibodies[71,76,77].

The influenza NA protein is another target of interest for influenza vaccine development. NA protein is antigenically more conserved than the HA protein[75]. Several studies in mice and chickens have shown that vaccination with recombinant NA can induce higher protective immunity against homologous virus infection and partial protection against heterologous virus infection[78,79]. Antibodies targeting NA generally inhibit NA activity and interfere with virus release from the cell surface, reducing the amount of virus progeny produced by the infected cell. In addition, anti-NA antibodies can also mediate ADCC[75]. Moreover, NA has also been a major target for multiple antiviral drugs in humans. NA inhibitor (NAI) drugs such as oseltamavir, zanamivir, ianinamivir, and peramivir are all competitive inhibitors of SA and have been approved for human use[80]. However, the widespread use of these drugs has allowed influenza virus to develop NAI resistance in recent years[81].

Recently, the extracellular domain of influenza M2 protein (M2e) has been extensively explored as a ‘universal’ antigen candidate for vaccines[75]. The antibodies against M2e do not neutralise the virus, instead these antibodies prevent viral budding. In addition, anti-M2e antibodies mediate killing of infected cells by natural killer (NK) cells or macrophages through ADCC, Fc-opsonisation, or complement activation[82]. Although, M2e protein is relatively well conserved, it is poorly immunogenic[83]. Various strategies have been used to improve the immunogenicity of M2e vaccine candidates in mammals. These strategies include using adjuvants, multimeric forms of M2e, co-immunisation with other influenza vaccines, or fusion with carrier proteins[84–86]. Human clinical trials have also demonstrated the safety and immunogenicity of different M2e vaccine candidates[87,88].

In the context of influenza infection, vaccines inducing cellular immunity is as important as the ones eliciting antibody responses. Hence, T cell inducing vaccines have been increasingly explored. A vaccine candidate based on Modified Vaccinia Ankara (MVA) viruses expressing influenza virus NP and M1 antigens (MVA-NP+M1) has been developed[89]. Individuals vaccinated with MVA-NP+M1 showed an increased IFN-γ producing $CD8^+$ T cells, consequently increasing the protection against influenza virus infection[90,91]. The co-administration of MVA-NP+M1 with the seasonal inactivated influenza vaccine resulted in a boost of memory T cells capable of recognising a range of IAV subtypes[92]. Recently, FP-0.001 (Flunisyn™) T cell inducing vaccine candidate was developed by Immune Targeting Systems Limited (London, UK)[93]. This vaccine consists of six peptides derived from NP, M1, PB1 an PB2 proteins of IAVs. These peptides are linked to fluorocarbon that can self-assemble into micelles, eliminating the need of adjuvants and increasing the half-life of the peptides. Healthy volunteers vaccinated with FP-0.01 only showed a slight increase in the IFN-γ producing $CD8^+$ T cells. However, peripheral blood mononuclear cells (PBMCs) isolated from these vaccinated individuals showed an increased IFN-γ production compared to the unvaccinated individuals[93]. Another T cell inducing candidate vaccine, Multimeric-001 is developed by BiondVax Pharmaceuticals Ltd (Ness Ziona, Israel). It is composed of a large protein containing nine highly conserved peptides derived from IAV HA, NP and M1 proteins[94]. PBMCs from vaccinated individuals showed an increased proliferation and cytokine production compared to PBMCs from non-vaccinated individuals, after stimulation with peptides derived from NP and HA proteins[94].

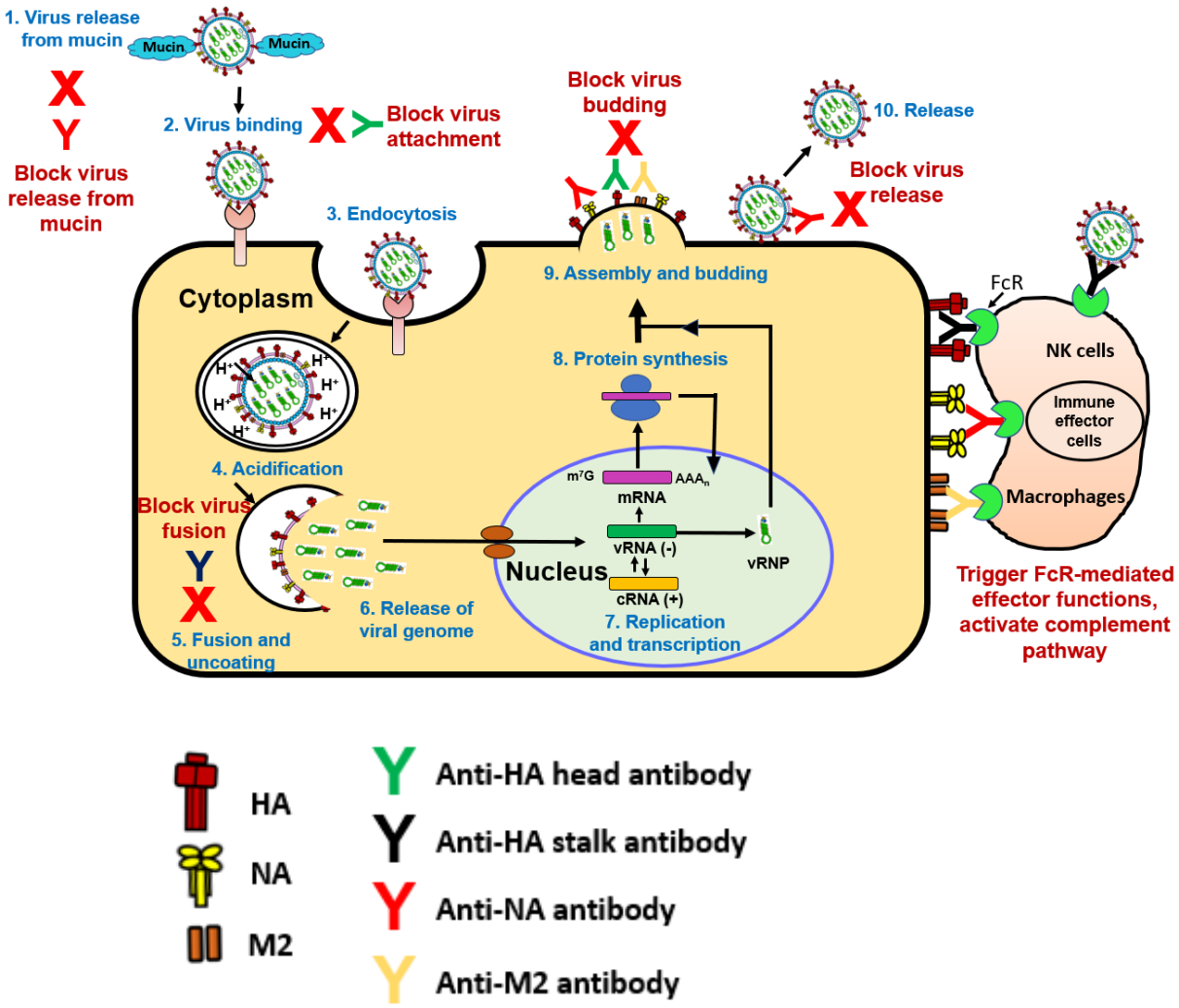


Figure 1.5. Some of the key influenza vaccine targets for inducing antibody responses. Antibodies against the head domain of HA inhibit the attachment of virus to the host cell receptors. On the other hand, antibodies against the stalk domain of HA can block the fusion of virus and host membranes, and mediate antibody dependent cell-mediated cytotoxicity (ADCC) or complement cytotoxicity. Anti-NA antibodies can prevent the release of progeny virions from the host cell surface as well as mediate ADCC. Anti-M2 antibodies can interfere with virus assembly and budding and facilitate complement activation and ADCC. **HA**: Haemagglutinin **NA**: Neuraminidase **M2**: Matrix 2 protein **FcR**: Fc receptor

1.1.4. Avian influenza viruses (AIVs)

Avian influenza viruses are highly contagious and widely spread in birds. Wild aquatic birds such as ducks, geese, swans, and gulls are thought to be their natural reservoir hosts[95]. Within their natural host, AIVs mainly replicate in the

respiratory and gastrointestinal tract, and are usually asymptomatic. Faecal-oral transmission is the predominant means of the virus spread in aquatic wild bird reservoirs. However, depending upon the host or virus subtypes respiratory transmission is also possible[96]. AIVs can readily infect domesticated poultry, with numerous subtypes such as H5Nx, H6N1, H7Nx and H9N2 jumping from the natural aquatic bird reservoir to the poultry species such as chickens, leading to severe economic losses for the poultry industry[97]. Once AIVs enter poultry flocks, it can spread on the farm by faecal-oral route and drinking water[95]. The pathogenicity of AIVs can be assessed using the intravenous pathogenicity index (IVPI) test, whereby chickens are inoculated with a virus and mortality and morbidity is observed for 10 days. Birds are observed each day for the clinical signs and scored accordingly (normal=0, sick=1, paralysed=2, dead=3). The IVPI index is calculated as the mean score per group per observation. An IVPI score between 0.0-1.2 indicates low pathogenicity avian influenza (LPAI) virus, and an IVPI score between 1.2-3.0 (>75% mortality) indicates high pathogenicity avian influenza (HPAI) virus[98]. LPAI viruses generally exhibit low mortality and morbidity in poultry; characterised by mild respiratory signs, depression, and egg production problems in layers. However, co-infections or other environmental factors have been associated with severe disease signs and high mortality in the field[99]. On the other hand, HPAI viruses can cause up to 100% mortality in chicken flocks. The key determinant of avian influenza pathogenicity in avian hosts is the sequence of amino acids at HA cleavage site[100]. The HA of most LPAI viruses typically contain mono- or dibasic cleavage sites (e.g., I-Q-S-**R**/G-L-F or **R**-S-S-**R**/G-L-F) that are cleaved by trypsin and trypsin-like proteases with monobasic specificity[101]. Therefore, the replication of LPAI viruses is generally confined to trypsin expressing

epithelial cells of the respiratory and gastrointestinal tracts. In contrast, HA of most HPAI viruses contains polybasic cleavage site (PBCS) (e.g., **R-R-K-K-R**/G-L-F) that is cleaved by ubiquitous intracellular subtilisin-like proteases, such as furin, with polybasic specificity[102]. Therefore, HPAI viruses can cause a systemic infection with broad tissue and organ tropism, which is considered to be a major factor responsible for high pathogenicity in poultry[103,104]. The influenza polymerase is error prone thus, it can result in accidental repetition of nucleotides through polymerase slippage. Mutations generated through polymerase slippage are often random and most commonly do not affect the cleavage site. However, mutations generating PBCS are advantageous as they allow the virus to replicate in various host tissues and organs. Such advantageous mutations are selected and passed onto the next generation. Over time, viruses with PBCS continue to replicate and become increasingly common in a population. Thus, the mutation and selection process following polymerase slippage has been proposed to be the main reason for acquisition of a PBCS in HPAI viruses[105].

AIVs can remain viable in the environment for a long time, especially at low temperatures[106]. Under controlled laboratory conditions, AIVs survived in faeces for 1 to 7 days at 15-35°C, whereas at low temperature (4°C), the virus survived for 8 weeks[107]. Similarly, water inoculated with infected duck faeces retained infectious virus over 30 days at 0°C whereas, at 22°C the infectious virus was retained for only 4 days[108]. Most cases of human infection due to AIVs have involved close contact with infected poultry, particularly sick or dying chickens[109]. Rarely, human infections after consumption of undercooked poultry products and fresh duck blood have been observed[110]. In 2004, H5N1 HPAI outbreak occurred in Thai tiger zoo which was caused by feeding tigers with raw infected chickens. Limited tiger-to-

tiger transmission of H5N1 HPAI viruses was also reported[111]. AIVs that infect species other than their usual hosts are transmitted inefficiently, and generally do not continue to circulate in that population. Many human cases after acquiring AIVs are limited to conjunctivitis or mild respiratory disease[112]. In rare cases, AIVs can evolve and adapt to circulate in a mammalian species resulting in pandemics[113].

1.2. Effects of avian influenza virus in poultry

Poultry is one of the most globalised livestock sectors. Over the past decades, poultry production and trade have been on the rise globally as the public demand for poultry meat and eggs has been increasing steadily (**Figure 1.6**). Currently, over 55 billion birds are raised for meat and eggs, with chicken meat accounting for approximately 90% of total poultry meat[114]. The Food and Agriculture Organisation (FAO) and the World Organisation for Animal Health (OIE) have classified poultry production systems into four sectors (Sectors 1 to 4). Sector 1 includes industrial poultry with high biosecurity. It is the system from which majority of internationally traded poultry is derived. Sector 2 includes large scale commercial producers with good biosecurity or farmers under contract to big companies. Sector 3 consists of small- to medium- scale commercial farms with low biosecurity investment. Sector 4 includes backyard, scavenging system where small poultry flocks are kept in houses. The productivity level of sector 4 flocks is low however, they significantly contribute to the household income and food security. Sectors 1 and 2 predominate in developed industrialised countries whereas, Sectors 3 and 4 predominate in developing nations.

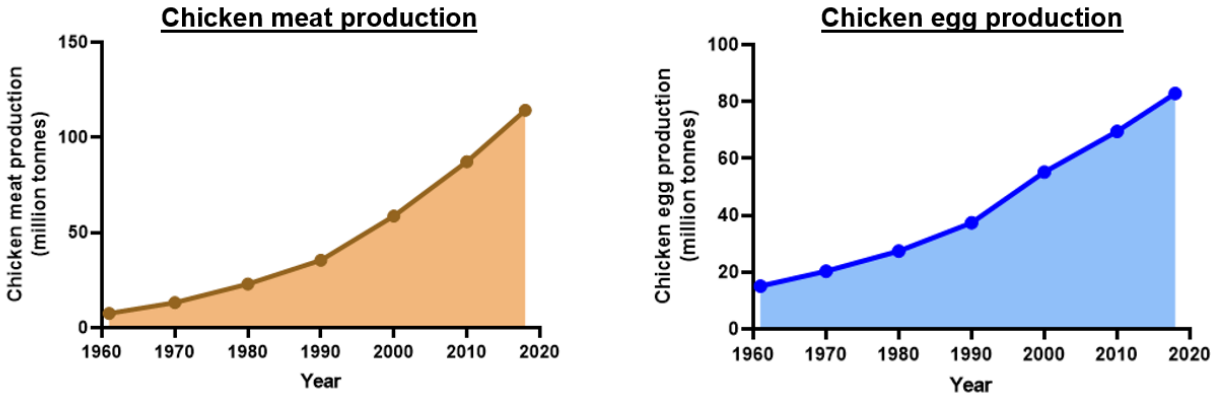


Figure 1.6. Global rise in chicken meat and egg production from 1961 to 2018. **Source**: United Nations Food and Agricultural Organisation (FAO).

AIV has become a disease of great concern for both animal and human health. The occasional 'spill-over' events allow these viruses to expand their transmission cycle to include a non-natural host. Such transmission to domestic poultry has led to the current status of AIVs being enzootic in many countries in Asia, Africa, and the Middle East. These enzootic infections are mainly caused by H5N1, H7N9 and H9N2 AIVs[115–119]. A hallmark of influenza virus adaptation to chickens is deletion of several amino acids within the NA stalk domain. This can contribute to an altered viral tropism from the gastrointestinal tract to the respiratory tract[120,121]. Over time these viruses can adapt to their new host and transmit between each-other. Depending upon the pathogenicity, AIVs infecting poultry can be divided into two groups: HPAI and LPAI. HPAI viruses are restricted mainly to H5 and H7 subtypes and can result in 100% mortality[122]. On the other hand, LPAI viruses generally cause mild disease symptoms. However, LPAI-associated morbidity can be exacerbated by co-infecting pathogens[123–126]. Furthermore, LPAI viruses include broad range of HA subtypes e.g., H9N2 and H5/H7 LPAI viruses[122]. The outbreaks of HPAI and LPAI viruses seriously affect poultry production and

trade through direct impact of disease related morbidity and mortality, and the cost associated with disease control strategies. According to the OIE reports, H5N1 HPAI outbreaks in domestic birds resulted in the loss of approximately 120 million birds worldwide from January 2013 to January 2018[127]. In 2013, the prevalence of H7N9 LPAI dropped poultry sales revenue in China by US$69 million[128]. Similarly, in 2017, around 17 million chickens were culled in Iran due to H5N8 outbreak. This outbreak inflicted losses of more than US$477 million on production units in Iran[129]. In addition, additional costs arise from the implementation of disease prevention and control programmes e.g., investments in improving biosecurity enhancements, vaccination, upgrade of diagnostic capacity, cost of culling and disposal of carcasses, disinfection of affected premises, intensive surveillance, and enforcement of movement control to poultry production and trade[130]. The AIV outbreaks also develop anxiety and fear in consumers. This reduces the national demand for poultry products as well as imposes international ban of potentially risky products from affected countries.

In lower- and middle-income countries (LMICs), live bird markets (LBMs) are culturally important component of poultry rearing. LBMs facilitate the storage and sale of live poultry including chickens, ducks, quails, and pigeons. In LBMs, different species of birds from range of sources are brought together, slaughtered, and sold[131]. Hence, LBMs act as major hubs for disease transmission in poultry as well as hotspot for zoonotic infection[132–134]. Several studies on chickens sampled in LBMs of LMICs like Vietnam, Bangladesh, Pakistan, and Egypt have found H9N2 to be the most dominant AIV subtype in LBMs[133–137]. The enzootic nature of H9N2 AIVs in LBMs is reported to be due to their LPAI phenotype allowing repeated re-infections of the same birds and silent spread between farms and small holdings[138].

Various control strategies including monthly ‘rest’ period where LBMs are cleaned and kept bird free, ban to overnight storage of leftover live birds, ban to the sale of live quails (potential intermediate host supporting reassortant AIVs), public awareness and biosecurity have proven to be important for the management of LBMs and prevention of AIV outbreaks[139–142].

The current spread of AIVs in domestic poultry and wild birds across the globe and their demonstrated ability to cross the species barrier and infect humans, heightens the pandemic concern. The antigenic shift is a key mechanism for zoonosis. Antigenic shifts can occur either through direct avian-to-human transmission or through mixing of human and animal influenza virus genes through a process called genetic reassortment[113]. The deadliest influenza pandemics in history include 1918 Spanish flu (H1N1 AIV), 1957 Asian flu (H2N2 AIV) and 1968 Hong Kong flu (H3N2 AIV) which killed approximately 50 million, 2 million and 1 million people, respectively[143]. Human cases reported with other subtypes of AIVs are shown in **Table 2**. Human pandemics are of significant concerns to public health and global economy. Therefore, controlling the disease at its source in poultry and livestock is the most effective way to prevent the occurrence of human pandemic of animal origin.

Table 2: Avian influenza virus subtypes that circulated in birds and infected humans.

Subtype	Disease in humans	Impact in animals	Refs
H5N1	Fatal human cases reported	High mortality	144,145
H5N6	Fatal human cases reported	High mortality	146,147
H6N1	Mild flu-like symptoms including fever and shortness of breath	High mortality and decrease in egg production	148,149
H7N3	Mild symptoms reported in humans	Varying mortality	150
H7N7	Mild/Fatal human cases reported	Varying morbidity and mortality	151,152
H7N9	Fatal human cases reported	Varying morbidity and mortality	153–155
H9N2	Mild symptoms reported in humans	Varying morbidity and mortality	138
H10N3	Mild symptoms reported in humans	Mild clinical signs	156
H10N7	Mild symptoms including conjunctivitis	High mortality and decrease in egg production	157
H10N8	Fatal human cases reported	Mild clinical signs	158,159

1.3. Avian immune system and its role in immunity against avian influenza virus

The avian immune system diverged from that of mammals over 200 million years ago[160]. Although, the basic structure and function of avian immune system is largely like that of mammals, several differences can be observed which is discussed in **section 1.3.1**. Like any other vertebrates, the avian immune system also has two main functional arms: innate and adaptive immunity, which are involved in recognising and eliminating infection. The innate immunity forms the first line of defence against pathogens. The innate immune system uses receptors that are encoded by genes inherited through the germline. These receptors recognise conserved pathogen-associated molecular patterns (PAMPs) such as nucleic acids, flagellin and carbohydrates[161]. The innate immune responses occur immediately or within hours of encountering an antigen and has no immunological memory. On the other hand, adaptive immunity allows the host to fight a 'specific' infection. The adaptive immune system uses antigen-specific B and T cell receptors which are formed by a tightly regulated rearrangement of antigen-specific receptor gene elements, a process which leads to each individual cell expressing a receptor of unique specificity[162]. Thus, adaptive immune responses are slower to develop (occurs over days to weeks). Unlike, the innate immune receptors which occur abundantly throughout the body, the antigen-specific B and T cell receptors are found on a very small population of cells derived from a specific clone that was successfully selected during B or T cell development[163]. Moreover, the adaptive immunity has immunological memory which enables the host to mount a rapid and efficient immune response upon subsequent exposure to the antigen[164]. There is a great deal of synergy between the innate and adaptive immunity, as the innate

immune system is not only involved in initiating and directing adaptive responses but is often used as an effector arm of the adaptive response. For example, macrophages are one of the important components of the innate immune system and are involved in phagocytosis and production of antimicrobial compounds. The T cell responses often involve the production of interferon gamma (IFN-γ) which can activate macrophages, increasing their capacity to phagocytose and kill the invading microorganisms. Here, the macrophages can be regarded as one of the effector arms of the T cell response[163]. Similarly, a range of phagocytic cells including macrophages and polymorphonuclear cells, express Fc receptors that bind immunoglobulin produced by B cells. This enhances the recognition and engulfment of foreign pathogen-derived material. In such cases, the phagocytic cells of the innate system act within the context of antibody and B cell mediated immunity[163]. These examples highlight the complexity of the interactions that occur between the innate and adaptive immune systems.

1.3.1. Similarities and differences between avian and mammalian immune systems

There are many similarities between the mammalian and avian immune systems as they evolved from a common reptilian ancestor however, there are also some important differences. Most research on avian immunology has been carried out in domesticated chickens because of their economic importance[165]. The avian lymphoid system is divided into primary lymphoid organs; where lymphocytes are formed and mature, and secondary lymphoid organs, where lymphocytes are activated by exposure to the antigen initiating the adaptive immune response. In mammals, bone marrow and thymus are regarded as primary lymphoid organs. Both B and T lymphocytes originate in the bone marrow. B lymphocytes mature in

the bone marrow whereas T lymphocytes migrate to the thymus to undergo their maturation[166]. In birds, thymus, and Bursa of Fabricius (hereinafter referred to as bursa) constitute as primary lymphoid organs[167]. Unlike mammals, the maturation of B lymphocytes occurs in bursa. Bursa is the central organ for B cell development in birds and is located in the cloaca[168] (**Figure 1.7**). The importance of bursa in developing antibody response has been demonstrated by studies involving bursectomised (removal of bursa) birds. Such bursectomised birds was shown to have impaired ability to produce antibodies[169]. In mammals, bone marrow is a major site for antibody formation[162]. The contribution of avian bone marrow to antibody formation is not fully understood however, many studies have shown that immunoglobulin-secreting cells can be produced in chicken bone marrow. Lawrence et al detected large numbers of immunoglobulin-secreting cells (IgM, IgY and IgA) in chicken bone marrow by day 31 of age, using a reverse hemolytic plaque assay[170]. Furthermore, Geus et al detected IgM, IgY and IgA producing cells in chicken bone marrow after infection with H7N1 LPAI virus. Similarly, the immunisation of chickens with Newcastle disease virus (NDV) resulted in a primary and secondary immunoglobulin-secreting cell response in the bone marrow which was similar in magnitude to that in the spleen[171]. A fundamental difference between mammalian and avian immune system is the absence of draining lymph nodes in the latter. Mammals have draining lymph nodes (secondary lymphoid organ) which are highly organised sites for interactions between B cells, T cells, macrophages, and dendritic cells (DCs) that are important for activation of adaptive immunity[166]. Lymph nodes appear to be present in water, marsh, and shore birds but not in chickens or higher species of birds[172]. However, mural lymphoid nodules associated with lymph vessels have been described in numerous species of birds,

including chickens[173]. These lymphoid nodules are found throughout the body: gall bladder, pancreas, lungs, skin, endocrine glands, peripheral nerves, and gonads. The lymphoid nodules arise in the same way as the nodes and are sometimes found to protrude into the lumen of the lymph vessel and consist essentially of a tube with lymphoid tissue on its wall[173]. They also have similar geminal centres as that of the nodes[173]. Due to these similarities, the avian lymphoid nodules are considered functional equivalent of mammalian lymph nodes. It is possible that the lymph nodules found in birds represent a precursor of the avian and mammalian node[173]. Apart from the lymphoid nodules, spleen, Peyer's patch and cecal tonsils are also regarded as important secondary lymphoid organs in birds. One of the most important lymphoid tissue in the paranasal area of birds is the Harderian gland. The Harderian gland is located directly behind the eye orbit and is the major site for the formation of antiviral IgA-antibody forming cells[174]. This gland is important for local immune responses of the eye, nasal turbinate, and upper respiratory tract areas. Vaccination of poultry using eyedrops stimulates mucosal immune responses through the Harderian gland[175].

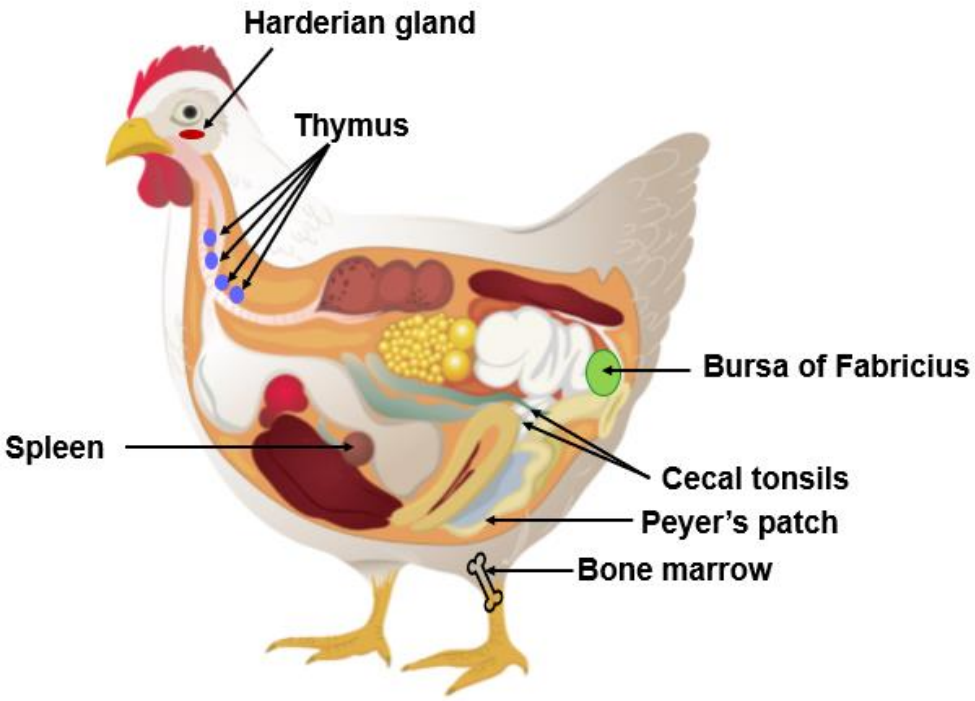


Figure 1.7. Key avian lymphoid tissues. Image adapted from mypetchicken.com/backyard-chickens.

A key difference is observed between mammalian and avian immune system in the mechanism by which the B cell receptor repertoire is generated. In humans and mice, the antibody repertoire of B cells is generated by a process known as immunoglobulin gene rearrangement[176]. The immunoglobulin light chain genes encode the variable region (V_L gene segment), a joining region (J_L gene segment) and the constant region (C_L gene segment). In the case of the immunoglobulin heavy chain, a further diversity (D) gene is involved, making a V_HDJ_H rearrangement[176]. There are multiple copies of V, J and D genes in the mammalian immunoglobulin locus. Therefore, a vast antibody repertoire (~10^{11}) can be obtained through (1) random recombination of the different gene segments; (2) different combinations of heavy and light chains paired together; and (3) point mutations introduced into the rearranged sequence (somatic hypermutation)[162]. In contrast, the cluster of genes encoding the chicken immunoglobulin light chain has

only a single copy of the functional V_L and J_L genes[177]. Hence, diversity due to V_LJ_L joining is very limited. Similarly, a single functional V_H and J_H genes in the immunoglobulin heavy chain locus means that only little diversity can be generated through V_HDJ_H rearrangement[177]. Clusters of pseudogenes existing upstream of the heavy and light chain immunoglobulin loci have an important role in generating chicken antibody diversity through a process known as somatic gene conversion, whereby the V_L and V_H sequences are replaced with pseudogene sequences[163,178]. The light chain locus includes 25 pseudogenes, whereas the heavy chain locus encompasses around 80 pseudogenes[179]. There is a great diversity in the hypervariable regions of the donor pseudogenes. Thus, gene conversion ensures that a wide range of functional immunoglobulin repertoires are generated in the chicken B cells[179].

In terms of the immunoglobulin classes, only three classes are present in birds: IgM, IgY and IgA, compared to five in mammals: IgM, IgG, IgA, IgD and IgE[163]. Avian IgY is homologous to mammalian IgG and has functional similarities with both mammalian IgG and IgE[180]. Unlike mammalian IgG, avian IgY has a longer heavy chain and lacks a hinge region. Instead, they have switch regions with limited flexibility at the constant region of heavy chain 1 (CH1)-CH2 and CH3-CH4 domain interfaces[163]. The limited flexibility of the avian IgY accounts for some of the unique biochemical properties, such as the inability to precipitate antigens at physiological salt concentrations[167]. Ducks produce two isoforms of IgY[181]. The larger is structurally analogous to chicken IgY and the smaller is referred to as truncated IgY (consists of CH1-CH2 only), structurally resembling fragment antigen binding $F(ab)_2$ fragment of IgY[181,182]. In contrast to mammalian IgG, IgY does not bind to Protein A and Protein G. On the other hand, chicken IgM and IgA are

structurally and functionally homologous to their mammalian counterparts[163]. No IgD and IgE isotypes have been described in birds. However, there were some early reports of chicken homologue of IgD on the surface of chicken lymphocytes[183]. With regards to the T-cell receptor (TCR) subtypes, birds have three TCRs, one more than mammals. Avian homologues of the mammalian αβ and γδ TCRs, TCR1 and TCR2 have been identified[184]. In addition, a third TCR subtype, TCR3 has also been identified in birds which emerges relatively late in ontogeny and can undergo expansion in secondary lymphoid tissues[185].

1.3.2. Innate immune response to avian influenza virus

The innate immunity is the first immunological mechanism for fighting against an intruding pathogen (**Figure 1.8**). It contains four primary components: i) physical and chemical barriers like skin, epithelia, and production of mucus ii) innate cells like macrophages, heterophils and NK cells iii) complement proteins and mediators of inflammation iv) cytokines[186]. The germline-encoded receptor, pattern recognition receptors (PRRs) also play an important role in innate immunity during viral infections. They recognise evolutionarily conserved molecular markers of infectious pathogens, known as PAMPs. One important family of PRRs that has been well characterised in viral infection is the Toll-like receptors (TLRs)[187]. To date 13 mammalian TLRs and 10 chicken TLRs have been described[188]. The chicken TLR repertoire includes at least one TLR1/6/10-like molecule, two TLR2, TLR3, TLR4, TLR5, and TLR7 orthologs, and two TLRs that are found only in birds (TLR15 and TLR21)[163]. The consequences of TLR activation are diverse; they include cytokine, chemokine and interferons production, cell maturation, induction of degranulation and the direct production of antimicrobial peptides. The binding of

TLRs by PAMPs on macrophages and heterophils initiates innate immune effector function such as production of pro-inflammatory cytokines, while stimulation of TLRs on DCs induces T-cell activation cytokines (e.g., interleukin-12 (IL-12))[187,189]. In pigs, chickens, turkeys and ducks an early innate response to influenza virus is characterised by an increased expression of pro-inflammatory cytokines, interferons, chemokines, acute-phase proteins (APPs) and PRRs. The intensity and duration of the response are dependent on the susceptibility of the animal, viral load, and virus strains[190]. In most species, interferons (IFNs); interferon-alpha (IFN-α) and interferon-beta (IFN-β), tumour necrosis factor alpha (TNF-α) and IL-1 are involved in the early cascade of responses, followed by IL-8, IL-6 and interferon-gamma (IFN-γ)[186]. IFNs play a central role in the defence against viral infections. Virus-induced IFNs represent a mixture of type I and type III IFNs which are important for innate immunity[191]. In chickens, both type I IFNs (IFN-α, IFN-β) and type III IFN (IFN-λ) are key in combating influenza viruses[191,192]. Type I IFNs can induce the expression of hundreds of IFN-stimulated genes (ISGs) that block viral replication and reduce virus spread[188]. Similarly, chicken type III IFN (IFN-λ) has been shown to inhibit the replication of influenza viruses in primary embryonic tracheal organ cultures and in the chicken lung cell line CLEC-123[193]. Consistent with these results, treatment with chicken IFN-λ resulted in a strong induction of the IFN-stimulated myxovirus-resistance protein (Mx) gene in epithelial-rich tissues, such as intestine, trachea, and lung in chickens[193]. Additionally, treatment of 3-week-old chickens with recombinant IFN-λ delayed H5N1 HPAI viral excretion via the upper respiratory tract and resulted in a reduced spread of the virus to the brain[193]. The antiviral response to AIVs is rapid and is typically induced by the viral replicative intermediate double-stranded RNA (dsRNA) and ssRNA genome[194,195].

Cytosolic PRRs known as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are RNA helicases which are essentially involved in the recognition of viral RNA in the cytoplasm[196]. Three of these helicases have been described in mammals; RIG-I, melanoma differentiation associated protein-5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA-5 have a tandem N-terminal caspase activation and recruitment domains (CARDs), the central DExD/H box helicase domain, and a C-terminal regulatory or repressor domain (CTD) which binds RNA whereas, LGP2 contains only the helicase domain and the CTD[196]. RIG-I recognises only short, blunt 5'-triphosphate (ppp) dsRNA, whereas MDA-5 binds long dsDNA in mammals[197–199]. Since, LGP2 lacks the interacting CARD domain, it does not have signalling capacity[200] however, it acts as a regulator of RLR-specific antiviral response[201]. The chicken genome contains MDA-5 and LGP2, but lacks RIG-I[202,203]. Furthermore, chicken MDA-5 can sense both long and short dsRNA, unlike mammalian MDA-5 which senses only long dsRNA, implying that chicken MDA-5 may compensate some of the functions of RIG-I in chickens[204,205]. Ruggli et al demonstrated that chicken MDA-5 is one of the key PRRs for recognition of AIV in chicken cells and senses AIV in 5'-ppp-independent manner[206]. Like the mammalian LGP2, chicken LGP2 acts as a positive regulator of MDA-5 and is considered important to mediate RLR-specific antiviral immune responses[207]. The binding of dsRNA to MDA-5 or TLR3 initiates downstream signalling mediated through mitochondrial antiviral-signalling protein (MAVS) and TIR-domain-containing adapter-inducing interferon-β (TRIF), respectively. Similarly, the binding of ssRNA to TLR7 initiates downstream signalling through Myleoid differentiation primary response protein (MyD88). The induction of type I IFN transcription occurs by the assembly and activation of transcription factors like

interferon regulatory factor (IRF)7, IRF3, nuclear factor kappa B (NF-κB), and activating protein 1 (AP-1) (ATF2/JUN). The secreted type I IFNs further bind to type I IFN receptors and activate Janus kinases/ signal transducer and activator of transcription proteins (JAK-STAT) pathway, resulting in the production of numerous IFN-stimulated genes (ISGs), that inhibit the invading virus through antiviral activities. Some of the key ISGs include Mx, PKR, IFN-induced protein with tetratricopeptide repeats 5 (IFIT5) and OAS[208–210]. Previous studies in mice have shown that RIG-I is essential for induction of higher type I IFN response against IAV infection[209,210]. Unlike chickens, ducks possess functional RIG-I[202]. It was suggested that the lack of RIG-I mediated IFN induction in chickens could be responsible for the increased susceptibility of chickens to AIV as compared to the ducks[202]. However, it has been demonstrated that infection of chickens with H5N1 HPAI viruses rapidly induces high amount of type I IFNs, but this does not mediate protection against the virus[211]. In addition, the presence of TLRs and other RLRs (MDA-5/LGP2) show that chicken cells possess functional sensors to detect AIV infection and induce IFNs production. Hence, the lack of RIG-I does not seem to be the reason for the high AIV susceptibility in chickens and more research is required to support this concept.

A variety of proteins are involved in the acute-phase response induced after entry of IAVs. These innate inhibitors include acute-phase proteins (APPs), collagenous lectins (e.g., collectins), pentraxins, alpha-macroglobulin and antimicrobial peptides (e.g., defensins)[186]. Alpha-2-macroglobulin is a major neutralising inhibitor of IAVs in pig and horse serum[212]. In addition, collectins can bind to mannose-rich glycans on the viral HA and NA proteins mediating range of antiviral activities like neutralisation of virus infectivity, virus aggregation, increased

virus uptake by neutrophils and increased virus opsonisation[213–215]. Furthermore, members of pentraxin family provide sialylated ligands that mimic the structure of the cellular SA receptors, thus blocking the receptor binding site of HA[216]. Moreover, respiratory DCs and macrophages are among the first cells to respond to IAV infection and are important for controlling innate and adaptive immune responses[186]. Human and mice respiratory macrophages and DCs are abortively infected by LPAI viruses and contribute to the effective host defence[217]. However, some HPAI viruses can cause productive infection of respiratory macrophages and DCs resulting in a 'cytokine storm' characterised by the presence of elevated levels of pro-inflammatory cytokines. This is believed to play a role in disease pathogenesis[218]. In chickens and pigs, cytokine storms are rare and appear to be dependent on the virus strains[219,220]. Another important innate lymphocyte is the NK cell. NK cells elicit antiviral responses by killing virus-infected cells without prior sensitisation[186]. *In vivo* studies in mice have shown that NK cells are required for the clearance of IAVs[221]. Indeed, chickens infected with H9N2 LPAI viruses demonstrated enhanced activation of NK cells in lungs, contributing to better clearance of the virus. In contrast, infection of chickens with H5N1 HPAI viruses resulted in decreased activation of NK cells in lungs, contributing to the severe virus pathogenicity[222].

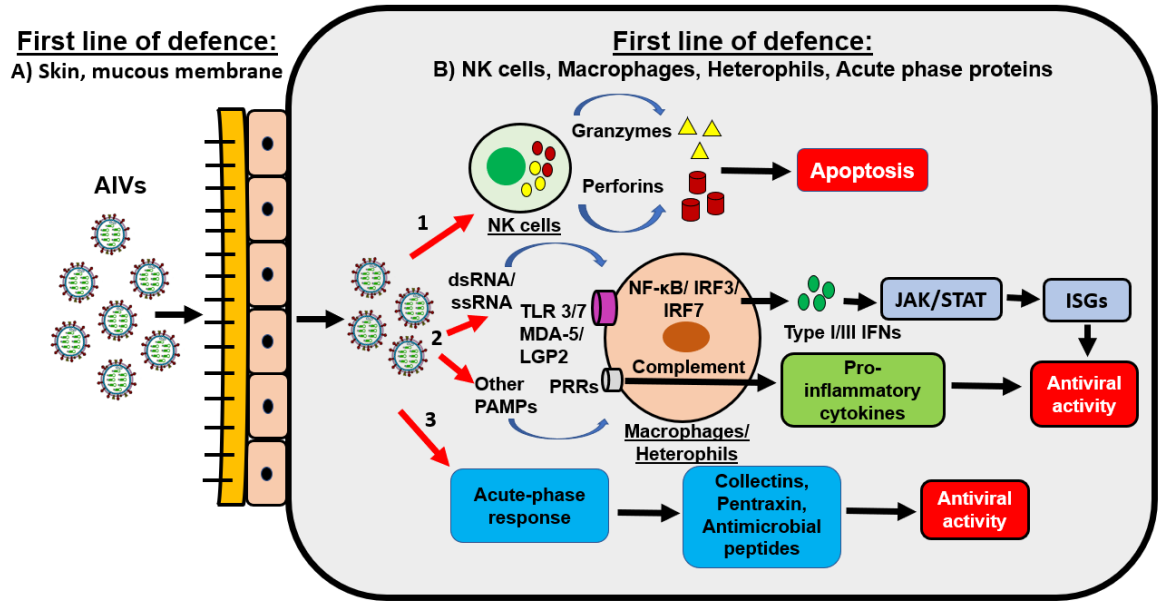


Figure 1.8. Innate immunity against avian influenza virus. It is the first line of immune defence against wide variety of pathogens. It includes physical and chemical barriers **(A)** like skin and mucosal epithelium; innate protector cells **(B)** like NK cells, macrophages and heterophils that can induce apoptosis of infected cells **(1)**, activate various RLR and TLR receptors for upregulating ISGs, activating complement system and pro-inflammatory cytokines **(2);** acute phase response involving various proteins like collectin and pentraxin with antiviral activities **(3)**. **NK**: Natural killer **TLR**: Toll Like **RLR**: Retinoic acid-inducible gene-I-like receptors (RLRs) **PAMPs**: Pathogen associated molecular patterns **PRRs**: Pattern recognition receptors **IRF:** Interferon regulatory factor **JAK/STAT**: Janus kinases/ signal transducer and activator of transcription proteins **ISGs**: Interferon-stimulated genes **IFN**: Interferon

1.3.3. Adaptive immune response to avian influenza virus

The immune system of mammals can be polarised functionally into type 1 (T helper 1 (Th1)) and type 2 (T helper 2 (Th2)) adaptive immune pathways that can resolve infections with intracellular and extracellular pathogens, respectively[223]. This polarisation of the responses is largely regulated by antigen-specific Th cells. Th1 cells drive cell mediated immunity (CMI); Th2 cells drive humoral immunity[224]. Until recently, the applicability of the Th1/Th2 paradigm to non-mammalian species was less clear. Degen et al demonstrated that chickens

are able to mount a typical Th1 and Th2 biased response to experimental viral and helminth parasite infections, respectively[225]. This provides evidence that the Th1/Th2 paradigm extends beyond mammals. Therefore, the antigen processing and presentation pathways in birds are likely to be similar to those of mammals. Upon encountering antigens, DCs undergo maturation which is characterised by redistribution of MHC molecules from intracellular endocytic compartments to the cell surface, increase in the expression of co-stimulatory molecules required for the T cell activation, secretion of distinct cytokines and chemokines, cytoskeletal reorganisation and morphological changes including the proliferation of dendrites from the membrane of DCs[226]. The exogenous antigens and cytosolic (endogenous) antigens are processed by matured DCs in different ways. DCs utilise endocytic pathways to internalise exogenous antigens into an early endosome. The early endosome matures into a late endosome and fuses with a lysosome that contains proteases and hydrolases. These enzymes degrade antigens into small peptides, which are processed and loaded onto MHC II molecules. MHC II-peptide complexes are recognised by $CD4^+$ T cells, which mature and differentiate into Th1 or Th2 cells. Like in mammals, chicken Th1 cells predominantly facilitate a pro-inflammatory response, which involves secretion of IFN-γ and IL-2. These cytokines can activate macrophages, NK cells and cytotoxic T lymphocytes (CTLs). On the other hand, chicken Th2 cells typically produce IL-4 and IL13, which can stimulate B cells to produce antibodies. This type of immune response involving antibodies is termed as humoral immunity. Unlike mammals, IL-5 mRNA expression is switched off during Th2 response in chickens[227].Moreover, some pathogens including viruses and bacteria are processed by proteasomal proteolysis followed by translocation into the endoplasmic reticulum (ER) via

Transporter Associated with antigen Processing (TAP) for further trimming and loading of optimised peptides onto MHC I molecules. These MHC I-peptide complexes are recognised by $CD8^+$ T cells, which can directly kill the infected cells. This is termed as CMI[228,229]. In mammals, the exogenous antigens can also be presented on MHC I molecules by a process called cross-presentation. Cytosolic and vacuolar pathways have been proposed for cross-presentation in mammals. The cytosolic pathway involves transport of endocytosed antigen into the cytosol for proteasomal degradation followed by translocation of peptides into ER via the TAP transporter and onto the MHC I molecule[230]. On the other hand, in vacuolar pathway MHC I molecules are delivered into the peptide loading compartment by the constitutive internalisation of cell surface MHC I molecules[231]. It is proposed that a conserved tyrosine residue in the cytosolic tail of MHC I is needed for MHC I endocytosis and its targeting to lysosomal vesicles containing internalised peptides[232]. The exact mechanism of cross-presentation in birds is unknown.

With regards to the AIV infection in chickens, viral proteins synthesised in the cytoplasm are processed by proteasomal proteolysis, facilitating the presentation by MHC I molecules to $CD8^+$ T cells (**Figure 1.9**). This results in the development of CTLs with the ability to lyse virus-infected cells. In addition, newly released virions are taken up by professional APCs by endocytosis. The processed viral peptides are presented by MHC II molecules to $CD4^+$ T cells, resulting in B cell activation for antibody production[186,190]. Following the administration of AIV vaccines, the host immune system directs the uptake and processing of the antigen. It is primarily the adaptive immune response to AIV vaccines that produces virus neutralising antibodies and AIV-specific cytotoxic lymphocytes, resulting in recognition and clearance of virus in the host following virus exposure[186]. The

protective humoral immunity of poultry against AIVs is generally the result of antibody response directed against HA protein which prevents the attachment of the virus to host cells[64]. Furthermore, the CMI has also been suggested to be an important factor in the development of protection in poultry against AIVs[233].

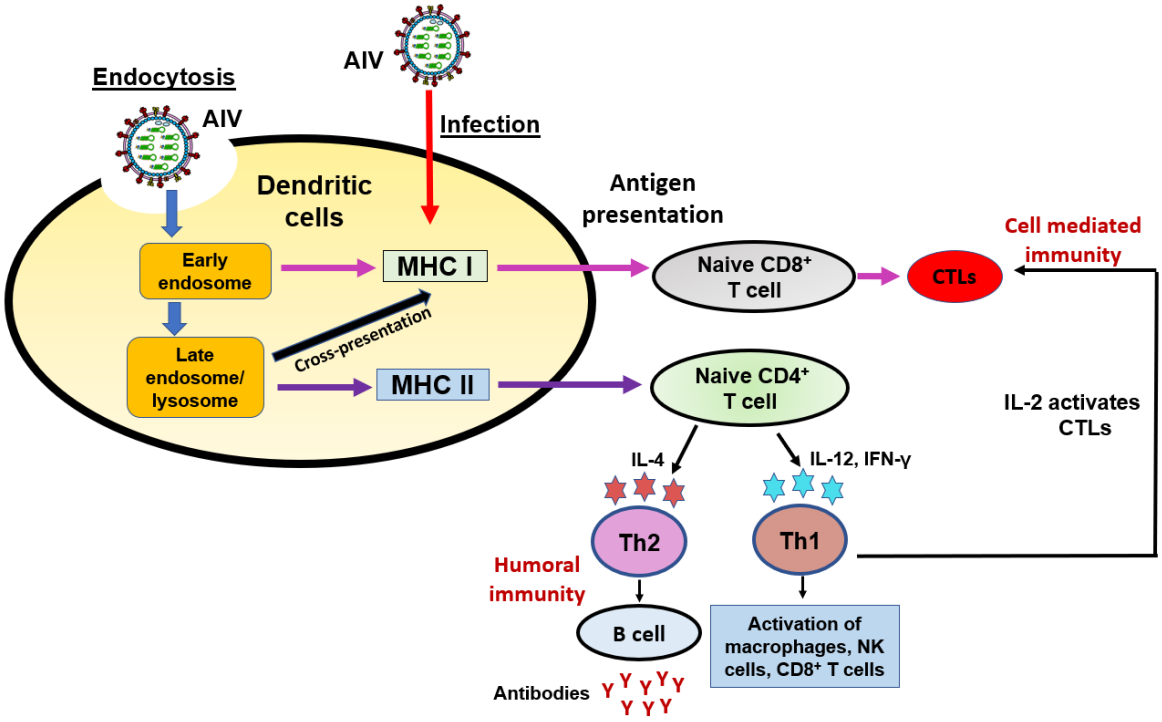


Figure 1.9. Adaptive immunity against avian influenza virus. AIVs can be phagocytosed by dendritic cells and presented via MHC II pathway, activating naive $CD4^+$ T cells for Th1 and Th2 responses. On the other hand, dendritic cells can also be directly infected by the virus resulting in antigen presentation via MHC I pathway, activating naive $CD8^+$ T cells. **CTLs**: Cytotoxic T lymphocytes

1.3.4. Mucosal immune response to avian influenza virus

The respiratory mucosa is the first target of influenza virus, constituting the first line of defence against the infection that can effectively prevent the virus entry[234]. The host responds to the initial IAV infection by producing antiviral and immunomodulatory components like mucins, lectins, antimicrobial peptides and complement molecules[195]. Upon the virus entry to epithelial cells, the local immune

cells can detect viral PAMPs via PRRs. This leads to the induction of antigen-specific immune responses, producing different cytokines and chemokines as well as activation and recruitment of adaptive effector cells[195]. The primary antibodies that mediate protection at mucosal surface are IgA and IgG (IgY in chickens). IgG is secreted into blood and diffuses onto the mucosal tissue, where they can exert antiviral activity[235]. In the mucosa, mammalian IgG has demonstrated a major role in decreasing viral pneumonia rather than preventing upper respiratory tract infection[236]. In contrast, IgA is the major mucosal antibody that can eliminate viruses from the infected secretory epithelium thus, preventing human upper respiratory tract mucosa infection[237] (**Figure 1.10**). Moreover, secretory IgA on the surface of respiratory mucosa can neutralise the virus prior to their attachment to epithelium and penetration into the epithelial surface[195]. The transforming growth factor (TGF)-β signalling is important for activating the B cells for IgA class switching and IgA secretion in mammals[238]. Studies in mice have shown that secretory IgA is more cross-reactive than serum IgG antibody[239,240]. Hence, it can contribute to cross-protection against different virus subtypes. Since, AIVs infecting poultry invade at mucosal surfaces in the respiratory tract and gastrointestinal tract, vaccines that can induce strong mucosal immunity are highly preferred[241]. Mucosal vaccines are designed to mimic the natural process of virus infections hence, they can induce better mucosal immune response than parenteral vaccines[242]. Furthermore, mucosal vaccines can enhance innate immunity by increasing the expression of various cytokines and chemokines. In addition, it can also enhance adaptive immunity by increasing the expression of co-stimulatory molecules like CD40, CD80, CD86 and B cell-activating factor[243]. The majority of currently licensed AIV vaccines in poultry are inactivated whole AIV vaccines which are

administered parenterally hence, they are poor inducers of mucosal immunity[244]. Therefore, efforts are being made to develop AIV vaccines that could be given via aerosol or intranasal route to confer protection at mucosal sites.

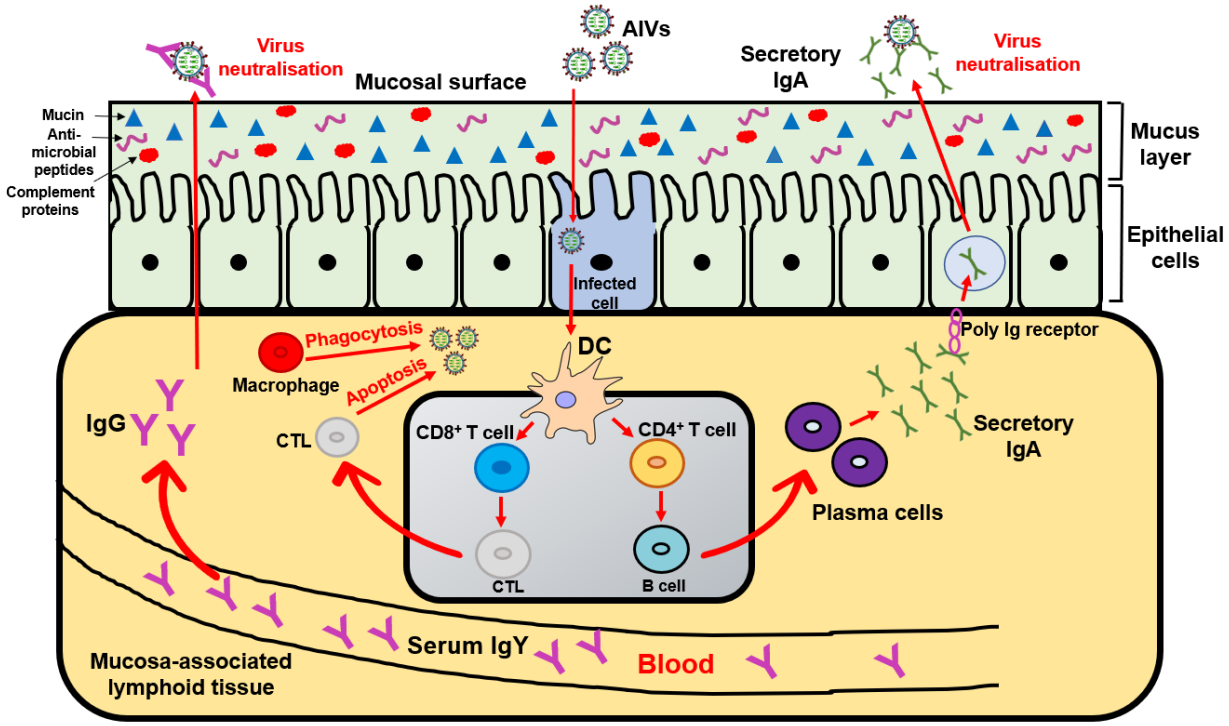


Figure 1.10: Mucosal immunity against avian influenza virus. The respiratory mucosal immune system consists of clusters of lymphoid cells beneath the mucosal epithelium, hosting both innate and adaptive immune cells. Dendritic cells can uptake the antigens and activate naive $CD4^+$ and $CD8^+$ T cells. Activated $CD4^+$ T cells can differentiate into Th1 and Th2 subsets. The latter can further activate and differentiate B cells into plasma cells for secretory IgA formation. Secretory IgA can prevent the attachment and penetration of virus into the mucosal epithelium. Furthermore, serum IgG can be diffused onto the mucosal tissue and facilitate virus neutralisation. The layer of mucus consisting of mucin, anti-microbial peptides and complement proteins is involved in host defence mechanism. Image inspired from Wang et al[195].**CTL**: Cytotoxic T lymphocyte

1.4. Avian influenza vaccines in poultry

The outbreak of avian influenza viruses in poultry results in severe economic losses, threatens animal welfare, and poses a zoonotic risk to humans. The control programmes for AIV aim to prevent, manage, and eradicate the disease[245]. The

initial response to poultry AIV outbreaks involves culling infected birds through stamping out campaigns. However, blanket culling of farmed birds is expensive and not always feasible. Therefore, different strategies are combined to manage AIV in poultry. These strategies include biosecurity practices, diagnostics and surveillance, elimination of infected animals, increasing host resistance and education of personnel in AIV control strategies[246]. Vaccination is one of the important tools incorporated in the AIV control programme. The use of vaccines increases host resistance to AIV infection[247]. The practice of AIV vaccination programmes in poultry varies around the world. The developed countries do not use the vaccines frequently, whereas some developing countries routinely use AIV vaccines to control LPAI H9N2 (Asia and Middle East), LPAI H5N2 (Central America) and HPAI H5N1 (Asia and Africa)[246]. However, various concerns have been raised regarding the inconsistencies of the AIV vaccines in field protection, with major issues being the quality of vaccines and inadequate administration[247].

The ideal poultry AIV vaccine should have the following features[248]: i) inexpensive ii) antigenically close to field virus for better protection iii) usable in multiple avian species iv) safe v) compatible with single dose protection vi) administration in the hatchery at 1-day-old or *in ovo* vii) easy administration by mass application viii) compatible with methods for easy differentiation of infected and vaccinated animals (DIVA strategy) ix) overcome maternally derived antibodies (MDA) interference **(Table 3)**. All these features should be taken into consideration when developing a new AIV vaccine for commercial use. None of the currently licensed AIV vaccines satisfy all the above criteria for an ideal vaccine hence, there is still much room for vaccine innovation and improvement.

Table 3: Features of ideal avian influenza vaccines and vaccination methods for poultry (modified from Swayne and Kapczynski[248]).

Desired property	Current situation	Refs
Inexpensive	Current cost of inactivated AIV vaccine: US $0.05-0.10 per dose, cost of administration: US $0.05-0.07 per dose.	248
Usage in multiple avian species	Most AIV vaccines are used in broilers, layers, and breeder chickens, also used in ducks, turkeys, geese, and quails.	64
Single dose protection	Inactivated AIV vaccines require a minimum of two doses: prime and boost, additional boost needed for layers and breeders at 6- to 12- months interval.	249
Mass application	Inactivated AIV vaccines are administered by handling and injecting individual birds, some vector vaccine (rNDV-vector) can be given by mass spray.	250, 251
DIVA compatibility	Inactivated AIV vaccines have poor DIVA compatibility, recombinant subunit and recombinant vector vaccines are DIVA compatible.	249
Overcome MDA interference	rHVT vaccines can overcome MDA interference but inactivated AIV vaccines cannot. Hence, inactivated AIV vaccines are given after the decline of MDA titres to allow optimal primary immune response.	251, 252
Administration to 1-day old in hatchery or *in ovo*	Inactivated AIV vaccines provide poor protection if given at 1-day of age. Vector vaccine (e.g., rHVT) can be given at 1-day of age or *in ovo*, however a field boost with inactivated vaccine is required.	253, 254
Antigenically close to field virus	Majority of inactivated AIV vaccines use reverse-genetic technology to generate vaccine seed strains antigenically similar to field viruses.	255

Safety	**Chickens**: Inactivated AIV vaccines contain killed viruses hence, they cannot cause any disease to chickens. Furthermore, they do not affect the broiler productivity (final body weight, body weight gain, feed intake). However, some inactivated AIV vaccines can cause a drop in egg production in layer hens. **User safety**: Inactivated AIV vaccines are generally given parenterally. Therefore, the main risk for people administering the product to birds is the possibility of accidentally injecting themselves. **Consumers**: Inactivated AIV vaccines do not contain any ingredients that are likely to pose a risk for consumers of vaccinated birds.	256,257

DIVA: Differentiation of infected and vaccinated animals **rNDV**: Recombinant Newcastle disease virus **rHVT**: Recombinant Herpesvirus of Turkey **MDA**: Maternally derived antibodies

1.4.1. Types of avian influenza vaccines in poultry

The avian influenza vaccines used in poultry fall into four categories based on experimental studies and field usage (**Figure 1.11**): i) Inactivated whole AIV vaccines ii) Live recombinant viral vector vaccine iii) Recombinant subunit vaccines iv) Nucleic acid vaccines.

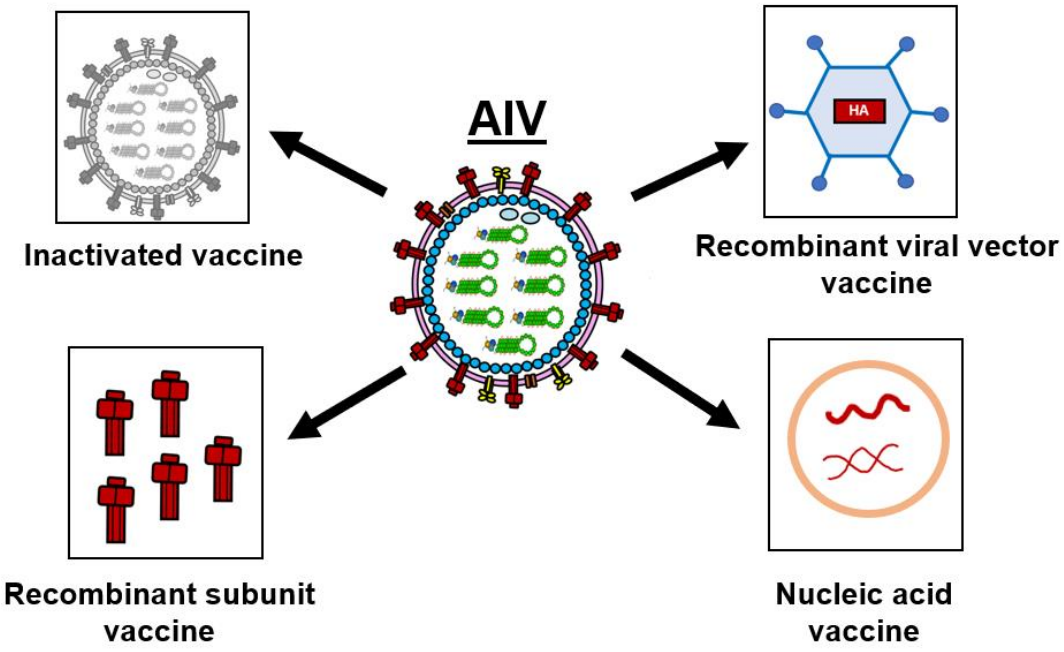


Figure 1.11. Major types of poultry vaccines that are either commercially available or currently under development for treating avian influenza virus.

1.4.1.1. Inactivated whole AIV vaccine

The majority of avian influenza vaccines that are currently licensed for use in poultry are inactivated whole AIV vaccines administered via parenteral route[248] (subcutaneous or intramuscular) (**Table 4**). The vaccine seed viruses are grown in specific- pathogen free (SPF) embryonated hen's eggs, and the infective allantoic fluid is collected and then chemically inactivated using either β-propiolactone, formalin or binary ethyleneimine[245,249,258]. This chemical inactivation cross-links viral proteins and nucleic acids such that viral replication cannot occur[259]. The degree of purification of the allantoic fluid might affect the overall immune response generated following vaccination however, poultry AIV vaccines use crude allantoic fluid without purification to reduce the production cost[248]. Most of the licensed poultry inactivated AIV vaccines are emulsified in a mineral oil adjuvant system.

Such oil-based emulsions stimulate the immune system and can extend the release of antigens, further enhancing the antibody production[260,261]. Moreover, the non-replicating nature of inactivated AIV vaccine increases the safety of the vaccine. However, inactivated AIV vaccines provide sub-optimal immunity hence, they need to be repeatedly administered with high antigen quantities. Furthermore, inclusion of adjuvants is required to enhance the humoral and cellular immune responses[249]. There is a huge risk of transmission of HPAI viruses to humans when producing them in a large scale for inactivation. Therefore, LPAI viruses isolated from poultry outbreaks are typically used to make inactivated AIV vaccines. In rare cases, HPAI viruses e.g. H7N3 in Pakistan and H5N1 in Indonesia have been used as seed viruses for making inactivated AIV vaccines[246]. The development of reverse genetic system for influenza viruses has made it possible to make inactivated AIV vaccines using HPAI viruses by replacing the polybasic cleavage site of HA to a mono-, or di-basic cleavage site, thus converting a HPAI to a LPAI[262,263]. In addition, reverse genetic systems allow incorporation of HA and NA of recent field viruses and the remaining six gene segments from a high growth AIV virus such as PR8. Thus, the vaccine seed strains are antigenically similar to the field strains, increasing the overall vaccine efficacy[264]. However, the usage of inactivated AIV vaccines is limited because of poor DIVA compatibility and interference from MDAs[246].

Table 4: List of some inactivated whole AIV vaccines available for commercial use in poultry.

Name	Subtype	Description	Supplier
Layermune® AIV H5N9	H5N9 LPAI	Monovalent, oil adjuvant	CEVA Animal Health
Gallimune® Flu H5N9	H5N9 LPAI	Monovalent, oil adjuvant	Boehringer Ingelheim
Volvac® AI KV	H5N2 LPAI	Monovalent, oil adjuvant	Boehringer Ingelheim
CEVAC®FLU-KEM	H5N2 LPAI	Monovalent, oil adjuvant	CEVA Animal Health
Nobilis® Influenza H5N2	H5N2 LPAI	Monovalent, oil adjuvant	MSD Animal Health
ITA-FLU	H5N2 LPAI	Monovalent, oil adjuvant	Laprovet S.A.S, France
Biovac®-AI	H5N1 HPAI	Monovalent, oil adjuvant	Biolabs (pvt) Ltd, Pakistan
Fluvac®-H5	H5N1 HPAI	Monovalent, oil adjuvant	Ottoman Pharmaceuticals, Pakistan
Gallimune® H9	H9N2 LPAI	Monovalent, oil adjuvant	Boehringer Ingelheim
CEVAC®NEW-FLU-KEM	H5N2 LPAI/ NDV	Bivalent, oil adjuvant	CEVA Animal Health
Poulvac® Flufend I-AI H5N9 H7N1	H5N9/H7N1 LPAI	Bivalent, oil adjuvant	Fort Dodge Animal Health, USA
Nobilis® Influenza H9N2+ND	H9N2 LPAI/ NDV	Bivalent, oil adjuvant	MSD Animal Health
Nobilis® Influenza H9N2	H9N2 LPAI	Monovalent, oil adjuvant	MSD Animal Health
Nobilis® H9N2+ND+ P	H9N2 LPAI/ NDV	Bivalent, oil adjuvant with PAMP	MSD Animal Health

Nobilis® H9N2 P	H9N2 LPAI	Monovalent, oil adjuvant with PAMP	MSD Animal Health
Nobilis® Influenza H5N2	H5N2 HPAI	Monovalent, oil adjuvant	MSD Animal Health

ND: Newcastle Disease **LPAI**: Low pathogenicity avian influenza **HPAI**: High pathogenicity avian influenza

1.4.1.2. Live recombinant viral vector vaccine

The live recombinant viral vector vaccine is based on the *in vivo* expression of antigens using viral vectors. These viral vectors are recombinantly engineered to incorporate genetic material from the AIV genome, typically the HA gene, into a viral backbone for *in vivo* gene expression[248]. The viral vectors are considered as live vaccines, but their replication is attenuated or even deficient. This increases safety of the vaccine. Various viral vector vaccines engineered to express AIV genes have been reported in literature for poultry. These include recombinant fowl poxvirus (FPV), Newcastle disease virus (NDV), herpesvirus of infectious laryngotracheitis (ILT), adenoviruses, herpesvirus of turkeys (HVT) and duck enteritis virus (DEV)[265]. The viral vector vaccines have several advantages over inactivated AIV vaccines. They do not induce antibodies against viral proteins such as NA or NP hence, enzyme-linked immunosorbent assay (ELISA) can be performed against these antibodies to differentiate vaccinated and infected birds[266]. Furthermore, viral vector vaccines are known to induce both humoral and CMI, with the latter reported to be important for clearance of virus from infected cells[248,267]. Some viral vectors like recombinant FPV (rFPV) and recombinant NDV (rNDV) often show sub-optimal protective efficacy in vaccinated birds carrying pre-existing vector virus-specific MDA. For example, most poultry raised in the developing

countries would have been immunised against NDV[268]. This will limit or restrict the immunogenicity of the rNDV vaccines. On the other hand, the recombinant HVT (rHVT) vaccines are known to overcome the MDA interference hence, they can be administered in the hatchery at 1-day of age or *in ovo* which improves the biosecurity and quality control of the vaccination[269]. The rFPV and rHVT vaccines are administered via parenteral injection like the inactivated AIV vaccines. However, rNDV vaccines can be applied by mass administration (drinking water, aerosol spray or eye drop) thus, reducing the cost of administration[270]. The host range is an important factor to consider when immunising bird species with different vector vaccines. For example, rFPV can only be used in chickens due to the host restriction of the vector, whereas rHVT has wider host range (chickens, turkeys, ducks and geese)[248,271]. The rFPV, rNDV and rHVT vaccines have been licensed for use in different countries (**Table 5**).

Table 5: Recombinant viral vector AIV vaccines available for commercial use in poultry.

Viral vector	Subtype	Countries licensed	Supplier
rFPV	H5N8/H7N3	China, Mexico, Guatemala, USA	Boehringer Ingelheim
rHVT	H5N1	Bangladesh, Egypt, Mexico, Vietnam, USA	CEVA Animal Health
rNDV	H5N1	China, Mexico	Harbin veterinary research institute, China

1.4.1.3. Recombinant subunit vaccines

Recombinant subunit vaccines are based on the production of different AIV proteins (typically HA) using various *in vitro* expression systems like insect cells,

bacteria, yeast, plants, and eukaryotic cell cultures[246]. The expressed antigen is recovered from the supernatant or cell lysates, purified, and emulsified in a similar manner to the inactivated whole AIV vaccine[248]. Subunit vaccines maintain the highest safety profile compared to other vaccine types due to the absence of infectious viruses. The HA protein is the major surface glycoprotein of AIV and the most used antigen for preparation of recombinant subunit influenza vaccines[272]. The recombinant subunit vaccine based on full-length H5HA protein, produced using baculovirus expression system protected chickens and mice from a lethal challenge with H5N1 HPAI virus[273]. Similarly, another recombinant H1HA subunit vaccine (HAC1) produced in a plant virus-based transient expression technology in *Nicotiana benthamiana* was demonstrated to be safe and immunogenic in mice and ferrets[274]. A phase I study of HAC1 vaccine was also conducted in humans (healthy adults, 18-50 years of age). The HAC1 vaccine was shown to be immunogenic and safe in humans[275]. The recombinant subunit influenza vaccines based on HA protein are licensed for commercial use in humans and poultry[276,277](**Table 6**). Moreover, the HA2 subunit of HA protein is more conserved than the HA1 subunit. Therefore, immune responses induced by the HA2 subunit are expected to elicit broadly cross-protective antibodies, suggesting its development as a universal vaccine[278]. Indeed, vaccination with a synthetic HA2 peptide-based subunit influenza vaccine protected mice against divergent viral subtypes H3N2, H1N1 and H5N1[279]. However, fewer antibodies are produced against the HA2 subunit compared to HA1 subunit due to the steric shielding by HA1 globular head domain, thereby decreasing the accessibility to HA2 epitopes[66]. The antibodies against the HA2 subunit are non-neutralising as they do not inhibit the receptor binding activity

of HA protein. However, these antibodies can prevent the fusion of virus and host membranes, and also mediate ADCC and complement cytotoxicity[278].

Other AIV proteins that have been studied in mammals as a potential for development as influenza subunit vaccines include M1, M2e, NP and NA[280], indicating that these could also be applied for poultry. *Escherichia coli* (*E. coli*) expressed M1 protein formulated with chitosan adjuvant provided 100% protection against homologous H9N2 challenge, and 70% and 30% protection against heterologous H1N1 and H5N1 viruses in mice, respectively[281]. Similarly, a recombinant fusion protein containing three tandem H5N1 M2e proteins was able to provide significant M2e-specific immune responses and cross-clade protective immunity against divergent H5N1 viruses in mice[282]. In addition, phase I clinical studies with recombinant M2e vaccine candidates have demonstrated safety and immunogenicity in humans[283]. NP is a highly conserved internal protein of influenza virus and was shown capable of providing cross-protection against heterosubtypic influenza virus challenge in mice[284]. On the other hand, immunity induced by NA was shown to be effective against homologous viruses, but was less cross-reactive and cross-protective against heterologous viruses in mice[285,286]. However, a universal monoclonal antibody targeting the conserved epitope in NA (residues 222-230) was able to inhibit different subtypes of IAVs and provide heterosubtypic protection in mice[287]. One of the challenges in developing effective recombinant subunit influenza vaccines is finding the balance between epitope variability and protective immunogenicity. The most protective epitopes are HA and NA however, they are also the most variable viral proteins. M1, M2e and NP have more conserved epitopes, but they provide lesser protective immunity (**Figure 1.12**). Although, the production of recombinant subunit influenza vaccines is rapid,

economic, stable, and scalable, such vaccines have poor immunogenicity and often require additional adjuvants to enhance the humoral and CMI[272]. In recent years, various strategies have been developed to enhance the overall immunogenicity of subunit vaccines. One such strategy is developing targeted antigen delivery vaccines (TADV) which is the primary subject of this thesis and explained in **section 1.5.**

Table 6: Recombinant subunit AIV vaccine available for commercial use in poultry.

Name	Expression system	Description	Supplier
Volvac® B.E.S.T AI+ND	Baculovirus Sf9 insect cells	Bivalent vaccine containing HA from H5N1 AIV and inactivated NDV	Boehringer Ingelheim

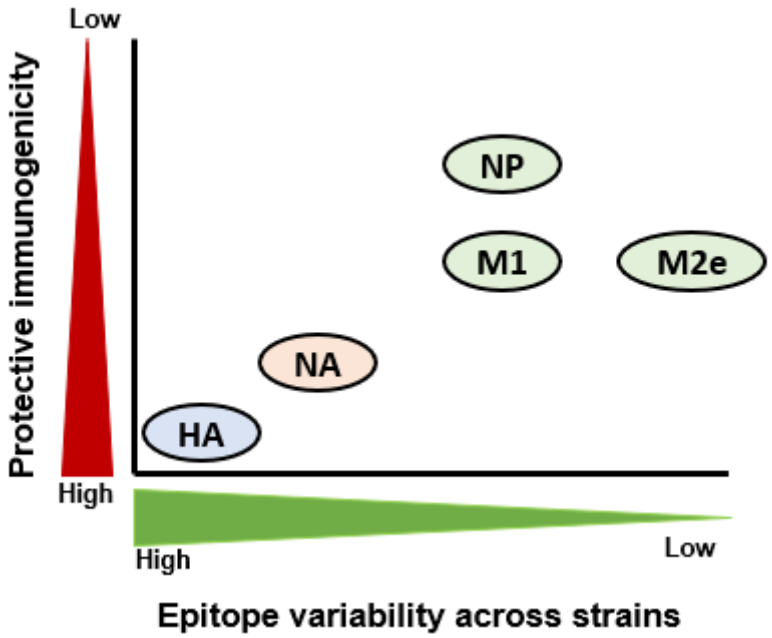


Figure 1.12: The dilemma of recombinant subunit influenza vaccines. HA and NA are the most protective epitopes, but they are very variable. On the other hand, M1, NP and M2e are more conserved epitopes however, they are less protective. The challenge is to find the right balance between immunogenicity and epitope variability. Figure adapted from Houser and Subbarao[288].

1.4.1.4. Nucleic acid vaccine

Nucleic acid vaccines for AIV are primarily deoxyribonucleic acid (DNA) plasmids that contain complementary DNA (cDNA) inserts that code for different AIV proteins (typically HA). DNA vaccines expressing HA have demonstrated a protective immune response in chickens against variety of H5 and H7 HPAI viruses[289,290]. Such vaccines produce antigen *in situ*, inducing both humoral and CMI[291]. Furthermore, DNA vaccines can integrate multi-epitopes or modified epitopes and are stable even at room temperature[292]. Studies investigating protective efficacy of DNA vaccines in chickens have reported the need for multiple immunisations compared to inactivated whole AIV and recombinant subunit vaccines[291,293,294]. This is one of the major limitations of the use of DNA vaccines in poultry. Currently, DNA vaccines are economically prohibitive for use in poultry. There is a need for additional research to identify better adjuvants and stronger promoters, for reducing the number of immunisations and quantity of nucleic acid needed per bird[246]. In addition, very few plasmid backbones (pcDNA, pVax, pCI, pIRES or pCAGGS) are developed for poultry DNA vaccination[291]. The plasmid backbones can be optimised for protein expression by using strong promoters. In chickens, the CMV promoter or chicken β-actin promoter-based plasmids have been used[291]. Apart from optimising promoters, the transcription terminator can also be optimised. Transcription termination of RNA polymerase requires a polyA signal and a downstream terminator sequence. It was established that transcription termination acts as a driving force for optimal gene expression and increases the efficiency of pre-mRNA processing in HeLa cells[295]. Therefore, different transcription terminators can be tested for optimising the plasmid backbones for protein expression in chickens. Moreover, several other improvements in DNA

vaccination have been demonstrated in poultry. DNA vaccine priming followed by protein boost was shown to be more effective than a prime/boost strategy with DNA vaccine alone in chickens[296,297]. Furthermore, the concurrent use of immunomodulators (e.g., IL-2 or IFN-γ) with DNA vaccines improved the immunological response in ducks and chickens compared to DNA vaccine alone[291,298,299]. Other strategies for increasing poultry DNA vaccine efficacy include routes of inoculation, plasmid DNA dose, improving uptake of plasmids by the host cells and host codon usage[291].

Apart from DNA vaccines, the use of alphavirus replicon RNA (repRNA) vaccine has also been studied in poultry. Contrary to the conventional mRNA vaccines that are directly translated from the incoming RNA molecules, introduction of repRNA into cells initiates continuous biosynthesis of antigen-encoding RNA, often requiring only a single administration to be effective[300,301]. This increases both the amount and duration of antigen expression eliciting more robust and long-lasting immune responses. In the context of AIV, alphavirus repRNA vaccine coding HA protein of H5N8 virus performed better than the inactivated virus vaccine in chickens. Chickens receiving a single dose of this vaccine at 7-day-old demonstrated higher HI antibody titres, greater reduction of viral shedding from buccal and cloacal cavities, and higher protection of chickens from mortality compared to the inactivated virus vaccine[302]. This alphavirus-repRNA AIV vaccine has been conditionally licensed in the USA (Merck Animal Health, Madison, NJ, USA).

1.4.2. Limitation of field usage of avian influenza vaccines

An ideal AIV vaccines should induce complete resistance to infection, prevent virus replication and excretion, prevent clinical signs and death, and allow identification of infected animals within a vaccinated population[248]. However, few if any commercially available or experimentally tested AIV vaccines satisfy all of the above requirements. Most AIV vaccines provide consistent protection against clinical signs and death however, immunity is not absolute in the field. Thus, even the vaccinated birds can potentially become infected with field virus and shed the virus into the environment[246]. Therefore, it is important to design monitoring programs to detect infections among vaccinated populations. Such monitoring can be done by routine serological test detecting antibodies against NP, heterologous NA, or NS-1 proteins[303]. However, not all the AIV vaccines are compatible with such serological differentiation tests. Some countries prohibit the use of AIV vaccines that have poor DIVA compatibility as these vaccines can interfere with the disease surveillance system[304]. Only AIV vaccines that are licensed by a country's national veterinary authority should be considered for use in the field.

1.5. Avian antigen presenting cells

Antigen presenting cells (APCs) are group of immune cells that mediate immune responses by processing and presenting antigens for the recognition by T cells or B cells. The professional APCs in mammals include DCs, macrophages and B cells. Of the three types of mammalian professional APCs, DCs and macrophages are considered principle professional APCs in birds. The B cells are considered less important for antigen presentation in birds since bursectomised

birds which lacks B cells, can mount normal T cell responses[305]. Avian DCs and macrophages are described in detail below.

1.5.1. Dendritic cells

DCs are important APCs with the unique capacity to initiate immune responses. They are derived from haematopoietic stem cells in the bone marrow and seed in non-lymphoid tissues where they develop into immature DCs[306]. Immature DCs are specialised in antigen capture (phagocytosis, micropinocytosis, and endocytosis) and processing but are poor T cell stimulators[306]. Upon activation, they migrate out of non-lymphoid tissues into T cell regions of secondary lymphoid tissues where they complete their maturation[306]. The maturation of DCs is characterised by the redistribution of MHC molecules from intracellular endocytic compartments to the cell surface, increase in the expression of co-stimulatory molecules required for the T cell activation, secretion of distinct cytokines and chemokines, cytoskeletal reorganisation and morphological changes including the proliferation of dendrites from the membrane of DCs[226]. Like in mammals, different subtypes of DCs have been identified in birds. A possible DC was first described in chickens using light and electron microscopy in the bursa and in the cecal tonsil's germinal centre (GC)[307–309]. Due to their highly elongated shape with short dendrite-like processes and presence of secretory granules in the cytoplasm, the cell was named the bursal secretory dendritic cells (BSDCs)[307,309]. BSDCs express vimentin intermediate filament, MHC II molecules, dendritic cell receptor for Endocytosis-205 (Dec205), cluster of differentiation 83 (CD83), cluster of differentiation 11c (CD11c), macrophage colony-stimulating factor 1 receptor (CSF1R), and constitutively express IgY binding receptor[309–311]. The BSDC play an important role

in B cell maturation after the hatch by displaying immune complexes formed from maternal derived antibodies and environmental antigens on their surface[312,313]. The thymus is the primary site of T cell development which arises at day 5 of chick embryogenesis[306]. Thymic dendritic cells (TDCs) were characterised using 74.3 monoclonal antibody (mAb) and shown to be concentrated in thymic medulla[306]. TDCs express vimentin, MHC II and CD83[306]. The adult avian TDCs occur as cell clusters and do not appear to undergo extensive local proliferation suggesting that TDCs are generated from circulating DC progenitors which continuously colonise the thymus stroma without further cell division[306]. Avian follicular DCs (FDCs), like mammalian FDCs occur in GC of the secondary lymphoid organs[306]. They display ramified morphology, contain cytoplasmic granules, and can trap antigens for presenting to B cells in the form of immune complexes[308,314,315]. Chicken FDCs have been identified in the GC of the spleen, oesophageal, pyloric and cecal tonsils and pineal gland, and resemble BSDCs in terms of their cytological structure and cell surface phenotypes[316–319]. The spleen is the largest peripheral lymphoid organ in chickens[306], and it plays a significant role in both antibacterial and antiviral immune responses against acquired antigens. The adult chicken spleen consists of FDCs of the GC (B cell zone) and interdigitating dendritic cells (IDCs) of the periarteriolar lymphoid sheath (PALS, T cell zone)[306]. The cytological structure and immunophenotype of FDCs and IDCs are highly similar to that of the ellipsoid-associated cell (EAC), which are highly phagocytic and responsible for the clearance of antigens from the circulation[317,318,320,321]. Various experimental evidence in chickens have proven that intravenously injected antigens (serum bovine albumin, β-galactosidase) or intracellular tracers (colloidal carbon, horse-radish peroxidase) are trapped by EACs[314,322,323]. The internalisation of antigens by

the EACs or the retention of immunocomplexes on the surface of EACs triggers their detachment from the ellipsoid surface, and transport of antigens from the ellipsoid into the PALS and GC. This migration pathway has led to the hypothesis that EAC could be the precursor cells of FDCs and IDCs[323].

The detailed immunohistochemical staining and enzyme histochemistry have demonstrated that avian skin contains about 2000 cells/mm^2 Langerhans cells (LCs)[324]. The avian LCs express CD45, vimentin, MHC II, and CD1 that overlap with the intense membrane ATPase activity[325–328]. The avian LCs share many aspects in common with the mammalian LCs. The most prominent one is the presence of the rod-like cytoplasmic organelles, the Birbeck granules[306]. Moreover, three sub-populations of DCs can be distinguished using monocyte, macrophage, and T cell specific markers in the chicken skin[325]. In addition to the epidermis, ATPase/vimentin/MHC II and 74.3+LCs like DCs have also been identified in the mucosal surface of the eye, tongue and esophagus of the chicken[319,329]. The immunisation experiments using haptens and fluorescent molecules applied to the mammalian skin have shown that LCs carrying antigens migrate to the nearest peripheral lymph node where they initiate an adaptive immune response[330]. Moreover, the cutaneous fluorescein isothiocyanate administration in birds demonstrated that the LCs loaded with hapten migrated out of the epidermis and appeared in the subcutaneous lymph nodules located around small veins and lymphatics[325]. The proliferating LCs were identified in the chicken epidermis maintaining the equilibrium against antigen stimulated emigration of LCs[325].

Chicken bone marrow DCs (BMDCs) have been successfully generated *in vitro* from chicken bone marrow precursor (chicken femur)[331]. Chicken bone marrow cells were cultured in the presence of recombinant chicken IL-4 and

granulocyte-macrophage colony stimulating factor (GM-CSF) for 7 days. The cultured cells had the morphology of typical mammalian DCs[331]. The majority of non-stimulated BMDCs showed high surface expression of MHC II and CD11c, moderate or low levels of co-stimulatory molecules and no surface expression of CD83 or Dec205. They could endocytose fluorescein isothiocyanate (FITC)-labelled dextran and phagocytose fluorescent latex beads but were poor stimulators of peripheral blood lymphocytes. Thus, they were defined as immature chicken BMDCs. Upon stimulation of the immature chicken BMDCs with chicken CD40L and lipopolysaccharides (LPS), the surface expression of co-stimulatory molecules (CD40 and CD86), CD83 and Dec205 were elevated compared to those seen on non-stimulated chicken BMDCs[331]. Furthermore, there was a significant reduction in their capability to uptake FITC-dextran, indicating matured DC phenotype[331]. Therefore, the higher expression of surface markers and the reduction in the ability of DCs for uptaking antigens could be used to characterise matured chicken DCs *in vitro*.

1.5.2. Macrophages

The term macrophage was introduced in 1884 by Ilya Metchnikoff to describe leukocytes that are capable of ingesting and destroying foreign substances, including micro-organisms[332]. They play a key role in tissue homeostasis, innate and adaptive immunity, inflammation, and immunopathology[333]. Macrophages can detect environmental signals through specific receptors, phagocytose apoptotic and necrotic cells as well as invading pathogens, and respond to these stimuli by the secretion of signalling and effector molecules[334]. The knowledge of avian macrophages is limited in comparison to the

mammals. Over the years, new technologies to generate avian macrophage cultures *in vitro* and generation of new antibodies have advanced our understanding of avian macrophage biology. The mononuclear phagocytes consist of monoblasts, promonocytes and macrophages[335]. The general morphology and distribution of different sub-populations of mononuclear phagocytes in birds and mammals are very similar. Like in mammals, mononuclear phagocytes in birds originate from bone marrow stem cells[336]. Bone marrow derived monoblasts consecutively differentiate into promonocytes and monocytes[336]. These monocytes migrate from the peripheral blood to the tissues and differentiate into macrophages[336]. The morphology and function of these macrophages are dependent on the organ and tissue in which they are present. Avian macrophages are adherent and highly phagocytic[337]. They were identified based on their phagocytic uptake of neutral red, acridine orange and immune complexes, among others[338–340]. They also contain endogenous enzymatic activities such as non-specific esterase activity[338] and acid phosphatase activity[320] involved in processing of the phagocytosed antigen. Phagocytosis is the best-known function of macrophages that has been used to identify these cells *in situ* and to discriminate them from other leukocytes in mixed-cell preparations[337]. In chicken embryos, macrophages with phagocytic activity have been observed as early as embryonic incubation day (EID) 12 in the liver and EID 16 in the spleen[188]. In mammals, macrophages are equipped with a range of receptor systems such as scavenger receptors, complement receptors, Fc receptors, C-type lectins and mannose receptors aiding binding of microorganisms prior to phagocytosis[334]. Some of these molecules are also present on primary avian macrophages and macrophage cells lines (HD11, MQ-NCSU and HTC). E.g., chicken macrophage-like cell line HD11

contains cell surface antigens like MHC I/II, transferrin receptor, CD40, K1, K55, KULO1 and different TLRs[337]. Specific antibodies against some of these cell surface antigens have been developed to characterise avian macrophages. However, in contrast to the mammals, only few mAb reactive with the cells of the monocyte/macrophage lineage of chickens are available. The first mAb reacting with myeloid cells of the chicken were developed by immunising mice with avian myelocytomastosis virus (AMV) and MC29-transformed chicken myeloblasts and macrophages[341]. Trembicki et al established mAb CMTD-1 and CMTD-2 that reacted with mature macrophages but did not react with blood monocytes or myeloid precursors[337]. The mAb CVI-ChNL-68.1 specifically reacts with an unknown cytoplasmic antigen of monocytes, macrophages, and interdigitating cells in tissue sections[342]. The mAb CVI-ChNL-74.2 recognises only a sub-population of macrophages in lymphoid tissues[320]. The mAb K1 was obtained by immunising mice with chicken spleen cell suspensions and was originally described as monocyte/macrophage specific[343]. This mAb has been used in flow cytometry and immunohistology to identify monocytes and macrophages, and to distinguish lymphocytes from thrombocytes in peripheral blood leukocyte preparations[344,345]. The mAb K1 has also been used in other avian species, including duck and guinea fowl[346,347]. A cell lineage-specific mAb for cells of the mononuclear phagocyte system of the chicken was developed by Mast et al and designated KUL01[335]. Chicken mannose receptor has been identified as KUL01 antigen[348]. Thus, KUL01 is the first monocyte/macrophage lineage-specific mAb in chicken for which the antigen has been defined at the molecular level[348].

Activated macrophages release reactive oxygen and nitrogen intermediates which are important microbicidal mechanism[333]. Dietert et al was the first to

demonstrate nitric oxide (NO) production by chicken macrophages[349]. The stimulation of Sephadex-elicited macrophages or HD11 cells by LPS led to the secretion of maximal NO_2^- into the cell culture medium within 24 hours[349]. Due to the simplicity of NO_2^- quantification, the measurement of nitric oxide synthase (NOS) II activity in chicken macrophages has become a frequently used method for the analysis of avian macrophage activation. NO secretion has also been used as a readout system for the identification of PAMPs with macrophage activating activity[337]. Furthermore, activated macrophages can also produce range of cytokines and chemokines. It has been established that activation of chicken macrophage cell lines and primary macrophages with different PAMPs induce the secretion of IL-6 and IL-1β[350–353]. In addition to pro-inflammatory cytokines, LPS-activated HD11 cells and monocyte-derived macrophages also produce the anti-inflammatory cytokine IL-10, thereby exerting multiple immunoregulatory effects during infections[354].

Moreover, avian macrophage biology has been studied *in vitro* utilising transformed cell lines or primary macrophage cultures. A new technology was introduced to generate avian macrophage cultures using essential growth and differentiation factors for macrophages[355]. This involves culturing of bone marrow cells in the presence of recombinant chicken macrophage colony stimulating factor (M-CSF) or IL-34 for 12 days[355]. The functional properties of these bone marrow derived chicken macrophages are not fully characterised.

1.6. Strategies for targeting antigens to antigen presenting cells

Professional APCs like dendritic cells and macrophages are important regulatory factors of humoral and cellular immune responses[356]. Hence, they are valuable for enhancing the vaccine efficacy. In recent years, the most extensively studied approach in the field of vaccine development has been the design of APC targeted vaccines (i.e., TADV). Such vaccines have shown to induce durable and effective immune responses. Additionally, APC targeted vaccines have also been used for the development of immunotherapies against cancer, autoimmunity, and infectious diseases in humans[357]. At present either ligand- or antibody-based methods are used for targeting antigens to APCs in mammals. These methods are described in detail below.

1.5.1. Ligand-Based APC targeting

The surface of antigen presenting cells e.g., DCs consist of PRRs which are specialised to recognise conserved structures on pathogens, termed as PAMPs. Major PAMPs include nucleic acids, proteins, sugars, and lipids. The type and intensity of the immune responses depend upon the recognition between PRRs and PAMPs. In ligand-based APC targeting, antigens are conjugated either chemically or recombinantly to the PRR ligands (PAMPs) resulting in antigen–PRR ligand conjugate vaccines. The conjugated PRR ligand is targeted to the associated PRRs on APCs, which in turn ensures that APCs process the antigen (**Figure 1.13**). Out of the four PRR families: TLRs, nucleotide-binding oligomerisation domain-like receptors (NLRs), RLRs, and C-type lectin receptors (CLRs), the TLRs have been

extensively used in developing antigen-PRR ligand conjugate vaccines in mammals[357].

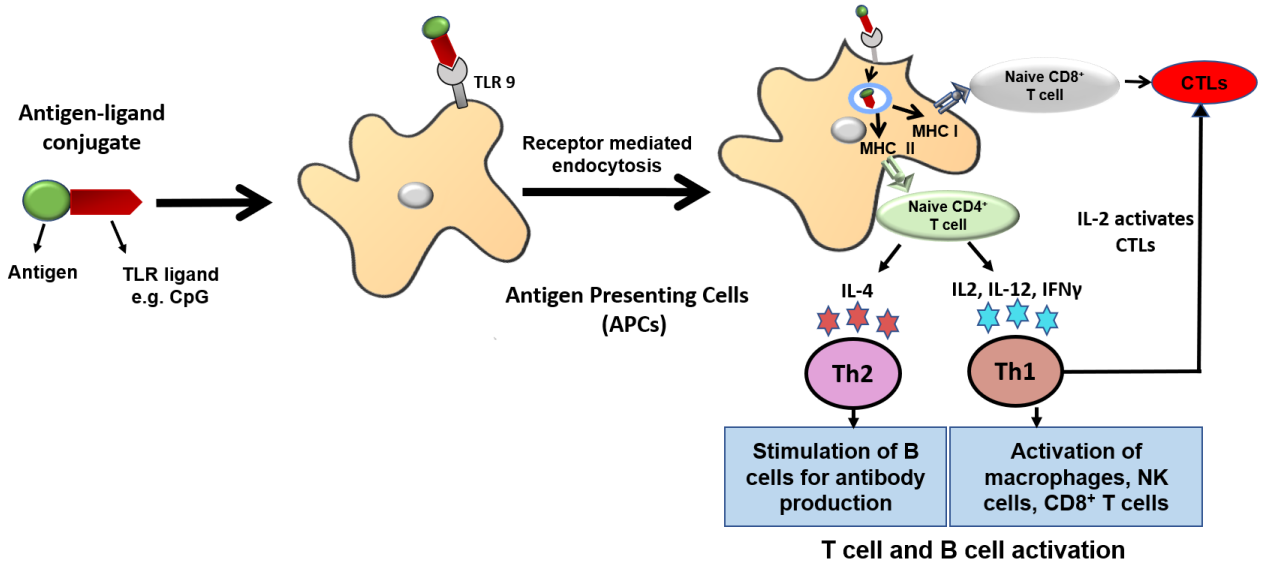


Figure 1.13. Schematic representation of ligand-based antigen targeting. Antigen can be conjugated to TLR ligands e.g., CpG oligodeoxynucleotides. Upon binding of the antigen-PRR ligand conjugate to the cognate receptor, it undergoes receptor mediated endocytosis resulting in the activation of both $CD4^+$ and $CD8^+$ T cell responses.

TLRs are transmembrane proteins present either on the cell surface or on endosomal vesicles. They recognise evolutionary conserved molecular motifs of infectious microbes such as bacterial cell wall components such as LPS, peptidoglycan, lipopeptides, flagellin, bacterial DNA and viral RNA[358]. To date, 13 and 10 TLRs have been identified in mammals and chickens, respectively[359,360]. TLRs are known to exhibit wide range of functions in host defence system hence, they are regarded as a key target for vaccine development. One of the major PRR ligands used in mammals for developing antigen–PRR ligand conjugate vaccines is CpG oligodeoxynucleotide (ODNs) (TLR 9 ligand)[357]. Model antigen ovalbumin (OVA) chemically crosslinked to CpG demonstrated more potent humoral immunity and CMI compared to free antigen and ligand in mice[361]. Similarly, immunisation of mice with OVA fused to flagellin (TLR 5 ligand) resulted in a better antigen-specific

T and B cell responses compared to OVA emulsified in complete Freund's adjuvant (CFA)[362]. Furthermore, antigen–flagellin conjugate vaccine has been studied before in chickens. Song et al generated a H7HA influenza subunit vaccine recombinantly fused to *Salmonella typhimurium* flagellin (H7HA-fliC). The immunisation of chickens with H7HA-fliC showed robust antibody responses leading to a significant reduction in viral loads compared to the chickens receiving only H7HA[363]. Moreover, TLR2 is usually described as the TLR recognising the largest range of ligands[364]. These include various bacterial cell wall components such as lipoproteins, peptidoglycan, LPS and lipoteichoic acids. Antigens coupled to TLR2 targeting lipid moieties have shown to induce a balanced T helper response and promote the development of antibody responses in mice[364]. Furthermore, one of the TLR 2 ligand $(Pam_3C)SK_4$ stimulated chicken splenocytes *in vitro* to produce both Th1 and Th2 associated cytokines, making it a potential ligand for PRR targeting[365]. TLR ligands such as Polyinosinic: polycytidylic acid (Poly I:C, TLR 3), LPS (TLR 4), flagellin (TLR 5) and CpG ODNs (TLR 21, equivalent of mammalian TLR 9) have been used as vaccine adjuvants for various viral diseases in chickens[366]. Therefore, TLR ligands are promising candidates for ligand-based APC targeting in chickens. A summary of studies relating to ligand-based APC targeting is provided in **Table 7**.

Table 7: Ligand-Based APC targeted vaccines.

Antigen	Ligands	Targeted receptor	Species	Key findings	Refs
Amb a 1	CpG	TLR 9	Rabbit	Enhanced antibody production	367
HIV gp120	CpG	TLR 9	Mouse	Enhanced $CD8^+$ T cells	368
Recombinant LLO and p60	CpG	TLR 9	Mouse	Induction of $CD8^+$ T cell mediated protection	369
HIV gp120	CpG	TLR 9	Mouse	Induction of Th1 cells, CTLs and HIV neutralising antibodies	370
OVA	3M-042/ 3M-044	TLR 7/8	Mouse	Induced potent Th1 $CD4^+$ and $CD8^+$ T cell responses.	371
HIV Gag	3M-012	TLR 7/8	Rhesus macaques	Enhanced antibody and Th1 responses	372
OVA and LLO	Flagellin	TLR 5	Mouse	Enhanced IgG1, IgG2a antibody and $CD8^+$ T cell responses.	362
Influenza H7HA	Flagellin	TLR 5	Mouse and Chickens	Induction of robust humoral and cellular responses and protection.	363, 373
gp100	Poly I:C	TLR 3	Mouse	Induction of potent $CD4^+$ T cell and CTL responses.	374
Various peptides	Pam2Cys	TLR 2	Mouse	Induction of potent antibody and $CD8^+$ T cell responses.	375
ESAT-6	Rv1411c	TLR 2	Mouse	Higher $CD4^+$ T cell response.	376

TLR: Toll-like receptor, **Amb a 1**: Ragweed allergen (*Ambrosia artemisiifolia*), **OVA**: ovalbumin, **LLO**: Listeriolysin O, **p60**: murein hydrolase, **Pam2Cys**: S-[2,3-bis(palmitoyloxy)propyl] cysteine, **3M012/42/44**: Synthetic TLR 7/8 agonist from 3M pharmaceutical **HIV**: Human Immunodeficiency virus **Poly IC**: Polyinosinic-Polycytidylic Acid, **Rv1411c**: *Mycobacterium tuberculosis* lipoprotein functioning as a TLR2 agonist, **CTLs**: Cytotoxic lymphocytes, **ESAT-6**: early secreted antigenic target of 6 kDa (also known as EsxA)

1.5.2. Antibody-Based APC targeting

In antibody-based APC targeting, antigens are delivered to APCs using antibodies specific to APC receptors. Here, antigens can be conjugated to either mAbs specific for the selected APC surface molecules or antibody fragments such as single chain fragment variable (scFv), fragment antigen binding (Fab) or nanobodies, specific for the APC receptors[377]. The advancement in recombinant antibody technology has greatly facilitated the genetic manipulation of antibody fragments. These small antibody fragments have several advantages in clinical practices including better tissue penetration, rapid blood clearance, lower retention times in non-target tissues and reduced immunogenicity compared to the whole antibodies[378]. The scFv antibodies have been popular for their use in antigen targeting due to their small size. They are the smallest unit of immunoglobulin molecule that hold a complete binding domain of an antibody and are 25-30 kDa in size. They can be synthetically generated by connecting the polypeptides of variable regions of heavy (vH) and light (vL) chains with a flexible peptide linker (**Figure 1.14**). In terms of expression, scFv can be produced cost-effectively in large quantities with correct folding properties in different cell types compared to the whole antibodies which require expensive cell culture techniques due to complicated folding and glycosylation patterns[378]. The targeting of antigens to specific receptors on APCs using antibodies can prevent the uptake of antigens by

non-target cells. This increases the amount of antigen reaching to APCs. Thus, lesser vaccine doses can be administered, which is an effective way of inducing strong immune responses cost-effectively. Moreover, different aspects of the targeting antibodies need to be considered when delivering antigens to APCs. Some of these aspects include intracellular routes taken by antigen-antibody upon internalisation, speed of internalisation, isotypes of antibodies and the use of full-length antibodies or antibody fragments, which are discussed in Chapter 4.

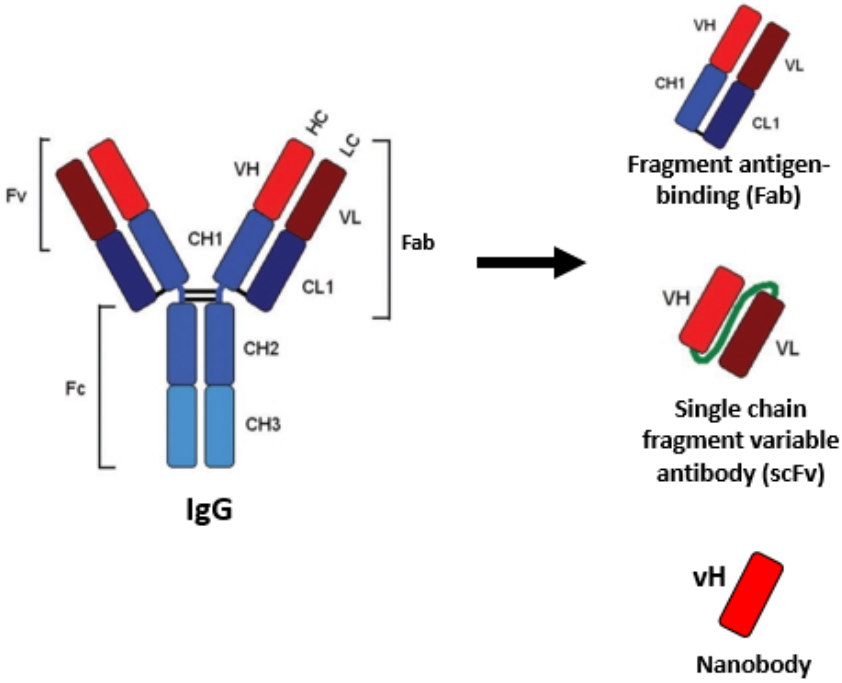


Figure 1.14. Different antibody fragments. Recombinant scFv antibodies are generated by linking vH and vL chains with glycine and serine peptide linkers. **vH**: Variable region of heavy chain **vL**: Variable region of light chain

The antibody-based APC targeting in mammals has been mainly focussed on receptors like CLRs, integrins and Fc receptors (**Figure 1.15**). Among CLRs, Dec205, NK lectin group receptor-1 (DNGR-1) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) are the major receptors being targeted. Various HIV, malaria, *Leishmania spp* and tumour-

associated antigens targeted to Dec205 receptor with its specific antibodies have shown a significant increase in the proliferation of antigen-specific T cells and the production of IFN-γ compared to the non-targeted antigen in mice[357,379,380]. Furthermore, the *in vivo* targeting of antigens to DCs via CD11c has also resulted in the efficient processing and presentation of antigens on both MHC I and MHC II molecules, inducing a robust $CD4^+$ and $CD8^+$ T cell responses in mice[381]. Similar observations were made with prostate-specific antigen conjugated with mAb to Fc-gamma receptor (FcγR) of the humanised THP-1 cells (human monocytic cell lines)[382]. Several other studies have explored antibody-based antigen targeting to APCs, a summary of which is provided in **Table 8**.

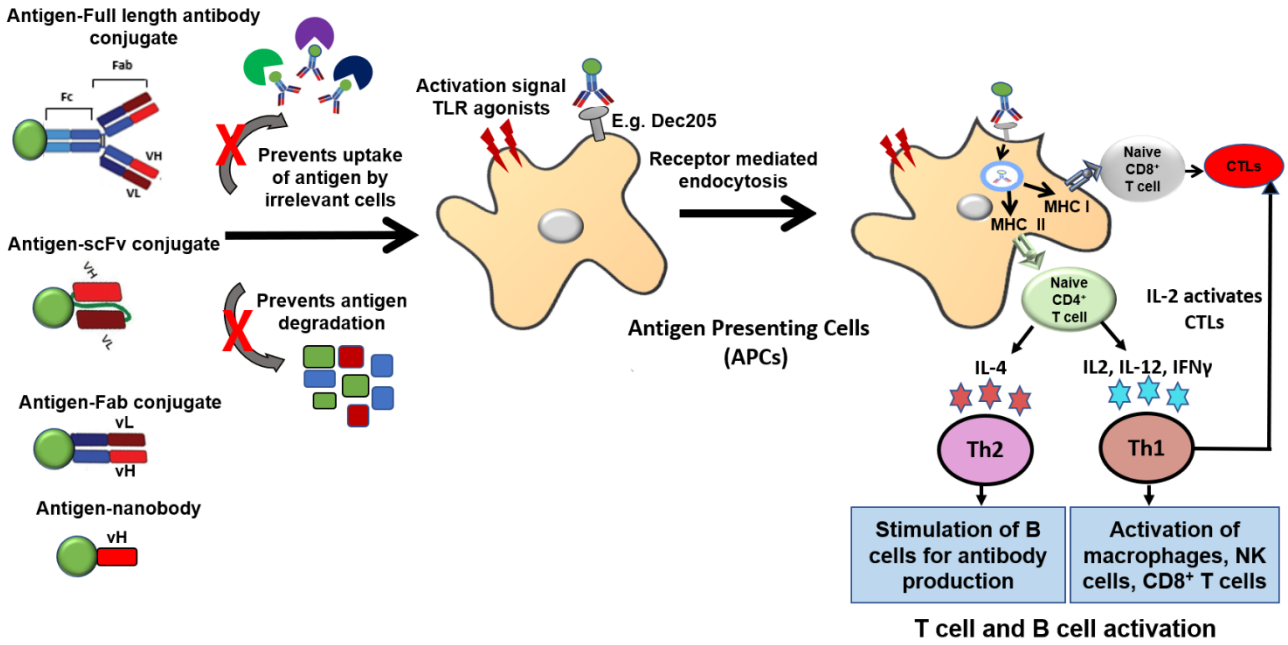


Figure 1.15. Schematic representation of antibody-based antigen targeting. Antigen can be conjugated to antibodies specific to the receptor on antigen presenting cells e.g., Dec205. Upon binding of the antigen-antibody conjugate to the cognate receptor, it undergoes receptor mediated endocytosis resulting in the activation of $CD4^+$ T cells, primarily inducing the antibody response. Depending on the specific receptor targeted, the antigen-antibody conjugate can also activate $CD8^+$ T cells.

Table 8: Antibody-Based APC targeted vaccines.

Antigen	Target	Species	Adjuvant	Key findings	Refs
OVA	Dec205	Mouse	CD40 mAb	Enhanced resistance to rapidly growing tumour and mucosal viral infection.	383,384
HIV gag p24	Dec205	Mouse	Poly I:C	Induction Type I IFN response.	385,386
OVA	Dec205	Mouse	CpG	Potent at inducing CTL response.	387
Dengue NS1	Dec205	Mouse	Poly I:C	Protection from lethal intracranial challenge with DENV2 NGC strain compared to DCIR targeting.	388
Mycobacterial ESX Ag	Dec205	Mouse	Poly I:C	Significant protection against virulent *Mycobacterium tuberculosis*.	389
EBNA1	Dec205	Mouse	Poly ICLC	Induction of $CD4^+$ T cell response	390
Leishmania LmSTI1a	Dec205	Mouse	Poly ICLC	Generation of IFN-γ producing $CD4^+$ T cells, Protection from challenge	391
Tumour antigen: NY-ES01	Dec205	Human	Resiquimod and Poly ICLC	Phase I trial for cancer, Robust humoral and cellular immunity, Stabilization of disease in 13 out of 48 patients.	392

Antigen	Target	Species	Adjuvant	Key findings	Refs
Tumour antigen: HER2/neu	Dec205	Mouse	Poly I:C/CD40 mAb	Targeted antigen induced high levels of T and B cell immunity with long-term survival benefit to mice challenged with neu-expressing tumour.	393
HIV gag	Dec205	Mouse	Poly I:C	$CD4^+$ and $CD8^+$ T cell response, Generation of long-lived T cell memory	394
HIV gag	Dec205	Human	None	Effective cross-presentation of antigens, Stimulation of proliferation and IFN-γ production by $CD8^+$ T cells	395
Influenza H5HA	Dec205	Chicken	LPS	Higher antibody response	396
MSP1a	Dec205	Calves	CD40L	Expansion of IFN-γ secreting $CD4^+$ T-cells, $CD4^+$ T-cell proliferation, and IgG antibody responses	397
KLH	DC-SIGN	Human	None	Induction of antigen specific naïve as well as recall T-cell responses.	398

Antigen	Target	Species	Adjuvant	Key findings	Refs
HIV gp120	DC-SIGN	Human	Poly I:C	Expression of high levels of pro-inflammatory cytokines and increased presentation of both MHC-I and MHC II restricted epitopes.	399
Tumour antigen: gp100	DC-SIGN	Human-*in vitro*	Poly I:C	Cross-presentation efficiency similar as cell penetrating peptides	400
TT	DC-SIGN	Human	None	Targeted delivery induced durable T cell responses in vaccinated donors	401
OVA	DC-SIGN	Mouse	CD40 mAb	Protective against infection with OVA expressing *Listeria monocytogenes*	402
OVA	DNGR1 (Clec9A)	Mouse	Poly I:C	Enhanced $CD4^+$ T cells, $CD8^+$ T cells and antibodies.	403
Tumour peptides	DNGR1 (Clec9A)	Mouse	CD40 mAb	Induction of CTL response, eradication of B16 melanoma lung pseudo metastases.	404
KLH	DNGR1 (Clec9A)	Human-*in vitro*	CD40 mAb and Poly I:C	Induction of recall $CD4^+$ T cell response	405
OVA	CD11c	Mouse	None	Induction of both $CD4^+$ and $CD8^+$ T cell response	381
Polyclonal antibody	CD11c	Mouse	None	Rapid and high antibody response	406

Antigen	Target	Species	Adjuvant	Key findings	Refs
FV gag	CD11c	Mouse	LPS	Improved the activation of virus-specific CTL response	407
OVA	CD11c	Mouse	None	Anti-CD11c antibodies delivered a powerful adjuvant effect by boosting humoral immunity	408
OVA	MR	Mouse	CpG	Enhanced effector T cell immunity and antigen specific protective tumour immunity	409
hCGβ	MR	Human *in vitro*	CD40L	Capable of generating CTLs that could kill human cell lines expressing hCGβ.	410
hCGβ	MR	Human	Poly ICLC and resiquimod	Phase I studies in patients with advanced malignancies. Humoral and T cell responses seen.	411
HIV peptide	CD40	Human *in vitro*	None	Multifunctional $CD8^+$ T cells generated with cytotoxic effects, ability to suppress viral replication *in vitro*	412
HSV glycoprotein D	CD40	Mouse	None	Small amount of CD40 mAb can exhibit potent adjuvant effects when attached to soluble antigen	413

Antigen	Target	Species	Adjuvant	Key findings	Refs
Tumour antigen MSLN	CD40	Mouse	None	Anti-tumour potency was observed with limited systemic toxicity in mice carrying MSLN tumours	414
HIV gag	CD40	Rhesus macaques	Poly ICLC	Broad and sustained HIV specific T cell responses were detected as early as two weeks after the first vaccination	415
Synthetic peptide	CD40	Chicken	None	Accelerated and enhanced antibody response	416
Hen avidin/hen egg lysozyme	MHC II	Mouse	None	Induction of serum IgG and IgA to the target antigen, targeting to intestinal mucosa possible	417
Influenza H1HA	MHC II	Mouse, pigs and ferrets	None	Rapid and increased antibody response	418
Influenza H3HA	MHC II	Rabbits	None	Induction of virus blocking IgG responses more effectively than with alum adjuvant	419

Antigen	Target	Species	Adjuvant	Key findings	Refs
Influenza H1HA	MHC II	Mouse	None	Antibody production and protection was long-lasting, Enhanced induction of T cells that cross-protected between different H1 strains of influenza	420
Tumour antigen Her-2/neu	CD19	Mouse	None	Induction of full B cell activation to produce a high titre of antibodies and enhanced $CD4^+$ Th2 response and $CD8^+$ T cell activation	421
PCC	CD19	Mouse	None	This work illustrated previously unrecognised mechanism for targeting proteins to B lymphocytes for antigen presentation and activation of $CD4^+$ T cells.	422
OVA	FcγR	Mouse	LPS	Stimulation of $CD4^+$ and $CD8^+$ lymphocytes	423
HCMV peptide	FcγR	Human *in vitro*	Poly I:C, LPS	Stimulation of $CD8^+$ T cells, IFN-γ and TNF	424

OVA: Ovalbumin, **MSP1a**: *Anapiasma marginale* antigen, **Her-2/neu**: Human c-ErbB-2, **KLH**: Keyhole limpet hemocyanin, **TT**: Tetanus toxide, **FV**: Friend virus (mouse model for retroviral infection), **HIV**: Human Immunodeficiency virus, **MR**: Mannose receptor, **hCG**: human chorionic gonadotropin, **Poly IC**: Polyinosinic-Polycytidylic Acid, **Poly ICLC**: Polyinosinic-Polycytidylic Acid Stabilised with Polylysine and Carboxymethylcellulose, **Human *in vitro***: Monocyte derived human DCs or human peripheral blood mononuclear cells (PBMCs) obtained from blood of healthy donor, **HSV**: Herpes simplex virus, **CTLs**: Cytotoxic T lymphocytes, **MSLN**: Mesothelin, **HA**: Haemagglutinin, **PCC**: Pigeon cytochrome c, **FcγR**: Fc receptor for IgG, **DC-SIGN**: DC-specific ICAM3-grabbing non-

integrin, **Dec205**: Dendritic cell receptor for endocytosis, **DNGR-1**: Dendritic cell NK lectin group receptor-1, **TNF**: Tumour necrosis factor, **HCMV**: Human cytomegalovirus

1.7. Different APC receptors focussed in this thesis

The work described in this thesis investigates the development of TADV by targeting HA antigen of AIV to three chicken APC receptors: Dec205, CD11c and CD83. To the best of our knowledge, this is the first report of CD11c and CD83 receptors being used in chickens for antigen targeting studies. The details of each of these receptors are given below:

1.7.1. Dec205

CLRs belong to a large family of transmembrane and soluble receptors that can recognise wide variety of glycans on the pathogens in a calcium dependent manner[425]. Dec205 is a type I CLR, first cloned in mice[426]. It consists of a single polypeptide chain: the extracellular portion contains N-terminal cysteine-rich domain, a fibronectin type II domain and 10 carbohydrate recognition domains (CRDs)[427,428] **(Figure 1.16**). It has a molecular weight of 205 kDa. Staines et al, first cloned and sequenced chicken Dec205 cDNA and used the sequence information for generating monoclonal antibodies specific to chicken Dec205[324]. It was found that chicken Dec205 has a highly conserved structure with its human and mouse orthologs. Chicken Dec205 shares about 51% and 48% amino acid similarity to human and mouse Dec205, respectively[324]. The cytosolic domain of mouse Dec205 consists of two important motifs: a coated pit localisation 'FSSVRY' sequence and triad of acidic amino acids 'EDE'. In chicken Dec205, the arginine

‘R’ residue in ‘FSSVRY’ sequence is replaced by histidine[324]. The significance of this amino acid change is not clear yet. The distal triad of acidic amino acids in chicken Dec205 is ‘DDE’ indicating that the triad of acidic amino acids remains conserved between different species[324]. In the same study, the quantitative reverse transcription polymerase chain reaction (qRT-PCR) results indicated that the tissues most closely associated with immune responses had the highest levels of Dec205 transcripts. In chicken spleen, $Dec205^{+}$ cells were found at the centre of the B cell rich peri-ellipsoid lymphoid sheath (PELS) and the T cell rich peri-arteriolar lymphoid sheath (PALS). In the thymus, Dec205 was seen to be expressed on cells with a reticular phenotype within both the cortex and medulla. These cells are involved in the clearance of apoptotic thymocytes. In the bursa, Dec205 was expressed on cells involved in presentation of antigen to B cells and regulation of B cell migration[324]. Furthermore, Dec205 expression has been observed in cells other that APCs. Low level expression of Dec205 has been shown on human T and B lymphocytes, NK cells and macrophages[429]. In chickens, low-level expression of Dec205 has been detected on $CD4^{+}$, $CD8^{+}$ and γδ T lymphocytes and B lymphocytes[324].

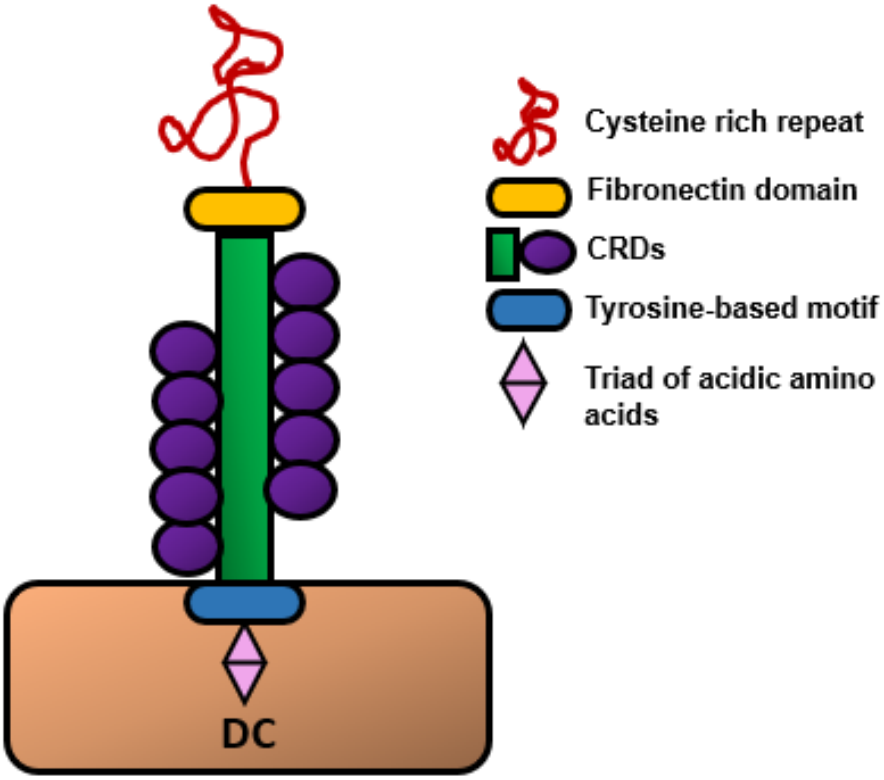


Figure 1.16. Structure of Dec205 receptor. Image inspired from Keler et al[430]. **CRD**: Carbohydrate recognition domain **DC**: Dendritic cells

Due to the structural similarities, it is predicted that the function of avian Dec205 is similar to that of the mammalian Dec205. The function of Dec205 in antigen uptake, processing and presentation has been characterised in mammals[431]. When antigens are targeted to DCs by conjugation to anti-Dec205 antibody, they are endocytosed, processed and presented on both MHC II and MHC I (cross-presentation) molecules with high efficiency[432,433]. There is a tyrosine containing FSSVRY motif in mammalian Dec205 which is responsible for initial clathrin-coated pit-mediated endocytosis. Furthermore, an acidic 'EDE' triad motif in its carboxyl terminus allows direct trafficking to late endosomes/early lysosomes that are rich in MHC class II molecules[433]. It has been reported that mammalian Dec205 receptor recognises dead cells in a pH dependent manner[428]. However,

the ligand specificity of this receptor is unknown. Some studies have identified keratins as cellular ligands of human Dec205 receptors as keratins act as markers of dead cells in an acidic environment[434], whereas others have demonstrated Dec205 to be a receptor for CpG oligonucleotides[435]. Since, the nature of Dec205 ligands has not been determined, most studies rely on the use of monoclonal antibodies as surrogate ligands[432].

Dec205 is one of the most common receptors that has been used for antibody-based APC targeting in mammals. The first antigens that were included in antigen-anti-Dec205 construct were peptides: hen egg lysozyme (HEL46–61) (anti-DEC205-HEL) and myelin oligodendrocyte glycoprotein (MOG35–55) (anti-DEC205-MOG)[436–438]. The studies involving anti-DEC205-HEL and anti-DEC205-MOG demonstrated that specific antigens may be presented by DCs to $CD4^+$ T cells *in vivo* and also revealed that, under "steady state", presentation of such targeted antigens by DCs induced T cell tolerance in mice[437]. Hence, in the absence of DC activation, APC targeting could be exploited to develop immunosuppressive antibody targeted vaccines that have useful application for treating transplantation, allergy or autoimmune disease[439–443]. Dec205 antigen targeting has also been successfully applied as a promising vaccine approach in tumour models in mammals. Dec205 targeted melanoma antigen tyrosinase-related protein-2 co-administered with CpG adjuvant protected mice against tumour growth and substantially slowed the growth of previously implanted melanoma cell line B16[444]. Furthermore, the CDX-1401 vaccine (Celldex Therapeutics) was used in the first phase I study of a protein vaccine targeting DCs *in vivo* in human[392]. This was an anti-cancer vaccine and comprised of tumour antigen NY-ESO-1 fused to anti-DEC205 antibody. This vaccine was proven to be safe and induced both

humoral and cellular response against the tumour antigens. Similarly, a decreased tumour burden was seen when survivin (antigen involved in tumorigenesis) was targeted to Dec205[445] in mice.

Moreover, Dec205 antigen targeting has also been investigated in a model of viral infection. Targeting rotavirus VP6 protein to Dec205 has shown to induce protection against the infection in mice[446]. Furthermore, the *in vivo* administration of Dec205 targeted Epstein-Barr virus or HIV antigens together with the adjuvant Poly I:C has shown to elicit antiviral T cell responses in mice[447,448]. In addition, the first antigen targeting study in chickens was also directed towards Dec205 receptor. AIV H5N2 HA was chemically conjugated to anti-chicken DEC-205 antibody[396]. A single dose of this vaccine was shown to elicit a strong immune response in chickens.

1.7.2. CD11c

Beta 2 (β_2) integrins are the most abundant leukocyte integrins and the first integrins to be studied functionally and structurally on human leukocytes[449]. The avian β_2 integrin has not been characterised yet. Most of the studies relating to the structure and function of β_2 integrins have been carried out in mammals (human and mice). In mammals, β_2 integrins are expressed only on leukocytes however, their expression pattern varies among the leukocyte sub-population. E.g., CD11a(α_L) predominates on lymphocytes. CD11b(α_M) is the most abundant integrin on neutrophils, and is also expressed on NK cells, fibrocytes, B cells, $CD8^+$ T cells and γδ T cells[449–451]. Furthermore, CD11c(α_X) is most abundant on myeloid dendritic cells and macrophages, with distribution like that of CD11b on NK, B and T cells[452]. CD11d(α_D) is basally expressed on the majority of circulating human

neutrophils and monocytes, NK cells, and on a small fraction of circulating T cells[453]. Integrins are very important for innate immunity in mammals. They are involved in the interaction of phagocytic cells with endothelium and extracellular matrix, ingestion of complement opsonized pathogens and cytokine production. In addition, they are also involved in the proliferation, survival and differentiation of lymphocytes during adaptive immunity[449,454]

The mammalian integrin receptor is a heterodimer made up of an alpha (α) and a beta (β) subunit. The integrin family consists of 18 α and 18 β subunits which can non-covalently associate into different heterodimers[454]. The majority of integrin heterodimers can recognise and bind to more than one ligand, subsequently the same ligand can also bind to different heterodimers[455,456]. CD11c is an integrin α_x chain (α subunit) protein with molecular weight of 145-150 kDa, and associates with CD18 (β subunit)[455–457]. The mammalian CD11c consists of a large extracellular domain, a transmembrane domain, and a cytoplasmic domain (**Figure 1.17**). The extracellular domain of the α subunit consists of a β-propeller, thigh, calf 1 and calf 2 domains, whereas the extracellular domain of the β subunit consists of an I/A domain, an immunoglobulin-like hybrid domain, a plexin-semaphorin-integrin (PSI) domain, four EGF-like repeats (EGF1-4) and a β-tail domain. The I/A domain contains a metal ion-dependent adhesion site (MIDAS) that acts as a ligand binding site. Metal ions such as magnesium, calcium or manganese can bind in the MIDAS[458,459]. In terms of ligands, mammalian CD11c can bind to intercellular adhesion molecules 1 and 4 (ICAM1, ICAM4), iC3b, vascular cell adhesion protein 1 (VCAM-1), heparin, fibrinogen and various polysaccharides[450,456,457,460–462]. The 8F2 mAb generated in chicken is referred to as putative chicken CD11c antibody[306]. This antibody has been used by several groups to enrich and phenotype chicken

DCs[337]. In particular, the 8F2 mAb has been used to stain chicken BSDCs[306], distinguish isolated splenic DC-like cells from KUL01$^+$ macrophages[463] and also for the phenotypic analysis of chicken BMDCs[331].

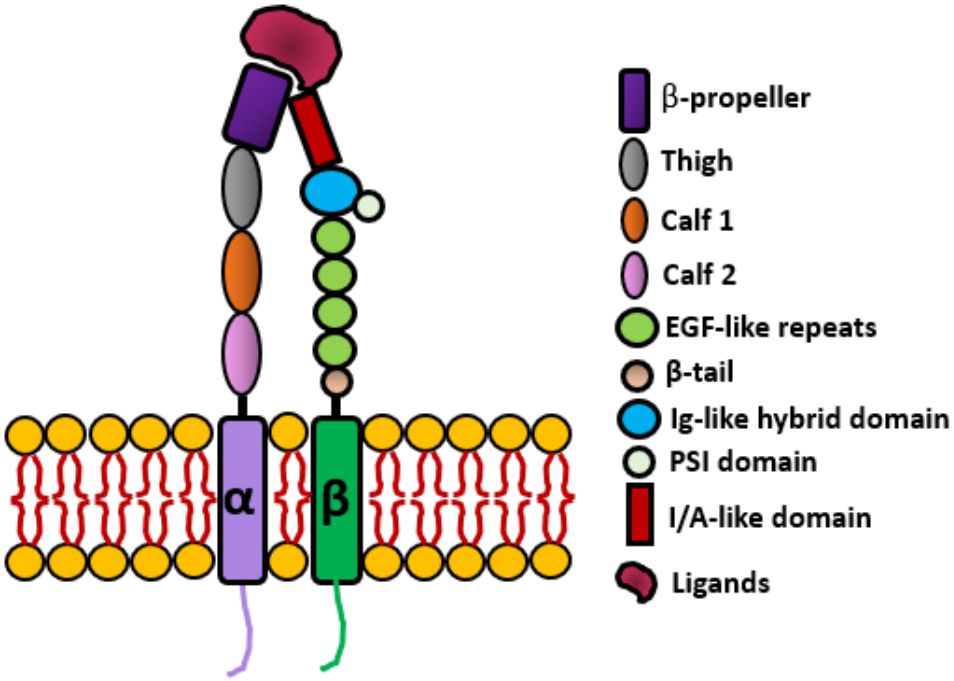


Figure 1.17. Structure of CD11c receptor. Image inspired from Cheah et al[464]. **EGF**: Epidermal growth factor, **Ig**: Immunoglobulin, **PSI**: Plexinsemaphorin-integrin

CD11c is one of the important receptors of interest for antigen targeting in mammals. Studies have shown that targeting antigens to CD11c can promote rapid, high-titre antibody response in mice[381,406]. It was shown that targeting as little as 0·5 μg of the model antigen OVA to CD11c resulted in robust antigen-specific CD4$^+$ and CD8$^+$ T cell responses in mice[381]. Furthermore, 100 ng OVA conjugated to anti-CD11c Fab was able to generate anti-OVA titres greater than those produced by a 100-fold higher dose of OVA (with CFA) in mice[408]. This adjuvant effect of anti-CD11c was reported to be due to its ability to enhance the GC and extrafollicular plasma cell formation upon ligation to DCs. It was postulated that anti-CD11c ligation to DCs can directly activate antigen-specific B cells through

several ways: i) change in DC phenotype that enhances DC-B cell interaction ii) enhancement of antigen presentation by DCs iii) increase in DC-B cell migration to facilitate their contact iv) enhancement of DCs for B cell differentiation and survival through the increased production of B-lymphocyte-activating factor (BAFF)[408]. Alternatively, anti-CD11c could have direct effect on plasma cells expressing integrins. Various comparative studies on CD11c antigen targeting have been carried out in mice, which demonstrated that CD11c is very efficient at inducing both antibody and CTL responses[381,408]. Therefore, CD11c could be a potential receptor to target antigen for vaccine development in chickens.

1.7.3. CD83

CD83 is a transmembrane glycoprotein that belongs to the member of immunoglobulin superfamily. In mammals, CD83 is expressed on the surface of most DCs including thymic DCs, LCs in the skin, monocytes derived DCs following culture with GM-CSF and IL-4, and interdigitating reticulum cells within the spleen[465,466]. Furthermore, human lymphocytes also show detectable CD83 expression upon activation *in vitro*. Low level CD83 expression is also detected on many human B and T cell lines[465,467,468]. The high expression of cell surface CD83 often correlates with the upregulation of MHC II expression and serves as a selective marker for mammalian DC activation/maturation[469]. In mice, both $CD4^+$ and $CD8^+$ T cells highly upregulate CD83 expression on the cell surface, following stimulation through CD3 and CD28[470]. Furthermore, CD40, cell surface IgM and TLR 4 signalling can induce rapid CD83 expression on B cells, which correlates with the increased CD86 and MHC II expression[470]. In addition, lymphocyte activation *in vivo* also induces CD83 expression on B cells prior to CD69

expression. This demonstrates that CD83 is an early marker for B cell activation in mammals[470,471]. Hansell et al, identified chicken CD83 as the avian equivalent of the human and mouse DC marker, CD83[472]. Chicken CD83 shares about 39% and 40% amino acid sequence similarity to human and mouse CD83, respectively[472]. In terms of the structure, four domains were identified in the chicken CD83 sequence: a signal peptide, a single extracellular V-type immunoglobulin like domain, transmembrane domain, and cytoplasmic domain (**Figure 1.18).** The predicted structure of the chicken CD83 was similar to the structure of human and mouse CD83[472]. Furthermore, the N-linked glycosylation sites of chicken CD83 was found to be in similar locations compared with human and mouse CD83 as reported previously[473]. The same study also assessed the mRNA expression of CD83 in normal chicken tissues. Higher level of chicken mRNA expression was seen in spleen, thymus, and bursa. The chicken CD83 transcripts were undetectable in the skin and brain. Furthermore, chicken $CD83^+$ cells were identified to have similar characteristics to DCs and FDCs. These characteristics include localisation in the chicken GCs and dendritic morphology[472]. The association of $CD83^+$ cells with GCs indicate that chicken CD83 may define a subset of DC specialised in the activation of chicken B cells[472]. Moreover, low levels of soluble CD83 were detected in chicken serum, similar to those detected in human serum[474]. The soluble form of CD83 protein produced by the cleavage of membrane bound CD83 is reported to have an immune suppressive function, thereby having a therapeutic potential in the regulation of immune disorders in humans[475]. Human CD83 gene is expressed as a 205 amino acid polypeptide with a predicted molecular weight of 23 kDa[465,467]. However, the mature human CD83 protein expressed by normal cells has a molecular weight of 45-50 kDa due to the N-linked glycosylation of its

ectodomain[465]. De-glycosylation studies have confirmed the highly glycosylated status of native human CD83[476–479]. Lee et al, identified a 53 kDa protein in the lysates of chicken spleen cells and HD11 macrophages, which was higher than the observed 23 kDa of chicken CD83 recombinant protein purified from CHO cells[472]. This was reported due to the hypo-glycosylation of the recombinant protein in CHO cells compared to the native molecule[480].

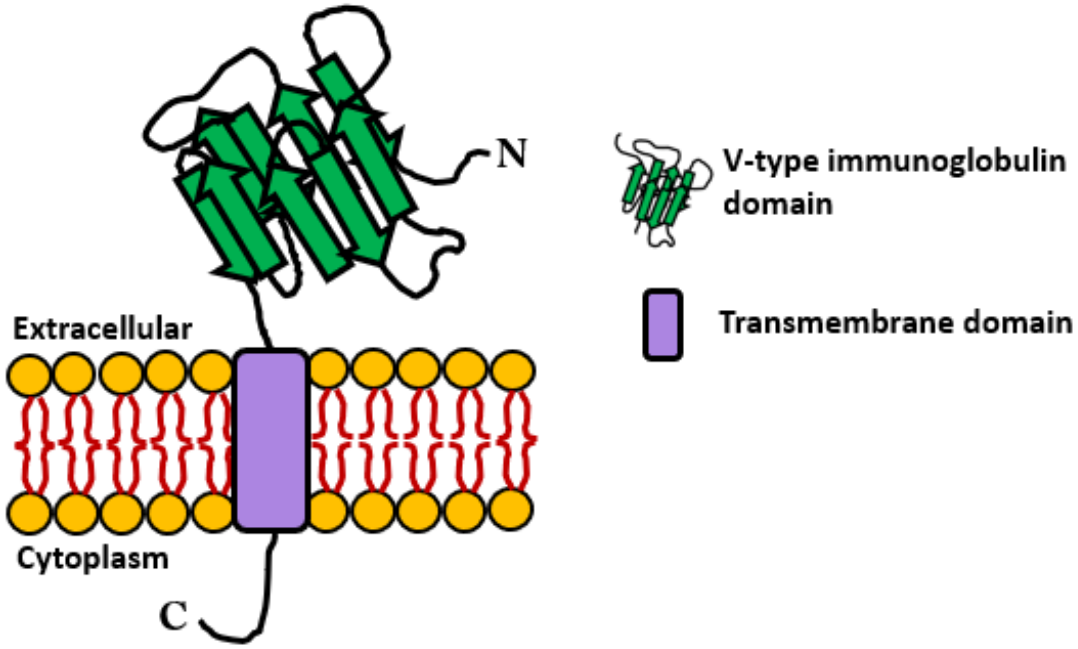


Figure 1.18: Structure of CD83 receptor.

Till date, the exact nature of mammalian or avian CD83 ligands remains undiscovered. Some have shown that human recombinant CD83 protein binds to myeloid differentiation factor-2 (MD-2), a co-receptor associated with TLR4 signalling complex on monocytes[481], while others showed that human CD83 binds to a carbohydrate epitope[482]. The role of CD83 in developing immune response is unclear. However, its structural similarity with B7 family members (e.g., CD80/CD86) led to predict that it has co-stimulatory function[483]. Indeed, the downregulation of CD83 expression on human DCs using RNA interference

reduces T cell proliferation and IFN-γ secretion. In addition, CD83 mRNA electroporated human DCs are stronger T cell stimulator. These observations indicate that CD83 represents a co-stimulatory receptor for T cell activation like CD80 and CD86 molecules[468]. Furthermore, human CD83 binding to its ligands can deliver a significant signal that enhances the *in vitro* generation of CTLs, and also enables long-term survival of antigen-specific T cells by enhancing the proliferation and inhibiting apoptosis[484]. Consistent with this observation, lymphocytes from CD83$^{-/-}$ mouse showed shortened lifespan *in vivo*[470]*.* Various evidences in mice suggest that CD83 is involved in the regulation of B cell function *in vivo*[485,486] since i) CD83 is up-regulated on activated B cells *in vivo* ii) ubiquitous transgenic (Tg) expression of CD83 *in vivo* on the B cells strongly interfered with the production of antigen-specific immunoglobulin iii) premature over-expression of CD83 on CD83Tg B cells resulted in reduced calcium signalling, reduced immunoglobulin secretion and increased anti-inflammatory IL-10 cytokines upon LPS stimulation. In mice, it has been predicted that CD83 is involved in the modulation of B cell receptor (BCR) signalling through association with other signalling receptors upon binding to its ligand[485]. Recently, it was reported that CD83 expression plays a major role in B cell function for antibody production in response to AIV in mice[487]. Although, CD83 receptor has not been explored before for antigen targeting studies, its role in T cell and B cell function makes it a potential target for developing TADV.

1.7. Thesis aims and objectives

The targeting of antigen to APC receptors is an efficient strategy for enhancing the vaccine efficacy. However, such antigen targeting strategies are poorly explored in chickens. Therefore, the aims and objectives of this thesis are:

<u>Aim 1</u>: Develop antibody-based antigen targeting system for the prevention of AIV infection in chickens.

Objective 1: Synthesise TADV by conjugating HA antigen of AIV with scFv antibodies specific to chicken Dec205, CD83 and CD11c receptors.

Objective 2: Evaluate the immunogenicity and protective efficacy of TADV.

Objective 3: Evaluate the mechanism of TADV.

<u>Aim 2</u>: Further evaluation of the immunogenicity of TADV in SPF and MDA positive chickens

Objective 1: Investigate the cross-reactivity, vaccination regimes and stability of TADV in SPF chickens.

Objective 2: Investigate the immunogenicity of TADV in HA-specific MDA positive chickens.

<u>Aim 3</u>: Explore the use of Herpesvirus of Turkey as a viral vector for *in vivo* expression of antigen-antibody construct.

Objective 1: Compare the efficiency of recombinant HVT for *in vivo* expression of membrane bound and secreted forms of HA antigen.

Objective 2: Evaluate the *in vitro* and *in vivo* stability of different recombinant HVT constructs.

Objective 3: Evaluate the antibody responses generated by different recombinant HVT constructs.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Cells, Eggs and Chickens

Table 9: Cell lines, eggs, and chickens used in this thesis.

Item	Description	Source
CEF cells	Primary chicken embryo fibroblasts (CEF)	The Pirbright institute CSU
DF-1 cells	Continuous cell line of chicken embryo fibroblasts	MSD Animal Health, The Netherlands
DH5α chemically competent cells	*Escherichia coli* (*E. coli*) DH5α for bacterial transformation	Life Technologies (Cat no: 18265017)
Embryonated hen's eggs	SPF VALO embryonated eggs (Embryonic day 10) for growth of influenza virus.	VALO BioMedia
Insect S2 cells	*Drosophila melanogaster* Schneider 2 cells for protein expression	Life Technologies (Cat no: R69007)
MDCK cells	Madin-Darby Canine Kidney cells	ECACC
Chickens	Rhode Island Red chickens (SPF, for use in animal study, collection of blood for HA and HI assays, collection of spleen for harvesting splenocytes)	National Avian Research Facility
	Dekalb white chickens (Medical grade, for use in animal study)	Henry Stewart & Co.Ltd
	White Leghorn Layers (SPF, for use in animal study)	MSD Animal Health, The Netherlands

2.1.2 Viruses

2.1.2.1. Avian Influenza Viruses

Table 10: Avian influenza viruses used in this thesis.

Strain	Alias	Description
A/chicken/Pakistan/UDL-01/2008 (H9N2)	UDL 01/08	Wildtype, Iqbal lab
A/chicken/Wenzhou/606/2013 (H9N2)	WZ/606	1:1:6 recombinant with WZ/606 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/chicken/Israel/239/2013 (H9N2)	Isr/239	1:1:6 recombinant with Isr/239 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/chicken/Emirates/R66/2002 (H9N2)	Em/R66	2:6 recombinant with Em/R66 HA, Em/R66 NA and PR8 internals, Iqbal lab
A/chicken/Egypt/D7100/2013 (H9N2)	Egy/D7100	1:1:6 recombinant with Egy/D7100 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/chicken/India/WB-NIV1057169/2010 (H9N2)	Ind/WB	1:1:6 recombinant with Ind/WB HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/chicken/SKP/827/2016 (H9N2)	SKP/2016	2:6 recombinant with SKP/2016 HA, SKP/2016 NA and PR8 internals, Iqbal lab
A/chicken/Egypt/D7100/2013 (H9N2)	Egy/D7100	1:1:6 recombinant with Egy/D7100 HA, UDL 01/08 NA and PR8 internals, Iqbal lab

A/Chinese hwamei/Vietnam/38/2006 (H9N2)	VN/38	1:1:6 recombinant with VN/38 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/environment/Bangladesh/10306/2011 (H9N2)	Env/BD	1:1:6 recombinant with Env/BD HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/Hong Kong/3239/2008 (H9N2)	HK/3239	1:1:6 recombinant with HK/33982 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/Hong Kong/33982/2009 (H9N2)	HK/33982	1:1:6 recombinant with HK/33982 HA, UDL 01/08 NA and PR8 internals, Iqbal lab
A/quail/United Arab Emirates/D1556/2011 (H9N2)	UAE/D1556	1:1:6 recombinant with UAE/D1556 HA, UDL 01/08 NA and PR8 internals, Iqbal lab

2.1.2.2. Herpesvirus of Turkey (HVT)

Table 11: HVT used in this thesis.

Constructs	Alias	Description
Herpesvirus of Turkey	HVT	Wild-type, FC-126 strain, MSD Animal Health.
Herpesvirus of Turkey-GFP	rHVT-GFP	Recombinant HVT containing GFP insert in UL45/46 region, Iqbal lab
Herpesvirus of Turkey-H9HA Full-length	rHVT-H9HA FL	Recombinant HVT containing full-length SKP/2016 H9HA protein insert in UL45/46 region
Herpesvirus of Turkey-H9HA Foldon	rHVT-H9HA	Recombinant HVT containing SKP/2016 H9HA ectodomain with foldon in UL45/46 region
Herpesvirus of Turkey-H9HA Foldon-CD83 scFv	rHVT-H9HA CD83 scFv	Recombinant HVT containing SKP/2016 H9HA ectodomain

		with foldon fused to chicken CD83 scFv in UL45/46 region

2.1.3. Plasmids

Table 12: Plasmids used in this thesis.

Name	Description	Source
pMT-BIP-V5-6xHis (Hereinafter referred to as Plasmid A)	*Drosophila melanogaster* expression vector for inducible and secreted recombinant protein expression. **Plasmid Features:** *Drosophila melanogaster* metallothionein (MT) promoter, *Drosophila* melanogaster immunoglobulin heavy chain binding protein (BIP) secretion signal, multiple cloning sites, V5 epitope for recombinant protein detection, polyhistidine (6xHis) tag for recombinant protein purification	Life Technologies Version: A
pMT- BIP-Dec205 scFv-V5-6xHis	Dec205 scFv cloned into plasmid A	This work
pMT-BIP-CD83 scFv-V5-6xHis	CD83 scFv cloned into plasmid A	This work
pMT-BIP-CD11c scFv-V5-6xHis	CD11c scFv cloned into plasmid A	This work
pMT-BIP-H9HA Foldon scFv-V5-6xHis (hereinafter referred to as Plasmid B)	H9HA Foldon cloned into plasmid A	This work
pMT-BIP-H9HA Foldon Dec205 scFv-V5-6xHis	Dec205 scFv cloned into plasmid B	This work
pMT-BIP-H9HA Foldon CD83 scFv-V5-6xHis	CD83 scFv cloned into plasmid B	This work
pMT-BIP-H9HA Foldon CD11c scFv-V5-6xHis	CD11c scFv cloned into plasmid B	This work

pExpreS2-V1 (pS2V1)	*Drosophila melanogaster* expression vector for constitutive recombinant protein expression	ExpreS2ion Biotechnologies
pS2V1-BIP-Ctag (hereinafter referred to as Plasmid C)	*Drosophila melanogaster* expression vector for constitutive and secreted recombinant protein expression **Plasmid Features:** *Drosophila melanogaster* pActin promoter, *Drosophila* BIP secretion signal, multiple cloning sites, Ctag for recombinant protein purification	Iqbal lab
pS2V1-Dec205 CTLD 4-5-6-Ctag	Dec205 C-type lectin domains (CTLD) 4-5-6 cloned into plasmid C	This work
pS2V1-CD83 ED-Ctag	CD83 ectodomain (ED) cloned into plasmid C	This work
pGEMT-H9HA Full-length-UL45-46	Plasmid containing full-length SKP/2016 H9HA flanked by UL45/46 region. Glycoprotein B of pseudorabies virus promoter at 5' end and felid alphavirus 1 polyadenylation (polyA) at 3' end. Used as a donor plasmid during HVT transfection.	This work
pGEMT-H9HA Foldon-UL45-46	Plasmid containing SKP/2016 H9HA ectodomain with foldon flanked by UL45/46 region. Glycoprotein B of pseudorabies virus promoter at 5' end and felid alphavirus 1 polyadenylation (polyA) at 3' end. Used as a donor plasmid during HVT transfection.	This work
pGEMT-H9HA Foldon-CD83 scFv-UL45-46	Plasmid containing SKP/2016 H9HA ectodomain with foldon fused to chicken CD83 scFv flanked by UL45/46 region. Glycoprotein B of pseudorabies virus promoter at 5' end and felid alphavirus 1 polyadenylation (polyA) at 3' end. Used as a donor plasmid during HVT transfection.	This work
pHVT-BAC3	Stable infectious bacterial artificial chromosome (BAC) clone of the whole genome of HVT FC126. Used	Susan J. Baigent, The

	as a standard for calibration of HVT genome copy number.	Pirbright Institute
pGEMT-Ovo	Plasmid containing 1 kb region of chicken ovotransferin gene (4001 bp-5000 bp). Used as a standard for calibration of chicken genome copy number.	Susan J. Baigent, The Pirbright Institute

2.1.4. Enzymes

Table 13: Enzymes used in this thesis.

Name	Supplier	Purpose
DNA restriction enzymes	New England Biolabs (NEB)	Restriction digestion of DNA for cloning
DNase I recombinant RNase free	Merck Life Science (Cat no: 04716728001)	RNA extraction
GoTaq®Green master mix (DNA polymerase)	Promega (Cat no: M7122)	Colony polymerase chain reaction (PCR)
Pfu Ultra II Hotstart 2x master mix (DNA polymerase)	Agilent (Cat no: 600850)	PCR amplification
PNGase F	NEB (Cat no: P0704S)	De-glycosylation of proteins
T4 DNA ligase	NEB (Cat no: M0202S)	DNA ligation

The enzymes were used according to the manufacturer's instructions unless otherwise stated.

2.1.5. Antibodies

2.1.5.1. Primary antibodies

Table 14: Primary antibodies used in this thesis.

Antibody	Description	Application	Source
Anti-H9HA monoclonal antibodies (1 mg/mL)	Combination of mouse anti- H9HA (UDL 01/08) monoclonal antibodies: JF7, CG12, JF8, IB3, HA9	**Immunohistochemistry** (1:2000)	Iqbal lab[488]
CD11c scFv (0.5-1 mg/mL)	Recombinant chicken CD11c scFv	**Western blot** (Equimolar concentration) **Flow cytometry** (5 µg/mL)	This work
CD83 scFv (0.5-1 mg/mL)	Recombinant chicken CD83 scFv	**Western blot** **ELISA** (Equimolar concentration)	This work
Ctag (1 mg/mL)	Biotinylated camelid antibody fragment against Ctag	**Western blot** (1:3000) **ELISA** (1:1000)	Life Technologies (Cat no: 7103252500)
Clone 8F2 (1 mg/mL)	Mouse anti-chicken CD11c (Monoclonal)	**Western blot** (1µg/mL) **Flow cytometry** (10 µg/mL)	The Pirbright institute
Chicken IFN-γ (0.5 mg/mL)	Anti-chicken IFN-γ (Capture antibody)	**ELISA** (1:250) **ELISPOT** (1:250)	Life Technologies (Cat no: CAC1233)
Chicken IFN-γ-Biotin (0.155 mg/mL)	Biotinylated anti-chicken IFN-γ (Detection antibody)	**ELISA** (1:150) **ELISPOT** (1:150)	Life Technologies (Cat no: CAC1233)

Dec205 scFv (0.5-1 mg/mL)	Recombinant chicken Dec205 scFv	**Western blot** **ELISA** (Equimolar concentration)	This work
F890 (Clone GE8) (1 mg/mL)	Mouse anti-chicken CD83 (Monoclonal)	**Western blot** (1µg/mL) **ELISA** (1 µg/mL)	The Pirbright institute
H9HA Foldon-CD11c scFv (0.5-1 mg/mL)	Chicken CD11c scFv conjugated to H9HA Foldon	**Western blot** **ELISA** (Equimolar concentration)	This work
H9HA Foldon-CD83 scFv (0.5-1 mg/mL)	Chicken CD83 scFv conjugated to H9HA Foldon	**Western blot** **ELISA** (Equimolar concentration)	This work
H9HA Foldon-Dec205 scFv (0.5-1 mg/mL)	Chicken Dec205 scFv conjugated to H9HA Foldon	**Western blot** **ELISA** (Equimolar concentration)	This work
ID2 scFv (0.5-1 mg/mL)	Recombinant anti-H9HA UDL 01/08 (scFv)	**Flow cytometry** (Equimolar concentration)	Iqbal lab
JF7 (1 mg/mL)	Mouse anti-H9HA UDL 01/08 (Monoclonal)	**Western blot** (1:1000) **ELISA** (1:1000)	Iqbal lab[488]
Polyclonal SKP/2016 H9N2	SKP/2016 H9N2 virus infected chicken serum (HI titre of 1024 ($log_2$10)	**Western blot** (1:500) **ELISA** (1:500)	Iqbal lab
Polyclonal UDL 01/08 H9N2	UDL 01/08 H9N2 virus infected chicken serum (HI titre of 256 ($log_2$8)	**Western blot** (1:500) **ELISA** (1:500)	Iqbal lab
Polyclonal HVT	HVT infected chicken serum	**Immunohistochemistry** (1:500)	Iqbal lab
V5 (1 mg/mL)	Mouse anti-V5	**Western blot** (1:1000) **ELISA** (1:1000)	Bio-Rad (Cat no: MCA1360)

2.1.5.2. Secondary antibodies

Table 15: Secondary antibodies used in this thesis.

Antibody	Application	Source
Alexa Fluor® 488 goat anti-mouse IgG (2 mg/mL)	**ELISA** (1:500 dilution)	Life Technologies (Cat no: A11001)
Alexa Fluor® 568 goat anti-chicken IgY (Heavy+Light chains) (2 mg/mL).	**Immunofluorescence assay** (1:500 dilution)	Life Technologies (Cat no: A11041)
Goat anti-chicken IgG (Heavy chain)-Horseradish peroxidase (HRP) (2 mg/mL). Recognises chicken immunoglobulin IgY (especially epitopes within the Fc region and shows no cross-reactivity with other immunoglobulin classes.	**ELISA** (1:6000)	Bio-Rad (Cat no: AAI29P)
Goat anti-chicken IgM (Heavy chain)-HRP (1 mg/mL). Recognises chicken immunoglobulin M and shows no cross-reactivity with other chicken immunoglobulin classes.	**ELISA** (1:3000)	Life Technologies (Cat no: PA1-84676)
Goat anti-chicken IgA (Heavy chain)-HRP (1 mg/mL). Recognises chicken immunoglobulin A and shows no cross-reactivity with other chicken immunoglobulin classes.	**ELISA** (1:3000)	Life Technologies (Cat no: PA1-84679
Goat anti-mouse-HRP (1 mg/mL)	**Western blot** (1:1000) **ELISA** (1:1000)	Bio-Rad (Cat no: 0300-0108P
Goat anti-mouse IgG Fluorescein isothiocyanate (FITC) (0.5 mg/mL)	**Flow cytometry** (1:200)	Bio-Rad (Cat no: STAR70)
IRDye® 680RD goat anti-mouse antibody (1 mg/mL)	**Western blot** (1:10000) **Immunohistochemistry** (1:10000)	LI-COR biosciences (Cat no: 926-68070)
IRDye® 800CW donkey anti-chicken antibody (1 mg/mL)	**Western blot** (1:10000) I**mmunohistochemistry** (1:10000)	LI-COR biosciences (Cat no: 926-32210)

IRDye® 800CW Streptavidin (1 mg/mL)	**Western blot** (1:10000)	LI-COR biosciences (Cat no: 926-32230)
Mouse anti-V5 tag- HRP (1 mg/mL)	**Western blot** (1:1000) **ELISA** (1:1000)	Bio-Rad (Cat no: MCA1360P)
Mouse anti-V5 tag-FITC (0.1 mg/mL)	**Flow cytometry** (1:200)	Bio-Rad (Cat no: MCA1360F
Mouse IgG1-PE (0.2 mg/mL)	**Flow cytometry** (1:50)	BioLegend (Cat no:406607)
Mouse IgG2b-PE (0.2 mg/mL)	**Flow cytometry** (1:50)	BioLegend (Cat no: 406707)
Rabbit anti-mouse IgG -HRP (1 mg/mL)	**Immunohistochemistry** (1:500)	Life Technologies (Cat no: 61-6520)
Streptavidin-HRP (1.25 mg/mL)	**ELISA** (1:1000)	Life Technologies (Cat no: N100)

2.1.6. Reagents

2.1.6.1. General reagents

Table 16: Reagents and kits used in this thesis.

Reagents/Kits	Supplier	Catalogue number
7-AAD Viability staining solution	Biolegend	420404
30% Acrylamide: bisacrylamide	Bio-Rad	1610158
3-amino-9-ethylcarbazole (AEC) substrate	Fisher Scientific	15836738
Absolute Blue qPCR low ROX mix kit	Fisher Scientific	AB4318
Agarose for plaque assay	Oxoid	LP0028
Agarose for gel electrophoresis	Eurogentec	18037G
Ammonium persulphate (APS)	Merck Life Science	A3678
β-mercaptoethanol (BME)	Merck Life Science	444203
β-propiolactone (BPL)	Alfa Aesar	B23197.03
Bis[sulfosuccinimidyl] suberate (BS3)	Life Technologies	A39266
Bovine serum albumin (BSA)	Merck Life Science	05470

Calcium phosphate transfection kit	Life Technologies	K278001
Carbonate-bicarbonate buffer capsules	Merck Life Science	C3041
Chloroform	Merck Life Science	288306
Concanavalin A (ConA)	Merck Life Science	L7647
Corning® cell strainer	Merck Life Science	CLS431752
Crystal violet solution	Merck Life Science	V5265
3,3'-diaminobenzidine (DAB) substrate	Life Technologies	34000
DNA gel loading dye (6x)	Life Technologies	R0611
DNA plasmid miniprep kit	Qiagen	27106
DNA plasmid maxiprep kit	Qiagen	12162
DNeasy Blood and Tissue Kit	Qiagen	69504
GelRed®Nucleic acid gel stain	Biotium	41001
Histopaque 1083	Merck Life Science	10831
HyperLadder™ 1kb DNA ladder	Bioline	BIO-33053
ID Screen® Influenza H9 indirect kit	ID.Vet	FLUH9S-10P
IFN-γ chicken matched antibody pair kit	Life Technologies	CAC1233
Ionomycin	Merck Life Science	IO634
Lipopolysaccharides (LPS)	Merck Life Science	L2630
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit	Fisher Scientific	L34963
Luria broth (LB) powder	Life Technologies	12795027
MagNA-Lyser green beads tubes	Roche	03358941001
MagNA Pure LC RNA isolation tissue lysis buffer	Roche	03604721001
MagNA Pure 96 processing cartridge	Roche	06241603001
MagNA Pure 96 Cellular RNA large volume kit	Roche	05467535001
Marvel milk powder	Spencer Holt	CH0097

Montanide™ ISA 71R VG	Seppic	36514P
Millipore Multiscreen 96 well plate	Life Technologies	MAIPS4510
Nitrocellulose membrane	Bio-Rad	1610800
Paraformaldehyde (PFA)	Merck Life Science	P6148
PageBlue™ protein staining solution	Life Technologies	24620
Phosphate-buffered saline (PBS)	The Pirbright institute CSU	In house
Phorbol myristate acetate (PMA)	Merck Life Science	P8139
Polyester tip swab in tube	Technical service consultant Ltd	TS/19-H250
Precision Plus Protein All Blue standards	Bio-Rad	1610373
QIAquick Gel extraction kit	Qiagen	28115
QIAquick PCR purification kit	Qiagen	28104
RNA Later stabilisation solution	Life Technologies	AM7021
RNeasy Mini kit	Qiagen	74104
Sucrose	Life Technologies	15503022
Sulphuric acid (H_2SO_4)	Merck Life Science	258105
Superscript III platinum One-step qRT-PCR kit	Life Technologies	11732020
Tetramethylbenzidine (TMB) substrate	Fisher Scientific	BD555214
Tetramethyl ethylenediamine (TEMED)	Bio-Rad	1610801
TransIT-X2® dynamic delivery system	Mirus Bio	MIR6003
Trizol reagent	Life Technologies	15596026
Tween 20	Merck Life Science	P1379-1
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Life Technologies	L23105
Vybrant™ CFDA SE Cell Tracer Kit	Life Technologies	V12883

2.1.6.2. Cell culture reagents

Table 17: Cell culture reagents used in this thesis.

Name	Supplier	Catalogue number
Ampicillin	Merck Life Science	A9393
Bovine serum albumin (BSA)	Merck Life Science	A7906
Chicken serum	Life technologies	16110082
Dextran hydrochloride	Merck Life Science	30461-25G
Dimethyl sulfoxide (DMSO)	Merck Life Science	D4540
Dulbecco's modified eagle medium (DMEM)	Merck Life Sciences	D6429
EX-CELL® 420 serum-free medium for Insect Cells	Merck Life Science	14420C
Fetal bovine serum (FBS)	Merck Life Science	F0926
Gentamicin	Merck Life Science	G1397
Hepes	Life Technologies	15630080
L-Glutamine	Life Technologies	A29168-01
10x Minimum essential medium eagle (MEM)	Merck Life Science	M02075
Medium 199	Life Technologies	21180-02
Non-essential amino acid solution (NEAA)	Life Technologies	11140035
N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) treated trypsin	Life Technologies	20233
Nystatin	Merck Life Science	N6261
Ofloxacin HCl	Merck Life Science	O8757-1G
Penicillin/Streptomycin (Pen/Strep)	Life Technologies	15140-122
Polymyxin B	Merck Life Science	92283
Roswell Park memorial institute 1640 (RPMI)	Merck Life Science	R8758

Schneider's (S2) insect cell medium	Merck Life Science	S0146
Sodium bicarbonate ($NaHCO_3$)	Life Technologies	25080094
Sulfamthoxazole	Merck Life Science	S7507
Super optimal broth with catabolite repression (SOC) medium	Merck Life Science	S1797
2.5% Trypsin-EDTA	Life Technologies	15090-046
Tris-Borate-EDTA (TBE) buffer (10x)	Life Technologies	15581044
Tryptose phosphate broth (TPB)	Merck Life Science	T8159
Versene	Life Technologies	15040033
Zeocin	Life Technologies	R25005

2.1.7. Buffers and media

Table 18: Buffers and media used in this thesis.

Name	Constituents	Use
CEF media	Medium 199 5% (v/v) FBS 10% TPB 100 U/mL Pen/Strep	Maintenance of CEF cells
Complete DMEM media	DMEM media 10% (v/v) FBS 100 U/mL Pen/Strep	Maintenance of MDCK cells
Complete RPMI media	RPMI media 10% (v/v) FBS 100 U/mL Pen/Strep 1% NEAA	Maintenance of chicken splenocytes
Complete S2 cell media	S2 media 10% (v/v) FBS	Maintenance of *Drosophila melanogaster* S2 cells
Crystal violet solution	In distilled water:	Staining cells after plaque assay

	0.1% crystal violet solution 20% Methanol	
ELISA coating buffer	In distilled water: 100 mM Na_2CO_3 100 mM $NaHCO_3$ (adjusted to pH 9.6)	Coating ELISA plates
Fluorescence-activated cell sorting (FACS) buffer	PBS 1% BSA	Flow cytometry
Freezing media	90% FBS 10% DMSO	For freezing/storing cells
LB agar	Pre-made LB 1.5% (w/v) Agar (+100 µg/mL ampicillin or 50 µg/mL kanamycin or zeocin)	Plating bacteria
NP-40 lysis buffer	50 mM Tris-HCl pH 8 150 mM NaCl 1% NP-40	Lysis buffer for chicken splenocytes
PBS-T	PBS 0.05% Tween 20	Used for washing ELISA plates/western blot membrane
Plaque assay overlay media	10x Minimal essential medium 0.2% (w/v) BSA 2 mM L-glutamine 0.15% (w/v) $NaHCO_3$ 10 mM Hepes 0.005% (w/v) dextran hydrochloride 100 U/mL Pen/Strep 0.6% (w/v) agarose	Plaque assays
8% resolving gel	For 1 gel (5 mL): 1.3 mL 30% acrylamide bisacrylamide 1.3 mL 1.5 M Tris-HCl pH 8.8 2.3 mL distilled water 50 µL 10% APS (w/v) 5 µL TEMED 50 µL 10% SDS	SDS-PAGE

10% resolving gel	For 1 gel (5 mL): 1.7 mL 30% acrylamide: bisacrylamide 1.3 mL 1.5 M Tris-HCl pH 8.8 1.9 mL distilled water 50 µL 10% (w/v) APS 5 µL TEMED 50 µL 10% SDS	SDS-PAGE
12% resolving gel	For 1 gel (5 mL): 2 mL 30% acrylamide: bisacrylamide 1.3 mL 1.5 M Tris-HCl pH 8.8 1.6 mL distilled water 50 µL 10% (w/v) APS 5 µL TEMED 50 µL 10% SDS	SDS-PAGE
5x SDS-PAGE Protein loading buffer	In distilled water: 10% w/v SDS 50% Glycerol 30% 1 M Tris HCl pH 6.8 0.5% (v/v) bromophenol blue 2% (v/v) β-mecaptoethanol	SDS-PAGE
Stacking gel	For 1 gel (3 mL): 2 mL 30% acrylamide: bisacrylamide 1.3 mL 1M Tris-HCl pH 6.8 1.6 mL water 30 µL 10% (w/v) APS 3 µL TEMED 30 µL 10% SDS	SDS-PAGE
Squishing buffer	10 mM Tris-HCl 1 mM EDTA 25 mM NaCl 200 µg/mL proteinase K (adjusted to pH 8)	Lysis buffer for extracting HVT genome
Tris/borate/EDTA (TBE) buffer	10x pre-made TBE diluted to 1x in distilled water	Gel electrophoresis
Tris-glycine SDS running buffer (Pre-made, SLS)	In distilled water: 0.025 M Tris 0.192 M glycine 0.1% (w/v) SDS (adjusted to pH 8.5)	SDS-PAGE

Virus transport media	In Medium 199: 0.5% (w/v) BSA Penicillin G $2x10^6$ U/L Streptomycin 200 mg/L Polymyxin B $2x10^6$ U/L Gentamicin 250 mg/L Nystatin $5x10^5$ U/L Ofloxacin HCl 60 mg/L Sulfamthoxazole 0.2 g/L (adjusted to pH 7.4)	Isolating virus from infectious swabs
Western blot/ELISA blocking buffer	PBS 5% w/v marvel milk powder	Blocking western blot membranes/ELISA plates
Western blot/ELISA antibody dilution buffer	PBS-T 1% w/v marvel milk powder	Antibody dilution in ELISA and western blot
Western blot transfer buffer	In distilled water: 25 mM tris 192 mM glycine 20% (v/v) methanol (adjusted to pH 8.3)	Transferring western blot

2.1.8. Protein purification and concentration reagents and buffers

2.1.8.1. Protein purification columns

Table 19: Protein purification columns used in this thesis.

Name	Supplier	Catalogue number	Purpose
CaptureSelect™ C-tag Affinity Matrix	Life Technologies	191307005	Ctag protein purification
Profinity™IMAC uncharged column	Bio-Rad	1560123	His tag protein purification
Profinity™IMAC Nickel charged column	Bio-Rad	1560133	His tag protein purification

2.1.8.2. Protein purification and concentration reagents

Table 20: Protein purification and concentration reagents used in this thesis.

Name	Supplier	Catalogue number	Purpose
Amicon Ultra-15 Centrifugal Filter Units (3 kDa)	Merck Life Science	UFC9003	Concentrating proteins
Copper sulphate	Fisher Scientific	4561657	Induction of recombinant protein expression
Ethylenediaminetetraacetic acid (EDTA)	Merck Life Science	E9884	Preparation of washing buffer (His tag column)
Glycine	Merck Life Science	G8898	Preparation of washing buffer (Ctag column)
Imidazole	Merck Life Science	I0250	Preparation of elution buffer (His tag column)
30 metre dialysis tubing (3.5 kDa MWCO)	Fisher Scientific	12797486	Removing impurities after protein purification
Magnesium chloride ($MgCl_2$)	Merck Life Science	M8266	Preparation of elution buffer (Ctag column)
Pierce™ BCA assay kit	Life Technologies	23227	Measurement of protein concentration
Sodium chloride (NaCl)	Merck Life Science	S9888	Preparation of elution buffer (His tag column)
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck Life Science	106346	Preparation of elution buffer (His tag column)
Stericup (0.22 µM pore size)	Merck Life Science	S2GPU11RE	Filter sterilisation of the protein expression supernatants

2.1.8.3. Protein purification buffers

Table 21: Protein purification buffers used in this thesis.

Name	Constituents	Purpose
Cleaning buffer (Ctag column)	In distilled water: 0.1 M glycine, pH: 2	Cleaning the column (Ctag column)
Elution buffer (Ctag column)	In distilled water: 20 mM Tris, 2 M $MgCl_2$ pH: 7 -7.4	Protein elution (Ctag column)
Cleaning buffer (His tag column)	In distilled water: 0.2 M EDTA, 0.5 M NaCl	Cleaning the column (His tag column)
Elution buffer 25 mM imidazole (His tag column)	In distilled water: 50 mM $NaH_2PO_4.H_2O$ 300 mM NaCl 25 mM imidazole pH: 8	Protein elution (His tag column)
Elution buffer 50 mM imidazole (His tag column)	In distilled water: 50 mM $NaH_2PO_4.H_2O$ 300 mM NaCl 50 mM imidazole pH: 8	Protein elution (His tag column)
Elution buffer 100 mM imidazole (His tag column)	In distilled water: 50 mM $NaH_2PO_4.H_2O$ 300 mM NaCl 100 mM imidazole pH: 8	Protein elution (His tag column)
Wash buffer (Ctag column)	PBS (pH: 7-7.4)	Washing (Ctag column)
Wash buffer (His tag column)	In distilled water: 50 mM $NaH_2PO_4.H_2O$ 300 mM NaCl 5 mM imidazole pH: 8	Washing (His tag column)

2.1.9. Primers

The cloning and sequencing primers were designed using IDT Oligoanalyser tool (https://eu.idtdna.com/calc/analyzer). For cloning primer pairs, the following properties were taken into consideration: GC content of 40%-60%, length of about 25-30 bases, melting temperature of primers pairs within 5°C of each other, if possible, start and end with 1-2G/C pairs.

2.1.9.1. Cloning primers

Table 22: Cloning primers used in this thesis.

Name	Sequence (5'-3')	Purpose
CD11c scFv_For	GGGGTACCTGAAGTGCAACTGCAACAAAGC	Forward primer for cloning CD11c scFv into pMT/BIP/H9HA Foldon/V5/His plasmid
CD11c scFv_Rev	CTCTAGACTTGATTTCCAGCTTGGTGCCTCCGCCG	Reverse primer for cloning CD11c scFv into pMT/BIP/H9HA Foldon/V5/His plasmid
CD83 ED_For	GGAATTCTGCATCTCAGTGCAACTAAAGATGAAGTTATGCATATTACTGG	Forward primer for cloning CD83 ED into pS2V1/Ctag plasmid
CD83 ED_Rev	GTCAGATATCGATCGATCCGCGGAGCCTCTGGCTCTTTAATTAA	Reverse primer for cloning CD83 ED into pS2V1/Ctag plasmid
CD83 scFv_For	GTGGCGGCCGCTCGAGGGAAGTGGATATCGTGATG	Forward primer for cloning CD83 scFv into pMT/BIP/H9HA Foldon/V5/His plasmid
CD83 scFv_Rev	CCCTCTAGAACTACTAACGGTCACACTGGTGCCCTGTC	Reverse primer for cloning CD83 scFv into pMT/BIP/H9HA Foldon/V5/His plasmid
Dec205 CTLD 4-5-6_For	GGAATTCTGCATCTCAGTGCAACTAAAGATGAAGTTATGCATATTACTGG	Forward primer for cloning Dec205 CTLD 4-5-6 ectodomain into pS2V1/Ctag plasmid
Dec205 CTLD 4,5,6_Rev	GATCCGCGGAGCCTCTGGCTCTTTAATTAAGTCAGCGGC	Reverse primer for cloning Dec205 CTLD 4-5-6 ectodomain into pS2V1/Ctag plasmid

Dec205 scFv_Rev	GGCCCTCTAGAACTACTAACT GTCACGGTGGTTCCGGTGC	Reverse primer for cloning Dec205 scFv into pMT/BIP/H9HA Foldon/V5/His plasmid
H9HA_For	CGGGGTACCGGTAAAACGCA GCGATAAGATCTGCATCGGC	Forward primer for cloning H9HA Foldon into pMT/BIP/V5/His plasmid
H9HA_Rev	TTCTTAATTAACAGGAAGGTG CTCAGCAGCACCCACTCGCC AT	Reverse primer for cloning H9HA Foldon into pMT/BIP/V5/His plasmid
M13_F	GTAAAACGACGGCCAGTG	Colony PCR (pMT vector construct, Forward primer)
M13_R	CAGGAAACAGCTATGAC	Colony PCR (pMT vector construct, Reverse primer)

2.1.9.2. Sequencing primers

Table 23: Sequencing primers used in this thesis.

Name	Sequence (5'-3')	Purpose
CD11c scFv_seq1	TCGATTACTGGGGCCAGGGAACGAC	Sequencing CD11c scFv (Forward primer)
CD11c scFv_seq2	TCCACCGCCACCACTTCCACCAC	Sequencing CD11c scFv (Reverse primer)
CD83 ED_seq1	GGAATTCTGCATCTCAGTGCAACTAA AGATGAAGTTATGCATATTACTGG	Sequencing CD83 ectodomain (Forward primer)
CD83 ED_seq2	GTCAGATATCGATCGATCCGCGGAG CCTCTGGCTCTTTAATTAA	Sequencing CD83 ectodomain (Reverse primer)
CD83 scFv_seq1	AGCGTGAAGATCAGCTGC	Sequencing CD83 scFv (Forward primer)
CD83 scFv_seq2	CACGCTGGCACCTGGTTTGA	Sequencing CD83 scFv (Reverse primer)
Dec205 CTLD 4,5,6_seq1	GATAAGCCAGTGACAAGCCTGGT	Sequencing Dec205 CTLD 4,5,6 (Forward primer)

Dec205 CTLD 4,5,6_seq2	ATCGAGAAGCTGTCCAACAGCGAGC AGA	Sequencing Dec205 CTLD 4,5,6 (Reverse primer)
Dec205 CTLD 4,5,6_seq3	GATTTCATCACCAGCCTGCTGC	Sequencing Dec205 CTLD 4,5,6 (Forward primer)
Dec205 CTLD 4,5,6_seq4	GTTTTCAGGGCCACGCAATCGCGGG TATCG	Sequencing Dec205 CTLD 4,5,6 (Forward primer)
Dec205 scFv_seq1	AAGTGCAGCTGGTTGAAAGCGG	Sequencing Dec205 scFv (Forward primer)
Dec205 scFv_seq2	CAACTCAGCTTCAGGCTTCCG	Sequencing Dec205 scFv (Reverse primer)
H9HA_seq1	GTACACCAACAATCGCGGCAAGGAC	Sequencing H9HA Foldon (Forward primer)
H9HA_seq2	GCTTCAGCACGCTCCAATAGTAGTTG ATGCG	Sequencing H9HA Foldon (Reverse primer)
H9HA_seq3	AAACTGCCCCAAGTACATCGGCG	Sequencing H9HA Foldon (Forward primer)
H9HA_seq4	TTGTAGGCCCACACATCCTGGATCTG GTCG	Sequencing H9HA Foldon (Forward primer)

2.1.9.3. Quantitative reverse transcription PCR (qRT-PCR) primers and probes

Table 24: qRT-PCR primers and probes used in this thesis.

Gene	Primer sequences (5'-3')	Probe sequence (5'-3')	Purpose
CxCLi2	**For:** GCCCTCCTCCTGG TTTCAG	**FAM**-TCTTTACCAGCGTCCTACC TTGCGACA-**TAMRA**	To amplify chicken CxCLi2 gene
	Rev: TGGCACCGCAGCT CATT		
IFN-α	**For:** GACAGCCAACG		

	CCAAAGC **Rev**: GTCGCTGCTGT CCAAGCATT	**FAM**-CTCAACCGGATCCACCGCTACACC-**TAMRA**	To amplify chicken IFN-α gene
IFN-γ	**For**: GTGAAGAAGGTGAAAGATATCATGGA **Rev**: GCTTTGCGCTGGATTCTCA	**FAM**-TGGCCAAGCTCCCGATGAACGA-**TAMRA**	To amplify chicken IFN-γ gene
IL-4	**For**: AACATGCGTCAGCTCCTGAAT **Rev**: TCTGCTAGGAACTTCTCCATTGAA	**FAM-**AGCAGCACC TCCCTCAAGGCACC-**TAMRA**	To amplify chicken IL-4 gene
IL-6	**For**: AACATGCGTCAGCTCCTGAAT **Rev:** TCTGCTAGGAACTTCTCCATTGAA	**FAM-**AGCAGCACCTCCCTCAAGGCACC-**TAMRA**	To amplify chicken IL-6 gene
IL-10	**For**: CATGCTGCTGGGCCTGAA **Rev**: CGTCTCCTTGATCTGCTTGATG	**FAM-**CGACGATTCGGCGCTGTCACC-**TAMRA**	To amplify chicken IL-10 gene
IL-1β	**For**: GCTCTACATGTCGTGTGTGATGAG **Rev**: TGTCGATGTCCCGCATGA	**FAM-**CCACACTGCAGCTGGAGGAAGCC-**TAMRA**	To amplify chicken IL-1β gene
RPLPO-1	**For:** TTGGGCATCACCACAAAGATT	**FAM-**CATCACTCAGAATTTCAATGGTCCCTCGGG-**TAMRA**	To amplify chicken ribosomal protein

	Rev: CCCACTTGTCTCCG GTCTTAA		lateral stalk subunit PO-1 (RPLPO-1) gene
RPL13	**For**: TCGTGCTGGCAGG ATC **Rev:** TCGTCCGAGCAAA CCTTTTG	**FAM-** TAATGCCCGCCAGTTTAAG CTCTTCTAGGC-**TAMRA**	To amplify chicken ribosomal protein L13 (RPL13) gene
28S	**For:** GGCGAAGCCAGAG GAAACT **Rev**: GACGACCGATTTG CACGTC	**FAM-** AGGACCGCTACGGACCTC CACCA-**TAMRA**	To amplify chicken 28S ribosomal RNA (rRNA) gene
TGF-β4	**For**: AGGATCTGCAGT GGAAGTGGAT **Rev**: CCCCGGGTT GTGTTGGT	**FAM-** ACCCAAAGGTTATATGGC CAACTTCTGCAT-**TAMRA**	To amplify chicken transforming growth factor (TGF)-β4 gene

2.1.9.4. Recombinant HVT vectors

Table 25: Primers, probes, and guide RNA (gRNA) for recombinant HVT vector used in this thesis.

Name	Sequence (5'-3')	Purpose
Chicken ovotransferrin gene_For[489,490]	CACTGCCACTGGGCTCTGT	qRT-PCR: To amplify chicken ovotransferrin gene
Chicken ovotransferrin gene_Rev[489,490]	GCAATGGCAATAAACCTCCAA	qRT-PCR: To amplify chicken ovotransferrin gene
Chicken ovotransferrin gene_Probe[489,490]	**VIC-** AGTCTGGAGAAGTCTGTGCAGCCTC CA**-TAMRA**	qRT-PCR: To amplify chicken ovotransferrin gene

GFP gRNA	AGCTGGACGGCGACGTAAACGG	Directing Cas9 nuclease to the target DNA sequence for editing HVT-GFP genome
HVT sORF1 gene_For[491]	GGCAGACACCGCGTTGTAT	qRT-PCR: To amplify HVT sORF1 gene
HVT sORF1 gene_Rev[491]	TGTCCACGCTCGAGACTATCC	qRT-PCR: To amplify HVT sORF1 gene
HVT sORF1 gene_Probe[491]	**FAM-** AACCCGGGCTTGTGGACGTCTTC- **TAMRA**	qRT-PCR: To amplify HVT sORF1 gene
SKP H9HA_For	CACAAGTGCGATGACCAG	Used for screening positive HVT plaques
UL45/46_For	GTCTTCCGGTTAAGGGACAG	For PCR analysis of the HA insert in UL45/46 region of HVT
UL45/46_Rev	CGAACAAGTCGGGAAGTACG	For PCR analysis of the HA insert in UL45/46 region of HVT, Used for screening positive HVT plaques
UL45/46 _seq1	CTGTACCTGACCTACGAGG	Sequencing primer (Forward) for SKP/2016 H9HA insert in UL45/46 region
UL45/46 _seq2	GACGCATCAATTACTACTGGAG	Sequencing primer (Forward) for SKP/2016 H9HA insert in UL45/46 region
UL45/46 _seq3	CACAAGTGCGATGACCAG	Sequencing primer (Forward) for SKP/2016

		H9HA insert in UL45/46 region

2.2. Methods

2.2.1. Sequencing, cloning, DNA preparation and analysis

2.2.1.1. Hybridoma sequencing

The hybridomas; clone: IAH F877: AD6 (Dec205), clone: IAH F890:GE8 (CD83) and clone: IAH 8F2 (CD11c) were sequenced commercially (Absolute Antibody Ltd, UK). Rapid amplification of cDNA ends (RACE) method was used whereby mRNA was harvested, reverse transcribed, tailed, amplified and sequenced as described by Doenecke et al[492].

2.2.1.2. Synthesis of scFv antibodies and construction of plasmids expressing H9HA Foldon fused scFv antibodies

The gene sequences comprising the vL and vH chain of chicken Dec205, CD83 and CD11c mAb were joined by a flexible peptide linker $(Gly_4Ser)_4$ to construct the respective scFv antibodies. The scFv antibody gene sequences were manufactured commercially (Geneart, Life Technologies). The respective scFv antibodies were then cloned into *Drosophila melanogaster* expression vector pMT-BIP-V5-6xHis (Version A, Life Technologies) using *NotI* and *XbaI* restriction sites. The resultant plasmids named pMT-BIP-Dec205 scFv-V5-6xHis, pMT-BIP-CD83 scFv-V5-6xHis and pMT-BIP-CD11c scFv-V5-6xHis were used to insert H9HA Foldon gene using *KpnI* and *PacI* restriction sites, generating pMT-BIP-H9HA Foldon-Dec205 scFv-V5-6xHis, pMT-BIP-H9HA Foldon-CD83 scFv-V5-6xHis and pMT-BIP-H9HA Foldon-CD11c scFv-V5-6xHis plasmids. Furthermore, H9HA

Foldon gene was also cloned into pMT-BIP-V5-6xHis plasmid between *KpnI* and *PacI* restriction sites to yield pMT-BIP-H9HA Foldon-V5-6xHis plasmid.

2.2.1.3. PCR amplification

All PCR reactions were performed using PfuUltra II Hotstart 2x Master mix (Agilent). About 1 µL of 10 µM forward and reverse primers (**section 2.1.9**) were added to 100 ng of DNA and 12.5 µL of master mix. The reaction was then made up to 25 µL with nuclease-free water. Thermocycling conditions involved an initial denaturation stage at 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 60°C-65°C for 20 seconds (optimised according to the melting temperature of forward and reverse primers) and 72°C at 15 seconds/kb (optimised according to template size), finishing with a final extension step at 72°C for 3 minutes.

2.2.1.4. PCR purification

The PCR products were purified using QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Products were eluted in 30-50 µL of nuclease free water and quantified using NanoDrop™ Lite Spectrophotometer (Life Technologies). DNA was stored at -20°C.

2.2.1.5. Agarose gel extraction

After separation by electrophoresis, a band representing a PCR product of an expected size was excised, melted, and extracted using the QIAquick Gel extraction kit (Qiagen), according to the manufacturer's instructions. Products were eluted in 30-50 µL of nuclease free water, quantified using NanoDrop™ Lite Spectrophotometer (Life Technologies) and stored at -20°C.

2.2.1.6. Restriction enzyme digest of DNA

All the DNA digestion were carried out in appropriate buffers using restriction enzymes purchased from NEB, as per the manufacturer's instructions.

2.2.1.7. Ligation, colony PCR and bacterial transformation

The digested plasmids and gene inserts were ligated using the T4 DNA ligase kit (NEB), according to manufacturer's instructions. The total reaction volume for ligation was 15 µL (3 µL of plasmid, 7 µL of gene insert, 1.5 µL of T4 DNA ligase buffer, 0.5 µL of T4 DNA ligase and 3 µL of nuclease free water). The ligation mixture was incubated at room temperature for 2 hours.

The ligated product was then transformed into DH5α competent *E.coli (*Life Technologies*).* Briefly, 25 µL of *E.coli* cells (thawed on ice) were mixed gently with 3 µL of ligation product and incubated for 30 minutes on ice. Cells were transformed by a 45 second heat shock at 42°C followed by immediate placement on ice for a further 2 minutes. About 450 µL of pre-warmed SOC medium (Merck Life Science) was added to the cells and allowed to recover for 1 hour at 37°C, shaking. The cells were then spread in appropriate antibiotic selection plates and incubated at 37°C overnight.

Colony PCR was carried out to select the positive clones after ligation. This was done using GoTaq®Green master mix (Promega). Briefly, 10 colonies were picked and diluted in 25 µL of nuclease free water. The total volume of the reaction was 25 µL consisting of 12.5 µL GoTaq®Green master mix, 1 µL each forward and reverse primers (10 µM, **section 2.1.9**), 2 µL of diluted DNA template and 8.5 µL nuclease free water. Thermocycling conditions involved an initial denaturation

stage at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C-65°C for 30 seconds (optimised according to the melting temperature of forward and reverse primers) and 72°C at 1 minute/kb (optimised according to template size), finishing with a final extension step at 72°C for 5 minutes.

The positive clones were then grown in LB broth (5 mL for miniprep, 500 mL for maxiprep) containing the appropriate antibiotic (37°C, overnight shaking). Bacterial cells were pelleted via centrifugation at 2500 xg for 20 minutes and supernatants were removed. Cells were used to harvest the plasmid DNA.

2.2.1.8. Plasmid DNA purification

The cells obtained in **section 2.2.1.7** were used to harvest the plasmid DNA using DNA miniprep/maxiprep kit (Qiagen), according to the manufacturer's instructions. DNA was eluted in nuclease free water (50 µL for miniprep and 500 µL for maxiprep). The quality and concentration of the purified plasmid DNA was assessed using NanoDrop™ Lite Spectrophotometer (Life Technologies).

2.2.1.9. Agarose gel electrophoresis

Gel electrophoresis was done to separate DNA. Agarose was boiled in 1xTBE buffer (Life Technologies) at typically 1.0% (w/v) and 1x GelRed® Nucleic acid stain was added to the cooled molten agarose for DNA visualisation. Agarose was cast into a horizontal gel tank (SCIE-PLAS) and left to solidify. Once solid, 1x TBE buffer was added to the gel tank. 3 µL of Hyperladder™ 1kb (Bioline) was used for DNA size confirmation. DNA samples were mixed with 6x DNA gel loading dye (1x final concentration, Life Technologies) and loaded in the set agarose gel. Gels

were run at 50-100 volts for about 45 minutes to 1 hour. DNA was visualised using a Gel Doc™ EZ Imager (Bio-Rad).

2.2.1.10. Sanger sequencing

To ensure that the cloned plasmids or PCR products contained the expected amino acid sequences, they were sent to Cambridge branch of Source BioScience for Sanger sequencing. For a single reaction, which involved generating about 1000 bp of sequencing data, 5 µL of 100 ng/µL plasmid sample and 3.2 pmol/µL per primer (**section 2.1.9**) were sent. Alternatively, when sending a PCR product for sequencing, 10 ng/µL of PCR product and 3.2 pmol/µL primer (**section 2.1.9**) were sent. The sequencing data generated by Source BioScience was assembled and analysed using Vector NTI® advance 11.5.4 software.

2.2.2. Recombinant protein expression, purification, and characterisation

2.2.2.1. Transfection of the expression plasmid into *Drosophila melanogaster* Schneider 2 (S2) cells

To facilitate the expression of recombinant proteins, the expression plasmids pMT-BIP-Dec205/CD11c/CD83 scFv-V5-6xHis or pMT-BIP-H9HA Foldon-V5-6xHis or pMT-BIP-H9HA Foldon-Dec205/CD11c/CD83 scFv-V5-6xHis were transfected into *Drosophila melanogaster* S2 cells using calcium phosphate transfection kit, according to the manufacturer's instruction. Prior to the transfection, $1x10^6$/mL of S2 cells were pre-seeded in 5 mL of complete S2 cell growth media (**section 2.1.7**) for 6 to 16 hours at 28°C.Transfection solution was prepared by adding 60 µL 2 M $CaCl_2$, 32 µg of expression plasmid DNA, 1.5 µg pCoHygro plasmid and sterile water to bring the total volume up to 500 µL. The

transfection solution was slowly added to the equal volume of 2xHepes buffered saline (HBS) and incubated at room temperature for 30 minutes. The resulting solution was slowly added dropwise to the pre-seeded S2 cells and incubated for 24 hours at 28°C. After 24 hours post transfection, the transfection media was replaced with fresh complete S2 cell media and the cells were incubated for further 3 days at 28°C. Stable S2 transfectants were generated by adding complete growth media containing hygromycin (final concentration 250 µg/mL) every week for at least 4 weeks.

Alternatively, for the transfection of ps2v1-BIP-Dec205 CTLD 4-5-6-Ctag and ps2v1-BIP-CD83 ED-Ctag expression plasmids into *Drosophila melanogaster* S2 cells, everything was performed identical as described above except the removal of pCoHygro plasmid from the transfection solution and the replacement of hygromycin with zeocin (final concentration 750-1000 µg/mL) for the selection of stable S2 transfectants.

2.2.2.2. Selection of a single clone of S2 cell expressing the recombinant proteins

For a single clone selection, dilution cloning method[493] was used in which 2 × 10^3 S2 transfectants were mixed with gamma-irradiated 10^6 parental S2 cells as feeder cells. About 100 µL of this mixture of cells were seeded into each well of 96-well plates. The single clones within each well became clearly visible after 4 weeks of incubation at 28°C. About 10-15 single clones were screened per construct. The single clones expressing higher recombinant proteins were selected by indirect ELISA (**section 2.2.2.5.3**).

2.2.2.3. Large scale production of the recombinant proteins

The single cell clones expressing higher amount of the recombinant proteins were grown in a 2 litres roller bottle (Corning) containing 400 mL of Ex-Cell® 420 serum-free media (Merck Life Science) for expression and purification. For pMT plasmid transfected cells, protein expression was induced by adding $CuSO_4$ at a final concentration of 500 µM. After 4 days post $CuSO_4$ induction, the cell supernatants were harvested via centrifugation at 1000 xg for 20 minutes and dialysed to remove the excess copper ions. The dialysed supernatants were filtered using stericup having 0.22 µM pore size. On the other hand, the pS2V1 plasmid allows constitutive expression of the recombinant proteins hence, no induction was required.

2.2.2.4. Recombinant protein purification

2.2.2.4.1. His tag protein purification

The recombinant proteins expressed using pMT/6xHis expression plasmids were purified using this technique. Briefly, the dialysed and filtered supernatants containing recombinant proteins were loaded in 10 mL Profinity™IMAC uncharged resin column (Bio-Rad) and washed with 5 column volumes of wash buffer (**section 2.1.8.3**). Copper bound proteins were eluted with elution buffers containing various concentration of imidazole (25 mM/ 50mM/ 100 mM/ 500 mM, **section 2.1.8.3**). The purified proteins were analysed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (**section 2.2.2.5.1**). The protein fractions were combined and concentrated using 15 mL Amicon Ultra-15 Centrifugal Filter column (3 kDa MWCO, Merck Life Science) by centrifuging at 1500 xg for 30 minutes. The concentration of the purified proteins

was measured using Pierce™ BCA Protein Assay Kit (Life Technologies) according to the manufacturer's protocol.

2.2.2.4.2. Ctag protein purification

The recombinant proteins expressed using pS2V1/Ctag expression plasmids were purified using this technique. Briefly, 10 mL of CaptureSelect™ C-tag affinity matrix column (Life Technologies) was equilibrated with 10 column volumes of wash buffer (**section 2.1.8.3**). The filtered expression supernatants containing recombinant proteins were loaded onto the equilibrated column and washed with 5 column volumes of wash buffer (**section 2.1.8.3**). C-tagged proteins were eluted using elution buffer containing 2 M $MgCl_2$ (**section 2.1.8.3**). The purified proteins were analysed, concentrated, and quantified in the same way as described in **section 2.2.2.4.1.**

2.2.2.5. Characterisation of the recombinant proteins

2.2.2.5.1. Protein electrophoresis

Proteins were separated by molecular weight using SDS-PAGE. The SDS-PAGE equipment was supplied by Bio-Rad (Mini-PROTEAN tetra system). The gels were assembled according to the manufacturer's protocol. Furthermore, the resolving and stacking gels were made according to the recipes mentioned in **section 2.1.7**. Samples were denatured in protein loading buffer **(section 2.1.7)** and heated at 95°C for 8 minutes. The samples were loaded in the wells of the SDS-PAGE gels and run for 1.5 hours at 120 volts. Pre-stained Precision Plus All Blue protein standards (Bio-Rad) were used as a guide for protein migration and

protein molecular weight confirmation. Gels were then stained with PageBlue™ protein staining solution (Life Technologies) for protein band visualisation.

2.2.2.5.2. Western blot

Proteins separated via SDS-PAGE were transferred onto nitrocellulose membranes (Bio-Rad) for western blot using a semi-dry blotting system (Trans-Blot Turbo Transfer System, Bio-Rad). Protein transfers were run for 20 minutes at 25 volts, 2.5 ampere. Nitrocellulose membranes were blocked with western blot blocking buffer (**section 2.1.7**) for at least 1 hour at room temperature on a rocker. Blocked membranes were probed with primary antibody (**section 2.1.5.1**) diluted in antibody dilution buffer (**section 2.1.7**) overnight at 4°C with rocking. Membranes were then washed with PBS-T thrice and incubated with secondary antibody (**section 2.1.5.2**) for 1 hour at room temperature, rocking and covered from light by aluminium foil. Finally, membranes were washed for 5 minutes (3x) with PBS-T before imaging on the Odyssey CLX (LI-COR Biosciences).

Alternatively, if the secondary antibody used was HRP conjugated then the nitrocellulose membranes were incubated with DAB substrate-chromogen solution (Agilent) for 5 minutes and imaged using a digital camera (Canon) or Odyssey CLX (LI-COR Biosciences). For the protein expression analysis, supernatants from the untransfected S2 cells were used as negative controls.

2.2.2.5.3. Enzyme-linked immunosorbent assay (ELISA)

A serial 2-fold dilution of the purified proteins or expression supernatants were made in ELISA coating buffer (**section 2.1.7**). The plates were then incubated at 4°C overnight. The next day plates were blocked with ELISA blocking buffer (**section 2.1.7**) for 1 hour at room temperature, before probing with primary

antibody (**section 2.1.5.1**) diluted in ELISA dilution buffer (**section 2.1.7**) for 1 hour at room temperature. If scFv or H9HA Foldon-scFv were used as primary antibodies, they were used in equimolar concentration. This was followed by incubation with HRP-conjugated secondary antibody (**section 2.1.5.2**) for 1 hour at room temperature. The plates were then washed thrice with PBS-T. The colorimetric detection was carried out by adding TMB substrate (Fisher Scientific) and the absorbance at wavelength 450 nm was measured in ELx808 absorbance microplate reader (BioTek). For the protein expression analysis, supernatants from the untransfected S2 cells were used as negative controls. In some cases, PBS was used as a negative control.

2.2.2.5.4. Bis[sulfosuccinimidyl] suberate (BS3) crosslinking

To determine the oligomeric structure of the recombinant H9HA protein containing trimerisation foldon domain, cross linking was performed using BS3 (Life Technologies) as described by Weldon et al[494] with the following modifications. Briefly, 15 µg of the recombinant protein was incubated at room temperature in the presence of BS3 (final concentration 10 mM) for one hour. Crosslinking was stopped by adding1 M Tris-HCl pH 8.0 to a final concentration of 50 mM. The cross-linked products were separated on SDS gel under reducing conditions, blotted and immunodetected using anti-H9HA mAb (JF7, **section 2.1.5.1**).

2.2.2.5.5. Haemagglutination assay

Haemagglutination assay was used to determine the activity of recombinant H9HA Foldon, H9HA Foldon-Dec205/ CD11c/ CD83 scFv proteins and to titrate AIVs. This assay was performed as previously described[495]. Briefly, recombinant proteins (35 µg) or AIVs were serially diluted 2-fold in PBS in a V-bottom 96-well

plate. Chicken red blood cells (RBCs) were diluted to 1% in PBS and added to each well of the plates. Plates were incubated at 4°C for 1 hour then tipped 90°C in a biosafety cabinet to visualise haemagglutination.

2.2.2.5.6. Preparation of chicken splenocyte extracts

About $1x10^6$ chicken splenocytes (**section 2.2.3.1**) were stimulated with 200 ng/mL LPS (Merck Life Science) for 24 hours. The splenocytes were centrifuged, resuspended in 300 µL of NP-40 lysis buffer (**section 2.1.7**) and vortexed. This was followed by incubation at room temperature for 30 minutes with constant vortexting. The splenocytes were then centrifuged at 1000 xg for 10 minutes and the supernatant was harvested and subjected to western blot (**section 2.2.2.5.2**).

2.2.2.5.7. Flow cytometry

The CD11c mAb and CD11c scFv antibodies were tested for their ability to stain chicken splenocytes. Briefly, $1x10^5$ chicken splenocytes (**section 2.2.3.1**) were stimulated with 200 ng/mL LPS (Merck Life Science) for 24 hours. The splenocytes were centrifuged and resuspended in 50 µL of FACS buffer (**section 2.1.7**) containing 5 µg/mL of CD11c scFv or 5 µg/mL of ID2 scFv antibodies (anti-H9HA, control scFv antibody) for 45 minutes at 4ºC. After the incubation with respective scFv antibodies, the splenocytes were washed with 150 µL of FACS buffer and resuspended in 100 µL of FACS buffer containing secondary antibody (FITC conjugated anti-V5 tag, 0.1 mg/mL diluted to 1:200) and LIVE/DEAD™ fixable blue dead cell stain (Life Technologies). The mixture was incubated in the dark for 30 minutes at 4ºC. This was followed by fixing of labelled splenocytes with 50 µL of 1% paraformaldehyde (Merck Life Science) for 20 minutes in the dark. Alternatively, if CD11c mAb (10 µg/mL) or JF7 mAb (IgG1 isotype control, 10

µg/mL) were used as primary antibodies, splenocytes were incubated for 20 minutes at 4°C, followed by incubation with FITC conjugated goat anti-mouse secondary antibody (0.5 mg/mL diluted to 1:200) and LIVE/DEAD™ fixable blue dead cell stain (Life Technologies) for 30 minutes at 4°C. The plates were read the next day using MACSQuant flow cytometer and analysed with FCS Express 6 software. About 10,000 events were acquired for the analysis. In terms of the gating strategy, cells were first gated based on forward scatter area (FSC-A) and side scatter area (SSC-A), followed by exclusion of doublets on SSC-A vs SSC-height density plot. Next, live cells were gated for the detection of $CD11c^+$ cells.

2.2.2.5.8. PNGase F treatment of proteins

The CD83 ED protein was de-glycosylated using PNGase F (NEB), according to the manufacturer's protocol. Briefly, 20 µg of the protein was combined with 2 µL of glycoprotein denaturing buffer (10x) in a total reaction volume of 20 µL and denatured at 100°C for 10 minutes, then chilled on ice for 2 minutes. The denatured protein was mixed with 4 µL of glycobuffer 2 (10x), 4 µL of 10% NP-40 and 2 µL of PNGase F enzyme and incubated at 37°C for 6 hours. The deglycosylated protein was analysed using SDS-PAGE and western blot assays as described in **section 2.2.2.5.1** and **section 2.2.2.5.2,** respectively**.**

2.2.3. Preparation, stimulation, and analysis of chicken splenocytes

2.2.3.1. Preparation of chicken splenocytes

Splenocytes were prepared from the chicken spleens via density gradient centrifugation using Histopaque 1083 (Merck Life Science), according to the

manufacturer's instructions. Briefly, spleens were mashed through 100 µm cell strainer (Merck Life Science) by adding 10 mL of complete RPMI media (**section 2.1.7**). After centrifugation at 400 xg for 10 minutes, the cells were resuspended in 15 mL of complete RPMI media, carefully layered with 7 mL Histopaque 1083, and centrifuged at 400 xg for 45 minutes at 4°C with low acceleration and deceleration. The splenocytes were then harvested from the 'buffy coat' interface in the density gradient and resuspended in a fresh complete RPMI media. Similarly, for the isolation of peripheral blood mononuclear cells (PBMCs), chicken whole blood collected in heparin tube was layered onto Histopaque 1083 (Merck Life Science). This was followed by the exact same protocol as described above.

2.2.3.2. Stimulation assay

About $2x10^6$ splenocytes were plated on each well of 24-well plate. Cells were treated with 10 µg of H9HA Foldon or 14 µg of H9HA Foldon-Dec205/CD11c/CD83 scFv (containing 10 µg H9HA Foldon according to the molecular weight) or 10 µg of Dec205/CD11c/CD83 scFv. Cells were also stimulated with PMA/Ionomycin (final concentration 10 µg/mL) as a positive control for IFN-γ cytokine production. All cells were stimulated for 5 hours, 22 hours and 30 hours *in vitro* at 41°C. The stimulated cells/supernatants were harvested by centrifugation at 10,000 xg for 10 minutes. For assessing the induction of type I interferon (IFN-α), the same protocol was followed as above with the following changes: LPS (200 ng/mL) was used as a positive control and cells were stimulated for 6 and 18 hours.

2.2.3.3. IFN-γ sandwich enzyme-linked immunosorbent assay

Supernatants from the stimulated splenocytes (**section 2.2.3.2**) were harvested and examined by sandwich ELISA. Briefly, anti-chicken IFN-γ (2 µg/mL) was coated on 96-well maxisorp ELISA plates (Life Technologies). The coated plates were blocked at room temperature with PBS containing 3% BSA for 1 hour. A 2-fold dilution of the supernatant was made in PBS buffer containing 3% BSA prior to use. The plates were then incubated with the diluted supernatants for 2 hours at room temperature. Detection was carried out using biotinylated anti-chicken IFN-γ detection antibody (1 µg/mL) for 1 hour at room temperature, followed by HRP conjugated streptavidin (1.25 mg/mL diluted to 1:1000) for another 1 hour at room temperature. About 100 µL of TMB substrate (Fisher Scientific) was added and the plates were incubated for 10 minutes at room temperature. The reaction was stopped using 2 M H_2SO_4 and read at wavelength 450 nm in ELx808 absorbance microplate reader (BioTek). The splenocytes stimulated with RPMI media and PMA/Ionomycin were used as negative and positive controls, respectively.

2.2.3.4. IFN-γ ELISPOT

ELISPOT plates (96-well, Life Technologies) were coated with anti-chicken IFN-γ (2 µg/mL) coating antibody diluted in carbonate-bicarbonate buffer solution (Merck Life Science) and incubated overnight. The coated plates were then blocked with PBS containing 3% BSA for 1 hour at room temperature. The plates were washed with PBS-T (5x). About $5x10^5$ chicken splenocytes were added on each well of the plate carefully. This was followed by an addition of 100 µL of the respective stimulants as described in **section 2.2.3.2**. The plates were incubated

at 41°C for 18 hours without any disturbance. After the incubation, the cells were discarded by flicking and further washing was carried out with PBS-T (5x). Detection was carried out using biotinylated anti-chicken IFN-γ detection antibody (1 µg/mL) for 1 hour at room temperature, followed by HRP conjugated streptavidin (1.25 mg/mL diluted to 1:1000) for another 1 hour at room temperature. AEC substrate solution (Fisher Scientific) was prepared by mixing one drop of AEC chromogen in 1 mL of AEC substrate no more than 15 minutes prior to use. About 100 µL of AEC substrate solution was added to the plates and incubated for 30-45 minutes in the dark. The plates were washed with copious amount of water and dried before reading and counting spots using EliSpot reader (Autoimmun Diagnostika GMBH). The splenocytes stimulated with RPMI media and PMA/Ionomycin were used as negative and positive controls, respectively.

2.2.3.5. Cell proliferation assay

Chicken splenocytes were harvested as mentioned in **section 2.2.3.1**. About $1x10^7$ cells were resuspended in 1 mL of pre-warmed PBS. Carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) dye was diluted in 2 mL of pre-warmed PBS to give 5 µM final concentration. About 1 mL of $1x10^7$ cells in PBS was mixed with 1 mL of 5 µM CFSE to give a final concentration of $5x10^6$ cells/mL and 2.5 µM CFSE. This mixture was incubated in water bath at 37°C for 10 minutes and centrifuged at 300 xg for 10 minutes to harvest the cells. The CFSE labelled cells were then resuspended in 10 mL of complete RPMI media and incubated at 37°C for 30 minutes. This was followed by further centrifugation at 400 xg for 10 minutes, resuspension in 2 mL complete RPMI media and seeding in 96-well U bottom plate (~$5x10^5$ cells per well). Finally, cells were treated with 25 µg of H9HA

Foldon or 35 µg of H9HA Foldon-Dec205/CD11c/CD83 scFv (containing 25 µg H9HA Foldon according to the molecular weight) and 10 µg/mL ConA (positive control). Cells were incubated at 41°C for 4 days.

CFSE labelled cells were prepared for analysis using flow cytometry as described in **section 2.2.2.5.7** with the following modifications: no antibody incubation steps, no paraformaldehyde fixation, use of 7-aminoactinomycin D live dead dye (7AAD, Biolegend) and reading of the plates just after adding the live dead dye.

2.2.4. RNA preparation and qRT-PCR

2.2.4.1. Extraction of RNA from chicken splenocytes using trizol

Chicken splenocytes were resuspended in 750 µL of trizol (Life Technologies) and incubated at room temperature for 5 minutes. About 150 µL of chloroform (Merck Life Science) was added to the mixture. The mixture was then vortexed and centrifuged at 10,000 xg for 30 minutes. Then, the upper clear phase was carefully transferred into a new tube and 100% isopropanol was added. The mixture was further centrifuged at 10,000 xg for 30 minutes. RNA pellets were visible after washing with 75% ethanol. RNA was then resuspended in 5-20 µL of RNase free water and treated with DNase (Merck Life Science) to remove any residual DNA contaminants. Finally, DNase enzyme was inactivated at 75°C for 5 minutes and RNA was quantified using NanoDrop™ Lite Spectrophotometer (Life Technologies). RNA was stored at -80°C.

2.2.4.2. RNA extraction from chicken spleen

Between 20-30 mg of spleen tissue collected in RNA later solution (Life Technologies) was transferred to MagNA-Lyser green beads tubes (Roche) and 550 µL MagNA Pure LC RNA isolation tissue lysis buffer (Roche) was added to each sample tube. Homogenisation was performed using a MagNA-Lyser instrument (Roche) using the following setting: 3 runs of 30 seconds and 10,000 xg per run, cooling the sample tubes for approximately 90 seconds on ice between runs. Homogenised samples were incubated at room temperature for 30 minutes in order to properly lyse the materials. After incubation, tubes were centrifuged for 2 minutes at 10,000 xg in a tabletop microcentrifuge to pellet debris and 350 µL supernatant was transferred to a MagNA Pure 96 processing cartridge (Roche).

RNA extractions were performed automated using the MagNA Pure 96 instrument (Roche) in combination with the MagNA Pure 96 Cellular RNA large volume kit (Roche) and protocol 'RNA Tissue FFStandard LV' (350 µL input, 50-200 µL output) according to the latest version of the MagNA Pure 96 Cellular RNA large volume kit manual. The concentration of the extracted RNA samples was measured using Nanodrop2000 equipment (Life Technologies). RNA samples were stored at -80°C.

2.2.4.3. qRT-PCR

RNA extracted from chicken splenocytes (**section 2.2.4.1**) and chicken spleen (**section 2.2.4.2**) were used for qRT-PCR experiment. All qRT-PCR experiments were performed using the Superscript III platinum One step qRT-PCR kit (Life Technologies) following the manufacturer's protocol. The sequences of primers and probes used are outlined in **Table 2.1.9.3**. The qRT-PCR protocol was

run in a 7500 FAST ABI RT-PCR thermocycler (Applied Biosystems). Cycling conditions used were as follows: i) 5 minutes hold step at 50°C ii) 2 minutes hold step at 95°C iii) 40 cycles of 95°C (3 seconds) and 60°C (30 seconds). Cycle threshold (CT) values were obtained using 7500 softwarev2.3. Negative controls were included within each plate to determine any non-specific amplification or contamination.

For qRT-PCR experiments relating to cytokine production, three housekeeping genes were included per sample (RPLPO-1, RPL13 and 28S). Out of the three reference genes (RPLPO-1, RPL13 and 28S) selected for normalisation, RPLPO-1 was the most stable gene across the samples hence, chosen for normalisation. Data was calculated either using $2^{-\Delta\Delta CT}$ approach (n-fold change compared to the media only control group) and reported as values normalised to the expression level of a housekeeping gene RPLPO-1 or calculated as 40-ΔCT value compared to RPLPO-1 reference gene.

2.2.5. Cell culture and eggs

2.2.5.1. Passaging of continuous cell lines

MDCK cells were maintained in DMEM media supplemented with 10% (v/v) FBS and 100 U/mL Penicillin-Streptomycin. Cells were grown at 37°C, 5% CO_2. Cells were typically passaged every 3-4 days by first washing with PBS and trypsinising with 0.25% (v/v) trypsin/versene.

Drosophila melanogaster S2 cells were maintained in S2 cell media supplemented with 10% (v/v) FBS and grown at 28°C, 5% CO_2. Cells were

passaged once a week by centrifuging at 400 xg for 10 minutes, and resuspending in fresh complete S2 cell media (**section 2.1.7**).

2.2.5.2. Embryonated hen's eggs

Embryonated eggs were delivered from the supplier (VALO BioMedia) at embryonic day 0 and stored at 16°C. Eggs were put at room temperature for 8-36 hours prior to setting. In the laboratory, eggs were maintained in a rocking incubator at 37°C for 10 days before they were infected with AIVs.

2.2.6. Avian influenza virus growth, titration, and purification

2.2.6.1. Propagation of virus in embryonated eggs

For growing high titres of AIVs, 10-day-old embryonated hen's eggs were inoculated with the virus diluted in PBS (1:5000-1:10000). After 72 hours post infection, eggs were euthanised by refrigeration at 4°C. The viruses were obtained by harvesting the allantoic fluid and then carrying out centrifugation at 1500 xg for 20 minutes. Finally, the viruses were aliquoted and stored at -80°C until use.

2.2.6.2. Virus titration

2.2.6.2.1. Plaque assay

Plaque assay was performed in order to titrate AIVs from the egg allantoic fluid (**section 2.2.6.1**) or from virus swab samples (**section 2.2.8.3**) as described elsewhere[496]. Briefly, confluent MDCK cells were washed with PBS, then infected with a serial 10-fold dilutions of AIVs or virus swab supernatants performed in serum-free DMEM media. The MDCK cells treated with serum-free DMEM media

were used as negative controls. Virus inoculum was removed from the cells after 1 hour. Cells were then overlaid by plaque assay overlay media (**section 2.1.7**) containing 2 µg/mL of TPCK trypsin (Life Technologies). After solidification of the overlay media, plates were inverted and kept in incubator at 37°C for 48-72 hours. When plaques were visible, agar plugs were removed, and cells were stained with 0.1% crystal violet solution (Merck Life Science).

2.2.6.2.2. $TCID_{50}$ (50% tissue culture infective dose)

The $TCID_{50}$ assay was performed as described elsewhere[497]. Briefly, confluent MDCK cells were washed with PBS. Then, AIVs were serially diluted in serum-free DMEM media and inoculated onto the confluent monolayers of MDCKs in quadruplicate. After 1 hour, cells were washed with PBS and complete DMEM media **(section 2.1.7**) containing 2 µg/mL of TPCK trypsin (Life Technologies) was added. Plates were incubated at 37°C for 72 hours. Finally, the media was removed, and cells were fixed and stained with 0.1% crystal violet solution (Merck Life Science).

The virus titre was calculated using the Spearman-Karber formula:

$$\boldsymbol{Log10(TCID50) = X - \frac{d}{2} + d\sum \frac{ri}{ni}}$$

where X = lowest dilution that all wells are positive, d = $\log_{10}$ of the dilution factor used to dilute virus, *ni* = number of replicates, and *ri* = number of positive wells

2.2.6.3. Virus inactivation, concentration, and purification

The AIVs from allantoic fluid were inactivated, concentrated, and purified for use as vaccines. About 500 mL of clarified infectious allantoic fluid from ~50 embryonated hen's eggs (**section 2.2.6.1**) was inactivated chemically using 0.1% Beta-propiolactone (Alfa Aesar) and three blind passages were performed in 10-day old embryonated chicken eggs (Rhode Island Red) to confirm inactivation. This was followed by ultracentrifugation at 207,600 xg for 2 hours at 4°C for virus concentration using Sorvall™ WX90 ultracentrifuge (Life Technologies). Pellets were then homogenised using a glass homogeniser and run down a continuous 30-60% sucrose gradient by ultracentrifugation at 207,600 xg for 2 hours. The virus-containing layer within the density gradient was harvested, re-suspended in PBS, and pelleted again at 207,600 xg for 2 hours using the ultracentrifuge. Purified virus was re-suspended in PBS.

2.2.7. Generation and characterisation of recombinant HVT vector vaccine

2.2.7.1. Construction of guide RNA and donor plasmids

The gRNA against green fluorescent protein (GFP) was adapted from a previous publication[498]. The DNA oligo of gRNA was synthesised (Merck Life Science) and cloned into plasmid pX459-v2 (Addgene) using *BbsI* cloning site. Donor plasmids were generated by cloning a complete coding sequence of SKP/2016 H9HA (H9HA Full-length, Genbank accession number: AVX19091.1, HA:1-560 amino acids), ectodomain of SKP/2016 H9HA with trimerisation foldon domain from T4 bacteriophage (H9HA Foldon, HA:1-525 amino acids) and H9HA

Foldon fused to chicken CD83 scFv (H9HA Foldon-CD83 scFv, HA:1-525 amino acids) into plasmid pGEMT-UL45-46 using *PacI* restriction sites, with glycoprotein B of pseudorabies virus promoter at 5' end and felid alphavirus 1 polyadenylation (polyA) at 3' end. The resulting plasmids were termed as pGEMT-H9HA Full-length-UL45-46, pGEMT-H9HA Foldon-UL45-46 and pGEMT-H9HA Foldon-CD83 scFv-UL45-46.

2.2.7.2. Homology Directed Repair (HDR)-Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9-mediated gene insertion

The primary CEF cells (~$4x10^5$) were plated on each well of 12-well plate one day before transfection. About 0.5 µg of GFP gRNA was co-transfected with 0.5 µg of pGEMT-H9HA Full-length-UL45-46 or pGEMT-H9HA Foldon-UL45-46 or pGEMT-H9HA Foldon-CD83 scFv-UL45-46 donor plasmids into CEF cells using TransIT-X2® Reagent (Mirius Bio), according to the manufacturer's protocol. At 12 hours post transfection, the CEF cells were infected with recombinant HVT (rHVT)-GFP at a multiplicity of infection (MOI) of 0.01. Virus was harvested at 72 hours post infection and subjected to plaque purification. The resulting viruses were termed as rHVT-H9HA FL (H9HA Full-length), rHVT-H9HA (H9HA Foldon) and rHVT-H9HA CD83 scFv (H9HA Foldon-CD83 scFv).

2.2.7.3. HVT genome extraction

The CEF cells infected with HVT were harvested and lysed in squishing buffer (**section 2.1.7**) at 65°C for 30 minutes. To screen positive HVT plaques primers SKP H9HA_For and UL45/46_Rev (**section 2.1.9.4**) were used during PCR analysis. Furthermore, the integrity of the HA gene insert after different

passages were examined by PCR using primers UL45/46_For and UL45/46_Rev (**section 2.1.9.4**).

2.2.7.4. Immunohistochemistry and Immunofluorescence assays

The CEF cells were infected for 72 hours with a serial 10-fold dilutions of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv performed in PBS. Cells treated with CEF media were used as negative controls. The cells were then fixed with ice cold methanol/acetone (1:1) for 15 minutes at room temperature. The expression of H9HA antigen was visualised by incubating the fixed cells with mouse anti-H9HA monoclonal antibodies (1 mg/mL diluted to 1:2000) for 1 hour at room temperature, followed by HRP-conjugated rabbit anti-mouse secondary antibody (1 mg/mL diluted to 1:500) for another 1 hour at room temperature. Furthermore, the cells were also stained with HVT serum raised in chicken (polyclonal, 1:500), followed by HRP-conjugated goat anti-chicken secondary antibody (2 mg/mL diluted to 1:6000). The staining was developed by adding DAB substrate-chromogen solution (Agilent) and incubating the plaques for 5 minutes at room temperature. Finally, the stained plaques were analysed using light microscope and counted to obtain the virus titre.

Concomitantly, the HVT plaques were also stained with the combination of mouse anti-H9HA monoclonal antibodies and HVT serum as described above (primary antibodies), followed by the combination of IRDye®800CW goat anti-mouse antibody (1 mg/mL diluted to 1:10000) and IRDye®680RD donkey anti-chicken antibody (1 mg/mL diluted to 1:10000) as secondary antibodies, respectively. Plaques were then imaged on Odyssey CLX (LI-COR biosciences) and analysed for HA insert kick-outs.

For the detection of viral content in chicken splenocytes and PBMCs after vaccination, about $5x10^6$ chicken splenocytes/PBMCs were co-cultivated with $1.75x10^6$ secondary CEF cells (DF-1 cells) in a 60 mm petri dish for 5 days. After 5 days, the cells were fixed with ice-cold 96% ethanol. For visualisation of HVT plaques, fixed cells were incubated with the combination of mouse anti-H9HA monoclonal antibodies and polyclonal HVT antibody (primary antibodies) as described above. This was followed by incubation with the combination of Alexa Fluor® 488 goat anti-mouse IgG (2 mg/mL diluted to 1:500) and Alexa Fluor® 568 goat anti-chicken IgY (2 mg/mL diluted to 1:500) secondary antibodies, respectively. The dishes were read using fluorescence microscope (Leica, DMI6000).

2.2.7.5. *In vitro* growth kinetics

2.2.7.5.1. Plaque assay

About $2x10^6$ CEF cells were seeded on each well of 6-well plate. Then, 100 pfu of either wildtype HVT or rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv were added to the cells gently. Cells treated with CEF media were used as negative controls. At 12, 24 ,48, 72, 96, 120 and 144 hours post HVT infection, the cells were trypsinised and harvested in two 100 µL aliquots. The titres of the viruses at each time point were obtained by following the protocol described in **section 2.2.7.4**.

2.2.7.5.2. HVT genome copy number

The DNA was harvested from one of the 100 µL aliquots of HVT infected CEF cells obtained at different time points in **section 2.2.7.5.1** using DNeasy Blood

and Tissue Kit (Qiagen). The method used was essentially as described previously[489], using a duplex PCR to detect both the virus and the host ovotransferin gene. Two constructs pHVT BAC3 and pGEMT-ovo were used as standards for calibration of HVT genome copy number and host genome copy number, respectively. The qRT-PCR reactions were set up by adding 2x Absolute Blue qPCR low ROX master mix (Fisher Scientific), HVT sORF1 gene_For (0.4 µM) / HVT sORF1 gene_Rev (0.4 µM) / HVT sORF1 gene_Probe (0.2 µM), Ovotransferin_For (0.4 µM)/ Ovotransferin_Rev (0.4 µM) / Ovotransferin_Probe (0.2 µM) and 4 µL of standard/test DNA. The reactions were run in a 7500 FAST ABI RT-PCR thermocycler (Applied Biosystems). Cycling conditions were as follows: i) 2 min hold step at 50°C ii) 10 min hold step at 95°C iii) 40 cycles of 95°C (15 seconds) and 60°C (1 minute). The CT values were obtained using 7500 softwarev2.3 and exported to Microsoft excel for further analysis.

2.2.8. *In vivo* studies

2.2.8.1. Ethics statement

All animal studies and procedures at The Pirbright institute were carried out in strict accordance with the guidance and regulations of European and United Kingdom Home Office regulations under project licence number P68D44CF4. All animal work was reviewed and approved by the Animal Welfare Ethical Review Body at The Pirbright Institute. Furthermore, all animal studies and procedures at MSD Animal Health were carried out in strict accordance with Directive 2010/63/EU on the protection of animals for scientific purposes. All animal work was approved by the central committee on animal trials in the Netherlands.

2.2.8.2. Generation of mouse monoclonal antibodies against chicken Dec205 and CD83

Chicken CD83 ED (GenBank accession number: XM_040663657.1) and Dec205 CTLD 4-5-6 (GenBank accession number: AJ574899) were used as immunogens for raising monoclonal antibodies against chicken CD83 and Dec205, respectively[324]. Mice were immunised intramuscularly with 100 µg of the antigen expressing plasmids, followed by a boost with an intraperitoneal injection of 50 µg of the purified antigen after four weeks. After four days following boost, the mice were sacrificed for the preparation of splenocytes for fusion with myeloma cells to generate hybridomas. Hybridomas secreting antibody specifically recognising the respective antigen were screened using ELISA.

2.2.8.3. Chicken vaccination, blood, tissue, and swab sample collection

2.2.8.3.1. Evaluation of the immunogenicity of H9HA Foldon-Dec205 scFv (rH9HA-Dec205 scFv), H9HA Foldon-CD83 scFv (rH9HA-CD83 scFv) and H9HA Foldon-CD11c scFv (rH9HA-CD11c scFv) vaccines

Groups of 7-day-old Dekalb White chickens (Medical grade, Henry Stewart & Co. Ltd, n=8 per group) were immunised with vaccine dose containing 2.8 µg, 28 µg and 49 µg of rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins, equivalent to 2 µg, 20 µg and 35 µg of rH9HA protein (equimolar concentration). Additionally, one group of chickens (n=8) was immunised with inactivated H9N2 vaccine (UDL 01/08,1024 haemagglutinin antigen units (HAU)/dose). Control groups were immunised with PBS. All vaccines were formulated in Montanide™ ISA 71 VG (Seppic) as water-in-oil emulsion. The ratio

of protein to adjuvant volume was 1:2. About 0.2 mL of each of the vaccines were given to the chickens subcutaneously at the back of the neck. All the vaccinated groups received a booster dose at 14-day-old (day 7 post primary vaccination (ppv)). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 ppv. All chickens were culled via cervical dislocation and decapitation methods. The serum was separated from whole blood by centrifugation at 1500 xg for 30 minutes at 4°C.

2.2.8.3.2. Evaluation of the protective efficacy of rH9HA-CD83 scFv vaccine

Groups of 7-day-old Rhode Island Red SPF chickens (National Avian Research Facility, n=7 per group) were immunised with 0.2 mL of vaccines via subcutaneous injection at day 7 and day 14 of age. The vaccine groups include PBS control (Group 1), inactivated H9N2 virus (UDL 01/08, 1024 HAU/dose, Group 2), 25 µg of rH9HA (Group 3) and 35 µg of rH9HA-CD83 scFv (equivalent to 25 µg of rH9HA in molecular weight, Group 4). The vaccines were formulated in Montanide™ ISA 71 VG (Seppic) as described in **section 2.2.8.3.1**. All chickens in group 1 to group 4 were inoculated intranasally with $1x10^6$ plaque forming units (PFU)/100 µL of H9N2 virus (UDL 01/08) at 22-day-old (day 14 ppv) and was designated as 'direct' groups. Two additional groups (Group 5 and Group 6) were also included to serve as 'contact' which received PBS and rH9HA-CD83 scFv vaccine, respectively. Chickens were housed in two separate isolators. The groups in isolator-1 include inactivated H9N2 virus vaccine (direct), rH9HA (direct) and rH9HA-CD83 scFv (direct). The groups in isolator-2 include PBS control (direct), PBS control (contact) and rH9HA-CD83 scFv (contact). All chickens were culled via intravenous injection with pentobarbital followed by cervical dislocation.

Swab samples from buccal and cloacal cavities were collected daily until day 7 post infection. The swab samples were transferred into the virus transport media (**section 2.1.7**), vortexed and centrifuged at 1500 xg for 10 minutes to clarify the medium. Swab samples were stored at -80^{o}C until further analysis.

Chickens were scored based on the clinical signs displayed using the clinical scoring system described in **Table 26.**

Table 26: Clinical scoring system.

Clinical score	Description of clinical score	Clinical signs
0	None	None
1	Mild	Ruffled feathers, pale comb/waffles, eye and nasal discharge, red eyes, snicking, weak response to capture
2	Moderate	Dropped wings, half eyes shut, swollen head, diarrhoea
3	Severe	Severe difficulty in breathing (laboured breathing), weight loss (>20%), sitting alone, no attempt to evade capture, paralysis, unconsciousness, convulsions
Moderate disease signs persisting for longer than 72 hours with no signs of improvement and recovery was taken as humane endpoint and the birds were euthanised. Chickens with any of the severe signs were taken as humane endpoint and were euthanised.		

2.2.8.3.3. Further evaluation of the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines in SPF chickens

Groups of 1-day-old White Leghorn SPF chickens (MSD Animal Health, n=50 per group for rH9HA and rH9HA-CD83 scFv groups and n=40 for inactivated H9N2 vaccine) were vaccinated with 0.2 mL of rH9HA (35 µg/dose), rH9HA-CD83 scFv (49 µg/dose equivalent to 35 µg/dose of rH9HA according to the molecular weight) and inactivated H9N2 (UDL 01/08, 1024 HAU/dose) vaccines. Control groups were left unvaccinated. The rH9HA and rH9HA-CD83 scFv groups were further divided into single and double vaccine groups (n=10 per group) whereby the latter received a second dose at 7-day-old. All vaccines were formulated as water-in-oil emulsion (MSD Animal Health) containing glycine and polysorbate 80 as stabilisers. All the vaccines were administered subcutaneously at the back of the neck. In all cases, blood samples were collected on day 6, 14, 21, 28 and 35 days ppv and serum was harvested as described in **section 2.2.8.3.1**. Furthermore, some chickens (n=6 per group, single vaccine group) were culled, and spleen samples were collected in RNA-Later solution (Life Technologies) on day 2, 4, 6, 8 and 21 post vaccination (pv) followed by RNA extraction as described in **section 2.2.4.2**. All chickens were culled via cervical dislocation and decapitation methods.

2.2.8.3.3. Generation of hatchlings with H9HA-specific MDA

Groups of White Leghorn SPF layer hens (MSD Animal Health, n=40) were immunised with 0.5 mL of inactivated H9N2 vaccine (UDL 01/08) containing 520 HAU/dose. The inactivated virus vaccine was administered by intramuscular route in the leg muscle. The first dose of vaccine was given to the hens at 17 weeks of age (T=0 weeks post first dose), followed by second and third doses at 20 weeks of age (T=3 weeks post first dose) and 50 weeks of age, respectively (T=24 weeks

post first dose). Blood samples were collected from the wing vein of the hens at 5, 11, 18, 29 and 36 weeks after first dose for serological monitoring. Fertilised eggs were collected at 36 weeks after first dose (~11 weeks post third dose) and set to incubate until hatch.

2.2.8.3.4. Vaccination of chickens with H9HA-specific MDA

The MDA positive chickens were divided into two groups: high MDA positive (MDA++, HI titre: 588 (9.2 log_2), 1-day-old) and medium MDA positive (MDA+, HI titre: 181 (7.5 log_2), 14-day-old). Groups of MDA++ chickens (n=10 per group) were immunised at 1-day-old with 0.2 mL of rH9HA (35 µg/dose) and rH9HA-CD83 scFv vaccines (49 µg/dose equivalent to 35 µg/dose of rH9HA in equimolar concentration). Furthermore, groups of MDA+ chickens were immunised at 14-day-old with 0.2 mL of inactivated H9N2 vaccine (UDL 01/08, 1024 HAU/dose) and 0.2 mL of rH9HA and rH9HA-CD83 scFv vaccines (concentration same as that given to MDA++ groups). All vaccines were formulated as water-in-oil emulsion (MSD Animal Health) containing glycine and polysorbate 80 as stabilisers. Single vaccination was administered for all the groups via subcutaneous route. Unvaccinated MDA positive chickens were also included in the study to follow the natural decline in anti-H9HA MDA levels. Blood samples were collected from the wing veins of chickens at 2, 3, 4, 5, 6, 7 ,8, 10- and 12-weeks pv. All chickens were culled via cervical dislocation and decapitation methods.

2.2.8.*3.4.* Evaluation of the immunogenicity of rHVT vaccines

Groups of 1-day-old White Leghorn SPF chickens (MSD Animal Health, n=20 per group) were immunised with 0.2 mL of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv vaccines, each dose containing 4000 pfu of the viruses

(Passage 10). All the vaccines were given subcutaneously at the back of the neck. Blood samples were collected on day 6, 14, 21, 28 and 35 pv for serological analysis. Furthermore, spleen samples (n=10 per group) were collected from the chickens on day 7 pv and splenocytes were harvested as described in **section 2.2.3.1** for the analysis of viral content. Similarly, PBMCs were also harvested from the chicken whole blood (n=10 per group) as described in **section 2.2.3.1** on day 21 pv to analyse the viral content. On day 42 pv, spleen samples (n=10 per group) were collected and splenocytes were harvested. All chickens were culled via cervical dislocation and decapitation methods.

2.2.8.5. Analysis of the immunised chicken serum

2.2.8.5.1. Haemagglutination inhibition (HI) assay

The AIVs were titrated by haemagglutination assay (**section 2.2.2.5.5**) and diluted in PBS to 4 HAU for the use in HI assay as described previously[497]. Briefly, a 2-fold serial dilution of the serum was prepared by mixing 25 µL of serum with 25 µL PBS. The diluted serum was then incubated with 4 HAU of virus for 1 hour at 37°C. This was followed by an addition of 1% chicken RBCs and further incubation for 1 hour at 37°C. The HI titres were expressed as reciprocal of the highest dilution of serum that causes total inhibition of 4 HAU of virus haemagglutination activity.

2.2.8.5.2. Measurement of serum anti-HA IgM, IgY and IgA antibodies

The amount of anti-HA IgM, IgY and IgA antibodies in the serum was determined by ELISA assay as described in **section 2.2.2.5.3** with some modifications. Plates were coated with 0.5 µg or 1 µg of recombinant H9HA Foldon protein overnight at 4°C. Then, 1:100 or 1:200 dilutions of serum were made in

ELISA antibody dilution buffer (**section 2.1.7**) prior to use (primary antibody). The HRP conjugated goat anti-chicken IgM, IgY and IgA antibodies were used as secondary antibodies (1 mg/mL diluted to 1:3000 for IgM and IgA, 2 mg/mL diluted to 1:6000 for IgY). A standard reference serum (serum collected from 35-day-old chicken challenged with UDL 01/08 H9N2 virus) was included in all assays. The amount of anti-HA IgM, IgY or IgA antibodies were expressed as sample to reference ratio (relation of absorbance of tested serum sample to absorbance of the reference serum). The sera from unvaccinated chickens/PBS treated chickens were used as negative controls.

Furthermore, ELISA (anti-H9HA IgG) was performed according to the manufacturer's protocol, if ID Screen® Influenza H9 indirect kit (ID. Vet) was used for the assay. The antibody titre was calculated as follows:

Sample (S)/Positive (P) ratio:

$$\frac{S}{P} = \frac{OD_{Sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}}$$

Antibody titre:

$$log_{10}(titre) = 1.3 \times log_{10}\left(\frac{S}{P}\right) + 3.256$$

$$Antibody\ titre = 10^{log_{10}(titre)}$$

2.2.8.5.3. Virus micro-neutralisation (MNT) assay

MDCK cells were pre-seeded in 96-well plate to reach 90-95% confluency. The serum was inactivated at 56°C for 30 minutes. Then, 1:200 dilution of the inactivated serum was carried out in serum-free DMEM media. This was followed by a further 2-fold serial dilution of the serum in triplicates. The diluted serum was

then mixed with 90 µL of virus containing 150 $TCID_{50}$. The serum-virus mixture was incubated at 37°C for 1 hour. Cells were washed with PBS and inoculated with serum-virus mixture at 37˚C for 1 hour. After the incubation, cells were again washed with PBS and serum-free DMEM containing 2 µg/mL TPCK trypsin was added. Cells were left at 37ºC for 72 hours. After 72 hours medium was removed, and cells were stained in 0.1% crystal violet solution (Life Technologies) for 30 minutes. The virus MNT titres were expressed as reciprocal of the highest dilution of serum that blocks the virus infectivity in cultured cells inoculated with 150 $TCID_{50}$ of the virus. The sera from unvaccinated chickens/PBS treated chickens were used as negative controls.

2.2.9. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical significance (p-values) was determined using one-way ANOVA followed by post hoc Tukey's multiple comparison test, Log rank (Mantel-Cox) test (survival plot) and paired/unpaired student's t-test using Prism 8.3.0 (GraphPad Software). Differences were considered statistically significant if $p<0.05$.

Chapter 3: Synthesis and characterisation of recombinant targeted antigen delivery vaccines

Data from this chapter have been published in research articles: ‘‘Targeting haemagglutinin antigen of avian influenza virus to chicken immune cell receptors Dec205 and CD11c induces differential immune-potentiating responses’’ by Shrestha et al. Vaccines, 9, 784 (2021) and “Selectively targeting haemagglutinin antigen to chicken CD83 receptor induces faster and stronger immunity against avian influenza” by Shrestha et al. npj Vaccines, 6, 1-12 (2021).

Chapter summary

In this chapter, targeted antigen delivery vaccines were synthesised for avian influenza virus, by recombinantly fusing the soluble form of H9HA protein with trimerisation foldon domain to scFv antibodies specific for chicken APC receptors; Dec205, CD83 and CD11c. These vaccine constructs were successfully expressed in a native trimeric form in *Drosophila melanogaster* S2 cells yielding about 10-20 mg of the recombinant proteins per litre of the culture supernatants. Both scFv antibodies and H9HA protein fused scFv antibodies were characterised using HA assay, ELISA, western blots, and flow cytometry. The results demonstrated that the fusion of HA antigen to scFv antibodies does not abrogate the functional activity of both HA as well as the scFv antibodies.

Chapter aims

- To purify and sequence the hybridomas producing monoclonal antibodies recognising chicken Dec205, CD83 and CD11c receptor proteins.

- To construct scFv antibodies specific for chicken APC receptors; Dec205, CD83 and CD11c.
- To construct a modified form of HA protein by removing the TM domain and replacing it with trimerisation foldon domain of fibritin protein from T4 bacteriophage, to be used as a model antigen.
- To synthesise TADV for avian influenza virus by recombinantly fusing the modified HA protein with scFv antibodies targeting chicken APC receptors; Dec205, CD83 and CD11c.
- To express TADV candidates in *Drosophila melanogaster* S2 cells.
- To characterise the functional activity of TADV candidates using HA assay, ELISA and western blots.

3.1. Introduction

Avian influenza viruses are one of the major threats to poultry production, inflicting severe loses to poultry industry as well as posing a credible zoonotic and pandemic threats[499]. Various outbreaks of LPAI and HPAI viruses have resulted in severe economic losses to poultry industry due to the expenses associated with culling, quarantine, emergency vaccination programs and loss of consumer confidence[500–502]. Surveillance and strict biosecurity measures are important for controlling AIV outbreaks. However, the biosecurity measures in poultry farms can be compromised, resulting in the invasion of novel virus strains causing disease outbreaks[127]. Therefore, vaccination is being used as an additional measure to control AIVs. Many studies have shown that vaccines can reduce shedding of virus, prevent contact transmission of the virus, and protect against clinical signs and

death[241,503,504]. Currently, majority of the commercial avian influenza vaccines in poultry are inactivated whole viruses produced in eggs. Such inactivated vaccines have several drawbacks: reliance on eggs for production which is affected by egg shortage during AIV outbreaks, sub-optimal immunogenicity, need for repeated vaccination, poor DIVA compatibility and MDA interference[505]. The emerging pandemic threats have stimulated the development of new strategies for producing influenza vaccines that could overcome existing limitations. One highly promising strategy is the development of recombinant subunit vaccines. Recombinant subunit vaccines can maintain the highest safety profile due to the absence of infectious viruses. Furthermore, the rapid, stable, consistent, and scalable production of recombinant subunit vaccines provide an effective strategy for meeting the demands of a possible influenza pandemic[280].

The HA protein of influenza virus is involved in two important events in the life cycle of the virus: i) binding to sialic acid receptors on the target cells and ii) fusion of virus and endosomal membranes causing the release of viral genome into the cytosol of the target cell[31]. Due to these key roles of HA protein in the virus life cycle, and its relative abundance on the surface of the viral membrane, this protein is an ideal target antigen for developing recombinant subunit vaccines. It has been reported that substantial proportion of the antibody response following influenza infection in mice and humans is directed against the HA protein[506,507]. Most importantly, these antibodies were shown to be sufficient for protection against virus infection[508,509]. The full-length HA protein is included in currently approved human and poultry recombinant subunit influenza vaccines[276,510]. Recently, various truncations and/or modifications have been performed to the HA protein to enhance the stability, ease the purification process, and increase the production yield. Such

modifications include removal of TM domain and replacement with trimerisation domain[494,511], genetic engineering to produce different monomeric and dimeric forms of HA protein[512] and truncations of HA0 to produce HA1/HA2 subunit vaccines[513,514]. The selection of protein expression system is important to produce HA protein in correctly folded form. This is key as the presentation of correct conformational epitopes is needed to enhance the immune responses. The recombinant subunit HA protein has been produced in mammalian cells[515,516], insect cells[163,170,171,421], plant cells[519] and bacterial cells[520,521]. Bacterial cell expression system is not preferred as it is unable to perform important post-translational modifications like glycosylation and disulphide bond formation[522], which are required for the correct folding and oligomerisation of HA protein. The baculovirus-insect cell expression system has been extensively explored for manufacturing various recombinant subunit vaccines for human and animal use[523]. Several of these vaccines for human and veterinary use are commercially available[524]:i) Cervarix®, a bivalent human papillomavirus (HPV) vaccine against cervical cancer (GlaxoSmithKline) ii) FluBlok®, a recombinant HA-based seasonal influenza vaccine for human (Protein Sciences Corporation) iii) Porcilis® against porcine circovirus type 2 (MSD Animal Health) iv) Volvac® B.E.S.T. bivalent vaccine based on recombinant H5HA and Newcastle disease virus (Boehringer Ingelheim). However, baculovirus-insect expression system has some disadvantages including an extra baculovirus infection stage, chances of protein degradation from viral proteases and lytic nature which can induce considerable stress to the infected cells[525]. Another insect cell expression system that could be an alternative to the baculovirus-insect expression system is *Drosophila melanogaster* S2 cells. The *Drosophila* S2 expression system allows stable and high-level expression of

recombinant proteins. They present several advantages like fast growing capacity and the use of culture media that do not require expensive additives[526]. They can be easily maintained in loosely adherent or suspension culture at room temperature and do not require carbon dioxide (CO_2). Furthermore, it is non-lytic insect expression system that uses simple plasmid vectors[527]. Biological products already expressed by these cells include antibodies[525], enzymes[528], growth factors[529], receptors[530,531], and viral antigens[532,533]. Various candidate vaccines produced to current good manufacturing practice (cGMP) in *Drosophila* S2 cells have entered clinical testing. These includes candidate vaccines for SARS-CoV-2[534], West Nile virus[535], dengue virus[536] and malaria[537,538] supporting the use of *Drosophila* S2 cell system for manufacturing recombinant protein-based vaccines for human and veterinary use.

Although, recombinant technology has enabled a safer and efficient scalable production of recombinant subunit influenza vaccines, very few of these have been commercialised for human and veterinary use. This could be due to low immunogenicity, the need for repeated vaccination and requirement of adjuvants to enhance the cellular immunity[272]. Over the past few years, various strategies have been developed to enhance the immunogenicity of recombinant subunit vaccines. One such strategy is the recombinant TADV whereby antigens are selectively delivered to professional APCs such as DC, macrophages and B cells[357]. Targeting antigens to professional APCs can be carried out using ligand or antibody-based methods. In ligand-based APC targeting, antigens are conjugated to ligands of PRRs e.g., LPS, CpG and flagellin whereas in antibody-based targeting, antigens are conjugated to antibodies specific to the receptors on professional APCs[357]. There has always been concerns over the safety of using

TLR agonists like LPS or CpG in vaccine formulation, particularly the possibility that they might increase susceptibility to toxic shock or autoimmune disease[539]. Hence, for our work antibody-based antigen targeting strategy was used for enhancing the vaccine efficacy. In this approach, antigen can be targeted by either using full-length antibody or different antibody fragments such as scFv, Fab or nanobodies[377]. For the purpose of our work, scFv antibodies were used because they are smaller in size and can be genetically engineered according to our need for easy expression and purification of antigen-antibody constructs. As described in **section 1.6.1**, the most commonly used antigen targeting receptor in mammalian studies is Dec205. In fact, the first-in-human study of a protein vaccine targeting APCs was directed towards human Dec205 receptor[392]. Similarly, the first antigen targeting study in chickens was also directed towards Dec205 receptor[396]. Several other studies have explored antibody-based antigen targeting to mammalian APCs via CD11c, CD40, Clec9A and MHC II receptors[418,540–545]. There are limited data available for targeting chicken APCs for modulating efficacy of poultry vaccines. In this chapter, antigen-scFv antibody vaccine candidates targeting chicken Dec205, CD83 and CD11c receptors were synthesised using modified HA protein as a 'model antigen'. In addition, the functional activity of all vaccine candidates was also tested.

3.2. Results

3.2.1. Monoclonal antibodies against chicken Dec205, CD83 and CD11c were successfully produced, purified and sequenced

Prior to the start of this work, respective hybridomas; clone: IAH F877: AD6 and clone: IAH F890:GE8 producing monoclonal antibodies recognising chicken Dec205[324] and CD83[324] receptors, respectively were produced in mice at the Pirbright institute (formerly known as Institute of Animal Health (IAH) as described in **section 2.2.8.2 (Figure 3.1)**. Hybridoma for chicken CD11c (clone: IAH 8F2) was gifted to the institute by Bernd Kaspers (University of Munich)[331]. The hybridoma clones secreting specific mAb were screened using ELISA and flow cytometry[324]. The mAbs were produced and purified commercially (BioServUK) using Protein G affinity chromatography. The purity of mAbs was confirmed by SDS-PAGE. The molecular weights of heavy (H) and light (L) chains were approximately 50 kDa and 25 kDa, respectively (**Figure 3.2**). Furthermore, the mAbs were sequenced commercially (Absolute Antibody Ltd, UK) to derive the vH and vL chain sequences. The nucleotide and amino acid sequences of vH and vL chains of the respective mAbs are shown in **Table 27**. Isotyping of mAbs showed that Dec205 mAb, CD83 mAb and CD11c mAb were IgG2b, IgG2a and IgG1, respectively and all mAbs contained kappa light chains. The complementarity-determining regions (CDRs) of the variable chains of mAbs were determined using the Kabat definition[546] (Absolute Antibody Ltd, UK) and shown in **Table 28**.

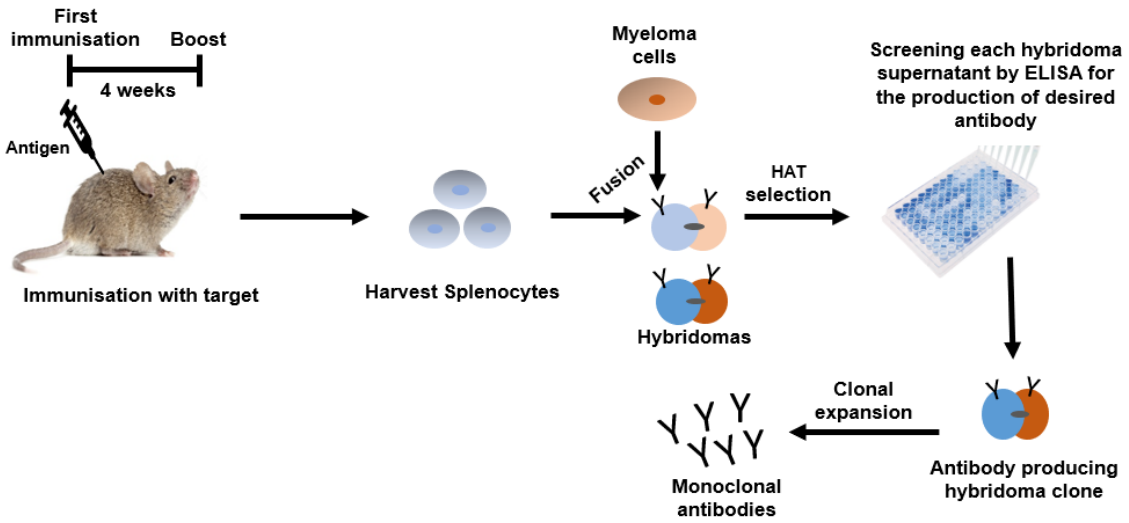


Figure 3.1: Schematic representation of the hybridoma method used to produce monoclonal antibodies recognising chicken Dec205 and CD83 receptors. Briefly, mice were immunised with the target immunogen. Splenocytes were harvested and fused with immortalised myeloma cells. The fused cells (hybrid cell lines) called hybridoma has both the antibody producing ability of B-cell and longevity and reproducibility of the myeloma cells. The HAT selection media containing Hypoxanthine, Aminopterin, & Thymine was used to eliminate the unfused myeloma cells. This was followed by hybridoma screening for specific antibody production using ELISA technique.

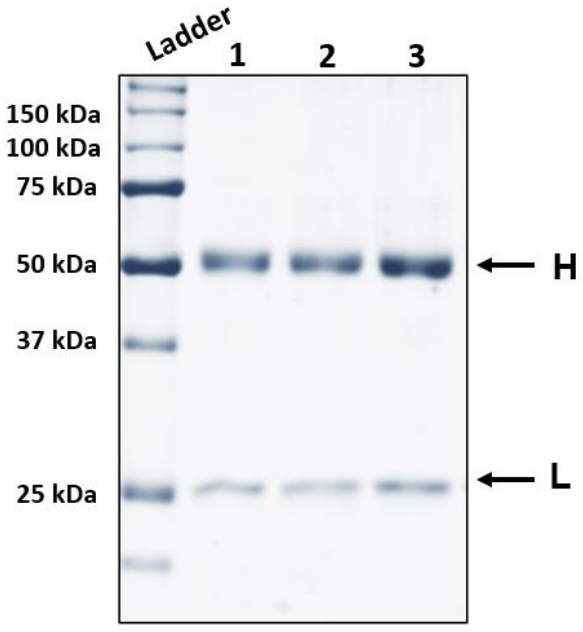


Figure 3.2: SDS-PAGE analysis to confirm the purity of monoclonal antibodies. **Lane 1**: Dec205 mAb **Lane 2**: CD83 mAb **Lane 3:** CD11c mAb. 'H' and 'L' refers to heavy and light chains, respectively. 5 µg of each purified mAb was denatured by boiling in SDS-PAGE protein loading buffer containing 2% Beta-mercaptoethanol and run on 10% SDS-PAGE.

Table 27: Nucleotide and amino acid sequences of heavy and light chains of Dec205 mAb, CD11c mAb and CD83 mAb.

scFv		Nucleotide sequence
Dec205	Heavy	GAGGTGCAACTGGTGGAGTCTGGGGGAGACTTAGTGAAGCCTGG AGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGT AGCTATGGCATGTCTTGGGTTCGCCAGACTCCAGACAAGAGGCTG GAGTGGGTCGCAACCATTAGTAGTGGTGGTAGTTACACCTACTATC CAGACAGTGTGAAGGGGCGATTCACCATTTCCAGAGACAATGCCA AGAACATCCTGTATCTGCAAATGAGCAGTCTGAAGTCTGAAGACAC AGCCATGTATTACTGTGCAAGACTTTCAACCTGGGACTGGTACTTC GATGTCTGGGGCACAGGGACCACGGTCACCGTCTCCTCA
	Light	GAAATTGTGCTCACCCAGTCTCCAGCACTCATGGCTGCATCTCCA GGGGAGAAGGTCACCATCACCTGCAGTGTCAGCTCAAGTATAAGT TCCGGCAACTTTCACTGGTACCAGCAGAAGTCAGGAACCTCCCCC AAACTCTGGATTTATGGCACATCCAACCTGGCTTCTGGAGTCCCTG TTCGCTTCAGTGGCAGTGGATCTGGGACCTCTTATTCTCTCACAAT CAGCAGCATGGAGGCTGAAGATGCTGCCACTTATTACTGTCAACA GTGGAGTAGTTACCCATTCACGTTCGGCTCGGGGACAAAGTTGGA AATAAAA
CD11c	Heavy	GAGGTCCAGCTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGG GGCTTCAGTGAAGATGTCCTGCAAGGCTTCTGGATACACATTCACT AACTATGTTCTGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTT GAGTGGATTGGATATATTAATCCTTACAATGATGGTACTAAGTTCAA TGAGAAGTTCAAAGGCAAGGCCACACTGACTTCAGACACATCCTC CAGCACAGCCTTCATGGAACTCAGCAGCCTGACCTCTGAGGACTC TGCGGTCTATTACTGTGCAAGAGGAGATAATCTACGGCCCTACTAC TTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
	Light	CAAATTGTTCTCACCCATTCTCCAGCAATCATGTCTGCATCTCCAG GGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTT TCATGTACTGGTACCAGCAGAAGCCAGGATCCTCCCCCCGACTCC TGCTTTATGACACATCCAGCCTGTCTTCTGGAGTCCCTGTTCGCTT CAGTGGCAGTGGCTCTGGGACCTCTTACTCTCTCACAATCAGCCG AATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAG TCGTTACCCACCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAA A
CD83	Heavy	GAGGTCCAGCTGCAACAATCTGGACCTGAGCTGGTGAAGCCTGG GGCTTCAGTGAAGATATCCTGTAAGGCTTCTGGATACACGTTCACT GACTACTACATAAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTT GAGTGGATTGGAGATATTAATCCTACTAATGGTGATTCTACCTACA GCCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAGTCCT CCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGTCT CTGCAGTCTATTACTGTGCAAGAGACTATGCTATGGACTACTGGGG TCAAGGAACCTCAGTCACCGTCTCCTCA

	Light	GACATTGTGATGACCCAGTCTCCATCCTCCCTGGCTGTGTCAGTC GGACAGAAGGTCACTATGAGCTGCACGTCCAGTCAGGTCCTTTTA CATAGTCCCAATCAAAAGAACTATTTGGCCTGGTACCAGCAGAAAC CAGGACAGTCTCCTAAACTTCTGGTATACTTTGCATCCACTAGGGA ATCTGGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGA TTTCACTCTTACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTT TATTACTGTCAGCAACATTATAGCACTCCGCTCACGTTCGGTGCTG GGACCAAGCTGGAGCTGAAA

scFv		Amino acid sequence
Dec205	Heavy	EVQLVESGGDLVKPGGSLKLSCAASGFTFSSYGMSWVRQTPDKRLEWVA TISSGGSYTYYPDSVKGRFTISRDNAKNILYLQMSSLKSEDTAMYYCARLST WDWYFDVWGTGTTVTVSS
	Light	EIVLTQSPALMAASPGEKVTITCSVSSSISSGNFHWYQQKSGTSPKLWIYGT SNLASGVPVRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSYPFTFGSG TKLEIK
CD11c	Heavy	EVQLQQSGPELVKPGASVKMSCKASGYTFTNYVLHWVKQKPGQGLEWIG YINPYNDGTKFNEKFKGKATLTSDTSSSTAFMELSSLTSEDSAVYYCARGD NLRPYYFDYWGQGTTLTVSS
	Light	QIVLTHSPAIMSASPGEKVTMTCSASSSVSFMYWYQQKPGSSPRLLLYDTS SLSSGVPVRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSRYPPTFGGGT KLEIK
CD83	Heavy	EVQLQQSGPELVKPGASVKISCKASGYTFTDYYINWVKQSHGKSLEWIGDI NPTNGDSTYSQKFKGKATLTVDKSSSTAYMELRSLTSEVSAVYYCARDYA MDYWGQGTSVTVSS
	Light	DIVMTQSPSSLAVSVGQKVTMSCTSSQVLLHSPNQKNYLAWYQQKPGQSP KLLVYFASTRESGVPDRFTGSGSGTDFTLTISSVQAEDLAVYYCQQHYSTP LTFGAGTKLELK

Table 28: Isotypes and complementarity-determining region sequences of the monoclonal antibodies.

mAb	Isotype (Heavy/ light)	CDR-H1	CDR-H2	CDR-H3	CDR-L1	CDR-L2	CDR-L3
Dec205	IgG2b/κ	SYGMS	SSGGSYTYYPDSVKGRF	LSTWDWYFDV	SVSSSISSGNFH	GTSNLAS	QQWSSYPFT
CD83	IgG2a/κ	DYYIN	DINPTNGDSTYSQKFKG	DYAMDY	TSSQVLLHSPNQKNYLA	FASTRES	QQHYSTPLT
CD11c	IgG1/κ	NYVLH	YINPYNDGTKFNEKFKG	GDNLRPYYFDY	SASSSVSFMY	DTSSLSS	QQWSRYPPT

CDR-H1, CDR-H2, CDR-H3: CDRs of variable heavy chain

CDR-L1, CDR-L2, CDR-L3: CDRs of variable light chain

3.2.2. Synthesis, production, and purification of model antigen, scFv antibodies and antigen-scFv antibody

3.2.2.1. The model antigen consists of a soluble H9HA protein without a transmembrane domain

The H9N2 viruses are enzootic in poultry throughout Asia, Middle East and North Africa[138]. It has been reported that since 2016 this virus has also been established in poultry in Morocco and Turkey with occasional incursions to Europe[138,547]. Hence, H9HA antigen was used as a model antigen for developing TADV. The H9HA sequence used for generating the recombinant TADV was synthetically produced incorporating consensus sequence of HA of H9N2 viruses

derived from analysis of over 200 H9HA sequences of G1-like H9 virus lineage using Minimum Sphere Consensus (MScon) method[548]. However, this synthetic H9HA has about 98% amino acid sequence similarity to HA ectodomain of UDL 01/08 H9N2 virus (GenBank accession number: ACP50708.1, HA1: 19-338 and HA2: 339-560). Moreover, in order to produce soluble recombinant H9HA protein that could be secreted in higher amount in the culture medium for ease in downstream processing, the TM domain was removed. However, the native trimeric structure of H9HA protein needs to be maintained to get a high immune response. Hence, the soluble H9HA protein was modified at its C-terminus by adding the 30 amino acid trimerisation foldon domain of fibritin protein from T4 bacteriophage[549]. The schematic representation of recombinant H9HA ectodomain without TM domain and with trimerisation foldon domain is shown in **Figure 3.3** (hereinafter referred to as rH9HA).

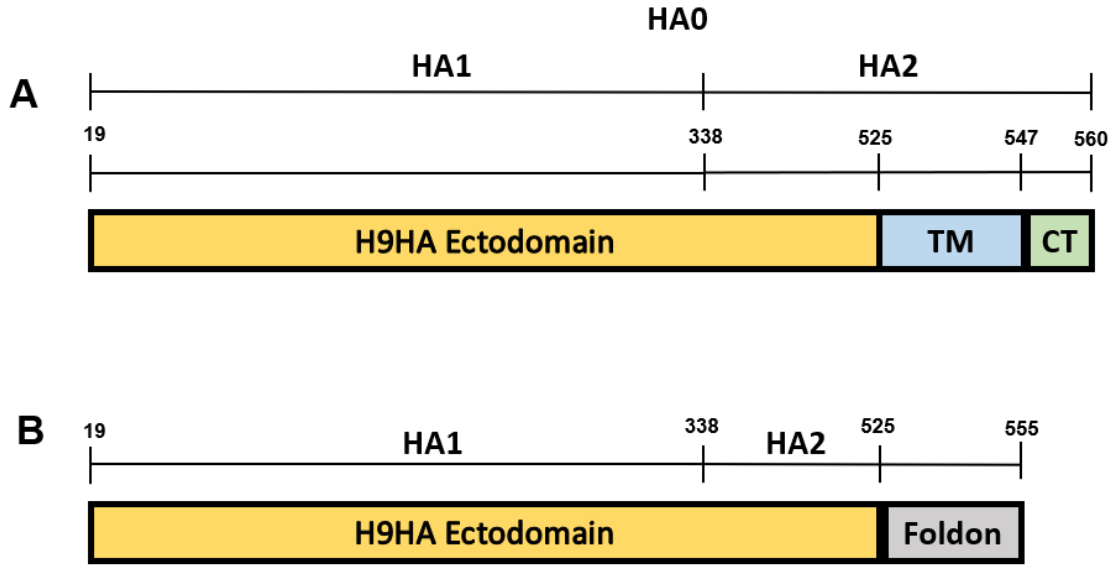


Figure 3.3: Schematic representation of recombinant H9HA constructs showing different domains**. (A)** Full-length HA of H9N2 (MScon) virus. Precursor HA0:1-560 amino acids (aa), HA1:19-338 aa, HA2: 339-560 aa, TM=Transmembrane domain (525-547 aa) CT=Cytosolic Tail domain (548-560 aa) **(B)** Soluble HA of H9N2 (MScon) virus. Soluble H9HA construct was generated by removing the TM and CT domains (525-560 aa) and conjugating the C-terminus of HA to 30 aa long trimerisation foldon sequence of the trimeric protein fibritin from T4 bacteriophage.

3.2.2.2. The plasmids expressing scFv antibodies, rH9HA and rH9HA-scFv antibody were successfully synthesised

The gene sequences comprising the vL and vH chains of Dec205 mAb, CD83 mAb and CD11c mAb were joined by $(Gly_4Ser)_4$ linker peptide sequence and manufactured commercially (Geneart, Life Technologies). The respective scFv antibody sequences were cloned into *Drosophila melanogaster* expression vector pMT-BIP-V5-6xHis (Version A, Life Technologies) as described in **section 2.2.1.2**. The resultant pMT-BIP-scFv-V5-6xHis plasmid was used to clone rH9HA protein. This generated pMT-BIP-rH9HA-scFv-V5-6xHis plasmids that could express recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv proteins. The schematic representation of all the expression cassettes is shown in **Figure 3.4.** The expression plasmids were then transformed into DH5α competent *E.coli* (Life Technologies) and the positive clones were screened by colony PCR using the respective scFv and M13 primers described in **Table 22** (**Figure 3.5**). The colony PCR positive clones were further confirmed by Sanger sequencing (Source Bioscience) before using them for protein expression.

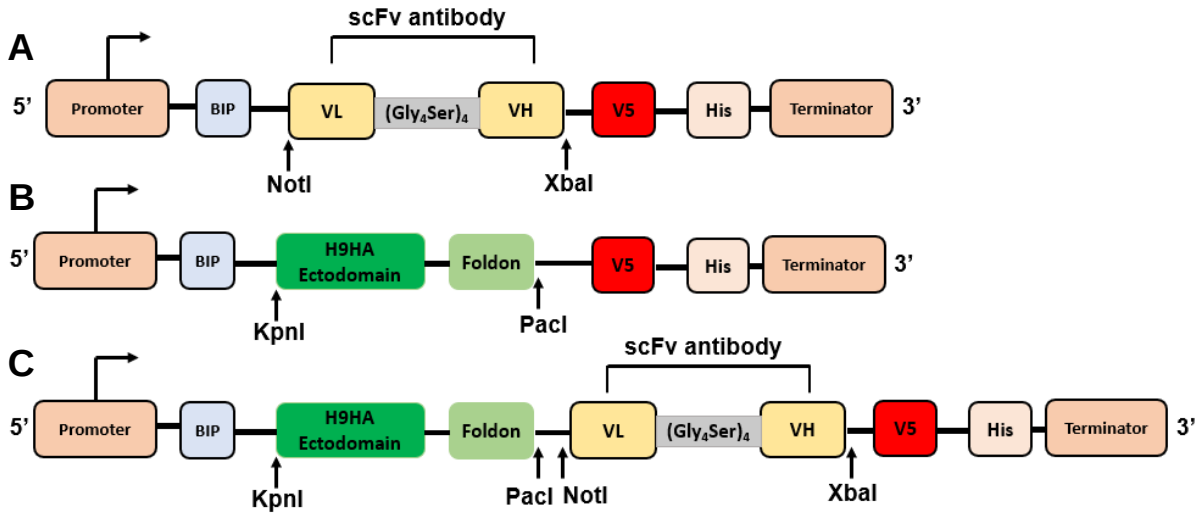


Figure 3.4. Schematic representation of scFv, rH9HA and rH9HA conjugated scFv antibody expression cassettes. (**A**) scFv antibody expression cassette. It includes *Drosophila melanogaster* immunoglobulin heavy chain binding protein (BIP) secretion signal sequence at 5' end followed by vL chain, linker peptide $(Gly_4Ser)_4$ and vH chain. The V5 tag is for detection and His tag for purification. (**B**) rH9HA expression cassette. It includes BIP secretion signal sequence at 5' end followed by H9HA ectodomain, trimerisation domain (indicated as foldon), V5 tag and His tag. (**C**) rH9HA-scFv expression cassette. It includes BIP secretion signal sequence at 5' end followed by H9HA ectodomain, trimerisation domain (indicated as foldon), vL chain, linker peptide $(Gly_4Ser)_4$ and vH chain, V5 tag and His tag.

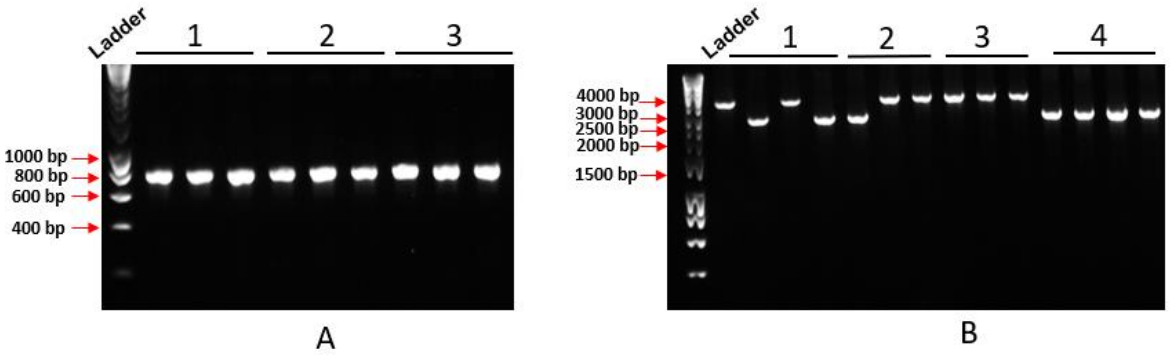


Figure 3.5. Colony PCR for the selection of positive clones after plasmid-insert ligation. (**A**) Colony PCR for the selection of positive clones containing scFv antibodies after ligation. **Lanes 1**: Dec205 scFv **Lanes 2:** CD83 scFv **Lanes 3**: CD11c scFv. The positive clones are about 760 bp in size. (**B**) Colony PCR for the selection of positive clones containing rH9HA or rH9HA-scFv after ligation. **Lanes 1**: rH9HA-Dec205 scFv **Lanes 2**: rH9HA-CD83 scFv **Lanes 3**: rH9HA-CD11c scFv. The positive clones are about 3500 bp in size. **Lanes 4**: rH9HA. The positive clones are about 2600 bp in size. The pMT-BIP-V5-6xHis vector and respective inserts were ligated and transformed into DH5α chemically competent *E.coli*. The next day 5-10 colonies were picked, and PCR was carried out with the respective scFv cloning primers (**A**) and M13_For and M13_Rev primers (**B**) as shown

in Table 22. The PCR products were run on 1% agarose gel for 1 hour at 65 volts and visualised using a Gel Doc™ EZ Imager (Bio-Rad) using the 'GelRed' setting.

3.2.2.3. Selection of S2 cell single clones expressing the recombinant proteins using ELISA

To express scFv antibodies, rH9HA and rH9HA-scFv, the respective expression plasmids generated in **section 3.2.2.2** were transfected into *Drosophila melanogaster* S2 cells as described in **section 2.2.2.1**. For a single clone selection, dilution cloning method was used in which $2x10^3$ S2 transfectants were mixed with gamma-irradiated 10^6 parental S2 cells as feeder cells. The single clones became clearly visible after 3 weeks of incubation at 28°C (**Figure 3.6**). The S2 cell single clones expressing recombinant proteins were selected by screening the culture supernatants (10-15 clones per construct) using ELISA (**Figure 3.7**). The ELISA results indicated that majority of the single clones were able to express the recombinant proteins. Furthermore, different single clones of the same construct showed varying levels of recombinant protein expression. Therefore, the top three S2 cell single clones expressing the highest amount of recombinant proteins were stored for future use and the rest were discarded.

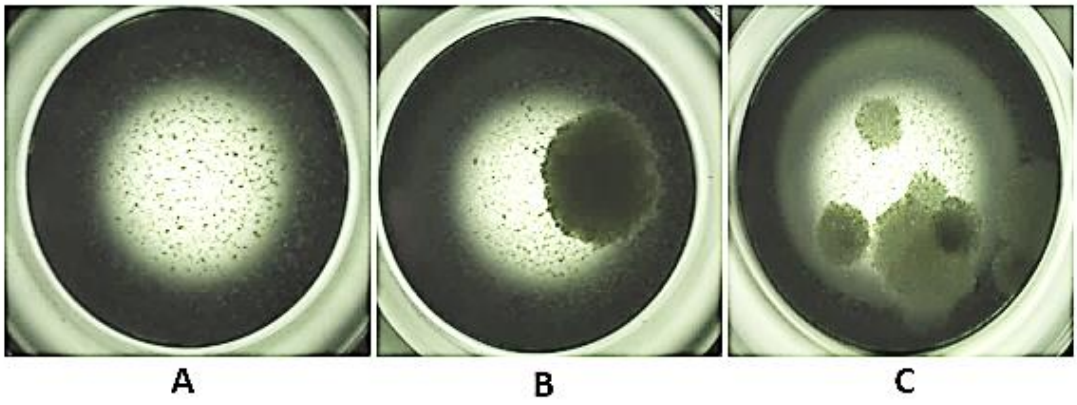


Figure 3.6. Generation of single clones expressing scFv antibodies, rH9HA and rH9HA-scFv. (**A**) Well with no clone (**B**) Well with a single clone (**C**) Well with multiple clones. About 2 $x10^3$ stable S2 transfectants were mixed with gamma-irradiated 10^6 parental S2

cells (feeder layer cells) in 10 mL of complete S2 cell growth media. A 100 µL portion of cell mixture was added to each well in 96 well plates, followed by incubation at 28°C for about 3 weeks. After 3 weeks, single clones were isolated for further expansion.

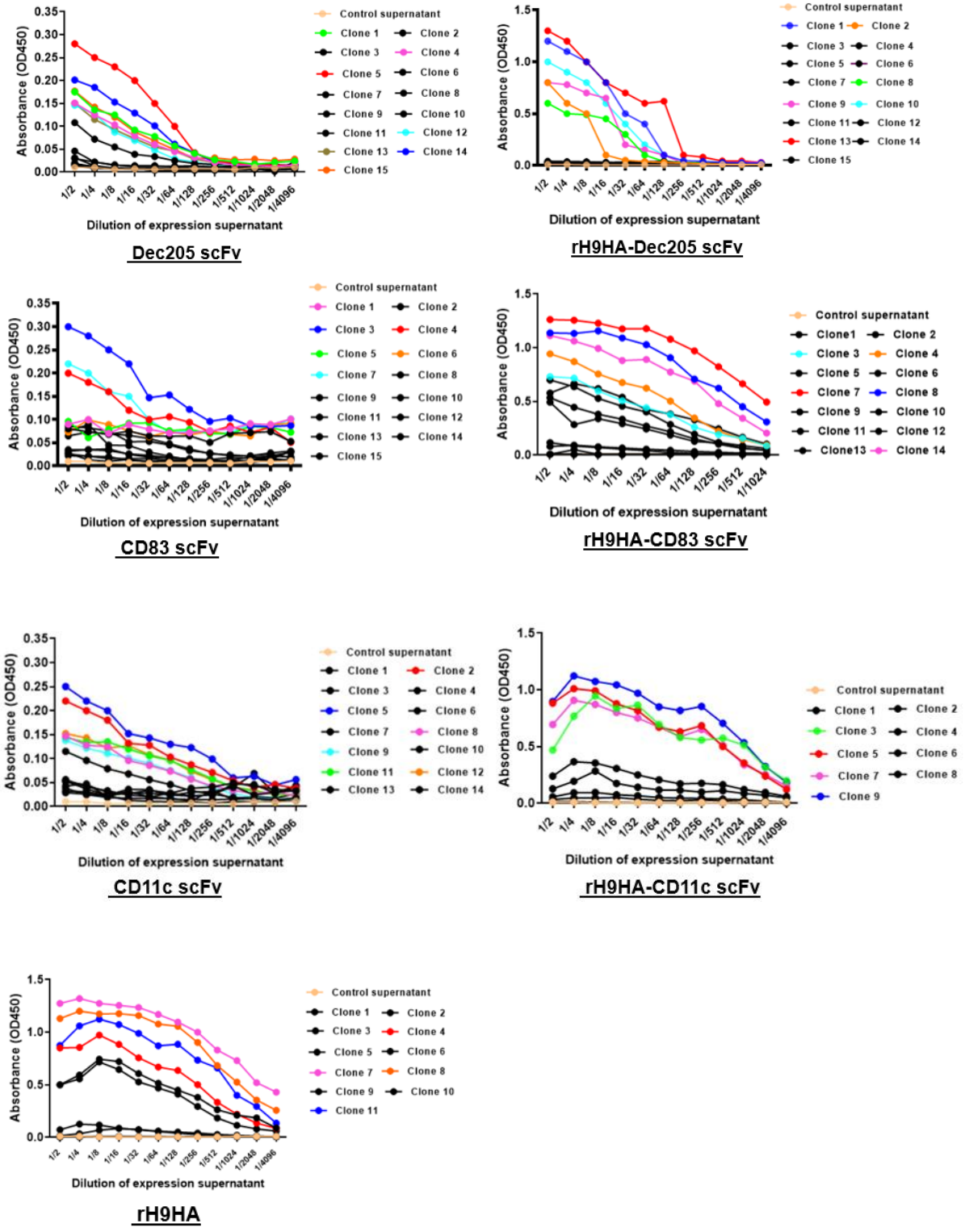


Figure 3.7. Selection of the highest recombinant protein expressing S2 cell single clones. The expression supernatant containing the respective protein of interest was coated onto

the ELISA plate and incubated overnight for 4ºC. The wells were then incubated with mouse anti-V5 primary antibody (1 mg/mL diluted to 1:1000) for 1 hour at room temperature. This was followed by 3x wash with PBS-T and further incubation with HRP-conjugated goat anti-mouse secondary antibody (1 mg/mL diluted to 1:1000). The colorimetric detection was carried out by adding TMB substrate. The single clone giving the highest colorimetric readout was selected for further expansion and purification. The clones shown in black are low recombinant protein expressing single clones and thus discarded.

3.2.2.4. The rH9HA protein is expressed as the HA precursor (HA0) in *Drosophila melanogaster* cells

The highest protein expressing single clones obtained from **section 3.2.2.3** were grown in a 2 litres roller bottle (Corning) containing 400 mL Ex-Cell® 420 serum-free media (Merck Life Science) for 5-6 days. All the recombinant proteins were successfully expressed in *Drosophila melanogaster* S2 cells and secreted as indicated by western blot results of the culture supernatants (**Figure 3.8**). Subsequent purification of the recombinant protein containing supernatants by His-tag affinity chromatography yielded proteins of molecular weights of approximately 30 kDa for scFv, 70 kDa for rH9HA and 100 kDa for rH9HA-scFv (**Figure 3.9**, results shown for rH9HA, CD83 scFv and rH9HA-CD83 scFv). The molecular weight observed for rH9HA and rH9HA-scFv were slightly higher than expected (expected molecular weight for rH9HA: 66 kDa and rH9HA-scFv: 94 kDa). This difference could be due to glycosylation. Most of the recombinant proteins were eluted with 25 mM or 50 mM imidazole as evident on the gel (**Figure 3.9**). Furthermore, most of the purification fractions showed a single band corresponding to the recombinant proteins indicating a relatively high level of purity. Moreover, the purified rH9HA protein migrated as a single polypeptide of about 70 kDa on SDS-PAGE under reducing denaturing conditions. This indicates that the rH9HA protein is expressed as the HA precursor (HA0). This observation is in line with the previous

reports of soluble HA expression as precursor HA0[494,455]. The precursor HA0 was not processed in *Drosophila melanogaster* S2 cells to yield cleavage products HA1 and HA2. However, the treatment of rH9HA protein with exogenous trypsin resulted in two bands representing HA1 (~45 kDa) and HA2 (~25 kDa) (**Figure 3.10**). This demonstrates that *Drosophila melanogaster* S2 cells do not have endogenous trypsin-like proteases compatible with the cleavage of HA0 precursor.

Based on the recovered purified proteins, the expression yield of the recombinant proteins was estimated to be between 10-20 mg per litre of the culture supernatants.

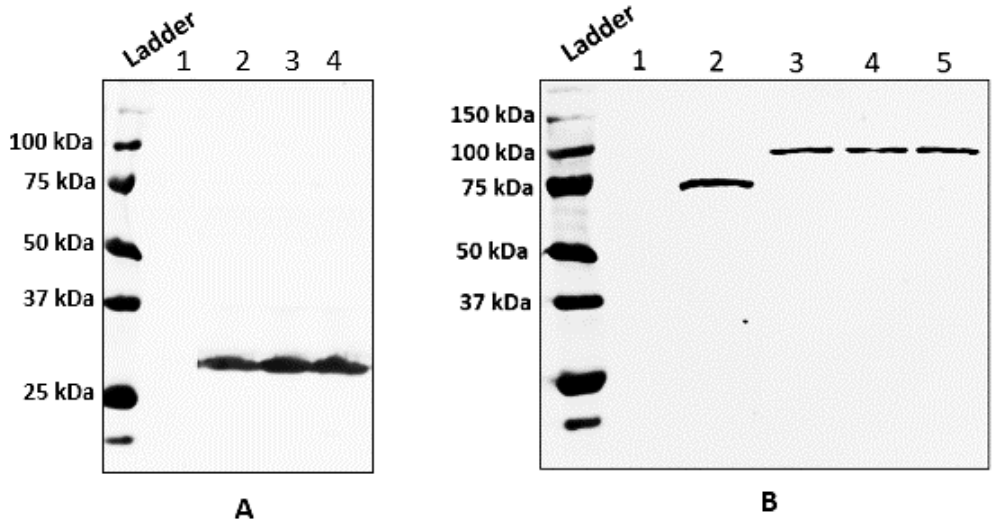


Figure 3.8. Western blot analysis of scFv, rH9HA and rH9HA-scFv culture supernatants under reducing condition. The culture supernatants were run on 10% SDS-PAGE and analysed by western blot. (**A**) Analysis of culture supernatant containing scFv antibodies. The molecular weight is about 30 kDa. For detection, mouse anti-V5 primary antibody (1 mg/mL diluted to 1:1000) and IRDye® 800CW goat anti-mouse secondary antibody (1 mg/mL diluted to 1:10000) were used. **Lane 1**: Control supernatant from the untransfected cells **Lane 2**: Dec205 scFv **Lane 3**: CD83 scFv **Lane 4**: CD11c scFv. (**B**) Analysis of culture supernatant containing rH9HA and rH9HA-scFv. The molecular weight of rH9HA is about 70 kDa whereas the molecular weight of rH9HA-scFv is about 100 kDa. For detection, mouse anti-H9HA JF7 primary antibody (1mg/mL diluted to 1:1000) and IRDye® 800CW goat anti-mouse secondary antibody (1 mg/mL diluted to 1:10000) were used. **Lane 1**: Control supernatant from the untransfected cells **Lane 2**: rH9HA **Lane 3**: rH9HA-Dec205 scFv **Lane 4**: rH9HA-CD83 scFv **Lane 5**: rH9HA-CD11c scFv

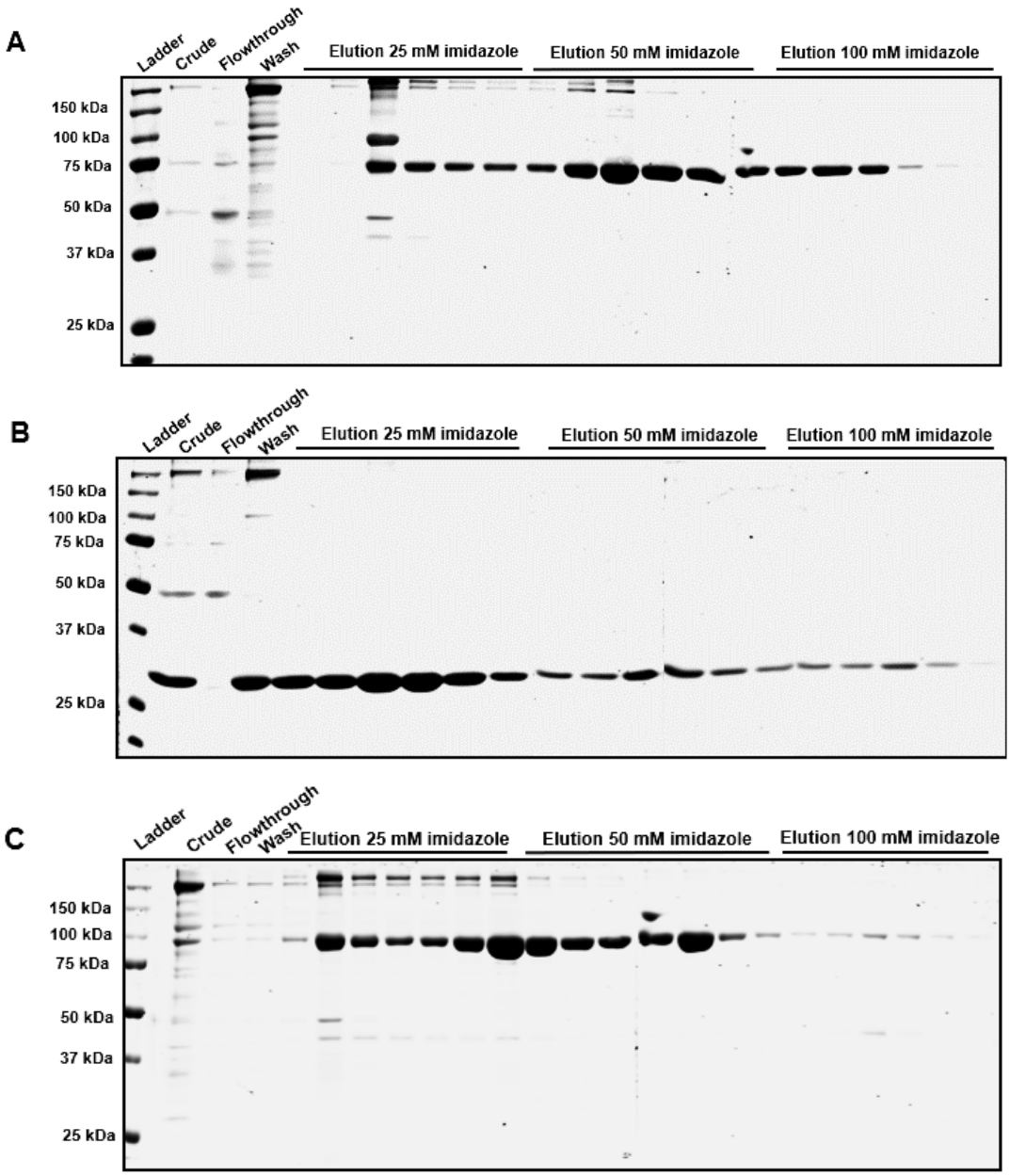


Figure 3.9. His tag purification of rH9HA, CD83 scFv and rH9HA-CD83 scFv. (**A**) His tag purification of rH9HA. The molecular weight of the purified protein is about 70 kDa (**B**) His tag purification of CD83 scFv. The molecular weight of the purified protein is about 30 kDa (**C**) His tag purification of rH9HA-CD83 scFv. The molecular weight of the purified protein is about 100 kDa. The harvested culture supernatants containing recombinant proteins bound to the copper ions (copper sulphate was used as an inducer of metallothionein promoter) were loaded onto the uncharged "UNOsphere" resin derivatised with iminodiacetic acid functioning as chelating ligand (Profinity™IMAC, Bio-Rad). Proteins were eluted with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 25/50/100 mM imidazole. The purified proteins were analysed using 10% SDS-PAGE followed by Coomassie staining.

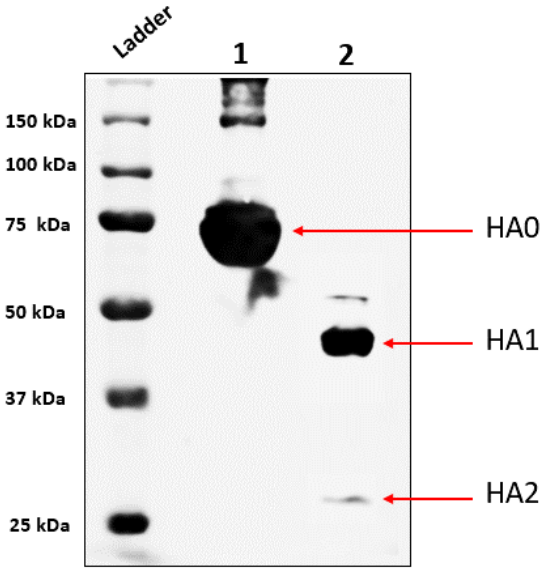


Figure 3.10. Western blot analysis of rH9HA protein before and after trypsinisation. **Lane 1**: rH9HA (-Trypsin) **Lane 2**: rH9HA (+Trypsin). The rH9HA protein was treated with 50 µg/mL of TPCK trypsin, run on 10% reducing SDS-PAGE gel and analysed by western blot. For detection, chicken serum raised by inoculating UDL 01/08 H9N2 virus (polyclonal, 1:500) was used as primary antibody, followed by IRDye® 800CW donkey anti-chicken secondary antibody (1 mg/mL diluted to 1:10000).

3.2.3. Functional characterisation of rH9HA-scFv

3.2.3.1. The H9HA ectodomain fused to T4 bacteriophage trimerisation foldon can trimerise

The HA protein is trimeric in nature and this trimeric structure is needed to retain the biological activity of the protein[550]. The native form of H9HA ectodomain containing trimerisation foldon domain was determined by performing a cross-linking reaction using a BS3 cross-linker. BS3 is a water-soluble cross-linker which reacts with primary amines of target proteins to form stable amide bonds[551]. When multimeric proteins are exposed to BS3, each subunit will be cross-linked together with the formation of amide bonds. This provides direct evidence for their close proximity[551]. This also helps to stabilise the structure of oligomers[552], allowing them to be resolved on SDS denaturing gels for western blot analysis. The rH9HA and

rH9HA-scFv proteins were exposed to BS3 cross linker and the cross-linked products were separated on SDS gel under reducing and denaturing conditions, blotted and immunodetected using anti-H9HA monoclonal antibody[488]. The result is shown in **Figure 3.11**. Without BS3 crosslinking, three species; monomer, dimer and trimer are observed (major band at monomer; Lane 1, Lane 3, Lane 5, Lane 7 corresponding to about 70 kDa and 100 kDa for rH9HA and rH9HA-scFv respectively). With crosslinking, stable trimeric form is observed (Lane 2, Lane 4, Lane 6 and Lane 8 corresponding to approximately 210 kDa and 300 kDa for rH9HA and rH9HA-scFv respectively). This indicates that the native structure of recombinant H9HA protein with foldon is a trimer, and three molecules of scFv antibodies are attached to one trimeric H9HA protein (**Figure 3.12**).

Furthermore, the oligomeric structure of the scFv antibodies was also determined by treating with BS3 cross-linker. Monomeric form was dominant both with and without BS3 cross-linking. However, some dimeric scFv antibodies were also observed when treated with BS3 cross-linker (**Figure 3.13**). This indicates that majority of the scFv antibodies were produced as monomers in *Drosophila melanogaster* S2 cells. This observation has also been reported elsewhere[525].

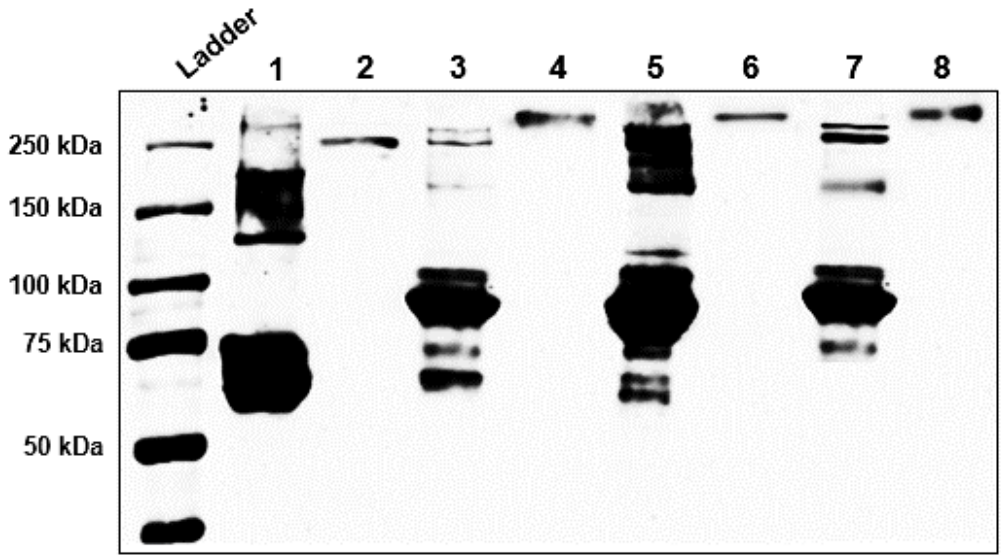


Figure 3.11. BS3 cross-linking experiment to determine the oligomeric form of rH9HA and rH9HA-scFv. **Lane 1**: rH9HA (-BS3) **Lane 2**: rH9HA (+BS3) **Lane 3**: rH9HA-Dec205 scFv (-BS3) **Lane 4**: rH9HA-Dec205 scFv (+BS3) **Lane 5**: rH9HA-CD83 scFv (-BS3) **Lane 6**: rH9HA-CD83 scFv (+BS3) **Lane 7**: rH9HA-CD11c scFv (-BS3) **Lane 8**: rH9HA-CD11c scFv (+BS3). Molecular weights: **rH9HA** (Monomer: 70 kDa, Dimer: 140 kDa, Trimer: 210 kDa), **rH9HA-scFv** (Monomer: 100 kDa, Dimer: 200 kDa, Trimer: 300 kDa). About 15 µg of the recombinant proteins were mixed with BS3 to a final concentration of 10 mM and incubated for 1 hour at room temperature. The cross-linking reaction was stopped by the addition of 1 M Tris-HCl pH 8.0 to a final concentration of 50 mM and incubated for 15 minutes at room temperature. After cross-linking, proteins were separated on 8% SDS-PAGE under reducing conditions, blotted and immunodetected using anti-H9HA JF7 antibody (1mg/mL diluted to 1:1000).

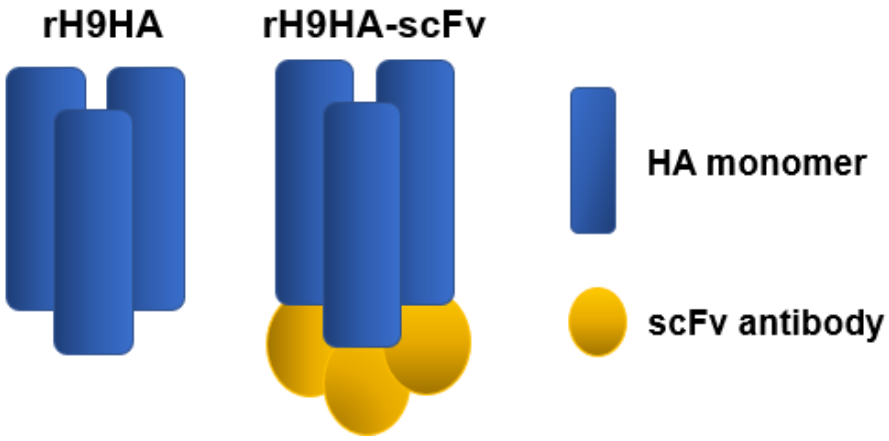


Figure 3.12. Illustration of the oligomeric structure of rH9HA and rH9HA-scFv.

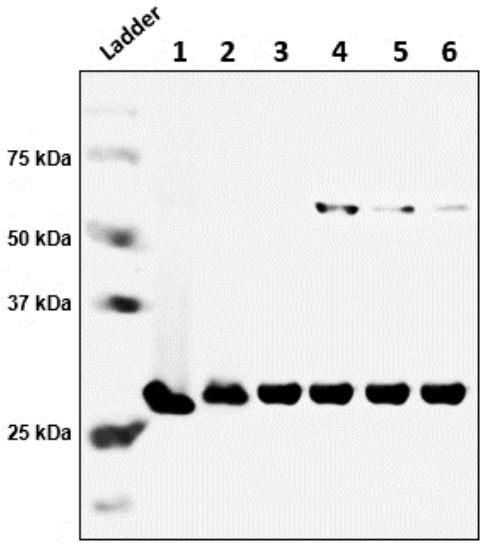


Figure 3.13. BS3 cross-linking experiment to determine the oligomeric form of recombinant scFv antibodies. **Lane 1**: Dec205 scFv (-BS3) **Lane 2**: CD83 scFv (-BS3) **Lane 3**: CD11c scFv (-BS3) **Lane 4**: Dec205 scFv (+BS3) **Lane 5**: CD83 scFv (+BS3) **Lane 6**: CD11c scFv (+BS3). About 20 µg of scFv antibodies were mixed with BS3 to a final concentration of 20 mM and incubated for 1 hour at room temperature. The cross-linking reaction was stopped by the addition of 1 M Tris-HCl pH 8.0 to a final concentration of 50 mM and incubated for 15 minutes at room temperature. After cross-linking, proteins were separated on a 12% SDS-PAGE under reducing conditions, blotted, and analysed by western blot using mouse anti-V5 antibody (1 mg/mL diluted to 1:1000).

3.2.3.2. The rH9HA fused to scFv antibodies retains haemagglutination activity

The function of HA protein can be determined by haemagglutination assay (HA assay). Here, HA assay was used to investigate the biological activity of rH9HA and rH9HA-scFv proteins (**Figure 3.14**). The rH9HA protein, both on its own and when fused to scFv antibodies was able to agglutinate chicken RBCs. This demonstrates that the modification of H9HA protein does not affect its ability to bind sialic acid receptors on chicken RBCs. Furthermore, on average the minimum concentration of rH9HA and rH9HA-scFv proteins required to agglutinate 50 µl of chicken RBCs was 0.14 µg.

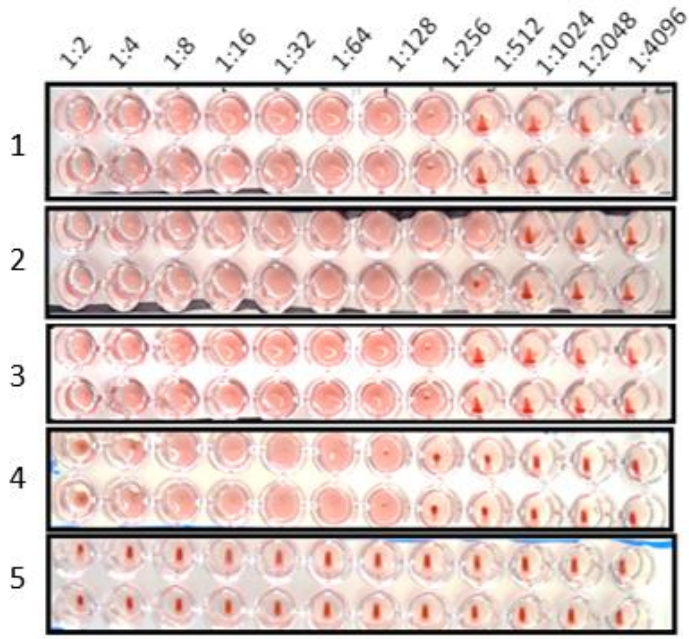


Figure 3.14. Haemagglutination assay to test the activity of rH9HA and rH9HA-scFv proteins. **1)** rH9HA **2) r**H9HA-Dec205 scFv **3)** rH9HA-CD83 scFv **4)** rH9HA-CD11c scFv **5)** Negative control (PBS). For haemagglutination assay, a 2-fold serial dilution of 35 µg of the recombinant HA proteins were carried out in 96-well plates. 50 µL of 1% chicken RBCs were added. The plates were incubated at 4°C for one hour and the highest dilution of the protein causing the agglutination of the RBCs was noted.

3.2.3.3. The fusion of scFv antibodies to rH9HA protein does not affect their function

The molecular weight of rH9HA monomer is about 70 kDa. The fusion of a large molecule like rH9HA to scFv antibody could jeopardise the function of the antibody fragment. To assess the binding activity of rH9HA-scFv to their respective cognate receptor antigens, ELISA and western blot assays were performed. The coding sequences of chicken Dec205 CTLD 4-5-6 and CD83 ED used to generate Dec205 mAb and CD83 mAb, respectively were cloned into pS2V1-Ctag vector for expression in *Drosophila melanogaster* S2 cells[324]. These recombinant receptor proteins were coated onto the ELISA plates for assessing the ability of rH9HA-scFv antibodies to recognise and bind to their respective receptor proteins. Dec205 mAb

and CD83 mAb were also included in the ELISA assay as a positive control. Furthermore, rH9HA-CD11c scFv was used as a negative control to assess any non-specific binding to chicken Dec205 CTLD 4-5-6 and CD83 ED. No non-specific binding by rH9HA-CD11c scFv was observed indicating that any positive signal is purely down to Dec205 scFv or CD83 scFv binding. Moreover, both Dec205 scFv and rH9HA-Dec205 scFv were able to detect and bind to Dec205 CTLD 4-5-6 (**Figure 3.15 A**). Similarly, CD83 scFv and rH9HA-CD83 scFv were also able to detect and bind to CD83 ED (**Figure 3.15 B**). No significant differences were observed in the binding between Dec205 scFv and rH9HA-Dec205 scFv. However, the binding to Dec205 CTLD 4-5-6 by rH9HA-Dec205 scFv was lower than Dec205 scFv at lower concentration (<0.125 µg) of Dec205 CTLD 4-5-6. Furthermore, a slightly lower binding to chicken CD83 ED by rH9HA-CD83 scFv antibodies was observed compared to CD83 scFv antibodies, although this was not statistically significant.

In addition, western blot analysis was also carried out to verify the results obtained from ELISA assay. Dec205 mAb, Dec205 scFv and rH9HA-Dec205 scFv showed a single band at approximately 53 kDa, which is the expected molecular weight of chicken Dec205 CTLD 4-5-6 (**Figure 3.16 A**). On the other hand, two bands were detected (15 kDa-20 kDa) for chicken CD83 ED probed with CD83 mAb, CD83 scFv and rH9HA-CD83 scFv (**Figure 3.16 B**). However, the PNGase F digestion of N-linked carbohydrates resulted in a single band around 15 kDa (**Figure 3.16C**). This band corresponds to the CD83 polypeptide lacking N-linked glycosylation.

The immunogen used for the generation of hybridoma for chicken CD11c (clone: IAH 8F2) could not be identified (unpublished data). However, it has been

reported that this chicken CD11c mAb (clone: IAH 8F2) stains chicken DCs and have been used by several groups to enrich and phenotype these cells[337]. Furthermore, CD11c mAb (clone: IAH 8F2) has been applied to stain chicken bursal secretory dendritic cells[306], distinguish isolated splenic DC-like cells from KUL01+ macrophages[463] and also for the phenotypic analysis of chicken bone marrow derived DCs[331]. The CD11c mAb and CD11c scFv were also able to stain chicken splenocytes (**Figure 3.17**). When chicken splenocyte extract was probed with CD11c mAb, CD11c scFv and rH9HA-CD11c scFv in western blot analysis, three bands with approximate molecular weights of 35 kDa, 50 kDa and 150 kDa were detected (**Figure 3.18**). The band corresponding to 150 kDa represents expected molecular weight of CD11c receptor protein[553]. The identity of the bands corresponding to 35 kDa and 50 kDa is unknown. It has been reported that CD11c mAb (clone: IAH 8F2) reacts with a still uncharacterised cell surface heterodimer protein on monocytes/macrophages and chicken NK cells[337].

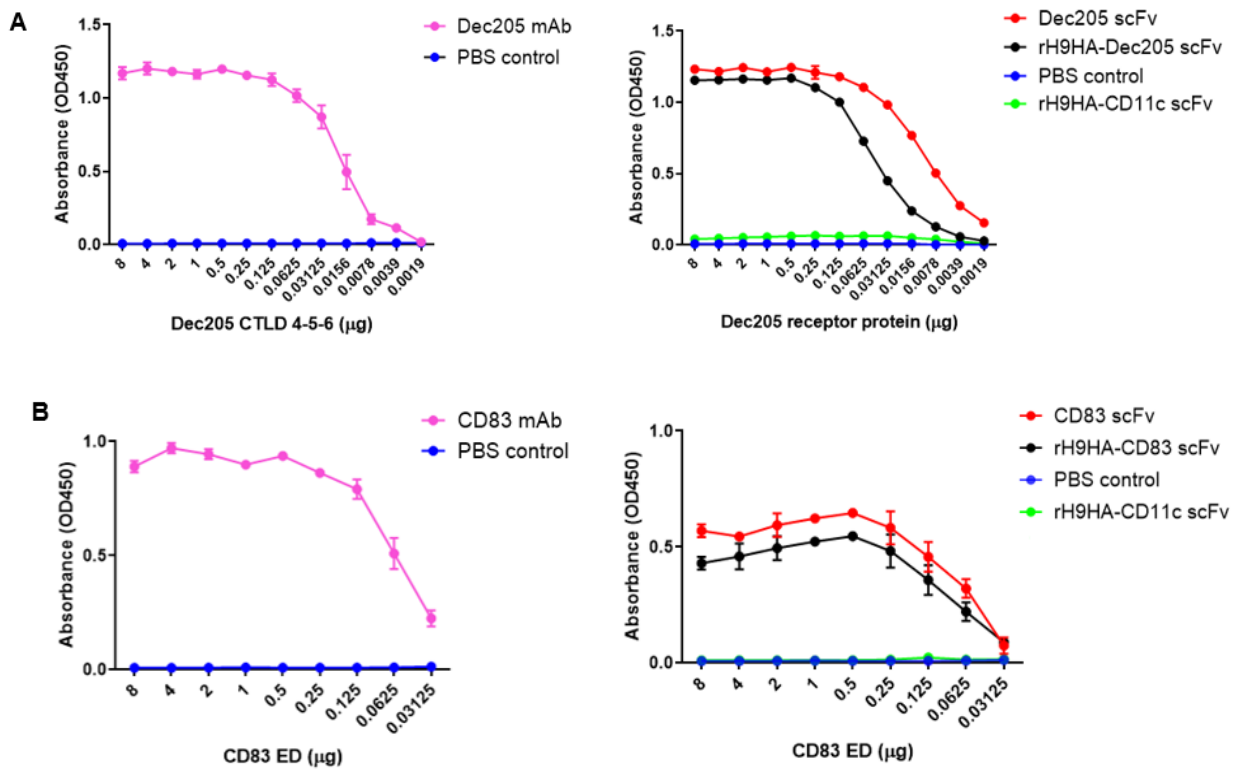


Figure 3.15: Characterisation of mAb, scFv and rH9HA-scFv using ELISA assay. (**A**) ELISA analysis for testing the ability of Dec205 mAb, Dec205 scFv and rH9HA-Dec205 scFv to bind to chicken Dec205 CTLD 4-5-6. (**B**) ELISA analysis for testing the ability of CD83 mAb, CD83 scFv and rH9HA-CD83 scFv to bind to chicken CD83 ED. About 8 µg of purified chicken Dec205 CTLD 4-5-6 and CD83 ED was coated onto each well of the ELISA plate, a 2-fold serial dilution was carried out and the plate was incubated overnight for 4ºC. For detection, the plates were incubated with 1 µg/mL of Dec205/CD83 mAb or equimolar concentration of Dec205/CD83 scFv and rH9HA-Dec205/CD83 scFv. This was followed by incubation with HRP conjugated goat anti-mouse secondary antibody for mAb (1 mg/mL diluted to 1:1000) and HRP conjugated anti-V5 secondary antibody for scFv and rH9HA-scFv (1 mg/mL diluted to 1:1000). The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. Data are represented as mean±SD from three independent experiments.

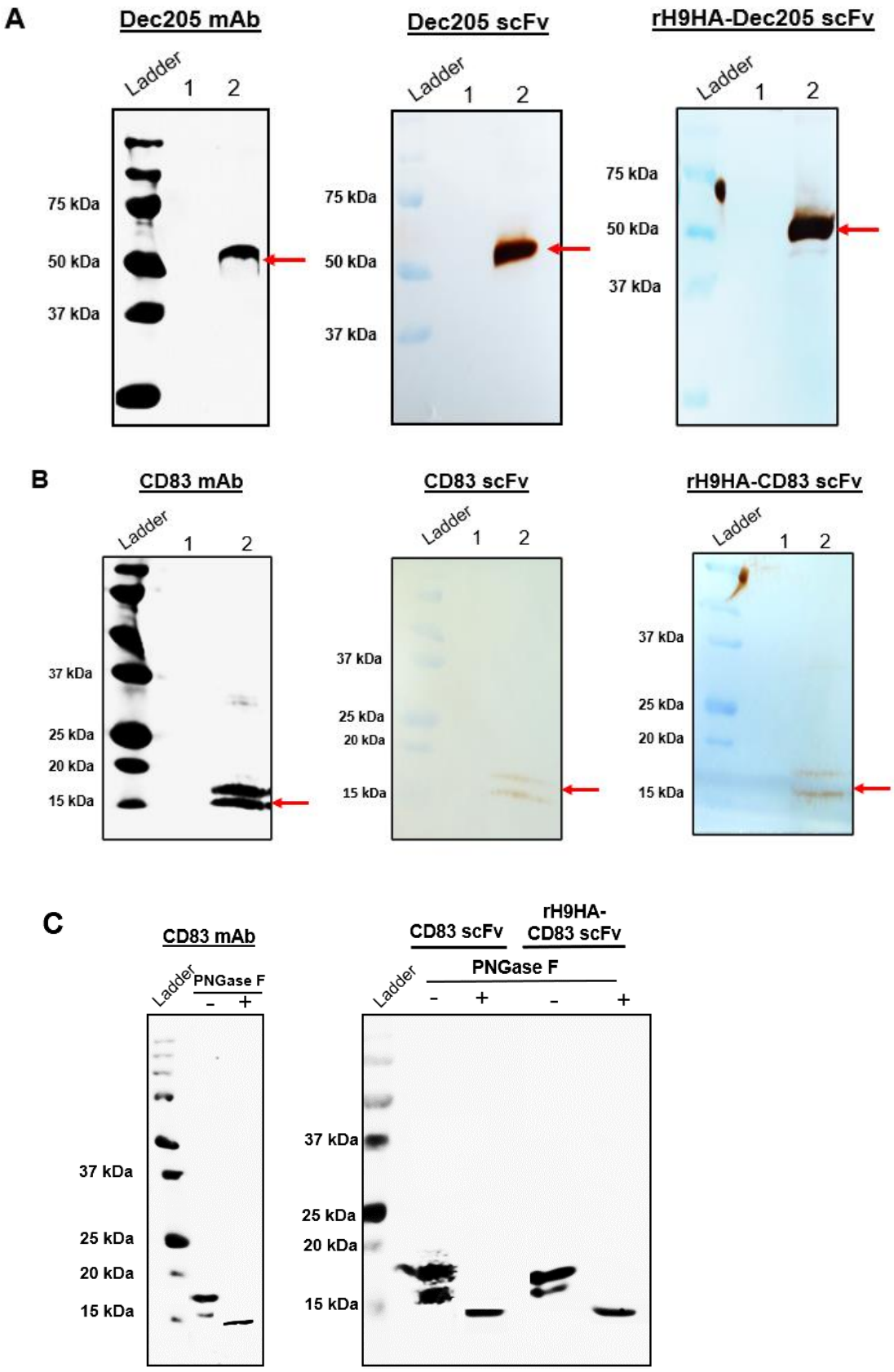


Figure 3.16. Characterisation of mAb, scFv and rH9HA-scFv using western blot assay. (**A**) Western blot analysis for testing the ability of Dec205 mAb, Dec205 scFv and rH9HA-

Dec205 scFv to detect chicken Dec205 CTLD 4-5-6. In all cases, **Lane 1** represents untransfected control cell supernatant whereas **Lane 2** represents chicken Dec205 CTLD 4-5-6 (red arrow). (**B**) Western blot analysis for testing the ability of CD83 mAb, CD83 scFv and rH9HA-CD83 scFv to detect chicken CD83 ED (red arrow). In all cases, **Lane 1** represents untransfected control cell supernatant whereas **Lane 2** represents chicken CD83 ED. The chicken Dec205 CTLD 4-5-6 and CD83 ED was run on 10% SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with either 1 µg/mL of Dec205/CD83 mAb or equimolar concentration of Dec205/CD83 scFv and rH9HA-Dec205/CD83 scFv overnight at 4°C. This was followed by the incubation with either IRDye® 800CW goat anti-mouse secondary antibody (1 mg/mL diluted to 1:10000, if mAb was used as primary antibody) or anti-V5 HRP secondary antibody (1 mg/mL diluted to 1:1000, if scFv or rH9HA-scFv antibodies were used as primary antibodies) for 1 hour at room temperature. For the latter, DAB substrate was added and development of brown colour product on the membrane was observed. **(C)** PNGase F digestion of CD83 ED. The chicken CD83 ED was treated with PNGase F for 6 hours at 37°C. The PNGase F treated CD83 ED was run on 10% SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with either 1 µg/mL of CD83 mAb or equimolar concentration of CD83 scFv and rH9HA-CD83 scFv overnight at 4°C. This was followed by the incubation with either IRDye® 800CW goat anti-mouse secondary antibody (1 mg/mL diluted to 1:10000, if mAb was used as primary antibody) or anti-V5 HRP secondary antibody (1 mg/mL diluted to 1:1000, if scFv or rH9HA-scFv antibodies were used as primary antibodies) for 1 hour at room temperature. For the latter, DAB substrate was added and development of brown colour product on the membrane was observed. The image was taken using Odyssey CLX (LI-COR biosciences)

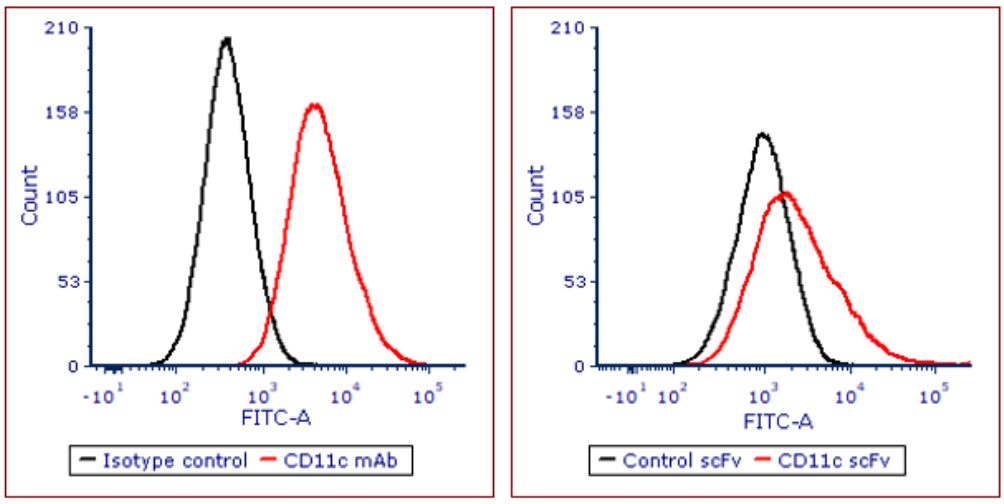


Figure 3.17. Staining of chicken splenocytes by CD11c mAb and CD11c scFv using flow cytometry. About 1x10^5 chicken splenocytes were stimulated with 200 ng/mL of LPS for 24 hours. The splenocytes were centrifuged and resuspended in FACS buffer containing 1 µg/mL CD11c mAb/IgG1 isotype control for 20 minutes at 4°C or 5 µg/mL of CD11c scFv/ID2 control scFv for 45 minutes at 4ºC. The splenocytes were washed with 150 µL of FACS buffer and resuspended in FACS buffer containing secondary antibodies (FITC conjugated goat anti-mouse secondary antibody (0.5 mg/mL diluted to 1:200) if mAb was used or FITC conjugated anti-V5 tag (0.1 mg/mL diluted to 1:200 dilution) if scFv was used)

along with LIVE/DEAD™ fixable blue dead cell stain, and incubated in the dark for 30 minutes at 4ºC. This was followed by fixing of labelled splenocytes with 50 µL of 1% PFA for 20 minutes in the dark. The plates were read the next day using MACSQuant flow cytometer and analysed with FCS Express 6 software. About 10,000 events were acquired for the analysis. Cells were first gated based on forward scatter area (FSC-A) and side scatter area (SSC-A), followed by exclusion of doublets on SSC-A vs SSC-height density plot. Next, live cells were gated for the detection of $CD11c^{+}$ cells.

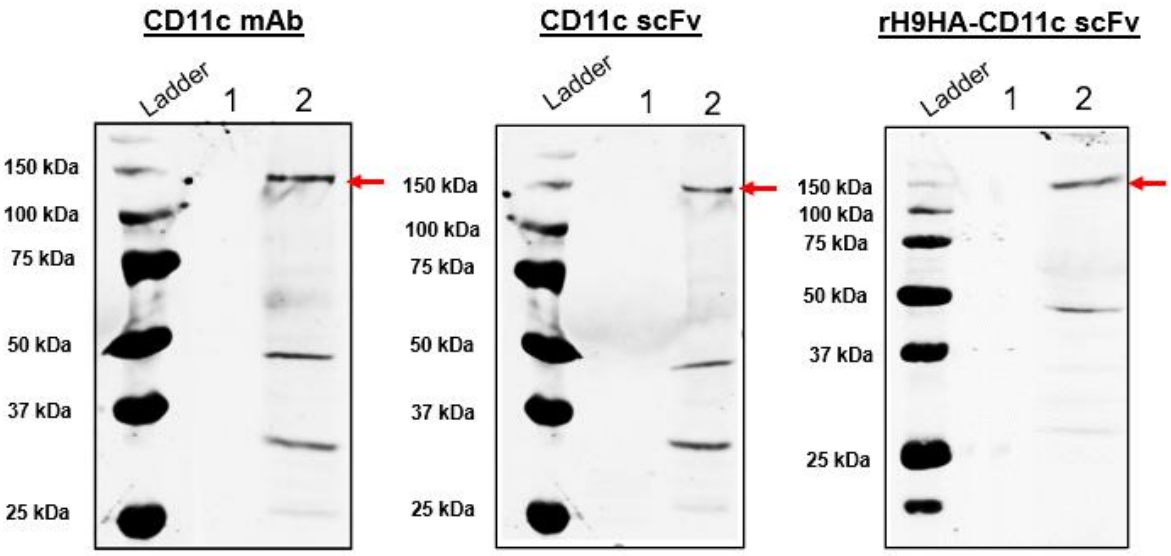


Figure 3.18. Detection of CD11c receptor protein in chicken splenocyte extract by CD11c mAb, CD11c scFv and rH9HA-CD11c scFv. In all cases, **Lane 1** represents *Drosophila melanogaster* S2 cell extract (control) and **Lane 2** represents chicken splenocyte extract. The chicken splenocyte extract was run on 8-10% SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with either 1 µg/mL of CD11c mAb or equimolar concentrations of CD11c scFv and rH9HA-CD11c scFv overnight at 4°C. This was followed by incubation with either IRDye® 800CW goat anti-mouse secondary antibody (1 mg/mL diluted to 1:10000, if mAb was used as primary antibody) or anti-V5 HRP secondary antibody (1 mg/mL diluted to 1:1000, if scFv or rH9HA-scFv antibodies were used as primary antibodies) for 1 hour at room temperature. For the latter, DAB substrate was added and development of brown colour product on the membrane was observed. The image was taken using Odyssey CLX (LI-COR biosciences). The red arrow indicates CD11c receptor protein.

3.3. Discussion

Most of the commercially available avian influenza vaccines for poultry are inactivated whole virus vaccines that are largely produced in the embryonated

hen's eggs. This egg-based technology is unable to respond quickly to pandemic crisis since, a minimum of 3-6 months is required for the vaccine development and production process using this technology[554]. Furthermore, some HPAI viruses are lethal to chicken eggs hence, they require additional genetic modifications to grow effectively in eggs. This further prolongs the production process[512]. In addition, inactivated whole virus vaccines have several drawbacks including sub-optimal immunogenicity, need for multiple dose administration and interference from MDAs[505]. Thus, innovative ideas for the rapid production of highly effective AIV vaccines are needed to prevent the impact of these viruses in poultry and reduce zoonotic threat. In this context, the recombinant protein-based technology has shown a great promise to replace the egg-based technique[555]. The use of recombinant protein as vaccine antigen has several advantages, including no need to handle potentially dangerous viruses, no need for eggs, easy upscaling of production and rapid production of new vaccines[512]. The HA is the major host protective antigen and a good candidate for making recombinant subunit vaccines against influenza virus[556]. The antibodies directed towards HA protein can prevent attachment and fusion of viral and host cell membranes thus, conferring protection against viral infection[557,558]. Over the last two decades, H9N2 AIVs have become globally widespread in poultry representing a genuine threat not just to the global poultry industry, but also to humans through their higher rates of zoonotic infection and pandemic potential[138]. Therefore, H9HA from H9N2 MScon virus (UDL 01/08) was used as a model antigen for generating recombinant subunit AIV vaccines.

Various modifications were done to the recombinant H9HA protein for better expression, secretion and purification, as well as enhancement of the immunogenicity: i) removal of native HA protein signal sequence and replacement

with *Drosophila melanogaster* immunoglobulin heavy chain binding protein (BIP) secretion signal ii) removal of TM and cytoplasmic tail domains and replacement with trimerisation foldon domain of fibritin protein from T4 bacteriophage iii) conjugation of scFv antibodies specific for receptors on chicken APCs at C-terminus iv) His-tag to facilitate rapid purification. This modified recombinant H9HA protein was referred to as TADV. Such TADV can selectively deliver antigens to APCs, thereby enhancing the immunogenicity and protective efficacy of the vaccine[559]. Three TADV constructs were generated, each targeting one of chicken Dec205, CD83 and CD11c receptors: rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv. The scFv antibodies were fused to rH9HA protein by genetic engineering instead of using chemical conjugation process. Different crosslinking reagents like thiolating agent (Traut's reagent) and Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate have been used before to create antigen-antibody constructs[381,396]. However, chemical conjugation may not be always feasible since, amino acid modifications can reduce or even eliminate biological activity of a protein[560]. On the other hand, genetically engineered antigen coupled antibodies are much preferred as it is possible to genetically modify targeting antibodies (e.g., solubility, glycosylation sites) according to the need. Furthermore, recombinant protein engineering techniques are more reproducible and scalable in terms of production[561].

The full-length HA is included in all the currently approved human and poultry recombinant subunit influenza vaccines[276,277,562]. However, the full-length HA protein containing TM domain needs to be extracted from cell membranes using several detergents, complicating the purification process, as well as reducing the protein yield[563]. Thus, different genetic manipulation of the HA gene are performed

to incorporate various truncations and/or modifications, to improve the stability and increase the production yield of recombinant HA proteins[518]. The modification involving removal of TM domain and addition of trimerisation domain is of particular relevance to our work. This modification allows the expression of soluble, secretable form of recombinant HA protein therefore, greatly facilitating the purification process and increasing the protein yield[511]. The TM domain of HA contributes to HA protein trimer stability and oligomerisation. It has been reported that removal of TM domain destabilises the trimeric structure of HA protein[564,565]. Studies have shown that recombinant HA protein expressed without TM domain are monomeric and leads to destabilisation of important neutralising epitopes[494,516]. In addition, the stability of protein is reduced and degradation by host cell proteases occurs[511]. This highlights the importance of trimerisation domains like foldon of fibritin from T4 bacteriophage[516,518,549] and isoleucine zipper of GCN4 transcriptional activator[494,515,566] from *Saccharomyces cerevisiae* in the generation of stable soluble HA proteins. These trimerisation domains are important because they preserve the trimeric structure of soluble HA protein, stabilising the conformational epitopes[511].

All the recombinant proteins generated here (scFv, rH9HA and rH9HA-Dec205/CD83/CD11c scFv) were expressed in *Drosophila* melanogaster S2 cells. The *Drosophila* expression system (DES) has been previously used for the production of many human vaccines in clinical trials[567]. The use of *Drosophila* S2 cells for protein expression has several advantages. They can reach very high cell densities; at least ten-fold higher than any other insect or mammalian cell lines (up to 70 million cells/mL) without aggregation and toxic metabolite formation[567]. This provides high volumetric productivity facilitating ease in up-scaling. Furthermore,

protein production time can be reduced to weeks from months. Unlike baculovirus expression vector system (BEVS), the DES system undergoes non-lytic expression whereby an expression vector is stably integrated into the chromosome of S2 cells and the recombinant protein is produced without subsequent cell lysis[567]. This non-lytic expression is advantageous as lytic infection can induce stress to the infected cells, leading to the saturation of the endoplasmic reticulum quality control and production of partially folded proteins[525]. The average yield of recombinant scFv, rH9HA and rH9HA-Dec205/CD83/CD11c scFv proteins ranged from 10-20 mg/L of the culture supernatant. However, in some instances it was higher than 30 mg/L. Similar expression yield of scFv antibodies (5-12 mg/L) using DES system has been reported before[525]. Moreover, the expected yield of recombinant HA protein using BEVS system in *Trichioplusia ni* insect cells (commonly known as High Five) is in the range of 0.2-30 mg/L of culture supernatant[511], which is also similar to the yield we observed with DES system. The SDS-PAGE and western blot analysis of purified rH9HA and rH9HA-Dec205/CD83/CD11c scFv proteins detected bands around 70 kDa and 100 kDa, which are slightly higher than the expected sizes of 66 kDa and 94 kDa, respectively. This could be due to the glycosylation effect which is one of the important post-translational modification. Indeed, S2 cells have been shown to produce paucimannose N-glycans (three terminal mannose) which is different from that of mammalian cells like Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK), which produce complex terminally galactosidated and/or sialylated N glycans[568–570]. Such differences in glycosylation pattern have shown to affect the molecular sizes of recombinant soluble HA proteins. The soluble HA proteins produced in *Drosophila* S2 cells were found to be slightly smaller in size compared to the ones produced in HEK293 cells, and the

glycosylation states in the latter also affected the receptor-binding specificity[515]. Moreover, rH9HA protein was expressed as a precursor HA (HA0, ~70 kDa) which is similar to the previous observations[494,550]. Upon treatment of the recombinant rH9HA protein with exogenous trypsin, two bands were observed each corresponding to the molecular weights of HA1 and HA2 subunits. This indicates that the HA0 precursor is not processed into HA1 and HA2 subunits under the S2 cell culture conditions. Similar observations were made when full-length H5HA[396] and H3HA[517] proteins were expressed using BEVS in *Spodoptera frugiperda* (Sf9) insect cells. Moreover, the native trimeric structure of HA is required for bioactivity and to get higher immune responses. Here, soluble H9HA protein was fused with T4 trimerisation foldon domain for the purpose of expressing stable trimeric H9HA soluble protein. A water soluble crosslinker, BS3 was employed which links monomers within an oligomeric structure to characterise the oligomerisation state of rH9HA and rH9HA-Dec205/ CD83/ CD11c scFv proteins[551,571]. Crosslinking provides the advantage of being able to determine the approximate ratio of oligomers without the need to use complex size exclusion chromatography techniques[494]. The BS3 crosslinking reaction indicated that the native structure of recombinant H9HA protein with foldon is a trimer, and three molecules of scFv antibodies are attached to one trimeric H9HA protein to form rH9HA-scFv constructs. Furthermore, recombinant scFv antibodies were mainly produced as monomers in the DES system however, some dimers were also present. This was in line with the observation made elsewhere[525]. It was also demonstrated that the trimeric rH9HA and rH9HA-Dec205/CD83/CD11c scFv proteins were biologically active through haemagglutination assay. Similar finding was observed when GCN4pII trimerisation repeats[494] and T4 trimerisation foldon[550] were used to

stabilise the trimeric structure of recombinant soluble HA protein. It has been reported that recombinant soluble HA proteins produced without trimerisation domains are not able to agglutinate RBCs due to the lack of stable trimeric structure[494,518]. This greatly affects the immunogenicity. One study reported that GCN4pII modified soluble H3HA (trimeric form) was more immunogenic than unmodified soluble H3HA (monomeric form) and increased the survival in mice following lethal challenge with virus. It was suggested that this enhanced immunogenicity of modified H3HA was due to the presentation of native trimeric epitopes in GCN4pII stabilised protein[494]. Similarly, modification of soluble H5HA by T4 trimerisation foldon also generated soluble trimeric HA in the baculovirus system which elicited stronger neutralising antibodies compared to the unmodified monomeric form[516]. Moreover, the fusion of scFv antibody to rH9HA did not affect the binding ability of scFv antibody. This was determined using western blot and ELISA assays, which use denatured linear form and native conformational form of the detection protein, respectively. Immunogenic epitopes on denatured polypeptides can be different from those on native protein. Some antibodies are specific to only denatured linear form or native conformational form of the antigen, while others can bind to both denatured linear and native conformational form depending on the nature of antigenic epitope structure, quality and composition of antigen administered to raise the antibodies[572–574]. The Dec205/CD83 scFv and rH9HA-Dec205/CD83 scFv were able to recognise and bind to their respective receptor proteins in both western blot and ELISA assays, indicating their ability to detect both the denatured linear form and native conformational form of the receptor proteins. Furthermore, CD11c scFv and rH9HA-CD11c scFv were able to detect a band corresponding to CD11c receptor molecule in chicken splenocytes

extract in western blot analysis. However, two smaller size bands were also detected whose identity is unknown.

The protein expression system is one of the important factors that can potentially influence the structure and antigenic properties of recombinant HA proteins. Various studies with bacterially expressed HA proteins based on the H5N1/H7N9 AIV and one clinical trial with a bacterially expressed fusion protein between HA fragment and flagellin from *Salmonella typhimurium* type 2 (STF2), a TLR5 agonist were reported[521,566,567,575]. Bacterially expressed HA proteins are not subjected to post-translational modifications like disulphide bond formation and glycosylation, which are important for proper folding of the HA protein[19]. Thus, they may not present native conformational epitopes crucial for the generation of an effective protective immune response. However, several studies have demonstrated that bacterially expressed HA can fold properly using efficient refolding procedures and elicit neutralising antibodies[426,485-580]. The major challenge of the recombinant technology is to ensure that recombinant HA protein resembles the native HA protein and can produce strong antibody responses targeting protective conformational epitopes on HA. In order to address this, several eukaryotic expression systems like baculovirus based Sf9 insect cells[396], HEK293 cells[550] and plant-based systems[519] have been researched. Out of all these systems, baculovirus based Sf9 insect cell has become one of the most widely used systems for the production of recombinant HA proteins[581,582]. The high-level expression of functional soluble recombinant HA protein in the DES system reported in this work suggests that this could be an alternative expression system for producing recombinant HA proteins, without the need for an extra baculovirus infection step.

In conclusion, rH9HA and Dec205/CD83/CD11c scFv fused rH9HA proteins were successfully expressed in a native trimeric form in *Drosophila melanogaster* S2 cells. Furthermore, the fusion of rH9HA with scFv antibodies did not abrogate the functional activity of both rH9HA as well as the scFv antibodies.

Chapter 4: Evaluation of the immunogenicity, protective efficacy, and mechanism of targeted antigen delivery vaccines in chickens

Data from this chapter have been published in research articles: ‘‘Targeting haemagglutinin antigen of avian influenza virus to chicken immune cell receptors Dec205 and CD11c induces differential immune-potentiating responses” by Shrestha et al. Vaccines, 9, 784 (2021) and “Selectively targeting haemagglutinin antigen to chicken CD83 receptor induces faster and stronger immunity against avian influenza” by Shrestha et al. npj Vaccines, 6, 1-12 (2021).

Chapter summary

In this chapter, the immunogenicity of rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines generated in Chapter 3 was assessed. Immunisations of chickens with rH9HA-scFv vaccines induced faster (as early as day 6 post primary vaccination) and stronger antibody responses compared to the untargeted counterpart or the inactivated H9N2 vaccine, as measured by HI and ELISA assays. Out of the three targeted antigens tested, rH9HA-CD83 scFv induced higher virus neutralising antibodies compared to the untargeted rH9HA and inactivated H9N2 vaccine. Furthermore, chickens vaccinated with rH9HA-CD83 scFv also showed reduced clinical disease signs and reduced shedding of the virus from buccal cavities when challenged with H9N2 virus compared to the untargeted rH9HA vaccine, as measured by plaque assay. Following on, the mechanism of the enhancement of TADV performance was

determined by examining their potential for inducing cytokines and chemokine. The rH9HA-CD83 scFv and rH9HA-CD11c scFv were able to induce higher mRNA levels of IFN-γ, IL-6, IL-1β and CXCLi2 upon *in vitro* stimulation of chicken splenocytes compared to rH9HA.

Chapter aims

- To investigate the antibody responses elicited by rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines in chickens.
- To investigate the protective efficacy of rH9HA and rH9HA-CD83 scFv vaccines in chickens.
- To investigate the ability of rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv in activating chicken splenocytes for producing different cytokines.

4.1. Introduction

The professional APCs are important for induction of potent antigen-specific immune responses. Among APCs, DCs are the key regulatory factors of humoral and cellular immunity. They possess specialised features including uptake receptors and downstream endocytic system for capturing, processing, and presenting antigens. They can efficiently process both foreign and self-antigens, inducing different immune responses thus, regulating the development of immunity or tolerance, respectively[357]. Their role in initiating and regulating immunity as well as generating distinct T cell responses make them valuable in enhancing the efficacy of the vaccines. Therefore, targeting DCs to improve the immunogenicity

of vaccines has been an important strategy in the field of vaccine development[583]. In recent years, DC targeted vaccines have been the most extensively studied approach to enhance both humoral and cellular immune responses following vaccination[584]. There are several factors that determine the efficacy of targeting DCs *in vivo* for vaccination purposes. One such factor is the choice of targeted receptor. The DC surface molecules like Dec205, Clec9A and DC-SIGN have been mainly focussed in mammals because they are restricted to DCs and are able to efficiently internalise and deliver antigens to endocytic compartments for antigen processing and presentation[585]. Other receptors like CD11c, which is not restricted to DCs but is also expressed on activated T cells, macrophages and NK cells has also been a popular choice for TADV[381,407,408,586]. In such case, antigen is delivered not just to DCs but also to other cell types. Studies have shown that efficient immune responses are still generated when DCs as well as other cell types receive the targeted antigens. Thus, although they are not specific to DCs, targeting CD11c[381,408,586], mannose receptor[409,587] and CD40[413,414,543] have all produced immune responses higher than the untargeted counterparts. Individual APC receptors differ in their expression levels, internalisation patterns and speed, and downstream intracellular trafficking pathways. These factors could also affect the efficiency of TADV[585]. It was proposed that the amount of internalised antigen correlates with antigen presentation levels hence, highly internalised receptors could be a good target for antigen targeting. However, this concept was dismissed by several studies. Chatterjee et al. measured the uptake of antibodies specific for Dec205, mannose receptor and CD40 by human and mouse DCs. Antibodies to mannose receptor and Dec205 were more efficiently accumulated and internalised compared to anti-CD40. However, CD40 was most efficient at cross-

presentation[588]. Similarly, a fluorescent DNA-based-coupled probe was used to quantitatively measure the amount of antigens delivered into the intracellular compartment via the targeted molecules. It was observed that matured mouse DCs internalised similar antigen load via Dec205, CD11c and CD40 receptors however, Dec205 was superior for MHC I and MHC II antigen presentation[587]. With regards to the speed of antigen internalisation, it was thought that targeting a receptor that is slowly internalised may act as an antigen depot, preserving important epitopes from enzymatic degradation and prolonging antigen presentation. However, it was observed that Dec205 undergoes rapid internalisation in mouse DCs whereas CD11c is endocytosed slowly. Yet, targeting Dec205 was shown to be better than CD11c at eliciting $CD8^+$ T cell responses[587]. Furthermore, antigen presentation can also be affected by the intracellular trafficking route of antigen once inside the cell. In this context, it was proposed that early endosome trafficking receptor targeted antigen can maintain reduced proteolysis thus, it can be efficiently processed for MHC I cross-presentation. In contrast, antigens that are trafficked to late endosomes are actively degraded by lysosomal proteases favouring MHC II presentation[585]. CD11c is known to traffic to early endosome in human DCs thus, it can efficiently elicit antigen cross-presentation[588,589]. However, antigen delivered to early endosome via CD40 was more efficiently presented on MHC II molecules than antigens delivered to lysosomes via Dec205[588,589]. Furthermore, antigen internalised via Dec205 normally traffics to late endosome, yet it can still undergo efficient cross-presentation[589]. In matured human monocyte derived DCs, CD83 was shown to be localised in lysosomal compartments rich in MHC II molecules[478], indicating that antigen could be delivered to lysosome via CD83 which favours MHC II presentation. However, as mentioned before no clear correlation was observed

between the intracellular trafficking route of targeted receptor and effectiveness of antigen presentation. Therefore, the intracellular trafficking of antigen delivered to DCs via specific receptors, including antigen load, speed of receptor internalisation or the destination of internalised receptor in the endocytic pathways are not reliable criteria to predict the antigen presentation outcomes in the context of APC targeting. Most of the studies mentioned above were performed in mice. Since, differences in mammalian and avian immune system exist, some of these factors could affect the fate of targeted antigens differently in chickens.

It is well known that the activation of adaptive immune system normally requires not only the presentation of foreign antigens, but also 'danger signals' from microbial products or damaged cells[590]. Hence, most vaccines using purified antigens need additional adjuvants to mimic these signals and activate DCs[366]. Immature DCs specialise on the uptake of antigen. Due to the low levels of co-stimulatory molecules and sub-optimal levels of MHC I/MHC II molecules, immature DCs are poor at antigen presentation[590]. Upon activation, DCs shut down their antigen uptake system and upregulate the expression of antigen presenting machineries like MHC and co-stimulatory molecules[591]. In the context of TADV, it is considered that the DCs need to be activated by co-administration of adjuvants such as TLR ligands for an effective immune response[590]. In fact, targeting antigen to Dec205 on steady-state DCs in the absence of DC-activating factors has been associated with T cell *tolerance* in mice[439] (**Figure 4.1**). Although, initial proliferation of antigen-specific T cells occurs, but these cells do not differentiate into effector T cells and are ultimately deleted or rendered unresponsive[432,592]. Furthermore, it has been demonstrated that Dec205 targeting without DC activating stimuli results in the activation of $CD25^+$ regulatory T cells (Tregs) suggesting the induction of T cell

tolerance[593]. However, co-administration of anti-Dec205-antigen complexes with Poly I:C resulted in a strong antibody and CTL responses[446]. The same was true when targeting another C-type lectin receptor, Clec12A[594]. However, many studies targeting surface receptors like MHC I[595], MHC II[596], Clec9A[594], mannose receptor[409] and CD45[597] in mice showed that DC activation stimuli was not required to induce strong antibody responses. Although, most studies targeting Dec205 reported poor antibody response in the absence of DC activating adjuvants, one study observed a potent humoral response following adjuvant free immunisation in mice[586]. This further led to hypothesise that targeting outcomes could also be impacted by inherent properties of the targeting antibodies themselves. Different mAbs have different ability to initiate a signalling response within the DCs[590,598]. It was reported that mAb recognising the neck region of C-type lectin receptor DC-SIGN showed different intracellular routing compared to mAb recognising the lectin domain of the same molecule. This greatly influenced the immunological outcome of the targeted antigen in mice[598]. Similarly, one anti-Clec9A mAb (IgG2a) induced T follicular helper cells (Tfh) and strong humoral responses in the absence of adjuvant[594,599]. On the other hand, a different anti-Clec9A mAb (IgG1) induced T cell tolerance in the absence of adjuvant in mice[600], indicating that the isotype of mAb used for targeting the antigen may also affect the immunological outcome. Furthermore, the Fc domain of mAb can make a crosslink with specific Fc receptor on APCs leading to the activation of effector functions including phagocytosis, ADCC, cytokine production, and modulation of T and B cells[601,602]. Therefore, differences in immune responses could be observed when using antibody fragments like scFv and Fab for antigen targeting compared to the full-length mAb.

However, some studies reported that Fc portion of the targeting mAb plays no role in the enhancement of immune responses during APC targeting[599,603].

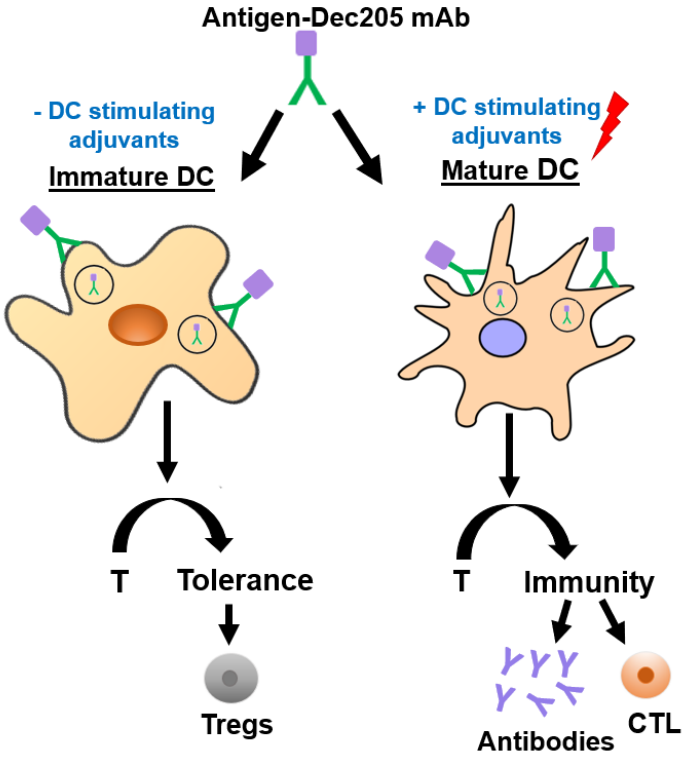


Figure 4.1. Immune responses elicited by antigen targeting to Dec205. Adjuvant free immunisation with antigen-Dec205 mAb leads to regulatory T cells (Tregs) dependent tolerance. On the other hand, co-administration of antigen-Dec205 mAb with adjuvant primes cytotoxic T lymphocyte (CTL) response and activates $CD4^+$ T cell response leading to the generation of T helper (Th) cells that support the humoral response.

Another potential factor affecting the outcome of antigen targeting could be the route of vaccine administration. The choice of injection site could result in different distribution patterns of targeting mAb influencing the access to different subsets of DCs, ultimately affecting the antibody responses[604]. However, antigen targeting directed towards CD11c receptor in mice assessed the effects of intravenous, intraperitoneal, and subcutaneous administration and noted no significant differences in the antibody levels[586]. Overall, there are several factors that can affect the immunological outcomes of targeted antigens however, these

factors seem to be situation dependent and thus, impact targeted antigens differently.

In Chapter 3, functional rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv proteins were successfully generated in *Drosophila melanogaster* S2 cells. The target receptors were chosen based on a combination of different factors e.g., expression pattern, endocytic function, targeting effect reported by others and the availability of suitable targeting antibody. Here, we investigated the immune responses elicited by the respective proteins constituted as recombinant subunit vaccines in chickens, via the subcutaneous administration route.

4.2. Results

4.2.1. Investigation of the antibody responses elicited by rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines in chickens

To evaluate the antibody response generated by TADV, groups of 7-day-old Dekalb White chickens (Medical grade, n=8 per group, Henry Stewart & Co. Ltd) were immunised with vaccine doses containing 2.8 µg, 28 µg and 49 µg of rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins, equivalent to 2 µg, 20 µg and 35 µg of rH9HA protein (equimolar concentration) as described in **section 2.2.8.3.1**. Additionally, one group of chickens was vaccinated with inactivated H9N2 vaccine (UDL 01/08, 1024 HAU/dose). All the vaccinated groups received a second dose at 14-day-old (day 7 post primary vaccination (ppv), **Figure 4.2**). As expected, no clinical signs were observed in the vaccinated chickens. All chickens were culled via cervical dislocation and decapitation methods. The serum

samples were obtained from the immunised chickens on day 6,14, 21 and 28 ppv to analyse the HI antibody titres (**section 4.2.1.1**), anti-HA IgM, IgY and IgA antibodies (**section 4.2.1.2**) and virus neutralising antibodies (**section 4.2.1.3**).

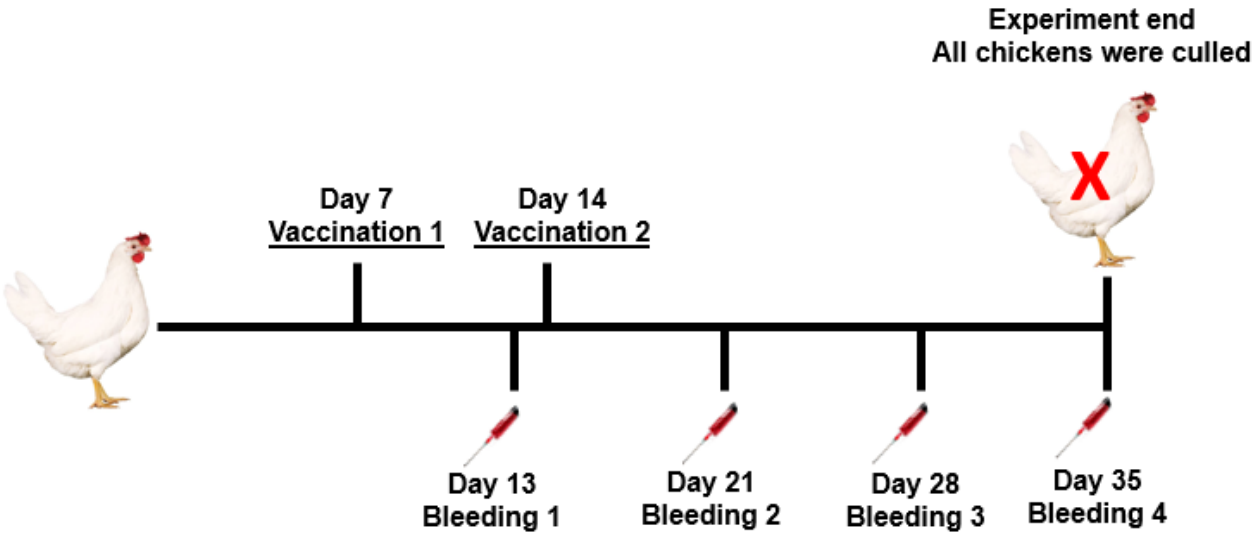


Figure 4.2*:* Experimental design to evaluate the immunogenicity of TADV. Groups of Dekalb White chickens (Medical grade, n=8 per group) were immunised twice (7-day-old and 14-day-old) with 2.8 µg, 28 µg and 49 µg of recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins equivalent to 2 µg, 20 µg and 35 µg of rH9HA (equimolar concentration). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 post primary vaccination.

4.2.1.1. The rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines induce higher haemagglutination inhibition antibody titres compared to rH9HA vaccine

A standard HI assay was conducted to test the ability of rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv to generate HI antibodies. The chickens vaccinated with rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv were able to induce significantly higher HI antibody titres compared to rH9HA group as early as day 6 ppv with the 20 µg ($p<0.05$ for rH9HA-CD83 scFv,

p<0.01 for rH9HA-CD11c scFv) and the 35 µg (p<0.001 for rH9HA-Dec205 scFv, p<0.0001 for rH9HA-CD83 scFv, p<0.05 for rH9HA-CD11c scFv) vaccine doses (**Figure 4.3A, 4.3B and 4.3C**). The HI antibody titres on day 6 ppv ranged from 8 to 30, which is below the predictive protective value of ≥32, according to the OIE[98]. Furthermore, none of the rH9HA-scFv vaccines were able to induce detectable HI antibodies with the 2 µg vaccine dose on day 6 ppv. Interestingly, there were no significant differences between the three doses of vaccination in rH9HA immunised groups on all the time points tested. On the contrary, the HI antibody titres obtained with rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv groups were higher with the 20 µg and 35 µg doses compared to the 2 µg dose after day 14 ppv.

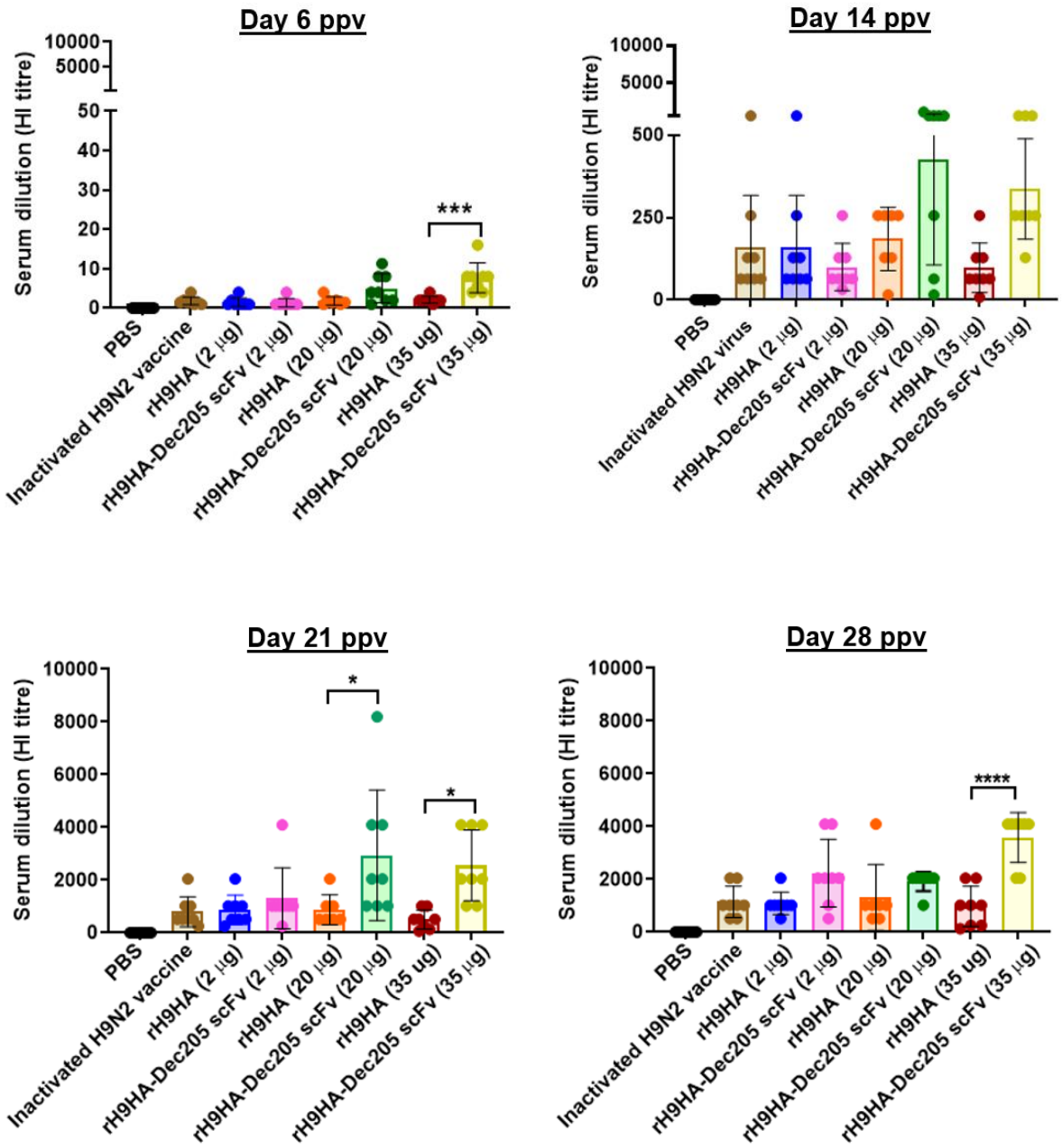
With regards to the 2 µg vaccine dose, no significant differences were observed in the HI antibody titres between rH9HA, inactivated H9N2 vaccine and rH9HA-Dec205/CD83/CD11c scFv groups on all the time points tested. All the vaccinated groups showed an increase in the HI antibody titres on day 21 ppv, after which it plateaued (**Figure 4.4**). Overall, the 2 µg dose of rH9HA and rH9HA-Dec205/CD83/CD11c scFv groups showed a delay in inducing HI antibodies however, by the end of the experiment (day 28 ppv) the HI antibody titres of 2 µg dose groups were similar to that of the 20 µg and 35 µg dose groups.

Moreover, there were significant differences in the HI antibody titres between rH9HA and rH9HA-Dec205 scFv (p<0.05 on day 21 ppv) or rH9HA and rH9HA-CD83 scFv (p<0.05 on day 6 ppv, p<0.01 on day 14 ppv, p<0.001 on day 21 ppv, p<0.0001 on day 28 ppv) or rH9HA and rH9HA-CD11c scFv (p<0.01 on day 6 ppv, p<0.05 on day 14 ppv, p<0.01 on day 21 ppv) groups on most of the time points tested with the 20 µg dose (**Figure 4.3A, 4.3B and 4.3C**). Furthermore, 20 µg of rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv also induced

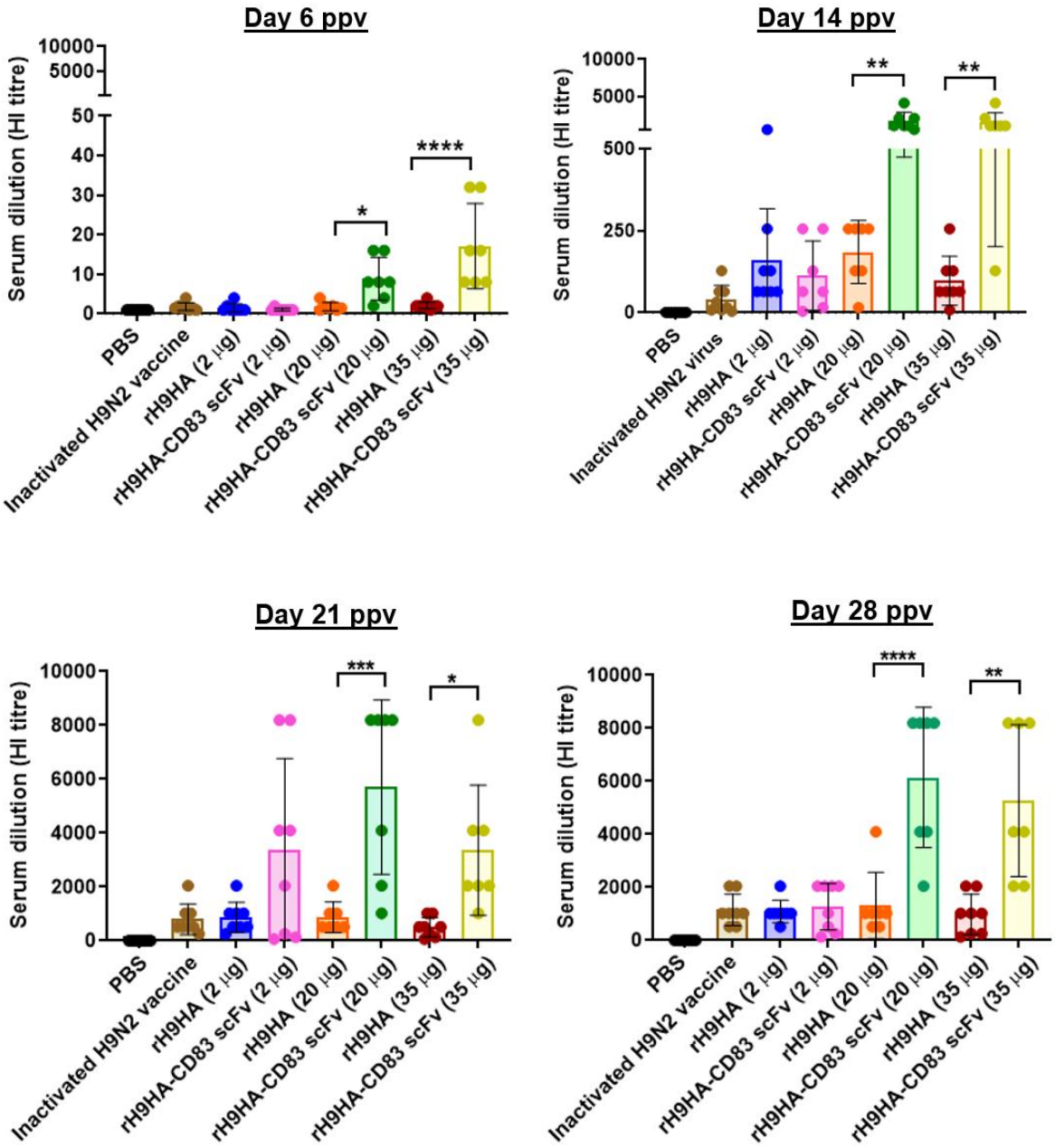
higher HI antibody titres compared to the inactivated H9N2 vaccine group after day 14 ppv. Unlike the 2 µg dose, a sharp rise in the HI antibody titres occurred on day 14 ppv in the chickens vaccinated with rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv groups, with the average HI antibody titres continuing to increase until the end of the experiment period (day 28 ppv) for rH9HA-CD83 scFv and rH9HA-CD11c scFv groups (**Figure 4.4**).

The highest vaccine dose of 35 µg showed a trend similar to the 20 µg dose with respect to the HI antibodies. A significantly higher HI antibody titres were observed between rH9HA and rH9HA-Dec205 scFv ($p<0.001$ on day 6 ppv, $p<0.05$ on day 21 ppv, $p<0.0001$ on day 28 ppv) or rH9HA and rH9HA-CD83 scFv ($p<0.0001$ on day 6 ppv, $p<0.01$ on day 14 ppv, $p<0.05$ on day 21 ppv, $p<0.01$ on day 28 ppv) or rH9HA and rH9HA-CD11c scFv ($p<0.05$ on day 6 ppv, $p<0.001$ on day 21 ppv, $p<0.0001$ on day 28 ppv) groups on most of the time points tested (**Figure 4.3A, 4.3B and 4.3C**) . Furthermore, the HI antibody titres in chickens vaccinated with 35 µg of rH9HA-Dec205 scFv, rH9HA-CD83 scFv, rH9HA-CD11c scFv groups were also higher than the inactivated H9N2 vaccine group after day 14 ppv. In addition, a sharp increase in the HI antibody titres was observed on day 14 ppv in chickens vaccinated with rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv groups, which was comparable to the 20 µg dose, with the average HI antibody titres continuing to increase until the end of the experiment period (day 28 ppv) (**Figure 4.4**).

A


Day 6 ppv
Serum dilution (HI titre)
10000
5000
50
40
30
20
10
0
***
PBS
Inactivated H9N2 vaccine
rH9HA (2 μg)
rH9HA-Dec205 scFv (2 μg)
rH9HA (20 μg)
rH9HA-Dec205 scFv (20 μg)
rH9HA (35 ug)
rH9HA-Dec205 scFv (35 μg)
Day 14 ppv
Serum dilution (HI titre)
10000
5000
500
250
0
PBS
Inactivated H9N2 virus
rH9HA (2 μg)
rH9HA-Dec205 scFv (2 μg)
rH9HA (20 μg)
rH9HA-Dec205 scFv (20 μg)
rH9HA (35 μg)
rH9HA-Dec205 scFv (35 μg)
Day 21 ppv
Serum dilution (HI titre)
10000
8000
6000
4000
2000
0
*
*
PBS
Inactivated H9N2 vaccine
rH9HA (2 μg)
rH9HA-Dec205 scFv (2 μg)
rH9HA (20 μg)
rH9HA-Dec205 scFv (20 μg)
rH9HA (35 ug)
rH9HA-Dec205 scFv (35 μg)
Day 28 ppv
Serum dilution (HI titre)
10000
8000
6000
4000
2000
0
****
PBS
Inactivated H9N2 vaccine
rH9HA (2 μg)
rH9HA-Dec205 scFv (2 μg)
rH9HA (20 μg)
rH9HA-Dec205 scFv (20 μg)
rH9HA (35 ug)
rH9HA-Dec205 scFv (35 μg)

B


Day 6 ppv
Day 14 ppv
Day 21 ppv
Day 28 ppv
Serum dilution (HI titre)
PBS
Inactivated H9N2 vaccine
Inactivated H9N2 virus
rH9HA (2 μg)
rH9HA-CD83 scFv (2 μg)
rH9HA (20 μg)
rH9HA-CD83 scFv (20 μg)
rH9HA (35 μg)
rH9HA-CD83 scFv (35 μg)

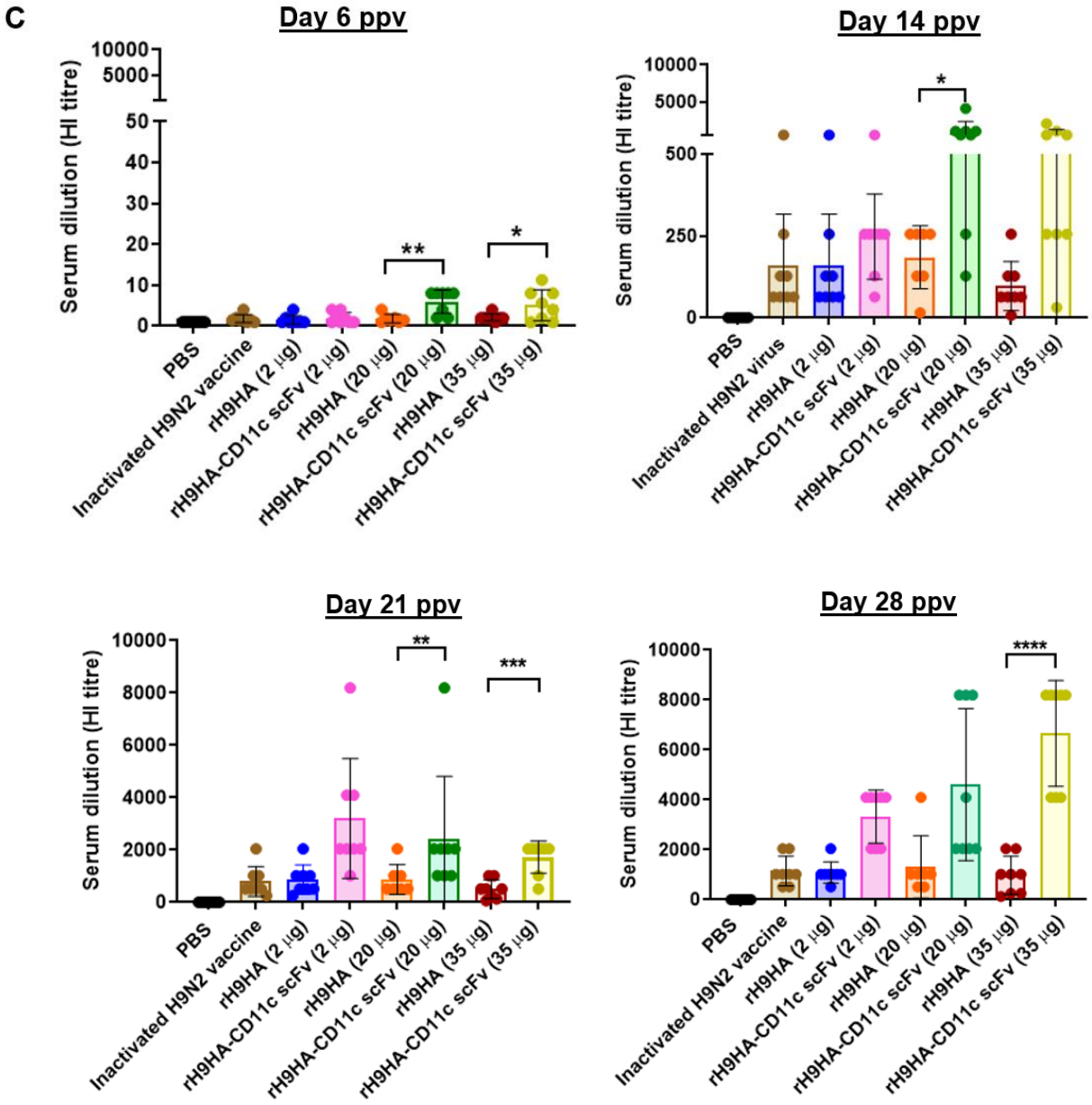


Figure 4.3: Analysis of HI antibodies in the serum of vaccinated chickens. **(A)** HI antibody titres in chickens vaccinated with rH9HA, rH9HA-Dec205 scFv and inactivated H9N2 vaccines. **(B)** HI antibody titres in chickens vaccinated with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines. **(C)** HI antibody titres in chickens vaccinated with rH9HA, rH9HA-CD11c scFv and inactivated H9N2 vaccines. Groups of Dekalb White chickens (Medical grade, n=8 per group) were immunised twice (7-day-old and 14-day-old) with 2.8 µg, 28 µg and 49 µg of recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins equivalent to 2 µg, 20 µg and 35 µg of rH9HA (equimolar concentration). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 post primary vaccination (ppv). The highest dilution of serum inhibiting the agglutination of chicken RBCs by UDL 01/08 H9N2 virus was recorded. Data are presented as mean ± SD (n=8 per group) and analysed by one-way ANOVA followed by Tukey's

multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001 **p<0.01 *p<0.05.

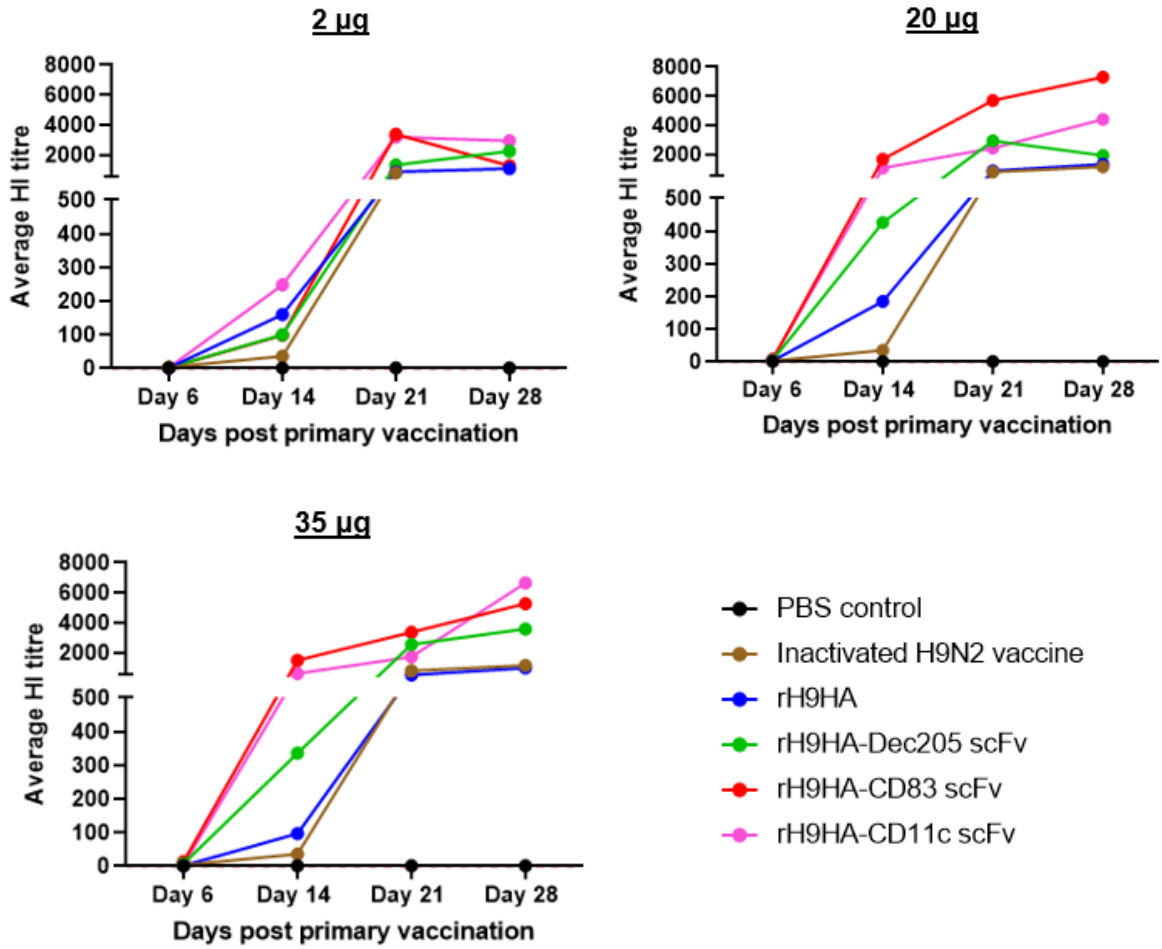
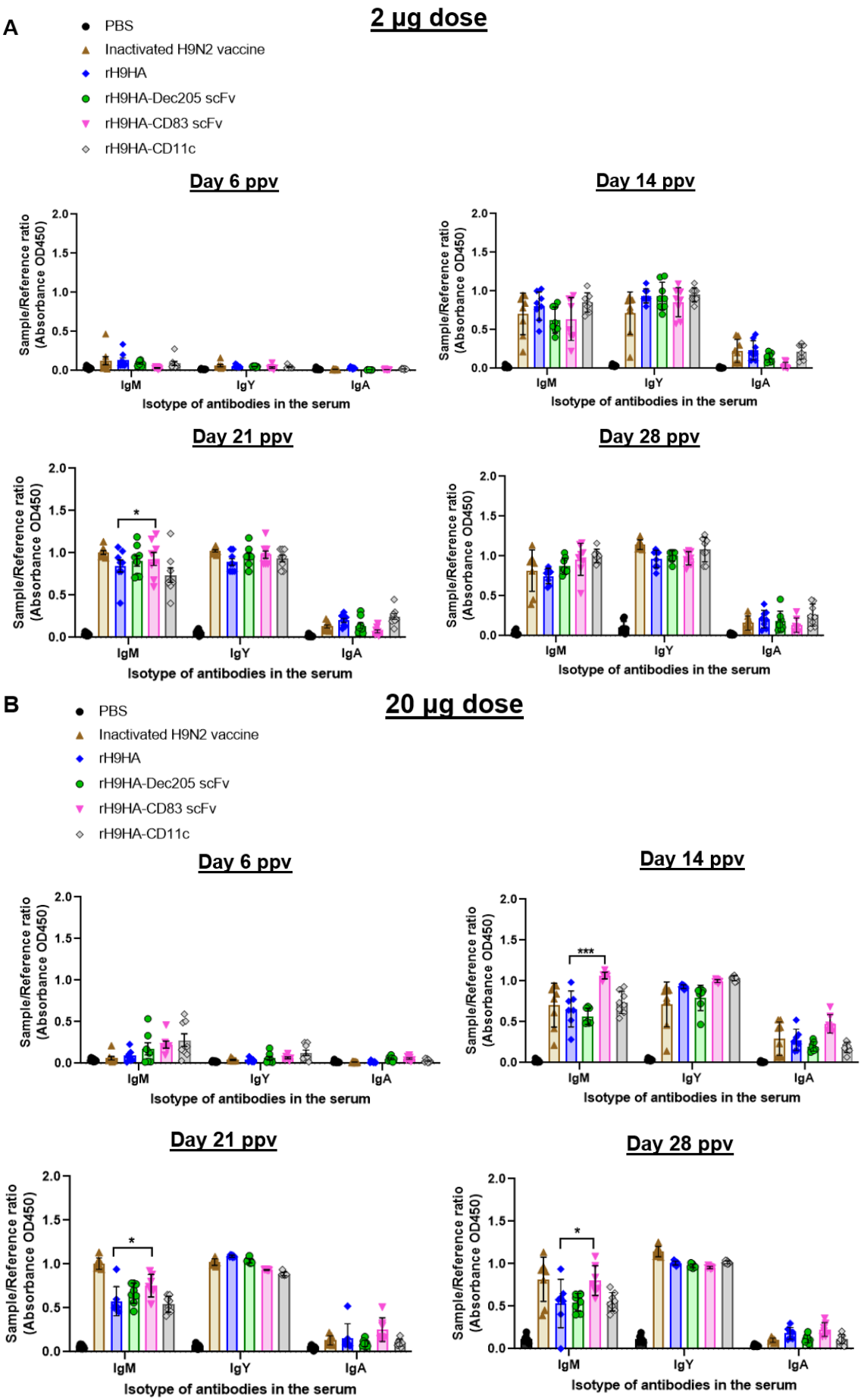


Figure 4.4: Average HI antibody titres with the 2 µg, 20 µg and 35 µg vaccine doses on different time points post vaccination. Groups of Dekalb White chickens (Medical grade, n=8 per group) were immunised twice (7-day-old and 14-day-old) with 2.8 µg, 28 µg and 49 µg of recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins equivalent to 2 µg, 20 µg and 35 µg of rH9HA (equimolar concentration). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 post primary vaccination.

4.2.1.2. The rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines induce higher anti-HA IgM and IgY antibodies compared to rH9HA vaccine

An ELISA approach was used to determine the total anti-HA reactivity for different classes of antibody (IgM, IgY and IgA) in the immunised serum. The serum was titrated to obtain the optimal dilution for the ELISA assay that gave good positive signal and low background noise. Overall, the amount of anti-HA IgM and IgY antibodies were higher than IgA antibodies in the immunised serum with all the vaccines tested (**Figure 4.5**). There were no differences observed in the amount of anti-HA IgM, IgY and IgA antibodies between rH9HA and rH9HA-Dec205 scFv or rH9HA and rH9HA-CD83 scFv or rH9HA and rH9HA-CD11c scFv with the 2 µg dose on all the time points tested (**Figure 4.5A**). However, with the 20 µg dose, significantly higher anti-HA IgM antibodies were observed in chickens vaccinated with rH9HA-CD83 scFv compared to rH9HA group on day 14 ppv ($p<0.001$), day 21 ppv ($p<0.05$) and day 28 ppv ($p<0.05$) (**Figure 4.5B**). In addition, rH9HA-CD83 scFv also induced higher anti-HA IgM and IgY antibodies compared to inactivated H9N2 vaccine on day 14 ppv ($p<0.01$). On all the other time points tested, no significant differences were observed in the anti-HA antibodies between any of the vaccinated groups. The highest dose of 35 µg induced significantly higher anti-HA IgM and IgY antibodies in rH9HA-Dec205 scFv ($p<0.01$) and rH9HA-CD83 scFv ($p<0.0001$) groups compared to rH9HA group on day 6 ppv (**Figure 4.5C**). Similarly, the anti-HA IgM and IgY antibodies were also significantly higher in rH9HA-Dec205 scFv ($p<0.05$) and rH9HA-CD83 scFv ($p<0.0001$) groups compared to the inactivated H9N2 vaccine group on day 6 ppv. Furthermore, rH9HA-CD83 scFv group also showed a significantly higher anti-HA IgM antibody

compared to rH9HA on day 14 ppv ($p<0.001$). Moreover, the chickens in rH9HA-CD11c scFv group also showed significantly higher anti-HA IgM antibodies compared to rH9HA group on day 14 ppv ($p<0.01$), day 21 ppv ($p<0.05$) and day 28 ppv ($p<0.05$).


A
2 μg dose
PBS
Inactivated H9N2 vaccine
rH9HA
rH9HA-Dec205 scFv
rH9HA-CD83 scFv
rH9HA-CD11c
Day 6 ppv
Day 14 ppv
Day 21 ppv
Day 28 ppv
Sample/Reference ratio (Absorbance OD450)
IgM
IgY
IgA
Isotype of antibodies in the serum
*
B
20 μg dose
PBS
Inactivated H9N2 vaccine
rH9HA
rH9HA-Dec205 scFv
rH9HA-CD83 scFv
rH9HA-CD11c
Day 6 ppv
Day 14 ppv
Day 21 ppv
Day 28 ppv
Sample/Reference ratio (Absorbance OD450)
IgM
IgY
IgA
Isotype of antibodies in the serum
***
*

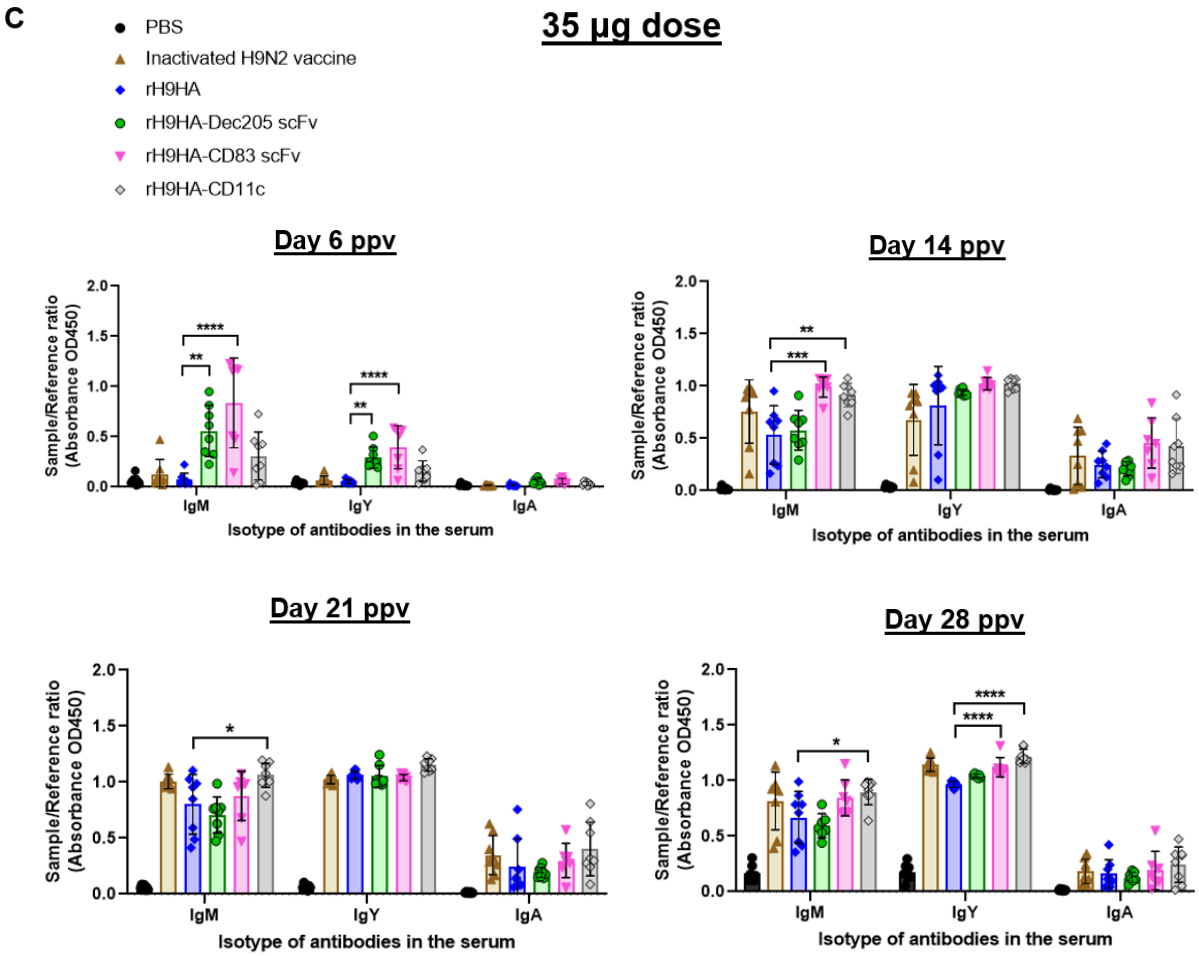


Figure 4.5. HA-specific IgM, IgY and IgA antibody levels in the serum of chickens immunised with rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv, rH9HA-CD11c scFv and inactivated H9N2 vaccine. **(A)** 2 µg vaccine dose **(B)** 20 µg vaccine dose **(C)** 35 µg vaccine dose. Groups of Dekalb White chickens (Medical grade, n=8 per group) were immunised twice (7-day-old and 14-day-old) with 2.8 µg, 28 µg and 49 µg of recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins equivalent to 2 µg, 20 µg and 35 µg of rH9HA (equimolar concentration). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 post primary vaccination (ppv). The HA-specific isotypes of antibodies were determined in 200-fold diluted sera by ELISA. Briefly, the plates were coated with 1 µg of recombinant H9HA Foldon protein overnight at 4°C. For detection, the plates were incubated with respective serum for one hour at room temperature. This was followed by further incubation for one hour with anti-chicken IgM (1 mg/mL diluted to 1:3000), IgY (2 mg/mL diluted to 1:6000), and IgA (1 mg/mL diluted to 1:3000) antibodies. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. The amount of HA specific IgM, IgY and IgA antibodies were expressed as sample to reference ratio (relation of absorbance of tested serum sample to absorbance of the reference serum). Data are presented as mean ± SD (n=8 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001 **p<0.01 *p<0.05.

4.2.1.3. The rH9HA-CD83 scFv vaccine induces higher virus neutralising antibodies compared to rH9HA, rH9HA-Dec205 scFv and rH9HA-CD11c scFv vaccines

The HI and virus MNT assays are two of the most common methods for measuring serum antibodies to influenza[605]. The virus MNT assay assesses the ability of the serum antibodies to block virus entry and productive replication[605]. Therefore, to determine the effect of serum antibodies on viral biology, virus MNT assay was performed on the immunised serum from the final time point of the experiment (i.e., day 28 ppv). No significant differences were observed in the virus neutralising antibody titres between any of the vaccinated groups with the 2 µg vaccine dose (**Figure 4.6**). Moreover, significantly higher virus neutralising antibody titres were observed in rH9HA-CD83 scFv group compared to rH9HA group with both the 20 µg and 35 µg doses ($p<0.0001$). Furthermore, the virus neutralising antibody titres in rH9HA-CD83 scFv group were also significantly higher than rH9HA-Dec205 scFv (20 µg and 35 µg doses), rH9HA-CD11c scFv (20 µg and 35 µg doses) and inactivated H9N2 vaccine (35 µg dose) groups ($p<0.01$).

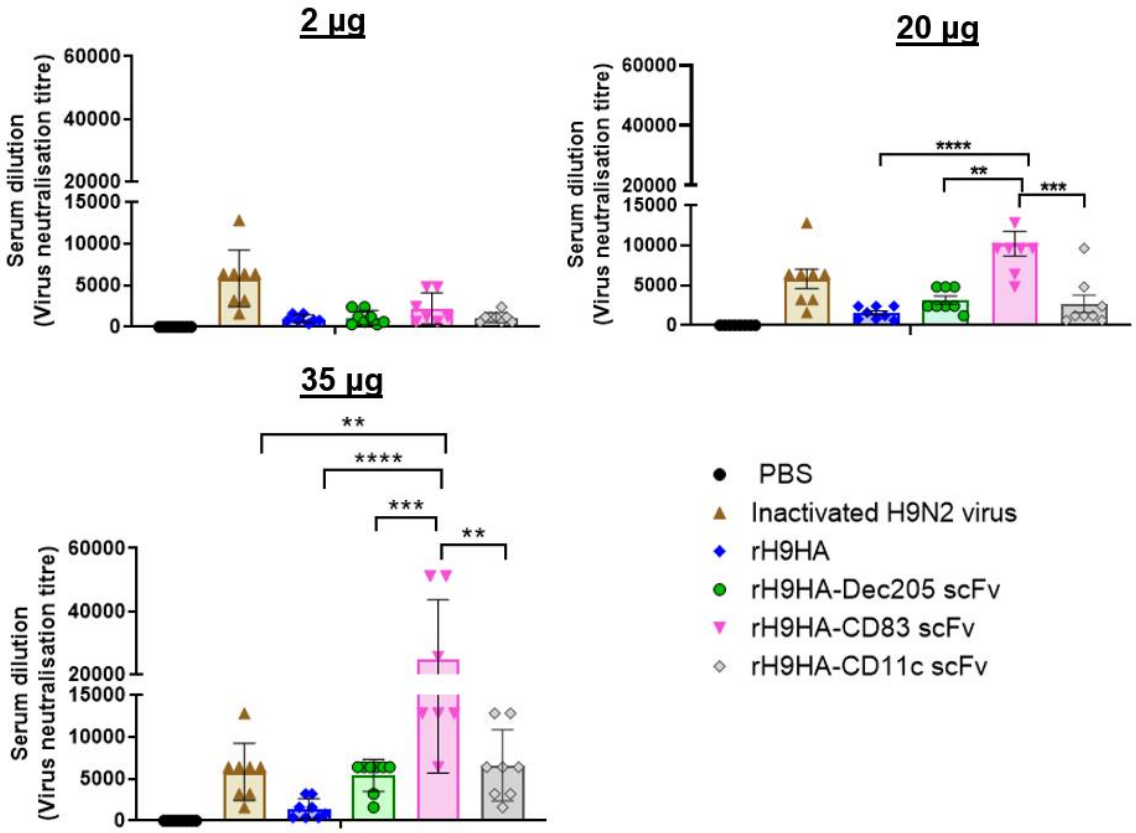


Figure 4.6: Virus neutralising antibody titres in the serum of chickens vaccinated with rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv, rH9HA-CD11c scFv and inactivated H9N2 vaccine. Groups of Dekalb White chickens (Medical grade, n=8 per group) were immunised twice (7-day-old and 14-day-old) with 2.8 µg, 28 µg and 49 µg of recombinant rH9HA-Dec205 scFv, rH9HA-CD83 scFv or rH9HA-CD11c scFv proteins equivalent to 2 µg, 20 µg and 35 µg of rH9HA (equimolar concentration). In all cases, blood samples were collected from the wing veins on day 6, 14, 21 and 28 post primary vaccination (ppv). Virus neutralisation assay was carried out on serum samples from day 28 ppv. Briefly, the serum was inactivated at 56°C and incubated with 150 $TCID_{50}$ of UDL 01/08 H9N2 virus for 1 hour at 37°C. The virus-serum mixture was then added onto the MDCK cells and further incubated for 1 hour at 37°C. This was followed by the removal of the virus-serum mixture from the cells and incubation with fresh media containing 2 µg/mL trypsin for about 3 days. The virus neutralisation titre was expressed as the reciprocal of the highest serum dilution at which virus infection is blocked and the cells survive. Data are presented as mean ± SD (n=8 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001 **p<0.01.

4.2.2. Investigation of the protective efficacy of rH9HA-CD83 scFv vaccine in chickens challenged with UDL 01/08 H9N2 virus

After the successful evaluation of the antibody responses generated by rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines, the rH9HA-CD83 scFv vaccine was taken forward for the protective efficacy study since it induced higher HI antibodies, anti-HA IgM and IgY antibodies and virus neutralising antibodies compared to the other vaccines tested (**section 4.2.1**). The experimental design for the challenge experiment is described in **section 2.2.8.3.2** and shown in **Figure 4.7**. Briefly, Rhode Island Red SPF chickens (n=7 per group, National Avian Research Facility) were immunised with inactivated H9N2 vaccine (1024 HAU/dose), rH9HA (25 µg/dose) and rH9HA-CD83 scFv (35 µg/dose equivalent to 25 µg/dose of rH9HA in equimolar concentration) at 7-day-old and 14-day old. The vaccinated chickens were inoculated intranasally with $1x10^6$ plaque forming units (PFU)/100 µL of H9N2 virus (UDL 01/08) at 22-day-old and were designated as 'direct' groups. The PBS and rH9HA-CD83 scFv vaccine 'contact' groups were also included as they provide evidence on whether the vaccinated chickens have a reduced chance of getting infection from the directly infected PBS treated chickens while sharing the same air space, food, and water. All chickens were culled via intravenous injection with pentobarbital followed by cervical dislocation.

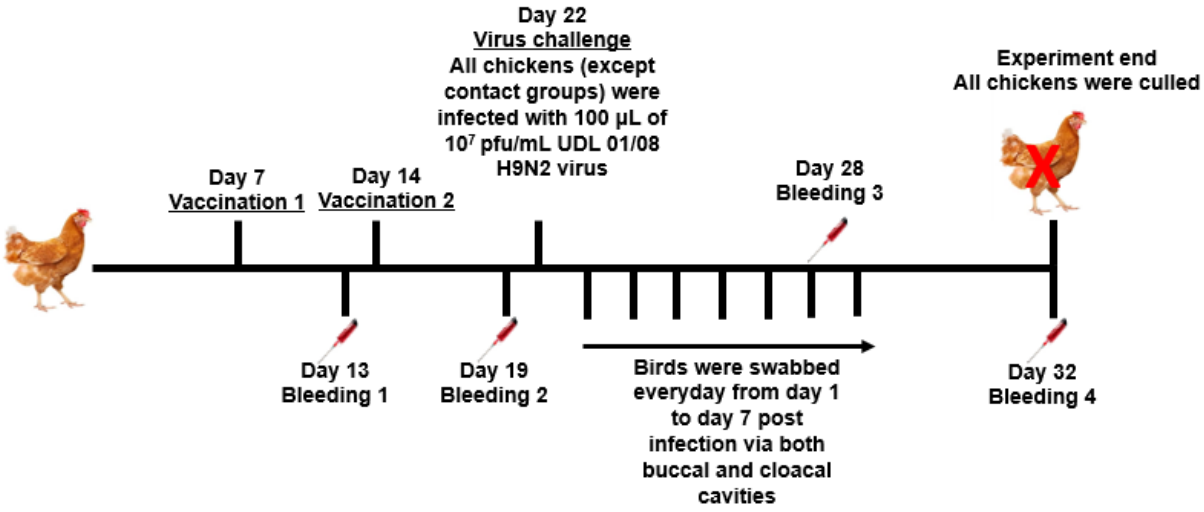


Figure 4.7: Experimental design for the virus challenge experiment. Rhode Island Red SPF chickens (n=7 per group) were divided into four groups: PBS control, inactivated H9N2 vaccine (1024 HAU/dose), rH9HA (25 µg/dose) and rH9HA-CD83 scFv (25 µg/dose equivalent of rH9HA). The PBS control and rH9HA-CD83 scFv groups were further divided into two sub-groups: 'direct' virus inoculated and 'contact'. The chickens were vaccinated twice: day 7 and day 14 of age. All chickens except the contact groups were challenged with $1x10^6$ pfu/100 µL of UDL 01/08 H9N2 virus intranasally at day 22 of age. Clinical signs were monitored throughout the study. If any signs of severe disease were observed, chickens were euthanised via a schedule one method (intravenous injection with pentobarbital /cervical dislocation).

4.2.2.1. The antibody response in the chickens increases after the virus infection

To assess the antibody response in vaccinated chickens, serum samples were collected on day 6 ppv, day 12 ppv, day 6 post virus infection (pi) and day 10 pi. The HI antibody titres in rH9HA-CD83 scFv vaccinated group were significantly higher compared to rH9HA group on day 6 ppv ($p<0.0001$) and day 12 ppv ($p<0.01$) (**Figure 4.8**). This verifies the results obtained in **section 4.2.1.1** whereby, the rH9HA-CD83 scFv group showed higher HI antibody titres compared to rH9HA group**.** Furthermore, there was an increase in the HI antibody titres in all the vaccinated groups (direct and contact) on day 6 pi, with the titre ranging from 1024 to 16,384. In addition, the PBS treated 'direct' and 'contact' groups also showed

high HI antibody titres ranging from 256 to 4096 on day 6 pi. This indicates that the virus infection can boost HI antibody titres in vaccinated and control groups. No significant differences were observed in the HI antibody titres between ‘direct’ and ‘contact’ PBS treated groups after the virus infection. Similarly, no significant differences were observed in the HI antibody titres between the ‘direct’ and ‘contact’ rH9HA-CD83 scFv groups after the virus infection. However, HI antibody titre of the latter was lower than the former on day 6 pi. This suggests that the PBS treated and rH9HA-CD83 scFv ‘contact’ groups also get infected with the virus resulting in similar HI antibody titres as their ‘directly infected’ counterparts.

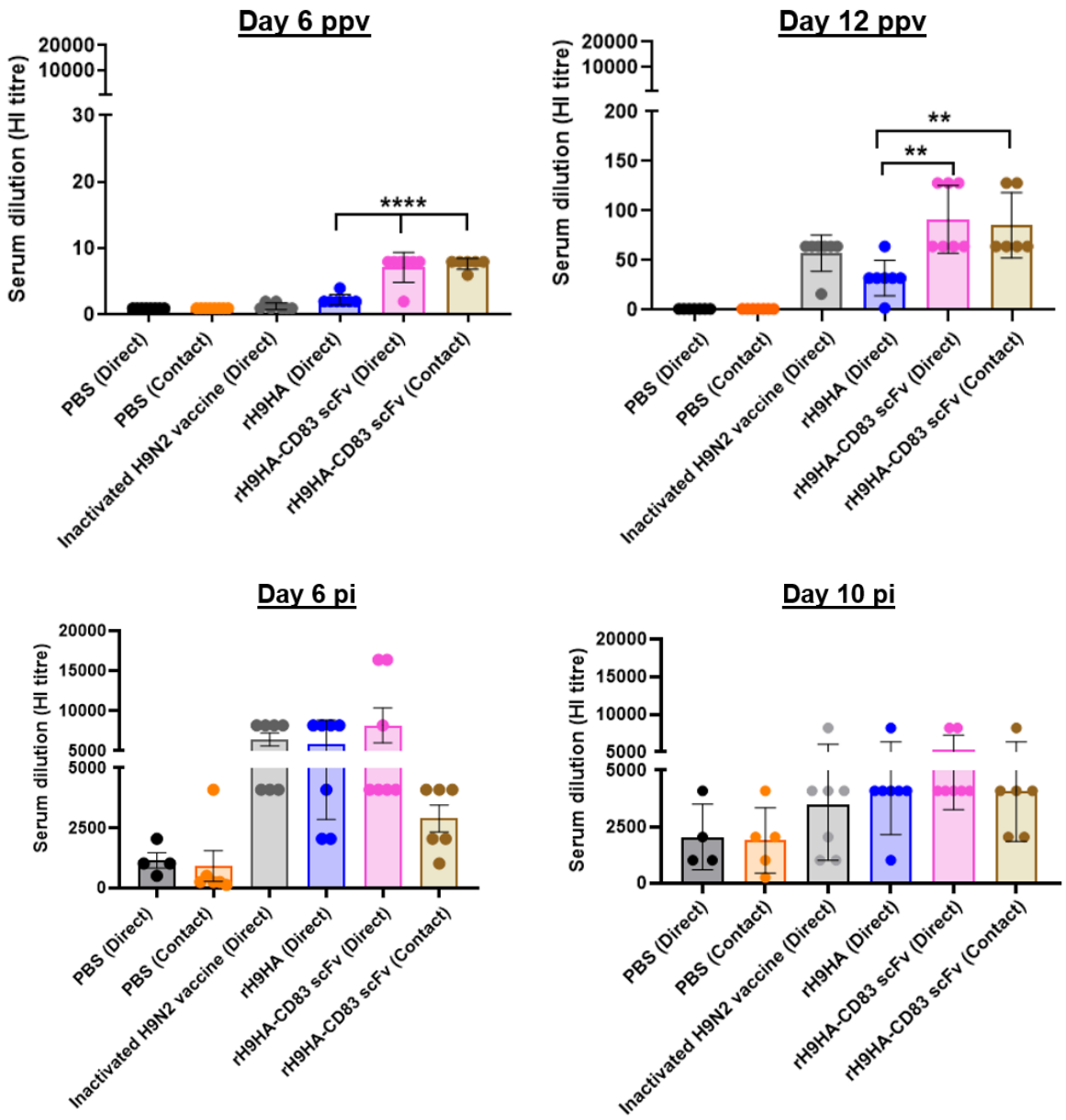


Figure 4.8: Analysis of HI antibodies in the serum of chickens before and after the UDL 01/08 H9N2 virus challenge. Groups of Rhode Island Red SPF chickens (n=7 per group) were vaccinated with inactivated H9N2 vaccine (1024 HAU/dose), rH9HA (25 µg/dose) and rH9HA-CD83 scFv (25 µg/dose equivalent of rH9HA) on day 7 and day 14 of age (double vaccination). All chickens except the contact groups were challenged with $1x10^6$ pfu/100 µL of UDL 01/08 H9N2 virus intranasally at day 22 of age. The serum samples were collected on day 6 post primary vaccination (ppv), day 12 ppv, day 6 post infection (pi) and day 10 pi. The highest dilution of serum inhibiting the agglutination of chicken RBCs by UDL 01/08 H9N2 virus was recorded. Data are presented as mean ± SD (n=8 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 **p<0.01

In terms of anti-HA IgM, IgY and IgA antibodies, no differences were observed between rH9HA and rH9HA-CD83 scFv groups on day 6 ppv (**Figure 4.9**). However, significantly higher anti-HA IgM antibodies were induced in chickens vaccinated with rH9HA-CD83 scFv (direct and contact) compared to rH9HA on day 12 ppv ($p<0.05$). Furthermore, increase in the anti-HA IgM and IgY antibodies were observed in all the 'direct' and 'contact' PBS treated and vaccinated groups on day 6 pi. Interestingly, all the 'direct' and 'contact' PBS treated, and vaccinated groups showed high anti-HA IgA antibodies on day 6 pi, with a significant difference between rH9HA (direct) and inactivated H9N2 vaccine (direct) groups ($p<0.01$) and rH9HA (direct) and rH9HA-CD83 scFv (direct) groups ($p<0.05$). This suggests that live H9N2 virus is better at inducing serum anti-HA IgA antibodies compared to the inactivated H9N2 and recombinant subunit vaccines. The anti-HA IgM and IgY antibodies remained consistent until day 10 pi whereas, anti- HA IgA antibodies were declined in all the groups by day 10 pi.

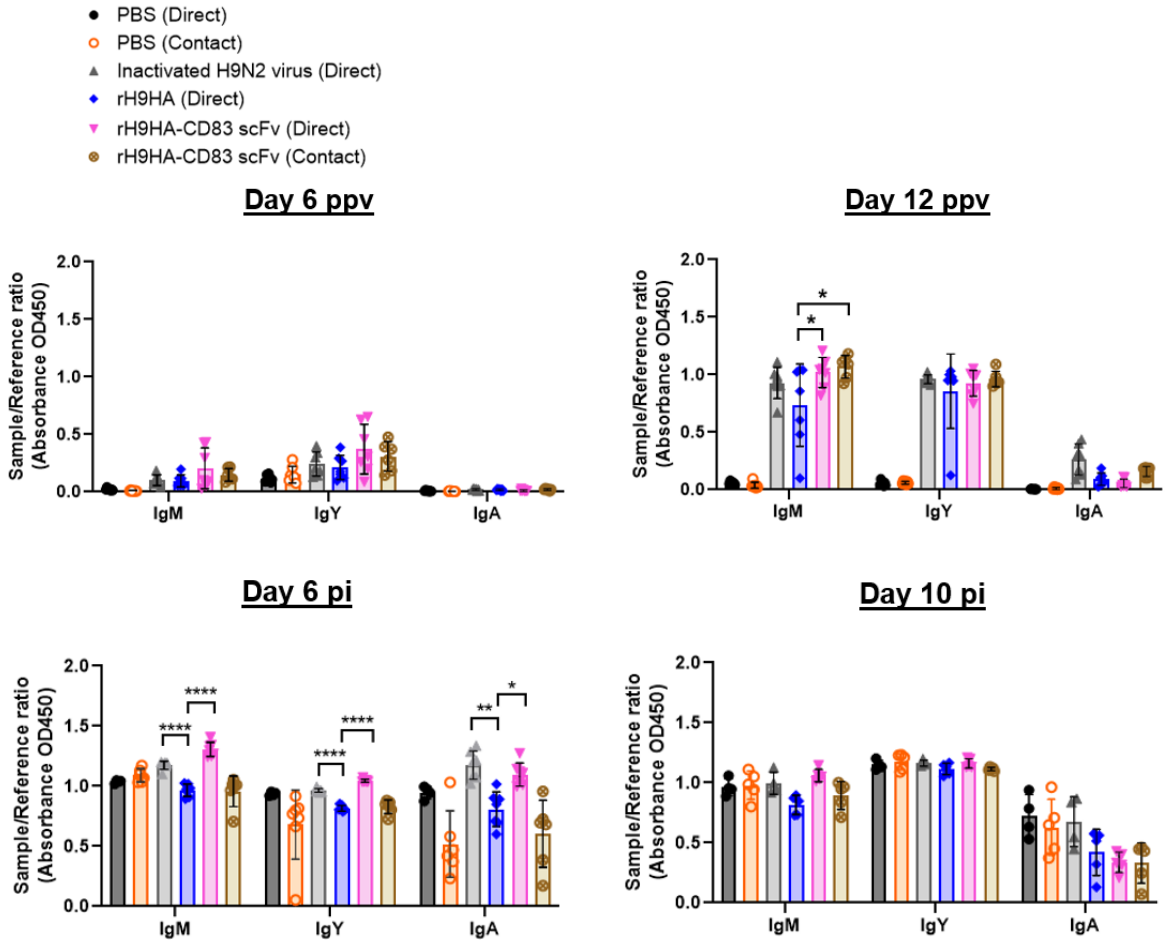


Figure 4.9. HA-specific IgM, IgY and IgA antibody levels in the serum of chickens before and after the UDL 01/08 H9N2 virus challenge. Groups of Rhode Island Red SPF chickens (n=7 per group) were vaccinated with inactivated H9N2 vaccine (1024 HAU/dose), rH9HA (25 µg/dose) and rH9HA-CD83 scFv (25 µg/dose equivalent of rH9HA) on day 7 and day 14 of age (double vaccination). All chickens except the contact groups were challenged with $1x10^6$ pfu/100 µL of UDL 01/08 H9N2 virus intranasally on day 22 of age. The serum samples were collected on day 6 post primary vaccination (ppv), day 12 ppv, day 6 post infection (pi) and day 10 pi. The HA-specific isotypes of antibodies were determined in 200-fold diluted sera by ELISA. Briefly, the plates were coated with 1 µg of recombinant H9HA Foldon protein overnight at 4°C. For detection, the plates were incubated with respective serum for one hour at room temperature. This was followed by further incubation for one hour with anti-chicken IgM (1 mg/mL diluted to 1:3000), IgY (2 mg/mL diluted to 1:6000), and IgA (1 mg/mL diluted to 1:3000) antibodies. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. The amount of HA specific IgM, IgY and IgA antibodies were expressed as sample to reference ratio (relation of absorbance of tested serum sample to absorbance of the reference serum). Data are presented as mean ± SD (n=8 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 **p<0.01 *p<0.05

4.2.2.2. The rH9HA-CD83 scFv vaccinated chickens show reduced clinical signs compared to rH9HA vaccinated chickens

The virus infected chickens were monitored daily for the presence of clinical signs of infection. The severity of the clinical signs was scored on a scale of 1-3 where 1 was 'mild', 2 was 'moderate' and 3 was 'severe' (**Table 26**). Clinical signs of disease were recorded for each chicken on each day resulting in an overall clinical score per day as described previously[606] (**Appendix B**). If any of the humane endpoint signs indicated in **Table 26** were observed, the chickens were euthanised via a schedule one method (intravenous injection with pentobarbital /cervical dislocation) and post-mortem examination was carried out. The chickens in all the vaccinated groups showed only mild and moderate clinical signs (mainly ruffled feathers, dropped wings and diarrhoea), whereas the chickens in PBS treated groups showed a combination of mild, moderate, and severe clinical signs (mainly ruffled feathers, diarrhoea, laboured breathing, and convulsions). The post-challenge clinical score was significantly reduced ($p<0.0001$) in vaccinated groups compared to PBS treated 'direct' group, especially on day 3 pi and day 4 pi (**Figure 4.10A**). Furthermore, the chickens in rH9HA group showed a significantly higher clinical score compared to inactivated H9N2 vaccine and rH9HA-CD83 scFv groups on day 3 pi ($p<0.01$, **Figure 4.10A**). In addition, the chickens in PBS 'contact' group showed a significantly higher clinical score compared to rH9HA-CD83 scFv 'contact' group on day 4 pi and day 5 pi ($p<0.0001$) (**Figure 4.10B**).

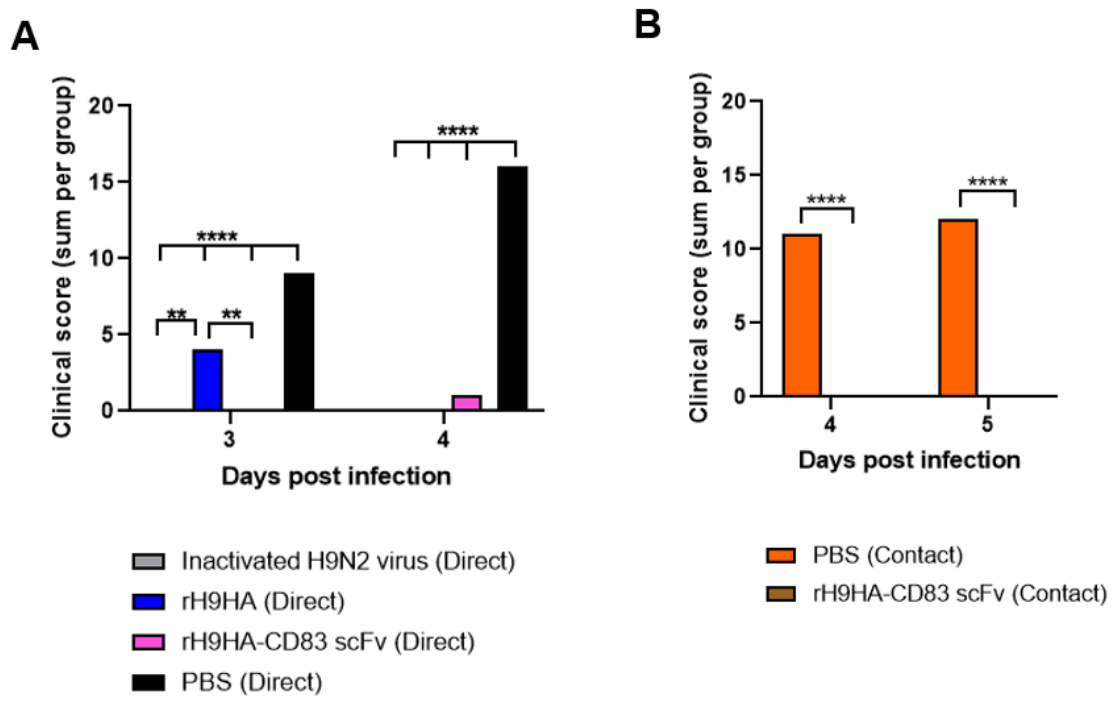


Figure 4.10: Total clinical scores for all the groups of chickens (n=7 per group) challenged with UDL 01/08 H9N2 virus. A total clinical score was calculated for each chicken by summing the daily values for mild, moderate, and severe clinical signs post virus infection. The chickens were euthanised if any moderate disease clinical signs persisted for more than 72 hours with no recovery, or if any of the severe disease clinical signs were observed.

4.2.2.3. Vaccination increases the survival rate and weight gain in chickens infected with UDL 01/08 H9N2 virus

As mentioned in **section 4.2.2.2**, the post-challenge clinical score was higher in PBS treated 'direct' and 'contact' chickens compared to the vaccinated chickens. In total, 4 chickens in PBS treated groups (3 in 'direct' and 1 in 'contact') died on day 4 pi. Out of the 4 chickens that were dead, 1 chicken had to be euthanised since it was showing a severe clinical sign (convulsions). All vaccinated groups ('direct' and 'contact') had 100% survival rates compared to 58% in the 'direct' PBS treated group. In addition, the survival rate of chickens in the PBS treated 'contact' group was 87% (**Figure 4.11A**).

With regards to the change in weight of the virus infected chickens, the average weight gain of all the vaccinated chickens (direct) remained consistent before and after the virus infection (**Figure 4.11B**). However, the average weight gain of the chickens in PBS treated 'direct' group was significantly reduced on day 3 pi and day 4 pi ($p<0.0001$) compared to the vaccinated groups. The chickens that survived showed a similar average weight gain pattern as the vaccinated groups. Furthermore, similar weight gain patterns were observed between the PBS treated 'contact' and rH9HA-CD83 scFv 'contact' groups however, on day 4 pi the latter showed significantly higher average weight gain ($p<0.001$**, Figure 4.11C**). These results indicate that vaccination can prevent weight loss in chickens during virus infection.

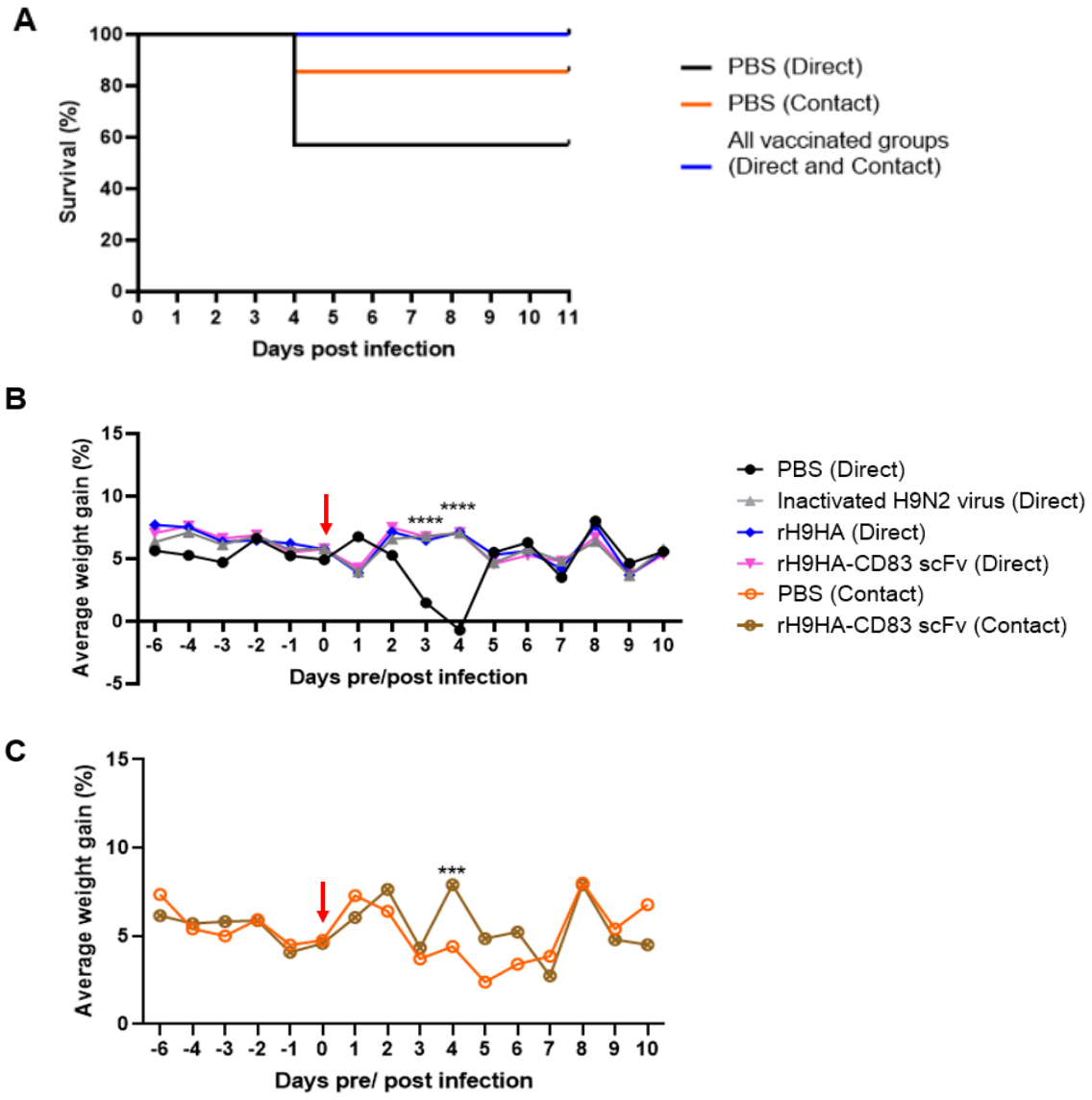
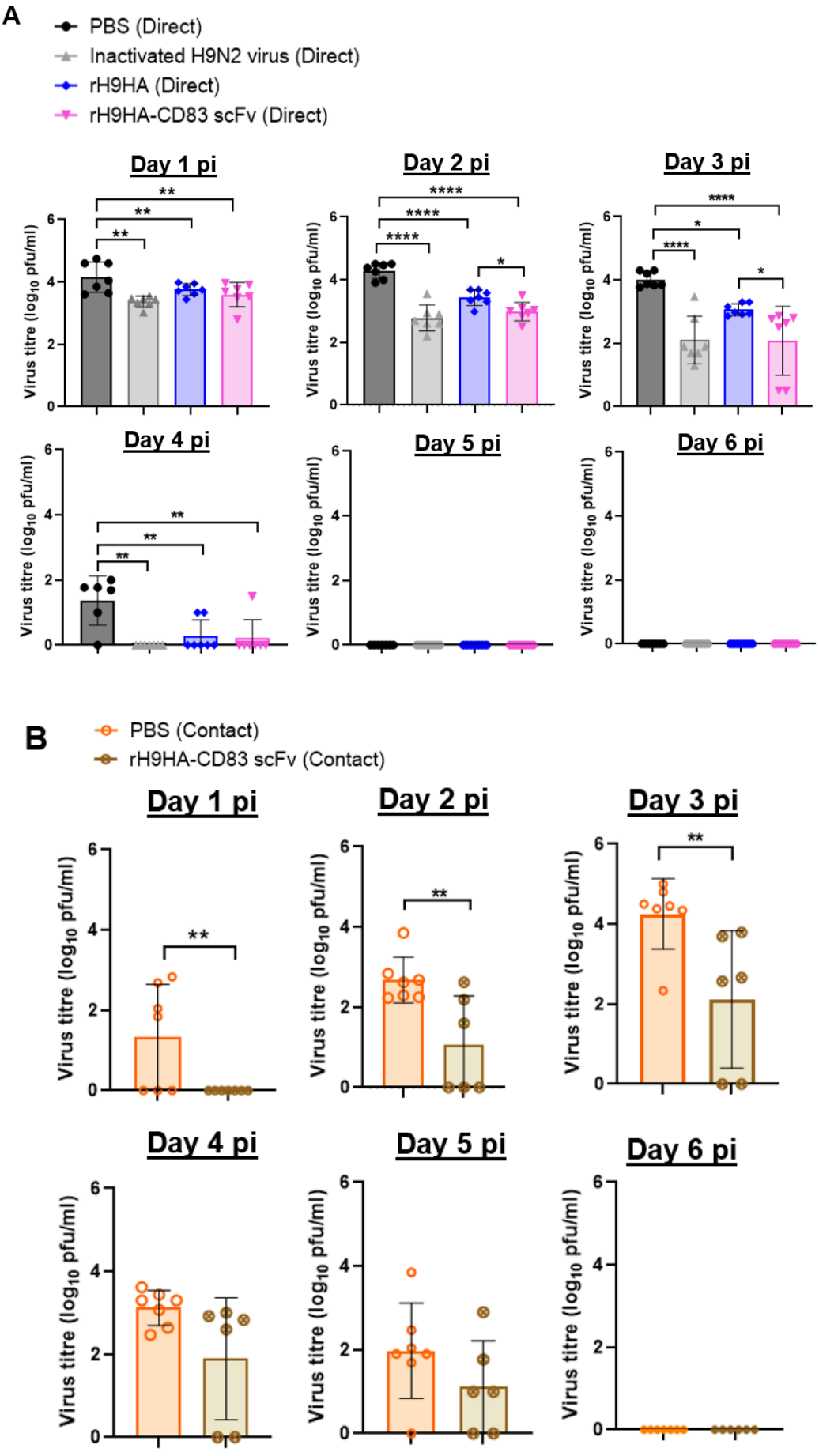


Figure 4.11. Survival and average weight gain of chickens after virus challenge. **(A)** Percentage survival between vaccinated and PBS treated chickens challenged with UDL 01/08 H9N2 virus. Directly infected and contact birds were monitored for clinical disease signs throughout the study duration. Birds that reached the humane endpoints were culled due to ethical reasons. Survival curves between PBS treated 'direct' (black line) and vaccinated groups 'direct'/'contact' (blue line) is significantly different ($p < 0.05$) (Log rank (Mantel-Cox) test). **(B, C)** Percentage average weight gain per day between vaccinated and PBS treated 'direct' or 'contact' chickens challenged with UDL 01/08 H9N2 virus. Chickens were weighted daily during the study duration. Data are analysed by one-way ANOVA followed by Tukey's multiple comparison test for **(B)** and unpaired t test for **(C).** Statistical significance between the vaccinated and PBS treated groups is shown with asterisks. The red arrow represents the day when the chickens were infected with UDL 01/08 H9N2 virus. ****$p<0.0001$ ***$p<0.001$.

4.2.2.4. The rH9HA-CD83 scFv vaccine is better at reducing viral load in H9N2 virus challenged chickens compared to rH9HA vaccine

To analyse the virus shedding from the vaccinated/PBS treated directly infected or contact chickens, swab samples were collected from buccal and cloacal cavities daily after the virus infection, and plaque assay was performed. No cloacal virus shedding was observed in chickens infected with UDL 01/08 H9N2 virus (data not shown). This was expected because cloacal shedding has been reported to be more sporadic in a number of viral subtypes (H9, H7 and H5) and the virus is not always shed via the cloacal route in chickens[54,607,608]. Viral titres in the buccal swabs collected from vaccinated groups (direct) on day 1 pi ($p<0.01$), day 2 pi ($p<0.0001$) and day 3 pi ($p<0.05$ for rH9HA, $p<0.0001$ for inactivated H9N2 vaccine and rH9HA-CD83 scFv) and day 4 pi ($p<0.01$) were significantly lower compared to the PBS treated (direct) group (**Figure 4.12A**). Furthermore, rH9HA-CD83 scFv vaccinated chickens (direct) had significantly lower viral load compared to rH9HA vaccinated chickens on day 2 pi ($p<0.05$) and day 3 pi ($p<0.05$) (**Figure 4.12A**). The average viral load in PBS treated (direct) group ranged from 23,000 pfu/mL to 12,000 pfu/mL on the first three days post virus infection, whereas the average viral load in vaccinated 'direct' groups ranged from 6200 pfu/mL to 390 pfu/mL (**Figure 4.12C**). No differences were observed between rH9HA-CD83 scFv and inactivated H9N2 vaccine groups in terms of viral load. By day 4 pi, the virus was almost cleared from all the directly infected vaccinated groups, whereas some virus remained detectable (<100 pfu /mL) in the directly infected PBS treated group. In addition, the chickens in rH9HA-CD83 scFv 'contact' group had significantly lower viral load compared to those in PBS treated 'contact' group on day 1 pi ($p<0.01$),

day 2 pi ($p<0.01$) and day 3 pi ($p<0.01$) (**Figure 4.12B**), indicating that rH9HA-CD83 scFv vaccine can reduce the chances of chickens getting infected with the virus while sharing the same air, water, and food space with directly infected chickens. The virus was cleared from both the 'contact' groups by day 6 pi.


A
PBS (Direct)
Inactivated H9N2 virus (Direct)
rH9HA (Direct)
rH9HA-CD83 scFv (Direct)
Day 1 pi
Day 2 pi
Day 3 pi
Day 4 pi
Day 5 pi
Day 6 pi
Virus titre (log10 pfu/ml)
B
PBS (Contact)
rH9HA-CD83 scFv (Contact)
Day 1 pi
Day 2 pi
Day 3 pi
Day 4 pi
Day 5 pi
Day 6 pi
Virus titre (log10 pfu/ml)

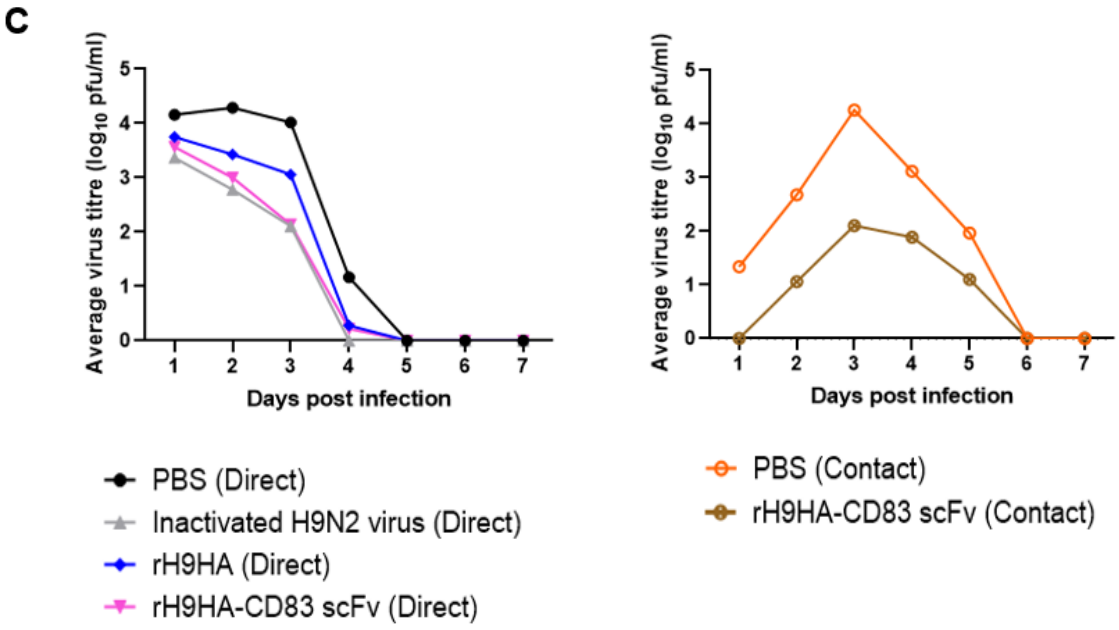


Figure 4.12. Challenge virus shedding profiles from the buccal cavity of vaccinated and PBS treated chickens infected with UDL 01/08 H9N2 virus. **(A, B)** Shedding profiles of 'directly infected' and 'contact' vaccinated, and PBS treated chickens at different days post infection. **(C)** The average shedding profile from the buccal cavity per group for 'directly infected' and 'contact' vaccinated, and PBS treated chickens. Groups of Rhode Island Red SPF chickens (n=7 per group) were vaccinated with inactivated H9N2 vaccine (1024 HAU/dose), rH9HA (25 µg/dose) and rH9HA-CD83 scFv (25 µg/dose equivalent of rH9HA) on day 7 and day 14 of age (double vaccination). All chickens except the contact groups were challenged with $1x10^6$ pfu/100 µL of UDL 01/08 H9N2 virus intranasally on day 22 of age. Swabs were taken from the buccal cavity until day 7 post virus infection (pi) and the amount of live virus particles recovered from swabs was titrated via plaque assays and presented as plaque forming units (pfu)/mL. Data are presented as mean ± SD (n=7 per group) for **(A)** and analysed by one-way ANOVA followed by Tukey's multiple comparison test for **(A)** and by unpaired t test for **(B).** Statistical significance is shown with asterisks. ****p<0.0001 **p<0.01 *p<0.05.

4.2.3. Investigating the mechanism of rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv in eliciting the immune response

It is evident from **section 4.2.1** that rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv vaccines can induce higher antibody response compared to rH9HA in chickens. In addition, rH9HA-CD83 scFv is better than rH9HA at

reducing clinical disease signs and virus shedding compared to rH9HA (**section 4.2.2**). It was hypothesised that targeting rH9HA protein to APC receptors could enhance the immunogenic potential of the protein antigen. Hence, the mechanism of rH9HA protein conjugated to APC-specific scFv was assessed by *in vitro* stimulation of chicken splenocytes.

4.2.3.1. The rH9HA-CD83 scFv and rH9HA-CD11c scFv can induce cytokine and chemokine production upon *in vitro* stimulation of chicken splenocytes

The *in vitro* stimulation of splenocytes can be used to determine the capacity of vaccine antigens to induce the expression of various cytokines and chemokines. The first cytokine tested was IFN-γ since it is a signature cytokine implicated in CMI[609]. Splenocytes (naive) were prepared from the spleen of 3-week-old Rhode Island Red SPF chickens (National Avian Research Facility) and stimulated with rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv for 18 hours as described in **section 2.2.3.2**. The analysis was done using IFN-γ ELISPOT assay (**Figure 4.13**). Interestingly, significantly higher IFN-γ production was observed with rH9HA-CD83 scFv and rH9HA-CD11c scFv compared to rH9HA ($p<0.0001$). No IFN-γ production was observed with rH9HA-Dec205 scFv.

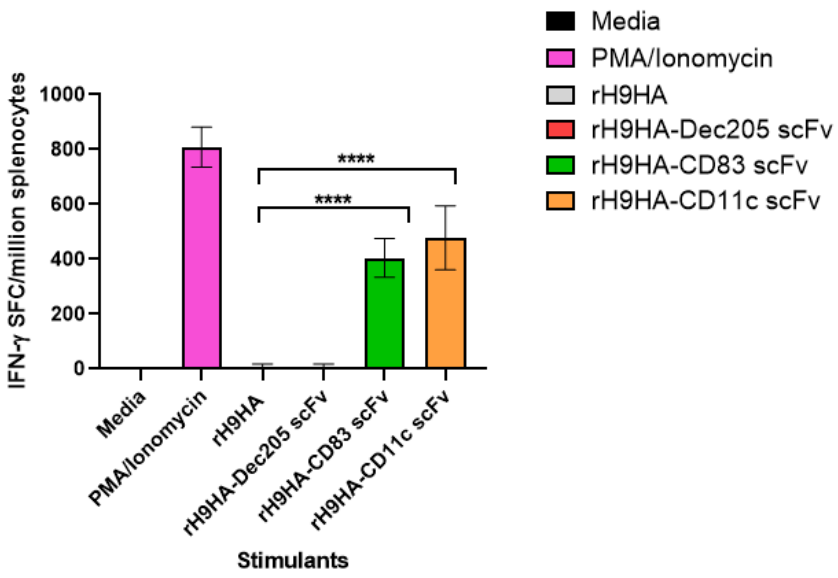
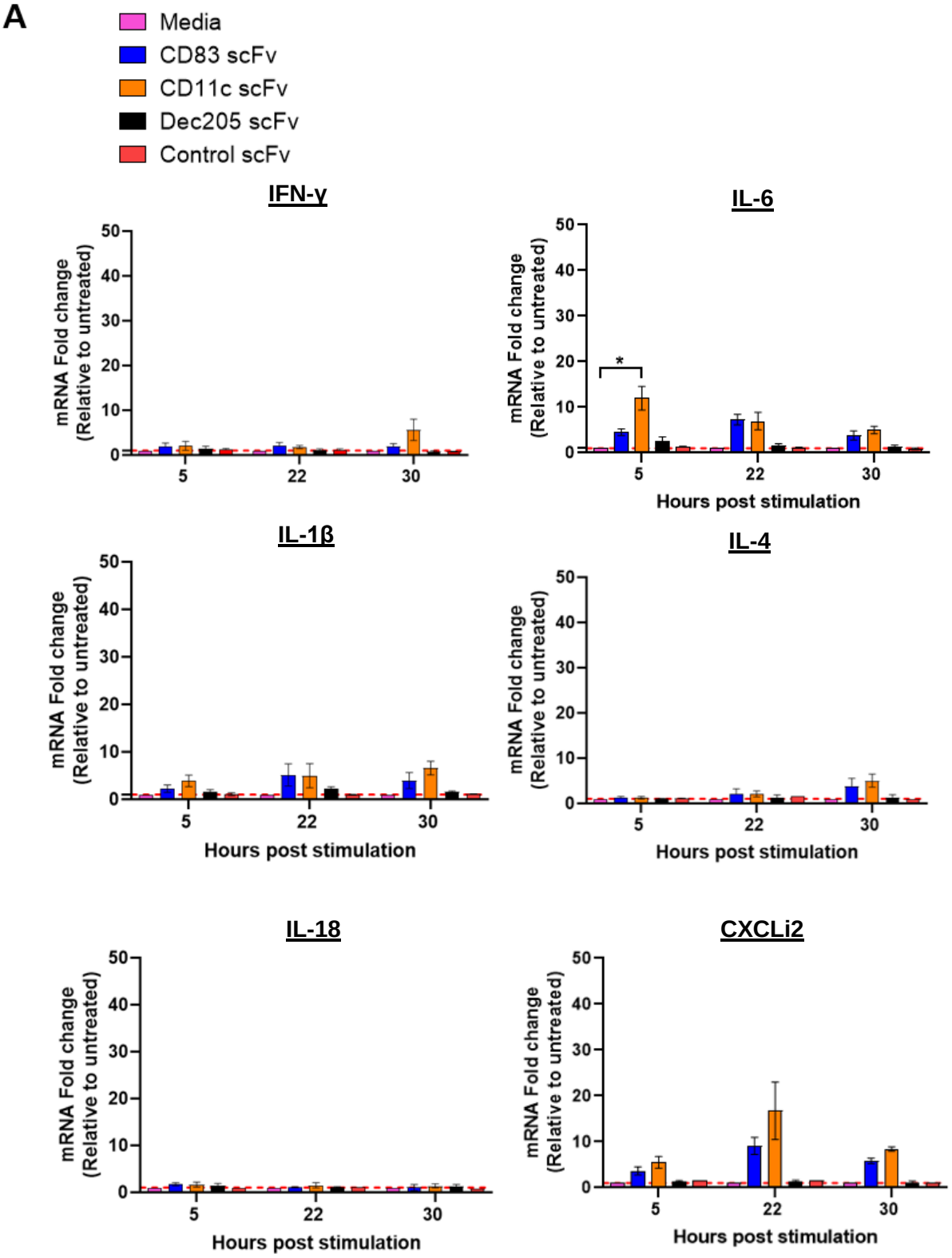


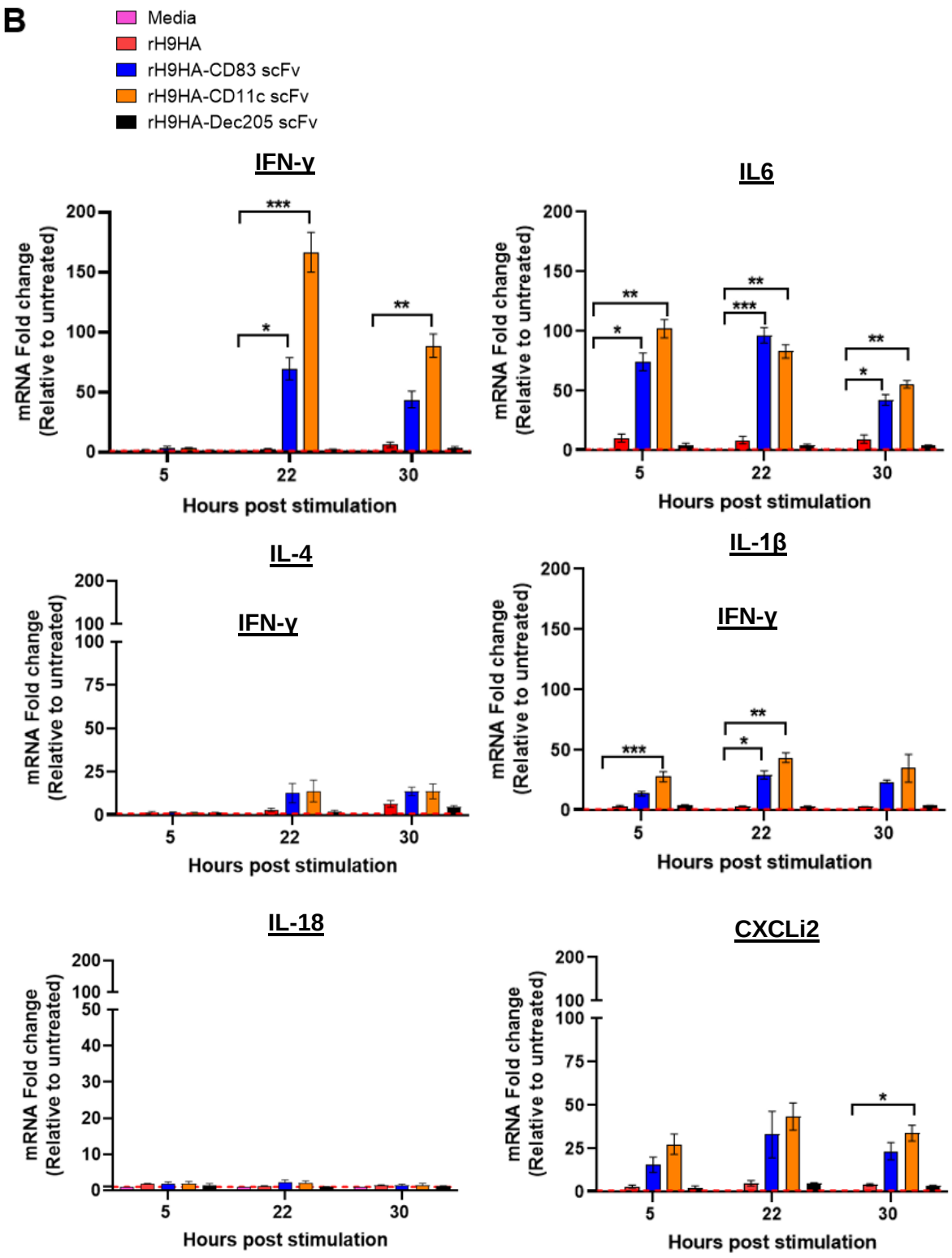
Figure 4.13. Analysis of IFN-γ production using IFN-γ ELISPOT assay. Briefly, ELISPOT plate (Life Technologies) was coated with anti-chicken IFN-γ (2 µg/mL) coating antibody overnight. The plate was washed with PBS containing 3% BSA and $5x10^5$ chicken splenocytes were added to each well of the plate. The chicken splenocytes were incubated with 10 µg rH9HA or 14 µg of rH9HA-Dec205/CD83/CD11c scFv (containing 10 µg rH9HA according to the molecular weight) for 18 hours. Detection was carried out using biotinylated anti-chicken IFN-γ detection antibody (1 µg/mL) for 1 hour at room temperature, followed by HRP conjugated streptavidin (1.25 mg/mL diluted to 1:1000) for another 1 hour at room temperature. Spots were developed by adding AEC substrate and counted using EliSpot reader (Autoimmun Diagnostika GMBH). Data are represented as mean±SD from three independent experiments using different spleen donor chickens. Statistical significance is shown with asterisks. ****p<0.0001

Next, we analysed the ability of scFv, rH9HA and rH9HA-scFv to induce the production of various cytokines and chemokine e.g., IFN-γ, IL-18, IL-4, IL-6, IL-1β , and CXCLi2 at different time points using qRT-PCR (**Appendix C**). With regards to scFv, Dec205 scFv was unable to induce the production of the tested cytokines. However, CD83 scFv and CD11c scFv stimulation showed moderate mRNA levels of IL-6 and CxCLi2 compared to the control scFv (**Figure 4.14A**). There was little or no induction of IFN-γ, IL-1β, IL-4 and IL-18. Furthermore, IFN-γ ELISA assay also confirmed no stimulation of IFN-γ production by different scFv (**Figure 4.14C**).

Moreover, rH9HA-Dec205 scFv was also unable to induce the production of tested cytokines just like Dec205 scFv. Interestingly, stimulation with rH9HA-CD83 scFv and rH9HA-CD11c scFv showed an overall higher mRNA levels of most of the cytokines tested compared to CD83 scFv and CD11c scFv, respectively. The rH9HA-CD83 scFv and rH9HA-CD11c scFv also showed significantly higher mRNA levels of IFN-γ, IL-6, IL-1β and CXCLi2 compared to rH9HA ($p<0.05$, **Figure 4.14B**). Furthermore, the induction of IL-6, IL-1β and CXCLi2 occurred earlier (after 5 hours post stimulation (ps)) whereas IFN-γ and IL-4 were induced later (after 22 hours ps). There was no induction of IL-18. In addition, the expression of IFN-γ in the culture supernatant after rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv stimulation was also verified using IFN-γ ELISA assay (**Figure 4.14C**). These results suggest that rH9HA-CD83 scFv and rH9HA-CD11c scFv can promote the stimulation of chicken splenocytes *in vitro*.

A


Media
CD83 scFv
CD11c scFv
Dec205 scFv
Control scFv
IFN-γ
IL-6
IL-1β
IL-4
IL-18
CXCLi2
mRNA Fold change (Relative to untreated)
Hours post stimulation
5
22
30
*


B
Media
rH9HA
rH9HA-CD83 scFv
rH9HA-CD11c scFv
rH9HA-Dec205 scFv
IFN-γ
IL6
IL-4
IFN-γ
IL-1β
IFN-γ
IL-18
CXCLi2
mRNA Fold change (Relative to untreated)
Hours post stimulation
5
22
30

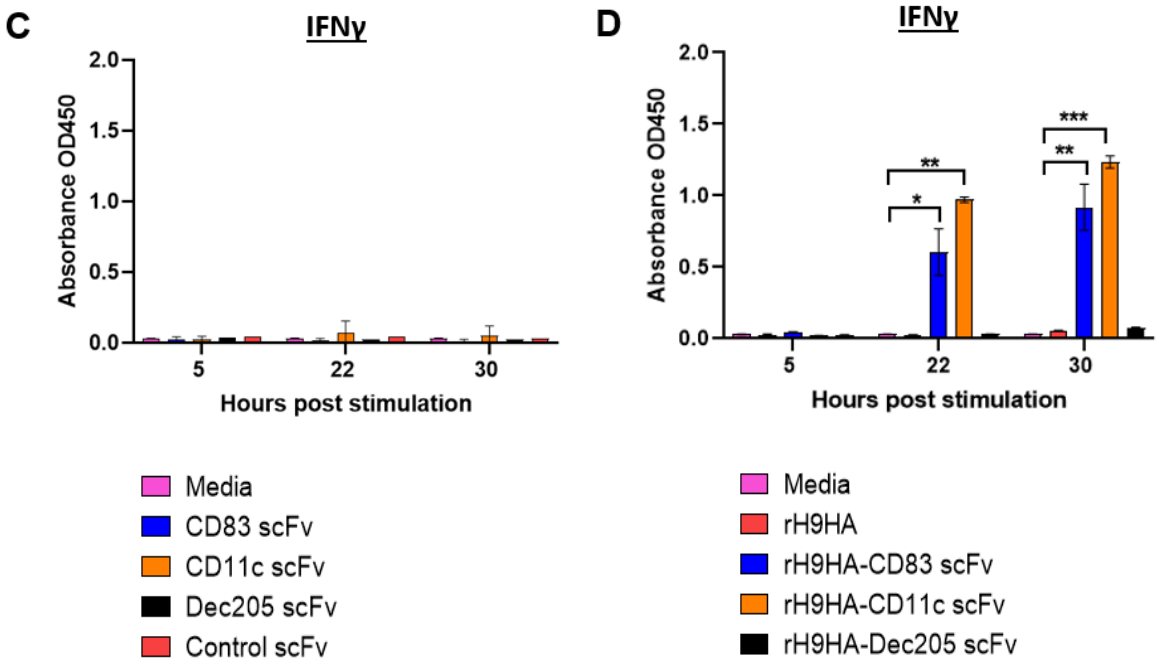


Figure 4.14: Analysis of cytokines (IFN-γ, IL-6, IL-1β, IL-4 and IL-18) and chemokine (CXCLi2) production by chicken splenocytes upon stimulation with scFv, rH9HA and Dec205/CD83/CD11c scFv analysed using qRT-PCR. **(B)** Cytokines and chemokine mRNA levels in chicken splenocytes stimulated with rH9HA and rH9HA-Dec205 scFv/ rH9HA-CD83 scFv/ rH9HA-CD11c scFv analysed using qRT-PCR **(C)** IFN-γ protein level upon stimulation of chicken splenocytes with Dec205/CD83/CD11c scFv analysed using ELISA. **(D)** IFN-γ protein level upon stimulation of chicken splenocytes with rH9HA and rH9HA-Dec205 scFv/ rH9HA-CD83 scFv/ rH9HA-CD11c scFv analysed using ELISA. For **A, B**; Splenocytes were isolated from the spleen of 3-week-old Rhode Island Red SPF chickens using Histopaque 1083 and stimulated with 10 µg of scFv/ rH9HA/ rH9HA-scFv for 5 hours, 22 hours and 30 hours *in vitro*. Stimulated splenocytes were harvested for RNA extraction and expression levels of the respective cytokines and chemokine were measured by qRT-PCR. Data were calculated using $2^{-\Delta\Delta CT}$ approach (n-fold change compared to the media only control group) and reported as values normalised to the expression level of a housekeeping gene RPLPO-1. For **C, D**; Supernatants from the stimulated splenocytes were analysed for the presence of IFN-γ by ELISA. Data are represented as mean±SD from three independent experiments using different spleen donor chickens and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ***p<0.001 **p<0.01 *p<0.05.

4.2.3.2. The rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv cannot proliferate chicken splenocytes

After assessing the ability of rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv to stimulate chicken splenocytes for the induction of various cytokines and chemokine, their potential for proliferating chicken splenocytes *in vitro* was also evaluated. Since, rH9HA-CD83 scFv and rH9HA-CD11c scFv were able to stimulate cytokines and chemokine production in splenocytes from antigen naive chickens, a test was done to analyse if they could proliferate splenocytes from antigen naive chickens. Therefore, splenocytes (naive) were harvested from the spleen of 3-week-old Rhode Island Red SPF chickens (National Avian Research Facility) and labelled with CFSE dye as described in **section 2.2.3.5**. The CFSE labelled cells were treated with rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv and incubated for 4 days. No proliferation of chicken splenocytes was observed with any of the treatments, except ConA which was used as a positive control (**Figure 4.15**).

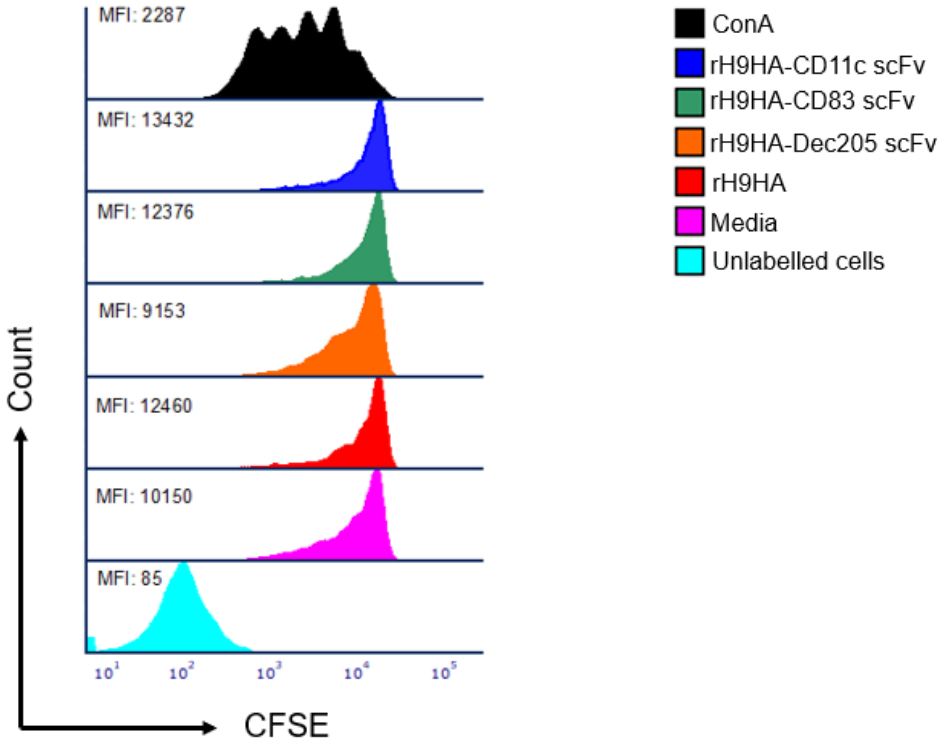


Figure 4.15. Analysis of the effects of rH9HA and rH9HA-scFv on the proliferation of chicken splenocytes. Chicken splenocytes were labelled with CFSE dye and incubated with 25 µg of rH9HA or 35 µg of rH9HA-Dec205/CD83/CD11c scFv (containing 25 µg rH9HA according to the molecular weight) and 10 µg/mL ConA (positive control) at 41°C for 4 days. The cell proliferation was measured using MACSQuant flow cytometer and analysed with FCS Express 6 software. About 10,000 events were acquired for the analysis. Cells were first gated based on forward scatter area (FSC-A) and side scatter area (SSC-A), followed by exclusion of doublets on SSC-A vs SSC-height density plot. Next, live cells were gated for the detection of $CFSE^+$ cells. ***CFSE****: Carboxyfluorescein succinimidyl ester* ***ConA****: Concanavalin A* ***MFI****: Mean Fluorscence intensity*

4.3. Discussion

Previous studies on targeting antigens to APCs using antibodies have shown to enhance the immunogenicity and protective efficacy of vaccines[396,446,540]. Such strategy is thought to facilitate the internalisation and activation of signalling pathways, which induce the maturation of DCs ultimately increasing antigen presentation and enhancing the induction of T cell responses[610–612]. To explore the immunogenicity and protective efficacy of TADV in chickens, and understand how

it compares to the mammalian studies, SPF chickens were immunised with rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv generated in Chapter 3. Antigen targeting to mammalian Dec205 and CD11c receptors have been reported before[276,280,281,483, 587]. Similarly, antigen targeting to chicken Dec205 receptor has also been carried out before[396] however, the study used full-length Dec205 mAb for antigen targeting which differed in the sequences of heavy and light chain CDRs compared to the one used in this work. This suggests that Dec205 mAb used in the previous work and generated here might recognise different chicken Dec205 receptor epitopes. Moreover, CD83 receptor has not yet been explored for antigen targeting in both mammalian and avian systems.

The overall results from *in vivo* dose titration vaccination study showed that serum HI antibody titres, anti-HA IgM and IgY antibodies and virus neutralisation antibody titres were higher in chickens vaccinated with the 20 µg and 35 µg doses of rH9HA-Dec205/CD83/CD11c scFv compared to the 2 µg dose of the respective vaccines. No significant differences were observed between the three doses of rH9HA, in terms of antibody titres. Furthermore, serum HI antibodies were detected as early as day 6 ppv with the 20 µg and 35 µg doses of rH9HA-Dec205/CD83/CD11c scFv vaccines compared to the untargeted rH9HA. The observed HI antibody titres on day 6 ppv were below the predictive protective value of $\geq$32 however, HI antibody titres between 10-40 have been previously associated with no prevention of viral shedding but complete prevention of mortality in H5N1 AIV infection[613]. It has been reported that inactivated virus vaccines are better at eliciting higher HI antibody titres compared to the subunit vaccines[614,615]. Here, it was observed that the 20 µg and 35 µg doses of recombinant subunit rH9HA-Dec205/CD83/CD11c scFv vaccines produced higher serum HI antibodies

compared to the inactivated H9N2 vaccine. An earlier study in chickens assessed the targeting of H5HA antigen to Dec205 receptor using full-length Dec205 mAb[396]. The study reported high serum antibody titres with 50 µg and 100 µg of the targeted H5HA protein on day 14 ppv. A direct comparison between the previous and current studies cannot be made because the previous study used Dec205 full-length mAb instead of scFv antibody, LPS as adjuvant and a single vaccination regime. Nevertheless, using lower vaccine dose can provide additional advantage of dose sparing, which is more cost-effective as well as reduces adverse reactions at the site of injection[616]. Moreover, anti-HA IgM and IgY antibodies were predominantly detected in the serum of chickens vaccinated with inactivated H9N2 vaccine and recombinant subunit targeted and untargeted rH9HA vaccines. Low detection of anti-HA IgA antibodies was observed in the immunised serum. This is expected as IgY (5-15 mg/mL) and IgM (1-3 mg/mL) antibodies are found in higher concentration than IgA (0.3-0.5 mg/mL) in the chicken serum[617]. Furthermore, inactivated and subunit influenza vaccines are known to induce high serum and mucosal IgG in humans, but not IgA. It is normally live vaccines administered via oral or nasal route that induce serum IgA and secretory IgA[618]. Indeed, upon administration of live UDL 01/08 H9N2 virus intranasally as 'challenge virus', serum anti-HA IgA antibody levels were significantly increased in both vaccinated and unvaccinated groups of chickens (**section 4.2.2.1**). The first antibodies to be produced in a humoral immune response are always IgM, because IgM can be expressed without isotype switching. Other isotypes like IgG and IgA are formed after isotype class switching at later phases of antibody responses[619]. The results of class switched antibody indicate that DCs, $CD4^+$ T cells and B cells are being stimulated and are involved in processing the antigen. The rH9HA-CD83 scFv was

better in inducing higher anti-HA IgM and IgY antibodies with the 20 µg and 35 µg doses compared to rH9HA, followed by rH9HA-CD11c scFv and rH9HA-Dec205 scFv. In addition, among all the vaccine groups tested, rH9HA-CD83 scFv group induced the highest virus neutralisation antibody titres with both the 20 µg and 35 µg doses. This suggests that targeting antigen to chicken CD83 induces higher humoral response compared to Dec205 and CD11c targeting. Moreover, variation was observed in the antibody responses to vaccines in the chickens within the same group. This could be due to the outbred nature of chickens, as outbred animals are expected to demonstrate more trait variability than inbred animals[620]. Such variability can be observed in the immune related genes including those involved in antigen processing and presentation. Hence, heterogeneity in DC subsets, macrophages and lymphocyte population, and MHC genes could have contributed to the variable immune responses in chickens within the same group.

Upon comparison of different targeting antibodies, including those specific for mouse MHC class II, CD11a, CD11b, CD11c, Dec205 and CD40, CD11c targeting was found to be the most potent among all the receptors targeted[408]. Furthermore, another study demonstrated that OVA antigen targeted to CD11c was 3-4-fold more efficient in priming T cells for proliferation compared to Dec205 targeting in mice[381]. This adjuvant effect of anti-CD11c targeting was reported to be due to the enhanced germinal centre and extrafollicular plasma cell responses, indicating that anti-CD11c binding to DCs can have direct stimulatory effect on the activation of antigen-specific B cells[408]. On the contrary, some studies have demonstrated either equal effectiveness of both CD11c and Dec205 targeting[621,622] or superior efficiency of Dec205 targeting over CD11c targeting in mice[587]. Overall, similar humoral responses were elicited by CD11c and Dec205 antigen targeting in

our work although, CD11c targeting induced slightly higher serum HI antibody titres. The chicken CD11c mAb (clone: IAH 8F2) used to generate the targeting CD11c scFv for our work also binds to some uncharacterised proteins other than CD11c receptor protein in chicken splenocytes. Therefore, this could have influenced the antibody responses obtained with rH9HA-CD11c scFv. In future, it is important to characterise all the additional proteins bound by CD11c mAb (clone: IAH 8F2) as this could potentially have unknown effects *in vivo*. The rH9HA-CD83 scFv demonstrated the highest antibody responses measured using HI assay, ELISA and VNT assay. It has been reported that anti-CD83 mAb can acquire adjuvant effect by modulating the regulation of B cell function[471,486]. Mice injected with nitroiodophenol conjugated to Ficoll (NIP-Ficoll) along with anti-CD83 mAb showed a dramatic increase in the production of NIP specific IgG1 antibodies, indicating that the ligation of anti-CD83 mAb to naturally induced CD83 receptor protein strongly affects antigen-specific IgG responses of B cells *in vivo*[486]. This could be a potential reason for the enhancement of antibody responses by CD83 scFv targeted rH9HA vaccine. In majority of studies involving antigen targeting to APC receptor using antibodies, the targeted antigen has always been administered in combination with DC activating agents, mostly TLR ligands[396,603,623,624]. In fact, antigens targeted to Dec205 in the absence of DC activating stimuli has shown to induce immune tolerance[432,592]. However, one study reported that enhanced humoral responses could be induced in mice with APC receptor-specific antigen targeting without the need of adjuvant[586]. In the current work, all the vaccines were formulated in Montanide™ ISA 71 VG (Seppic) water-in-oil (W/O) adjuvant. The W/O adjuvant has depot effect, enables enhanced recruitment of immune cells, facilitates the antigen uptake by APCs, and is considered safer than adjuvants

based on TLR ligands[261,625,626]. It has been suggested that some DC surface receptors can induce intracellular signalling and DC activation upon interaction with its natural ligand or agonistic mAb. This could likely enhance the immune responses without the need of DC activating adjuvants[627], benefitting the use of TADV.

The rH9HA-CD83 scFv vaccine was taken forward for the protective efficacy study since, it induced higher antibody responses compared to Dec205 and CD11c targeting. All chickens in the vaccinated groups: inactivated H9N2 vaccine, rH9HA and rH9HA-CD83 scFv survived the challenge, and showed lower clinical signs compared to the chickens in PBS control group. Furthermore, the inactivated H9N2 vaccine and rH9HA-CD83 scFv displayed lower clinical signs compared to rH9HA group in the first three days post virus infection. In addition, rH9HA-CD83 scFv was better at reducing the viral load in H9N2 virus infected chickens compared to rH9HA, and at a level comparative to the inactivated H9N2 vaccine. Moreover, rH9HA-CD83 scFv 'contact' birds also showed lower viral load compared to the PBS 'contact' group. This suggests that vaccination with rH9HA-CD83 scFv can lower the chance of getting the virus infection, while sharing the same air space, water, and food with the infected chickens. However, rH9HA-CD83 scFv vaccine does not provide sterile immunity as the vaccinated chickens still got infected and shed virus. Due to the limited number of isolators and space constraints within the isolators, we were unable to add inactivated H9N2 vaccine and rH9HA vaccine 'contact' groups. In future studies, it would be interesting to compare the viral load between different vaccinated 'contact' groups. It is well known that the inactivated influenza virus vaccines can produce antibodies not just against HA protein but also against other viral proteins like NA and M1/M2. Furthermore, they are also capable

of stimulating innate immunity via pattern recognition receptor-based activation of immune cells[614,628]. These factors could further enhance the protection following virus infection. On the other hand, recombinant subunit HA vaccine is only able to produce antibodies against HA protein and requires adjuvants to induce CMI[629]. It is therefore promising that the recombinant rH9HA-CD83 scFv vaccine was able to induce a similar level of protection to the industrial standard inactivated H9N2 vaccine. Previous studies have demonstrated that inactivated influenza virus vaccine can induce superior immune responses over those induced by subunit vaccine in mice[582,615,630]. In particular, they could generate Th1 response and induce cross-protective cytotoxic T cells[631]. This was attributed to triggering TLR 7 by single-stranded viral RNA which is present in inactivated virus vaccine but not in subunit vaccine[614]. It has been reported that antigen targeted to Dec205 and CD11c in mice can induce CMI, measured by the expression of IFN-γ[446,540]. In both cases, adjuvants were used: Poly I:C and anti-CD40 mAb, respectively. Here, the CMI induced by inactivated H9N2 vaccine and recombinant subunit rH9HA and rH9HA-Dec205/CD83/CD11c vaccines were assessed by IFN-γ ELISPOT assay using rH9HA, inactivated H9N2 virus and live H9N2 virus (MOI 1) as recall antigens. However, no spots were detected even with the positive control (splenocytes from chickens infected with live H9N2 virus) (data not shown). No IFN-γ stimulating effect with inactivated virus used as a recall antigen has been reported elsewhere[632]. On the contrary, several studies have used whole protein as a recall antigen with success[446,632,633]. The antigen peptides (~9-20 amino acid long) are generally preferred for the use as recall antigens for stimulating both $CD4^+$ and $CD8^+$ T cells, as these shorter peptides correspond to the length of MHC binding

sections[634]. Therefore, it is important to identify and optimise the recall antigen for chicken IFN-γ ELISPOT assay.

The mechanism of Dec205, CD83 and CD11c targeted rH9HA was assessed by the *in vitro* stimulation of chicken splenocytes. The *in vitro* responses might not be predictive for *in vivo* immunogenicity in all cases however, many studies have successfully used *in vitro* stimulation of immune cells to investigate the immunogenic potential of vaccines and adjuvants. This has been done by determining the capacity of the stimulant to induce the expression of maturation markers and/or cytokine production[615,628,635]. Here, the priming effects of scFv and scFv conjugated to rH9HA on chicken splenocytes was evaluated by assessing the mRNA expression levels of different cytokines (Th1: IFN-γ, Th2: IL-4, Pro-inflammatory cytokines: IL-1β, IL-18 and IL-6, Chemokine: CxCLi2). Dec205 scFv and rH9HA-Dec205 scFv were unable to induce the production of any of the cytokines tested. On the other hand, CD83 scFv and CD11c scFv induced low mRNA levels of IL-6, IL-1β and CXCLi2, but no IFN-γ. Interestingly, rH9HA-CD83/CD11c scFv showed a higher upregulation of mRNA encoding IFN-γ, IL-6, IL-1β and CxCLi2 compared to rH9HA. No significant differences were observed in the production of IL-4 between rH9HA and rH9HA-CD83/CD11c scFv however, the level of IL-4 mRNA was higher in the latter. The subunit HA vaccine has shown to have only minor effects on cytokine production by murine bone marrow derived DCs (BMDCs) *in vitro*[628]. Studies in mice have demonstrated that subunit influenza vaccines elicit Th2 dominated response characterised by IgG1 and IL-4 producing T helper cells[615,636]. According to our observation, subunit rH9HA-CD83/CD11c scFv was able to induce IFN-γ production *in vitro,* indicating Th1 response. Furthermore, a strong antibody response was generated by rH9HA-CD83/CD11c

scFv *in vivo*, indicating Th2 response. Hence, rH9HA-CD83/CD11c scFv vaccine might be able to evoke both Th1 and Th2 responses *in vivo*. However, Th1 response *in vivo* with rH9HA-CD83/CD11c scFv needs to be confirmed. This could be one of the reasons why rH9HA-CD83 scFv showed better protective efficacy compared to rH9HA *in vivo*. Overall, rH9HA-CD83/CD11c scFv was better at inducing higher mRNA levels of cytokines compared to CD83/CD11c scFv alone. It has been suggested that binding of antigen to antibody can result in the conformational changes in the antibody. This conformational change can enhance the uptake of antigen-antibody complexes by APCs compared to the free antibody hence, increasing the immunostimulatory potential of the antigen[637]. In addition, it is also possible that the observed stimulation effects could be attributed to the trimeric form of scFv in rH9HA-CD83/CD11c scFv compared to the monomeric form in CD83/CD11c scFv. Furthermore, the differences observed in the ability of Dec205 scFv, CD83 scFv and CD11c scFv to stimulate chicken splenocytes could also be attributed to the differential expression of the targeted receptors by different immune cells e.g. CD83 and CD11c is expressed not only on DCs but also on activated lymphocytes and macrophages, whereas Dec205 receptor is exclusively expressed on dendritic cells[306,324,638]. Previously, differences were observed in the mRNA levels of cytokines and chemokines upon stimulating chicken splenocytes and PBMCs by flagellin conjugated H7HA[633]. Therefore, it would be interesting to see how the mRNA levels of cytokines and chemokines might differ upon stimulating chicken PBMCs by rH9HA Dec205/CD83/CD11c scFv. Moreover, no proliferation of chicken splenocytes was observed upon stimulation with rH9HA and rH9HA-Dec205/CD83/CD11c scFv *in vitro.* It is possible that splenocytes from

vaccinated chickens are required to see the proliferation effect upon *in vitro* stimulation with rH9HA-Dec205/CD83/CD11c scFv[639,640].

In conclusion, the immunogenicity of rH9HA and rH9HA-Dec205/CD83/CD11c scFv vaccines were assessed, with rH9HA-CD83 scFv demonstrating the highest antibody response in chickens. Furthermore, rH9HA-CD83 scFv vaccine also evoked a higher protective efficacy compared to rH9HA, following H9N2 virus infection. The *in vitro* cytokine gene expression profile and *in vivo* strong antibody response indicate that rH9HA-CD83 scFv could induce both Th1 and Th2 immune responses *in vivo*. However, Th1 response by rH9HA-CD83 scFv *in vivo* further needs to be confirmed.

Chapter 5: Further evaluation of the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines in SPF and H9HA-specific MDA positive chickens

Chapter summary

In this chapter, a further evaluation of the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines was performed in SPF and H9HA-specific MDA positive chickens. Overall, rH9HA-CD83 scFv vaccine showed a higher serum antibody response in SPF chickens as measured by HI and virus MNT assays, against various heterologous H9N2 viruses compared to rH9HA and inactivated H9N2 vaccines. Furthermore, no differences were observed between a single and double vaccine doses of rH9HA-CD83 scFv on antibody responses in SPF chickens. Therefore, rH9HA-CD83 scFv vaccine can be recommended for a single dose administration to 1-day-old chickens. In addition, rH9HA-CD83 scFv vaccine was stable for at least 1 year when stored at 2-8°C. Most importantly, among all the vaccines tested only rH9HA-CD83 scFv was able to overcome the MDA interference.

Chapter aims

- To analyse the cross-reactivity of immunised serum against different H9N2 viruses.

- To analyse the cytokines produced in the spleen of vaccinated chickens at different time points.
- To evaluate the effects of vaccination regimes on antibody responses elicited by rH9HA and rH9HA-CD83 scFv vaccines.
- To evaluate the stability of rH9HA-CD83 scFv vaccine.
- To compare the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines in H9HA-specific MDA positive chickens.

5.1. Introduction

There are many factors that influence the outcome of AIV vaccination and challenge experiments. Such factors include type of chickens used, age of vaccination, vaccine formulation, vaccine dose and route of vaccination[641]. With regards to the type of chickens used, it is important to consider their 'pathogen' status and understand how it might affect the immunological outcome. The SPF chickens are guaranteed free of designated viral and bacterial pathogens. This ensures that specific pathogens or presence of antibodies to those pathogens do not interfere with the vaccination regime, providing 'standard' means of testing the vaccine efficacy[641]. However, this might not adequately reflect the situation in the field. On the contrary, the use of MDA positive chicks relates more closely to the field situation, as most hens in the field are vaccinated against various viral and bacterial pathogens. Different MDA levels in the hatched offspring either due to the age of the hens from which they are hatched and/or vaccination schedule used in those hens, introduce significant amount of variability between experiments[642,643]. Moreover, some studies demonstrated similar protection upon Infectious Bronchitis

(IB) virus challenge following vaccination to 1-day old SPF and MDA positive chickens[644]. However, others found that SPF chickens were fully protected with no virus detection following IB virus challenge, whereas the replication of challenge virus was reduced but not completely eliminated in MDA positive chickens[645]. Therefore, it is important to test the efficacy of poultry vaccines in both SPF and MDA positive chickens. Furthermore, it is necessary that all the AIV vaccines for commercial use are produced according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Evidence should demonstrate that the vaccine significantly reduces virus excretion from vaccinated birds if they are subsequently infected, and that the vaccine is able to prevent virus circulation in the target species. Storage and transportation conditions of the vaccines, and the vaccination schedule should be used in strict adherence to the manufacturer's recommendations. Currently, two major AIV vaccine types are commercially available for use in poultry: i) Inactivated virus vaccines (monovalent, bivalent, or multivalent) ii) Live recombinant viral vector vaccines (FPV, HVT, NDV). These vaccines are given either as emergency vaccination in the face of an epizootic, preventive vaccination if there is a high risk of virus incursion and routine vaccination in enzootic areas[646]. Only high-quality vaccines produced according to the OIE standards are used in poultry vaccination programmes.

The spread of LPAI H9N2 virus across Asia, Middle East and North Africa has resulted in the routine AIV vaccination programs using inactivated AIV vaccines in several countries in this region[647,648] . Furthermore, the outbreaks of H5 and H7 subtypes of HPAI viruses have resulted in devastating consequences for the commercial poultry sector, compelling many countries to enforce intensive AIV vaccination campaigns[642,649]. As a consequence of these vaccination programmes,

progeny chickens from vaccinated hens will have MDAs in the first weeks after hatch[650]. MDAs are vertically transferred from mothers to the hatching progeny[651]. In chickens, serum IgY antibodies are transported from the mother hen's blood stream into the oocyte (which becomes the egg yolk) through a specific receptor that recognises the Fc domain[652,653]. During the embryo development, IgY antibodies are transported from the egg yolk into the embryo's systemic circulation through receptor-mediated transcytosis[654,655]. The IgY antibodies are transported as early as seven days into incubation, increases over time and peaks towards the last couple of days before hatch[656,657]. On the other hand, IgM and IgA antibodies are secreted by the mucosa of the oviduct and incorporated in low amounts into the egg during egg formation[658]. It is reported that these IgM and IgA antibodies are ingested by the embryo from the amniotic fluid and transported to the gut in very low amounts. Therefore, maternal IgM and IgA antibodies in young chicks are thought to protect gastro-intestinal tract against pathogens without having effect on vaccine induced immune responses[567-660]. Hence, for the purpose of this thesis, the term 'maternally derived antibodies' will be used for passively transferred IgY antibodies. Such MDAs passed through the egg to the hatching progeny (passive immunity) are important for protection against pathogens in the neonatal stage, when immunocompetence is not fully developed in the chickens[661–663]. The amount of MDAs transferred to the offspring is dependent upon the circulating antibodies in the hens (produced from vaccination or natural infection) at the time of egg laying, and they naturally decline in concentration, usually between 2 to 3 weeks of age in chickens[664]. Even the low titres of MDAs are still able to inhibit vaccination against infectious diseases in chickens. It is this phase of decaying MDAs that presents a window of opportunity for infection by pathogens in neonatal chickens[660]. In

mammals, various mechanisms have been suggested to explain the lack of vaccination success in the presence of MDAs (**Figure 5.1**). MDAs can often interfere with vaccination by masking the specific epitopes of the vaccine antigen, affecting the antigen recognition and presentation by APCs[545]. Furthermore, it has also been reported that B cell activation can be inhibited through a cross-link between B cell receptor (BCR) and the Fcγ-receptor IIB (CD32) present on B cells by vaccine-MDA complex[665]. Other possible mechanism include neutralisation of the vaccine virus or opsonisation of vaccine antigens[660]. The MDA interference is thought to be one of the main reasons for the lack of virus eradication success using AIV vaccines in several HPAI virus enzootic countries such as Egypt and Mexico[666–668]. Several studies have reported poor induction of antibody titres after vaccination of chickens with MDAs[643,649,669]. Apart from the inactivated virus vaccines (which are most widely used in the field), some recombinant viral vector vaccines such as FPV or NDV are also shown to be impacted by MDA interference[670,671]. These observations make it necessary to further explore alternative vaccines that are lesser impacted by MDAs.

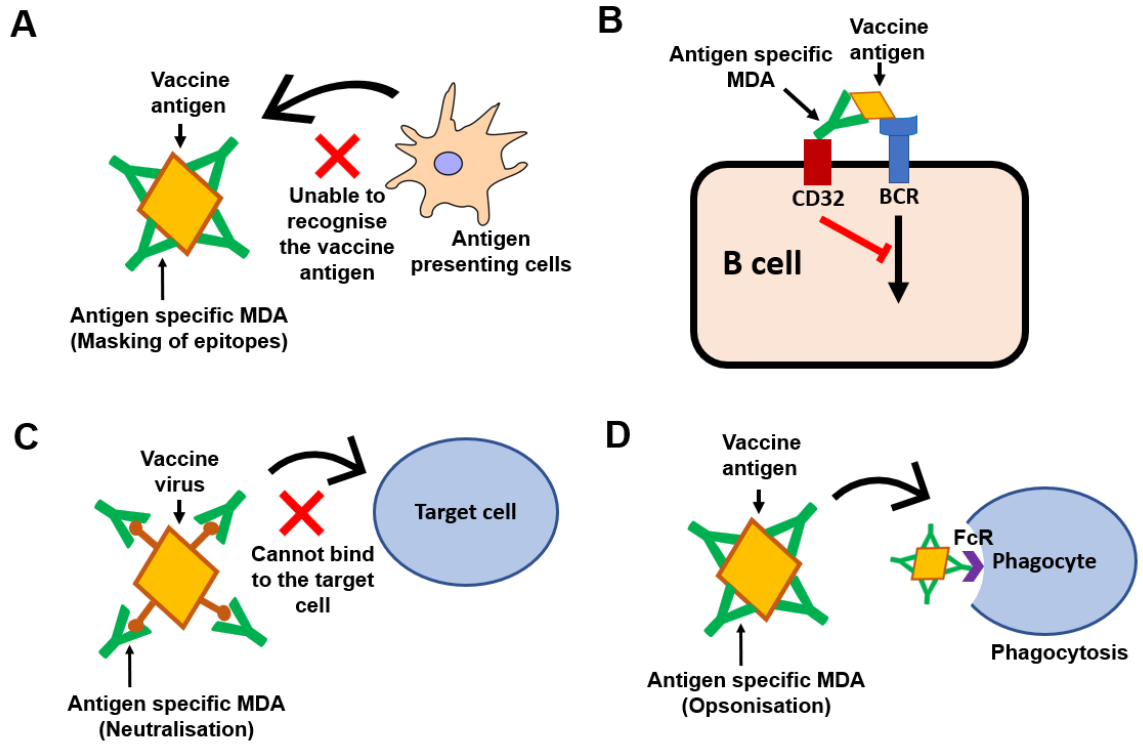


Figure 5.1. Possible mechanisms for the vaccine failure in the presence of MDAs. **A)** Epitope masking. Various epitopes on the antigen can be covered by the MDAs. This could prevent the antigen presenting cells from recognising and presenting the antigens. **B)** B cell inhibition. In the presence of MDAs, the antigen-MDA complex binds to FcγRIIB (CD32) receptor on B cells via the Fc region of MDA. CD32 is the only Fc receptor on B cells and does not bind to other immunoglobulins like IgM or IgA. The juxtaposition of the BCR and CD32 delivers a negative signal through BCR even after binding to the antigen. Thus, B cell activation is inhibited. **C)** Neutralisation. MDAs can bind to the vaccine virus and prevent them from binding to the target cells. **D)** Opsonisation. MDAs can bind to the vaccine antigens and tag them for phagocytosis, which in turn destroy the antigens. ***BCR***: B cell receptor ***MDA***: Maternally derived antibodies ***FcR***: Fc receptor

In Chapter 4, the immunogenicity and protective efficacy of rH9HA-CD83 scFv vaccine were investigated in SPF chickens. Furthermore, the immunogenic potential of rH9HA, rH9HA-Dec205 scFv, rH9HA-CD83 scFv and rH9HA-CD11c scFv for the induction of various cytokines upon *in vitro* stimulation of chicken splenocytes was also examined. Here, a further evaluation of the immunogenicity of rH9HA-CD83 scFv was made in SPF chickens in order to investigate its ability to produce broadly cross-reactive antibodies, vaccination regimes and stability.

Furthermore, the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine was also examined in H9HA-specific MDA positive chickens.

5.2. Results

5.2.1. Evaluation of the cross-reactivity, vaccination regimes and stability of rH9HA-CD83 scFv vaccine in SPF chickens

The experimental design for a further evaluation of the immunogenicity of rH9HA, rH9HA-CD83 and inactivated H9N2 vaccines in SPF chickens is described in **section 2.2.8.3.3** and shown in **Figure 5.2**. Briefly, groups of 1-day-old White Leghorn SPF chickens (MSD Animal Health) were vaccinated with rH9HA (35 µg/dose, n=50 per group), rH9HA-CD83 scFv (49 µg/dose equivalent to 35 µg/dose of rH9HA according to the molecular weight, n=50 per group) and inactivated H9N2 vaccine (UDL 01/08, 1024 HAU/dose, n=40 per group). The rH9HA and rH9HA-CD83 scFv groups were further divided into single and double vaccine groups, whereby the latter received a second dose at 7-day-old (day 7 ppv). In all cases, blood samples were collected on day 6, 14, 21, 28 and 35 days ppv for serological analysis. Furthermore, some chickens (n=6 per group, single vaccine group) were culled, and spleen samples were collected on day 2, 4, 6, 8 and 21 pv. Chickens were observed daily for signs of clinical disease. No clinical signs were observed in the vaccinated chickens. All chickens were culled via cervical dislocation and decapitation methods.

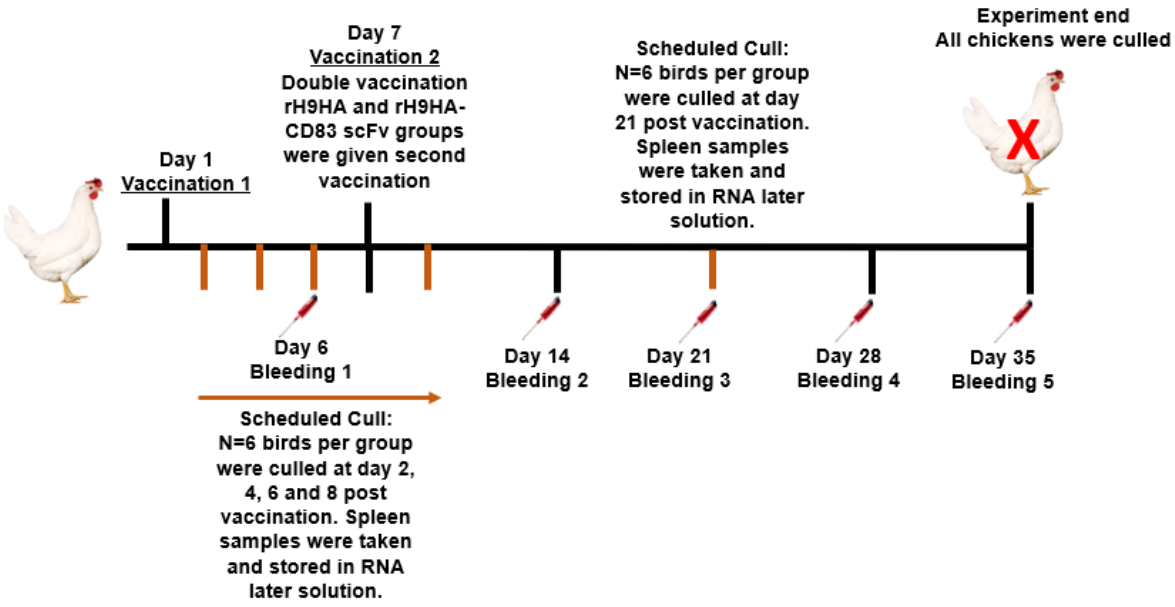


Figure 5.2. Experimental design to further evaluate the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine in SPF chickens. Groups of White Leghorn SPF chickens were immunised once at 1-day-old with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose) (n=50 per group for rH9HA, rH9HA-CD83 scFv groups and n=40 per group for inactivated H9N2 vaccine). The rH9HA and rH9HA-CD83 scFv groups were further divided into single and double vaccine groups (n=10 per group) whereby the latter received a booster vaccination on day 7 post primary vaccination (ppv). The vaccine dose for the booster vaccination was same as that of the primary vaccination. In all cases, blood samples were collected on day 6, 14, 21, 28 and 35 ppv. Furthermore, spleen samples (n=6 per group) were also collected in RNA-later solution on day 2, 4, 6, 8 and 21 post vaccination.

5.2.1.1. The rH9HA-CD83 scFv vaccine produces higher cross-reactive antibodies compared to rH9HA and inactivated H9N2 vaccines

The serum samples (day 35 pv) were obtained from the chickens immunised at 1-day-old with a single dose of the respective vaccines. To investigate the cross-reactivity of different serum samples against heterologous H9N2 influenza viruses, the HI and virus MNT assays were performed against a panel of both closely and distantly related viruses (**Table 29 and Figure 5.3**). The results indicate a general trend whereby the serum displayed higher cross-reactivity with more closely related

viruses and lower cross-reactivity with distantly related viruses (**Figure 5.4**). Interestingly, the serum from rH9HA-CD83 scFv group also showed significantly higher cross-reactive HI antibodies and virus neutralising antibodies compared to rH9HA and inactivated H9N2 vaccine groups with most of the viruses tested ($p<0.05$), despite of having the same HA content. Moreover, there was a positive correlation between the data obtained from HI and virus MNT assays (Pearson correlation coefficient 'r'=0.8). Overall, the serum from rH9HA-CD83 scFv group showed a higher cross-reactivity with different heterologous H9N2 viruses compared to other vaccinated groups.

Table 29: The HA amino acid sequence similarity (%) between UDL 01/08 and other H9N2 viruses tested for the serum cross-reactivity.

Virus strain name	Lineage	HA amino acid similarity (%) with UDL 01/08 virus
UDL 01/08	G1	100
Egy/D7100	G1	97
Ind/WB	G1	96
Isr/239	G1	96
BD/10306	G1	96
VN/38	G1	92
HK/3239	BJ94	91
Em/R66	G1	94
WZ/606	BJ94	89
UAE/D1556	G1	93
SKP/2016	G1	95
Wis/66	USA	87

The HA sequence similarity was computed using Clustal Omega (Multiple Sequence Alignment) software (EMBL-EBI).

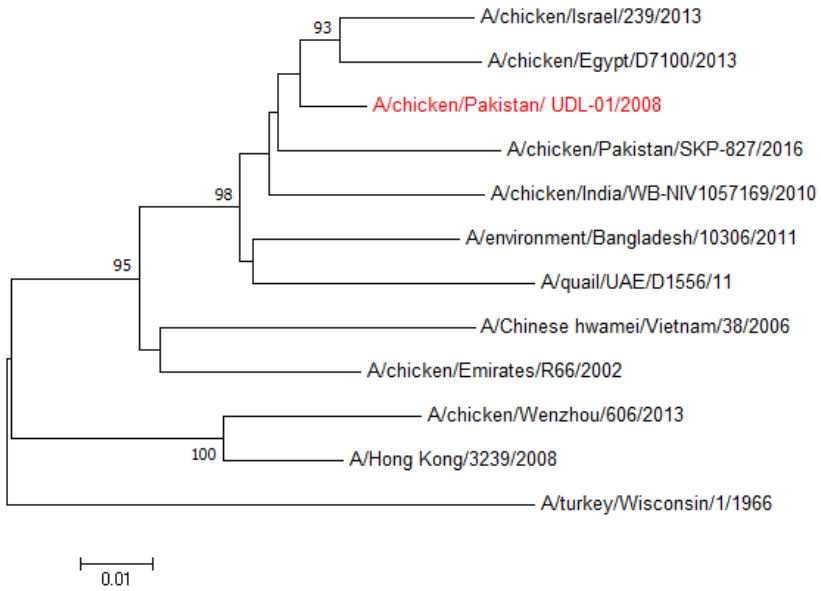


Figure 5.3. Phylogenetic tree of H9N2 viruses used for testing the cross-reactivity of the immunised serum. The HA sequences were aligned using the MUSCLE algorithm in Mega software version 7. Phylogenetic tree was created using the Neighbour-joining algorithm with 1000 bootstrap replications. Bootstrap values of <70 was excluded from the tree. The H9N2 virus that is homologous to rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines is represented in red.

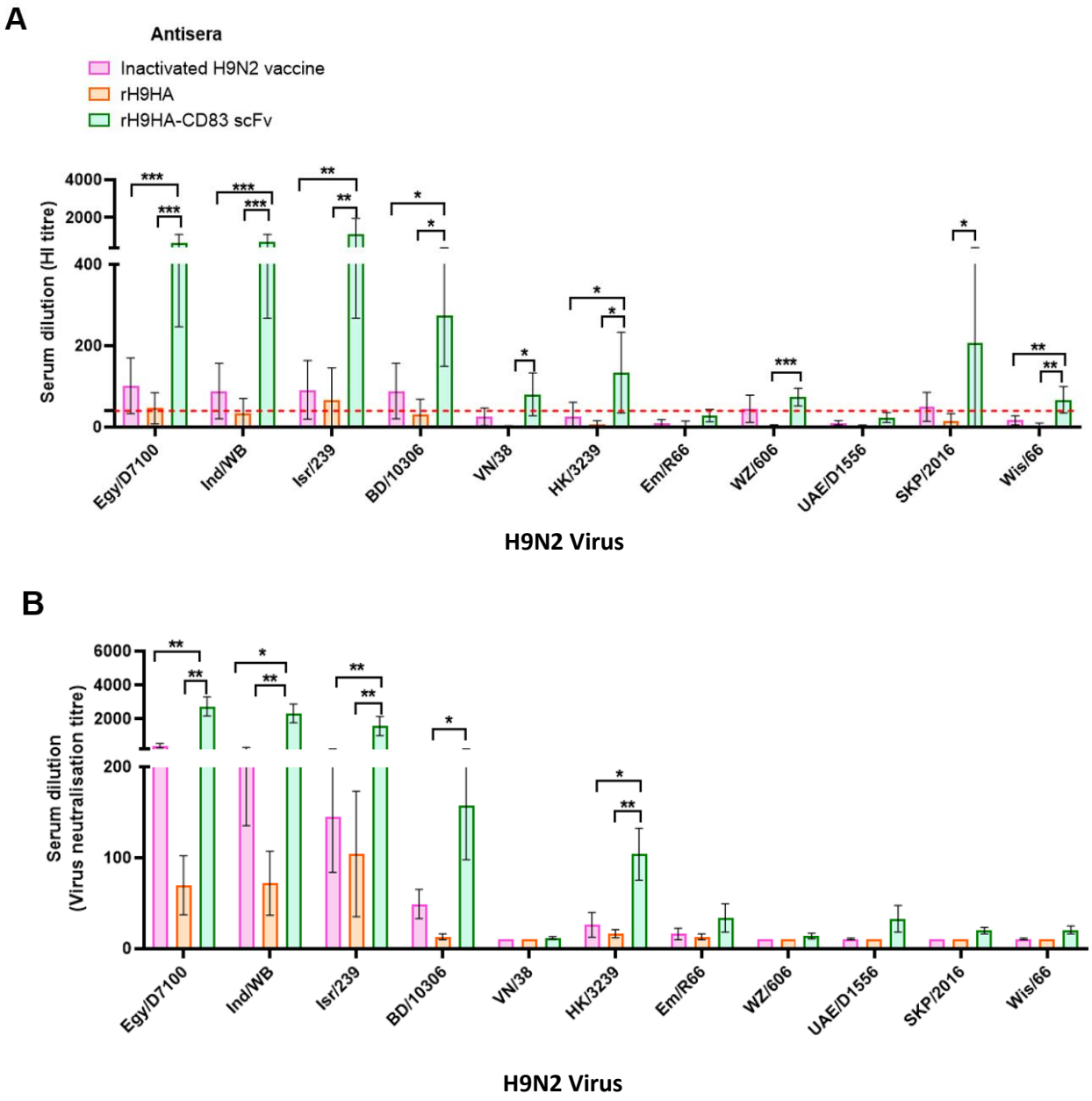


Figure 5.4. Cross-reactivity of the serum of chickens vaccinated with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines against different H9N2 viruses. **A)** Serum cross-reactivity test using HI assay. **B)** Serum cross-reactivity test using virus MNT assay. Groups of 1-day-old White Leghorn SPF chickens (n=10 per group) were vaccinated once with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose). In all cases, blood samples were collected on day 6, 14, 21, 28 and 35 post vaccination. For both assays, day 35 post vaccination serum was used. For **A**, HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of virus haemagglutination activity. The red dotted line indicates the predictive protective HI titre of 32. For **B**, virus MNT titres were expressed as the reciprocal of the highest serum dilution at which virus infection is blocked and the cells survive. Data are presented as mean ± SD (n=10 per group) and analysed by one-

way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ***p<0.001 **p<0.01 *p<0.05

5.2.1.2. Spleen samples from the vaccinated chickens do not show upregulation of cytokines

Monitoring the cytokine response after vaccination could provide an important tool for measuring the efficacy and safety of the vaccine. Here, the cytokine and chemokine responses elicited after immunisation with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines were investigated. Spleen samples were collected from the chickens vaccinated with a single dose of the respective vaccines on day 2, 4, 6, 8 and 21 pv, and RNA was extracted. The extracted RNA was then subjected to qRT-PCR for assessing the expression of different cytokines and chemokines; IFN-γ, IL-6, IL-1β, CxCLi2, IL-18, IL-4, IL-10 and TGF-β. No upregulation in the mRNA levels of cytokines was detected in the vaccinated spleen samples compared to the unvaccinated controls at different time points (**Figure 5.5, Appendix C**).

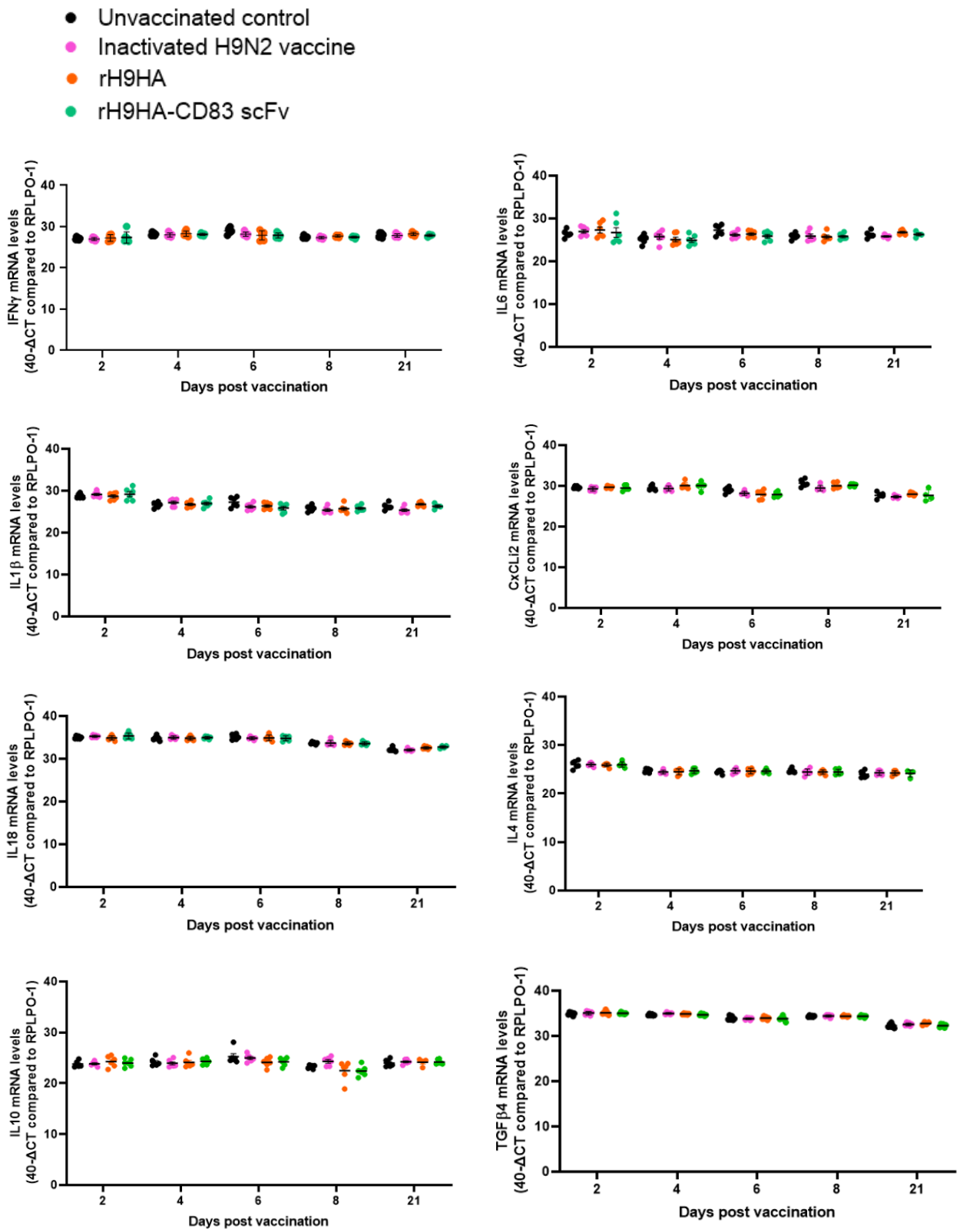
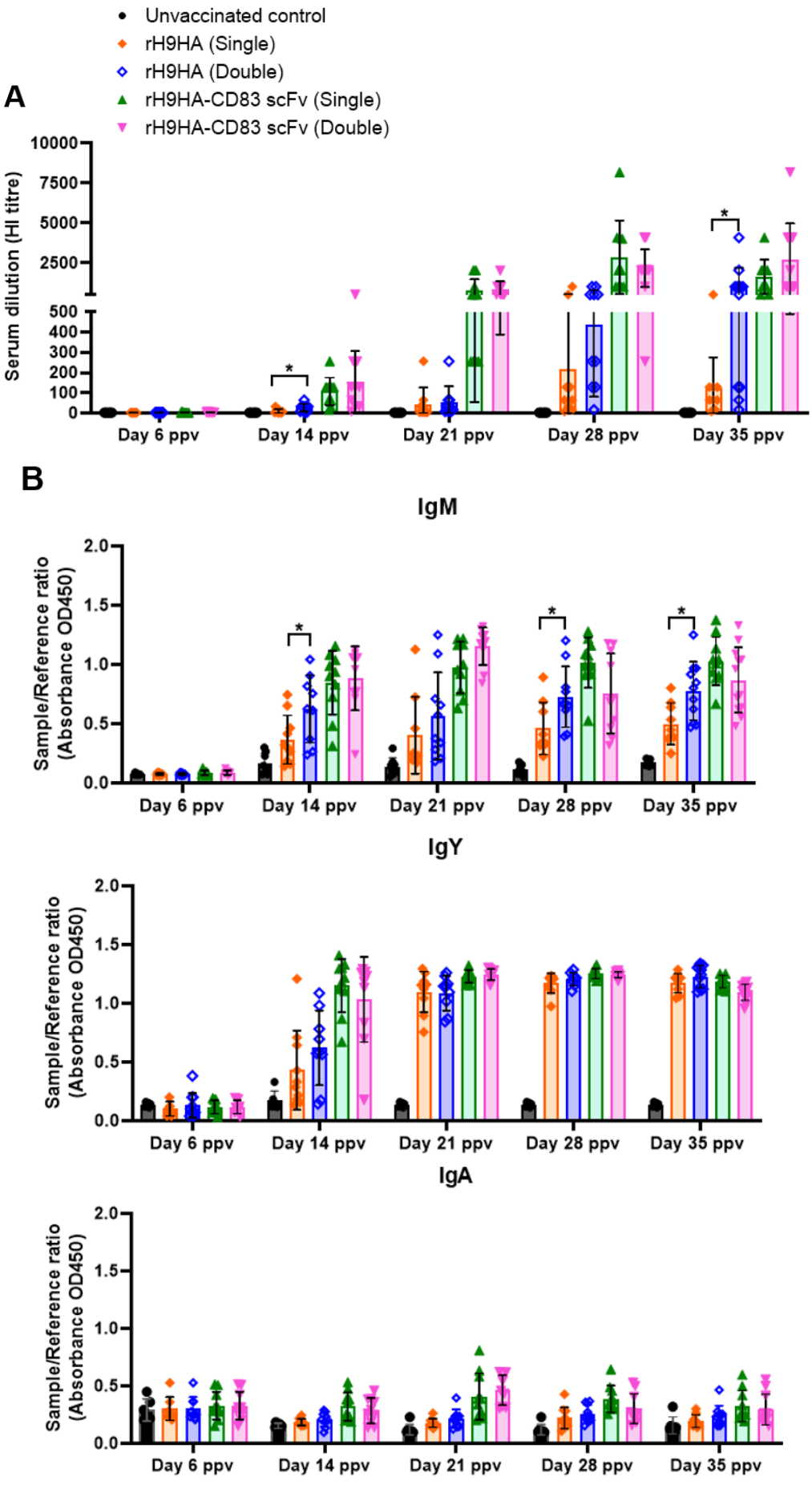


Figure 5.5. Cytokine profiles in the spleen of chickens vaccinated with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine. Groups of 1-day-old White Leghorn SPF chickens were vaccinated once with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose

equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose). Spleen samples (n=6 per group) were collected from the vaccinated chickens on day 2, 4, 6, 8 and 21 post vaccination and RNA was extracted. qRT-PCR was performed for a panel of cytokines and levels compared to RPLPO-1 reference gene were calculated as 40-ΔCT value. Data are presented as mean ± SD (n=6 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test.

5.2.1.3. Single and double doses of rH9HA-CD83 scFv vaccine elicit similar antibody responses

Following on, the effects of single and double vaccination regimes on antibody responses elicited by rH9HA and rH9HA-CD83 scFv vaccines were investigated. Chickens were vaccinated either once at 1-day-old (single vaccination group) or twice at 1-day-old and 7-day-old (double vaccination group). Blood samples were collected each week after vaccination for serological analysis using HI, ELISA and VNT assays (**Figure 5.6**). Single vaccinated rH9HA group showed lower HI antibody titres on day 14 and day 35 ppv compared to the double vaccinated rH9HA group ($p<0.05$, **Figure 5.6A**). Interestingly, no differences were observed in the HI antibody titres between single and double vaccinated rH9HA-CD83 scFv groups on all the time points tested. Similarly, single vaccinated rH9HA group also showed lower levels of anti-HA IgM antibodies compared to the double vaccinated rH9HA group on day 14 ppv, day 28 ppv and day 35 ppv ($p<0.05$, **Figure 5.6B**). There were no differences in the anti-HA IgM antibodies between single and double vaccinated rH9HA-CD83 scFv groups. Furthermore, no differences were observed in the anti-HA IgY and IgA antibodies, and virus MNT antibody titres between the single and double vaccinated rH9HA groups. The same observation was made with rH9HA-CD83 scFv vaccine (**Figure 5.6B and 5.6C**). Overall, rH9HA-CD83 scFv vaccine showed higher antibody responses compared to rH9HA in both single and double vaccinated groups.


A
Unvaccinated control
rH9HA (Single)
rH9HA (Double)
rH9HA-CD83 scFv (Single)
rH9HA-CD83 scFv (Double)
Serum dilution (HI titre)
10000
7500
5000
2500
500
400
300
200
100
0
Day 6 ppv
Day 14 ppv
Day 21 ppv
Day 28 ppv
Day 35 ppv
*
B
IgM
Sample/Reference ratio (Absorbance OD450)
2.0
1.5
1.0
0.5
0.0
IgY
IgA

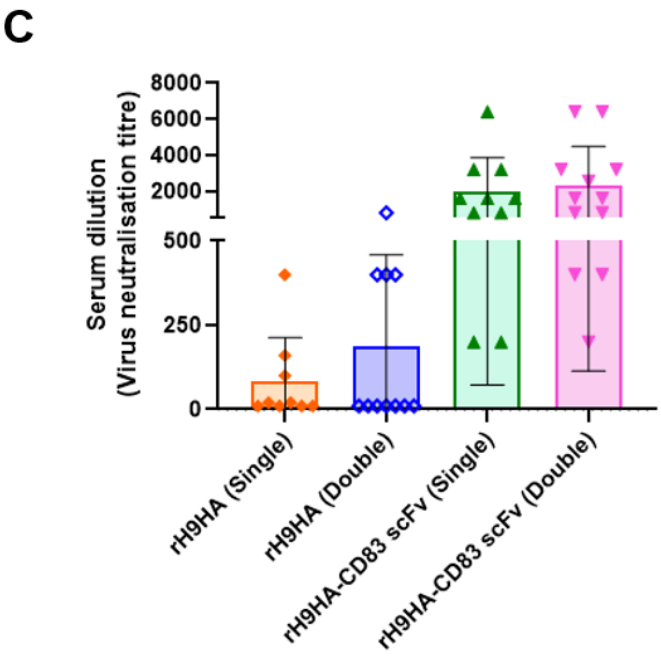


Figure 5.6. Effects of 'single' and 'double' vaccination regimes on the antibody response in chickens vaccinated with rH9HA and rH9HA-CD83 scFv vaccines. **A)** HI antibody titres. **B)** Anti-HA IgM, IgY and IgA antibodies. **C)** Virus MNT antibody titres. Groups of White Leghorn SPF chickens (n=10 per group) were vaccinated once or twice with rH9HA (35 µg/dose) and rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA). The single vaccination was given to the chickens at 1-day-old, whereas the double vaccination was given at 1-day-old and 7-day-old. The vaccine dose for the booster vaccination was same as that of the primary vaccination. Serum samples were obtained on day 6, 14, 21, 28 and 35 post primary vaccination (ppv), and HI, anti-HA ELISA and virus MNT assays were performed. For **C**, serum samples from day 35 ppv were used. For **A**, HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of UDL 01/08 H9N2 virus haemagglutination activity. For **B**, anti-HA antibody titres were expressed as sample to reference ratio (relation of absorbance of tested serum sample to absorbance of the reference serum). For **C**, virus MNT titres were expressed as the reciprocal of the highest serum dilution at which UDL 01/08 H9N2 virus infection is blocked, and the cells survive. Data are presented as mean ± SD (n=10 per group) and analysed by unpaired student t test. Statistical significance is shown with asterisks. *p<0.05

5.2.1.4. The rH9HA-CD83 scFv vaccine is stable for at least 1 year at 2-8°C

According to the stability requirement of OIE, the final vaccine product should maintain its potency for at least 1 year under the recommended conditions[98]. To determine the stability of rH9HA-CD83 scFv, formulation of the vaccine as

water-in-oil emulsion (MSD Animal Health) was carried out. Then, the formulated vaccine was stored at 2-8°C for 1 year. After 1 year storage, a single dose of the vaccine was administered to 1-day-old White Leghorn SPF chickens (n=10 per group, MSD Animal Health) and blood samples were collected for serological analysis using HI and virus MNT assays. No significant differences were observed in the antibody titres between the fresh and stored batches of rH9HA-CD83 scFv vaccines (**Figure 5.7**). This indicates that rH9HA-CD83 scFv vaccine is stable for at least 1 year when stored at 2-8°C. This satisfies the OIE stability requirement for regulatory approval of AIV vaccines[98].

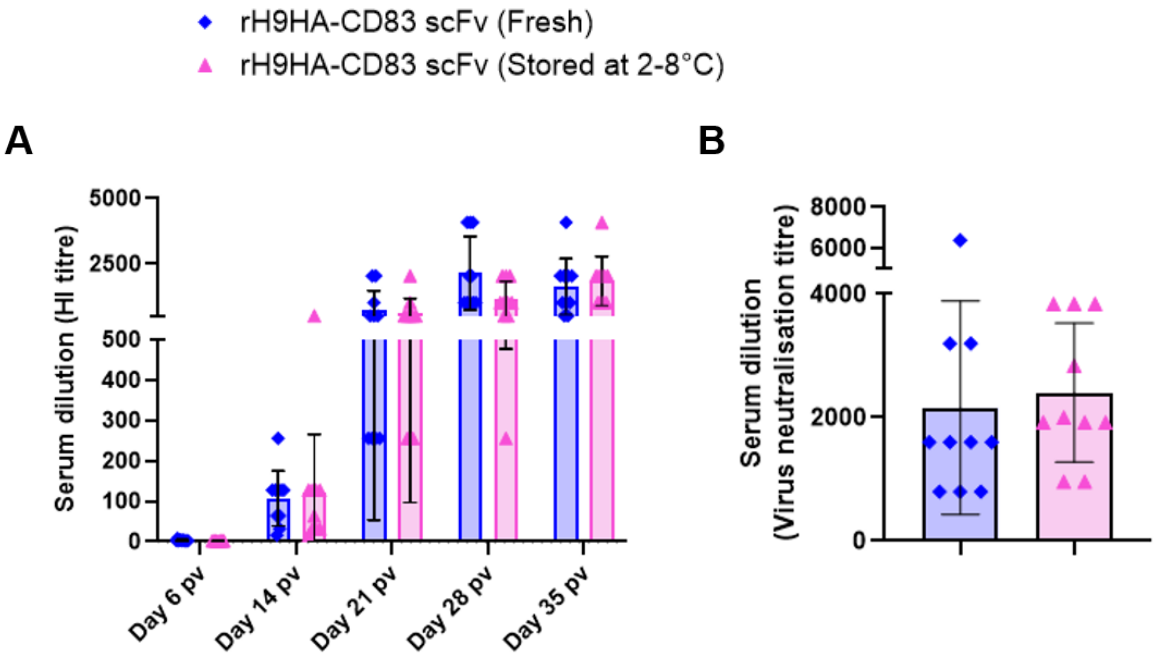


Figure 5.7. Analysis of the stability of rH9HA-CD83 scFv vaccine. **A)** HI antibody titres **B)** Virus neutralising antibody titres. The rH9HA-CD83 scFv vaccine (35 µg/dose) was formulated as water-in-oil emulsion (MSD Animal Health) and given to White Leghorn SPF chickens (1-day-old, n=10 per group). The same batch of vaccine was stored at 2-8°C for 1 year. After 1 year of storage, the vaccine was given to White Leghorn SPF chickens (1-day-old, n=10 per group). Serum samples were obtained on day 6, 14, 21, 28 and 35 post vaccination (pv) and HI assay and virus MNT assay (only day 35 pv samples) were performed. For **A**, the HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of UDL 01/08 H9N2 virus haemagglutination activity. For **B**, the virus neutralisation titres were expressed as the reciprocal of the highest

serum dilution at which UDL 01/08 H9N2 virus infection is blocked, and the cells survive. Data are presented as mean ± SD (n=10 per group) and analysed by paired student t test.

5.2.2. Comparison of the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine in MDA positive chickens

5.2.2.1. Antibody response in mother hens vaccinated with inactivated H9N2 vaccine

The experimental design of vaccination schedule of mother hens for the generation of anti-H9HA MDA positive hatchlings is described in **section 2.2.8.3.3** and shown in **Figure 5.8A**. A group of White Leghorn SPF layer hens (n=40) was immunised with inactivated H9N2 vaccine (520 HAU/dose) at 17, 20 and 50 weeks of age. Blood samples were collected at different time points post vaccination for serological monitoring of anti-H9HA antibodies using HI assay (**Figure 5.8B**). The first blood sampling was carried out at 5 weeks post first dose where the average HI antibody titre of serum from mother hens was 1351 (10.4 $\log_2$). As a significant drop in the HI antibody titre was observed in mother hens at 18 weeks post first dose compared to 11 weeks post first dose ($p<0.05$), a third dose was given at 24 weeks post first dose. This significantly increased the HI antibody titres in mother hens at 29 weeks post first dose ($p<0.01$). After the third dose of vaccine, the HI antibody titres remained consistent until the last blood sampling time point (36 weeks post first dose). Fertilised eggs were collected at 36 weeks post first dose and set to incubate until hatch. The average HI antibody titre of mother hens during lay was 4096 (12 $\log_2$).

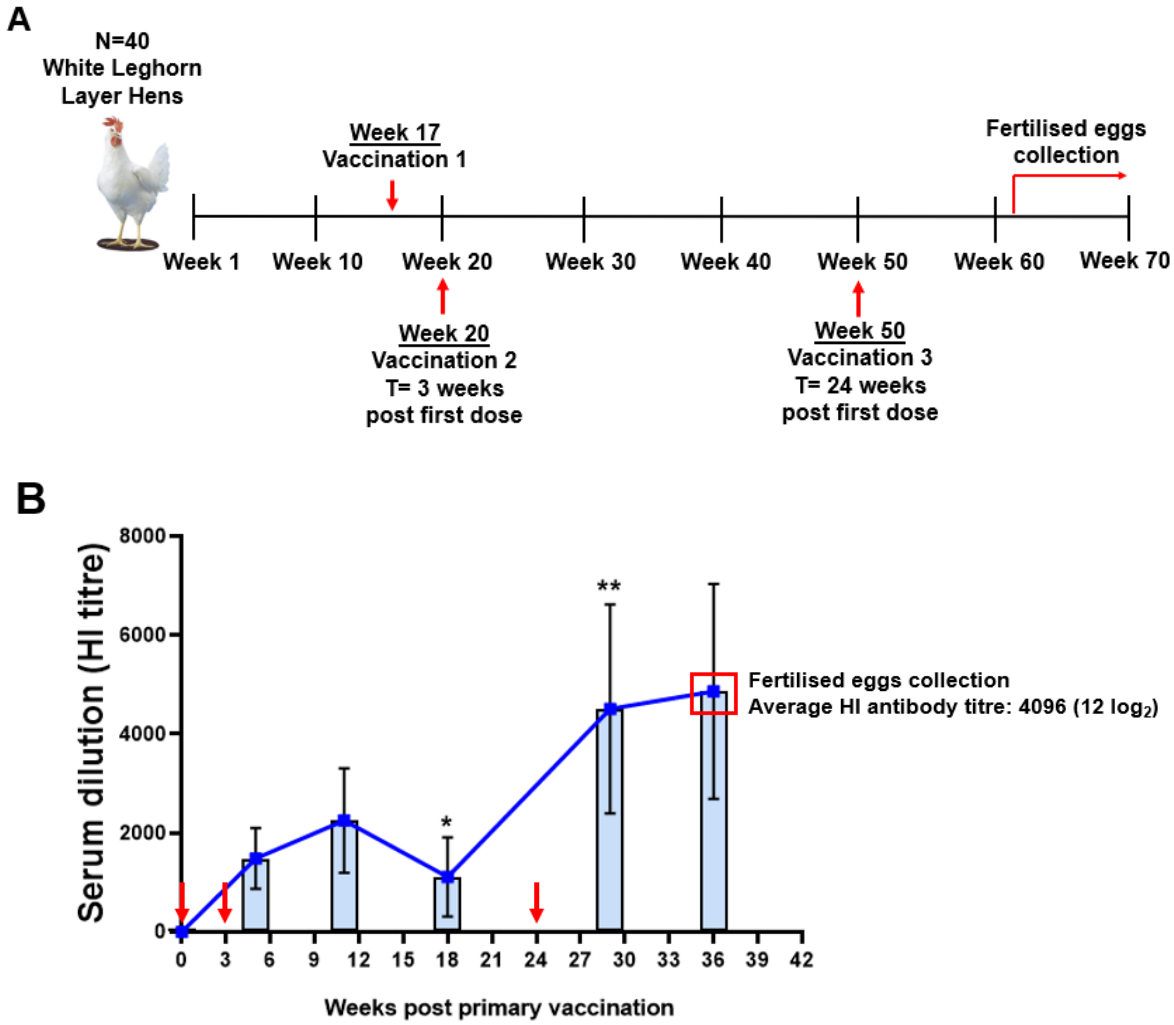


Figure 5.8. Generation of hatchlings with H9HA-specific MDAs. **A)** Vaccination schedule of mother hens. Forty SPF White Leghorn layer hens were vaccinated via intramuscular route at 17 weeks old with 0.5 mL of inactivated H9N2 vaccine (520 HAU/dose). Two booster vaccinations were given at 20 weeks of age and 50 weeks of age. Fertilised eggs were collected at 36 weeks post first vaccine dose and set to incubate until hatch. **B)** Anti-HA antibody titres measured by HI assay in the serum of mother hens. Blood samples were collected at different time points for the serological monitoring of mother hens. The HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of UDL 01/08 H9N2 virus haemagglutination activity. The red box indicates average HI antibody titre of mother hens at the time of laying eggs (HI: 4096 (12 $\log_2$)). For **B**, data are presented as mean ± SD (n=40) and analysed using paired t test. The asterisk represents significant difference between HI antibody titres at 11 and 18 weeks post first vaccine dose, and 18 and 29 weeks post first vaccine dose. **p<0.01 *p<0.05. ***(This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

5.2.2.2. Anti-H9HA MDA levels in the hatchlings reduces significantly by day 14 post hatch.

The fertilised eggs collected from mother hens were incubated and set to hatch. Ten hatchlings were sacrificed at 1-day-old and bled to confirm the presence of anti-H9HA MDA. The remaining hatchlings were bled either weekly or 2 weeks apart to monitor the decline in the MDA levels. The hatchlings from the vaccinated mother hens showed evidence of MDA against H9HA in both HI and ELISA assays (**Figure 5.9**). At 1-day-old, the hatchlings had an average HI antibody titre of 588 (9.2 log_2), which was approximately 7-fold lower than the serum HI antibody titre detected in the mother hens (HI: 4096 (12 log_2)). The antibody titres dropped slightly (not significant) by day 7 post hatch and was more than halved by day 14 post hatch (average HI antibody titre: 181 (7.5 log_2), average anti-H9HA ELISA antibody titre:10900). The antibody titres rapidly declined further after that, and by day 35 post hatch the MDA levels were very low (average HI antibody titre: 16 (4 log_2), average anti-H9HA ELISA antibody titre: ~1000). The MDAs in the hatchlings were undetectable in both HI and ELISA assays after day 42 post hatch.

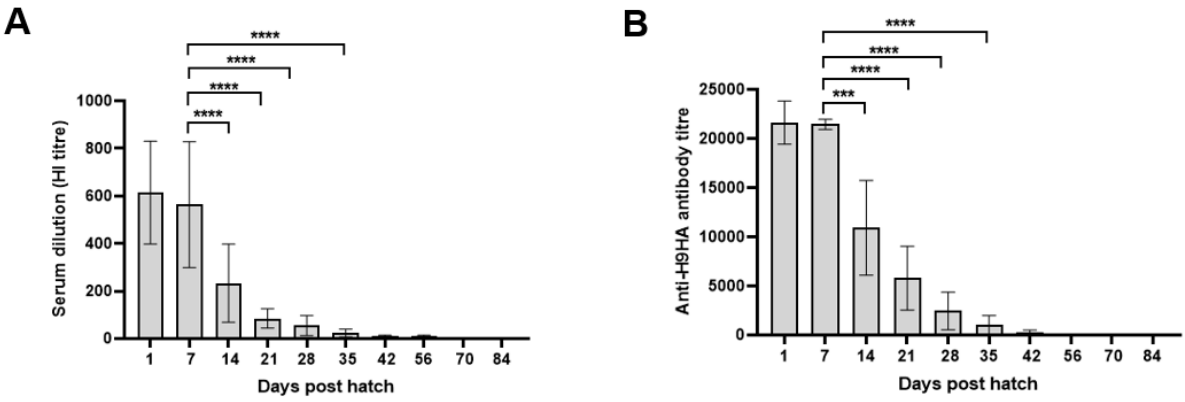


Figure 5.9. Monitoring the decline of anti-H9HA MDA in the unvaccinated MDA positive control chickens post hatch. Ten hatchlings were sacrificed at 1-day-old and bled to confirm

the presence of anti-H9HA MDA. The remaining hatchlings were bled either weekly or 2 weeks apart to monitor the decline in the MDA levels. **(A)** Anti-H9HA MDA measured by HI assay. The HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of virus haemagglutination activity. **(B)** Anti-H9HA MDA measured by indirect ELISA assay using ID Screen® Influenza H9 Indirect kit (ID. Vet) according to the manufacturer's protocol. Briefly, 1:500 dilution of the serum samples was carried out and added onto the recombinant H9HA pre-coated ELISA plates, followed by one hour incubation at 21°C. Plates were washed three times with wash buffer and 100 µL of anti-chicken HRP conjugate (1:10) was added. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. Antibody titres were calculated as $10^{(1.3*\log_{10}(\text{Sample/Positive})+3.256)}$. For **(A)** and **(B)** data are presented as mean ± SD (n=10) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001. **(*This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

5.2.2.3. The rH9HA-CD83 scFv vaccine can overcome the interference of high MDA levels (MDA++) compared to rH9HA vaccine

The 1-day-old hatchlings had average HI antibody titre of 588 (9.2 $\log_2$) and average anti-H9HA ELISA antibody titre of 21600. Hence, due to this high antibody titres we designated these chickens as MDA++. The chickens in MDA++ groups were vaccinated at 1-day-old with rH9HA and rH9HA-CD83 scFv vaccines. Blood samples were collected at different time points after vaccination and the antibody response in the serum of chickens with high MDA levels was analysed.

The HI assay was carried out to determine HI antibody titres. The first blood sampling point was day 14 pv during which no differences were observed in the HI antibody titres between rH9HA and rH9HA-CD83 scFv groups (**Figure 5.10**). The HI antibody titres in rH9HA and unvaccinated MDA positive groups steadily decreased after day 14 pv and were below the predictive protective value of 32. They did not show any significant rise in the HI antibody titres at any point after vaccination. On the contrary, chickens in rH9HA-CD83 scFv group showed an initial

decline in HI antibodies however, the antibody titres rapidly increased after day 35 pv until the last blood sampling point (day 84 pv). The average HI antibody titre in rH9HA-CD83 scFv group on day 84 pv was 832 (9.7 log_2).

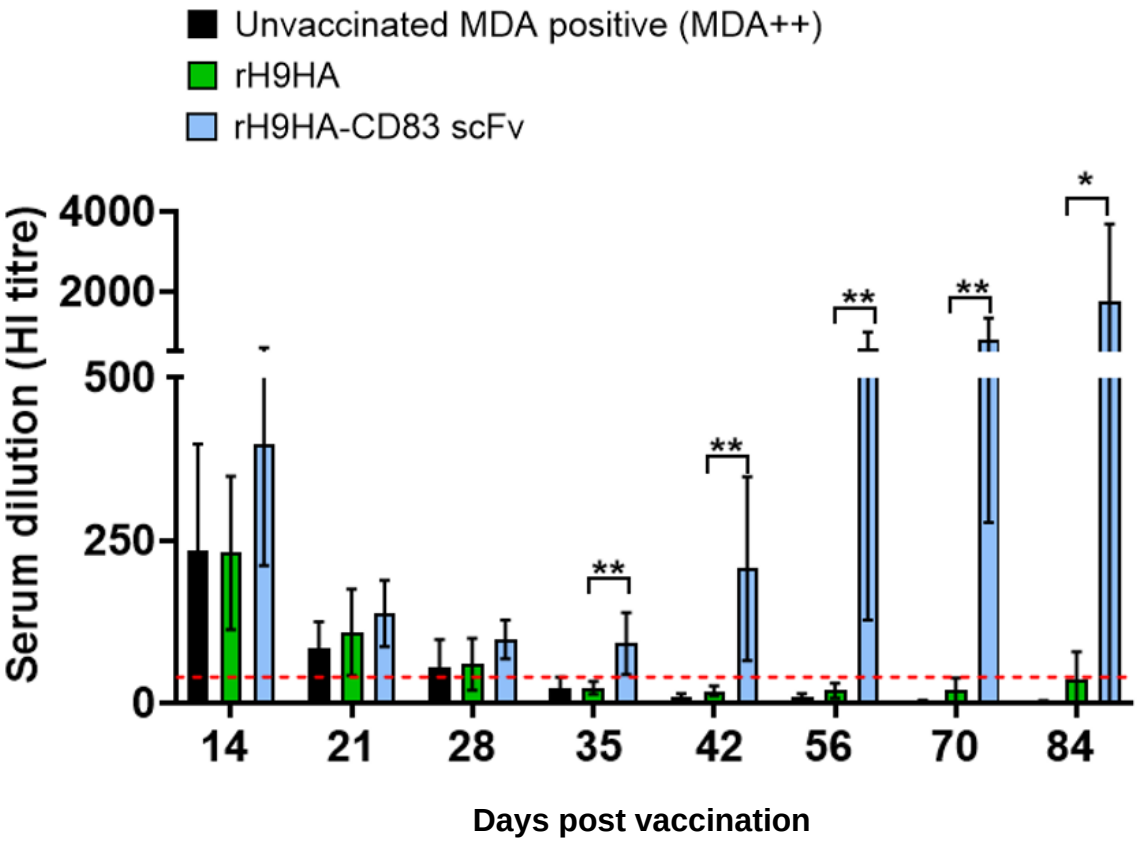


Figure 5.10. Anti-H9HA antibody titres measured by HI assay in the serum of MDA++ chickens vaccinated with rH9HA and rH9HA-CD83 scFv. The White Leghorn chickens in MDA++ group (n=10 per group) were vaccinated once at 1-day-old with rH9HA (35 µg/dose) and rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA). Blood samples were collected from the wing veins of chickens at 14, 21, 28, 35, 42, 56 ,70- and 84- days post vaccination. The HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of UDL 01/08 H9N2 virus haemagglutination activity. The red dotted line indicates the predictive protective HI titre of 32. Data are presented as mean ± SD (n=10 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. **p<0.01 *p<0.05**. (*This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

The antibody response was simultaneously monitored by measuring anti-H9HA antibodies by ELISA (ID. Vet). The results obtained from the ELISA assay were very similar to the HI assay. There were no significant differences in the anti-H9HA ELISA antibody titres between rH9HA and rH9HA-CD83 scFv groups on day 14 pv (**Figure 5.11**). The antibody titres gradually decreased in both the vaccinated groups by day 35 pv. However, rH9HA-CD83 scFv group showed significantly higher anti-H9HA ELISA antibody titres (average titre: 4656) compared to rH9HA vaccine group (average titre: 1200) on day 35 pv ($p<0.01$). In addition, most of the chickens in rH9HA vaccine group did not show detectable anti-H9HA antibodies after day 35 pv. The chickens in rH9HA-CD83 scFv vaccine group displayed rise in the anti-H9HA ELISA antibody titres after day 35 pv until the last blood sampling point (day 84 pv), mirroring the results observed in the HI assay.

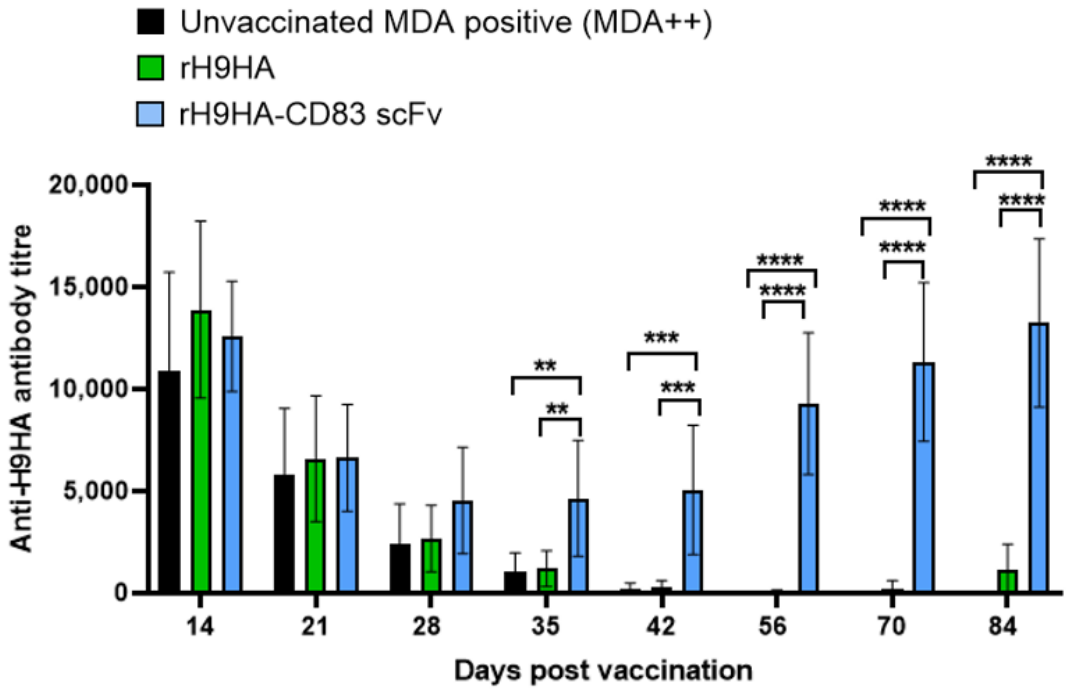


Figure 5.11. Anti-H9HA antibody titres measured by indirect ELISA assay in the serum of MDA++ chickens vaccinated with rH9HA and rH9HA-CD83 scFv. The White Leghorn chickens in the MDA++ group (n=10 per group) were vaccinated once at 1-day-old with

rH9HA (35 µg/dose) and rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA). Blood samples were collected from the wing veins of chickens at 14, 21, 28, 35, 42, 56 ,70- and 84-days post vaccination. The anti-H9HA antibodies were measured using ID Screen® Influenza H9 Indirect kit (ID. Vet) according to the manufacturer's protocol. Briefly,1:500 dilution of the serum samples was carried out and added onto the recombinant H9HA protein pre-coated ELISA plates, followed by 1 hour incubation at 21°C. Plates were washed three times with wash buffer and 100 µL of and anti-chicken HRP conjugate (1:10 dilution) was added. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. Antibody titres were calculated as $10^{(1.3 \times \log_{10}(Sample/Positive) + 3.256)}$**).** Data are presented as mean ± SD (n=10 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks ****p<0.0001 ***p<0.001 **p<0.01. ***(This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

5.2.2.4. The rH9HA-CD83 scFv vaccine can overcome the interference of medium MDA levels (MDA+) compared to rH9HA and inactivated H9N2 vaccines

The HI and anti-H9HA ELISA antibody titres in the hatchlings showed a significant decline by day 14 post-hatch (average HI titre: 181 (7.5 $\log_2$), average anti-H9HA ELISA antibody titre:10900, **Figure 5.9**). We designated these chickens as MDA+. The chickens in MDA+ groups were vaccinated at 14-day-old with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine. Blood samples were collected at different time points after vaccination to follow the antibody responses in chickens with medium MDA levels.

Unlike the MDA++ group, the chickens in MDA+ rH9HA-CD83 scFv group showed rise in the HI antibody titres after day 14 pv, and by the last blood sampling point (day 56 pv) the titres were significantly higher than rH9HA and inactivated H9N2 vaccine groups (p<0.001, **Figure 5.12**). On the contrary, there were no increase in the HI antibody titres in rH9HA and inactivated H9N2 vaccine groups after day 14 pv. Moreover, a similar trend was observed with indirect ELISA assay (**Figure 5.13**). The anti-H9HA ELISA antibody titres in rH9HA-CD83 scFv group

increased after day 14 pv and were higher than rH9HA and inactivated H9N2 vaccine groups on day 21 pv ($p<0.01$), day 28 pv ($p<0.001$), day 35 pv ($p<0.0001$), day 42 pv ($p<0.0001$) and day 56 pv (0.0001).

Overall, there were no significant differences observed in the HI antibody titres and anti-H9HA ELISA antibody titres in both MDA+ and MDA++ groups immunised with rH9HA-CD83 scFv vaccine at the end of the study period. However, the increase in antibody titres with rH9HA-CD83 scFv was more rapid in MDA+ group (after day 14 pv) compared to MDA++ group (after day 35 pv).

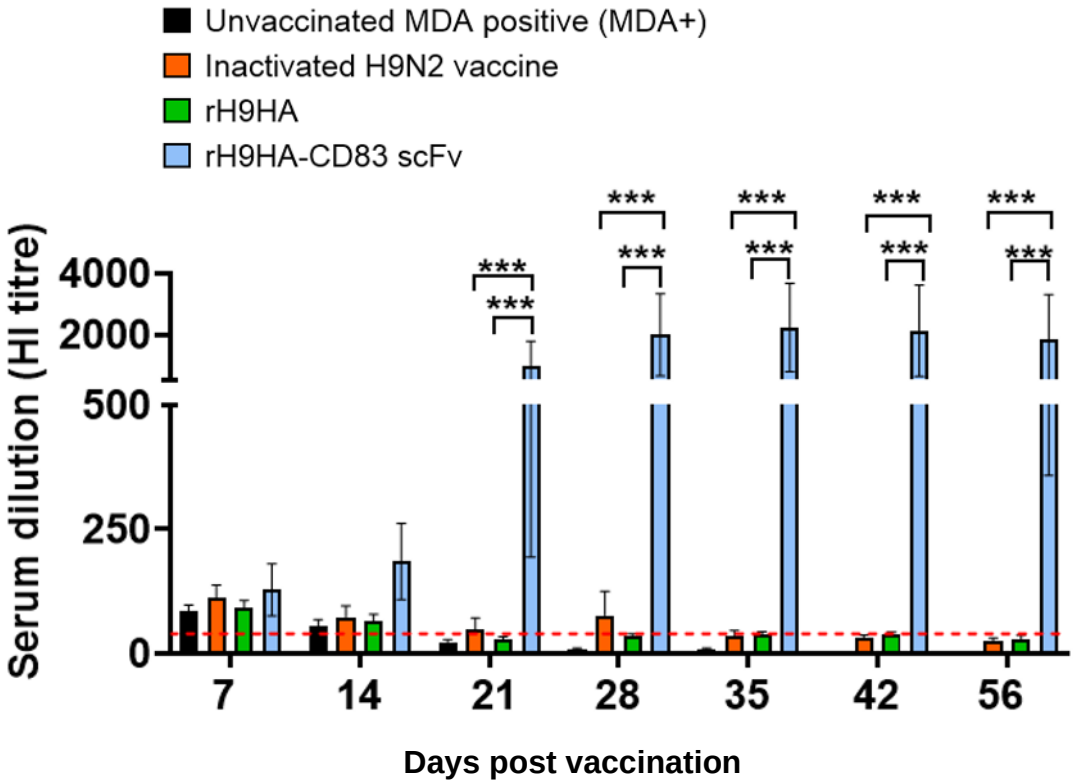


Figure 5.12. Anti-H9HA antibody titres measured by HI assay in the serum of MDA+ chickens vaccinated with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine. The White Leghorn chickens in the MDA+ group (n=10 per group) were vaccinated once at 14-day-old with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose). Blood samples were collected from the wing veins of chickens at 7, 14, 21, 28, 35, 42- and 56-days post vaccination. The HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of UDL 01/08 H9N2 virus haemagglutination activity. The red dotted line indicates the predictive protective HI tire of 32. Data are presented as mean ± SD (n=10 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison

test. Statistical significance is shown with asterisks ***p<0.001. **(*This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

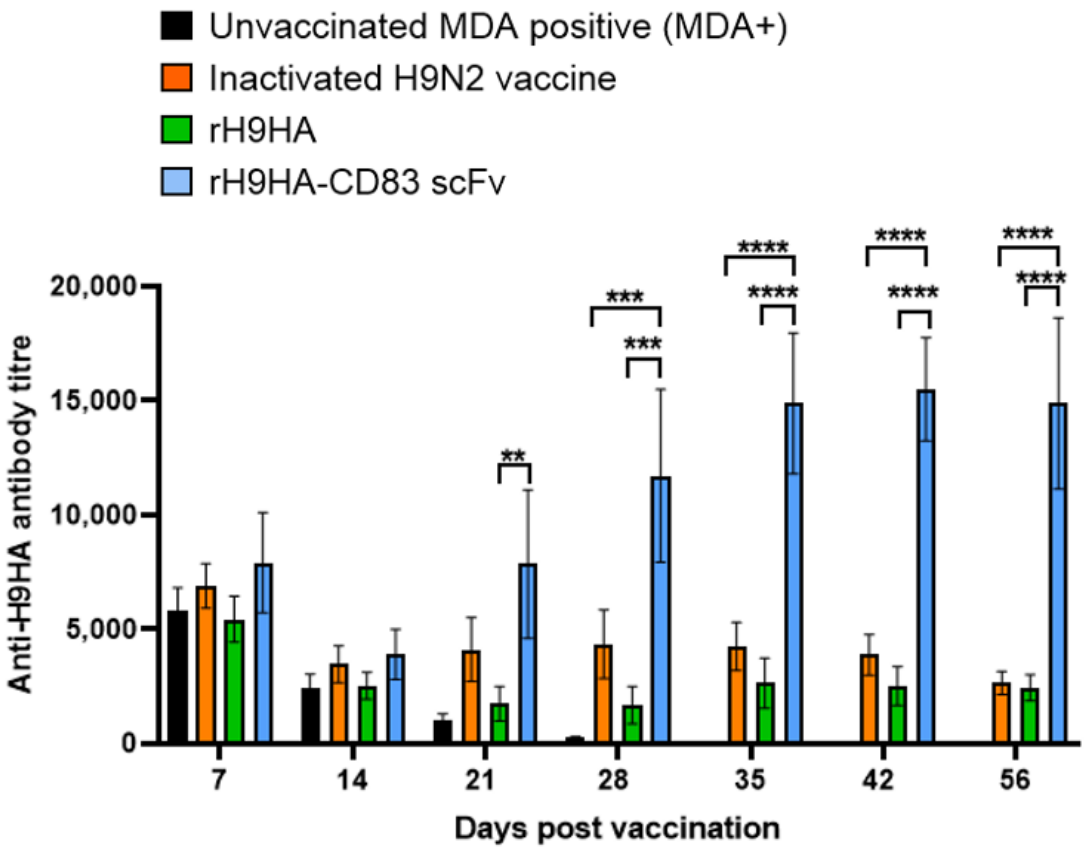


Figure 5.13. Anti-H9HA antibody titres measured by indirect ELISA assay in the serum of MDA+ chickens vaccinated with rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccine. The White Leghorn chickens in the MDA+ group (n=10 per group) were vaccinated once at 14-day-old with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose). Blood samples were collected from the wing veins of chickens at 7, 14, 21, 28, 35, 42- and 56-days post vaccination. The anti-H9HA antibodies were measured using ID Screen® Influenza H9 Indirect kit (ID. Vet) according to the manufacturer's protocol. Briefly,1:500 dilution of the serum samples was carried out and added onto the recombinant H9HA protein pre-coated ELISA plates, followed by 1 hour incubation at 21°C. Plates were washed three times with wash buffer and 100 µL of and anti-chicken HRP conjugate (1:10) was added. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. Antibody titres were calculated as $10^{(1.3 \times \log_{10}(\text{Sample/Positive}) + 3.256)}$**).** Data are presented as mean ± SD (n=10 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001 **p<0.01. **(*This experiment was done at MSD Animal Health, The Netherlands as mentioned in the declarations).***

5.2.2.5. The rH9HA-CD83 scFv cannot stimulate chicken splenocytes to produce type I interferon (IFN-α) *in vitro*

It has been reported that induction of type I interferon (IFN-α) can stimulate B cells for antibody production in the presence of MDAs in cotton rats[672]. Therefore, rH9HA and rH9HA-CD83 scFv were assessed for their ability to stimulate chicken splenocytes for the induction of IFN-α. Splenocytes (naive) were prepared from the spleen of 3-week-old Rhode Island Red SPF chickens (National Avian Research Facility) and stimulated with rH9HA and rH9HA-CD83 scFv for 6 hours and 18 hours as described in **section 2.2.3.2**. Both rH9HA and rH9HA-CD83 scFv were unable to stimulate the production of IFN-α *in vitro*, as shown by qRT-PCR **(Figure 5.14)**.

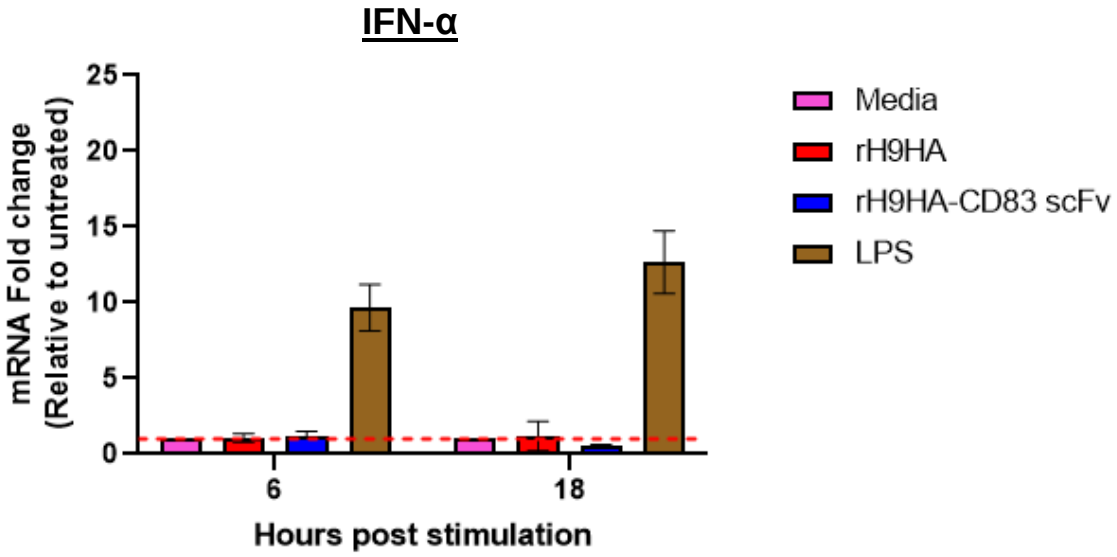


Figure 5.14: Analysis of type I interferon (IFN-α) production by chicken splenocytes upon stimulation with rH9HA and rH9HA-CD83 scFv using qRT-PCR. Splenocytes were isolated from the spleen of 3-week-old SPF chickens using Histopaque 1083 and stimulated with 10 µg of rH9HA and rH9HA-CD83 scFv for 6 hours and 18 hours. Stimulated splenocytes were harvested for RNA extraction and expression level of IFN-α was measured by qRT-PCR. Data were calculated using $2^{-\Delta\Delta CT}$ approach (n-fold change compared to the media only control group) and reported as values normalised to the expression level of a housekeeping gene RPLPO-1. Data are represented as mean±SD from three independent

experiments using different spleen donor chickens and analysed by one-way ANOVA followed by Tukey's multiple comparison test.

5.3. Discussion

In this chapter, the immunogenicity of rH9HA, rH9HA-CD83 scFv and inactivated H9N2 vaccines was further evaluated in SPF and H9HA specific-MDA positive chickens.

5.3.1. Immunogenicity in SPF chickens

In terms of cross-reactivity, the serum from rH9HA-CD83 scFv vaccine group showed significantly higher cross-reactivity to most of the H9N2 viruses tested compared to the serum from rH9HA and inactivated H9N2 vaccine groups, even though all the vaccines had similar HA content. There is a possibility that CD83 targeting could be playing a role in boosting cross-reactive antibodies targeting the conserved epitopes present in the vaccine antigen. It is well known that most of the neutralising antibodies against influenza virus are directed towards the conformational epitopes present on the globular head domain of HA protein. Although, the HA stalk domain represents relatively conserved region, very little antibodies targeting the stalk domain are produced during vaccination or natural infection[673]. Moreover, the head domain of HA protein is highly variable however, various conserved epitopes have been identified on the head domain that are thought to be responsible for cross-reactivity of antibodies[674–677]. Generally, antibodies against the variable epitopes on HA head domain are favoured. Herein, we propose a hypothetical structure model of CD83 scFv conjugated rH9HA (**Figure 5.15**), whereby each of the scFv molecule on HA monomer are occluding some parts of HA stalk domain. Such shielding could concentrate the overall

production of antibodies towards the head domain, maximising the chances of multiple conserved epitopes on the head domain to be targeted. Previously, attempt has been made to generate cross-reactive antibodies by shielding variable regions of HA, so that antibodies could be targeted towards conserved stalk regions[678]. Thus, the following factors could be contributing to the higher cross-reactivity of rH9HA-CD83 scFv: i) overall higher production of neutralising antibodies directed towards the variable HA head domain ii) higher production of antibodies towards conserved epitopes on the HA head domain. However, the actual structure of rH9HA-CD83 scFv needs to be verified with structural studies. It is also necessary to conduct heterologous virus challenge studies to investigate whether these cross-reactive antibodies can provide protection against heterologous H9N2 viruses. Furthermore, IgA isotype of antibody is more cross-reactive than IgG isotype[679–681]. The isotype of antibodies produced after vaccination is also affected by the adjuvants used. Therefore, adjuvants are selected to enhance the production of IgA antibodies, through their effects on APCs and T cell differentiation[682]. In our case, the inactivated H9N2 vaccine and recombinant subunit vaccines used were poor at inducing IgA antibodies in the serum. Also, no significant differences were observed in the amount of serum IgA antibodies between rH9HA-CD83 scFv and other vaccines tested. It is possible that differences could be observed in the amount of secretory IgA, which was not examined in this work. Moreover, HI antibodies and anti-HA IgM and IgY antibodies were not detected in the serum of chickens in rH9HA-CD83 scFv group on day 6 post vaccination. This contradicts our previous finding in Chapter 4. This could be due to the different vaccination schedule used i.e., vaccination to 7-day-old chickens previously compared to 1-day-old chickens here. In fact, the immune response is affected by the immaturity of the immune

mechanism thus, the older the chickens are at the time of vaccination, the better is their immune response[683]. Evidence suggests that the level of IgA and IgM antibodies are undetectable or low in plasma of chickens in first week of life indicating limited immunoglobulin class switch and production of antibodies[664]. In addition to the reduced B cell response after vaccination, the splenic T cells from 1-week-old chickens are less responsive to activation and proliferation compared to older chickens[684]. Furthermore, 1-week-old chickens demonstrated a reduced expression of immune related genes in the lungs and trachea after inoculation with H9N2 AIVs compared to 4-week-old chickens[685]. Hence, early exposure to pathogens or vaccines might induce sub-optimal immune responses in chickens. Moreover, the early induction of serum HI antibodies and anti-HA IgM and IgY antibodies in Chapter 4 could also be attributed to the use of different breeds of chickens i.e., Dekalb White chickens in previous studies compared to White Leghorn chickens here.

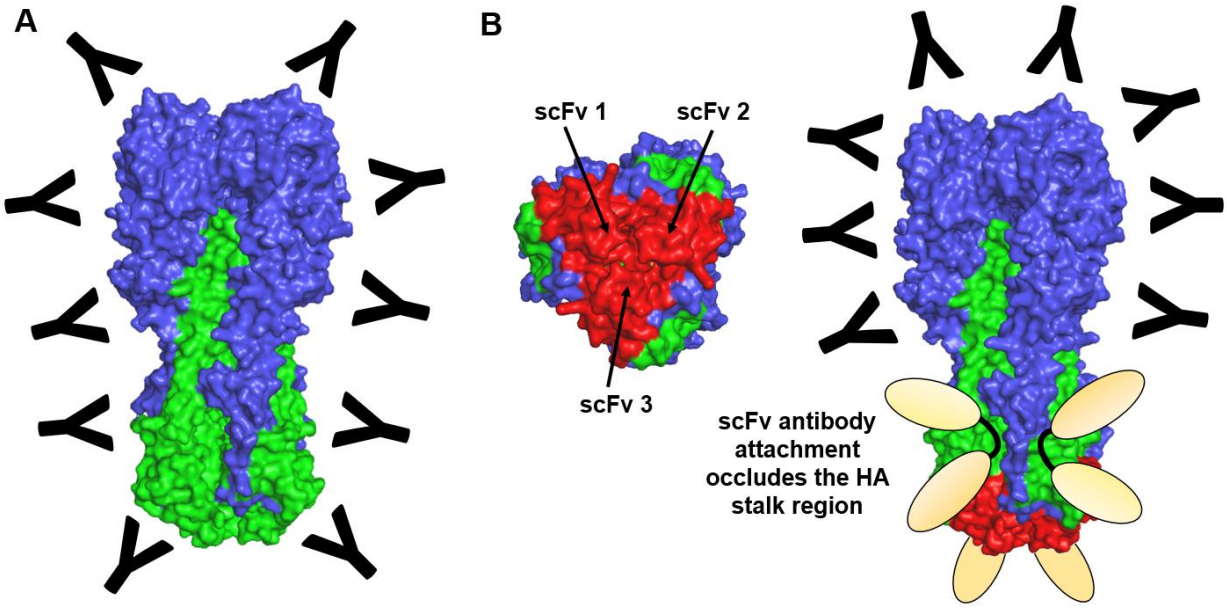


Figure 5.15. Hypothetical structural model of rH9HA-CD83 scFv. **A)** rH9HA. Antibodies are produced against different regions of HA head (HA1, blue) and stalk (HA2, green)

domains, with more antibodies targeting the variable head domain. **B)** rH9HA-CD83 scFv. The scFv antibodies attached to the HA2 stalk domain occlude some parts of HA2 domain. This concentrates the overall antibody production towards the HA1 head domain, increasing the chances of multiple highly conserved epitopes to be targeted. ***Blue:*** *HA1 domain* ***Green****: HA2 domain* ***Red****: Possible attachment sites for scFv antibodies.* The structure of rH9HA is predicted by SWISS-MODEL homology-modelling server[686] and made using Pymol[37].

The natural influenza virus infection normally induces a strong Th1 response that results in the generation of CTLs and activation of the macrophages. In mice, it has been demonstrated that the formulation of influenza vaccines can determine the type of cytokine response produced. Split influenza virus vaccine induced a Th2 biased cytokine response, whereas whole inactivated influenza vaccine induced a Th1 biased response[679,687]. Here, we investigated the effect of inactivated H9N2 virus vaccine and recombinant subunit vaccines on the gene expression levels of cytokines in the spleen samples. None of the cytokines tested showed any upregulation in their mRNA levels compared to the unvaccinated control at all the time points tested, as measured using qRT-PCR. This could be due to the absence of *in vitro* stimulation. Since, parenteral vaccination induces a strong systemic response[244], blood/PBMCs could be better samples for analysing cytokine responses after subcutaneous vaccination in chickens. Furthermore, our first sampling point was day 2 pv. Thus, testing in the time frame just after vaccination (few hours to day 1 pv) may be more appropriate to observe changes in the cytokine levels induced by vaccination without *in vitro* stimulation. In fact, analysis following the administration of trivalent inactivated influenza vaccine (Fluzone®, Sanofi-Pasteur) in humans identified temporal patterns of serum cytokine and chemokine changes which occurred as early as 3 hours pv, generally peaking at approximately 24 hours[688]. It is also possible that a response may have occurred between sample

points day 8 to day 21 pv. The separation between these sample points were made large due to the restrictions on the number of chickens used for the animal study.

Different vaccination regimes are practised in poultry for common diseases like AIV, NDV, IB and Infectious Bursal Disease virus (IBDV). The shorter lifespan of broiler chickens (6 to 7 weeks) makes the vaccination regimes less complex for broilers compared to layers and breeders. For broilers, a single vaccination with commercial inactivated AIV vaccine between day 7 and day 10 of age is preferred whereas, for layers and breeders additional booster vaccinations after 6-10 weeks are recommended, with the last vaccination taking place at least 4 weeks before the onset of lay[689–691]. It is important to identify the most suitable vaccination regime to maximise the growth performance, health status and protection of the chickens, as well as avoid any excessive and unnecessary use of vaccines. The increased frequency of vaccinations has been correlated with higher immune stress negatively affecting the growth performance, feed intake and meat quality in broilers[692,693]. Therefore, vaccination regimes involving less frequency of administration is highly preferred to improve the poultry production and reduce the cost. Here, two vaccination regimes were compared for rH9HA and rH9HA-CD83 scFv vaccines: single vaccination at 1-day-old or double vaccination at 1-day-old and 7-day-old. Overall, similar antibody responses were observed between single and double vaccinated rH9HA-CD83 scFv groups whereas, single vaccinated rH9HA group showed reduced antibody titres compared to double vaccinated rH9HA group. Importantly, the chickens in single vaccinated rH9HA-CD83 scFv group retained high HI antibody titres (average HI: 1663 (10.7 $\log_2$)) above the predictive protective level (HI$\geq$32 (5 $\log_2$)) until the last time point i.e., day 35 pv which is the approximate production lifecycle of broiler chickens. This indicates that

a single dose of rH9HA-CD83 scFv vaccine given at 1-day-old could potentially protect broilers throughout their lifetime. Further studies focussing on the duration of the antibody response can help us to determine the time and frequency of vaccinations required for layers and breeders with rH9HA-CD83 scFv vaccine.

5.3.2. Immunogenicity in H9HA-specific MDA positive chickens

In majority of the AIV enzootic countries, routine field vaccination in chickens is implemented to reduce the disease incidence. This results in the hatchlings bearing MDAs. For example, in the MENAP (Middle East, North Africa, Afghanistan and Pakistan) region, broiler breeders' flocks are almost always vaccinated against H9N2 AIV. Hence, most of the 1-day-old broiler chickens produced have MDAs against H9N2 AIV which compromises the use of inactivated H9N2 virus vaccines in this region[253]. To generate H9HA-specific MDA positive hatchlings, White Leghorn layer hens were vaccinated with three doses of inactivated H9N2 vaccine. The HI antibody titre in the mother hens increased significantly after the third dose of vaccine and remained consistent (average HI: 4096 (12 $\log_2$)) for about 12 weeks post third dose. Since, the serum HI antibody titre in the mother hens was not followed after 12 weeks post third dose, the exact duration of this antibody response is unknown. The progeny chickens from the vaccinated mother hens showed average HI antibody titre of 588 (9.2 $\log_2$) at 1-day-old, which was approximately 7-fold lower than the serum HI antibody titre detected in the mothers (average HI:4096 (12 $\log_2$)), consistent with previous observations[642,649]. The hatchlings displayed a significant drop in the HI antibody titre on day 14 post hatch, and by day 35 post hatch 90% of the chickens showed HI antibody titre below the predictive protective value of 32. The decline of anti-H5HA MDAs in broilers with

average HI titre of about 548 (9 log_2) at 1-day-old has also shown a similar trend[694]. Furthermore, previous study in White Leghorn layers with adjuvanted inactivated H7N3 virus vaccine showed a decrease in MDA-derived HI antibody titres below predictive protective value by day 14 post hatch[642]. Likewise, hatchlings from Lohmann layer flocks vaccinated with inactivated H9N2 virus vaccine showed MDAs for 3-4 weeks post hatch[643]. These differences observed in the decline of MDAs in progeny chickens could be attributed to vaccine antigen and formulation, vaccination protocol, the time between immunisation of hens and collection of eggs, level of MDAs present in mother hens during egg laying and chicken breeds.

Most vaccination programmes against AIV currently implemented worldwide use inactivated virus vaccines, which face high levels of interference with MDAs when administered within the first 10 days of age[642,669,695]. The impact of MDAs on the immunogenicity of recombinant subunit poultry AIV vaccines has not been studied until now. Here, we examined the immunogenicity of recombinant rH9HA and rH9HA-CD83 scFv vaccines in chickens with high H9HA-specific MDA (MDA++, 1-day-old, average HI:588 (9.2 log_2)) and medium H9HA-specific MDA (MDA+, 14-day-old, average HI:181 (7.5 log_2)). The inactivated H9N2 vaccine was also included for comparison in MDA+ group vaccinated at 14-day-old, as 1-day-old vaccination of such vaccine in MDA positive chickens has been previously reported with poor immune responses[695]. In both MDA++ and MDA+ groups, the induction of antibodies after vaccination with rH9HA vaccine was greatly inhibited. Similarly, in MDA+ group the induction of antibodies after vaccination with the inactivated H9N2 vaccine was also inhibited. However, rH9HA-CD83 scFv vaccine was lesser impacted by both high and medium MDA levels, with HI and anti-H9HA ELISA antibody titres significantly increasing after day 35 pv (MDA++ chickens) and

day 14 pv (MDA+ chickens). Furthermore, a long-lasting antibody response (at least 3 months) was observed when MDA++ chickens were vaccinated with rH9HA-CD83 scFv at 1-day-old. Moreover, a direct comparison between the amount of HA protein in the inactivated H9N2 vaccine and recombinant subunit vaccines could not be made. However, 35 µg of rH9HA and rH9HA-CD83 scFv (equimolar concentration) displayed haemagglutination titre of approximately 256 HAU which is four-fold lower than that present in a single dose of inactivated H9N2 vaccine given to the chickens (1024 HAU).

The recombinant live vector vaccine e.g., HVT expressing HA protein of AIV is reported to be less sensitive to MDAs thus, protecting chickens even in the presence of MDAs[694,696]. The HVT can replicate in a highly cell-associated manner in lymphocytes providing a pathway that can stimulate CMI[697,698]. Furthermore, HVT can continuously express the foreign antigen *in vivo*, enabling B cells to be stimulated even in the presence of MDAs[660]. It has been reported that vaccines can overcome MDA interference if they can stimulate type I interferon (IFN-α)[660] (**Figure 5.16A**). IFN-α can bind to both interferon receptor and CD21 (complement receptor 2) on B cells. This dual receptor usage leads to a strong positive signal stimulating antibody secretion by B cells even in the presence of MDAs[672]. This is one of the reasons why vaccines containing LPS or CpG oligonucleotide as adjuvant are able to breakthrough MDAs[660,699]. In addition, type I interferons (IFN-α/β) are also known to support $CD8^+$ T cell priming, by indirectly enhancing APCs to activate $CD8^+$ T cells or directly supporting the development of effector and memory $CD8^+$ T cell populations[700]. Following on, the ability of rH9HA-CD83 scFv to induce IFN-α upon *in vitro* stimulation of chicken splenocytes was assessed. However, rH9HA-CD83 scFv failed to stimulate the production of IFN-α. Weeratna

et al. showed that MDAs exerted inhibition of B cell responses when mice were immunised with hepatitis B surface antigen (+CpG adjuvant) vaccine or DNA vaccine. However, both the vaccines were able to produce a strong CTL response despite the presence of high MDAs[701]. This suggests that MDAs do not inhibit CMI. This might explain the need for vaccines that are capable of inducing CMI for providing protection against pathogens in the presence of MDAs. Another mechanism reported in mammals for overcoming MDA inhibited B cells is through IgM stimulation (**Figure 5.16B**). The antigen-specific IgM antibodies can form a complex with vaccine antigen and complement protein (C3d), which further cross-links the BCR and CD21. This cross-link has shown to stimulate B cells *in vitro* even in the presence of inhibitory IgG[660,702]. Hence, it is possible that vaccines can overcome MDA interference if they can induce higher antigen-specific IgM antibodies. The ability of rH9HA-CD83 scFv vaccine to generate IgM antibodies in MDA positive chickens was not tested. However, rH9HA-CD83 scFv was able to induce higher amount of anti-HA IgM antibodies compared to inactivated H9N2 vaccine and rH9HA vaccine in SPF chickens (**section 4.2.1.3 and section 5.2.1.2**). Therefore, this could be a potential mechanism by which rH9HA-CD83 scFv can stimulate B cell inhibited by MDAs. The exact mechanism needs to be further investigated. Overall, the results also indicate that rH9HA-CD83 scFv is highly stable even in the presence of MDAs, unlike rH9HA and inactivated virus vaccines which are easily cleared out. This high stability of rH9HA-CD83 scFv could also explain the rise in antibody titres after the decline in MDAs. Moreover, it could be important to consider the site of vaccine administration when developing and testing vaccines in the presence of MDAs. Inhibitory maternal antibodies are of IgY isotype in chickens, present in higher concentration in the blood compared to

respiratory and ocular fluids, and saliva[617]. Therefore, alternative routes of immunisation (other than subcutaneous or intramuscular) e.g., eyedrops or intranasal could be beneficial in this context.

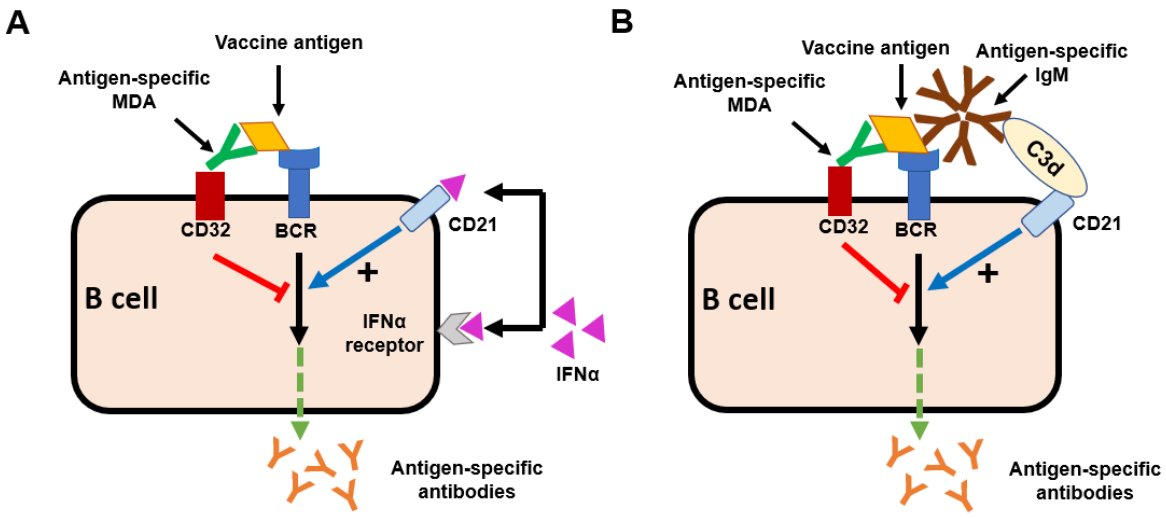


Figure 5.16. Model of B cell activation in the presence of maternal antibodies. **A)** Type I interferon (IFN-α) can bind to both the interferon receptor and CD21 on B cells. This dual usage of receptors on B cells can lead to a strong positive signal which cancels out the negative signal through CD32, stimulating antibody secretion by B cells in the presence of maternal antibodies. **B)** Antigen-specific IgM binding to the vaccine antigen can also bind to CD21 via complement component C3d. This provides a positive signal to overcome inhibition by CD32 signalling, resulting in B cell stimulation to produce antibodies even in the presence of MDAs. Image inspired from Niewiesk, S[569]. ***BCR****: B cell receptor*

The most common regime for AIV vaccination in enzootic countries like Egypt is to administer inactivated virus vaccines to day 1-5 day old chickens[695]. Hatchery vaccination administered to 1-day-old chickens is preferred as this would optimise vaccine coverage and flock protection levels, with the use of less resources[703]. However, the use of inactivated virus vaccines to 1-day-old MDA positive chickens has faced difficulties in developing good immune responses due to the interference from MDAs[695]. One of the key determinants for the success or failure of vaccines is the level of MDAs present in chickens at the time of

vaccination. Hence, it is important to determine the optimal age of vaccination depending upon the MDA titres in 1-day-old chickens. It has been reported that vaccination of MDA positive chickens with inactivated AIV vaccine at 7-10 day old (when MDA titre is low) results in higher immune response and protection compared to 1-day-old vaccination[669]. Several studies have demonstrated that MDAs have limited value in protecting young chickens against AIV infection[649,669]. In some case, progeny chickens from the vaccinated hens were partially protected from HPAI H5N1 infection only up to 2 weeks after hatch[669]. Thus, it becomes necessary to vaccinate chickens early, even in the presence of high MDAs. This helps to reduce the gap in protection due to the decline of MDAs and delay in the onset of an active immunity[704]. Therefore, there is a huge demand for vaccines that can be administered to 1-day-old chickens to induce an early immune response in high-risk situation. Here, a single dose of rH9HA-CD83 scFv vaccine was able to overcome the negative effects of high MDA levels when administered to 1-day-old chickens. Furthermore, rH9HA-CD83 scFv vaccine can also be administered to MDA positive chickens at later age (14-day-old), in situations where 1-day-old vaccination is not applicable. Therefore, rH9HA-CD83 scFv represents an alternative to inactivated H9N2 vaccine that can be administered at hatch inducing active immunity and an early protection in the face of MDAs in chickens.

Chapter 6: Generation of recombinant Herpesvirus of Turkey vector vaccines expressing H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv and evaluating their immunogenicity in chickens

Chapter summary

In this chapter, the recombinant Herpesvirus of Turkey (rHVT) vector vaccines expressing full-length H9HA protein and secreted forms of H9HA proteins i.e., H9HA Foldon and H9HA Foldon-CD83 scFv were generated using Homology Directed Repair-CRISPR/Cas9 system. Upon analysis by western blots, H9HA protein was detected only in the cell lysates of rHVT-H9HA Full-length (hereinafter referred to as rHVT-H9HA FL) infected CEF cells, whereas rHVT-H9HA Foldon (hereinafter referred to as rHVT-H9HA) and rHVT-H9HA Foldon-CD83 scFv (hereinafter referred to as rHVT-H9HA CD83 scFv) infected CEF cells showed H9HA protein expression in both the culture supernatants and cell lysates. Moreover, the rHVT was detected in spleen and blood samples collected from rHVT vaccinated chickens on day 7 pv and day 21 pv, respectively and had 99%-100% stability *in vivo*. Among all the rHVT constructs, rHVT-H9HA FL demonstrated the highest antibody responses. Furthermore, the antibody responses elicited by rHVT-H9HA CD83 scFv was higher than rHVT-H9HA, consistent with the observation made with the recombinant subunit vaccines.

Chapter aims

- To generate rHVT expressing H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv.
- To analyse the *in vitro* and *in vivo* stability of all the rHVT vaccine constructs.
- To analyse the *in vitro* and *in vivo* replication of all the rHVT vaccine constructs.
- To investigate the antibody responses elicited by rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv in chickens.

6.1. Introduction

The inactivated AIV vaccines are currently being used for routine vaccination programmes in poultry. As mentioned earlier, such vaccines have drawbacks such as sub-optimal immunogenicity, need for multiple dose administration, poor DIVA compatibility and MDA interference. Over the years, alternative strategies have been developed to produce new AIV vaccines by manipulating the attenuated avian viruses for use as vectors to deliver HA antigens of AIV[265]. The viral vectors are considered as live vaccines, but their replication is attenuated or even deficient. Therefore, vector-based vaccines are safer in general. Despite their attenuated phenotype, viral vectors are immunogenic and induce virus-specific antibody and T cell responses[265]. Additionally, recombinant viral vectors confer advantage of fighting against at least two pathogens with a single vaccine, one against the vector virus itself and another against the expressed antigens of other viruses[705]. Moreover, viral vector candidates such as FPV, NDV, infectious laryngotracheitis

virus (ILTV), and HVT have received the most attention for combating poultry diseases. Among these viral vectors, HVT has been a popular choice for developing poultry multivalent live vaccines. The HVT is a naturally occurring apathogenic alpha herpesvirus that was originally isolated from turkeys[706]. It belongs to the *Mardivirus* genus, which is known to cause Marek's disease (MD) in chickens. MD virus (MDV) serotype 1 (*Gallid herpesvirus 2* (GaHV-2)) is pathogenic and a causative agent for MD characterised by highly contagious T cell lymphoma. *Mardivirus* also includes two non-pathogenic serotypes: serotype 2 (GaHV-3) and serotype 3 (*Meleagrid herpesvirus 1* (MeHV-1), commonly known as HVT)[707]. Live HVT vaccine (FC-126 strain) is the first generation of MD vaccine that has been widely used in chickens since 1970s[708]. Although HVT is non-pathogenic in chickens, it does induce viremia which is associated with induction of protective immune responses against GaHV-2[709]. In addition, HVT appears to replicate less efficiently in skin, a phenotype which is thought to be involved in the relatively infrequent transmission of HVT among chickens compared to GaHV-2 and GaHV-3[710], which further enhances their safety. Unlike GaHV-2 and GaHV-3 which are strictly cell-associated, HVT is capable of producing both cell-associated and cell-free virus in infected tissue culture[711]. Both the cell-associated and cell-free forms of HVT vaccine has shown similar virus neutralising antibody titres in MDA positive chickens, with the former producing higher viraemia[712]. Furthermore, the cell-associated replication of HVT has shown to induce strong CMI[698,713]. It has been demonstrated that HVT can cause persistent infection in chickens even in the presence of MDAs, enhancing long term protection[714]. Another advantage of HVT vaccines is that they can be administered either *in ovo* at day 18 of embryonation

or on the day of hatch[254]. The *in ovo* vaccination can be carried out using automated methods, which significantly improves the speed and ease of vaccination.

The complete genome sequence of FC-126 strain of HVT has been determined. The HVT is a large double-stranded DNA virus with 159,160 bp long genome containing at least 99 functional genes[709]. The large genome size helps to accommodate many foreign DNA sequences without affecting their biology. Several non-essential sites such as the intergenic region between unique long (UL)45 and UL46, and unique short (US)2 and US10 have been identified in HVT genome as compatible sites for foreign gene insertions[714–716]. Among these sites, the intergenic region between UL45/46 has been frequently used as foreign gene insertion site[717–719]. Furthermore, a comparison between US2 and US10 region indicated that US2 region provides a favourable foreign gene insertion site for the generation of rHVT vaccines[715]. The HVT has been used as virus vector for expressing range of antigens including HA of AIV[717], fusion (F) protein of NDV[720], glycoprotein D (gD) and glycoprotein I (gI) of ILTV[721] and viral protein 2 (VP2) of IBDV[716]. In the context of AIV, rHVT vector vaccines have been generated expressing HA of H5N1, H7N1 and H9N2 AIVs, and their efficacy has been assessed[267,717,722]. Vectormune® HVT-AI (CEVA Animal Health) is a commercially available HVT vector vaccine expressing HA protein of H5N1 HPAI virus. It has demonstrated to be effective in preventing the development of clinical signs and suppressing virus shedding upon heterologous H5N1 virus challenge[723]. Furthermore, it was also shown to reduce the challenge virus transmission to vaccinated contact chickens[723]. In addition, Vectormune® HVT-AI also showed protection in turkeys and domestic ducks against highly pathogenic H5N1 AIV[724,725]. Moreover, rHVT-H7HA protected chickens from lethal challenge against

H7N1 HPAI virus and highly virulent MDV, and also supported DIVA vaccination strategy[717]. Similarly, rHVT-H9HA demonstrated strong humoral and cellular immune responses, as well as effective protection against H9N2 AIV challenge[267].

Most of the first generation rHVT vaccines were produced using conventional genome recombination techniques. This was followed by reverse genetic approaches allowing integration of HVT genome into cosmid or bacterial artificial chromosome (BAC) clones[254]. However, all these traditional methods are generally time-consuming and labour-intensive due to the requirement of several rounds of plaque purification and transfer vector cloning procedures[711]. In recent years, CRISPR associated Cas9 system has gained popularity due to its versatility and specificity in gene editing. The CRISPR/Cas9 system has been successfully used in the generation of genetically modified cells and animal models[726–728], as well as in the manipulation of several large DNA viral genomes[729–731]. This system uses a single gRNA recognising a 20-nucleotide target sequence adjacent to a protospacer adjacent motif (PAM). The Cas9 nuclease introduces a double strand break (DSB) in the target sequence. The DSB can then be repaired by either the non-homologous end-joining (NHEJ) or Homology Directed Repair (HDR) pathway[732] **(Figure 6.1)**. Both NHEJ and HDR methods have been used to generate recombinant vaccines, with improved speed and efficiency[719,733–736]. The NHEJ method is considered error-prone and unpredictable, as unintended insertion/deletion may be introduced during gene modification. On the other hand, HDR method provides high-fidelity in precision genome editing[732]. Having high fidelity is important for virus-vector vaccine, because even a small insertion or deletion might affect the stability of the recombinant viruses. Therefore, HDR

CRISPR/Cas9 system is well established, with extensive research being carried out to demonstrate the value of this system for vaccine development[729,734,735,737].

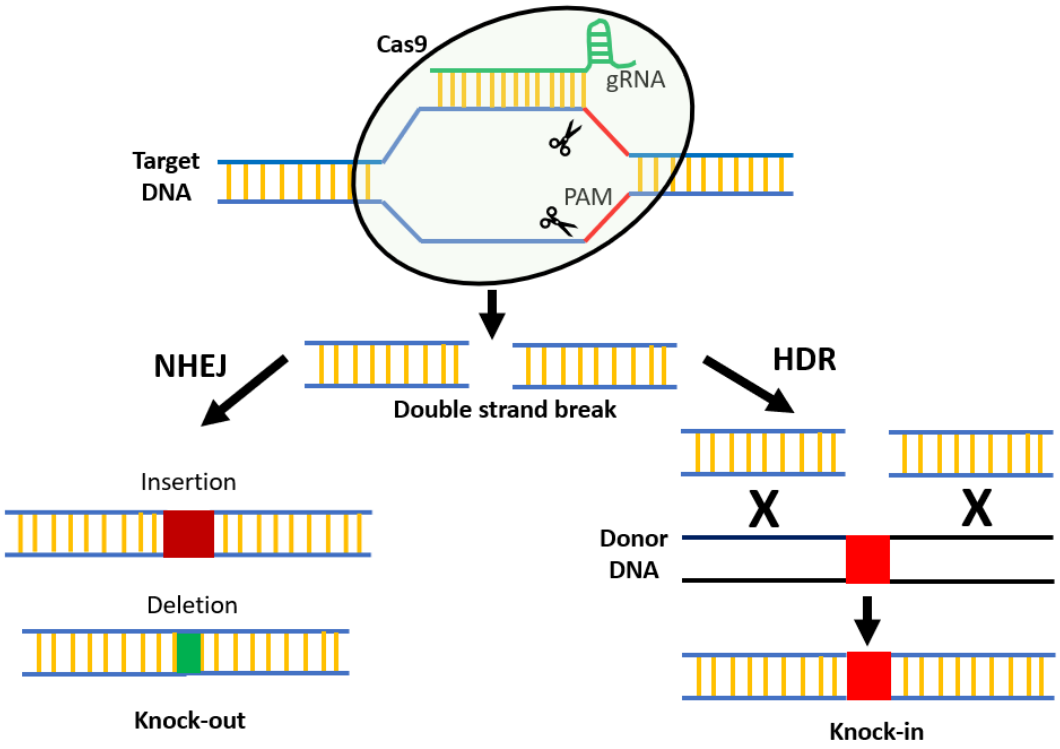


Figure 6.1. Pathways for repairing DSB created by Cas9 nuclease. In NHEJ pathway, the ends of DSB are re-joined which can result in random insertion or deletion (indel) at the site of junction. Such indel mutations occurring within the coding region of a gene can cause frameshifts and create premature stop codon resulting in gene knock-out. Alternatively, HDR pathway uses homologous donor DNA to create insertions or base substitutions between DSB sites. This allows high fidelity and precise editing.

In previous chapters, recombinant subunit rH9HA and rH9HA-CD83 scFv vaccines were generated and their immunogenicity and protective efficacy were evaluated. The rH9HA-CD83 scFv vaccine induced higher antibody responses, higher cytokine responses *in vitro* and reduced virus shedding compared to rH9HA vaccine. Furthermore, rH9HA-CD83 scFv vaccine was also able to enhance the production of cross-reactive antibodies and overcome the MDA interference. However, subunit vaccines have several drawbacks including short- term immunity

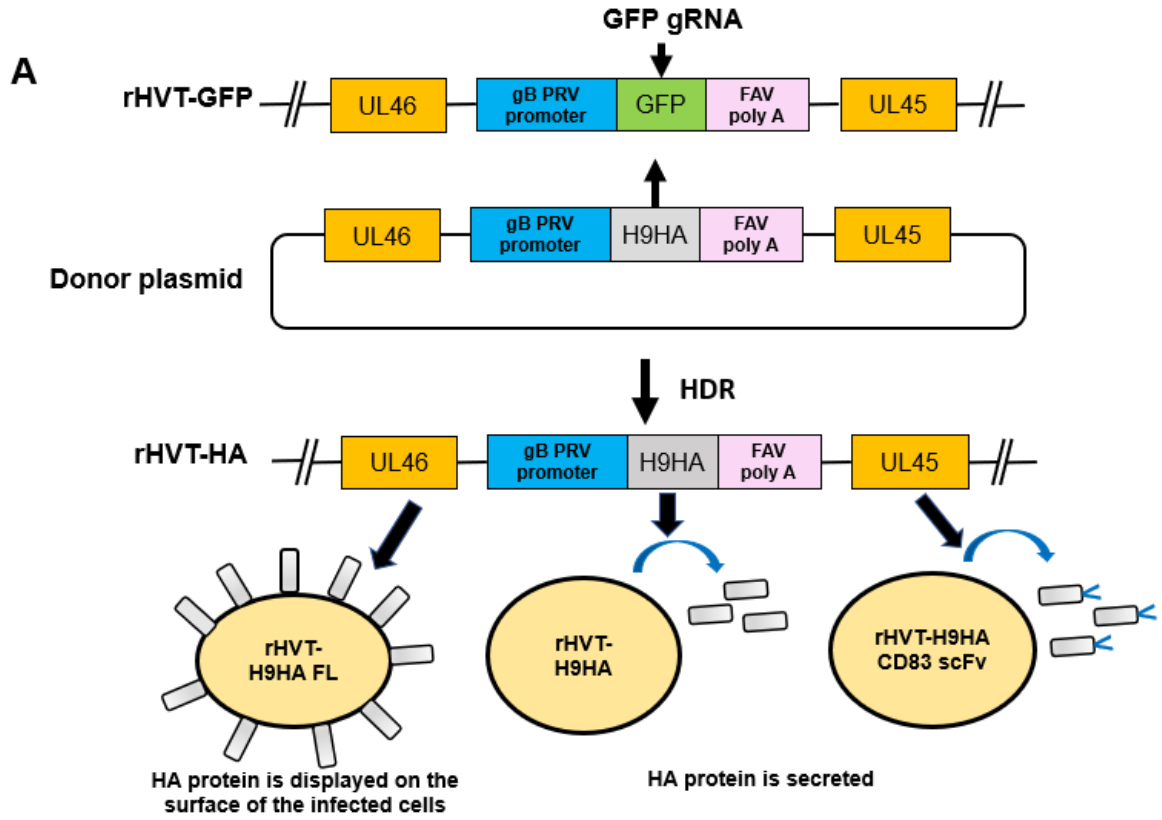
and the requirement of adjuvants for enhancing the humoral and cellular immune responses[272]. Several studies have been done to evaluate the immunogenicity and protective efficacy of HVT vector vaccine expressing the full-length HA protein containing TM domain[267,717,722]. Here, the ability of HVT to act as an efficient viral vector for the expression of secreted forms of HA protein i.e. H9HA Foldon and H9HA Foldon-CD83 scFv was evaluated, along with comparing their immunogenicity to those generated by rHVT-H9HA FL *in vivo*.

6.2. Results

6.2.1. The rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv were successfully generated using HDR CRISPR/Cas9 system

To generate the recombinant bivalent HVT-AIV vaccine, the intergenic region between UL45/UL46 was chosen as non-essential site for H9HA gene insertion, as this site has been frequently used as a foreign gene insertion site before[717–719,734]. The HA protein sequence of SKP/2016 H9N2 virus (Genbank accession number: AVX19091.1) was used for this work since, SKP/2016 H9N2 virus serum was shown to be more cross-reactive to heterologous H9N2 viruses compared to UDL 01/08 H9N2 virus serum (unpublished). The schematic representation of rHVT-H9HA generation using HDR-CRISPR/Cas9 is shown in **Figure 6.2A**. The glycoprotein B (gB) promoter of pseudorabies virus (PRV) and polyA terminator of felid alpha herpesvirus 1 were used for the foreign gene expression. The CEF cells were transfected with GFP gRNA plasmid and donor plasmid containing target HA flanked by regions homologous to the Cas9 cut sites.

This was followed by infection with rHVT-GFP at a MOI of 0.01 at 12 hours post transfection. Three rHVT constructs were generated: rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. The rHVT-H9HA FL is expected to display H9HA protein on the cell surface whereas rHVT-H9HA and rHVT-H9HA CD83 scFv are expected to secrete the respective H9HA proteins into the cell culture medium. Virus plaques formed by rHVT infected cells without green fluorescence were isolated **(Figure 6.2B)**. These GFP negative plaques are either correct rHVT-H9HA positive clones or false positive clones with silenced GFP. Viral DNA was extracted and subjected to PCR analysis using primers targeting the region within H9HA insert **(Figure 6.2C)**. In total, about 56%, 11% and 22% of the clones were positive for H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv insertions, respectively **(Figure 6.2D)**. One of the positive rHVT clone from each construct was taken forward for plaque purification and expansion.



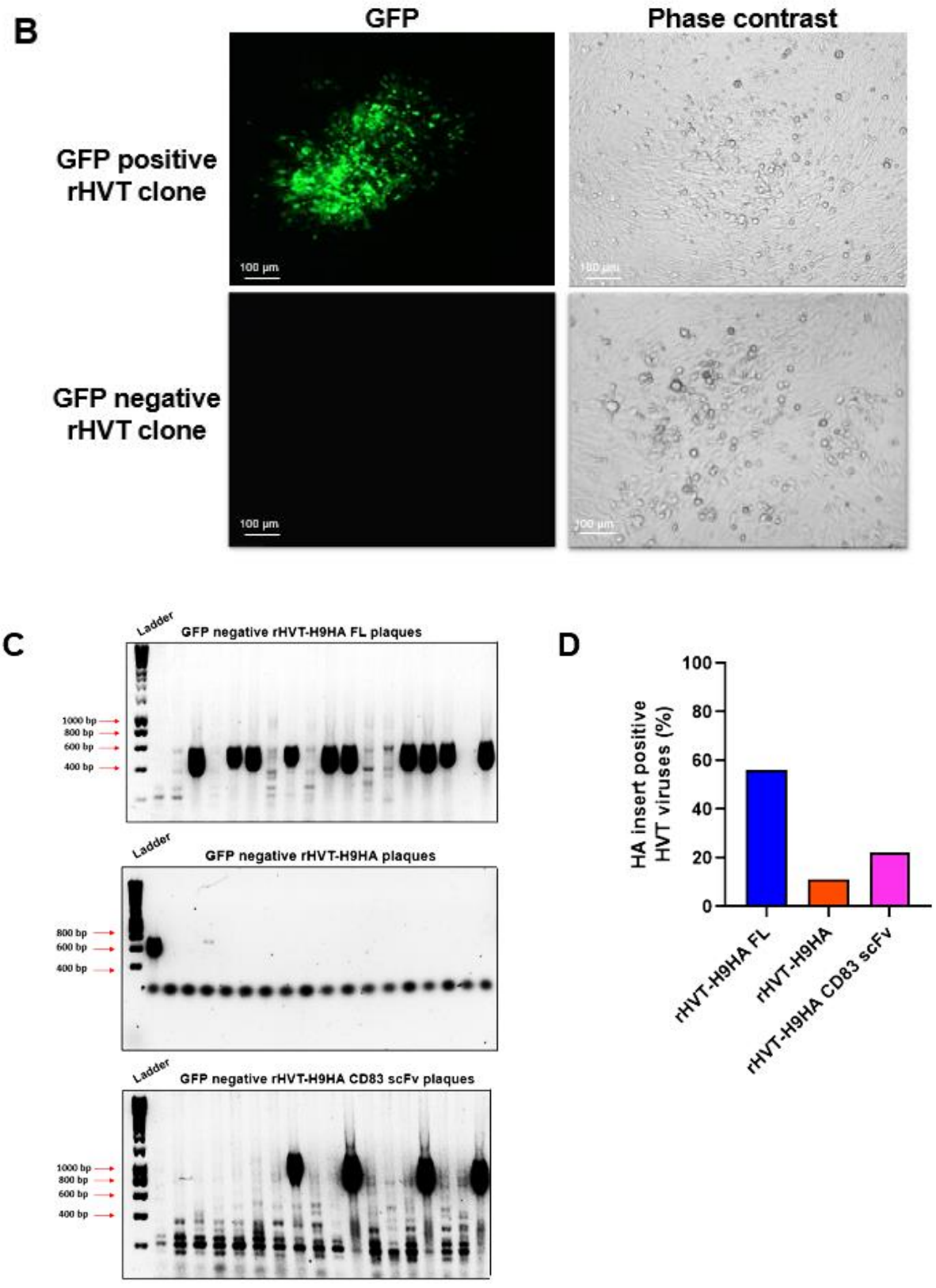


Figure 6.2. Recombinant HVT-H9HA generation using HDR-CRISPR/Cas9 system. (**A**) Schematic representation of the use of HDR-CRISPR/Cas9 system in the construction of rHVT-H9HA. The GFP expression cassette was replaced by H9HA expression cassette

via HDR-CRISPR/Cas9 with gRNA-targeted GFP. Three constructs were generated: rHVT-H9HA FL (H9HA displayed on the cell surface), rHVT-H9HA (H9HA secreted), rHVT-H9HA CD83 scFv (H9HA CD83 scFv secreted). **gB PRV**: Glycoprotein B promoter of pseudorabies virus **FAV**: Felid alpha herpesvirus1. **(B)** GFP and phase contrast images of rHVT plaques. The images were taken using the Leica fluorescence microscope (Leica, Germany**). (C)** PCR screening of H9HA insert positive rHVT plaques. The plaques formed by rHVT infected cells without green fluorescence were isolated, and then subjected to PCR screening using gene specific primers (~ 581 bp for H9HA Full-length positive rHVT plaques, (~ 517 bp for H9HA Foldon positive rHVT plaques, (~ 1266 bp for H9HA Foldon-CD83 scFv positive rHVT plaques). (**D**) The efficiency of rHVT–H9HA gene recombination. The indicated percentages of rHVT–H9HA positive plaques were calculated from the total number of 18 rHVT plaques without green fluorescence.

6.2.2. Replication and expression of HA antigen by rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv *in vitro*

To characterise rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv viruses, CEF cells were infected with the respective viruses for 72 hours. The supernatant and cell lysates were harvested for analysing H9HA protein expression using ELISA and western blot assays. The H9HA protein expression was assessed after every 5 passages until passage 20. Furthermore, virus titres at different passages were also noted using plaque titration. For all the rHVT constructs, virus titres were higher at passage 10 compared to passage 5 **(Figure 6.3A)**. The continued passage of the viruses could make them more adaptable to the *in vitro* cell culture conditions, improving their growth characteristics thus, increasing the infectious virus titres. However, after passage 10, the virus titres were consistent until passage 20. Moreover, at passage 5 low levels of H9HA Foldon and H9HA Foldon-CD83 scFv proteins were detected in the supernatants analysed using ELISA assay **(Figure 6.3B)**. However, after passage 5, significantly higher H9HA Foldon-CD83 scFv protein was detected in the supernatant compared to H9HA Foldon protein ($p<0.0001$). This correlated with the increase in the rHVT-H9HA CD83 scFv titres after passage 5, indicating that more viruses were present to infect

the cells for protein expression. On the contrary, no significant differences were observed in the levels of H9HA Foldon protein in the supernatant in all the passages tested, even though rHVT-H9HA titres increased after passage 5. Furthermore, no HA protein was detected in the supernatant of CEF cells infected with rHVT-H9HA FL virus. This was also confirmed by western blot assay **(Figure 6.3C)**. Bands were detected at approximately 70 kDa and 100 kDa in both supernatants and cell lysates of CEF cells infected with rHVT-H9HA and rHVT-H9HA CD83 scFv, respectively. These sizes were same as that detected for rH9HA and rH9HA-CD83 scFv proteins. Moreover, H9HA Full-length protein was detected only in the cell lysates (~70 kDa), indicating that the HA protein in this form is not secreted. No detectable band was observed in CEF cell lysates and supernatants infected with HVT wild-type virus (control).

Overall, the results indicate that rHVT-H9HA and rHVT-H9HA CD83 scFv can express HA protein in the secreted form, whereas rHVT-H9HA FL expresses non-secreted form of HA protein, most likely to be displayed on the cell surface. Furthermore, the results also suggest that the sequential passage of rHVT does not affect the protein expression, as H9HA protein was stably expressed until passage 20.

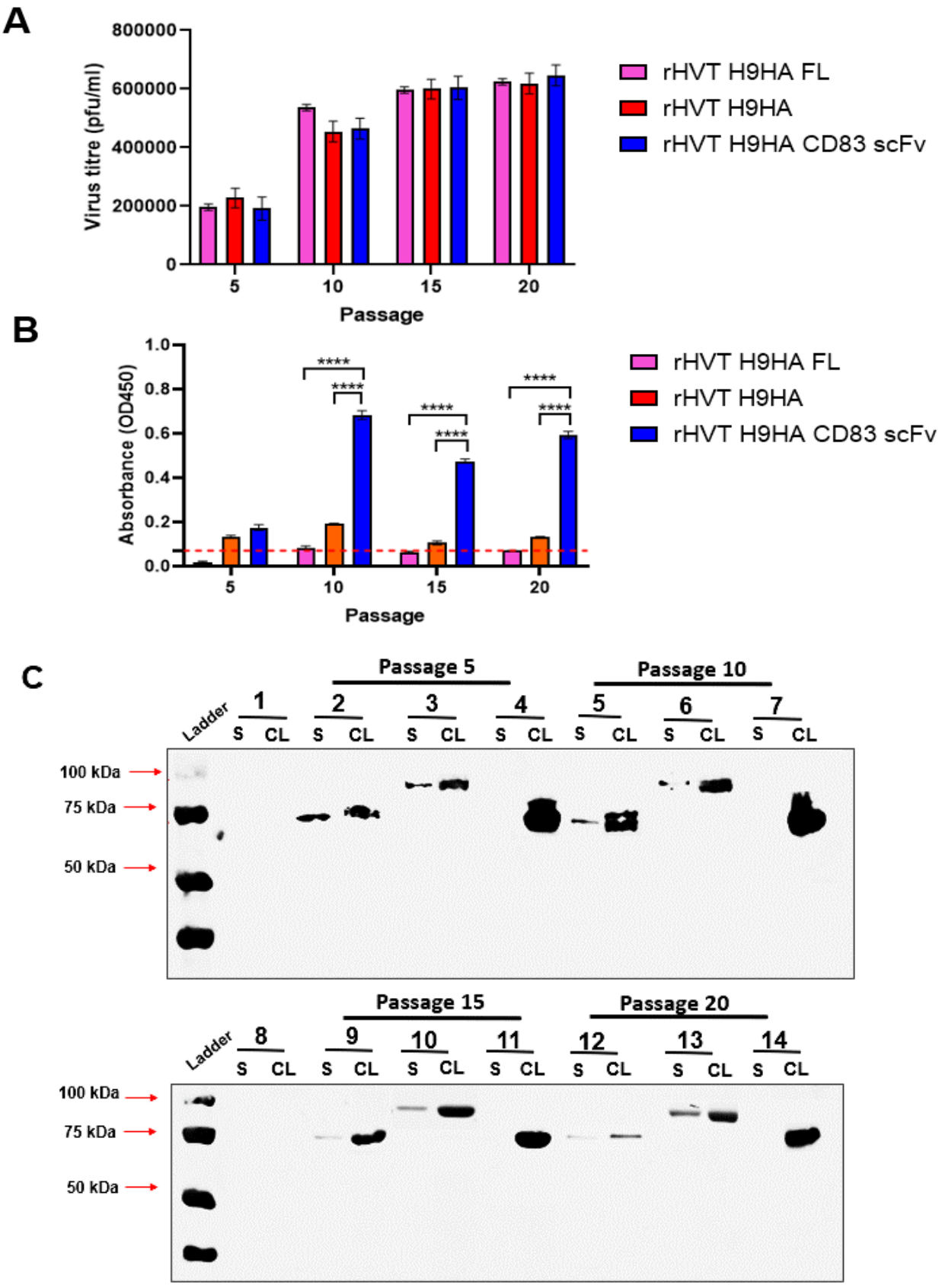


Figure 6.3. Characterisation of rHVT expressing H9HA protein. **A)** Titres of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv at different passages measured using plaque titration. At different passages, CEF cells were infected with the respective rHVT constructs

for 72 hours. The cells were then fixed with ice cold methanol/acetone (1:1) for 15 minutes at room temperature. The expression of H9HA was visualised by incubating the fixed cells with mouse anti-H9HA monoclonal antibodies (1 mg/mL diluted to 1:2000) for 1 hour, followed by HRP-conjugated rabbit anti-mouse secondary antibody (1 mg/mL diluted to 1:200) for another 1 hour. The staining was developed by adding DAB substrate-chromogen solution and stained plaques were counted to obtain the virus titres. **B)** Detection of H9HA Foldon and H9HA Foldon-CD83 scFv proteins in the supernatants of rHVT infected CEF cells using ELISA. The red dotted line indicates the threshold of non-specific background. **C)** Western blot detection of H9HA protein in the supernatants and cell lysates of rHVT infected CEF cells. The supernatants and cell lysates were harvested from the rHVT infected CEF cells at passages 5 ,10 ,15 and 20 and subjected to ELISA (supernatants only) **(B)** and western blots (supernatants and cell lysates) **(C)**. For **B** and **C**, detection of H9HA protein was performed by using polyclonal SKP/2016 H9N2 serum raised in chicken (HI titre: 1024 ($log_2$10), 1:500) as primary antibody, followed by HRP conjugated goat anti-chicken secondary antibody (2 mg/mL diluted to 1:6000) for **(B)** and IRDye® 800CW donkey anti-chicken secondary antibody (1 mg/mL diluted to 1:10000) for **(C).** For **C**, **Lanes 1 and 8**: HVT wild-type (Negative control); **Lanes 2, 5, 9 and 12**: rHVT-H9HA; **Lanes 3, 6,10 and 13**: rHVT-H9HA-CD83 scFv; **Lanes 4, 7 ,11 and 14**: rHVT-H9HA FL; **S**: Supernatant **CL**: Cell lysates. For **A** and **B**, data are presented as mean ± SD from three independent experiments and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001.

6.2.3. The rHVT-H9HA CD83 scFv is more stable compared to rHVT-H9HA FL and rHVT-H9HA *in vitro*

To analyse the stability of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv *in vitro*, the respective constructs were grown sequentially in CEF cells for 20 passages and viral DNA was extracted and analysed after every 5 passages using H9HA-specific PCR primers described in **Table 25** (**Figure 6.4A**). The expected sizes of H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv inserts are 3143 bp, 3079 bp and 3829 bp, respectively. No significant variability in size and amount of H9HA gene product following repeated passages of the virus in cultured CEF cells were found. However, extra bands were observed at ~400 bp (size similar to HVT wild-type control) for rHVT-H9HA FL and rHVT-H9HA at each passage analysed. The sequence analysis of the bands at lower molecular weight showed that they consist of ~200 bp of HVT wild-type sequence and ~200 bp of

H9HA sequence. This indicates that a part of H9HA insert is being knocked out from rHVT-H9HA FL and rHVT-H9HA constructs during sequential passages. No such lower molecular weight bands were observed for rHVT-H9HA CD83 scFv. Furthermore, the stability of rHVT constructs was also assessed by immunofluorescence assay (**Figure 6.4B**). Cells were double stained with anti-H9HA (green) and anti-HVT (red) antibodies and analysed for H9HA knock out. The loss of insert from any of the recombinant viruses during the passage would be apparent, as these cells would only show positive staining with anti-HVT (red) antibodies. Approximately, 500 plaques were counted for each rHVT constructs and 100% of the counted plaques showed both H9HA (green) and HVT (red) positive staining. As expected, cells infected with HVT wild-type showed only HVT positive staining (red). The results from PCR suggest that rHVT-H9HA CD83 scFv is the most stable construct *in vitro*. In addition, the results from immunofluorescence assay suggest that the rate of H9HA insert knock out from rHVT constructs is low (at least <1:500 plaques)

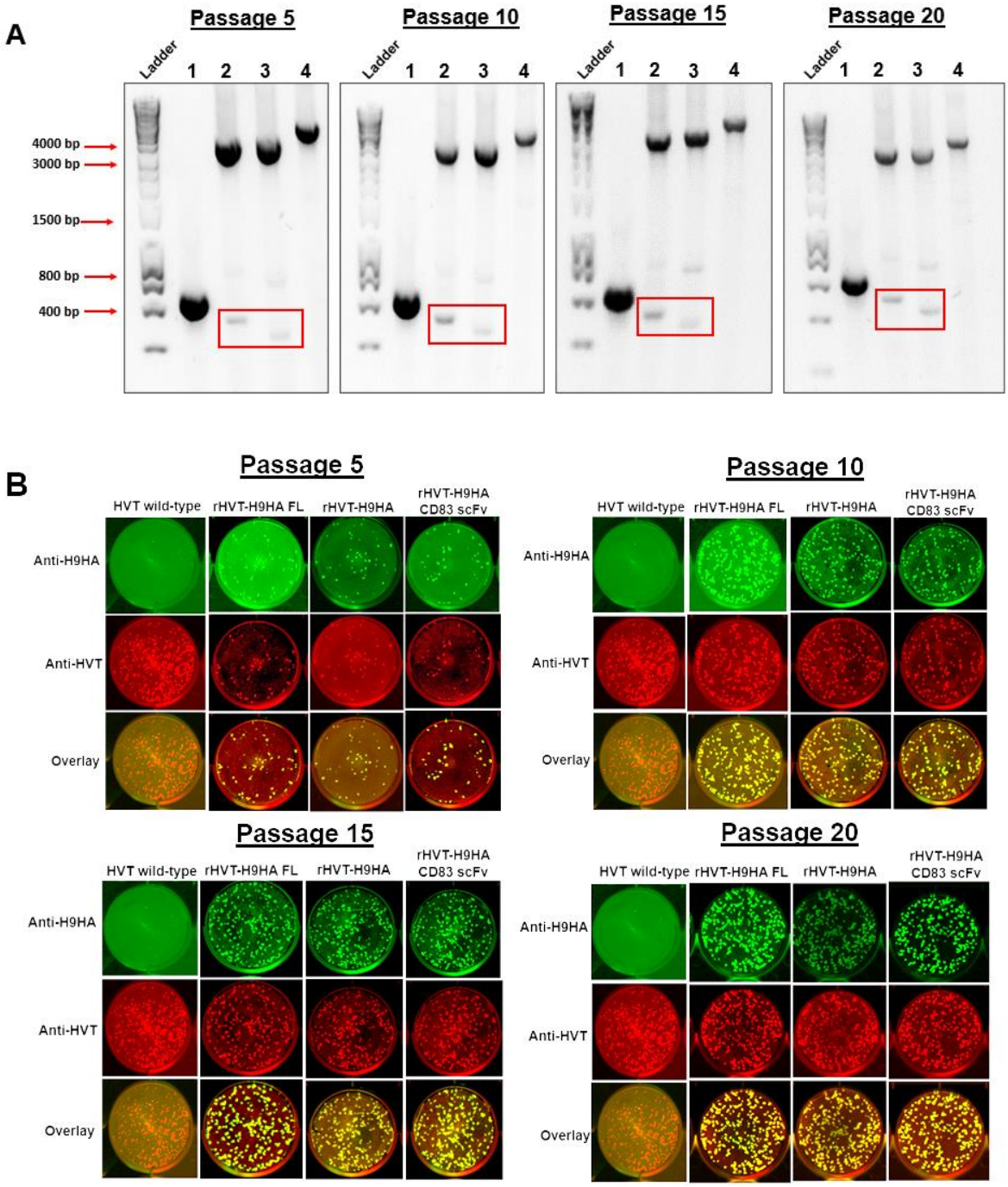


Figure 6.4. *In vitro* stability of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. **A)** PCR to confirm the presence of H9HA expression cassettes in rHVT at different passages. CEF cells infected with the respective recombinant HVT constructs were harvested and lysed in squishing buffer. PCR was performed using primers targeting the region within H9HA insert. **Lane 1**: HVT wild-type (Negative control), **Lane 2**: rHVT-H9HA FL **Lane 3**: rHVT H9HA **Lane 4**: rHVT-H9HA CD83 scFv. The expected sizes of bands for the inserts: H9HA Full-length~3143 bp, H9HA Foldon~3079 bp, H9HA Foldon-CD83 scFv~3829 bp. The red box represents extra bands around the size of HVT wild-type (negative control). **B)** Immunofluorescence assay. CEF cells were infected with the

respective rHVT constructs for 72 hours. The cells were then fixed with ice cold methanol/acetone (1:1) for 15 minutes at room temperature. The plaques were stained with a combination of mouse anti-H9HA monoclonal antibodies (green, 1 mg/mL diluted to 1:2000) and polyclonal HVT serum raised in chicken (red,1:500) (primary antibodies), followed by a combination of IRDye® 680RD goat anti-mouse antibody (1 mg/mL diluted to 1:10000) and IRDye® 800CW donkey anti-chicken antibody goat anti-mouse antibody (1 mg/mL diluted to 1:10000) as secondary antibodies, respectively. Plaques were then imaged on the Odyssey CLX (LI-COR biosciences) and analysed for HA insert knock out.

6.2.4. The rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv show similar *in vitro* replication kinetics as that of the wild-type HVT

Having characterised rHVT constructs for the expression of H9HA protein (**section 6.2.2**) and stability (**section 6.2.3**) *in vitro*, the effect of H9HA insertion on the replication of rHVT constructs was determined next. For this, primary CEF cells were infected with 100 pfu of either HVT wild-type or rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Virus replication rates were measured by qRT-PCR for genome copy numbers (**Figure 6.5A**) and plaque titration (**Figure 6.5B**). The virus titres were detectable by 12 hours post infection. The rate of virus replication measured by qRT-PCR showed no differences between the wild-type HVT and rHVT. Similar results were observed with plaque titration, except at 120 hours post infection where rHVT-H9HA FL showed significantly reduced virus titre compared to HVT wild-type ($p<0.05$). When the morphology of plaques was analysed at 120 hours post infection, the shape and sizes of the plaques were indistinguishable between wild-type HVT and rHVT **(Figure 6.5C)**. Overall, viral genome copy number was highly correlated with viral titre estimated by plaque titration (Pearson correlation coefficient ‘r’=0.77).

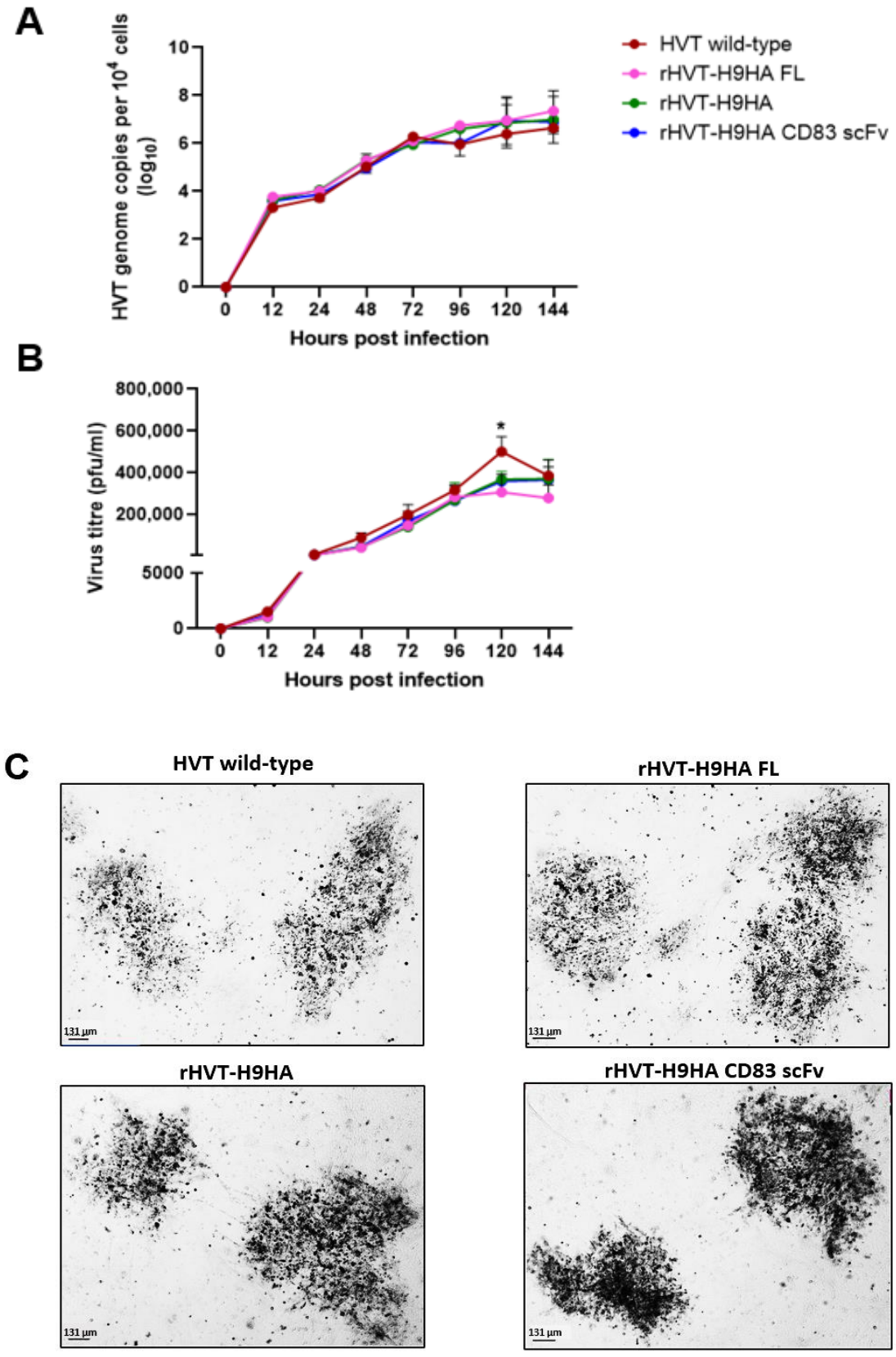


Figure 6.5. The replication kinetics and morphology of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Virus titres were measured at different time points after infection

with 100 pfu of each virus by qRT-PCR of HVT SORF1 gene on DNA extracted from CEF **(A)** and plaque titration **(B)**. **(C)** Morphology of the virus plaques on CEF cells at 120 hours post infection with of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Virus plaques were stained by polyclonal HVT serum raised in chicken (1:500) (primary antibody), followed by HRP conjugated goat anti-chicken secondary antibody (2 mg/mL diluted to 1:6000). The stained plaques were imaged using Leica fluorescence microscope (Leica, Germany). For **B**, the virus growth was determined by calculating HVT genome copies per 10,000 cells. For **A** and **B**, data are presented as mean ± SD from three independent experiments and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Asterisk represents significant differences between rHVT-H9HA FL and HVT wild-type. *$p<0.05$.

6.2.5. Evaluation of the immunogenicity of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv in chickens

After the characterisation of rHVT vaccine constructs *in vitro*, their immunogenicity in SPF chickens was assessed. The experimental design for the animal study is described in **section 2.2.8.3.4** and shown in **Figure 6.6.** Briefly, groups of 1-day-old White Leghorn SPF chickens (MSD Animal Health, n=20 per group) were immunised with rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv vaccines, each dose containing 4000 pfu of the viruses (Passage 10). Blood samples were collected on day 6, 14, 21, 28 and 35 pv for serological analysis. Furthermore, splenocytes and PBMCs were obtained from the chickens on day 7 pv and day 21 pv, respectively for the analysis of viral content. Chickens were observed daily. No clinical signs were observed in any of the groups. All chickens were culled via cervical dislocation and decapitation methods.

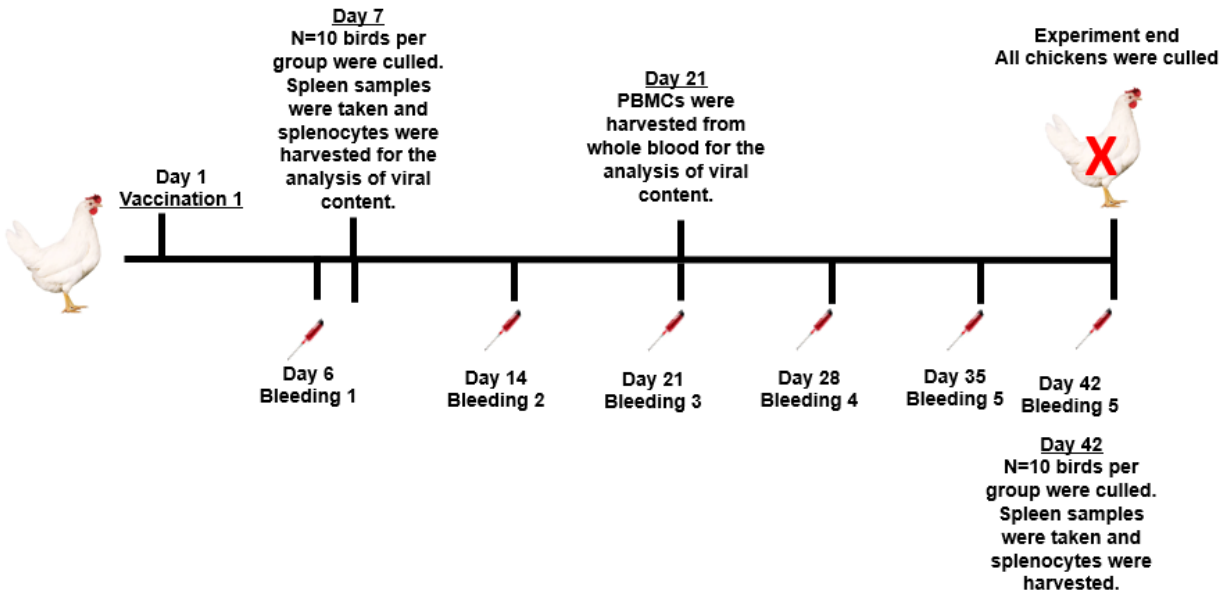
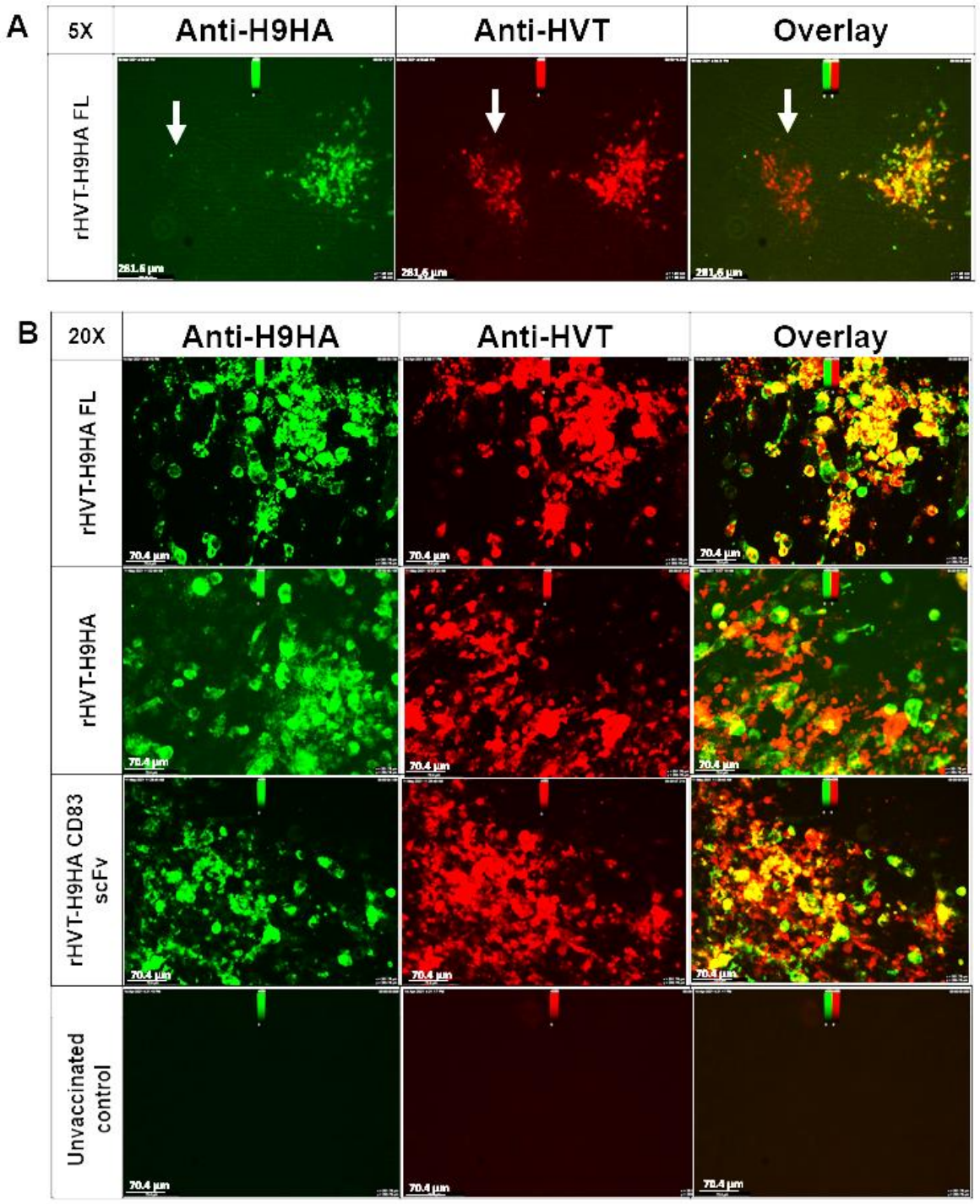


Figure 6.6: Experimental design to evaluate the immunogenicity of rHVT vaccines. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv, subcutaneously. Blood samples were collected from the wing veins on day 6, 14, 21,28 and 35 post vaccination (pv). On day 7 pv, spleen samples (n=10 per group) were collected and splenocytes were harvested for the analysis of viral content. Similarly, PBMCs were harvested from whole blood on day 21 pv (n=10 per group) for the analysis of viral content. Furthermore, splenocytes were harvested from spleen samples (n=10 per group) on day 42 pv.

6.2.5.1. The rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA-CD83 scFv show 99-100% stability *in vivo*

To analyse the replication and stability of the respective rHVT constructs *in vivo*, splenocytes and PBMCs were harvested from the spleen and blood samples of vaccinated chickens on day 7 pv and day 21 pv, respectively. The splenocytes/PBMCs were co-cultured with secondary CEF cells for 5 days. The cells were stained with both anti-HVT (red) and anti-H9HA (green) antibodies and analysed using fluorescence microscopy. An example of H9HA gene knock out from rHVT-H9HA FL construct is shown in **Figure 6.7A**, where no anti-H9HA (green) staining is observed. In chickens vaccinated with rHVT-H9HA FL, rHVT-

H9HA and rHVT-H9HA CD83 scFv, HVT virus was detected in splenocyte samples on day 7 pv and PBMC samples on day 21 pv **(Figure 6.7B and 6.7C)**. No HVT virus was detected in splenocyte and PBMC samples from unvaccinated control chickens. Overall, higher virus titres were observed in splenocytes compared to PBMCs. Furthermore, all the three rHVT constructs were able to produce H9HA protein, as indicated by the positive anti-H9HA (green) staining.



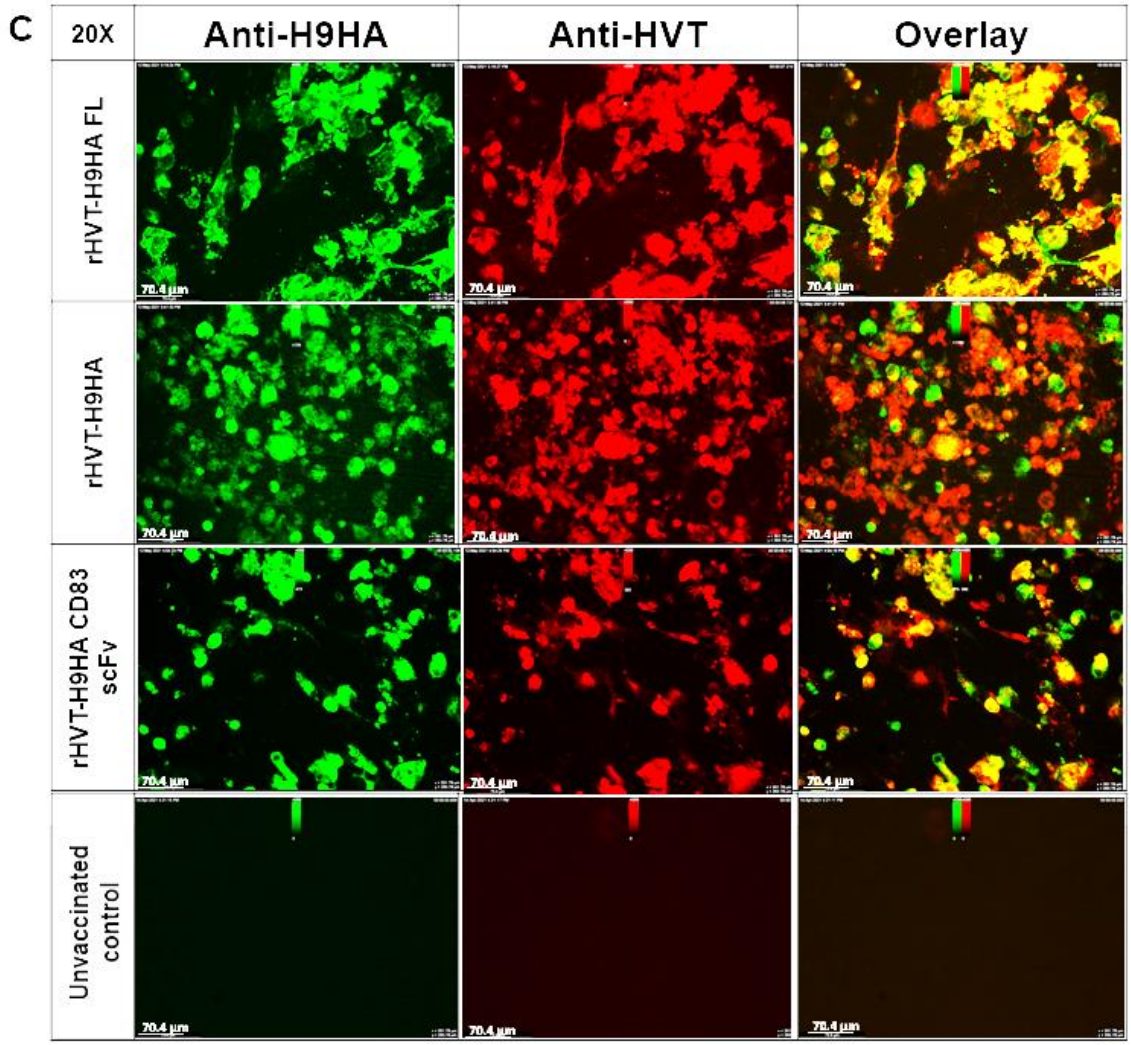


Figure 6.7: Immunofluorescence assay to detect the viral content in chickens after rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv vaccination. **A)** Example of H9HA gene knockout from rHVT-H9HA FL construct (white arrow). **B)** Detection of virus in chicken splenocytes on day 7 post vaccination (pv). **C)** Detection of virus in chicken PBMCs on day 21 pv. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Splenocytes and PBMCs were harvested from the spleen and blood samples of vaccinated chickens on day 7 pv and day 21 pv, respectively. Briefly, chicken splenocytes/PBMCs were co-cultivated with secondary CEF cells for 5 days. After 5 days, the cells were fixed with ice-cold 96% ethanol and stained with a combination of mouse anti-H9HA monoclonal antibodies (1 mg/mL diluted to 1:2000) and polyclonal HVT serum raised in chicken (1:500) as primary antibodies. This was followed by further incubation with a combination of Alexa Fluor® 488 goat anti-mouse IgG (2 mg/mL diluted to 1:500) and Alexa Fluor® 568 goat anti-chicken IgY (2 mg/mL diluted to 1:500) secondary antibodies, and imaged using fluorescence microscope (Leica, DMI6000).

In terms of virus titres, rHVT-H9HA CD83 scFv showed significantly higher titres in splenocytes compared to rHVT-H9HA FL and rHVT-H9HA on day 7 pv ($p<0.01$) **(Figure 6.8A)**. Furthermore, virus titres in PBMCs on day 21 pv was significantly higher in rHVT-H9HA group compared to rHVT-H9HA FL group ($p<0.01$) **(Figure 6.8C)**. No significant differences were observed in virus titres between rHVT-H9HA and rHVT-H9HA CD83 scFv groups on day 21 pv. This shows that all the rHVT constructs were able to replicate well in chickens, with rHVT-H9HA FL replicating lesser compared to rHVT-H9HA CD83 scFv and rHVT-H9HA in splenocytes and PBMCs, respectively. In order to determine the stability of H9HA expression cassette, respective rHVT plaques were stained with both anti-HVT and anti-H9HA antibodies, followed by analysis of rHVT plaques which showed negative anti-H9HA staining. It was difficult to detect anti-H9HA stain, particularly in rHVT-H9HA samples therefore, only 20 plaques per sample giving distinct anti-HVT and anti-H9HA staining were analysed. For rHVT-H9HA FL and rHVT-H9HA CD83 scFv samples, all the plaques obtained were analysed. About 99% to 100% H9HA insert stability was observed with all the rHVT constructs in both splenocyte and PBMC samples (**Figure 6.8B and 6.8D**).

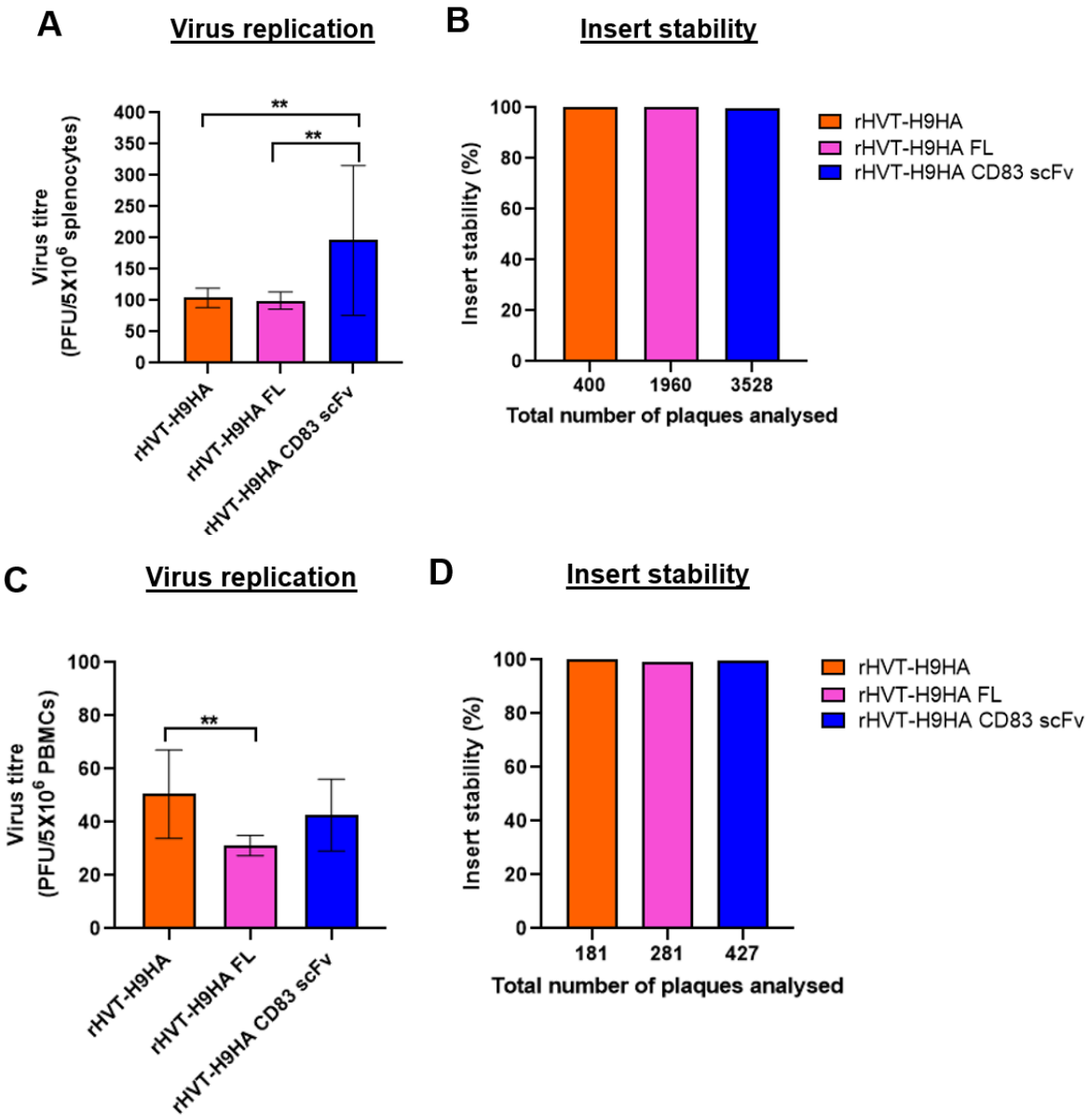


Figure 6.8. Replication and stability of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv *in vivo*. **A, B)** Virus replication and stability in chicken splenocytes on day 7 post vaccination (pv). **C, D)** Virus replication and stability in chicken PBMCs on day 21 pv. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Splenocytes and PBMCs were harvested from the spleen and blood samples of vaccinated chickens on day 7 pv and day 21 pv, respectively. Briefly, splenocytes/PBMCs obtained from vaccinated chickens were co-cultured with secondary CEF cells for 5 days. The virus plaques were fixed with 96% ice cold ethanol and stained with a combination of anti-H9HA monoclonal antibodies (1 mg/mL diluted to 1:2000) and polyclonal HVT serum raised in chicken (1:500) as primary antibodies, followed by incubation with a combination of Alexa Fluor® 488 goat anti-mouse IgG (2 mg/mL diluted to 1:500) and Alexa Fluor® 568 goat anti-chicken IgY (2 mg/mL diluted to 1:500) secondary antibodies. The dishes were read using fluorescence microscope (Leica, DMI6000). For the determination of insert stability, HVT

plaques showing negative anti-H9HA staining were noted. For **A** and **C**, data are presented as mean ± SD (n=20 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. **p<0.01

6.2.5.2. The rHVT-H9HA FL induces higher antibody responses compared to rHVT-H9HA and rHVT-H9HA CD83 scFv

The serum samples were obtained from immunised chickens on day 7,14, 21, 28, 35 and 42 pv. Serum antibody titres were analysed using HI, anti-HA IgM, IgY and IgA ELISA and virus MNT assays. Chickens vaccinated with rHVT-H9HA FL showed detectable HI antibodies on day 14 pv whereas those vaccinated with rHVT-H9HA CD83 scFv and rHVT-H9HA showed detectable HI antibodies only on day 21 and day 28 pv, respectively **(Figure 6.9A)**. Furthermore, the HI antibody titres in rHVT-H9HA FL group were significantly higher than rHVT-H9HA and rHVT-H9HA CD83 scFv groups in all the time points tested ($p<0.0001$). In addition, comparison of HI antibody titres between rHVT-H9HA CD83 scFv and rHVT-H9HA demonstrated that the former was able to induce significantly higher HI antibody titres than the latter after day 21 pv (day 28 pv: $p<0.05$, day 35 pv: $p<0.0001$, day 42 pv: $p<0.0001$) **(Figure 6.9B)**.

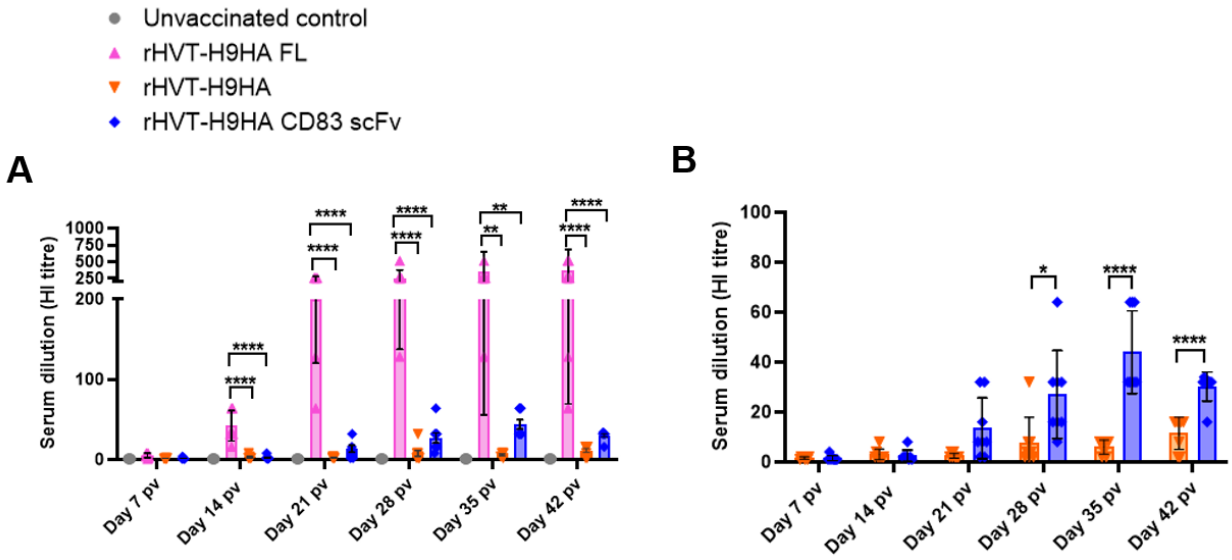


Figure 6.9. Analysis of HI antibody titres in the serum of chickens vaccinated with rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. **A)** Comparison between rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv immunised serum. **B)** Comparison between rHVT-H9HA and rHVT-H9HA CD83 scFv immunised serum. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. In all cases, blood samples were collected from the wing veins on day 7, 14, 21, 28, 35 and 42 post vaccination (pv). The HI titres were expressed as the reciprocal of the highest dilution of serum causing the total inhibition of 4 HA units of SKP/2016 H9N2 virus haemagglutination activity. Data are presented as mean ± SD (n=20 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test **(A)** and unpaired student t-test **(B)**. Statistical significance is shown with asterisks. ****p<0.0001 **p<0.01 *p<0.05

With regards to anti-HA IgM, IgY and IgA antibodies, rHVT-H9HA FL vaccinated chickens showed an overall higher amount of these antibodies in the serum compared to rHVT-H9HA and rHVT-H9HA CD83 scFv vaccinated chickens **(Figure 6.10)**. Moreover, chickens vaccinated with rHVT-H9HA FL showed detectable anti-HA IgM/IgY/IgA antibodies on day 14 pv, whereas those vaccinated with rHVT-H9HA and rHVT-H9HA CD83 scFv showed detectable anti-HA IgM/IgY/IgA antibodies on day 21 pv. There were no differences observed in the titres of anti-HA IgM and IgA antibodies between rHVT-H9HA and rHVT-H9HA CD83 scFv groups. However, rHVT-H9HA CD83 scFv group showed significantly

higher anti-HA IgY antibodies compared to rHVT-H9HA group on day 21 pv ($p<0.05$), day 28 pv ($p<0.001$) and day 35 pv ($p<0.05$). One key observation made with rHVT-H9HA FL vaccine was the higher induction of anti-HA IgA antibodies in the serum compared to the recombinant subunit vaccines and inactivated H9N2 vaccine tested in the previous chapters. The amount of anti-HA IgA antibodies was also significantly higher than rHVT-H9HA and rHVT-H9HA CD83 scFv groups ($p<0.0001$).

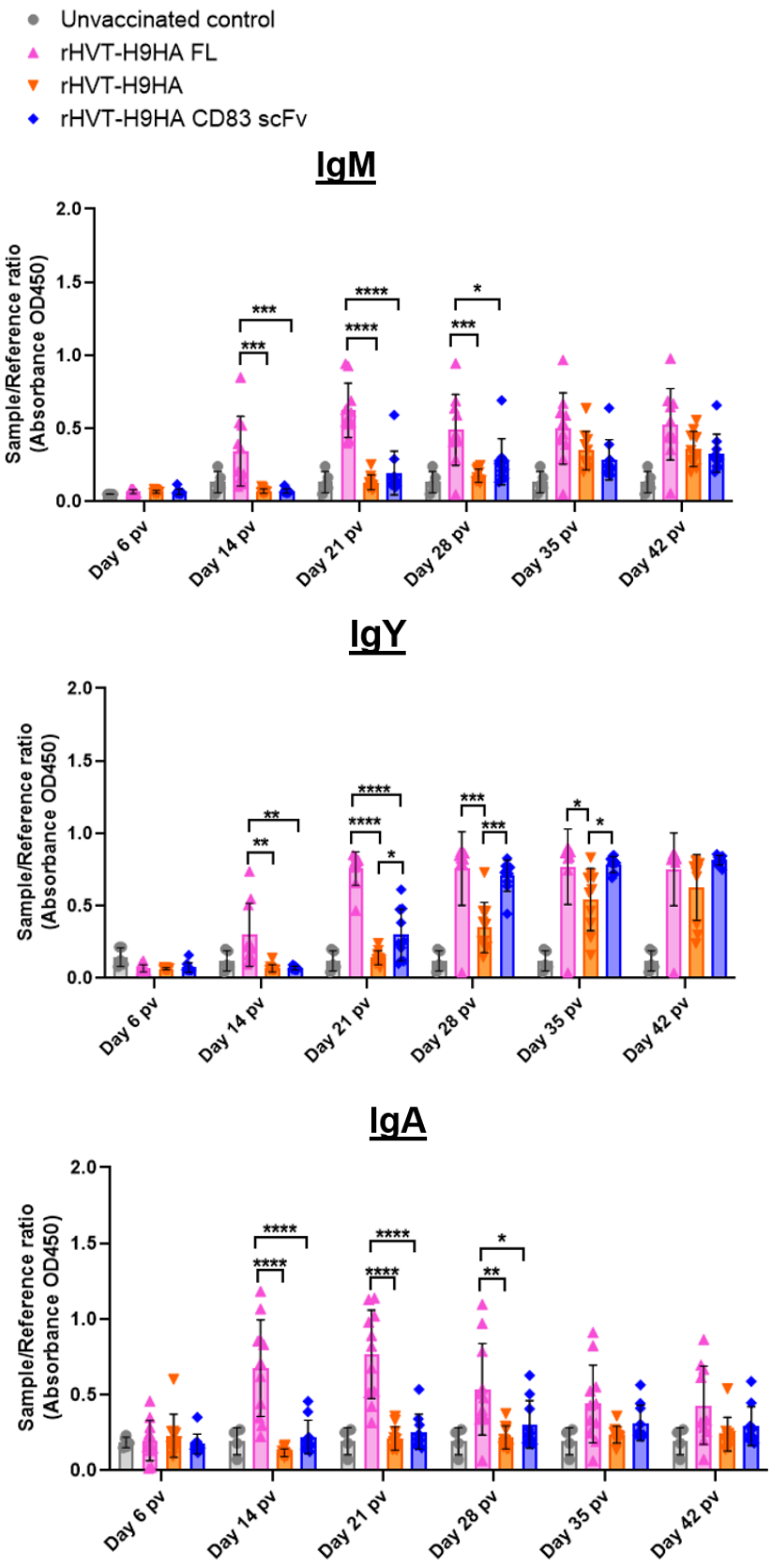


Figure 6.10. HA-specific IgM, IgY and IgA antibodies in the serum of chickens vaccinated with rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-

H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. In all cases, blood samples were collected from the wing veins on day 7, 14, 21, 28, 35 and 42 post vaccination (pv). The anti-HA antibodies were determined in 200-fold diluted sera by ELISA. Briefly, the plates were coated with 0.5 µg of recombinant H9HA Foldon protein overnight at 4°C. For detection, the plates were incubated with respective serum for one hour at room temperature. This was followed by further incubation for one hour with anti-chicken IgM (1 mg/mL diluted to1:3000), IgY (2 mg/mL diluted to 1:6000), and IgA (1 mg/mL diluted to 1:3000) antibodies. The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded. The amount of HA specific IgM, IgY and IgA antibodies were expressed as sample to reference ratio (relation of absorbance of tested serum sample to absorbance of the reference serum). Data are presented as mean ± SD (n=20 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is shown with asterisks. ****p<0.0001 ***p<0.001 **p<0.01 *p<0.05

Similarly, the virus MNT assay performed on day 42 pv serum samples also displayed significantly higher virus neutralising antibodies in chickens vaccinated with rHVT-H9HA FL compared to those vaccinated with rHVT-H9HA (p<0.0001) and rHVT-H9HA CD83 scFv (p<0.001) **(Figure 6.11A)**. Furthermore, rHVT-H9HA CD83 scFv showed significantly higher virus neutralising antibodies compared to rHVT-H9HA (p<0.01) **(Figure 6.11B)**.

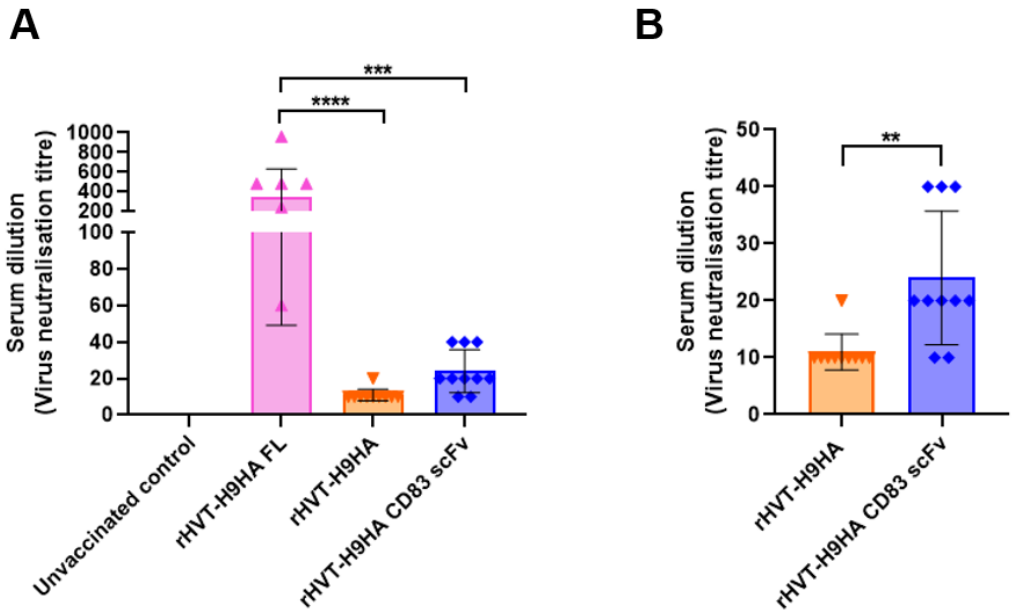


Figure 6.11: Analysis of virus neutralising antibody titres in the serum of chickens vaccinated with rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. **A)** Comparison

between rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv immunised serum. **B)** Comparison between rHVT-H9HA and rHVT-H9HA CD83 scFv immunised serum. Groups of 1-day-old White Leghorn SPF chickens (n=20 per group) were immunised once with 4000 pfu of rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv. In all cases, blood samples were collected from the wing veins on day 7, 14, 21, 28, 35 and 42 post vaccination (pv). Virus neutralisation assay was carried out on serum samples from day 42 pv. Briefly, the serum was inactivated at 56°C and incubated with 150 $TCID_{50}$ of SKP/2016 H9N2 virus for 1 hour at 37°C. The virus-serum mixture was then added onto the MDCK cells and further incubated for 1 hour at 37°C. This was followed by the removal of virus-serum mixture from the cells and incubation with fresh media containing 2 µg/ml trypsin for about 3 days. The virus neutralisation titre was expressed as the reciprocal of the highest serum dilution at which virus infection is blocked and the cells survive. Data are presented as mean ± SD (n=10 per group) and analysed by one-way ANOVA followed by Tukey's multiple comparison test **(A)** and unpaired student t-test **(B).** ****p<0.0001 ***p<0.001 **p<0.01

6.2.5.3. There is a negative correlation between the rHVT replication efficiency in PBMCs and HI antibody response

Next, the correlation between rHVT replication efficiency *in vivo* and HI antibody response was investigated. The plaque count in PBMCs and the respective HI antibody titre on day 21 pv were analysed. A positive correlation between HVT replication efficiency and HI antibody titre was hypothesised, as having a higher replication efficiency of rHVT meant that more viruses were present to infect cells for antigen production. This would eventually lead to a higher HI antibody response. However, there was a negative correlation between the virus titre and HI antibody titre (Pearson corelation coefficient r = **-** 0.55, **Figure 6.12**). The rHVT-H9HA showed higher virus titres in PBMCs compared to rHVT-H9HA FL on day 21 pv (p<0.01, **Figure 6.8C**). However, rHVT-H9HA FL demonstrated significantly higher HI antibody titre compared to rHVT-H9HA (p<0.0001, **Figure 6.9A**). Similarly, rHVT-H9HA displayed slightly higher virus titres however, it showed lower HI antibody titre compared to rHVT-H9HA CD83 scFv **(Figure 6.8C and Figure 6.9B)**.

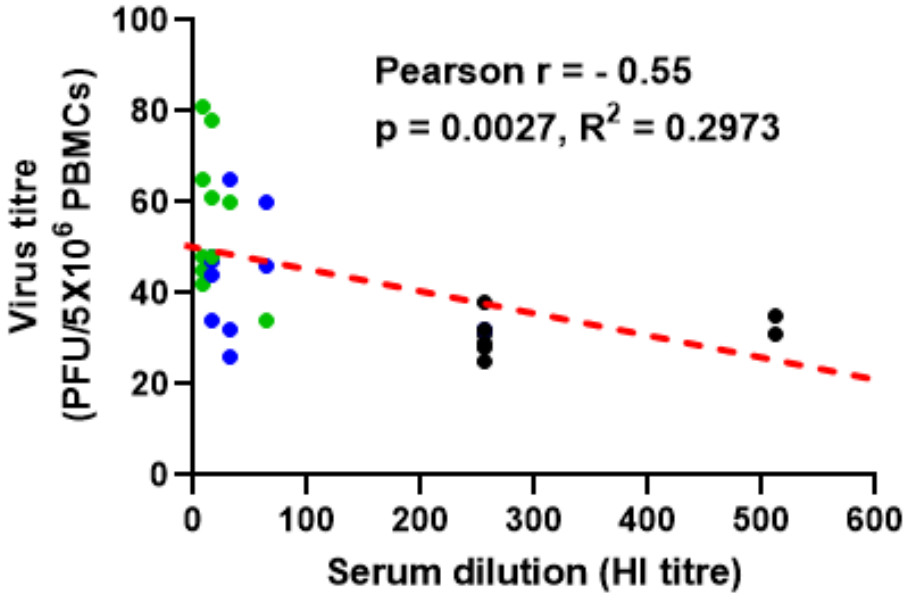


Figure: 6.12. Association of virus titres and HI antibody titres in chickens vaccinated with rHVT-H9HA FL (black), rHVT-H9HA (green) and rHVT-H9HA CD83 scFv (blue) on day 21 post vaccination.

6.3. Discussion

Over the last few years, commercialisation of HVT vector vaccines for multiple poultry diseases have increased significantly. The ability of HVT vectors to provide life-long protection even in the presence of MDAs has attracted its use over other viral vectors for the development of poultry vaccines[254]. The traditional technologies for the generation of rHVT vector-based vaccines like homologous recombination and BAC technology usually involves several rounds of vector cloning and selection, as well as large scale screening, which might take several months[730,738]. In addition, rHVT-BAC system is often unstable in bacteria, making it difficult to generate rHVT viruses[734]. Moreover, the discovery of CRISPR/Cas9 gene editing system has provided a better tool for generating HVT vector vaccines with higher specificity, versatility and speed[498]. Using this approach, the

recombinant viruses can be obtained in about 2.5 weeks[498,734]. Both NHEJ and HDR-dependent CRISPR/Cas9 system have been used for generating rHVT-HA vaccines[734,736]. NHEJ is a major form of mammalian DNA repair machinery that successfully joins broken DNA together[739]. Thus, when used alongside CRISPR/Cas9, NHEJ has a greater efficiency than HDR because genome repair occurs throughout the cell cycle, while HDR is limited to the S and G2 phase due to the availability of sister chromatids only on these cell cycle phases[740]. Another advantage of NHEJ CRISPR/Cas9 system is that the donor plasmid is free from the restriction of homology arms therefore, donor plasmids could be shared between different virus vectors or different insertion sites inside one virus vector[734]. However, NHEJ is error prone and unpredictable, as unspecific base deletion or insertion may be introduced during gene modification, lowering the fidelity[733]. In comparison, HDR CRISPR/Cas9 system uses homologous donor DNA sequences from sister chromatids or foreign DNA to create accurate insertions or base substitutions between DSB sites. Such precise modification increases the fidelity and is significant to genomic engineering in order to achieve the desired effect[741]. Therefore, HDR mechanism is favoured for gene modifications. The HDR CRISPR/Cas9 system was used for the first time by Chang et al. to generate a rHVT vaccine expressing influenza HA protein[734]. The same protocol was used to generate all the rHVT vaccines for this thesis.

The gene insertion site in the HVT genome has a major influence in the successful expression of foreign gene[742]. The HVT genome is very large, and includes many regions non-essential for viral growth, that can be chosen to insert and express foreign antigens. Such sites in the HVT genome include US2, US10, intergenic regions of UL45/UL46 and HVT065/HVT066[736]. The intergenic region

between UL45/UL46 was used to insert H9HA gene for our work. This site has been previously used to insert H7HA gene[734]. Here, instead of using fluorescent markers as an indicator for antigen gene incorporation, we used GFP gRNA mediated HDR CRISPR/Cas9 to replace GFP gene with the H9HA gene, and isolated plaques without green fluorescence. Plaques lacking green fluorescence are likely to be either correct rHVT-H9HA positive clones due to the successful replacement of the GFP gene with H9HA gene, or false positive clones with silenced GFP due to a disturbed open reading frame. About 11%-56% of successful rHVT-H9HA gene insertion efficiency was observed. This observation was different from the one observed by Chang et al. where successful rHVT-HA gene insertion rate was only about 6%[734]. However, the study used Pec promoter (Cytomegalovirus (CMV) enhancer and chicken β-actin chimera promoter) whereas we used gB promoter of pseudorabies virus. The large insert size generally impacts the cloning and transfection efficiency[743]. Although, H9HA Full-length and H9HA Foldon constructs were similar in size, the former observed higher gene insertion efficiency compared to the latter. Furthermore, H9HA Foldon-CD83 scFv is larger in size compared to H9HA Foldon however, the gene insertion efficiency of the former was higher than that of the latter. This implies that there is no proper correlation between the insert sizes and HVT gene insertion efficiency. One possible explanation for the lower knock-in efficiency of rHVT-H9HA is that the majority of CEF cells were somehow transfected with just GFP gRNA and no donor plasmid. This resulted in higher amount of false positive GFP disrupted HVT clones. It has been shown that knock-in of influenza HA into US2 site of HVT using the same HDR CRISPR/Cas9 approach and the same GFP gRNA, yielded successful rHVT-HA positive rates of ~50% (Iqbal lab, unpublished). A study by Zhang et al. compared the influence of

different insertion sites of HVT; US2, US10 and one of Meq gene on the transcription and expression levels of the AIV-H9N2 HA gene[742]. The US2 site showed the highest transcription activity, while US10 and Meq sites had lower transcription activity levels. Therefore, gene insertion sites should be carefully considered when generating HVT vector vaccines. Moreover, H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv expression cassettes were successfully integrated into the desired location and remained stable even after 20 passages *in vitro*, as confirmed by the immunofluorescence assay. However, the PCR results showed a band around 400 bp with rHVT-H9HA FL and rHVT-H9HA at every passage analysed. The sequence analysis of this band indicated that a part of H9HA insert might be knocked out from rHVT-H9HA FL and rHVT-H9HA constructs during sequential passages. However, no such deletion was observed in 500 plaques analysed using immunofluorescence assay. During the generation of rHVT, several rounds of plaque purification was carried out to obtain a single rHVT plaque expressing the protein of interest. Therefore, it is less likely that the lower bands are from other population of mixed plaques. It is also possible that the small PCR products are the result of PCR artefacts. In future, next generation sequencing can be performed to analyse the stability of rHVT at different passages. Moreover, the *in vivo* stability of rHVT constructs was about 99%-100%. The recombinant viruses with foreign gene insertions had overall similar replication characteristics to that of the wild-type HVT virus *in vitro*. This further confirms the suitability of the intergenic region between UL45/UL46 site for foreign gene insertion.

The activity of promoters has been one of the critical factors affecting the antigen expression and protective efficacy of recombinant vector vaccines. Two

rHVT viruses expressing VP2 protein of IBDV under the control of Pec promoter (rHVT-pecVP2) and CMV promoter (rHVT-cmvVP2) were compared. It was found that rHVT-pecVP2 expressed the VP2 antigen approximately four times more than rHVT-cmvVP2 *in vitro* and induced complete protection against a lethal IBDV challenge in chickens, whereas rHVT-cmvVP2 induced only 58% protection[744]. Similarly, another study showed that rHVT-H9HA constructed under the control of Pec promoter induced higher level of HI and virus neutralising antibodies and strong cellular immune response compared to rHVT-H9HA under CMV promoter[267]. Sonoda et al. reported a different relationship between foreign antigen expression levels and the protective efficacy of different types of recombinant Marek's disease virus (rMDV)[745]. In their study, rMDV-F expressing NDV-F protein under the control of simian virus 40 (SV40) promoter expressed more F protein *in vitro* compared to rMDV-F under the control of MDV gB promoter. However, only rMDV-gB F could protect MDA positive commercial chickens upon NDV challenge, despite of being a weaker promoter compared to SV40 in CEFs. This was speculated to be due to the differences in cell types in which antigens are expressed *in vivo* during MDV infection. It is reported that HVT gB promoter is active in the productive stage of MDV infection in spleen, thymus and feather follicle epithelium (FFE) but not in the latent stage[746]. Furthermore, some promoters might leakily express antigen even in latently infected T cells[744]. Therefore, further studies are needed to understand the expression sites of foreign promoters under HVT background *in vivo.* Overall, the combination of gene insertion sites, virus vector strains used, promoters and differences in cell types in which the antigens are expressed might dictate the efficacy of recombinant viral vector vaccines. For our work, gB promoter of PRV was used to express H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83

scFv proteins. All the proteins were successfully expressed *in vitro* as indicated by ELISA, western blots, and immunofluorescence assays. The H9HA Full-length protein was detected only in cell lysates whereas H9HA Foldon and H9HA Foldon-CD83 scFv were detected in both cell lysates and supernatants, indicating that the expression cassettes in the latter constructs are present in secreted forms. The H9HA Foldon and H9HA Foldon-CD83 scFv proteins were also detected in the cell lysates. This suggests that not all the H9HA Foldon and H9HA Foldon-CD83 scFv proteins are secreted out, and some remains in the cytoplasm. The native signal peptide sequence of HA gene was used for generating all the rHVT constructs. Other signal peptides like BIP or CD33 could be used to enhance the secretion of the foreign proteins. Upon comparison between rHVT-H9HA and rHVT-H9HA CD83 scFv, the latter secreted higher amount of respective protein *in vitro* as indicated by ELISA. The exact reason for this is unknown as both constructs showed similar virus titres *in vitro* indicating that the foreign gene insertion did not affect the virus replication efficiency. However, different rHVT clones of the same construct might differ in their ability to express foreign proteins. Therefore, it is better to screen many positive clones and take forward the highest protein expressing clone. In the case of rHVT-H9HA, the successful gene insertion efficiency was low (only 2 out of 18 plaques were positive for the insert) and both had similar expression level. In future, it might be better to select more GFP negative plaques (25-50) to increase the chances of obtaining higher foreign antigen expressing rHVT clones for all the constructs.

The viral distribution of rHVT-H9HA FL, rHVT-H9HA, rHVT-H9HA CD83 scFv vaccines was analysed in splenocyte and PBMC samples on day 7 pv and day 21 pv, respectively. The rHVT was detected in both splenocyte and PBMC

samples. In addition, majority of the rHVT detected (99%-100%) were able to express H9HA protein as indicated by double immunostaining assay. Furthermore, the replication efficiency of all the rHVT constructs *in vivo* was also assessed. Overall, rHVT-H9HA FL demonstrated lower virus titres in splenocytes and PBMCs compared to rHVT-H9HA CD83 scFv and rHVT-H9HA, respectively. However, all the rHVT constructs showed similar virus titres in CEF cells *in vitro*. This observed difference could be due to the heterogenous nature of different cell types that the rHVT infect *in vivo*. Moreover, a study demonstrated that the virus titres of rHVT-pecVP2 was lower than rHVT-cmvVP2 *in vivo*. However, rHVT-pecVP2 showed higher humoral immune response[744]. It was speculated that the strong protective immunity to VP2 antigens can suppress the rHVT-pecVP2 replication *in vivo*. A similar phenomenon was observed in which a strong protective immunity induced by booster vaccination suppressed the replication of rHVT in chickens[747]. Here, rHVT-H9HA FL demonstrated higher humoral immunity compared to rHVT-H9HA and rHVT-H9HA CD83 scFv. This could have suppressed the replication of rHVT-H9HA FL *in vivo*. Another possibility is that the over expression of HA protein on the surface of infected cells could directly induce apoptosis of the infected cells *in vivo*. This apoptotic effect might be lesser in rHVT-H9HA and rHVT-H9HA CD83 scFv where the HA proteins are secreted out. The tissue tropism of rHVT vaccines was not investigated in this thesis. Only spleen and blood samples were analysed for rHVT infection. Previous study explored the tissue tropism of two commercial vaccines: rHVT-IBD Vaxxitek® and rHVT-IBD Vectormune® both expressing VP2 protein of IBDV[748]. The rHVT was detected in spleen, bursa, and lung tissue samples at day 5 pv, with the virus level peaking at day 7 pv and dropping between day 9 pv and day 12 pv. Furthermore, low levels of virus were also detected in

PBMCs at day 7 pv and day 9 pv. Another study detected rHVT-NDV (F) vaccine virus in spleen up to 74 weeks of age[749]. This verifies life-long persistence of the virus in vaccinated chickens thus, ensuring strong and long-lasting immunity through the continuous antigen stimulus.

Moreover, live vector vaccines are known to induce strong mucosal, systemic, and cellular immunities against pathogens[195]. The range of immune responses provided depends upon the viral vectors used and the routes of vaccine administration[750]. The recombinant HVT vaccines are administered either *in ovo* or by subcutaneous injection at hatch[751]. Higher serum HI and anti-NDV (F) IgM/ IgY/ IgA antibodies were detected upon subcutaneously injecting 1-day-old chickens with Vectormune® HVT-ND (CEVA Animal Health) vaccine[752]. Similarly, higher serum HI and anti-HA IgM/ IgY/ IgA antibodies were observed upon subcutaneous administration of rHVT-H9HA FL vaccine compared to rHVT-H9HA and rHVT-H9HA CD83 scFv vaccines in our study. Both NDV 'F' and H9HA FL proteins are displayed on the cell surface after rHVT infection, mimicking the natural virus infection. This could be a potential reason for enhanced serum antibody responses with rHVT-H9HA FL compared to rHVT-H9HA and rHVT-H9HA CD83 scFv vaccines. It is also possible that the secreted form of H9HA protein is more prone to degradation and uptake by irrelevant cells **(Figure 6.13)**, reducing their immunogenic potential *in vivo*. The antibody responses induced by rHVT-H9HA CD83 scFv was higher than rHVT-H9HA, consistent with the results obtained with recombinant subunit vaccines. This implies that CD83 scFv targeting can improve the immunogenic potential of secreted H9HA protein. The earliest immune responses observed with rHVT-H9HA FL was two weeks post vaccination. Similar observation was made elsewhere when chickens were vaccinated with rHVT-H9HA

FL[267]. However, higher HI antibody titres were observed in this study compared to the previous study. This could be attributed to different promoters used i.e., gB of PRV in this study compared to Pec promoter in the previous study. Here, the antibody response after rHVT vaccination in chickens was only followed for 6 weeks post vaccination however, rHVT vaccines have a high potential for long-term induction of immune responses. The rHVT-VP2 vaccine demonstrated serum antibody responses for more than 30 weeks post vaccination[744]. Similarly, a single vaccination with Vectormune® HVT-ND (CEVA Animal Health) to 1-day-old layer chickens elicited antibody responses for 72 weeks post vaccination and provided almost complete (95%-100%) clinical protection against NDV challenge[749]. Besides antibody responses, rHVT vaccines can also induce CMI which plays an important role in protection against challenge viruses[698]. Several studies have shown AIV-, NDV- or IBDV-specific CMI in chickens vaccinated with rHVT vaccines, as measured by *ex vivo* specific antigenic stimulation of lymphocytes[267,753–755]. As mentioned in Chapter 4, due to the lack of a suitable recall antigen for IFN-γ ELISPOT assay, the evaluation of CMI induced by recombinant subunit vaccines was unsuccessful. Here, live SKP/2016 H9N2 virus (MOI 1) was used as a recall antigen. However, it too failed to induce IFN-γ when incubated with splenocytes from chickens immunised with rHVT vaccines (data not shown). In future, we can either test different MOI of live SKP/2016 H9N2 virus for splenocyte stimulation or generate H9N2-specific peptides. Our preliminary rHVT vaccine study demonstrated that rHVT-H9HA FL, rHVT-H9HA and rHVT-H9HA CD83 scFv can produce higher amount of $CD8^+$ T cells in splenocytes compared to the control unvaccinated group, indicating that rHVT vaccine could enhance the production of CTLs important for CMI (unpublished).

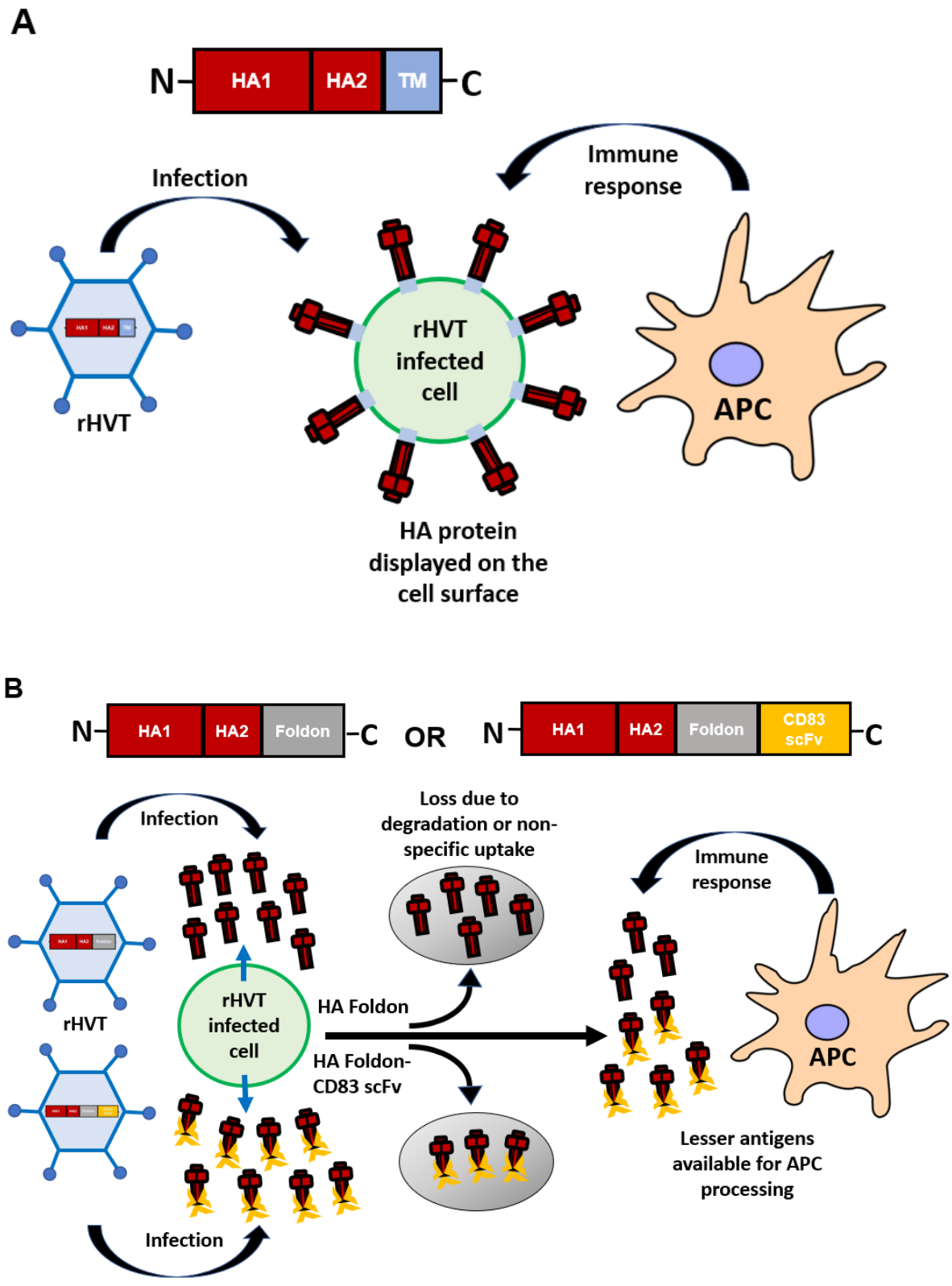


Figure 6.13. *In vivo* expression of antigen by different rHVT vaccine constructs. **A)** rHVT-H9HA FL. The HA antigens are displayed on the surface of rHVT infected cells. This can

protect the antigens from degradation or non-specific uptake by other cells. **B)** rHVT-H9HA or rHVT-H9HA CD83 scFv. The HA antigens are released out from the rHVT infected cells. Therefore, these antigens are more prone to degradation and non-specific uptake, reducing the amount of antigens available for APC processing. This can lower the immune response. Targeting the secreted form of HA antigen to CD83 receptor on APCs can protect the antigens from degradation and non-specific uptake. ***APC****: Antigen Presenting Cell*

Overall, there was a negative correlation between the virus replication efficiency in chickens and HI antibody response. The rHVT-H9HA had a higher virus titre in PBMCs compared to rHVT-H9HA FL on day 21 pv. However, rHVT-H9HA FL demonstrated higher HI antibody titres compared to rHVT-H9HA. Similarly, rHVT-H9HA CD83 scFv showed higher HI antibody titres, despite of having lower virus titres compared to rHVT-H9HA. The rHVT-H9HA CD83 scFv showed a higher production of H9HA Foldon-CD83 scFv antigen in CEFs *in vitro* compared to rHVT-H9HA as indicated by ELISA. This could have attributed to higher antibody responses by rHVT-H9HA CD83 scFv compared to rHVT-H9HA *in vivo*. It is possible that rHVT-H9HA FL can produce more antigens compared to rHVT-H9HA and rHVT-H9HA CD83 scFv *in vivo.* In addition, the H9HA protein displayed on the cell surface might be more protected from degradation and non-specific uptake compared to the secreted forms as described above. This could further enhance the antibody responses elicited by rHVT-H9HA FL. A study showed that rHVT-pecVP2 produced higher serum antibody responses compared to rHVT-cmvVP2 although, the latter showed higher virus titres *in vivo (*PBMCs*)*[744]. It was reported that rHVT-pecVP2 could produce more VP2 antigens compared to rHVT-cmvVP2. Thus, it was concluded that viral replication is not as important as the amount of antigen available to induce strong antibody responses. Furthermore, it has also been demonstrated that the correlation between vaccine virus titres and

humoral antibody responses differs with tissues. Among spleen, FFE, bursa and lungs, FFE showed stronger correlation between vaccine virus titre and NDV-specific humoral responses[752].

In conclusion, rHVT vaccines were generated expressing H9HA Full-length, H9HA Foldon and H9HA Foldon-CD83 scFv using HDR-CRISPR/Cas9 technology. All the rHVT vaccine constructs were able to replicate well in chickens and showed 99%-100% stability *in vivo*. Furthermore, all the rHVT constructs were able to express the HA protein. The rHVT-H9HA FL induced higher antibody responses compared to rHVT-H9HA and rHVT-H9HA CD83 scFv in chickens. In addition, targeting the secreted form of H9HA protein with CD83 scFv enhanced the antibody responses produced by rHVT-H9HA CD83 scFv. Overall, rHVT vector was more potent in delivering HA antigen expressed on the cell surface compared to those secreted out of the cell. The majority of work on poultry rHVT vaccines have been focussed on expressing viral cell surface proteins like HA (AIV)[267], F (NDV)[720] and gD and gI (ILTV)[756]. Future studies should focus on exploring other vector systems for delivering secreted form of antigens.

Chapter 7: Discussion, Limitation, Future work and Concluding remarks

7.1. Discussion

The main aim of this thesis was to understand the effect of targeting antigen to APCs on the immunogenicity and protective efficacy of recombinant subunit vaccines. Here, recombinant subunit TADV was generated by fusing chicken APC-specific scFv antibodies to HA antigen of AIV. The ability of the TADV to generate antibody responses, reduce viral shedding, produce cross-reactive antibodies, and overcome the MDA interference was assessed. Furthermore, the use of viral vector i.e., HVT for *in vivo* expression of antigen-antibody gene was also investigated. This information has given us insights on the potential use of TADV for commercial use.

7.1.1. Targeting vaccine antigen to chicken CD83 receptor: Next generation vaccine for poultry diseases

There are several bacterial (e.g., *Salmonella enteritidis*, *E.coli*, *Clostridium perfringens* and *Pasteurella multocida*) and viral diseases (e.g., AIV, NDV, MDV, IBDV and IBV) that impact poultry health. Most of the licensed vaccines for poultry diseases are inactivated virus vaccines which can often provide sub-optimal immunity. The ongoing research on poultry vaccines include recombinant subunit vaccines, DNA/RNA vaccines, as well as improving the existing vaccines like inactivated virus vaccines and viral vector vaccines. In this thesis, recombinant TADV was developed by fusing vaccine antigen (H9HA of AIV) to different chicken APC receptors; Dec205, CD83 and CD11c. Targeting H9HA antigen to chicken

CD83 demonstrated the highest antibody responses compared to Dec205 and CD11c targeting. In addition, rH9HA-CD83 scFv also showed a reduced virus shedding compared to rH9HA upon H9N2 AIV challenge. However, the chickens vaccinated with rH9HA-CD83 scFv still got infected and shed virus, indicating that the vaccine is unable to provide sterile immunity. Providing 'sterile immunity' is one of the major challenges in vaccine development. Hence, further research is needed to develop AIV vaccines that can provide sterile immunity to the vaccinated animals. Moreover, rH9HA-CD83 scFv vaccine was able to induce higher cross-reactive HI and virus neutralising antibodies against different heterologous H9N2 AIVs compared to rH9HA and inactivated H9N2 vaccines. Furthermore, a single dose of rH9HA-CD83 scFv vaccine given to 1-day-old chickens also showed reduced MDA interference. None of the other vaccines tested (i.e., rH9HA and inactivated H9N2 vaccine) were able to overcome the MDA interference. Therefore, rH9HA-CD83 scFv vaccine can overcome the limitations of conventional inactivated virus vaccine, and constitutes several features described in **Table 3** for an ideal poultry AIV vaccine. Similar technique can be used to develop recombinant TADV for various poultry diseases. Hence, such TADV can represent the next generation of poultry vaccines. A summary of the features of key subunit vaccines and inactivated H9N2 vaccine used in this thesis is shown in **Table 30**.

Table 30: Features of key subunit vaccines and inactivated H9N2 vaccine used in this thesis.

Features	rH9HA	rH9HA-CD83 scFv	Inactivated H9N2
Description	Subunit	Subunit	Inactivated
Adjuvant	Yes, Water-in-oil	Yes, Water-in-oil	Yes, Water-in-oil
DIVA compatibility	Yes	Yes	Poor
Route	Subcutaneous injection	Subcutaneous injection	Subcutaneous injection
Age of chickens	**One dose**: 1-day-old **Two doses**: 1-day-old and 7-day-old *or* 7-day-old and 14-day-old	**One dose**: 1-day-old **Two doses**: 1-day-old and 7-day-old *or* 7-day-old and 14-day-old	**One dose**: 1-day-old **Two doses**: 1-day-old and 7-day-old *or* 7-day-old and 14-day-old
Recommended vaccination regime	Multiple doses required	Single vaccination to 1-day-old chickens	Multiple doses required
Onset of immunity	**One dose/Two doses:** Between day 6 pv to day 14 pv	**One dose**: Between day 6 pv to day 14 pv **Two doses** (7-day-old and 14-day-old): Within day 6 ppv	**One dose/Two doses:** Between day 6 pv to day 14 pv
HI antibodies	**Two vaccinations: 7-day-old and 14-day old** No significant differences between 2 µg, 20 µg and 35 µg	**Two vaccinations: 7-day-old and 14-day old** 20 µg and 35 µg vaccine doses induced significantly	**Two vaccinations: 7-day-old and 14-day old** Only one dose (1024 HAU/dose) was tested. The HI antibody titres were similar to that

	vaccine doses. Initial detection of HI antibodies occurred between day 6 ppv to day 14 ppv, with all the vaccine doses.	higher/earlier (within day 6 ppv) HI antibody titres compared to the 2 µg vaccine dose (day 14 ppv).	induced by rH9HA. Initial detection of HI antibodies occurred between day 6 ppv to day 14 ppv.
Anti-HA antibodies in the serum	**Two vaccinations: 7-day-old and 14-day old** No significant differences observed between 2 µg, 20 µg and 35 µg vaccine doses with regards to the anti-HA IgM and IgY antibodies. Initial detection of anti-HA IgM and IgY antibodies occurred between day 6 ppv to day 14 ppv, with all the vaccine doses. Low anti-HA IgA detected.	**Two vaccinations: 7-day-old and 14-day old** Initial detection of anti-HA IgM and IgY antibodies occurred within day 6 ppv with 35 µg vaccine dose. No significant differences between 2 µg, 20 µg and 35 µg vaccine doses with regards to the anti-HA IgM and IgY antibodies after day 14 ppv. Low anti-HA IgA detected.	**Two vaccinations: 7-day-old and 14-day old** Only one dose (1024 HAU/dose) was tested. Initial detection of anti-HA IgM and IgY antibodies occurred between day 6 ppv to day 14 ppv. The level of anti-HA IgM and IgY antibodies were similar to that induced by rH9HA. Low anti-HA IgA detected.
Virus neutralising antibodies	**Two vaccinations: 7-day-old and 14-day old** 2 µg, 20 µg and 35 µg vaccine doses induced lower amount of virus neutralising antibodies, measured on day 28 ppv.	**Two vaccinations: 7-day-old and 14-day old** 20 µg and 35 µg vaccine doses induced significantly higher virus neutralising antibodies compared to the 2 µg vaccine dose,	**Two vaccinations: 7-day-old and 14-day old** Only one dose (1024 HAU/dose) was tested. Induced virus neutralising antibodies higher than 2 µg, 20 µg and 35 µg doses of rH9HA but lower than 20 µg and 35

		measured on day 28 ppv.	µg of rH9HA-CD83 scFv.
Cross-reactive antibodies	Fewer cross-reactive antibodies against heterologous H9N2 viruses, as measured by HI and MNT assays.	Higher cross-reactive antibodies against heterologous H9N2 viruses, as measured by HI and MNT assays.	Fewer cross-reactive antibodies against heterologous H9N2 viruses, as measured by HI and MNT assays.
Protection	Lower reduction in virus shedding	Higher reduction in virus shedding	Higher reduction in virus shedding
Affected by MDA	High	Very little	High
Cell-mediated immunity	Not able to test in this thesis	Not able to test in this thesis	Not able to test in this thesis. However, whole inactivated influenza virus vaccine has been reported to induce CMI[614,631].
In vitro stimulation of chicken splenocytes	Very low or no induction of IFN-γ, IL-6, IL-1β and CXCLi2	High induction of IFN-γ, IL-6, IL-1β and CXCLi2	Not tested in this thesis
Sterile immunity	No	No	No
Stability	Not tested in this thesis	At least 1 year at 2-8°C	Not tested in this thesis

pv: post vaccination **ppv**: post primary vaccination

7.1.2. Production of broadly cross-reactive antibodies using CD83 targeted vaccine

The antigenic drift facilitates the accumulation of mutations on HA protein over time, resulting in IAVs that are antigenically different from the vaccine strains[144]. This is the main reason for the failure of conventional inactivated AIV vaccines and recombinant vaccines based on HA protein. Several recombinant subunit AIV vaccines based on more conserved viral proteins like NP and M2e have been developed[283,284]. However, these vaccines have poor immunogenicity. In recent years, there has been surge of interest in developing 'universal' vaccine based on the conserved stalk domain of HA protein. One approach used 'headless' HA protein constituting only the stalk domain of HA[674] (**Figure 7.1 B**). These 'headless' HA proteins were difficult to generate due to the instability and poor folding. Another approach showed that the deletion of immunodominant region of HA could generate higher cross-reactive antibodies by targeting more conserved HA epitopes. Using this approach, 'glycan shielding' strategy was developed whereby many glycosylation sites are added at variable, immunodominant HA epitopes. The shielding of these variable epitopes on the HA head domain was shown to generate a more cross-reactive antibody response directed towards the stalk domain[678] (**Figure 7.1 C**). Furthermore, it has also been suggested that HA protein with high receptor binding avidity, which preferentially binds to sialic acid receptors instead of antibodies, could guide the immune response towards the stalk domain (unpublished, **Figure 7.1 D**). For H9N2 AIVs, a single amino acid change at residue 180 from threonine or alanine to valine was shown to increase the receptor binding avidity of the virus[757]. Therefore, HA protein having higher receptor binding avidity could be used to generate cross-reactive stalk antibodies.

In this thesis, additional methods of raising broadly cross-reactive antibodies are suggested. The rH9HA-CD83 scFv vaccine was shown to induce more cross-reactive antibody responses (**section 5.2.1.3**). As described in **section 5.3.1**, this could be due to the following reasons: i) overall higher production of neutralising antibodies directed towards the immunodominant variable HA head domain ii) changes in the HA structure with exposure of more conserved epitopes on the HA head domain (**Figure 7.1 E**). The rH9HA-CD83 scFv protein was constructed by fusing CD83 scFv antibody to the C-terminus of HA protein. It would be interesting to generate rH9HA-CD83 scFv protein whereby CD83 scFv antibody is fused to the N-terminus of HA protein (**Figure 7.1 F**). This would be similar to the 'glycan shielding' approach, whereby the CD83 scFv antibodies might shield the variable epitopes in the HA head domain, facilitating more efficient generation of stalk-reactive antibodies. However, fusing CD83 scFv antibody on the head domain of HA protein might also shield immunodominant epitopes needed for the generation of neutralising antibodies. It is possible that CD83 targeting could balance this limitation by enhancing the production of non-neutralising stalk-reactive antibodies superior in ADCC and complement cytotoxicity.

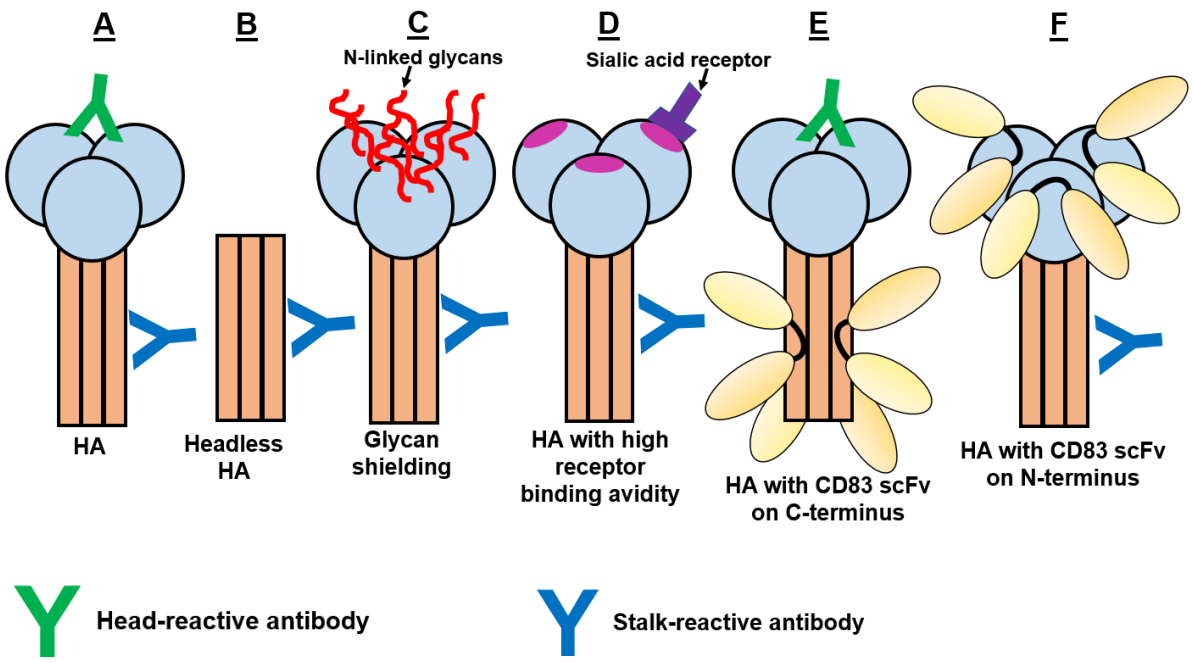


Figure 7.1: Schematic representation of the methods of generating cross-reactive antibodies against HA protein. **A)** A trimeric HA protein with most antibodies towards the variable head domain and some towards the conserved stalk domain. **B)** Headless HA inducing antibodies towards more conserved HA stalk domain. **C)** Glycan shielding approach where hiding immunodominant epitopes on the head domain directs antibodies towards the stalk domain**. D)** HA with higher sialic acid receptor binding avidity enhances the production of more stalk-reactive antibodies. **E)** HA with CD83 scFv fused to the C-terminus. The scFv antibodies could occlude some part of stalk domain, concentrating the antibodies towards the head domain. This can increase the chances of conserved epitopes being targeted**. F)** HA with CD83 scFv in the N-terminus. The scFv antibodies might shield some of the immunodominant epitopes on the head domain facilitating the generation of cross-reactive stalk antibodies.

7.1.3. Recombinant HVT as a viral vector for the expression of membrane bound and secreted forms of HA antigen *in vivo*

The HVT vector has been successfully used for the generation of recombinant vaccines against a number of poultry diseases such as AIV (Vectormune® HVT-AI (CEVA Animal Health), NDV (Innovax-ND, Innovax-ND-ILT, Innovax-ND-IBD (MSD Animal Health), Vectormune® HVT-ND (CEVA Animal

Health), IBDV (Innovax-ND-IBD (MSD Animal Health), Vectormune®HVT-IBD (CEVA Animal Health), Vaxxitek HVT+IBD (Boehringer Ingelheim) and ILTV (Innovax-ILT, Innovax-ND-ILT (MSD Animal Health). These vaccines facilitate the expression of membrane bound forms of HA (AIV)[723], F (NDV)[720,751,756] , gD and gI (ILTV)[756] and cytoplasmic VP2 (IBDV)[748] proteins. In this thesis, HVT vector was used to express both the membrane bound (rHVT-H9HA FL) and secreted forms (rHVT-H9HA and rHVT-H9HA CD83 scFv) of HA protein. In terms of antibody responses, rHVT-H9HA FL showed an early onset of immunity as well as higher HI, anti-HA IgM/ IgY/ IgA and virus neutralising antibodies compared to rHVT-H9HA and rHVT-H9HA CD83 scFv. An explanation for this could be that the display of antigens on the cell surface mimics the natural virus infection. It is also possible that the secreted form of H9HA protein has a reduced expression levels *in vivo* and is more prone to degradation and uptake by irrelevant cells hence, reducing the immunogenic potential. Therefore, we conclude that HVT vector is not ideal for expressing secreted form of HA antigen *in vivo*. However, this may be antigen dependent and other antigens could be better expressed in secreted form using HVT vector system, retaining high immunogenicity.

Adeno-associated virus (AAV) is an attractive vector platform for gene therapy as it allows long-term expression, and penetration through biological barriers like the blood-brain barrier[758]. AAV vector system has been successfully used in humans for expressing various monoclonal antibodies and antibody fragments to treat immune disorders, cancers, and infectious diseases[759]. Several other secreted proteins such as lysosomal enzyme acid α-glucosidase[760], neuroactive peptide galanin[761] and prostate apoptosis response-4 (tumour-suppressor protein)[762] have been expressed *in vivo* using AAV system. Numerous

studies in experimental animals and several clinical trials in humans have provided convincing results for the efficacy of this genetic delivery system[758]. However, there is limited research on the use of AAV in avian species. One study used avian AAV to express NDV haemagglutinin-neuraminidase protein for poultry vaccination. This vaccine was able to provide about 80% protection in chickens upon virulent NDV challenge[763]. Another study expressed calcitonin gene related peptide using avian AAV for treating chicken umbilical artery vasospasm[764]. It was also shown that avian AAV can transduce various type of chicken cell lines, as well as chicken neurons[765]. Therefore, avian AAV could be an ideal vector for expressing secretory proteins *in vivo.*

7.2. Limitation

One of the major limitations in this thesis is the availability of antibodies against different chicken APC receptors. Successfully targeted APC receptors in mammals include Dec205, CD11c, CD80, CD40, DC-SIGN, MR, MHC I/II, Dectin1/2, Clec9A and FcγR[766]. Since, only chicken Dec205, CD11c and CD83 antibodies were available at the beginning of the project, these three antibodies were used to generate TADV constructs. Having more options of chicken APC targeting antibodies would have allowed us to do a better comparison and choose the best target to take forward. Another limitation lies in the design of virus challenge animal study. Due to the lack of enough isolators and space constraints of the animal facilities at the Pirbright Institute, we were unable to perform protective efficacy study with all the TADV constructs. Furthermore, only one concentration of rH9HA and rH9HA-CD83 scFv vaccines (i.e.,25 μg) could be tested. In addition, most of the animal studies were designed to last for only 35 days due to the cost

associated with maintaining the chickens for a longer period. Thus, a complete duration of antibody responses after vaccination could not be investigated. Currently, we are liaising with MSD Animal Health (The Netherlands) to overcome some of these limitations. Despite of these limitations, we were able to obtain significant amount of information that allowed us to understand the immunogenicity and protective efficacy of TADV.

Another limitation in this thesis is the lack of results supporting the vaccine induced CMI. Various techniques are currently in use for the study of T cell responses in poultry. These include the measurement of antigen-specific proliferation by flow cytometry[767], intracellular cytokine staining[768], measurement of IFN-γ production by ELISA[769,770] and ELISPOT[769,771]. Here, IFN-γ ELISPOT was used to analyse the CMI induced by vaccines in chickens. The recombinant protein, inactivated virus and live virus have been used as recall antigens for IFN-γ ELISPOT assay in chickens with varying success[632,633,753]. As mentioned in Chapter 4, no spots could be detected upon stimulation of chicken splenocytes using the above recall antigens, even with the positive control (splenocytes from chickens infected with live H9N2 virus). Peptide antigens are considered better recall antigens for stimulating $CD4^+$ and $CD8^+$ T cells in IFN-γ ELISPOT assay[634]. Peptides have been successfully applied to study influenza antigen-specific T cell responses in birds[772,773]. Reemers et al identified novel AIV-derived $CD8^+$ T cell epitopes in NP and M1 proteins based on anchor residues described for MHC B4, B12, B19 and B21[778]. Upon stimulation of chicken lung cells by these peptide pools, an increase in the number of IFN-γ producing cells was observed in ELISPOT analysis[772]. Furthermore, Haghighi et al generated a library of 112 overlapping peptides covering the entire H5HA protein. These peptides were 15 amino acid

residues long and overlapped by 10 residues. Stimulation of the spleen cells from chickens vaccinated with TROVAC-AIV H5 vaccine (recombinant fowlpox virus vector expressing H5N8 HA antigen) with these peptide pools upregulated IFN-γ expression and cell proliferation[773]. In future, similar approaches could be taken to generate overlapping peptide library covering the entire H9HA protein for our work, so that they could be successfully used in IFN-γ ELISPOT assay.

7.3. Future work

There are numerous things that could be investigated in order to broaden our understanding of TADV and their potential for commercial use. Some of them are discussed below:

7.3.1. Targeting other chicken antigen presenting cell receptors

At the beginning of the project, we were limited by the availability of antibodies targeting chicken APC receptors. This led us to focus on antigen targeting to only three chicken APC receptors: Dec205, CD11c and CD83. Currently, additional antibodies against other chicken APC receptors: CD86, Bu1, CD40, CD80 and CD45 have been obtained. These antibodies could be tested for their ability to enhance the immunogenic potential of the targeted antigen. Furthermore, there are several other chicken receptors that could be used for this purpose. Some of these receptors include avian DC and macrophage receptors: mannose receptor C-type 1-like B (MRC1L-B, macrophage marker), 74.2 (macrophage marker), 68.2 (macrophage marker), K1 (macrophage marker), 74.3 (DC marker), T cell membrane protein 4 (Tim4, macrophage marker)[306,774]. In humans, mannose receptor is suggested to be a good target for antigen delivery

for vaccine development since it is highly expressed on macrophages and DCs, and plays a key role in antigen recognition[583]. It can be expected that some of these receptors might be better than others in terms of developing enhanced immune response after antigen targeting in chickens. The difference in the capacity of enhancing immune response upon targeting different APC receptors might depend on various factors discussed in Chapter 4. Therefore, the overall information obtained can aid us in developing a panel of successful antibodies that could be used for developing TADV against various poultry diseases. Moreover, future studies could also involve testing the cross-reactivity of these chicken APC targeting antibodies with other poultry species like turkeys, quails, and ducks. Our preliminary experiment has shown that chicken CD83 mAb is cross-reactive with turkey CD83 receptor protein *in vitro* (**Figure 7.2**). Therefore, it is likely that we could use chicken CD83 antibody to target antigens to turkey CD83 receptor and develop TADV for turkeys.

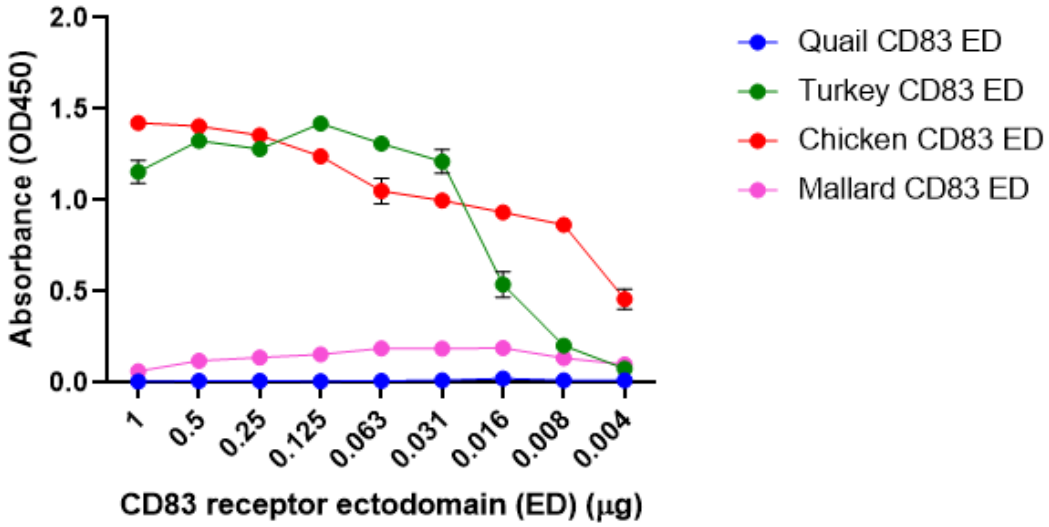


Figure 7.2: Analysis of the cross-reactivity of chicken CD83 mAb to quail, turkey, and duck CD83 ectodomain (ED) using ELISA. About 1 µg quail, turkey, chicken, and duck CD83 ED were coated onto each well of the ELISA plate, a 2-fold serial dilution was carried out and the plate was incubated overnight for 4ºC. For detection, the plates were incubated 1

μg/mL of mouse anti-chicken CD83 mAb. This was followed by incubation with goat anti-mouse HRP (1 mg/mL diluted to 1:1000). The colorimetric detection was carried out by adding TMB substrate and absorbance at 450 nm was recorded.

7.3.2. Expanding the understanding of TADV mechanism

The mechanism of TADV can be further explored by investigating different DC subsets up taking the targeted antigen after vaccination. Following on, the routes of antigen uptake after vaccination and how these uptake mechanisms might differ when targeting different APC receptors can also be investigated. Markers like Early endosomal antigen 1 (EEA-1) or Lysosomal associated membrane protein 1 (LAMP-1) could be used to assess whether the antigens are trafficked to early endosomes or lysosomes following internalisation via the targeted receptors[775]. Studies have shown that antigens trafficked to early endosomes are good at cross-presentation to MHC I molecules, whereas those trafficked to lysosomes are good at presentation via MHC II molecules[585]. Understanding the type of DC subsets up taking the antigens and the route followed after antigen internalisation could allow us to predict the type of immune response generated when targeting different APC receptors.

7.3.3. Production of multivalent vaccines

Currently, there are several poultry 'multivalent' vaccines that are available commercially. Such multivalent vaccines either contain mixture of different strains of the same pathogen or different pathogens in the same vaccine formulation, in order to broaden the protection against different pathogens. E.g., Nobilis H9N2+ND (MSD Animal Health, inactivated vaccine against H9N2 AIV and ND virus), Nobilis RT+IBMULTI+ND+EDS (MSD Animal Health, inactivated vaccine against avian

pneumovirus, IB, ND and Egg Drop Syndrome). Therefore, future work could involve constructing 'multivalent' TADV either containing different HA (H5/H7/H9) antigens or mixtures of HA antigens and other viral antigens like VP2 (IBDV) and F protein (NDV). The use of a single expression plasmid for multiple protein expression can be limited by the size of gene inserts (**Figure 7.3A**). Large size of the expression plasmid can reduce the transformation efficiency and copy number leading to lower protein expression[776,777]. In order to address this, multiple expression plasmids could be synthesised each containing different antigen, scFv antibody and detection tag (**Figure 7.3B**). Different antigen expressing plasmids can be co-transfected into *Drosophila* S2 cells and the single cell clone expressing all the antigens can be easily screened using their unique detection tags. The culture supernatant containing all the antigens can be directly formulated as multivalent vaccine. By avoiding the purification step, the time and cost involved in poultry vaccine production could be substantially reduced.

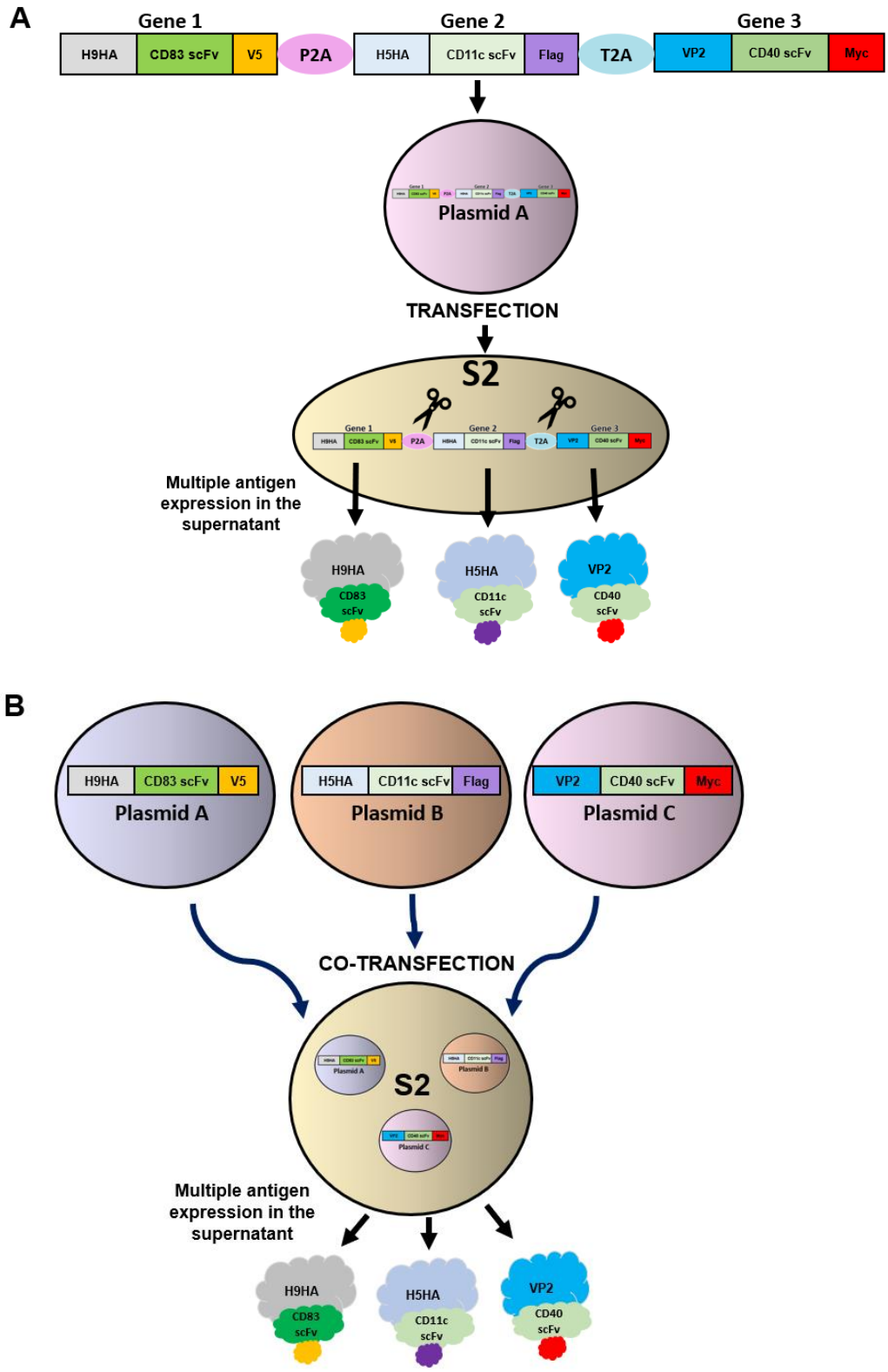


Figure 7.3. Production of multivalent vaccines in *Drosophila melanogaster* S2 cells. **A)** Co-expression of multiple proteins from a single plasmid using P2A/T2A cleavage peptide. P2A/T2A cleavage peptides are cloned between the gene of interests. During translation successful ribosomal skipping events result in the formation of cleaved proteins. The major limitation here would be the size of gene inserts. **B)** Expression of multiple proteins by co-

transfection of individual expression plasmids. Each expression plasmid consists of an antigen, scFv antibody and detection tag. The expression of individual protein in the supernatant can be analysed with the help of unique detection tags.

7.3.4. Routes of vaccination

The route of vaccine administration is one of the potential factors affecting the outcome of vaccination. Most vaccines are given via *in ovo*, spray/aerosol and drinking water route to the broilers, whereas layers are parenterally vaccinated (intramuscular or subcutaneous route). In this thesis, vaccines were administered subcutaneously. Such parenteral administration induces good systemic immune response but no mucosal immune response[244,778]. In contrast, intranasal delivery of vaccine induces both systemic as well as mucosal immune responses[681,779,780]. Mucosal immunity plays an important role in controlling AIV infection in the respiratory tract, which serves as the initial site for AIV entry[195]. It has been shown that mucosal immunisation can induce higher amount of secretory and serum IgA antibodies than parenteral vaccines[781]. Such IgA antibodies are known to induce cross-protection against heterologous virus infection, increasing the breath of disease protection[782,783]. Therefore, it would be interesting to assess the immunogenicity and protective efficacy of TADV given via intranasal route. Various studies have evaluated the administration of inactivated influenza vaccine by the intranasal route[778,784–786]. These studies have shown that administering inactivated influenza vaccine intranasally led to lower serum antibody responses but improved nasal secretory IgA antibodies. Several adjuvants like bacterial components and immunostimulating complexes (ISCOMS) have been developed to enhance the intranasal immunisations of inactivated and subunit vaccines[195]. A study by Saluja et al., described a safe adjuvant Gram-positive enhancer matrix (GEM) particle

derived from the food-grade bacterium *Lactococcus lactis* for intranasal vaccination with subunit influenza vaccine in mice. The intranasal immunisation with GEM subunit vaccine elicited superior systemic and mucosal immune responses compared to those induced by intramuscular and intranasal administered subunit vaccine alone[784].

7.3.5. Characterisation of B cell repertoire responding to rH9HA-CD83 scFv vaccine

The diverse range of B cell receptors (BCRs) expressed by the total B cell population in an organism is termed as B cell repertoire[787]. As mentioned in Chapter 1 (**section 1.3.1**), the diversity in chicken B cell receptor repertoire is generated by the process of somatic gene conversion, whereby the V_L and V_H sequences are replaced with diverse pseudogene sequences. In future, B cells expressing immunoglobulins with the desired specificity and functional characteristics can be isolated, following rH9HA-CD83 scFv vaccination in chickens. This can help us to identify cross-reactive antibodies produced with rH9HA-CD83 scFv vaccine. Single B cell expression cloning technique can be used for this purpose[788,789] (**Figure 7.4**). This method involves fluorescence-assisted cell sorting (FACS) of antigen-specific single B cells, followed by single cell reverse transcription PCR to generate 5'RACE-ready cDNA. Subsequently, the BCR genes (IgM, IgY, IgA, and IgL) are amplified, cloned, and expressed in eukaryotic cell lines (e.g., CHO or HEK293) for monoclonal antibody production. The monoclonal antibodies can then be screened for the desired characteristics. This technique allows very efficient and highly

selective isolation of B cells, with 80–90% of the cells sorted by flow cytometry producing immunoglobulins with the desired specificity[790].

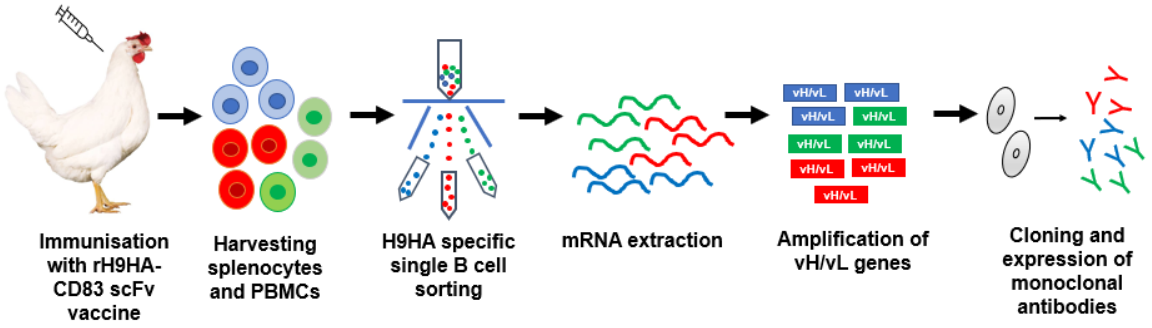


Figure 7.4: Single cell expression cloning technique for isolating monoclonal antibodies. Chickens are immunised with rH9HA-CD83 scFv vaccine. Splenocytes and PBMCs are harvested, and H9HA-specific B cell isolation is carried out using fluorescence activated cell sorting (FACS). This is followed by mRNA extraction and amplification of vH and vL genes using qRT-PCR. The vH/vL genes are cloned into expression vector for *in vitro* expression of monoclonal antibodies which are then screened for the desired characteristics.

7.3.6. Generation of the recombinant HVT vector expressing full-length HA protein conjugated to chicken CD83 scFv

Our work with HVT vector vaccine demonstrated that the membrane bound form of HA protein displayed on the cell surface was more immunogenic than the secreted forms (**Chapter 6**). Next, the HVT vector system could be used to test the *in vivo* expression of membrane bound HA protein fused to chicken CD83 scFv. There are two ways in which CD83 scFv can be fused to the membrane bound form of HA protein: i) at N terminus before HA1 domain ii) at C-terminus after HA2 domain. Both ways bear pros and cons. The fusion of CD83 scFv at the N-terminus of HA protein might mask some of the important immunodominant epitopes on the head domain. These immunodominant epitopes are essential for generating neutralising antibodies. However, the CD83 scFv would be free to bind and target

CD83 receptor (**Figure 7.5A**). On the other hand, conjugating CD83 scFv to the C-terminus might disclose immunodominant epitopes on the head domain but restrict the CD83 scFv binding since, scFv antibodies are not positioned at the free end of HA protein (**Figure 7.5B**). These could have different impact on the immunogenicity of HVT vector vaccines. The information obtained could also provide us a basis for generating influenza virus like particles (VLPs) consisting of full-length HA fused to CD83 scFv, NA and M1 proteins[791].

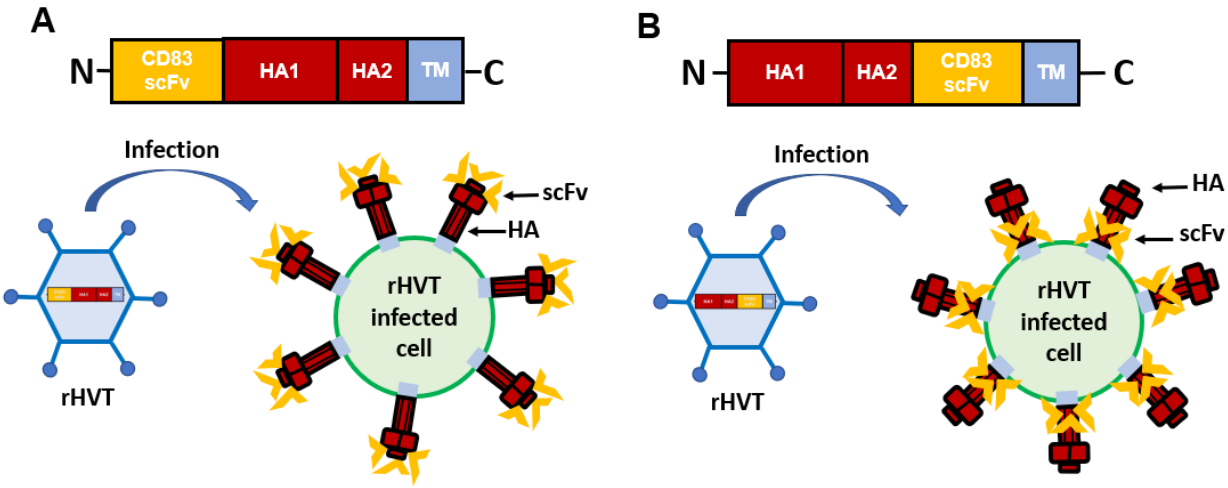


Figure 7.5: Generation of rHVT constructs expressing membrane bound form of HA protein conjugated to CD83 scFv. **A)** Fusion of CD83 scFv at the head domain of HA protein. Three CD83 scFv (one on each HA monomer) antibodies are free to bind to CD83 receptor on chicken APCs however, these scFv antibodies might mask some of the important immunodominant epitopes on the head domain. **B)** Fusion of CD83 scFv at the stalk domain of HA protein. Three CD83 scFv (one on each HA monomer) antibodies might be occluded which can restrict the CD83 receptor binding, however the immunodominant epitopes on the head domain are not shielded.

7.4. Concluding remarks

Avian influenza viruses are not only major threats to the poultry production, but also pose a credible zoonotic and pandemic threats. One of the most effective ways to prevent AIV infection is through vaccination. Therefore, there is a high

demand for novel vaccines which can overcome the limitations of the current AIV vaccines. Here, the specificity and high affinity binding properties of antibodies were exploited to create TADV for avian influenza in poultry. The approach using CD83 targeting led to high antibody responses and effective reduction of the viral load in chickens. Most importantly, such vaccine was able to produce higher cross-reactive antibodies and overcome the MDA interference. Therefore, CD83 targeting approach represents a powerful addition to the armoury of the developers of poultry vaccines. Similar approaches could be used in the development of safe and effective vaccines against other poultry, livestock and human diseases.

Appendix A: Antigen sequences

H9HA: Nucleotide and amino acid sequences of H9HA antigen used in this thesis (Trimerisation Foldon domain in red)

	Sequence (H9HA Foldon, MsCon (UDL 01/08)
NUCLEOTIDE	GATAAGATCTGCATCGGCCACCAGAGCACCAACAGCACCGAGACCGTGGATACC CTGACCGAGACCAACGTGCCCGTGACCCACGCCAAGGAGCTGCTGCACACCGA GCACAACGGCATGCTGTGCGCCACCAACCTGGGCCACCCCCTGATCCTGGATAC CTGCACCATCGAGGGCCTGATCTACGGCAACCCCAGCTGCGATCTGCTGCTGGG CGGCCGCGAGTGGAGCTACATCGTGGAGCGCCCCAGCGCCGTGAACGGCACCT GCTACCCCGGCAACGTGGAGAACCTGGAGGAGCTGCGCACCCTGTTCAGCAGC AGCAGCAGCTACCAGCGCATCCAGATCTTCCCCGATACCATCTGGAACGTGACC TACACCGGCACCAGCAAGAGCTGCAGCGATAGCTTCTACCGCAACATGCGCTGG CTGACCCAGAAGAGCGGCCTGTACCCCGTGCAGGATGCCCAGTACACCAACAAC CGCGGCAAGGATATCCTGTTCGTGTGGGGCATCCACCACCCCCCCACCGATACC GCCCAGACCAACCTGTACACCCGCACCGATACCACCACCAGCGTGACCACCGAG AACCTGGATCGCACCTTCAAGCCCGTGATCGGCCCCCGCCCCCTGGTGAACGG CCTGATCGGCCGCATCAACTACTACTGGAGCGTGCTGAAGCCCGGCCAGACCCT GCGCGTGCGCAGCAACGGCAACCTGATCGCCCCCTGGTACGGCCACGTGCTGA GCGGCGAGAGCCACGGCCGCATCCTGAAGACCGATCTGAACAGCGGCAACTGC GTGGTGCAGTGCCAGACCGAGAAGGGCGGCCTGAACAGCACCCTGCCCTTCCA CAACATCAGCAAGTACGCCTTCGGCAACTGCCCCAAGTACATCGGCGTGAAGAG CCTGAAGCTGGCCATCGGCCTGCGCAACGTGCCCGCCCGCAGCAGCCGCGGCC TGTTCGGCGCCATCGCCGGCTTCATCGAGGGCGGCTGGCCCGGCCTGGTGGCC GGCTGGTACGGCTTCCAGCACAGCAACGATCAGGGCGTGGGCATGGCCGCCGA TCGCGATAGCACCCAGAAGGCCGTGGATAAGATCACCAGCAAGGTGAACAACAT CGTGGATAAGATGAACAAGCAGTACGAGATCATCGATCACGAGTTCAGCGAGGT GGAGACCCGCCTGAACATGATCAACAACAAGATCGATGATCAGATCCAGGATGT GTGGGCCTACAACGCCGAGCTGCTGGTGCTGCTGGAGAACCAGAAGACCCTGG ATGAGCACGATGCCAACGTGAACAACCTGTACAACAAGGTGAAGCGCGCCCTGG GCAGCAACGCCATGGAGGATGGCAAGGGCTGCTTCGAGCTGTACCACAAGTGC GATGATCAGTGCATGGAGACCATCCGCAACGGCACCTACAACCGCCGCAAGTAC AAGGAGGAGAGCCGCCTGGAGCGCCAGAAGATCGAGGGCGTGAAGCTGGAGA GCGAGGGCACCTACAAGATCCTGGGCAGCGGCTACATCCCCGAGGCCCCCCGC GATGGCCAGGCCTACGTGCGCAAGGATGGCGAGTGGGTGCTGCTGAGCACCTT CCTGGGC
AMINO ACID	DKICIGHQSTNSTETVDTLTETNVPVTHAKELLHTEHNGMLCATNLGHPLILDTCTIEG LIYGNPSCDLLLGGREWSYIVERPSAVNGTCYPGNVENLEELRTLFSSSSSYQRIQIF PDTIWNVTYTGTSKSCSDSFYRNMRWLTQKSGLYPVQDAQYTNNRGKDILFVWGIH HPPTDTAQTNLYTRTDTTTSVTTENLDRTFKPVIGPRPLVNGLIGRINYYWSVLKPGQ TLRVRSNGNLIAPWYGHVLSGESHGRILKTDLNSGNCVVQCQTEKGGLNSTLPFHNI SKYAFGNCPKYIGVKSLKLAIGLRNVPARSSRGLFGAIAGFIEGGWPGLVAGWYGFQ HSNDQGVGMAADRDSTQKAVDKITSKVNNIVDKMNKQYEIIDHEFSEVETRLNMINN KIDDQIQDVWAYNAELLVLLENQKTLDEHDANVNNLYNKVKRALGSNAMEDGKGCF ELYHKCDDQCMETIRNGTYNRRKYKEESRLERQKIEGVKLESEGTYKILGSGYIPEAP RDGQAYVRKDGEWVLLSTFLG

	Sequence (H9HA Full length, SKP/2016)
NUCLEOTIDE	ATGGAAGCAATATCACTGATGATTATACTACTAGTAGTGACAACAAGCAAT GCAGACAAAATCTGCATCGGCCACCAATCAACAAATTCCACAGAAACAGT GGACACGCTAACAGAATCTAACATTCCTGTGACACAAGCCAAAGAATTGC TCCACACGGAACATAATGGAATGCTGTGTGCAACAAATCTGGGACGTCC CCTCATCCTGGACACATGCACTGTCGAAGGACTGATTTATGGCAATCCTT CTTGTGATCTGCTATTGGGGGGAAGAGAATGGTCATACATTGTTGAAAGA CCATCAGCGGTAAATGGAACATGTTACCCTGGGAACGTAGAAAACTTAGA GGAACTCAGAATGCTTTTTAGTTCTGCTAGTTCTTACCAAAGAATCCAAAT CTTCCCAGACGCAATCTGGAATGTGACTTATGATGGAACAAGCAAATCAT GCTCTAATTCGTTCTACAGGAATATGAGATGGCTGACTCAAAAGAATGGG AATTATCCTATTCAAGACGCCCAATACACAAACAACCGAGGGAAGGACAT TCTCTTCATTTGGGGCATACATCATCCACCCACTGATACTACGCAAACGA ATTTATACACAAGAACCGACACAACAACAAGCGTCACAACAGAAAATCTG GATAGGACCTTCAAACCATTAATAGGACCAAGGCCTCTTGTCAATGGTCT AATTGGAAGAATTAATTATTATTGGTCAGTATTAAAGCCAGGACAGACGTT GAGAGTAAGATCCAATGGGAATCTAATTGCTCCATGGTATGGACATGTCC TCTCAGGAGAGAGCCATGGGAGAATTCTGAAGACTGATTTAAAGAGTGGT AATTGCGTAGTGCAATGTCAGACTGAAAAAGGTGGCTTAAACAGTACACT ACCATTCCACAATATCAGTAAATATGCATTTGGGACATGCCCCAAGTATAT AGGAGTCAAGAGTCTCAAGCTGGCAATCGGTCTGAGAAACGTGCCTGCT AAGTCAAACAGAGGGCTATTTGGAGCCATAGCTGGATTCATAGAAGGGG GTTGGCCAGGGCTAGTTGCTGGTTGGTATGGTTTCCAGCATTCAAATGAC CAAGGGGTTGGTATGGCAGCAGACAGAGGTTCAACTCAGAAAGCAGTTG ATAAGATAACATCCAAAGTGAACAATATAATTGATAAGATGAACAGGCAAT ATGAAATAATTGATCATGAATTCAGTGAGATTGAAACTAGACTCAATATGA TCAACAACAAGATTGATGATCAAATACAAGACGTATGGGCATATAATGCA GAGTTGCTGGTACTACTTGAAAATCAGAAGACACTTGATGAACATGATGC AAACGTGAACAATCTATACAACAAGGTAAAAAGGGCATTGGGTTCCAATG CTATGGAAGATGGGAAGGGTTGTTTCGAGCTATACCATAAATGTGATGAT CAATGCATGGAAACAATTCGGAACGGGACATATAATAGGAGGAAATATAC AGAGGAGTCAAGGCTAGAAAGGCAGAAGATCGAGGGCGTGAAGCTGGA GAGCGAGGGCACCTACAAGATCCTGACCATCTACAGCACCGTGGCCAGC AGCCTGGTGCTGGCCATGGGCGTGGCCGCCTTCCTGTTCTGGGCCATG AGCAACGGCAGCTGCCGCTGCAACATCTGCATC
AMINO ACID	MEAISLMIILLVVTTSNADKICIGHQSTNSTETVDTLTESNIPVTQAKELLHTEH NGMLCATNLGRPLILDTCTVEGLIYGNPSCDLLLGGREWSYIVERPSAVNGT CYPGNVENLEELRMLFSSASSYQRIQIFPDAIWNVTYDGTSKSCSNSFYRNM RWLTQKNGNYPIQDAQYTNNRGKDILFIWGIHHPPTDTTQTNLYTRTDTTTS VTTENLDRTFKPLIGPRPLVNGLIGRINYYWSVLKPGQTLRVRSNGNLIAPWY GHVLSGESHGRILKTDLKSGNCVVQCQTEKGGLNSTLPFHNISKYAFGTCPK YIGVKSLKLAIGLRNVPAKSNRGLFGAIAGFIEGGWPGLVAGWYGFQHSNDQ GVGMAADRGSTQKAVDKITSKVNNIIDKMNRQYEIIDHEFSEIETRLNMINNKI DDQIQDVWAYNAELLVLLENQKTLDEHDANVNNLYNKVKRALGSNAMEDGK GCFELYHKCDDQCMETIRNGTYNRRKYTEESRLERQKIEGVKLESEGTYKIL TIYSTVASSLVLAMGVAAFLFWAMSNGSCRCNICI

	Sequence (H9HA Foldon, SKP/2016)
NUCLEOTIDE	GACAAAATCTGCATCGGCCACCAATCAACAAATTCCACAGAAACAGTGGA CACGCTAACAGAATCTAACATTCCTGTGACACAAGCCAAAGAATTGCTCC ACACGGAACATAATGGAATGCTGTGTGCAACAAATCTGGGACGTCCCCT CATCCTGGACACATGCACTGTCGAAGGACTGATTTATGGCAATCCTTCTT GTGATCTGCTATTGGGGGGAAGAGAATGGTCATACATTGTTGAAAGACCA TCAGCGGTAAATGGAACATGTTACCCTGGGAACGTAGAAAACTTAGAGGA ACTCAGAATGCTTTTTAGTTCTGCTAGTTCTTACCAAAGAATCCAAATCTT CCCAGACGCAATCTGGAATGTGACTTATGATGGAACAAGCAAATCATGCT CTAATTCGTTCTACAGGAATATGAGATGGCTGACTCAAAAGAATGGGAAT TATCCTATTCAAGACGCCCAATACACAAACAACCGAGGGAAGGACATTCT CTTCATTTGGGGCATACATCATCCACCCACTGATACTACGCAAACGAATT TATACACAAGAACCGACACAACAACAAGCGTCACAACAGAAAATCTGGAT AGGACCTTCAAACCATTAATAGGACCAAGGCCTCTTGTCAATGGTCTAAT TGGAAGAATTAATTATTATTGGTCAGTATTAAAGCCAGGACAGACGTTGA GAGTAAGATCCAATGGGAATCTAATTGCTCCATGGTATGGACATGTCCTC TCAGGAGAGAGCCATGGGAGAATTCTGAAGACTGATTTAAAGAGTGGTAA TTGCGTAGTGCAATGTCAGACTGAAAAAGGTGGCTTAAACAGTACACTAC CATTCCACAATATCAGTAAATATGCATTTGGGACATGCCCCAAGTATATAG GAGTCAAGAGTCTCAAGCTGGCAATCGGTCTGAGAAACGTGCCTGCTAA GTCAAACAGAGGGCTATTTGGAGCCATAGCTGGATTCATAGAAGGGGGT TGGCCAGGGCTAGTTGCTGGTTGGTATGGTTTCCAGCATTCAAATGACCA AGGGGTTGGTATGGCAGCAGACAGAGGTTCAACTCAGAAAGCAGTTGAT AAGATAACATCCAAAGTGAACAATATAATTGATAAGATGAACAGGCAATAT GAAATAATTGATCATGAATTCAGTGAGATTGAAACTAGACTCAATATGATC AACAACAAGATTGATGATCAAATACAAGACGTATGGGCATATAATGCAGA GTTGCTGGTACTACTTGAAAATCAGAAGACACTTGATGAACATGATGCAA ACGTGAACAATCTATACAACAAGGTAAAAAGGGCATTGGGTTCCAATGCT ATGGAAGATGGGAAGGGTTGTTTCGAGCTATACCATAAATGTGATGATCA ATGCATGGAAACAATTCGGAACGGGACATATAATAGGAGGAAATATACAG AGGAGTCAAGGCTAGAAAGGCAGGGCAGCGGCTACATCCCCGAGGCCC CCCGCGATGGCCAGGCCTACGTGCGCAAGGATGGCGAGTGGGTGCTGC TGAGCACCTTCCTGGGC
AMINO ACID	DKICIGHQSTNSTETVDTLTESNIPVTQAKELLHTEHNGMLCATNLGRPLILDT CTVEGLIYGNPSCDLLLGGREWSYIVERPSAVNGTCYPGNVENLEELRMLFS SASSYQRIQIFPDAIWNVTYDGTSKSCSNSFYRNMRWLTQKNGNYPIQDAQ YTNNRGKDILFIWGIHHPPTDTTQTNLYTRTDTTTSVTTENLDRTFKPLIGPRP LVNGLIGRINYYWSVLKPGQTLRVRSNGNLIAPWYGHVLSGESHGRILKTDL KSGNCVVQCQTEKGGLNSTLPFHNISKYAFGTCPKYIGVKSLKLAIGLRNVPA KSNRGLFGAIAGFIEGGWPGLVAGWYGFQHSNDQGVGMAADRGSTQKAV DKITSKVNNIIDKMNRQYEIIDHEFSEIETRLNMINNKIDDQIQDVWAYNAELLV LLENQKTLDEHDANVNNLYNKVKRALGSNAMEDGKGCFELYHKCDDQCME TIRNGTYNRRKYTEESRLERQKIEGVKLESEGTYKILGSGYIPEAPRDGQAYV RKDGEWVLLSTFLG

Appendix B: Clinical scores

Day post infection	Clinical signs	Inactivated H9N2 vaccine (Direct)							rH9HA (Direct)							rH9HA-CD83 scFv (Direct)							PBS (Direct)							PBS (Contact)							rH9HA-CD83 scFv (Contact)					
		801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841
1	Diarrhoea	0	0	0	2	0	0	0	0	2	0	2	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Diarrhoea	2	0	0	0	0	0	0	2	0	0	0	0	2	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Diarrhoea	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Diarrhoea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	3	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Day post infection	Clinical signs	Inactivated H9N2 vaccine							rH9HA (Direct)							rH9HA-CD83 scFv (Direct)							PBS (Direct)							PBS (Contact)							rH9HA-CD83 scFv (Contact)					
		801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841
5	Diarrhoea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	0	2	0	2	2	0	0	0	0	0	0	0	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	2	0	0	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	Diarrhoea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
	Ruffled feathers	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pale comb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eye and nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Red eyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dropped wings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Half eyes shut	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Swollen head	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weak response to capture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Laboured breathing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Paralysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Isolated behaviour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Description of clinical score	
None	0
Mild	1
Moderate	2
Severe	3

Appendix C: CT value for determining cytokine mRNA level (Chapter 4, Section 4.2.3.1)

Stimulants	Replicates	RPLPO-1			IFN-γ			IL-6			IL-1β			IL-4			IL-18			CxCLi2		
		CT value			CT value			CT value			CT value			CT value			CT value			CT value		
		Hours post stimulation			Hours post stimulation			Hours post stimulation			Hours post stimulation			Hours post stimulation			Hours post stimulation			Hours post stimulation		
		5	22	30	5	22	30	5	22	30	5	22	30	5	22	30	5	22	30	5	22	30
Media	1	17.6	19.4	19.4	27.8	29.0	29.0	28.3	29.4	29.3	27.5	28.7	29.2	25.7	29.5	31.1	22.9	24.4	24.3	25.2	27.7	27.9
	2	17.2	19.3	19.8	28.5	29.7	29.9	28.3	28.3	29.0	27.2	27.6	28.2	25.8	29.8	30.7	22.9	24.4	24.3	25.7	28.0	28.3
	3	18.0	18.3	20.0	29.6	29.7	30.4	29.4	30.2	30.6	28.0	29.7	29.7	26.9	27.4	28.7	23.6	24.6	24.1	24.3	25.6	26.8
rH9HA	1	17.7	19.9	19.4	27.4	27.7	27.3	26.3	26.9	26.7	26.1	27.5	27.4	25.5	28.1	28.1	22.6	24.6	23.7	23.9	25.5	25.7
	2	17.5	19.5	19.6	27.6	29.5	27.4	24.8	26.5	25.9	25.3	25.9	26.1	25.6	28.3	27.4	22.6	24.6	23.7	23.8	25.6	25.6
	3	18.2	18.5	19.9	28.9	28.5	27.4	26.0	26.3	27.0	26.7	27.9	28.6	26.0	26.4	27.0	22.9	24.5	23.9	23.5	24.5	25.7
rH9HA-Dec205 scFv	1	17.8	19.3	19.3	27.6	27.1	26.3	27.4	26.7	26.9	26.6	26.7	27.2	25.3	28.0	28.6	22.7	24.6	24.0	24.5	25.4	25.6
	2	17.5	19.4	19.3	28.1	28.7	28.3	25.7	26.8	26.6	25.2	25.9	25.9	25.8	28.4	28.3	23.2	24.6	24.0	23.9	25.6	26.4
	3	18.0	18.4	19.6	28.3	28.5	28.5	26.8	27.5	27.6	25.2	27.7	26.9	26.4	26.4	25.6	23.5	24.6	23.1	23.4	23.1	24.4
rH9HA-CD83 scFv	1	17.9	18.8	19.0	25.6	22.4	22.5	22.5	22.7	23.4	24.2	23.4	23.9	25.1	25.2	26.4	22.5	23.4	22.8	20.9	21.7	22.2
	2	17.8	19.2	19.3	27.3	23.8	23.9	23.1	21.4	22.9	24.3	22.6	23.3	25.5	26.5	26.5	22.5	23.0	23.5	22.6	23.6	23.6
	3	18.0	18.3	19.6	29.0	23.5	24.9	23.0	23.6	24.7	23.9	25.0	24.4	26.2	23.9	24.6	23.1	23.0	23.2	21.4	21.1	21.9
rH9HA-CD11c scFv	1	18.0	18.9	19.6	26.7	20.9	23.0	22.1	22.5	23.5	22.8	22.6	23.5	25.4	25.8	27.1	22.5	23.0	23.5	20.6	21.8	22.7
	2	17.8	19.3	19.4	27.0	22.2	22.8	22.1	21.7	22.7	22.9	22.4	23.7	25.7	25.5	26.7	22.8	23.0	23.5	21.2	22.2	22.9
	3	17.9	18.5	19.4	28.5	22.8	23.1	22.9	24.0	24.1	23.6	24.1	23.9	26.3	23.8	24.4	22.9	23.8	23.0	20.3	20.7	21.2
Control scFv	1	17.9	19.0	19.2	28.5	29.9	30.2	28.5	28.4	28.8	27.0	27.4	27.9	26.0	29.1	30.6	23.3	24.3	24.5	25.1	27.6	27.6
	2	17.3	19.1	19.2	28.4	29.7	30.3	28.3	27.9	29.2	26.8	27.4	28.0	25.7	29.6	31.2	23.5	24.6	24.5	25.3	27.1	28.3
	3	17.9	18.6	19.7	27.9	29.7	29.9	27.6	28.0	28.8	27.1	28.3	28.2	25.2	29.4	30.8	22.8	24.6	24.6	25.1	27.9	28.4
Dec205 scFv	1	17.9	19.0	19.6	28.1	28.6	29.5	29.2	29.0	29.8	27.6	27.4	28.5	25.9	30.1	31.6	22.8	23.7	24.7	25.5	27.1	28.0
	2	17.7	19.7	20.2	28.2	29.6	30.9	27.1	28.3	20.4	26.8	26.9	28.2	26.1	29.4	31.1	22.8	24.6	24.7	25.4	27.8	28.2
	3	17.7	18.4	19.8	28.4	29.7	29.9	27.1	28.9	29.2	26.8	28.3	28.6	26.4	27.0	27.2	23.2	24.6	22.7	24.0	24.8	26.2
CD83 scFv	1	18.0	19.4	19.6	27.9	28.1	28.5	26.3	27.1	28.5	27.5	27.2	27.9	26.2	29.2	30.8	22.7	24.5	25.4	23.5	24.8	25.6
	2	17.5	19.7	20.5	27.5	28.6	29.4	26.3	25.7	20.7	26.3	25.7	27.4	25.5	28.5	30.7	22.8	24.5	25.4	23.8	24.7	26.9
	3	17.8	18.5	19.7	28.7	28.9	29.2	27.5	27.1	28.0	26.2	26.9	26.7	26.5	26.5	27.4	22.7	24.5	22.8	23.1	22.9	23.6
CD11c scFv	1	17.9	19.3	19.9	27.7	28.2	27.1	24.9	27.1	27.9	26.3	26.8	27.3	25.8	28.9	30.2	22.7	24.5	24.9	23.1	23.8	25.5
	2	17.8	19.6	20.2	27.5	28.9	27.9	24.9	25.1	20.8	25.4	24.9	26.2	25.7	28.7	28.9	22.7	23.7	24.9	23.4	23.5	26.1
	3	17.8	18.2	19.8	28.7	28.9	28.2	26.3	27.6	27.9	25.9	27.8	26.6	26.8	26.1	26.2	23.3	23.7	22.8	22.3	22.6	23.4

Appendix C (Chapter 4, Section 4.2.3.1): CT value for determing the mRNA level of different cytokines. Splenocytes were isolated from the spleen of 3-week-old SPF chickens using Histopaque 1083 and stimulated with 10 µg of scFv/ rH9HA/ rH9HA-scFv for 5 hours, 22 hours and 30 hours *in vitro*. Stimulated splenocytes were harvested for RNA extraction and expression levels of the respective cytokines and chemokine were measured by qRT-PCR. The qRT-PCR runs were carried out in triplicates for each RNA sample and the CT values are represented as mean. Three independent experiments (replicates 1,2 and 3) were carried out using different spleen donor chickens.

Chapter 5, Section 5.2.1.2

Groups	Bird number	RPLPO-1 CT value					IFNγ CT value					IL-6 CT value					IL1β CT value				
		Days post vaccination					Hours post stimulation					Hours post stimulation					Hours post stimulation				
		2	4	6	8	21	2	4	6	8	21	2	4	6	8	21	2	4	6	8	21
Unvaccinated control	801	16.11	15.90	16.24	15.71	15.51	29.01	27.76	26.30	28.49	27.50	29.42	32.33	28.20	29.83	29.50	27.92	30.27	30.25	27.71	27.72
	802	16.05	15.52	16.05	15.88	16.02	29.13	27.57	26.97	28.65	27.47	29.86	30.24	29.61	29.03	28.46	27.05	29.39	29.39	26.87	27.06
	803	16.30	15.72	15.85	15.68	15.51	29.13	27.26	27.45	28.21	27.66	29.78	29.23	30.10	30.43	29.41	27.80	28.65	28.65	27.88	27.26
	804	16.42	15.39	15.79	15.42	15.75	29.42	27.58	27.11	27.91	28.49	30.96	29.90	27.17	29.43	29.80	27.90	28.58	28.58	26.64	25.92
	805	16.26	15.63	15.90	15.63	16.00	28.80	26.89	26.89	28.15	27.54	28.60	29.93	29.29	30.80	29.45	26.83	28.22	28.22	27.91	27.53
	806	16.10	15.26	16.17	15.94	15.65	29.40	27.46	24.27	27.91	28.20	30.11	30.62	27.85	29.81	30.41	27.37	28.57	28.58	27.28	27.17
Inactivated H9N2 vaccine	621	16.11	16.26	16.33	15.80	16.29	29.59	27.69	27.30	28.26	28.00	30.03	31.06	31.00	31.04	29.63	27.91	29.24	29.24	28.02	28.22
	622	15.85	15.90	15.93	15.51	15.72	29.07	27.68	27.55	27.01	27.84	30.60	31.04	30.13	28.99	28.76	28.61	28.96	28.96	27.33	26.78
	623	16.25	15.64	15.95	15.76	15.96	29.30	27.69	27.35	28.10	27.97	30.12	30.87	31.58	30.27	29.00	27.68	28.80	28.80	27.14	27.29
	624	16.57	15.59	16.25	15.58	15.83	29.34	27.74	27.72	27.77	27.81	29.97	30.67	30.89	30.46	29.73	27.09	28.48	28.52	27.38	27.30
	625	16.17	15.74	16.10	15.45	16.11	29.70	27.87	27.69	27.60	28.40	29.57	29.12	29.32	29.05	29.42	27.11	27.56	27.56	27.10	26.89
	626	16.33	15.68	15.88	15.36	15.80	29.88	27.97	27.96	27.70	28.17	30.68	31.18	31.42	29.77	28.21	27.26	27.84	27.84	27.05	27.72
rH9HA	831	15.99	15.78	16.06	16.01	15.90	29.62	26.86	26.81	28.07	27.66	30.49	29.02	28.90	30.13	29.00	28.35	28.79	28.78	27.87	27.48
	832	16.03	15.86	16.08	15.39	16.20	27.85	27.72	27.51	27.41	27.83	26.42	31.54	30.57	30.73	29.99	26.73	29.75	29.75	28.29	28.25
	833	16.38	15.60	15.93	15.37	16.70	29.45	28.03	26.78	27.52	27.92	30.41	31.76	28.69	29.99	30.14	28.56	29.68	29.63	27.06	27.64
	834	16.44	15.52	15.88	15.52	16.06	28.85	27.60	26.77	28.06	27.98	28.73	31.48	29.01	27.98	29.78	27.06	29.05	29.15	26.13	27.67
	835	16.50	15.80	16.08	15.75	15.94	28.41	27.78	27.54	27.85	27.90	27.48	30.96	30.20	30.21	28.52	26.97	28.65	28.65	27.37	26.37
	836	16.27	15.67	16.35	15.72	15.88	29.76	26.35	27.16	28.39	28.17	30.12	28.85	30.75	30.41	28.63	27.95	27.95	27.96	27.46	27.45
rH9HA-CD83 scFv	41	16.06	15.90	16.20	15.35	16.11	29.39	28.13	26.38	27.77	28.63	31.60	31.78	29.23	29.92	29.90	28.45	30.16	30.10	27.34	28.15
	42	16.87	15.75	16.32	15.53	15.95	26.82	27.52	27.89	28.14	27.91	25.69	29.82	30.59	29.78	29.63	25.65	28.65	28.62	27.71	27.87
	43	16.32	15.55	16.27	15.73	16.36	29.88	27.04	27.43	28.10	28.50	31.65	28.82	29.74	29.39	29.26	28.64	28.62	28.62	26.34	28.10
	44	16.82	15.76	15.92	15.86	15.96	29.72	27.56	27.61	28.39	27.82	30.72	31.68	31.13	30.83	30.46	27.50	29.32	29.31	27.95	27.10
	45	16.18	16.03	15.75	15.60	15.90	29.61	27.92	27.96	28.06	28.87	31.20	30.95	31.29	28.72	25.99	27.47	28.86	28.85	26.63	27.69
	46	16.24	15.61	16.74	15.41	15.70	28.66	27.70	26.82	28.40	32.95	27.28	32.06	30.04	29.98	29.00	26.09	27.34	27.34	27.05	27.30

Groups	Bird number	IL-4 CT value					IL-18 CT value					IL-10 CT value					TGF-β4 CT value					CxCLi2 CT value				
		Hours post stimulation					Hours post stimulation					Hours post stimulation					Hours post stimulation					Hours post stimulation				
		2	4	6	8	21	2	4	6	8	21	2	4	6	8	21	2	4	6	8	21	2	4	6	8	21
Unvaccinated control	801	29.81	30.75	31.45	30.75	31.90	21.55	21.20	20.31	22.21	23.80	33.07	31.98	31.29	33.00	32.45	21.45	20.95	22.39	21.45	21.10	26.83	26.57	27.10	23.79	27.70
	802	31.15	30.85	31.34	31.50	30.99	20.93	21.32	21.01	22.04	23.40	32.65	30.95	31.80	32.30	31.85	21.01	20.96	22.41	21.65	20.90	26.44	25.29	27.47	24.45	27.35
	803	30.48	30.80	31.02	31.35	32.16	21.34	20.74	21.31	22.39	23.45	31.95	31.80	31.26	32.52	31.50	21.36	21.15	22.32	21.46	20.95	26.72	25.25	27.98	25.51	28.00
	804	29.59	30.21	31.47	30.93	32.45	21.54	20.82	20.56	21.65	22.65	32.79	31.77	31.00	31.80	31.69	21.84	20.84	22.18	20.93	20.80	26.18	26.50	25.80	24.83	29.10
	805	29.45	31.31	32.09	30.95	31.50	21.09	19.88	21.45	21.98	23.68	32.58	30.00	30.89	32.70	31.90	21.10	20.78	21.50	21.22	21.05	26.38	26.44	26.99	26.03	27.95
	806	30.83	31.05	31.51	30.45	31.50	21.08	20.35	20.42	21.93	23.65	31.73	31.70	28.08	32.45	32.80	21.37	20.75	21.55	21.36	20.80	27.25	25.93	26.64	25.56	28.00
Inactivated H9N2 vaccine	621	30.40	30.73	30.99	31.64	31.70	21.27	21.61	21.50	22.49	23.98	31.50	32.68	32.92	32.95	32.05	21.67	21.20	22.26	21.36	21.30	26.09	24.80	28.66	25.98	27.28
	622	30.42	30.92	31.33	30.65	31.70	21.53	20.98	21.13	21.79	23.45	33.10	31.43	31.19	29.95	32.35	21.54	20.67	21.46	21.10	21.05	26.02	25.21	28.27	25.94	27.95
	623	30.07	31.14	31.56	31.70	31.45	21.28	20.95	21.72	21.96	23.75	31.37	31.84	32.46	31.28	32.80	21.33	20.87	21.86	21.12	21.10	25.78	25.02	28.15	25.60	28.95
	624	29.92	30.65	31.32	31.40	31.35	21.22	20.57	21.60	22.37	23.75	31.75	32.12	32.17	32.32	31.75	21.38	20.65	21.80	21.09	20.90	26.14	26.11	27.68	25.28	28.15
	625	30.58	30.99	32.64	32.13	32.07	20.99	20.09	20.96	21.60	23.29	31.58	30.38	32.09	30.47	32.45	21.22	20.69	22.11	21.64	20.95	26.70	25.91	26.28	26.17	28.98
	626	30.55	31.12	31.18	32.29	30.40	21.40	20.02	21.86	22.25	23.95	32.41	32.19	32.83	31.40	32.75	21.53	20.92	21.95	21.29	20.95	25.96	25.90	27.08	25.67	29.20
rH9HA	831	29.92	32.23	32.11	31.08	32.34	21.85	20.25	21.04	22.18	23.29	32.07	31.27	30.90	31.08	31.20	21.47	20.82	22.25	21.44	20.75	26.49	24.12	28.13	24.94	28.10
	832	30.14	31.01	30.82	30.70	31.94	20.93	21.56	21.49	22.18	23.85	31.51	32.37	31.62	30.00	32.60	21.00	20.83	22.14	21.43	21.20	26.72	25.94	29.56	25.63	28.25
	833	30.18	30.80	31.82	31.20	31.95	21.33	20.96	19.81	22.18	23.80	32.75	32.04	29.83	30.70	32.60	20.87	20.80	21.93	21.05	21.35	26.95	25.39	29.31	25.83	28.15
	834	30.35	30.76	30.75	31.80	31.85	21.34	20.79	20.79	21.34	23.80	32.42	32.28	31.08	32.18	33.00	21.15	20.71	22.35	21.02	20.55	26.61	26.17	27.32	24.61	27.70
	835	30.54	30.70	31.47	31.30	31.23	20.87	20.70	21.45	22.10	22.90	32.69	31.81	32.04	31.38	32.00	21.13	20.52	22.44	21.11	20.80	26.39	26.23	27.56	26.00	28.40
	836	31.11	31.77	31.57	31.04	31.85	21.74	20.18	21.62	22.22	23.50	33.06	30.62	31.04	32.35	32.10	21.53	20.58	22.61	21.11	20.80	26.38	25.96	27.13	26.45	28.00
rH9HA-CD83 scFv	41	30.08	30.66	30.95	30.15	31.60	21.12	21.36	20.92	22.20	23.50	31.35	32.18	31.01	31.50	31.70	21.08	20.99	22.25	20.97	20.95	26.77	25.77	27.78	25.57	29.10
	42	29.92	31.26	31.75	30.43	31.26	20.36	20.48	20.92	21.85	23.55	31.32	29.79	31.99	32.37	32.45	21.02	20.81	22.12	21.12	21.15	26.63	25.69	29.11	25.50	28.15
	43	31.05	31.56	31.55	31.60	33.25	21.53	20.54	21.55	21.52	23.24	33.59	31.06	32.21	31.84	32.35	21.07	20.81	22.52	21.30	21.65	27.29	25.41	28.49	25.23	26.75
	44	30.96	30.86	31.17	31.60	31.65	21.19	20.78	21.68	22.55	22.95	33.37	32.30	33.27	34.04	32.10	21.59	21.02	22.07	21.25	20.85	26.57	25.55	28.14	25.52	29.60
	45	30.36	31.72	31.43	31.60	30.20	21.40	21.26	21.70	21.77	23.00	32.09	32.74	32.04	32.33	32.00	21.53	21.27	22.23	21.26	21.20	27.01	24.79	28.45	25.10	27.90
	46	30.25	30.33	32.62	31.50	30.10	20.49	20.26	21.47	21.94	23.20	31.00	31.54	31.87	36.50	32.20	21.28	20.58	22.33	21.16	21.00	27.64	27.15	27.96	25.55	28.00

Appendix C (Chapter 5, Section 5.2.1.2) : CT value for determing the mRNA level of different cytokines after vaccination of chickens with inactivated H9N2 vaccine, rH9HA and rH9HA-CD83 scFv. Groups of 1-day-old White Leghorn SPF chickens were vaccinated once with rH9HA (35 µg/dose), rH9HA-CD83 scFv (35 µg/dose equivalent of rH9HA) and inactivated H9N2 vaccine (1024 HAU/dose). Spleen samples (n=6 per group) were collected from the vaccinated chickens on day 2, 4, 6, 8 and 21 post vaccination and RNA was extracted. The expression levels of the respective cytokines and chemokine were measured by qRT-PCR. The qRT-PCR runs were carried out in triplicates for each RNA sample (each bird) and the CT values are represented as mean.

Appendix D: Resulting publications

All publications associated with work presented in this thesis are listed below:

Accepted publications:

1. Shrestha, A.; Sadeyen, J.-R. & Iqbal, M. Enhancing Protective Efficacy of Poultry Vaccines through Targeted Delivery of Antigens to Antigen-Presenting Cells. *Vaccines.* **6**, 75 (2018).

2. Shrestha, A. *et al*. Targeting Haemagglutinin Antigen of Avian Influenza Virus to Chicken Immune Cell Receptors Dec205 and CD11c Induces Differential Immune-Potentiating Responses. *Vaccines.* **9**, 784 (2021).

3. Shrestha, A. *et al*. Selectively targeting haemagglutinin antigen to chicken CD83 receptor induces faster and stronger immunity against avian influenza. *npj Vaccines.* **6***,* 1–12 (2021).

Under review:

1. Shrestha, A. *et al*. Recombinant haemagglutinin antigen selectively targeted to chicken CD83 receptor can overcome the interference from maternally derived antibodies in chickens.

Appendix E: Resulting grants and studentships

BBSRC-LINK (2020-2023): Establishment of a novel poultry vaccine platform inducing rapid and strong immunity through targeted delivery of antigens to chicken immune cells. Total value £1,055,488.

PhD Studentship (2020-2024): Improving vaccines for poultry: targeted delivery of multivalent viral antigens to distinct immune cells of different avian species.

Appendix F: Resulting patents

The work carried out in this thesis has resulted in filing of two patents jointly by The Pirbright institute and MSD Animal Health (The Netherlands).

1. Patent Application: ‘Vaccine’, Date of filing: 26 October 2020.
2. Patent Application: ‘Overcoming antibody interference in avians’, Date of filing: 13 July 2021.

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