Manipulating the binding of the hemagglutinin protein to host sialic acids for next-generation influenza vaccine development

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ABSTRACT

Influenza vaccine programs can significantly reduce morbidity and mortality associated with infection. However, the efficacy of current vaccines is highly variable from one year to the next (0-70%) and vaccine-induced protection typically wanes rapidly. Vaccines targeting avian influenza strains are often even less effective unless given at very high doses with adjuvants, raising concerns about pandemic preparedness. New strategies are urgently needed to improve the quality of these vaccines and understanding the mechanisms underlying the poor immunogenicity is an active area of investigation. Our approach was to examine whether differences in the binding properties of the influenza hemagglutinin (HA) protein to the host sialic acid (SA) receptors influence vaccine immunogenicity. This approach was motivated by a serendipitous finding that plant-based virus like particle (VLP) vaccines bearing influenza HA readily bind to SA residues on the surface of human immune cells. In fact, SAs are present at high density in many human tissues including skeletal muscle, where almost all influenza vaccines are administered. We hypothesized that HA-SA interactions play a role in shaping the immune response to vaccination and that differences in binding properties among the various influenza HAs contribute to variable immunogenicity. Initial experiments focused on determining whether differences in SA binding properties influence the pattern of VLP-immune cell interactions and/or downstream immune responses *in vitro*. We examined VLPs targeting an avian strain (H5N1, H5-VLP) and a mammalian strain of influenza (H1N1, H1-VLP), which preferentially bind to distinct SA receptors (α(2,3)- and α(2,6)-linked, respectively), and found that the patterns of interactions were largely determined by the differential distribution of the SA receptors on immune cell subsets. These early interactions also strongly influenced downstream cell activation, prompting us to investigate the possible impact of HA-SA interactions on vaccine responses *in vivo*. As a proof of principle, we generated an H1-VLP (A/California/07/2009) bearing an HA that is unable to bind to SA by substituting tyrosine for phenylalanine at amino acid 98 of the HA protein (H1$_{Y98F}$-VLP). This well-known mutation prevents SA binding without interfering with the antigenicity of the HA, making it an ideal candidate for assessing the impact of receptor binding on vaccine responses. In mice, vaccination with the H1$_{Y98F}$-VLP resulted in significantly higher neutralizing antibody

titers, improved IgG avidity maturation, and enhanced antibody durability compared to mice vaccinated with wild-type H1-VLP ($H1_{WT}$-VLP). Furthermore, improved antibody responses significantly correlated with a reduction in viral load and pulmonary inflammation following challenge in mice vaccinated with $H1_{Y98F}$-VLP. Finally, we found that elimination of HA-SA interactions using the Y98F mutation also improves the immunogenicity of recombinant H1 trimers (H1N1 A/Brisbane/02/2018) and plant-based VLPs targeting avian strains of influenza (H7N9 A/Shanghai/02/2013 and H5N1 A/Indonesia/05/2005). Thus, ablation of HA-SA interactions represents a promising and easily implemented strategy in the development of next-generation influenza vaccines.

RÉSUMÉ

Les programmes de vaccination contre la grippe saisonnière permettent de réduire significativement la morbidité et la mortalité associées à cette infection. Cependant, l'efficacité des vaccins actuellement disponibles est très variable d'une année à l'autre (0-70%) et la protection qu'ils procurent diminue rapidement. Les vaccins ciblant les souches pandémiques d'origine aviaire sont souvent encore moins efficaces à moins d'être administrés à haute dose additionnée d'adjuvant, ce qui soulève de nombreuses inquiétudes concernant notre niveau de préparation aux pandémies. Il est donc urgent de développer de nouvelles stratégies pour améliorer la qualité de ces vaccins; et dans ce contexte les mécanismes d'action à l'origine de leur faible immunogénicité font l'objet d'intenses investigations. Notre approche fut d'examiner si les différences dans la liaison entre la protéine hémagglutinine (HA) du virus influenza et l'acide sialique (AS) à la surface des cellules de l'hôte impactent l'immunogénicité. Cette approche résulte de la découverte inattendue de la capacité des pseudo-particules virales (PPV) exprimant la protéine HA à leur surface et produites dans les plantes à se lier aux ASs à la surface des cellules immunitaires chez l'humain. En fait, les ASs sont fortement présents à la surface des cellules de nombreux tissus chez l'humain, y compris les muscles striés squelettiques dans lesquels sont injectés la vaste majorité des vaccins antigrippaux. Nous avons émis l'hypothèse selon laquelle la liaison HA-AS jouerait un rôle dans l'élaboration et la qualité de la réponse immunitaire; et que différentes interactions HA-AS pourraient contribuer aux différences observées quant à immunogénicité des différentes HA. Nous nous sommes tout d'abord intéressés à savoir si les différentes propriétés de liaison peuvent influencer les interactions entre PPV et cellules immunitaires ainsi qu'aux les conséquences sur l'activation de ces cellules *in vitro*. Nous avons examiné les interactions et leurs conséquences de l'exposition aux PPV exprimant l'HA de la souche aviaire H5N1 (H5-PPV) ainsi que celle d'une souche saisonnière H1N1 (H1-PPV) qui se lient respectivement de manière préférentielle aux récepteurs α(2,3) et α(2,6). Nous avons découvert que le modèle d'interaction était largement déterminé par la distribution des SA à la surface des différentes sous-populations des cellules immunitaires. Ces interactions influencent également l'activation subséquente de ces cellules, ce qui nous a encouragé à investiguer les effets potentiels de ces interactions spécifiques

sur la réponse immunitaire aux vaccins *in vivo*. Nous avons produit une PVV exprimant H1 (A/California/07/2009, H1-PPV) exprimant une H1 dont la capacité de liaison avec SA a été abrogée par la substitution d'une tyrosine par une phénylalanine en position 98 ($H1_{Y98F}$-PPV). Cette mutation bien caractérisée empêche la liaison avec AS sans interférer avec l'antigénicité, ce qui en fait le candidat idéal pour déterminer l'impact de la liaison avec le récepteur sur la réponse immunitaire au vaccin. Chez la souris, la vaccination avec $H1_{Y98F}$-PPV induit un niveau de titres d'anticorps neutralisants significativement supérieurs, une meilleure maturation de l'avidité des IgG ainsi qu'une amélioration de la durée de la réponse comparativement aux souris vaccinées avec H1 original (wild-type, $H1_{WT}$-PPV). De plus, cette réponse humorale supérieure est significativement corrélée à une réduction de la charge virale et de l'inflammation dans les poumons suite à une infection des souris vaccinées avec $H1_{Y98F}$-PPV. Finalement, nous avons découvert que l'abrogation de l'interaction HA-AS suite à la mutation Y98F améliorait également l'immunogénicité de trimères de la protéine H1 recombinante (H1N1 A/Brisbane/02/2018). Ces observations s'étendent également aux PPV exprimant la HA d'autres souches aviaires telles que H7N9 (A/Shanghai/02/2013) et H5N1 (A/Indonesia/05/2005). En conclusion, la suppression de la liaison HA-AS représente une stratégie encourageante et facilement implémentable afin de développer une nouvelle génération de vaccins antigrippaux plus efficaces.

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LIST OF ABBREVIATIONS

ACK	ammonium-chloride-potassium (buffer)
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
AI	avidity index
ALI	acute lung injury
ANOVA	analysis of variance
APC	antigen presenting cell
ARDS	acute respiratory distress syndrome
ASC	antibody secreting cell
BCR	B cell receptor
BEVS	baculovirus expression vector system
BM	bone marrow
BMGF	Bill and Melinda Gates Foundation
CDC	complement-dependent cytotoxicity
COBRA	computationally optimized broadly reactive antigen
CPE	cytopathic effects
CVV	candidate vaccine virus
DAPI	4’,6-diamidino-2-phenylindole
DiD	dialkylcarbocyanine dye
DMSO	dimethyl sulfoxide
DPI	days post-infection
DPV	days post-vaccination
eGFP	enhanced green fluorescence protein
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum

EV	empty vesicle
Fc	fragment crystallizable
FcR	fragment crystallizable receptor
GC	germinal center
GISRS	Global Influenza Surveillance and Response System
H&E	hematoxylin and eosin
HA	hemagglutinin
HBSS	Hank's balanced salt solution
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HRP	horseradish peroxidase
IAV	influenza A virus
IBV	influenza B virus
ICS	intracellular cytokine staining
ICV	influenza C virus
ID	intradermal
IDV	influenza D virus
IFN	interferon
IgG	immunoglobulin G
IIV	inactivated influenza vaccine
ILI	influenza-like illness
IM	intramuscular
IN	intranasal
IRAT	Influenza Risk Assessment Tool
ISG	interferon-stimulated genes
LAIV	live-attenuated influenza vaccine
LLPC	long-lived plasma cells
LN	lymph node
LRT	lower respiratory tract
m	month
M1	matrix protein 1

M2	matrix protein 2
M2e	ectodomain of matrix protein 2
MBC	memory B cell
MDCK	Madin-Darby Canine Kidney
MFI	mean fluorescence intensity
min	minute
mL	milliliter
mm	millimeter
MMR	measles, mumps and rubella
MN	mironeutralization
MPV	months post-vaccination
mRNA	messenger ribonucleic acid
NA	neuraminidase
NB	non-binding
NEP	nuclear export protein
NIAID	National Institute for Allergy and Infectious Diseases
NK	natural killer
NP	nucleoprotein
NS1	non-structural protein 1
PA	polymerase acidic subunit
PB1	polymerase basic subunit 1
PB2	polymerase basic subunit 2
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	plasma cell
PDISP	signal peptide of protein disulfide isomerase
PFA	paraformaldehyde
PHA	phytohemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
QIV	quadrivalent influenza vaccine

RBC	red blood cell
RBD	receptor binding domain
RdRp	ribonucleic acid-dependent ribonucleic acid polymerase
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Rosewell Park Memorial Institute medium
RT	room temperature
RU	resonance units
SA	sialic acid
SEM	standard error of the mean
SLO	secondary lymphoid organ
SPR	surface plasmon resonance
SRH	single radial hemolysis
ssRNA	single-stranded ribonucleic acid
TBST	tris-buffered saline with Tween 20
$TCID_{50}$	tissue culture infectious dose 50
TEM	transmission electron microscope
Thpp	primed-precursor T helper cell
TIV	trivalent influenza vaccine
TLR	toll-like receptor
UIV	universal influenza vaccine
µg	microgram
µl	microliter
URT	upper respiratory tract
VLP	virus-like particle
w	week
WHO	World Health Organization
wpv	weeks post-vaccination
WT	wild-type

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis contributes original knowledge to the fields of influenza vaccine immunology and the development of next-generation influenza vaccines. The specific contributions are as follows:

In **Chapter 2**, we describe the interactions plant-based virus-like particle (VLP) vaccines bearing hemagglutinin (HA) of mammalian or avian influenza with human immune cells *in vitro.* We demonstrated that:

1. HA-VLPs bind to sialic acid (SA) residues on the surface of human peripheral blood mononuclear cells (PBMC).
2. Mammalian (H1) and avian (H5) VLPs have distinct patterns of interaction with PBMC because of the differential distribution of their respective SA receptors.
3. Receptor-mediated interactions between VLPs and PBMCs lead to strain-specific patterns of cell activation.

In **Chapter 3**, we developed an H1-VLP that is unable to bind to SA ($H1_{Y98F}$-VLP) and evaluated its immunogenicity and efficacy compared to the wild-type H1-VLP ($H1_{WT}$-VLP) in mice. We demonstrated that:

1. The Y98F mutation does not impact HA-VLP expression or morphology but successfully ablates SA binding.
2. Vaccination with the $H1_{Y98F}$-VLP results in significantly stronger humoral responses compared to $H1_{WT}$-VLP, including higher functional antibody titers, improved IgG avidity maturation, and enhanced antibody durability.
3. Mice vaccinated with the $H1_{Y98F}$-VLP were better protected from severe infection, including reduced viral titers and pulmonary inflammation.

In **Chapter 4**, we demonstrated that reducing HA-SA interactions can improve the immunogenicity of VLPs bearing HAs from highly pathogenic strains of avian influenza (H5 and H7), which tend to be poorly immunogenic. We demonstrated that:

1. The Y98F mutation markedly reduces or eliminates SA binding by H5- and H7-VLPs.
2. Vaccination with H7$_{Y98F}$-VLP results in significantly stronger humoral responses compared to H7$_{WT}$-VLP, including higher hemagglutination inhibition (HI) titers, improved IgG avidity maturation and increased HA-specific PC in the BM.
3. Vaccination with H5$_{Y98F}$-VLP results in increased HA-specific PC in the BM but has little impact on HI titers or IgG avidity.
4. Delayed administration of the second vaccine dose results in markedly higher antibody titers and avidity maturation across all vaccine groups.

Taken together, we have demonstrated that HA-SA interactions play a previously unappreciated role in the context of influenza vaccines and that eliminating these interactions is a promising strategy to improve the quality and durability of influenza vaccine responses.

CONTRIBUTIONS OF AUTHORS

The candidate has chosen to present a manuscript-based thesis. This thesis contains three original manuscripts and is in accordance with the guidelines for thesis preparation provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. The candidate, Hilary Hendin, is recognized as the principal author and to have performed the majority of the work of the manuscripts presented. The specific contributions of authors are as follows:

CHAPTER 2

Hendin HE, Pillet S, Lara AN, Wu C-Y, Charland N, Landry N, Ward BJ (2017). Plant-made virus-like particle vaccines bearing the hemagglutinin of either seasonal (H1) or avian (H5) influenza have distinct patterns of interaction with human immune cells in vitro. *Vaccine* 35(19): 2592-2599.

This manuscript is reprinted from the journal Vaccine with permission from Elsevier. HEH designed and performed all experiments and prepared the manuscript with guidance from BJW, NL, NC, and SP. ANL assisted with imaging experiments. C-YW assisted with study design. BJW was responsible for research supervision and strategy.

CHAPTER 3

Hendin HE, Lavoie P-O, Gravett JM, Saxena P, Pillet S, Landry N, D'Aoust M-A, Ward BJ. Elimination of receptor binding by influenza hemagglutinin improves vaccine-induced immunity. Manuscript under review at *npj Vaccines*.

HEH designed and performed all experiments and prepared the manuscript with guidance from BJW, SP, M-AD, and NL. P-OL generated VLP expression cassettes and provided biomass for purification by HEH. PS coordinated analytic testing of VLP reagents and studies involving H1-Idaho VLPs. JMG assisted with germinal center experiments. BJW and M-AD were responsible for research supervision and strategy.

CHAPTER 4

Hendin HE, Lavoie P-O, Gravett JM, Pillet S, Charland N, D'Aoust M-A, Ward BJ. Reduced sialic acid binding by influenza hemagglutinin improves the immunogenicity of vaccines targeting highly pathogenic strains of avian influenza. Manuscript prepared for submission to *Human Vaccines & Immunotherapeutics.*

HEH designed and performed all experiments and prepared the manuscript with guidance from BJW, SP, M-AD, and NC. P-OL generated VLP expression cassettes and provided biomass for purification by HEH. JMG assisted with animal experiments. BJW and M-AD were responsible for research supervision and strategy.

*The literature review and general discussion (chapters 1 and 5, respectively) were written by HEH and edited by BJW

CHAPTER 1

General Introduction

1.1 INFLUENZA

Influenza is an acute viral respiratory disease caused by influenza viruses. In humans, infections range from mild to severe and are characterized by sudden onset fever, dry cough, myalgia and malaise[1]. Despite considerable advances in the prevention and treatment of influenza, annual epidemics remain a major cause of morbidity and mortality worldwide and carry a heavy socioeconomic burden[2,3]. These ongoing challenges combined with the ever-present threat of a pandemic make influenza a major public health concern[4]. Vaccination programs aim to mitigate the impact of influenza outbreaks but their success is hindered by limited vaccine efficacy and the rapid evolution of influenza viruses[4,5]. Thus, new strategies are required to reduce the immense impact of influenza on society. The following sections outline key aspects of influenza biology and pathophysiology that are central to the development of better vaccines.

1.1.1 A brief history

Although influenza virus was only isolated in the 20th century[6], influenza pandemics and epidemics have been identified throughout history on the basis of disease progression and epidemiological patterns. The 'fever of Perinthus' described by Hippocrates in 412 BC in the book of *Epidemics* is often cited as the first documented influenza epidemic[7], however, considerable doubt surrounding the etiology of this outbreak remains[8]. More convincing reports emerged in 1173 when an epidemic of influenza-like illness (ILI) described as 'a certain evil and unheard-of cough' tore through Europe[9,10]. This epidemic, known simply as 'the plague' due to its rapid spread and high mortality rate, is considered the first documented influenza epidemic[11]. Epidemics of this nature continued throughout the 12th and 13th centuries but the name *Influenza* only emerged in 1357 when an epidemic in Italy was dubbed *influenza di freddo* or 'influence of the cold' referencing the seasonal nature of influenza outbreaks[12]. Global dissemination of influenza virus

is believed to have occurred during the pandemic of 1580 when influenza spread from Asia to Africa, Europe, and America[13,14].

Influenza epidemics and pandemics were documented at regular intervals throughout the ensuing centuries[11], however, the etiology of the disease was not known. Most scientists at the time believed that the disease was caused by *Bacillus influenzae* (now known as *Haemophilus influenzae*), which was isolated from the nose of an influenza patient in 1892[15]. This belief remained unchallenged until the emergence of the 'Spanish' influenza in 1918, when researchers attempted to develop antisera and vaccines to curb the deadliest influenza pandemic in history. Failed attempts to isolate *B. Influenzae* from infected patients and the finding that nasal secretions remained infectious following fine filtration casted doubt on the theory that the disease was caused by bacteria, and eventually led to the isolation of influenza A virus from human nasal secretions in 1932[6,15-17]. This discovery laid the groundwork for the field of influenza virology and the development of early influenza vaccines.

1.1.2 Etiology and epidemiology

Influenza virus is an enveloped, negative-sense, single-stranded RNA virus belonging to the *Orthomyxoviridae* family[18]. There are four antigenically distinct types of influenza viruses: A, B, C and D. Influenza A, B and C viruses are all capable of causing respiratory disease in humans, however, infection with influenza C viruses (ICV) tends to be mild and is not considered a major threat to human health[18]. Influenza D viruses (IDV) primarily infect cattle and other farm animals and are not thought to cause disease in humans despite some evidence of human infection and transmission[19-21]. Thus, influenza A and B viruses (IAV and IBV, respectively) pose the greatest threat to public health and are the primary focus of influenza prevention efforts.

1.1.2.1 Influenza A and B virus classification

Influenza viruses are classified on the basis of genetic and antigenic differences that emerge as a result of evolutionary change[22]. Similarities in the structure and genetic organization of IAV and IBV suggest that both types of influenza evolved from a common ancestor, however, they can be distinguished by differences in core proteins (nucleoprotein and matrix protein 1)[22]. IAV are further categorized into subtypes according to the combination of their two main surface

glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There are 18 known HAs (H1-H18) and 11 known NAs (N1-N11) that together are used to define the IAV subtype (e.g. H1N1)[18]. Subtypes are also clustered into two groups (1 and 2) based on the genetic relatedness of the HA protein[23]. In contrast, IBV express a single type of HA and NA and are not divided into subtypes[24]. However, antigenic divergence beginning in the 1970s resulted in two distinct IBV lineages characterized by relatedness to the reference strains B/Yamagata/16/88 (Yamagata lineage) and B/Victoria/2/87 (Victoria lineage)[25,26]. IAV and IBV strains are named using a standard convention defined by the World Health Organization (WHO) in 1980: The virus type followed by the host (if non-human), the geographic location of the isolation, the isolate number, the year of isolation, and the HA and NA subtypes (IAV only) (e.g. A/California/07/2009 (H1N1))[23].

1.1.2.2 Seasonal epidemics

Influenza epidemics occur annually throughout the world and are a major cause of global morbidity and mortality. The WHO estimates these epidemics are responsible for 3-5 million cases of severe disease and 290,000-650,000 deaths anually[2]. Epidemics are caused by both IAV and IBV and typically occur during the winter months in temperate regions and during the rainy season in tropical regions[27]. Among IAV, H1N1, H2N2 and H3N2 are known to cause epidemics in humans, although H2N2 is no longer circulating. H1N1 and H3N2 have been co-circulating since 1977 along with both lineages of IBV[28]. Thus, these strains are the target of current seasonal influenza vaccines.

Although a large proportion of the population is exposed to seasonal influenza strains through natural infection or vaccination, the propensity of the virus to mutate results in variants capable of evading pre-existing immunity and causing new epidemics. This phenomenon, also known as antigenic drift, is caused by the gradual accumulation of mutations affecting antibody binding sites of the HA and NA proteins[27]. Antigenic drift occurs among all seasonal influenza strains and is driven by the absence of proofreading mechanisms during RNA replication by the viral RNA polymerase[29]. The extent of the antigenic change between or within influenza 'seasons' often correlates with the severity of influenza epidemics[30]. Global influenza surveillance data aid in identifying new circulating strains and inform decisions surrounding vaccine composition[31],

however, seasonal influenza epidemics continue to have a significant impact on public health and society.

1.1.2.3 Pandemics

Global epidemics of influenza, also known as pandemics, arise when influenza viruses to which people have little pre-existing immunity are introduced to the human population[22]. These novel strains emerge when an influenza virus obtains the entire HA gene segment (and sometimes the NA segment) from a virus of zoonotic origin through a process known as antigenic shift[22]. IBV are not known to cause pandemics because there are no known reservoirs outside of humans. Although sporadic infections have been detected in harbour seals and pigs, isolates were antigenically similar to circulating human strains[32-34]. In contrast, IAV have a broad host range, which results in sporadic emergence of reassortant viruses with pandemic potential[35]. Aquatic birds are the natural reservoir for IAV and host adaptation throughout history has resulted in major reservoirs in poultry, pigs, and humans[36]. Direct transfer of influenza viruses between humans and birds is relatively rare due differences in host susceptibility. However, pigs are susceptible to both human and avian influenza viruses, allowing them to serve as 'mixing vessels' for novel reassortant viruses to emerge[37]. Reassortants that are capable of sustaining human-to-human transmission pose a significant pandemic threat[38]. To date, only three IAV HA subtypes (H1, H2 and H3) have circulated widely among humans and they can all be traced back to pandemics of zoonotic origin[36].

There have been four pandemics since the start of the 20th century: H1N1 in 1918 ('Spanish influenza'), H2N2 in 1957 ('Asian influenza'), H3N2 in 1968 ('Hong Kong influenza') and another H1N1 in 2009 ('swine influenza')[39]. The 1918 H1N1 pandemic is widely recognized as most severe pandemic in documented history. Over the span of nearly 3 years, H1N1 infected roughly one third of the world's population and killed an estimated 50 million people[40,41]. At the time, the 'tools' needed to isolate and identify viruses (e.g. cell culture) were not yet available, and little was known surrounding the etiology or zoonotic origins of the pandemic. However, serologic analysis from survivors in the 1930s revealed that the outbreak was likely caused by an H1N1-subtype IAV[42] and this was recently confirmed by sequencing of viral RNA found in infected lung tissues preserved in the Alaskan permafrost[43,44]. Genetic similarity to avian H1N1 suggests that

the pandemic strain originated from wild waterfowl, however, it remains unclear whether a brief period of adaptation in pigs also occurred since outbreaks among pigs were detected throughout the pandemic[45]. Following the pandemic, drifted variants of the 1918 H1N1 continued to cause seasonal epidemics in humans while independently circulating and evolving in pigs (classical swine H1N1). Persistence of H1N1 in the pig population eventually led to the re-emergence of a triple reassortant H1N1 that caused the 'swine flu' pandemic in 2009 (pdm09 H1N1)[36] and drifted variants of this strain and the 1968 H3N2 continue to cause epidemics in humans today.

The emergence of new influenza strains that can infect humans is inevitable. While it is difficult to predict when this will occur and which strain(s) will be responsible, increased surveillance of influenza infections in humans and animals plays a crucial role in pandemic preparedness[35]. Today, much attention is focused on highly pathogenic avian influenza (HPAI) viruses of the H5 and H7 subtypes, which sporadically infect humans with high rates of mortality[46,47]. Infections typically occur among people in close contact with infected birds and human-to-human transmission remains rare[35,47,48]. However, the accumulation of mutations that allow these HPAI viruses to spread rapidly among humans is a real and significant threat[49,50]. Thus, continued surveillance combined with the development of more effective vaccines and therapeutics is essential to mitigate the impact of future pandemics.

1.1.3 Influenza A structure and life cycle

Influenza A is a pleomorphic enveloped virus. Virions are primarily spherical or elliptical (80-120nm diameter) but can also take on a filamentous morphology of ≥20μm in length. Spherical virions are most easily transmitted between people; however, filamentous virions are often found in clinical isolates and are thought to be important for viral survival in nature[51,52]. The IAV viral envelope is composed of a lipid membrane taken from the originating host cell and is studded with hemagglutinin (HA), neuraminidase (NA), and a smaller number of matrix ion channels (M2) that cross the membrane. Matrix protein 1 (M1) forms a matrix directly below the lipid envelope and is thought to bind together the membrane, the surface glycoproteins, and IAV genetic material[53,54]. The core of the virion contains the nuclear export protein (NEP), also known as non-structural protein 1 (NS1), and the ribonucleoprotein (RNP) complex composed of eight single-stranded, negative-sense RNA (-ssRNA) segments coated with nucleoprotein (NP). Each segment is also

bound by the RNA-dependent RNA polymerase (RdRp), which is comprised of two polymerase basic subunits (PB1 and PB2) and a polymerase acidic subunit (PA). Segments are numbered by decreasing size. The influenza viral structure and the protein(s) encoded by each gene segment are outlined in **figure 1.1a**.

An overview of the influenza virus replication cycle is depicted in **figure 1.1b**[55]. Influenza infection is initiated by the surface glycoprotein HA, which is expressed as trimers on the surface of all influenza viruses in its precursor form (HA0). In order to become infectious, HA0 must be cleaved into two subunits, HA1 and HA2, by host serine proteases[56]. These two subunits then act in concert to facilitate viral entry; HA1 binds to sialic acid (SA) on the surface of respiratory epithelial cells to trigger endocytosis and the HA2 fusion peptide is exposed upon acidification of the endosome to facilitate fusion of the viral and endosomal membranes. Acidification of the endosome also causes the M2 ion channel to open, which promotes acidification of the viral core and causes the M1 protein to release the RNP complex into the host cell cytoplasm[53]. Once inside the cytoplasm the RNP complex enters the nucleus via nuclear localisation signals to initiate viral replication[57]. Viral replication depends on expression of viral proteins and replication of the viral genome for progeny virions. To facilitate protein expression, the PB2 subunit of the RdRp binds to and cleaves the 5' capped end of host messenger RNA (mRNA) to be used as a primer for viral mRNA synthesis[58]. Viral mRNAs then exit the nucleus to be translated using host machinery. Surface proteins (HA, NA, and M2) are translated by membrane-bound ribosomes of the endoplasmic reticulum (ER) and are directly inserted into the ER membrane and translocated to the cell surface via the Golgi network. In contrast, core proteins are translated in the cytoplasm and then re-enter the nucleus to mediate genome replication. The newly synthesized RdRp transcribes +ssRNA copies of each RNA segment to serve as a template for new copies of the -ssRNA genome[57]. Viral RNPs then exit the nucleus in a complex with the M1 and NS2 proteins and localize near the cytoplasmic tails of the viral surface proteins via M1-glycoprotein interactions[59]. Once assembled, virions bud from lipid rafts in the host cell plasma membrane and NA catalyses the cleavage of SA residues to prevent HA from remaining tethered to the host cell[60]. Progeny virions may then infect other cells to further propagate infection.

1.1.3.1 HA binding properties impact host range

Influenza A viruses have a broad host range, however, only viruses of the H1, H2, and H3 subtypes are known to circulate in humans[36]. The ability of IAV to replicate and transmit efficiently among humans has been linked to specific features of several different viral proteins and understanding the determinants of host range is crucial for assessing the risk of emerging strains[36,61]. One of the most well-defined host species barriers is the ability of the virus to attach to the host cell via the HA protein[36,62]. All influenza viruses initiate infection by binding to sialylated N-linked glycans on the surface of host cells[63]. However, HA binding properties are strain-specific and there is considerable diversity among SA receptor types and their distribution in animal tissues[63-65]. Thus, HA binding properties are major determinants of host-range, transmissibility, and severity of infection[66,67] (**figure 1.2**).

Sialic acids are composed of a nine-carbon backbone linked to the terminal position of N- and O-linked glycans[68]. In the context of influenza, SA are primarily classified based on their linkage to the underlying galactose residue[68]. However, features such as the length of the underlying sugar chain have also been shown to be important determinants of HA binding[69-71]. In general, avian influenza viruses preferentially bind to α2,3-linked SA with short underlying sugar chains and mammalian strains preferentially bind to α2,6-linked SA with long underlying sugar chains (pentasaccharide or longer)[62,69]. 'Long' α2,6-linked SA are highly expressed in the nasal passages and upper airways[36,72]. As a result, human-adapted influenza strains typically cause mild upper respiratory tract (URT) infections with efficient human-to-human transmission. In contrast, avian-type α2,3-linked SA are primarily found in the lower respiratory tract (LRT) of humans, where they are expressed on the surface of alveolar epithelial cells and the junction between the bronchioles and alveoli[72]. Positioning of these receptors in the LRT acts as a significant barrier to human-to-human transmission[36]. However, infection of humans with avian influenza strains sporadically occurs (typically following close contact with birds) and results in high levels of mortality due to LRT involvement[46,47]. Thus, adaptive mutations that allow avian influenza viruses to bind to α2,6-linked SA in the URT and α2,3-linked SA in the LRT are particularly concerning. Interestingly, HPAI H5N1 strains capable of binding α2,3- and α2,6-linked SA have been reported, but increased transmissibility between humans was limited by the fact that virus still could not bind to 'long' α2,6-linked SA in the URT[73]. This finding highlights the importance of

looking beyond SA linkage when examining HA binding properties. Current efforts have shifted towards identifying mutations that allow binding to 'long' α2,6-linked SA to allow for better surveillance of emerging pandemic threats[49,50].

1.1.4 Pathogenesis in humans

Influenza viruses typically enter the human respiratory tract through inhalation of infectious aerosols or by self-inoculation following contact with the respiratory secretions of an infected individual[74]. Viruses then bind to SA on the surface of respiratory epithelial cells to initiate infection. Pathogenesis following infection is divided into two phases: early viral replication (days 1-3) and the inflammatory response to infection[75,76]. The extent of tissue damage at the site of viral replication and the magnitude of the inflammatory response both contribute to disease outcomes resulting in a broad spectrum of disease[75,77].

In uncomplicated influenza infection, viral replication is mostly limited to epithelial cells of the URT and results in a potent anti-viral immune response that can control infection[74,78]. Antiviral immunity is predominantly mediated by type I interferons (IFN), which facilitate expression of interferon-stimulated genes (ISG) that sense viral RNAs and induce apoptosis of infected cells[79]. This process can cause significant inflammation and damage to the respiratory epithelium, leading to the local and systemic symptoms that are typically associated with mild influenza infection (cough, fever, headache, myalgia, etc.)[78]. However, a tightly regulated innate response is highly effective at clearing URT infection and promotes the induction of adaptive immunity. In contrast, LRT infection and/or dysregulation of the innate immune response can lead to severe disease manifestations including viral pneumonia, secondary bacterial pneumonia, acute respiratory distress syndrome (ARDS), and death[78]. These severe outcomes occur due to a combination of viral and host factors[75].

Infection of the LRT is a common cause of increased morbidity and mortality following influenza infection. Infection of the alveolar epithelium disrupts the alveolar barrier leading to extensive pulmonary edema[80]. Furthermore, epithelial death results in exposure of the underlying endothelial layer to viral antigens, which leads to the recruitment of inflammatory cells and substantial immunopathology[81,82]. Taken together, these events greatly compromise gas exchange and can lead to the development of ARDS with a high case fatality rate (up to 60%)[81]. LRT

involvement is characteristic of infections with avian influenza viruses such as H5N1 and H7N9, since α2,3-linked SA are predominantly expressed on alveolar epithelial cells[72]. However, expanded tropism of human influenza viruses to infect alveolar epithelial cells can also occur and was a common feature of the last four pandemic influenza viruses (1918 H1N1, 1957 H2N2, 1968 H3N2 and 2009 H1N1)[83].

The immune response to influenza infection plays a large role in its pathogenesis in humans[84]. Thus, host factors that impact immune responses may also contribute to disease severity[75]. Increased severity is particularly pronounced among individuals over the age of 65, who account for 90% of influenza-related deaths[85]. Increased severity of infections in this population is thought to be largely attributable to immunosenescence, which is associated with insufficient type I IFN induction without reducing pro-inflammatory responses[86]. Thus, the immune system is often slow to effectively control viral replication, leading to excessive lung inflammation and impaired tissue repair[87]. Sex differences in influenza pathogenesis are also well-documented, including increased susceptibility of pre-pubescent and elderly males to severe disease following IAV infection and a higher incidence of severe disease in reproductive age females following H5N1 and H7N9 infection[75]. The exact mechanisms for these differences are not well understood. However, several pre-clinical studies have demonstrated an important role for sex hormones in modulating influenza-mediated immunopathology without necessarily impacting viral titers following infection[88,89]. Other factors such as pregnancy, obesity, genetics, and the presence of pre-existing immunity also have a marked impact on influenza pathogenesis and disease outcomes[75]. Thus, it is important to develop vaccines capable of eliciting protective immune responses, particularly in those who are more susceptible to severe disease.

1.2 PROTECTIVE IMMUNITY

In the absence of pre-existing immunity, innate immune mechanisms play a critical role in controlling early infection and mediating disease outcomes. However, influenza-specific antibody-mediated and T cell-mediated immunity are required to prevent reinfection. These adaptive immune responses can be elicited by natural infection or vaccination. The quality, breadth, and durability of protection can vary substantially based on the influenza strain, the exposure history of the individual, and the mechanism of exposure (infection vs. vaccination). The following

sections outline features of the adaptive immune response to influenza and how they contribute to protection.

1.2.1 Humoral Immunity

Antibodies have long been known to provide protection from influenza. The first report of antibody-mediated protection from influenza was in 1933, when Smith *et al.* discovered that serum from influenza-infected ferrets could neutralize the virus[6]. We now know that antibody-mediated immunity is crucial for controlling the spread of emerging influenza strains and mitigating pandemic threats[90].

In the absence of pre-existing immunity, antibody responses are initiated in the secondary lymphoid organs (SLO). Naïve B cells become activated upon recognition of cognate antigen and migrate to the border of the B cell follicle, where interactions with helper T cells drive their proliferation[91]. A portion of these cells differentiate into short-lived plasma cells (PC) that secrete low-affinity antibodies (primarily IgM), which are important for resolving the infection[90,92]. Simultaneously, antigen-specific B cells enter the germinal center (GC), where they undergo further proliferation, clonal selection, and avidity maturation[93]. The GC reaction gives rise to long-lived memory B cells (MBC) and affinity-matured PCs that secrete high affinity antibodies[93]. Some of these PCs migrate to the bone marrow (BM), where they can persist for decades and mediate long-term maintenance of serum antibody titers[94,95]. At sufficient levels, antibodies in the serum can reduce the impact of subsequent infections and can provide sterilizing immunity if they block viral entry into the host cell[93]. Furthermore, MBC maintained in the periphery can rapidly differentiate into PC and secrete high levels of antibodies upon re-infection. The level of protection provided by these antibodies is dependent on a number of factors including the types of antibodies produced (i.e. isotype and subtype), epitope specificity, and the quality of the GC reaction[90].

Infection with influenza viruses results in a diverse antibody landscape[90]. Responses tend to be biased towards the HA and NA proteins because their expression on the surface of the virus and host cells promotes efficient recognition by B cells[90]. Internal viral proteins are less accessible, resulting in reduced production of antibodies specific for these proteins[90]. The main antibody isotypes produced following infection are IgM, IgA and IgG, with IgG constituting ~75% of total influenza-specific antibodies[96]. These antibodies can mediate protection through direct antiviral

activity (neutralizing) or indirect effector functions that promote viral clearance by the innate immune system (mostly by IgG1 and IgG3)[90,97] (**figure 1.3**).

1.2.1.1 Neutralizing antibodies

The majority of neutralizing antibodies elicited by influenza infection are directed against the HA protein[98], which is comprised of a highly variable globular head domain and a more conserved stem (or stalk) domain[99,100]. Most HA-specific antibodies recognize conformational epitopes on the globular head and can prevent infection by blocking the interaction between HA and its SA receptor on target cells[101]. These antibodies can be detected and quantified using the hemagglutination inhibition (HI) assay, which measures antibodies that inhibit the hemagglutination of red blood cells (RBC) by influenza viruses[102]. The HI titer is the most well-recognized correlate of protection for influenza[103] and a titer ≥1:40 is thought to confer 50% protection against seasonal influenza infection[104]. However, these antibodies also tend to be highly strain-specific due to the constant antigenic drift of the globular head antigenic sites[105,106].

Antibodies directed against epitopes on the HA stem are elicited at much lower levels than those targeting the globular head but they are usually more broadly reactive because of the conserved nature of the stem domain[107-109]. Stem-specific antibodies can neutralize influenza viruses through a variety of mechanisms such as preventing HA fusion with the endosomal membrane[110], preventing proteolytic cleavage of the full-length HA0 into its fusion-competent configuration (HA1, HA2)[110,111], or inhibiting viral egress[112-114]. Furthermore, antibodies directed against the HA stem were recently found to independently correlate with protection from influenza infection in humans, but the relative contributions of neutralizing and non-neutralizing antibodies were not evaluated[115].

Influenza infection also results in the production of NA-specific antibodies, albeit at lower levels than the HA-specific antibodies[90]. These antibodies can interfere with infection by binding to the active site of NA or by sterically hindering the cleavage of SA to prevent viral egress[116]. Since NA-specific neutralizing antibodies do not prevent viral entry or replication, they do not provide sterilizing immunity. However, antibodies that inhibit the enzymatic activity of the NA protein have been shown to ameliorate the clinical symptoms of influenza infection in mice and to be correlated with protection from pdm09 H1N1 infection in humans[116-119]. Furthermore, NA-

specific antibodies elicited by influenza infection are highly cross-reactive and can be an important source of protective immunity against divergent influenza strains[117].

Antibodies targeting the ectodomain of the M2 protein (M2e) are also produced following infection with influenza viruses and have been shown to reduce viral titers in mouse and human challenge models[92,120]. However, these antibodies are non-neutralizing no M2-specific correlate of protection has been identified[92,120]. Similarly, antibodies targeting the internal viral proteins have been detected following infection, but they are also non-neutralizing and their protective role is unknown[121-123].

1.2.1.2 Non-neutralizing antibodies

In addition to direct antiviral activity, antibodies elicited by influenza can also have indirect antiviral effects through the activity of innate immune cells that recognize the constant region of the antibody (fragment crystallizable, Fc). These effector functions are dependent on both antibody recognition of the antigen and engagement of the Fc region, either by Fc receptors (FcR) on innate immune cells or the complement recognition molecule C1q[124,125]. Fc-mediated antiviral effects include antibody-dependent cellular cytotoxicity (ADCC) by natural killer cells, antibody-dependent cellular phagocytosis (ADCP) by neutrophils and macrophages, and complement-dependent cytotoxicity (CDC). These effector functions have not yet been shown to correlate with protection in humans, but several studies have demonstrated that they are necessary for protection in mice[126-129]. Furthermore, it is well known that antibodies with Fc-mediated antiviral activity are generated following influenza infection in humans. These antibodies were recently shown to mediate protection in mice upon passive transfer[130] and to be inversely correlated with disease severity in a human challenge model[131]. Thus, Fc-mediated effector functions likely play a bigger role in protection than previously recognized.

The Fc-mediated effector functions of antibodies directed against the HA protein are well-described. Antibodies targeting the HA stem domain have been shown to mediate ADCC, ADCP and CDC[132,133]. Although some stem-directed antibodies have direct neutralizing capacity, these Fc-mediated effector functions are thought to be the main mechanism of protection for anti-stem antibodies[115,132]. In fact, it was recently demonstrated the FcR engagement is required for protection by neutralizing antibodies targeting conserved epitopes on the HA stem domain[127]. This

was also demonstrated for broadly reactive neutralizing and non-neutralizing antibodies directed against the NA protein[127]. Furthermore, antibodies directed against the M2e protein are thought to contribute to viral clearance through Fc-mediated effector functions facilitated by high expression of M2e on the surface of infected cells prior to viral budding. These antibodies are non-neutralizing but have been shown to enhance viral clearance in mice and humans[120,134,135]. Taken together, it is clear that antibody engagement of FcRs is an important aspect of the immune response to influenza. However, the precise mechanism(s) that contribute to protection have not yet been determined[136,137].

1.2.2 Cell-mediated immunity

The second branch of the adaptive immune response to influenza infection is T cell-mediated immunity. Unlike antibodies, antigen-specific T cells cannot prevent infection[138]. However, they play important roles in viral clearance, limiting disease severity, and supporting the development of strong humoral immune responses[139-141].

During primary infection, naïve $CD4^+$ and $CD8^+$ T cells are primed in the mediastinal lymph nodes (LN), leading to their activation and expansion[142]. Some antigen-specific $CD4^+$ T cells remain in the LN to support B cell affinity maturation at the T-B border and within the GC[143]. At the same time, effector $CD4^+$ and $CD8^+$ T cells migrate to the lungs to promote viral clearance through direct cytotoxicity (perforin and granzyme B) and production of pro-inflammatory cytokines (mainly IFNγ, TNFα, and IL-2)[143-145]. These effector T cell populations are short-lived and significantly contract following resolution of the infection[146]. However, a small pool of influenza-specific T cells transition into long-lived memory cells that can limit the severity of subsequent infections[144].

Influenza-specific T cells typically target highly conserved epitopes on internal influenza proteins (e.g. M1, NP, PB1)[147-151]. As a result, cell-mediated immune responses tend to be considerably more cross-reactive than antibody responses and have been shown to reduce the severity of infection even in the absence of pre-existing humoral immunity[140,141,152-155]. The role of $CD8^+$ T cells in mediating protection was first demonstrated in a human challenge study in 1983[152]. In a more recent cohort study, pre-existing cross-reactive $IFN\gamma^+IL2^-$ $CD8^+$ T cells that recognized epitopes on internal influenza proteins were significantly correlated with decreased

symptom scores following natural infection with pdm09 H1N1[140]. In addition, production of granzyme B by antigen-specific $CD8^+$ T cells has been shown to correlate with protection in elderly individuals[156,157]. Antigen-specific $CD4^+$ T cells are also implicated in protection and IFNγ-secreting $CD4^+$ T cells were significantly correlated with protection in a recent human challenge study[139].

Taken together, T cell-mediated immunity likely plays an important role in protection from influenza, especially in the absence of pre-existing humoral immunity. Cellular IFNγ production has been reported to correlate with protection in a number of studies, including a large-scale trial evaluating the efficacy of a live attenuated influenza vaccine (LAIV) in over 2000 children[158]. However, thresholds for protective immunity varied between studies[159] and no correlate of protection for $CD4^+$ or $CD8^+$ T cell-mediated immunity has been established[160,161].

1.2.3 Immune responses to natural infection and vaccination

Influenza vaccines aim to stimulate protective immune responses without causing disease. However, patterns of immunity following vaccination differ substantially from those elicited by natural infection. One of the biggest differences is that vaccines tend to elicit quite narrow antibody responses that are predominantly directed against the highly variable globular head domain of the HA protein[98,117]. Infection-induced humoral immune responses are considerably more diverse and include antibodies directed against the more conserved stem domain of the HA protein and other viral proteins[98,107]. As a result, antibody responses elicited by natural infection tend to be significantly more broadly-reactive than vaccine-induced responses. Cell-mediated immunity is also important for mediating cross-protection[140,141]. However, current influenza vaccines (except live attenuated formulations) are poor inducers of T cell-mediated immunity[162-164] and provide limited protection from mismatched or drifted influenza strains[165].

Another important difference between natural infection and vaccination is the durability of immune responses and protection. Antibody responses following natural influenza infection can be very long-lived and have the potential to provide life-long protection[166-168]. To highlight this, Yu *et al.* demonstrated that individuals exposed to H1N1 during the 1918 pandemic still had detectable serum neutralization titers and seroprotective HI titers (mean HI titer 1:396) nearly 90 years later in 2008[167]. Furthermore, many individuals born before 1930 had cross-neutralizing

antibodies to pdm09 H1N1 and it is thought that this pre-existing immunity provided some protection to elderly individuals during the 2009 pandemic[168]. In contrast, antibody titers and protection decline rapidly following vaccination, sometimes within the same influenza season[5,169-172]. The mechanisms underlying this poor durability are not well understood. However, a recent study conducted by Davis *et al*. suggests that a failure to elicit long-lived plasma cell (LLPC) populations in the BM could contribute to rapidly waning immunity following vaccination[173]. One possible explanation for the poor LLPC induction is that vaccines elicit relatively short-lived GC reactions compared to natural infection[174], and LLPCs were recently shown to arise from 'late' GCs (≥18 days)[175]. Thus, new strategies that prolong GC reactions may be required to improve the durability of vaccine-induced immunity.

1.3 INFLUENZA VACCINES

The isolation of influenza virus in 1932 marked the beginning of a global effort to develop a vaccine capable of protecting against future epidemics and pandemics. These efforts yielded several promising vaccine candidates including live-attenuated and inactivated viruses that were capable of neutralizing infection in mice[176,177]. The first candidate that was approved for use in humans was a formalin-inactivated whole virion vaccine produced using egg-propagated virus[176,178]. This vaccine was licensed in 1945, marking a major milestone in the effort to prevent and control influenza outbreaks. To date, the majority of influenza vaccines are produced using similar techniques and vaccination remains the most effective means of preventing infection[179]. However, the efficacy of influenza vaccines continues to be hindered by constant antigenic drift that can result in mismatch between the vaccine antigens and circulating strains[180,181], variable immunogenicity of different HA subtypes[182,183], and unreliable production platforms[165,184]. The following sections describe the influenza vaccines that are currently licensed and the challenges that remain.

1.3.1 Vaccine composition

Seasonal influenza vaccines are formulated to provide protection from influenza strains that are predicted to circulate in the following influenza season. Vaccine strains are chosen based

on recommendations from the Global Influenza Surveillance and Response System (GISRS) coordinated by the WHO[185]. The GISRS monitors the spread and evolution of influenza strains in 114 countries[186] and provides recommendations for vaccine composition twice annually: in February for the Northern Hemisphere and in September for the Southern Hemisphere[187]. Recommendations are made for each of the four circulating influenza lineages (A/H3N2, A/H1N1, B/Yamagata and B/Victoria)[187]. Some seasonal influenza vaccines include antigens from all four of the predicted strains (quadrivalent) while others include one IBV lineage strain with the two IAV strains: H1N1 and H3N2 (trivalent)[187]. In contrast, pandemic influenza vaccines are monovalent formulations composed of antigens from the actively circulating pandemic strain. Emergence of pandemic influenza strains cannot be predicted[188]; however, identification of high-risk strains can be accomplished by monitoring the evolution of zoonotic influenza viruses[186]. In the United States, the Influenza Risk Assessment Tool (IRAT) was developed to assess the risk of each novel influenza virus and to inform the development of pre-pandemic vaccines[188].

Influenza vaccines are currently available in split-virion, subunit, live attenuated and recombinant formulations[189,190], which will be discussed in greater detail in the following sections. The amount of antigen contained in the vaccines varies based on the type of vaccine and the target population[190]. In addition, pandemic vaccines often include an adjuvant since immunogenicity tends to be low among immunologically naïve individuals[191,192]. Adjuvants are also included in some seasonal influenza vaccines to improve their immunogenicity in elderly individuals and young children[190].

1.3.2 Egg-based vaccines

The discovery that influenza viruses can be propagated in embryonated chicken eggs in the late 1930s was pivotal to the development of early influenza vaccines and continues to be the primary method of vaccine production today[177,178,193]. A small amount of the target influenza strain is injected into the allantoic fluid of the egg where the virus replicates for 2-3 days, and then is harvested and purified for vaccine production. Downstream processing techniques have evolved to optimize yield and immunogenicity, however, the viral propagation steps remain relatively unchanged from the protocol described by Stanley in 1945[193]. As of 2018, egg-based influenza vaccines accounted for 88% of the global influenza vaccine market[194] and were the only vaccines

licensed in Canada until 2019[195]. Today, egg-based influenza vaccines are commercially available in three different formulations: split-virus, subunit, and live attenuated influenza vaccines (LAIV)[196].

1.3.2.1 Split-virus and subunit vaccines

Although first-generation whole-inactivated influenza vaccines were highly immunogenic, they also resulted in substantial systemic reactogenicity, particularly in young children[197]. Thus, whole virus vaccines were largely discontinued in the 1970s in favor of more tolerable 'split' virus formulations[198,199]. Split-virus vaccines are produced by disrupting the lipid envelope of inactivated viral particles using detergents or diethyl ether. These vaccines may also be further purified to generate so-called subunit vaccines enriched in HA and NA[200]. In influenza-experienced individuals, split-virus and subunit influenza vaccines elicit similar immune responses to whole-inactivated vaccines[185]. However, two doses are required to confer seroprotection (HI titer ≥1:40) in influenza-naïve individuals such as infants and young children[185] and increased antigen dose or adjuvants are often used to improve immunogenicity in elderly individuals[190].

In Canada, the majority of commercially available vaccines are egg-based split-virus or subunit vaccines[190]. Trivalent and quadrivalent formulations are available and contain standardized quantities of each strain based on the HA content. Standard-dose inactivated vaccines contain 15µg of HA per strain. To improve vaccine responses in the elderly, a high-dose formulation containing 60µg of HA per strain is available for individuals ≥65 years old and results in improved seroconversion and protection compared to the standard-dose[201,202]. Adjuvanted formulations containing 7.5µg or 15µg of HA per strain with the oil-in-water, squalene-based emulsion MF59 are also available for young children (6-23 months) and the elderly (≥65 years), respectively[190]. The MF59 adjuvant is thought to improve immunogenicity by inducing a controlled degree of immune cell death, leading to more effective dendritic cell maturation and antigen presentation in the draining LN[203,204], and has been shown to improve the antibody responses elicited by influenza subunit vaccines in young children[205-207]. However, similar benefits have not been consistently demonstrated in the elderly[208]. Thus, use of the high-dose vaccine is preferred in adults ≥65 years old[190].

1.3.2.2 Live attenuated influenza vaccines (LAIV)

Live attenuated vaccines aim to stimulate an immune response similar to natural infection without causing disease. In general, live attenuated vaccines tend to be highly effective and are credited with eradicating or controlling diseases such as smallpox, polio and measles[209]. Live attenuated influenza vaccines (LAIV) were among the earliest experimental vaccines and showed clear immunological advantages over inactivated vaccines[210,211]. Traditionally, viruses were attenuated by serial passaging in mice and ferrets prior to propagation in embryonated hen eggs. However, the unpredictable nature of this attenuation technique and the frequent emergence of new influenza strains limited the use of these early LAIVs in favor of inactivated vaccines[176,178]. Nonetheless, efforts to develop an easily modifiable LAIV continued and the first successful vaccine candidate, FluMist®, obtained regulatory approval in Canada in 2010. Modern LAIVs such as FluMist utilize master donor viruses (A/Ann Arbor/6/60 or B/Ann Arbor/1/66) with mutations in several internal gene segments that render the virus temperature sensitive and cold-adapted[209,212]. As a result, the donor virus is not able to replicate at the temperature of the lower respiratory tract (37ºC) but replicates efficiently in the cooler temperatures of the nasopharynx (25ºC)[213]. The master donor virus is then reverse engineered to express the HA and NA proteins of the target influenza strain while retaining the internal proteins that confer attenuation[214]. Importantly, these reassortant LAIV strains are phenotypically stable, as the presence of attenuating mutations in several different gene segments prevents reversion to the wild-type phenotype[215,216].

Early clinical trials revealed that the cold-adapted LAIV was highly effective at preventing infection in children and adults[217-219]. However, these vaccines elicited poor HI titers in the serum, which are important for vaccine licensure in many juristictions[220]. Nonetheless, the cold-adapted LAIV was licensed on the basis of protective efficacy in a series of field trials[217,221]. Post-licensure trials also revealed that compared to inactivated vaccines, the LAIV was more effective in children[222,223] and equally or slightly less effective in adults[224,225]. However, LAIV is not recommended for older adults (≥60 years old) in Canada due to a reduction in the measurable immune responses[226,227] and limited evidence of protection[228]. To date, correlates of protection for the LAIV have not been established[229]. However, potential roles for cellular and/or mucosal immunity in mediating protection have been suggested and studies to identify biomarkers that correlate with protection are ongoing[229,230].

1.3.2.3 Ongoing challenges with egg-based vaccine production

Although the majority of influenza vaccines are manufactured in eggs, there are several limitations associated with continued reliance on this platform. One of the biggest issues is that expression of human-type SA receptors ('long' α2,6-linked SA) is limited in eggs, hindering the replication of many human influenza strains[189,231]. Viruses can adapt to growth in eggs by increasing their capacity to bind to avian-type receptors ('short' α2,3-linked SA)[232], however, adaptive-mutations in the receptor binding domain (RBD) can greatly hinder vaccine efficacy. This phenomenon has been demonstrated repeatedly, especially among H3N2 vaccine strains[165,233-238]. Furthermore, the inefficiency of egg-adaptation (i.e. low yield per egg) can sometimes lead to production delays[239] and reduced efficacy when poor growth causes a circulating strain to be excluded from the vaccine[165].

Additional concerns stem from the fact that HPAI viruses can be highly lethal to both hens and their eggs. Thus, vaccine production for HPAI viruses may be hindered by the inability to grow the wild-type virus in eggs or insufficient egg supply[185]. To mitigate this risk, considerable efforts have been made to develop attenuated candidate vaccine viruses (CVV) of avian origin that can be propagated in eggs[240]. These CVVs are developed for viruses known to pose a pandemic threat[241], however, the emergence of an unanticipated virus could lead to significant vaccine production delays. Furthermore, these CVVs would be of limited utility in the event of an outbreak among poultry that limits egg supply.

Taken together, it is evident that the continued reliance on egg-based vaccine manufacturing can lead to considerable delays in vaccine production timelines and a concerning impact on vaccine efficacy. Thus, new strategies for rapid and reliable vaccine production are urgently needed.

1.3.3 Cell-culture based vaccines

Due to the ongoing challenges associated with egg adaptation, there is increasing interest in growing influenza vaccine viruses using mammalian cell culture[184]. The first such vaccine, Flucelvax® (Optaflu® in Europe), has been available in the United States and Europe for several years and was recently authorized for use in Canada[196]. Flucelvax is the first egg-independent influenza vaccine to be approved in Canada and it is authorized for use in adults and children over

the age of two[196]. Cell-based vaccine production is similar in overall concept to egg-based subunit vaccines; however, the viruses are propagated in Madin-Darby Canine Kidney (MDCK) cells. The main advantage of the cell-based production platform is that compared to eggs, less viral adaptation is required for efficient growth[242,243]. As a result, production timelines and the risk of adaptative mutations that can impact vaccine efficacy are reduced[242,244]. Indeed, several recent studies have demonstrated that cell-based inactivated influenza vaccines are significantly more effective at reducing influenza-like illness and influenza-related medical encounters compared to egg-based vaccines[245-247]. However, the transition from egg-based vaccine production is limited by the high cost of cell-based vaccine manufacturing, which is estimated to be around 40% higher than egg-based methods[194].

1.3.4 Recombinant HA vaccines

In recent years, recombinant technologies have emerged that eliminate the need for viral propagation in vaccine production. These platforms allow for expression of wild-type gene sequences, which circumvents adaptive mutations that could impact immunogenicity and efficacy. Furthermore, these platforms do not depend on seed viruses and can typically produce vaccines more rapidly than egg- and cell culture-based technologies. To date, there is only one licensed recombinant influenza vaccine (FluBlok®) and it is not yet authorized for use in Canada[190]. However, availability of recombinant influenza vaccines is likely to increase in the coming years as several recombinant vaccines have yielded promising results in late-stage clinical trials[191].

1.3.4.1 Baculovirus-based vaccines

The first recombinant vaccine for influenza (FluBlok®) was licensed for use in the United States in 2013 and is composed of recombinant HA trimers expressed using the baculovirus expression vector system (BEVS). Baculoviruses are DNA viruses that infect insects and are non-pathogenic in humans[248]. They express abundant quantities of polyhedrin to provide protection in the environment, but this protein is not required for replication[249]. The BEVS technology exploits this feature of baculovirus biology by placing the gene of interest (in this case, HA) under the control of the polyhedrin promoter, resulting in high levels of expression in insect cell culture[250]. The BEVS-derived FluBlok vaccine is a quadrivalent formulation containing 45µg of HA per

strain that oligomerize to form rosettes[251]. It can be produced in as little as 45 days and utilizes wild-type HA sequences, eliminating the possibility for adaptive mutations[252].

Clinical trials and post-licensure studies have demonstrated that the BEVS-derived FluBlok® vaccine is well-tolerated, immunogenic, and can improve protection in adults ≥50 years old[253-255]. Furthermore, this vaccine was recently shown to elicit stronger $CD4^+$ T cell responses than egg-based and cell culture-based comparators[256]. However, immunogenicity in young children (≤35 months) is inferior to inactivated vaccines for reasons that are not yet understood[257]. Currently, FluBlok Quadrivalent is licensed for all adults (≥18 years) in the United States[190].

1.3.4.2 Plant-based virus like particle vaccines

Another emerging technology in the production of influenza vaccines is the expression of recombinant proteins and virus-like particles (VLP) in plants. To date, no such vaccine has been approved for use in humans[258]. However, plant-based VLP vaccines for influenza developed by the Canadian biopharmaceutical company Medicago Inc. have yielded promising results in late-stage clinical trials[259]. These VLPs are produced by transient transfection of *Nicotiana benthamiana* plants with *Agrobacterium tumefaciens* engineered to carry the gene encoding the HA protein of interest. Once expressed, the HA molecules gather on the surface of the plant cells and spontaneously bud from the plasma membrane to form HA-VLPs. The VLPs are purified from the plant leaf tissue after just 6-10 days[260]. An overview of this process is depicted in **figure 1.4**. The efficiency and scalability of plant-based vaccine production are highly attractive. Furthermore, several clinical trials have demonstrated that the HA-VLPs are safe, well-tolerated and immunogenic in humans[259,261-264]. Despite failing to meet the primary endpoint of 70% efficacy in the phase III trial, the plant-based VLP vaccine provided substantial protection and was non-inferior to an egg-based inactivated influenza vaccine in elderly individuals[259]. Thus, plant-based VLP vaccines for influenza represent a promising alternative to current influenza vaccines.

1.3.5 The need for better influenza vaccines

Cell-based and recombinant platforms for influenza vaccine production have shown great promise with respect to reducing or eliminating adaptive mutations and improving the efficiency and scalability of vaccine production. However, several challenges remain across all current

vaccine platforms. First, the protection offered by current influenza vaccines is quite narrow, particularly in children, and antigenic drift during the influenza season can lead to poor vaccine efficacy[90,170]. This narrow protection combined with the rapidly mutating nature of influenza HA is the reason that new vaccines must be formulated each year. Second, vaccine immunogenicity and efficacy vary greatly from season-to-season and from strain-to-strain[183,265]. In particular, vaccines for HPAI viruses tend to be poorly immunogenic in humans[262,266]. Finally, the antibodies generated by vaccination tend to be relatively low-avidity and vaccine efficacy tends to be short-lived, even in the absence of viral mutations[170,171,267]. Taken together, these challenges highlight the need for novel approaches that can lead to broadly protective and long-lasting immunity following vaccination.

1.4 NEXT-GENERATION INFLUENZA VACCINES

The poor efficacy of current influenza vaccines is well-recognized and raises significant concerns surrounding pandemic preparedness. Recognizing the need for better vaccines, there has been a tremendous global effort to develop new strategies to improve both the breadth and durability of vaccine-mediated protection. Much of this work has been supported by funding initiatives from public and non-governmental organizations such as the WHO, the Bill and Melinda Gates Foundation (BMGF), and the US National Institute for Allergy and Infectious Diseases (NIAID) that aim to accelerate the development of better influenza vaccines[268]. The ultimate goal is to develop a so-called 'universal' influenza vaccine (UIV) capable of providing long-term immunity against all influenza viruses. However, progress towards a 'true' UIV will likely occur in incremental steps and new strategies that can improve breadth and durability of protection provided by current influenza vaccines are also highly desirable (**figure 1.5**). The following sections describe important advances that have been made towards the development of rationally-designed, next-generation influenza vaccines.

1.4.1 Towards a universal influenza vaccine

In the last decade, several strategies for developing more broadly-protective influenza vaccines have emerged. To focus these research efforts, the NIAID recently released a strategic

plan that defines four target criteria for a successful UIV: (1) 75% efficacy, (2) protection against all influenza A viruses, (3) protection lasting for ≥1 year and (4) suitability for all age groups[269]. Similar targets have also been suggested by the WHO and the BMGF[270,271].

Development of a successful UIV is contingent on eliciting an immune response directed against antigens or epitopes that are highly conserved among influenza viruses. Several strategies for targeting these conserved regions are being investigated and have led to a number of promising UIV candidates at various stages of pre-clinical and clinical development. Common approaches include the exploration of new production platforms that can promote stronger cross-reactive T cell responses[272,273], targeting antigens that are highly conserved among influenza viruses (e.g. M2 and NP)[274,275], and targeting conserved regions of the HA protein[276]. However, the majority of UIV candidates continue to be based on the HA protein[194]. The following section outlines a number of strategies that have been evaluated with the potential to elicit broadly-reactive immune responses directed against the HA protein.

1.4.1.1 Targeting conserved HA epitopes

Current vaccines primarily elicit antibodies against the rapidly mutating antigenic sites on the globular head of the HA protein, which severely limits antibody recognition of drifted strains[90]. However, there are also several regions of the HA head and stem domains that are highly conserved among influenza viruses and antibodies targeting these epitopes can have broad neutralizing capacity[111,277]. For example, antibodies directed against conserved epitopes near the HA fusion peptide can fix HA in its pre-fusion form and prevent viral replication[111]. Antibodies targeting conserved HA epitopes can also provide protection through non-neutralizing effector functions such as steric hindrance of NA activity or ADCC[278,279]. Thus, the HA protein is a promising target for UIV development, and several novel approaches are being explored to direct immune responses against conserved but immunologically subdominant HA epitopes.

One strategy for eliciting stem-specific antibodies is removing the HA head domain altogether. However, this can result in conformational changes that impact antigenicity[280]. Recently, techniques for stabilizing the HA stem domain have been developed, allowing for expression of correctly folded HA stem trimers[281-283]. These so-called ‘headless’ HAs have been

shown to provide heterosubtypic protection to group 1 IAV in mice, ferrets, and non-human primates[281,282].

Stem-specific antibody responses can also be elicited by sequential exposure to HAs with the same stem domain but different head domains[276]. This is accomplished by generating chimeric HAs with the stem domain from currently circulating strains and the head domain from exotic HA subtypes (e.g. H8)[276,284]. These chimeric HAs have been shown to elicit robust and long-lasting heterosubtypic immunity in animal models[285-288] and a recent phase I clinical trial revealed that this approach successfully elicited broadly cross-reactive anti-stem antibodies against group 1 HAs in humans[289]. Although theoretically promising, further commercial development of this approach was recently abandoned by GSK in response to disappointing phase II clinical results[290].

Another strategy that has been used to target conserved epitopes in the HA head and stem domains is the manipulation of glycosylation patterns. N-glycosylation of the HA protein is an important mechanism that viruses use to evade host immunity by masking the underlying antigenic regions[291,292]. In one approach, this 'masking' effect was harnessed to redirect responses to the conserved stem of H1 (PR/8) by adding N-glycosylation sites near the immunodominant antigenic sites on the globular head[293]. This hyperglycosylated HA elicited robust stem-specific antibody responses that were broadly cross-reactive with distinct H1N1 and H5N1 viruses[293]. On the other hand, unmasking antigenic sites on H1 by truncating or removing the surrounding N-glycans has also been shown to elicit more broadly reactive antibodies since 'glycan shielding' tends to slow antigenic drift in the masked sites[294-297]. Thus, either approach could be used to improve the breadth of antibody responses directed against the HA protein.

In recent years, there has been a marked increase in the use of computational approaches to identify target antigens and epitopes for the rational design of HA-based vaccine antigens. In one approach, Computationally Optimized Broadly Reactive Antigens (COBRA) are generated using consensus sequences from all of the known HAs within a given subtype. In another approach, 'mosaic' HAs are generated by combining important antigenic sites and/or T cell epitopes from more than one HA protein. Both the COBRA and mosaic approaches have yielded promising results in pre-clinical studies[298-303]. Furthermore, their ability to elicit both stem- and head-directed antibodies is beneficial given that HI titers remain important for vaccine licensure[301,302].

1.4.2 Working with what we have

In the last decade, considerable progress has been made towards the development of more broadly protective influenza vaccines. However, evaluating these vaccines in the clinical setting remains challenging. One of the major obstacles to licensure is the lack of appropriate strategies to evaluate vaccine efficacy against viruses that are not currently circulating. Moreover, the currently accepted correlates of protection (HI and SRH) are unlikely to be indicative of vaccine efficacy, since the majority of universal vaccine candidates aim to elicit stem-directed and non-neutralizing functional antibodies[269,304]. Thus, new correlates for mucosal immunity, cell-mediated immunity, and/or non-neutralizing antibodies will likely need to be established and accepted by regulatory authorities before universal influenza vaccines can be properly evaluated and implemented[194,269,305]. In the meantime, identification of novel strategies to improve the immunogenicity and efficacy of existing influenza vaccines is highly desirable. The following sections outline several easily implementable approaches that have the potential to improve the quality and/or durability of responses to current influenza vaccines.

1.4.2.1 Modified quantity, timing, or route of administration

It is well known that the amount of antigen in a vaccine can greatly impact immune responses and protection. In elderly individuals, the use of high-dose inactivated influenza vaccines significantly improves protection from matched and mis-matched influenza strains compared to the standard dose[306-308]. Improved immunogenicity of the high-dose vaccine has also been observed in high-risk populations such as transplant recipients[309] and cancer patients[310]. On the other hand, reduced antigen doses may be justified to provide better protection at the population-level in the event of a vaccine shortage[311]. Interestingly, administration of inactivated influenza vaccines via the intradermal (ID) route instead of the intramuscular (IM) route has been shown to have a significant dose-sparing effect in all ages[312] and could be implemented in the event of a vaccine shortage. This effect is thought to be mediated by the relative abundance of antigen-presenting cells in the dermis compared to the muscle[313]. Although attractive in many respects, a quadrivalent ID influenza vaccine introduced by Sanofi Pasteur in 2011-2012 has not been a major commercial success[314,315].

The timing of vaccine administration may also have a considerable impact on vaccine outcomes. Several recent studies have found that waning efficacy of influenza vaccines leads to an increase in breakthrough infections towards the end of the influenza season[5,170,316]. This is particularly evident among high-risk populations such as the elderly, and some have suggested that delaying vaccination may reduce the impact of waning efficacy[5,317,318]. However, this approach may not be beneficial in seasons when waning is limited and could be detrimental if it leads to lower vaccine coverage[267,318,319]. Further research is required to optimize the timing of vaccination for optimal protection and uptake. The duration between doses in individuals that receive two vaccine doses may also be worth considering, since delaying the second dose has been shown to improve the immunogenicity of several non-influenza vaccines[320-323]. Currently, the National Advisory Committee on Immunization recommends an interval of at least four weeks between influenza vaccine doses[196]. However, evaluating the impact of increasing this interval may be warranted.

1.4.2.2 A 'mix and match' approach

One of the biggest challenges associated with current vaccines is the limited breadth of immune responses. However, several promising strategies for improving cross-reactivity within HA subtypes have emerged in recent years. For example, Darricarrière *et al*. demonstrated that vaccinating mice with a combination of HA nanoparticles derived from 3-4 distinct H1 influenza viruses stimulated immune responses that cross-reacted with diverse H1 proteins not included in the vaccine[298]. Ferrets vaccinated with the combination vaccine were also better protected from challenge with a mis-matched H1N1 strain[298]. In humans, heterologous prime-boost strategies with inactivated vaccines derived from distinct H5N1 viruses also results in cross-reactive antibody responses to related H5 proteins[324,325]. Thus, vaccination with distinct HAs of the same subtype, either in combination or sequentially, may represent a simple strategy to improve the breadth of immunity elicited by current influenza vaccines.

Combining different routes of vaccine administration (multi-modal vaccination) has also been shown to impact the magnitude and quality of immune responses[326-328]. In one model, simultaneous administration of a candidate influenza vaccine via IM and intranasal (IN) routes in pigs elicited more balanced immune responses and better protection than either route alone[329].

However, a similar study examining plant-based VLP vaccines in aged mice found that multimodal vaccination (IM and IN) resulted in similar immune responses and protection to IM only vaccination[330]. Thus, additional research will be required to determine whether this approach can improve the quality of influenza vaccine responses in humans.

1.4.2.3 Adjuvants

Adjuvants are substances that can be combined with vaccine antigens to boost the immune response[331]. There are many potential advantages to including adjuvants in vaccine formulations, such as improving responses to weak immunogens, dose sparing, and accelerating immune responses[331-333]. For these reasons, adjuvants are often included in pandemic influenza vaccine formulations[331]. Although the majority of seasonal influenza vaccines do not contain an adjuvant, adjuvanted formulations are available for poorly responding populations[331]. The oldest and most widely used vaccine adjuvant are aluminum salts including Alum[331]. However, use of aluminum-based adjuvants in influenza vaccines has largely been replaced by oil-in-water emulsions such as MF59, AS03, and Matrix-M, which have been shown to improve the magnitude, breadth, and durability of vaccine-elicited immune responses[334-338].

Adjuvants can also be used to modify the pattern of immunity elicited by a vaccine. For example, MF59 and Alum promote Th2 biased responses to enhance antibody production[331]. Newer adjuvants developed to target specific aspects of the immune response have yielded promising results and could potentially be used to improve the immunogenicity of existing influenza vaccines. For example, combining a toll-like receptor (TLR) 7/8 agonist with an oil-in-water adjuvant significantly enhanced the breadth and functionality of antibody responses elicited by recombinant H5 HA in mice[339]. Another promising adjuvant is the heat shock protein gp96, which was recently shown to provide protection from group 1 and group 2 influenza strains in mice when administered with a monovalent H1N1 split-virion vaccine[340]. These adjuvants are just two examples among many that have the potential to improve the quality and breadth of responses to current influenza vaccines. Although regulatory authorities may take some time to accept the concept, there is also growing enthusiasm for the potential of combining adjuvants with different mechanisms of action[341].

1.5 RATIONALE AND RESEARCH OBJECTIVES

Despite the emergence of several new influenza vaccine technologies and recent advances in our understanding of influenza vaccine immunology, current vaccines still face the challenges of rapidly waning protection and marked strain-to-strain variation in efficacy[171,182,265]. These issues contribute to the significant socioeconomic burden of influenza epidemics and raise concerns about pandemic preparedness. However, the mechanisms underlying the transient and variable immune responses to influenza vaccines are not well understood. Early in the candidate's doctoral studies, a serendipitous finding that plant-based HA-VLPs non-specifically and rapidly associated with and activated human immune cells motivated further investigation into the possibility that the receptor binding properties of HA could impact vaccine responses. The role of HA binding properties in mediating host restriction, viral transmission, and pathogenesis is well-studied[66,67]. However, the significance of the HA-SA interaction in the context of vaccination had never before been considered, despite the fact that these SAs are known to be widely distributed throughout the body[342-344]. Thus, the binding properties of HA may play a previously unappreciated role in the immune response to vaccination.

Both α2,3- and α2,6-linked SA are highly expressed on the surface of skeletal muscle cells[344-346], which has the potential to influence the trafficking of HA proteins to the draining lymph node following IM injection. These SA receptors are also abundant on the surface of immune cells, which may facilitate HA-immune cell interactions following vaccination. Importantly, α2,3- and α2,6-linked SA are differentially expressed on the surface of each immune cell subset[342,343,347]. In particular, human B cells express high levels of α2,6-linked SA but not α2,3-linked SA[348]. This inherent lack of binding sites for avian HA on B cells may help to explain the universally poor antibody responses elicited by vaccines against avian strains of influenza. This thesis addresses the central hypothesis that HA in vaccines leads to previously unappreciated receptor-mediated interactions that shape the immune response to vaccination. If true, the nature of these interactions would likely depend on HA binding preferences, which could theoretically contribute to strain-specific differences in immunogenicity.

The first objective of this thesis was to characterize HA-immune cell interactions and their potential impact on human immune responses *in vitro* (**Chapter 2**). We evaluated VLP-PBMC interactions and downstream immune responses following incubation with VLPs targeting a

mammalian influenza strain (H1 A/California/07/2009, H1-VLP) and an avian influenza strain (H5 A/Indonesia/05/2005, H5-VLP). Interactions and downstream immune responses clearly reflected the differential distribution of α2,3- and α2,6-linked SA on human immune cells, suggesting that differences in HA binding properties may influence vaccine immunogenicity. These findings prompted us to investigate the impact of HA-SA interactions on vaccine responses *in vivo*. The second objective of this thesis was to generate a novel HA-VLP bearing H1 (A/California/07/2009) that is unable to bind to SA and evaluate its immunogenicity and efficacy compared to WT H1-VLP using a murine model (**Chapter 3**). Ablation of binding resulted in markedly improved antibody titers, antibody durability and avidity as well as viral clearance, suggesting that eliminating HA binding is a promising and remarkably simple strategy to address common limitations of current influenza vaccines. Therefore, the third objective of this thesis was to determine whether this approach can be used to ameliorate the notoriously poor immunogenicity of vaccines targeting avian influenza strains with pandemic potential (**chapter 4**). To this end, we generated novel non-binding HA-VLPs bearing either H7 (A/Shanghai/02/2013) or H5 (A/Indonesia/05/2005) and evaluated their immunogenicity compared to WT HA-VLPs using a murine model. The findings outlined in this thesis highlight the profound impact of the HA-SA interaction on vaccine responses and suggests that this novel strategy may contribute to next-generation influenza vaccine development.

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1.7 FIGURES AND LEGENDS

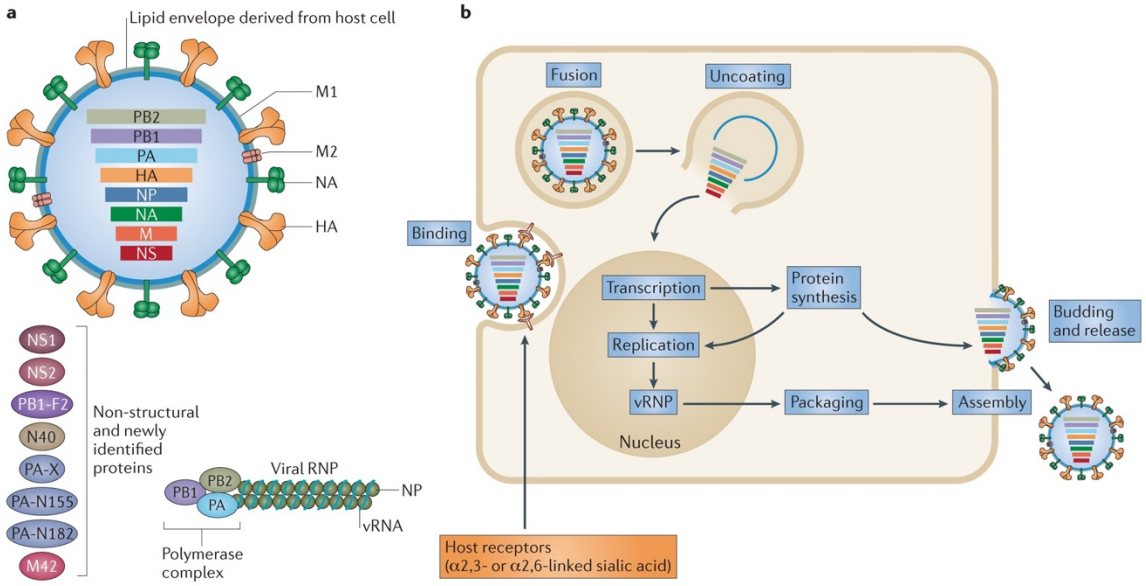

Figure 1.1 Structure and life cycle of influenza A viruses. (a) Schematic diagram of the influenza viral structure and the proteins encoded by each gene segment. (b) Schematic diagram of the influenza life cycle.

Reproduced from Shi, Y., Wu, Y., Zhang, W. *et al.* Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses. *Nat Rev Microbiol* 12, 822–831 (2014). https://doi.org/10.1038/nrmicro3362 with permission from Springer Nature Ltd.,

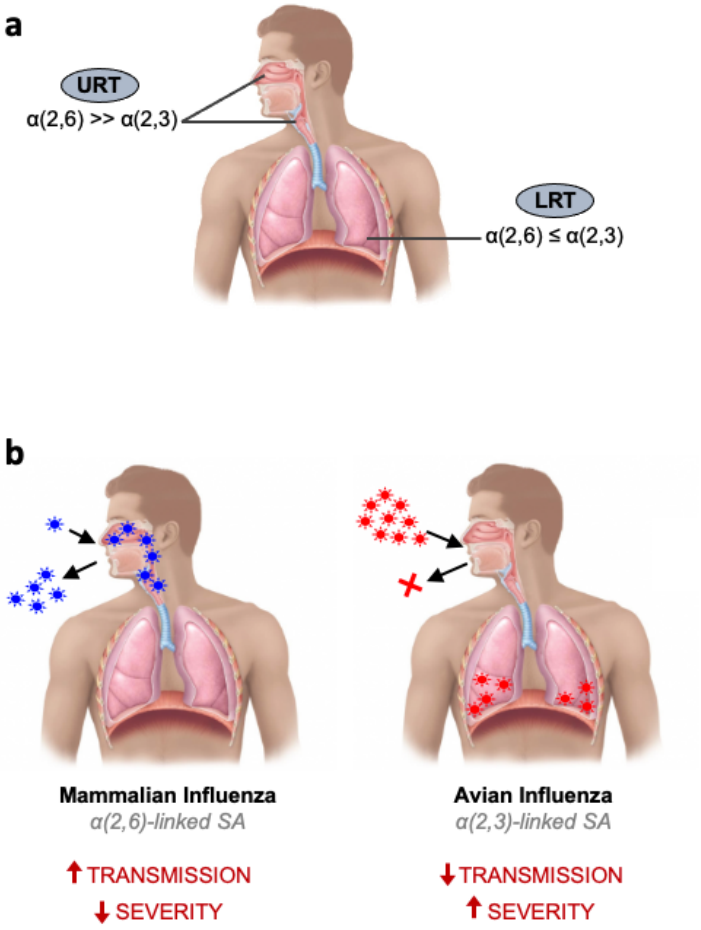

Figure 1.2 Impact of HA binding properties on the severity and transmissibility of influenza infection. (a) distribution of α(2,3)- and α(2,6)-linked SA in the human respiratory tract. (b) Impact of SA receptor specificity on influenza transmission and disease severity.

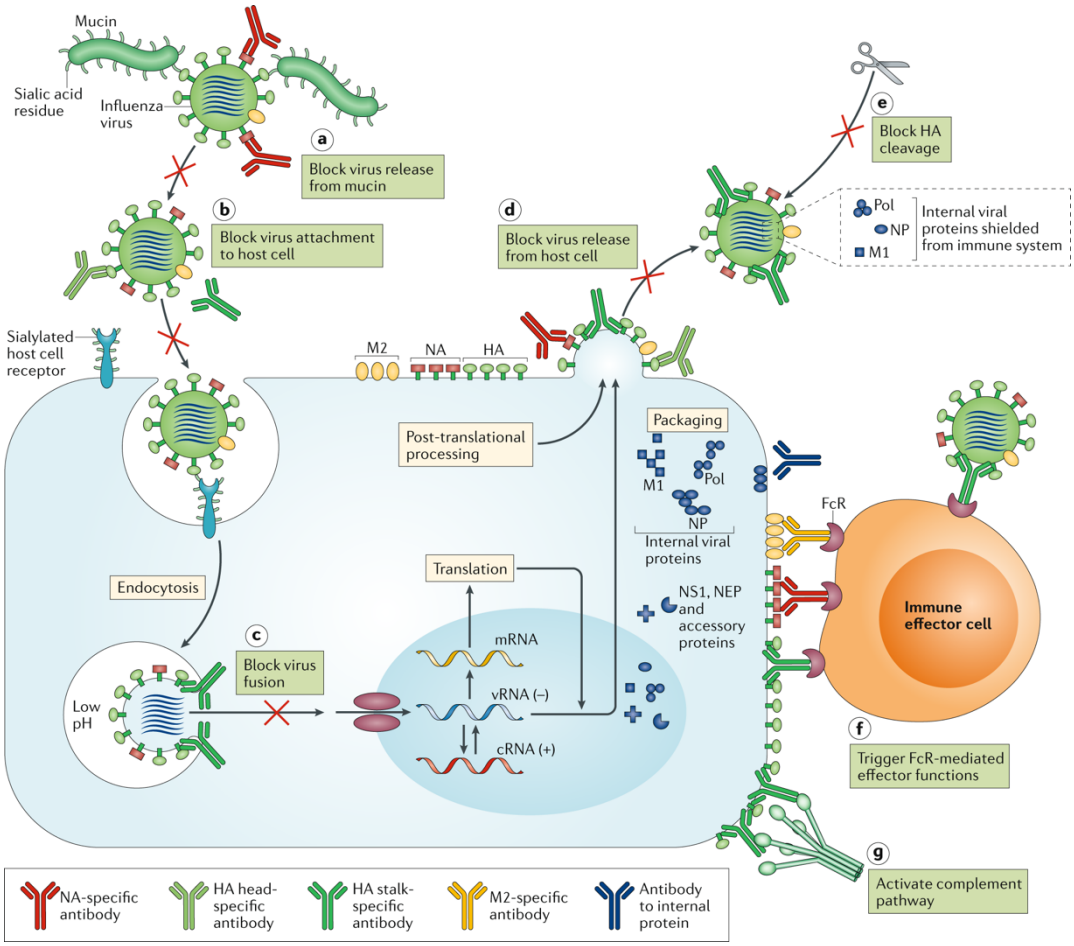

Figure 1.3 Mechanism of action of antibodies against influenza virus. Antibodies can interfere with different stages of the viral life cycle. Possible antigenic targets of antibodies and their mechanism(s) of action are shown.

Reproduced from Krammer, F. The human antibody response to influenza A virus infection and vaccination. *Nat Rev Immunol* 19, 383–397 (2019). https://doi.org/10.1038/s41577-019-0143-6Wei, CJ., with permission from Springer Nature Ltd., © Copyright Springer Nature Ltd. 2019

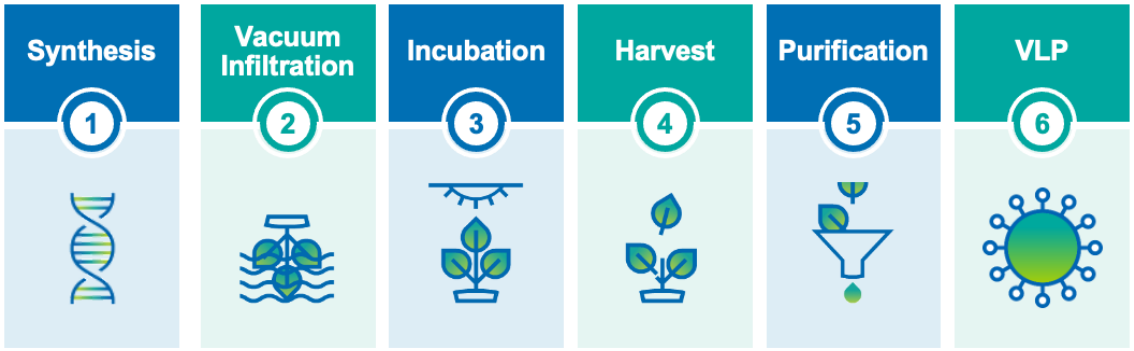

Figure 1.4 Influenza HA-VLP production in *Nicotiana benthamiana* plants. (1) The gene encoding the HA protein of interest is cloned into the *Agrobacterium tumefaciens* expression vector. (2) *Nicotiana benthamiana* plants are transfected with the bacterial expression vector by vacuum infiltration. (3) Plants are incubated for 7-10 days. During this time, HA is expressed within the plant cells and is embedded in the plasma membrane. Virus-like particles spontaneously bud from the plasma membrane and accumulate in between the plasma membrane and cell wall of the plant cells. (4) VLPs are harvested from the plant tissue by mechanical or enzymatic digestion and then (5) purified to obtain clinical grade material. (6) The resulting VLPs mimic the native structure of viruses but are non-infectious. Figure provided by Medicago Inc.

Vaccine	Coverage
Strain-specific	Circulating strains
Subtype-specific	Multiple strains within a single HA subtype
Multi-subtype	Multiple HA subtypes within group 1, group 2 or type B
Pan-group/lineage	Group 1 or 2 influenza A or influenza B lineages
Universal flu A	Influenza A
Universal flu A and B	Influenza A and B
'TRUE' universal	All strains. Single product. Multiple years.

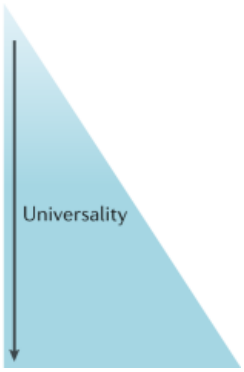

Figure 1.5 Incremental steps towards a 'true' universal influenza vaccine.

Reproduced from Wei, CJ., Crank, M.C., Shiver, J. *et al.* Next-generation influenza vaccines: opportunities and challenges. *Nat Rev Drug Discov* 19, 239–252 (2020). https://doi.org/10.1038/s41573-019-0056-x, with permission from Springer Nature Ltd., © Copyright Springer Nature Ltd. 2020

PREFACE TO CHAPTER 2

Current vaccines for influenza are known to exhibit highly variable immunogenicity. In particular, vaccines targeting avian influenza strains tend to be poorly immunogenic compared to those targeting seasonal influenza strains. However, the mechanisms underlying the poor immunogenicity of avian influenza vaccines are not well understood. We sought to determine whether differences in innate immune responses to influenza antigens could be contributing to this poor immunogenicity. Early in our investigations we found that plant-derived virus-like particle (VLP) vaccines bearing influenza hemagglutinin (HA) on their surfaces readily interact with human immune cells by binding to their sialic acid (SA) receptor on the cell surface. The following chapter describes the distinct interactions of VLPs targeting an avian influenza strain (H5) and a mammalian influenza strain (H1) with human immune cells. This chapter also describes how differences in these early interactions may influence downstream immune responses by evaluating immune cell activation and cytokine production following brief exposure to H1- or H5-VLPs.

This chapter was adapted from the following manuscript: Plant-made virus-like particles bearing the hemagglutinin of either seasonal (H1) or avian (H5) influenza have distinct patterns of interaction with human immune cells *in vitro*. **Hendin**, **H.E.**, *et al. Vaccine* 35, 2592-2599 (2017).

CHAPTER 2

Plant-made virus-like particle vaccines bearing the hemagglutinin of either seasonal (H1) or avian (H5) influenza have distinct patterns of interaction with human immune cells *in vitro*.

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2.1 ABSTRACT

Introduction: The recent emergence of avian influenza strains has fuelled concern about pandemic preparedness since vaccines targeting these viruses are often poorly immunogenic. Weak antibody responses to vaccines have been seen across multiple platforms including plant-made VLPs. To better understand these differences, we compared the *in vitro* responses of human immune cells exposed to plant-made virus-like particle (VLP) vaccines targeting H1N1 (H1-VLP) and H5N1 (H5-VLP).

Methods: Peripheral blood mononuclear cells (PBMC) from healthy adults were stimulated *ex vivo* with 2-5µg/mL VLPs bearing the hemagglutinin (HA) of either H1N1 (A/California/7/2009) or H5N1 (A/Indonesia/5/05). VLP-immune cell interactions were characterized by confocal microscopy and flow cytometry 30min after stimulation with dialkylaminostyryl dye-labeled (DiD) VLP. Expression of CD69 and pro-inflammatory cytokines were used to assess innate immune activation 6h after stimulation.

Results: H1- and H5-VLPs rapidly associated with all subsets of human PBMC but exhibited unique binding preferences and frequencies. The H1-VLP bound to 88.7±1.6% of the $CD19^+$ B cells compared to only 21.9±1.8% bound by the H5-VLP. At 6h in culture, CD69 expression on B cells was increased in response to H1-VLP but not H5-VLP (22.79±3.42% vs. 6.15±0.82% respectively: $p<.0001$). Both VLPs were rapidly internalized by $CD14^+$ monocytes resulting in the induction of pro-inflammatory cytokines (ie: IL-8, IL-1β, TNFα and IL-6). However, a higher concentration of the H5-VLP was required to induce a comparable response and the pattern of cytokine production differed between VLP vaccines.

Conclusions: Plant-made VLP vaccines bearing H1 or H5 rapidly elicit immune activation and cytokine production in human PBMC. Differences in the VLP-immune cell interactions suggest that features of the HA proteins themselves, such as receptor specificity, influence innate immune responses. Although not generally considered for inactivated vaccines, the distribution and characteristics of influenza receptor(s) on the immune cells themselves may contribute to both the strength and pattern of the immune response generated.

2.2 INTRODUCTION

Influenza viruses are members of the *Orthomyxoviridae* family (single-stranded, negative-sense RNA) that cause acute respiratory infection in humans. Seasonal outbreaks of influenza are responsible for approximately 250,000-500,000 deaths worldwide each year[1] and antigenic variants arising through inter-species genetic reassortment pose a significant pandemic threat[2]. Public vaccination programs help to minimize the morbidity and mortality associated with influenza infection, however several investigations have shown that current vaccine formulations are only effective in 50-60% of healthy adults[3,4] with marked strain-to-strain variation in immunogenicity. In particular, vaccines targeting avian strains of influenza generally elicit poor antibody responses compared to those targeting mammalian (ie: seasonal) strains. Candidate pandemic vaccines often require higher doses of antigen and/or the addition of adjuvants to achieve reasonable levels of seroconversion[5]. Combined with the inherent inefficiency of egg-based vaccine production and the vulnerability of this platform to the avian viruses themselves (ie: potentially lethal to hens and eggs), poor immunogenicity could be devastating in the event of a serious pandemic[6-8].

Recently, several novel approaches have been explored for rapid and scalable vaccine production that can address some of these concerns[9-11]. Among the most promising are recombinant platforms that generate virus-like particles (VLP), including those made in plants[12]. In the most commonly-used plant-based platform, transient transfection of *Nicotiana benthamiana* with *Agrobacteria tumefaciens* carrying the gene for the influenza HA protein results in the formation of HA-studded, enveloped particles that closely resemble influenza viruses[13]. These plant-made VLP vaccines elicit not only good antibody titres but also surprisingly strong cellular responses[14,15], making them a promising alternative to current vaccine formulations[6,14]. However, even these plant-made VLP vaccine candidates induce relatively low antibody responses when formulated with avian HA compared to virtually any seasonal HA[14,16].

To better understand the mechanisms underlying the differences in immunogenicity between VLP vaccines targeting seasonal and avian strains, we investigated the early events when plant-made VLP vaccines targeting an avian (H5-VLP) and a seasonal strain (H1-VLP) were mixed with human PBMC *ex vivo*. In the current work, we describe marked differences in the way

that these VLPs interact with human immune cells and unique features of the early innate immune response.

2.3 MATERIALS AND METHODS

2.3.1 Vaccines

Clinical-grade VLPs bearing H1 (A/California/7/09) or H5 (A/Indonesia/5/05) were produced by Medicago Inc. as previously described[14]. Empty vesicles (EV) were generated from homogenized *N. benthamiana* cell membranes. The EV were slightly smaller than the VLPs formulated with either H1 or H5 and more heterogeneous (median diameter 171 nm) but had a lipid profile similar to that of the HA-bearing VLPs (data not shown).

2.3.2 Subjects and ethical approval

Healthy adults aged 18-64 were recruited by the McGill Vaccine Study Centre and participants provided written consent prior to blood collection. This protocol was approved by the Biomedical D Research Ethics Board of the McGill University Health Centre.

2.3.3 PBMC isolation and handling

PBMC were isolated from peripheral blood by differential-density gradient centrifugation within one hour of blood collection. Briefly, blood was diluted 1:1 in phosphate-buffered saline (PBS) (Wisent) at RT prior to layering over Lymphocyte Separation Medium (Ficoll) (Wisent). PBMC were collected from the Ficoll-PBS interface following centrifugation (650x*g*, 45min, 22^{o}C) and washed 3 times in PBS (320x*g*, 10 min, 22^{o}C). Cells were resuspended in RPMI-1640 complete medium (Wisent) supplemented with 10% heat inactivated fetal bovine serum (Wisent), 10mM HEPES Wisent), and 1mM penicillin/streptomycin (Wisent).

2.3.4 Fluorescent labelling of VLP

VLP were stained with 5ng/mL DiD lipophilic dye (Fischer Scientific) reconstituted in DMSO for 30min. Excess DiD was removed using a Sephadex G-50 column (GE Healthcare Life Sciences).

2.3.5 Imaging

Freshly isolated PBMC (500µL at 4x10^6/mL) were co-incubated with 5 µg/mL unlabeled VLP or DiD-labeled VLP, EV (based on lipid content and volume) or VLP-free PBS containing DiD (30min, 37°C). Cells were transferred onto a 170µm thick glass coverslip (Sarstedt) coated with 0.005% poly-L-lysine (Sigma) and incubated for an additional 20 minutes (37°C). The remaining steps were carried out at room temperature. Cells were fixed with 2% PFA (Sigma) and washed 3x5min in PBS with 1% BSA (Sigma). Cells were stained with different combinations of the following anti-human antibodies (Biolegend): anti-CD56 Brilliant Violet 421 (HCD56), anti-CD19 Alexa Fluor 488 or Alexa Fluor 594 or Brilliant Violet 421 (HIB19), anti-CD3 Alexa Fluor 594 or Alexa Fluor 488 (UCHT1), anti-CD14 Alexa Fluor 647 or Alexa Fluor 594 or unconjugated (HCD14). Unconjugated anti-CD14 was detected using anti-mouse IgG1 DyLight 405 (Poly24091). Samples were washed 5 times prior to imaging using a Zeiss LSM780 laser scanning confocal microscope. Images and Z-stacks were acquired and processed using ZEN software (Zeiss).

2.3.6 Flow cytometry

PBMC (1x10^6/200µL) were incubated with either VLP or EV (as above) or PHA (5µg/mL, Sigma) for 30min or 6h (37°C). Cells were washed 3x in 200µL/well PBS (320x*g*, 8min, 4°C) in a 96-well round-bottom plate and labeled with Fixable Viability Dye eFluor 780 (eBioscience) (20min, 4°C). Cells were washed 3 times followed by surface staining with the following anti-human antibodies (30min, 4°C): anti-CD3 V500 (UCHT1, BD), anti-CD4 eFluor 450 (RPA-T4, eBioscience), anti-CD8a Brilliant Violet 605 (RPA-T8, Biolegend), anti-CD19 Brilliant Violet 650 (HIB19, Biolegend), anti-CD56 PE or APC (CMSSB, eBioscience), and anti-CD14 BUV395 (MφP9, BD). To measure PBMC activation, cells were stained with anti-CD69 (CH/4, Invitrogen). Cells were fixed (Fix/Perm solution, BD) for 30min prior to acquisition. For detection of intracellular cytokines, fixed cells were washed 3x in 200µL perm/wash buffer (BD) followed by intracellular staining with the following anti-human antibodies (30min, 4°C): anti-IL-1β PE (CRM56, eBioscience), anti-IL-6 PE-CF594 (MQ2-13A5, BD), anti-IL-8 FITC (E8N1, Biolegend), anti-TNFα Brilliant Violet 711 (MAb11, Biolegend), and IL-2 Alexa Fluor 700 (MQ1-17H12, Biolegend). PBMC were washed 3 times in perm/wash buffer and then resuspended in PBS. One hundred thousand events were collected to measure VLP binding and 200,000 events

were collected to measure PBMC activation and cytokine production using a BD LSRFortessa cell analyzer. Data was analyzed using FlowJo software (Treestar, Ashland) (gating strategy in **supp. figure 2.1**).

2.3.7 Statistical analysis

Statistical analyses were carried out using Prism 6 software (GraphPad). Pie charts were generated using SPICE software (NIAID). Specific tests and significance levels are indicated in the results and Figure legends.

2.4 RESULTS

2.4.1 H1- and H5-VLP vaccines bind to distinct subsets of human PBMC

Association of the DiD-labeled VLPs to different human PBMC subsets was readily detected by flow cytometry following 30min of co-incubation. Overall, the H1-VLP and H5-VLP bound to 63.19±1.66% and 40.05±3.63% of total live cells respectively (**figure 2.1a**). Binding of VLPs lacking a viral HA (EV) was detected on only 1.1±0.08% of PBMC suggesting that association with the immune cells was predominantly HA-mediated. Furthermore, PBMC treated with sialidase to remove surface-associated sialic acids exhibited a dramatic reduction in VLP binding (**supp. figure 2.2**).

When binding to individual PBMC subsets was evaluated, we observed clear differences between the H1- and H5-VLPs. Most strikingly, the H1-VLP bound to the surface of almost 90% of the $CD19^+$ B cells (88.7±1.6%) compared to only 21.9±1.8% for the H5 VLP ($P<0.0001$) (**figure 2.1b**). Similar trends were observed in the T cell subsets, in which 69.0±2.2% of $CD4^+$ T cells and 61.0±2.6% of $CD8^+$ T cells were bound by the H1-VLP compared to 31.9±2.3% and 44.1±3.97% respectively for the H5-VLP (CD4: $P<0.0001$; CD8: $P=0.0211$). The H1- and H5-VLPs bound in equal proportions to $CD56^+$ NK cells (H1: 60.5±1.9%, H5: 58.7±4.0%), $CD3^+$ $CD56^+$ NKT cells (H1: 69.9±2.9%, H5: 59.9±4.0%), and $CD14^+$ monocytes (H1: 84.7±1.1%, H5: 86.9±1.6%). Although the EV only interacted with a small proportion of the total PBMC, we found that HA-

independent binding was over-represented in the B cell and monocyte populations (6.2±0.3% and 10.0±1.6%, respectively) (**figure 2.1b**).

2.4.2 HA-mediated VLP binding facilitates unique intercellular interactions

Given that there are multiple HA trimers on the surface of each VLP, we were interested to determine if VLP binding could facilitate intercellular interactions. PBMC were visualized by confocal microscopy or flow cytometry following 30min co-incubation with DiD-labeled or unlabeled VLP. DiD fluorescence was absent following co-incubation with a VLP-free DiD control **(supp. figure 2.3).**

Co-incubation with the H1-VLP resulted in obvious clustering of the PBMC. This phenomenon was not observed following exposure to the H5-VLP (**figure 2.2a**). The frequency of clustering within each cell subset was determined by flow cytometry. The cells within each cluster cannot be directly quantified using this technique, however we found that a reduction in the single cell population was inversely related to the degree of clustering (**supp. figure 2.4a**). Because the VLP do not influence cell viability at the concentrations used, changes in the single cell population are an accurate reflection of the formation of clusters (**supp. figure 2.4b**). As expected, the frequency of single cells was unchanged following co-incubation with the H5-VLP (data not shown). The H1-VLP mediated significant clustering in all lymphocyte populations. This effect was concentration dependent and was most prominent among B cells, which exhibited a 75% reduction in single cells at 5µg/mL H1-VLP (**figure 2.2a**). Accordingly, the DiD-labeled H1-VLPs appeared to be localized predominantly on the surface of B cells and at points of intercellular contact. Distinct polarization of the actin cytoskeleton was observed in many VLP-rich foci (**supp. figure 2.5**). In contrast, DiD-labeled H5-VLP was not observed on B cells and was generally less prominent than the H1-VLP on the surface of other lymphocytes (**figure 2.2b**). The clustering caused by the H1-VLP was largely abrogated by sialidase treatment of the PBMC (**supp. figure 2.2**).

Both the H1- and H5-VLPs readily bound to the surface of $CD14^+$ monocytes, although the pattern of binding was very different. The H1-VLPs appeared to be present in distinct foci at the interfaces between monocytes and adjacent lymphocytes. The H5-VLP rapidly coated the surface of monocytes and many of these cells appeared to bind substantially more H5- than H1-

VLP but had similar morphology and viability (data not shown). Despite the marked difference in binding pattern, both the H1- and H5-VLPs were rapidly internalized by monocytes (**figure 2.3**).

2.4.3 The H1- and H5-VLP vaccines elicited distinct innate immune responses *in vitro*

To determine whether the HA-specific interactions described above influenced downstream innate immune responses, we evaluated PBMC activation (eg: CD69 expression) and pro-inflammatory cytokine production by flow cytometry following *in vitro* stimulation for 6h.

Stimulation with H1- or H5-VLPs did not result in increased CD69 expression on T cells, monocytes, NK cells, or NKT cells. However there was a striking increase in $CD69^{+}CD19^{+}$ B cells following exposure to H1-VLP but not H5-VLP (**figure 2.4a**) at any of the concentrations tested (**supp. figure 2.6**). As expected, CD69 was up-regulated in all PBMC subsets following 6h stimulation with PHA (5µg/mL) (**supp. figure 2.7**).

Pro-inflammatory cytokine production was absent in B cells, T cells, and NK cells following stimulation with either H1-VLP or H5-VLP (data not shown). However, there was a robust pro-inflammatory cytokine response in the majority of $CD14^{+}$ monocytes stimulated with 2µg/mL H1-VLP, including the production of IL-8 (72.8±2.8%), IL-1β (55.6±2.9%), TNFα (55.8±7.7%) and IL-6 (30.6±3.7%) (all $P<0.0001$ vs. unstimulated controls). Although production of these same cytokines tended to increase following stimulation with 2µg/mL of the H5-VLP, none of these increases reached statistical significance. At higher concentrations of the H5-VLP (5µg/mL), a more robust response as observed including significant increases in the proportion of monocytes producing IL-8 (62.9±9.1%, $P<0.0001$), IL-1β (24.9±5.6%, $P<0.0001$), and TNFα (31.3±5.6%, $P<0.01$) but not IL-6 (compared to unstimulated controls) (**figure 2.4b**).

When the cytokine signatures of the VLP-stimulated monocytes were evaluated by Boolean gating, 80.16±2.89% of the monocytes exposed to H1-VLP (2µg/mL) produced ≥2 cytokines compared to only 31.65±7.92% following H5-VLP stimulation ($P<0.0001$). The size of the population responding to H5-VLP stimulation with at least 2 cytokines increased to 67.66±7.7% at the higher dose (5µg/mL) (**figure 2.4c**).

2.5 DISCUSSION

The emergence of new avian influenza strains in the past decade has led to growing concern about pandemic preparedness, as vaccines targeting these viruses tend to be poorly immunogenic compared to those targeting seasonal strains. This relatively poor immunogenicity has been observed with candidate vaccines across multiple platforms including our own plant-made VLPs[5,14]. To better understand this phenomenon, we compared the early events following *in vitro* stimulation of human PBMC with VLPs bearing only the HA protein from either H1N1 A/California/7/09 (H1) or H5N1 A/Indonesia/5/05 (H5).

We found that both H1- and H5-VLPs interact rapidly with human immune cells but that they exhibited very different binding preferences and frequencies with immune cell subsets and promoted different responses. The association of 'naked' VLPs (ie: empty plant lipid vesicles or EV) with the PBMC was much more limited, suggesting that the VLP-PBMC interactions we observed were mediated largely by the different viral HA proteins. Terminal sialic acids (SA) are the primary receptors for HA-mediated influenza attachment to host cells, and both the specificity and affinity of this interaction are strain-dependent[17]; mammalian strains preferentially bind to α(2,6)-linked SA and avian strains typically bind to α(2,3)-linked SA. Although anatomic distribution of these SA receptors in the human respiratory tract has been the focus of much research (ie: α(2,6) in the upper respiratory tract and α(2,3) lower in the lung), there are also marked differences in terminal SA expression patterns on human immune cells[18-20]. The striking differences we observed in the behaviour of the H1- and H5-VLP with human PBMC subsets raised the possibility that differential expression of SA receptors on individual subsets may contribute to vaccine outcomes including the unusual immunogenicity of plant-made VLP vaccines.

The greatest difference in VLP-PBMC interactions that we observed was the strong binding of H1-VLP to B cells and the formation of multi-cellular clusters *in vitro*. Within minutes of mixing the H1-VLP with PBMC, almost all of the B cells were present in doublets or clusters (90%) compared to only 22% following exposure to equal concentrations of the H5-VLP. While most immune cell subsets express both α(2,3)- and α(2,6)-linked SA to varying degrees, human B cells exclusively express α(2,6)-linked SA in high quantities[21]. This expression pattern and the binding preferences of the seasonal versus avian HA proteins almost certainly explain the very

different behaviour of the H1- and H5-VLPs. The greater binding of the H1-VLP to T cells compared with the H5-VLP can also be explained by the fact that α(2,6)-linked SA are the predominant N-glycan on these cells as well[19]. In every PBMC subset, but most prominently in B and T cells, the populations binding H1-VLP had higher MFIs than their H5-VLP-bound counterparts, suggesting that a great proportion of cells bound a larger number of VLPs.

The H1-VLP not only bound to the surface of human PBMC, it also appeared to facilitate strong intercellular interactions that were capable of withstanding the relatively strong shear forces generated by vortexing or flow cytometry. Actin polarization towards VLP-rich foci suggests that reorganization of the plasma membrane allows the H1-VLP to form durable cell-to-cell interactions. This clustering effect was largely absent upon exposure to a similar concentration of the H5-VLP (2μg/mL). Given that low level binding of the DiD-labelled H5-VLP was readily detectible by flow cytometry on most cell subsets, the absence of PBMC clustering likely reflects a decrease in the relative binding affinity of H5 compared to H1. Although it is possible that the binding affinity of H1 is intrinsically higher than H5 for their respective SA receptors, previous studies have reported that glycan moieties on the globular head of HA can reduce affinity[22-24]. It is therefore interesting that H5 has two glycans directly adjacent to the SA binding site and binds to SAs with a lower affinity than H1, which has no glycans on the globular head[25]. Our observation that PBMC clustering could be induced at high concentrations of the H5-VLP (25μg/mL) supports this notion. Additional investigations will be required to determine whether this clustering phenomenon occurs *in vivo* and to assess the fate of the clustered PBMC. It will also be of considerable interest to assess the *in vitro* and *in vivo* behaviour of VLPs bearing H1 and H5 that have been modified to alter their SA binding specificity and glycosylation patterns.

Since the H1N1 strain targeted by the H1 VLP has been actively circulating since 2009-10, it is worth considering the possibility that the markedly different interactions of H1- and H5-VLPs with human PBMC we observed were driven by immune memory. It is likely that a large proportion of the healthy subjects whose PBMC were included in this study had been exposed to the H1 antigen through vaccination or natural infection while it is virtually certain that they were naïve to the H5 antigen. Although some degree of the B cell activation observed following stimulation with the H1- but not the H5-VLP may be attributable to antigen-specific B cells, cells responding to a particular antigen typically make up <1% of total B cells[26] and are unlikely to

proliferate within 6h[27]. It is therefore highly likely that the H1-VLP-induced B cell activation we observed was predominantly an innate rather than an adaptive response.

Despite the robust activation of B cells following stimulation with the H1-VLP, we did not detect cytokine production. Cytokine-producing B cells play a variety of roles in modulating immune responses, however several studies have demonstrated that engagement of the B cell receptor (BCR) is not sufficient to elicit cytokine production[28,29]. For example, Duddy *et al*. reported that BCR engagement and subsequent stimulation with CD40L for 72h resulted in production of IL-6, TNFα and lymphotoxin, but this effect was not observed with BCR engagement alone[28]. In contrast, engagement of the BCR alone is sufficient to elicit rapid cell activation. Villar *et al.* recently demonstrated that crosslinking the heavily sialylated BCR using multivalent HA results in robust cell activation, reminiscent of our results[30]. While it is possible that H1-VLP stimulated B cells eventually develop into cytokine-producing cells, this is unlikely to occur to any great extent within 6h in either naïve or antigen-experienced cells. Later time points will be required to determine whether or not early VLP-mediated clustering of PBMC influences B cell cytokine-production.

The relationship between early interactions of the HA-bearing VLPs and downstream immune responses is the principal subject of on-going work. However, our observations to date suggest that any such relationship will not be a simple 'domino' effect (ie: high binding leading to high activation leading to high response). For example, both of the HA-bearing VLPs interacted with >80% of monocytes and are were subsequently internalized. Assuming that DiD labelling was comparable between H1- and H5-VLPs, similar quantities of VLPs interacted with these cells based on mean fluorescence intensity (MFI $1.93\times10^4\pm1.4\times10^3$ vs $1.86\times10^4\pm2.24\times10^3$ respectively; data not shown) yet the H1-VLP elicited much higher levels of pro-inflammatory cytokines and broader co-expression of these cytokines than the H5-VLPs. Even though the magnitude of the pro-inflammatory cytokine response increased with 5µg/mL of the H5-VLP, the level of co-expression did not approach those elicited by the H1-VLP. Possible contributors to these observations include differences in HA-associated glycans that can play an important role in mediating influenza virus uptake by antigen presenting cells[31] and the fact that how a virus (or VLP) enters a cell can alter downstream responses[32,33]. Parallel investigations with primary human monocyte-derived macrophages have suggested the internalization of H1-VLPs by APCs is primarily SA-dependent while H5-VLP internalization requires additional interactions between

HA-associated glycans and glycan co-receptors on APCs (A. Markarkov, manuscript in preparation). This is consistent with our observation that a higher proportion of H5-VLP interactions were maintained following sialidase treatment of PBMC, and supports the notion that the mechanism of VLP entry may influence downstream responses. It is also possible that while the H5-VLPs bind to monocytes and are internalized, the pro-inflammatory response requires additional stimulatory signals from adjacent lymphocytes present in the H1-VLP induced clusters. Because potent quenching occurs with DiD[34], fusion events could powerfully influence the amount of apparent fluorescence (data not shown) resulting in misleading MFI values. For example, if H5-VLP were to fuse more efficiently than the H1-VLP with either cytoplasmic or endosomal membranes, then fewer H5-VLP would be required to generate similar MFI values as a result of dequenching[35]. Ongoing studies will determine the mechanism(s) of internalization of the different HA-VLPs and explore correlations between monocyte-VLP interactions and downstream immune responses.

To date, interest in HA-SA receptor interactions of seasonal and avian influenza strains has been limited largely to the anatomy of binding within the respiratory tract and how this binding influences the severity and transmissibility of infection. We found that VLP vaccine candidates bearing only H1 or H5 bind to different human PBMC subsets in an HA-dependent manner to induce strain-specific innate immune responses. Because such early events can powerfully influence subsequent adaptive responses[36,37], the binding properties of different HAs on immune cells may be a previously underappreciated factor contributing to vaccine immunogenicity and efficacy. Although the focus of the current work was possible differences between the monovalent plant-made VLP vaccines bearing avian- and seasonal-strain HAs, on-going studies suggest that immune cell interactions and innate responses also differ substantially between a quadrivalent plant-made VLP vaccine and a commercial split vaccine comparator (data not shown). Future studies will focus on determining how structural features of different HAs affect their interactions with human immune cells (eg: SA binding specificity, glycosylation) and how such interactions influence the downstream immune response. Through this work, we hope to contribute to the rational design of more effective influenza vaccines for both seasonal and avian strains.

2.6 ACKNOWLEDGEMENTS

The authors would like to thank Thomas di Lenardo for technical assistance with flow cytometry, Sabrina Chierzi for assistance with imaging, and members of collaborating laboratories (Piccirillo, Krawczyk, and Rouiller groups at McGill University) for helpful discussions during experimental planning and manuscript preparation. While generating this manuscript, H.E.H. held a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship (Master's Award).

2.7 CONFLICTS OF INTEREST

B.J.W. has been a principal investigator of vaccine trials for several manufacturers, including Medicago Inc., for which his institution obtained research contracts. Since 2010, B.J.W. has served as Medical Officer for Medicago Inc. In addition, B.J.W. has held and continues to hold peer-reviewed support from CIHR and other sources for collaborative, basic science work with Medicago Inc. B.J.W. has received honoraria from several vaccine manufacturers for participation on Scientific Advisory Boards (including Medicago Inc.). S.P. and N.L. are current employees of Medicago Inc.

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2.9 FIGURES AND LEGENDS

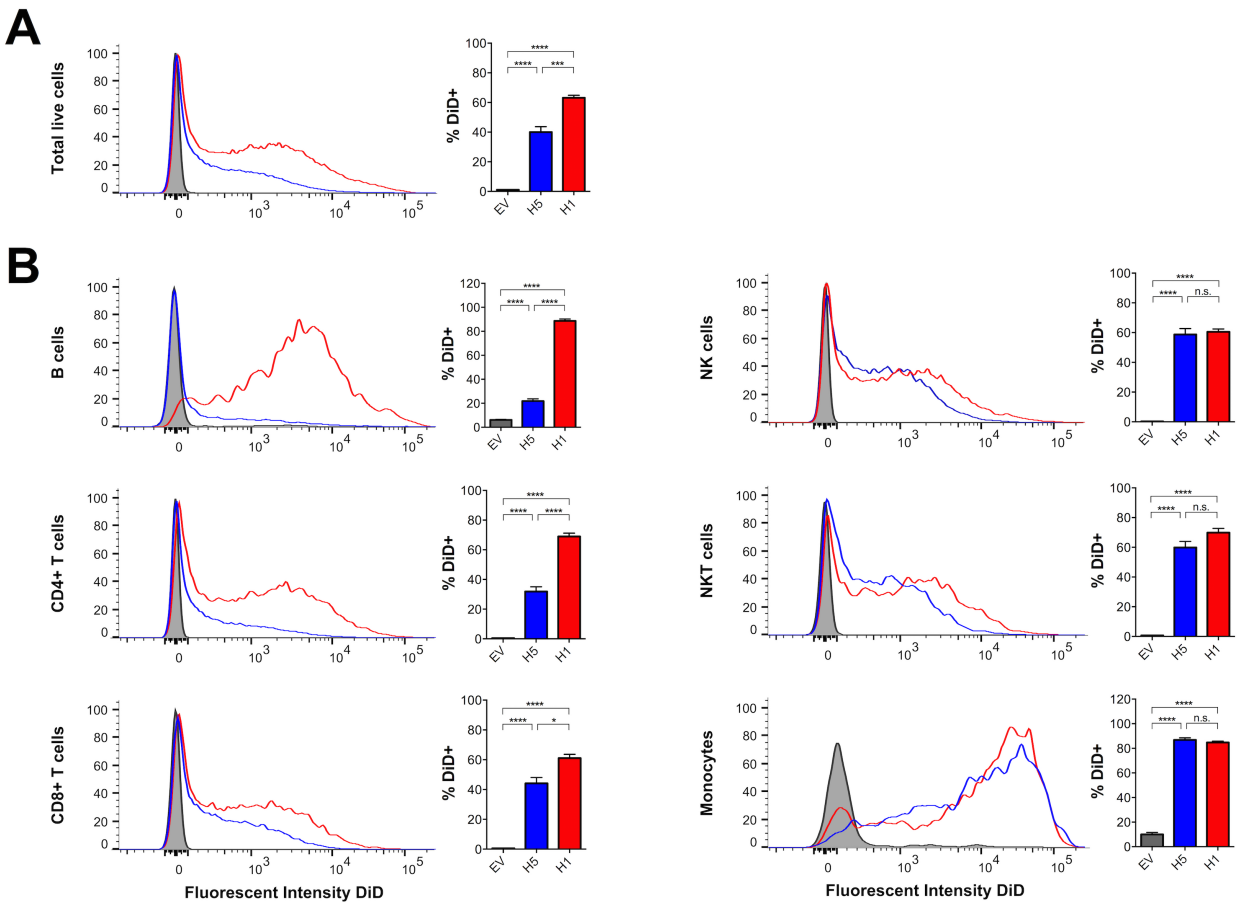


Figure 2.1. The H1- and H5-VLP vaccines exhibit distinct patterns of binding to human PBMC. 1x10^6 PBMC were co-incubated with DiD-labeled HA VLPs (5µg/mL in 200µL) or an equivalent volume of DiD-labeled EV for 30min (37°C). Flow cytometry was used to quantify binding to live PBMC (A) and within PBMC sub-populations (B). Fluorescent intensities are displayed as representative histograms (left panel) and binding was quantified as the proportion of DiD$^+$ cells within each population (right panel). Error bars represent the SEM, n=8. Statistical significance was determined by one-way ANOVA with the Greenhouse-Geisser correction (**P<0.05, **P<0.01, ***P<0.001, ****P<0.0001*).

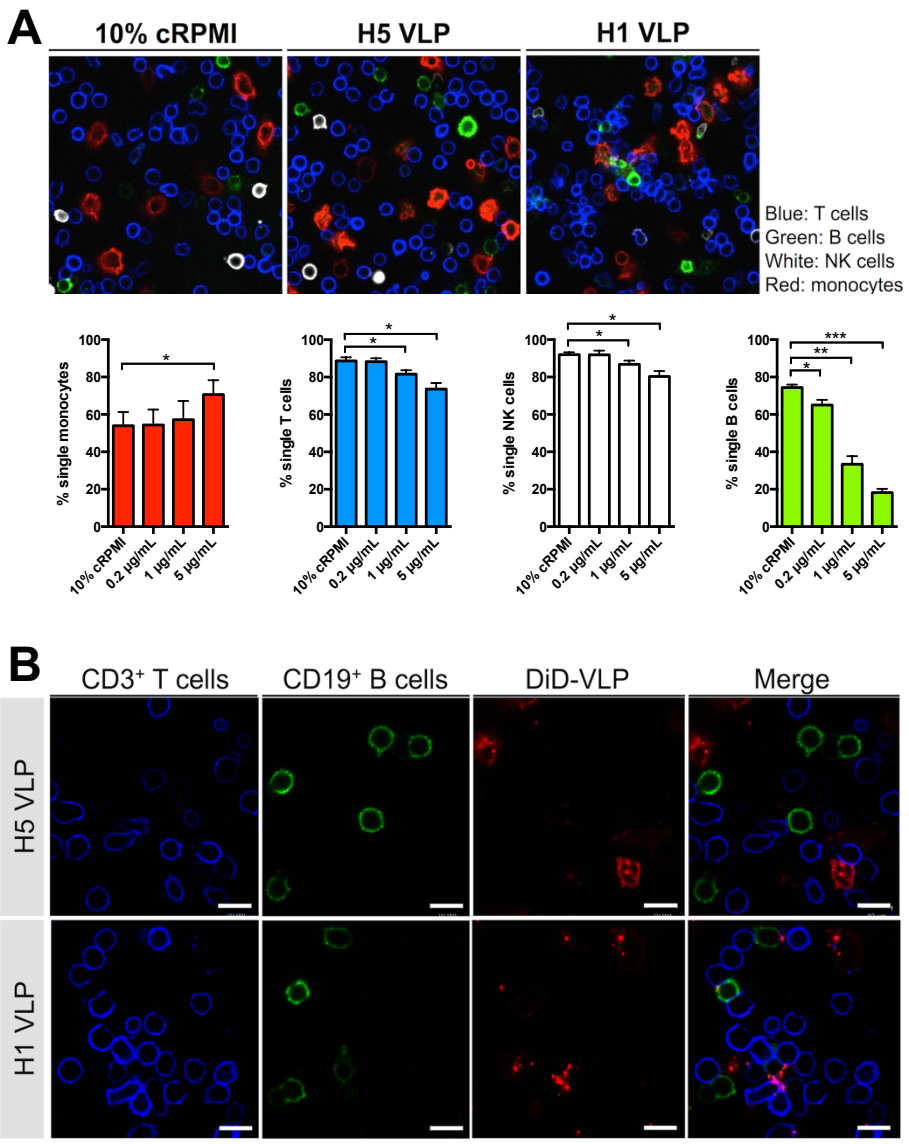


Figure 2.2. Binding of the H1-VLP facilitates intercellular interactions. For imaging, 1.5x10^6 PBMC were co-incubated with 5 µg/mL of unstained VLP (A; top panel) or DiD-labeled VLP (B) for 30 min (37°C). Cells were seeded on a 0.7mm coverslip coated with poly-L-lysine (0.005%) and then fixed with 2µg/mL PFA. PBMC were stained with monoclonal antibodies targeting: CD56, NK cells (A only); CD14, monocytes (A only); CD19, B cells; and CD3, T cells. Images were acquired using a Zeiss LSM780 laser scanning confocal microscope (A: 40X, B: 100X; scale bar: 10 µm). To quantify cell clustering (A; bottom panel), 1x10^6 PBMC were co-incubated with 0.2-5 µg/mL of unstained H1-VLP (30 min, 37°C). Flow cytometry was used to distinguish singlets from clustered cells, and data are presented as the proportion of single cells within each cell subset. Error bars represent the SEM, n=4. Statistical significance was determined by one-way ANOVA with the Greenhouse-Geisser correction (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

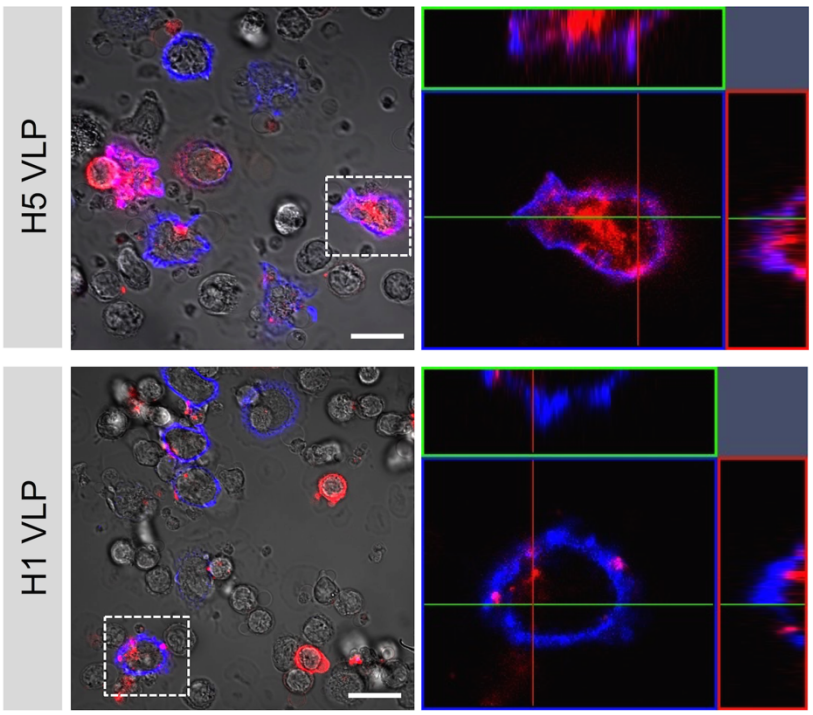


Figure 2.3. The H1- and H5-VLP vaccines are rapidly internalized by CD14^{+} monocytes. 1.5x10^{6} PBMC were co-incubated with DiD-labeled H1- or H5-VLPs for 30min (37°C). Cells were seeded on a 0.7mm coverslip coated with poly-L-lysine (0.005%) and then fixed with 2µg/mL PFA. Monocytes were stained with anti-CD14 and anti-mouse IgG1 DyLight 405. Left panel: Representative images demonstrating the interactions between VLP (red) and monocytes (blue). Z-stacks were acquired for 3D rendering of targeted cells and orthogonal views across these stacks demonstrate internalization of VLPs (right panel). Images were acquired using a Zeiss LSM780 laser scanning confocal microscope (100X, scale bar: 10 µm).

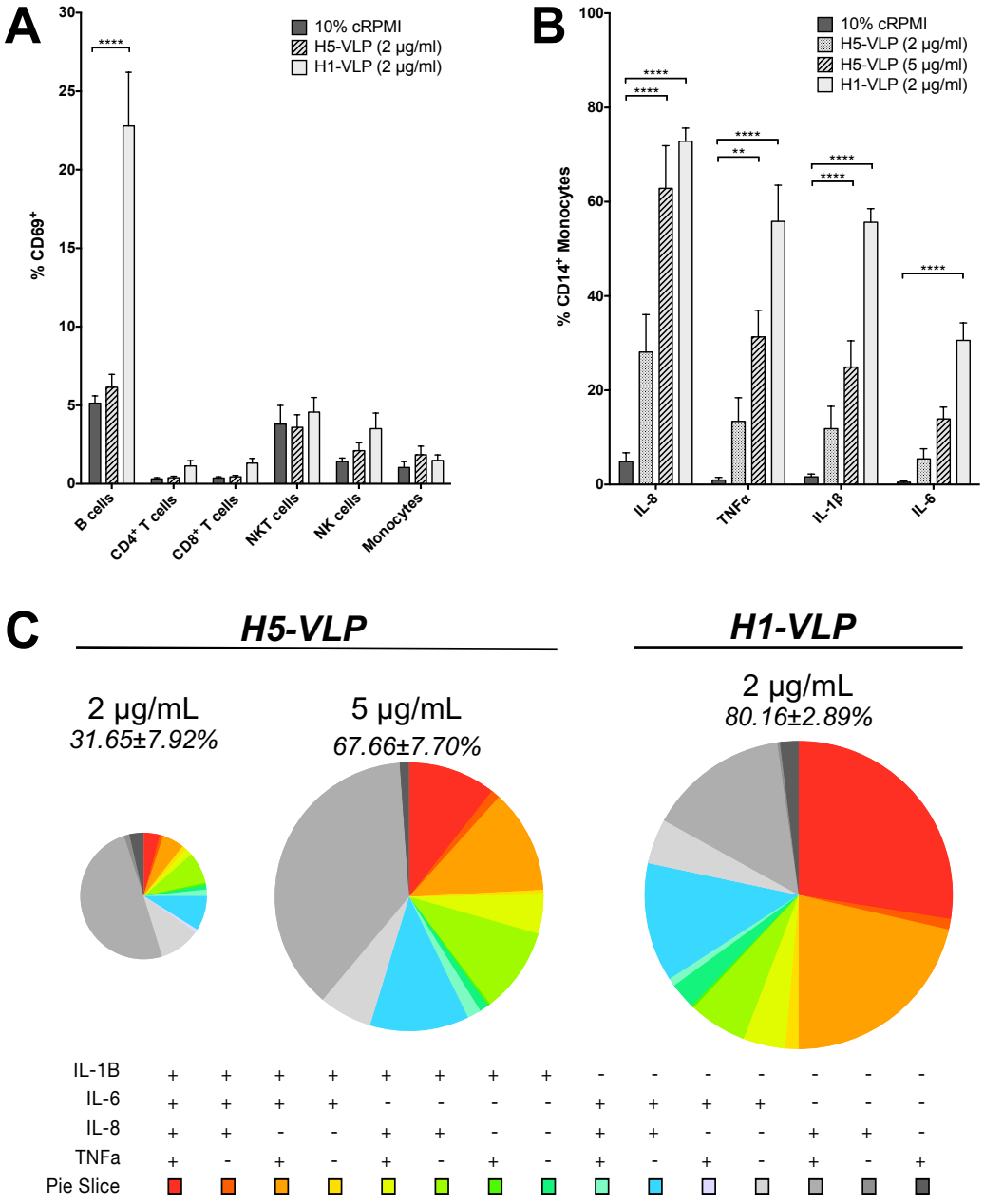
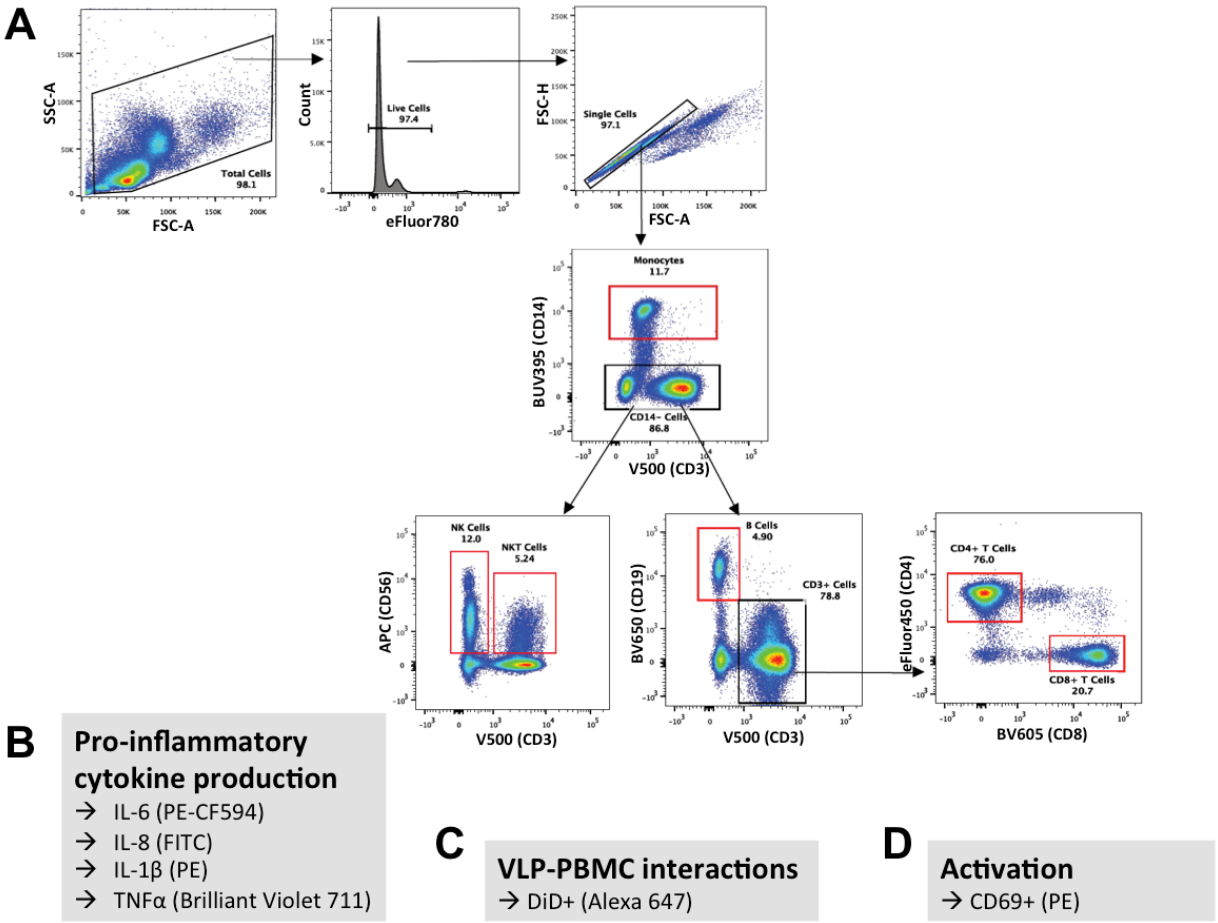
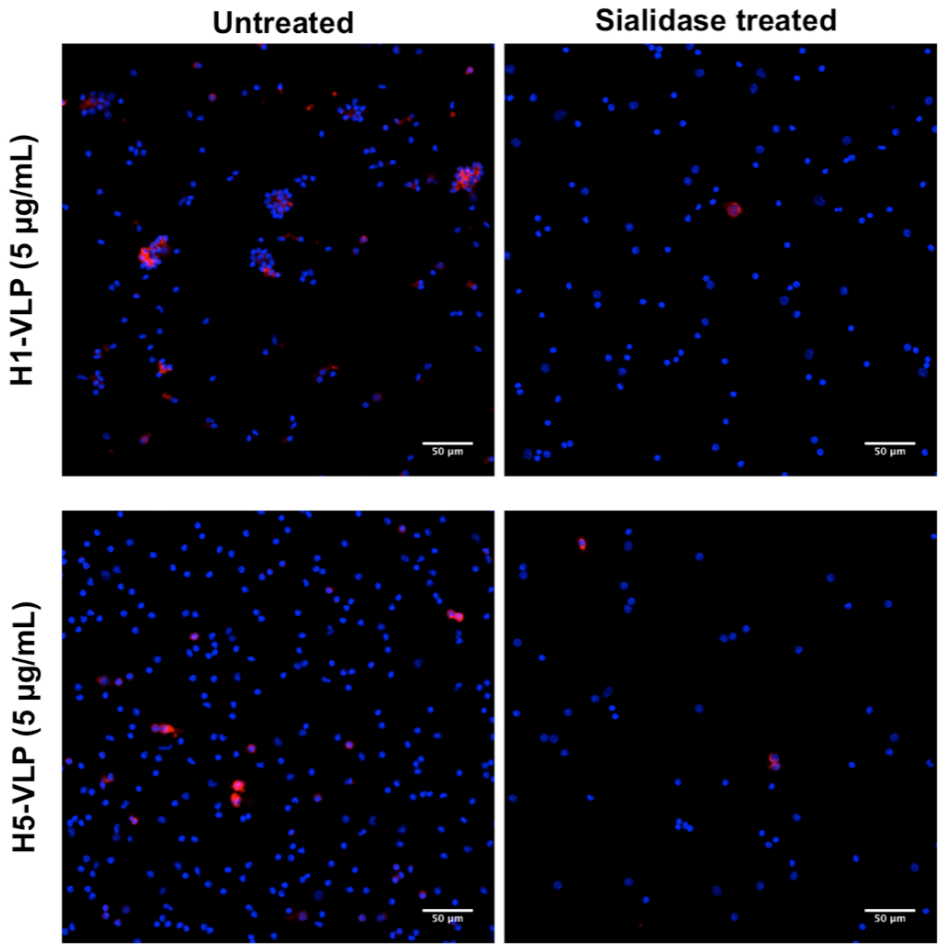


Figure 2.4. The H1- and H5-VLPs elicit distinct patterns of cell activation and pro-inflammatory cytokine production. 1x10^6 PBMC were stimulated with H1- or H5-VLP for 6h (37°C) and responses were detected by flow cytometry. (A) CD69 expression presented as the proportion of CD69$^+$ cells within each PBMC sub-population. (B) Frequency of CD14+ monocytes expressing IL-1β, IL-6, IL-8, or TNFα. (C) Cytokine signatures of CD14$^+$ monocytes that produce ≥1 cytokine. Populations were determined by Boolean gating (FlowJo, Treestar). The size of pie charts is adjusted to the size of the responding population (numerical value over each pie). Error bars represent the SEM, n=8. Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons test (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

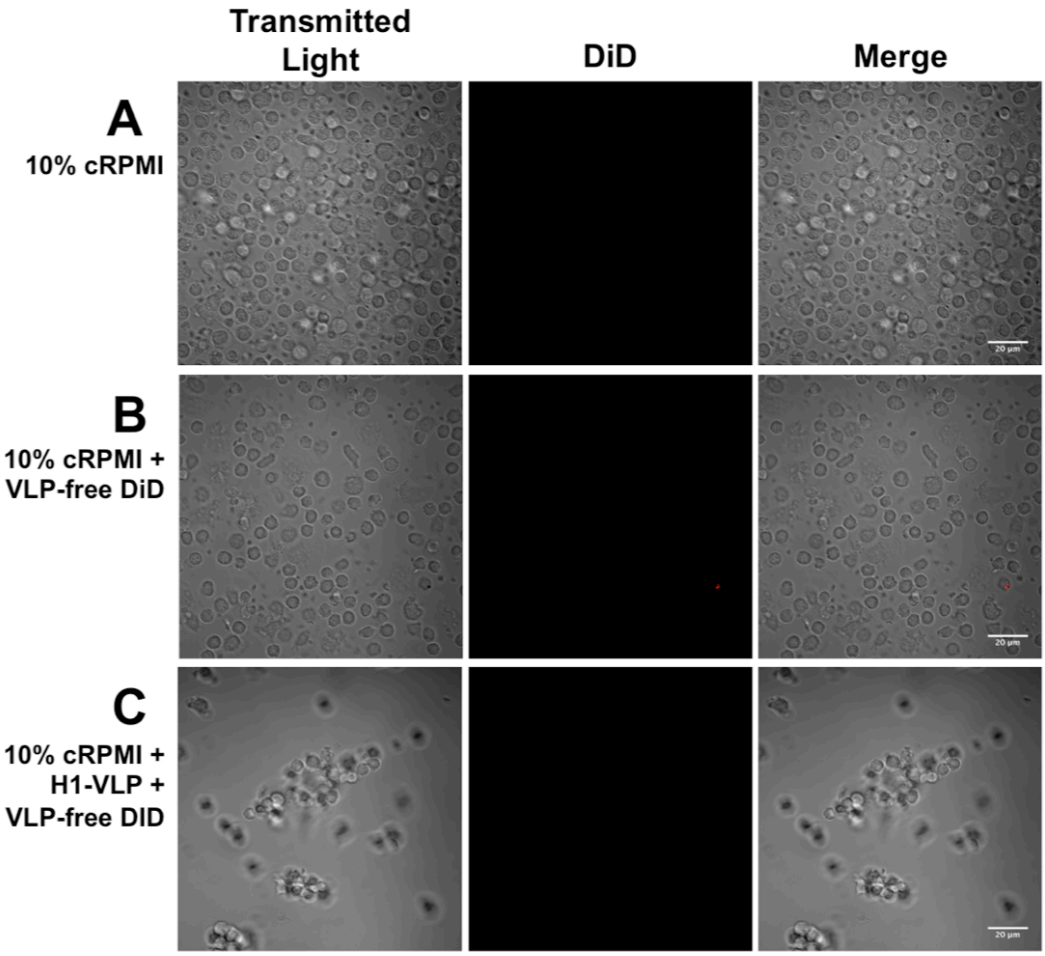
2.10 SUPPLEMENTAL FIGURES AND LEGENDS



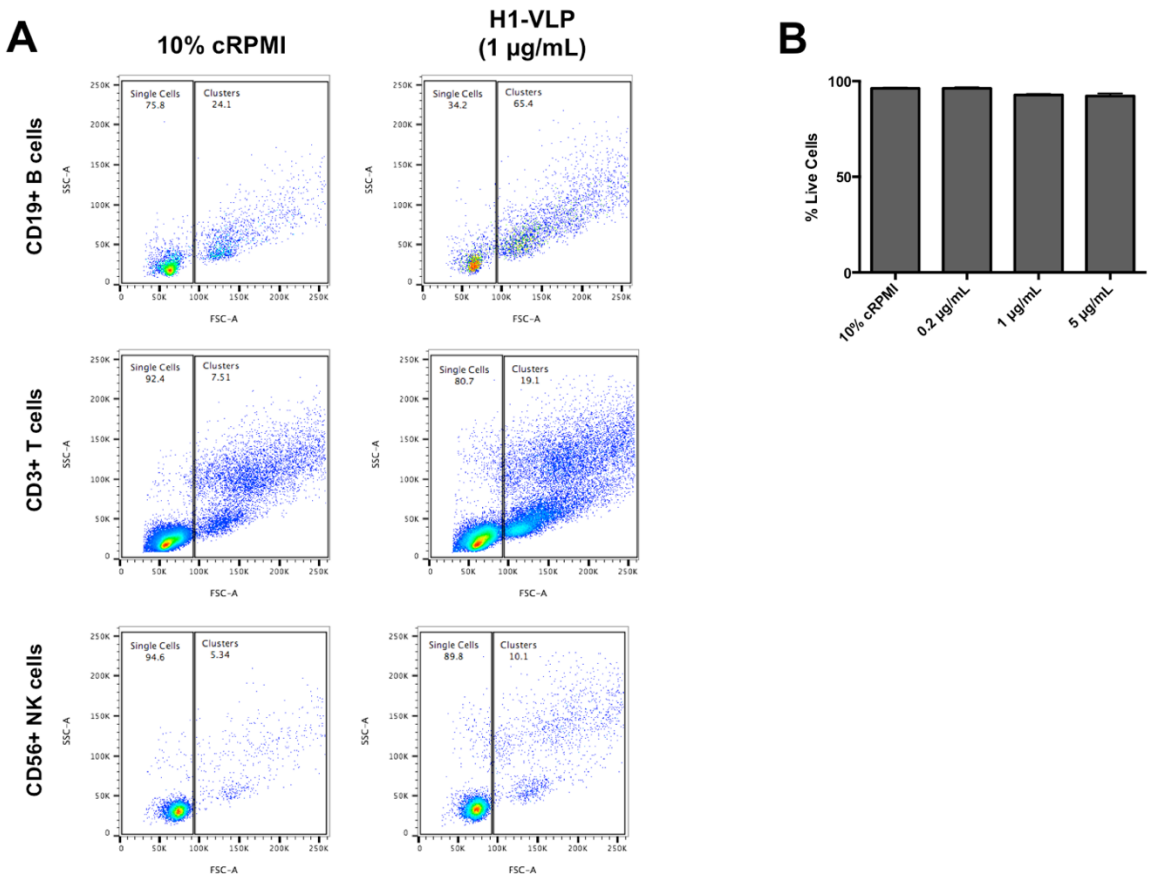
Supplemental figure 2.1. Flow cytometry gating strategy. All samples assessed by flow cytometry were initially gated as shown in (A). Cells were gated to exclude debris, viable events were selected (efluor780-), and doublets were excluded. The following markers were used to distinguish immune cell subsets (red gates): $CD14^+$ (monocytes), $CD19^+$ (B cells), $CD3^+CD4^+$ ($CD4^+$ T cells), $CD3^+CD8^+$ ($CD8^+$ T cells), $CD3^-CD56^+$ (NK cells) and $CD3^+CD56^+$ (NKT cells). Pro-inflammatory cytokine-producing cells (B) VLP-bound immune cells (C), and activated cells ($CD69^+$) (D) were measured within each immune cell subset.



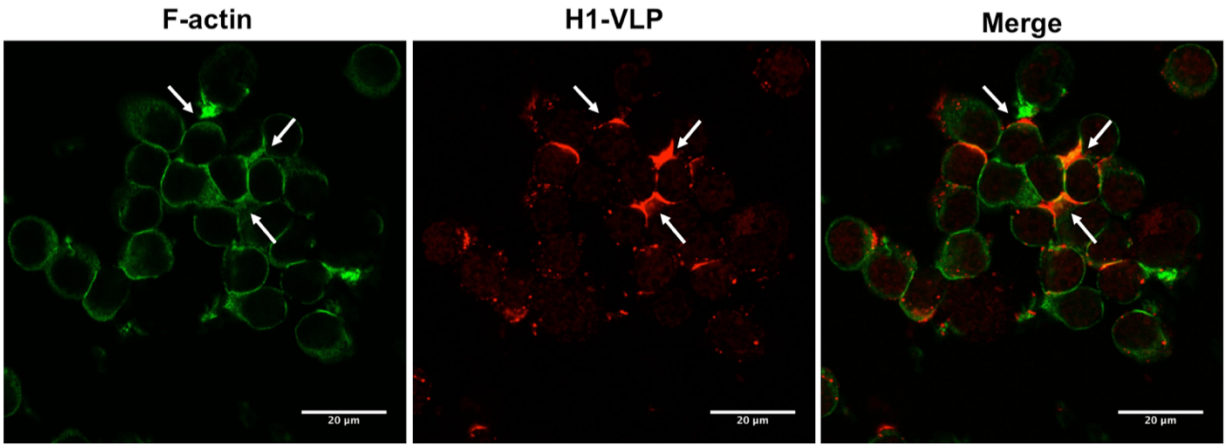
Supplemental figure 2.2. Sialidase treatment of PBMC reduces H1- and H5-VLP binding to human PBMC and eliminates cell clustering mediated by the H1-VLP. 1x10^6 PBMC were incubated with 250 mU sialidase from *Vibrio cholerae* (Sigma) or PBS for 2h at 37°C. Cells were washed 3x in PBS and then incubated with DiD-labeled H1- or H5-VLP (30 min, 37°C). Cells were fixed using 4% PFA and mounted on gelatin-coated microscope slides. Cells were counterstained using DAPI (Thermo Fisher). Images were acquired using a Zeiss LSM780 laser scanning confocal microscope (20X objective).



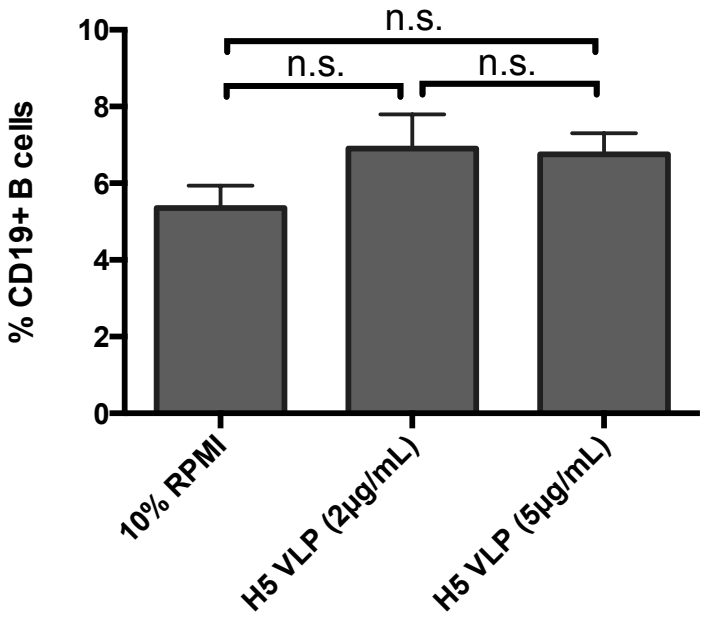
Supplemental figure 2.3. DiD-labelling controls for confocal microscopy. $1.5x10^6$ PBMC were co-incubated for 30 min with 10% cRPMI, VLP-free DiD, or unlabeled H1-VLP (5 µg/mL) in the presence of VLP-free DiD. VLP-free DiD was prepared by dilution in PBS and purification by size exclusion chromatography (see protocol for labeling H1- and H5- VLP). The volume of VLP-free DiD added to each sample was equivalent the volume required for stimulation with 5 µg/mL DiD-labeled H1-VLP. Images were acquired using a Zeiss LSM780 laser scanning confocal microscope.



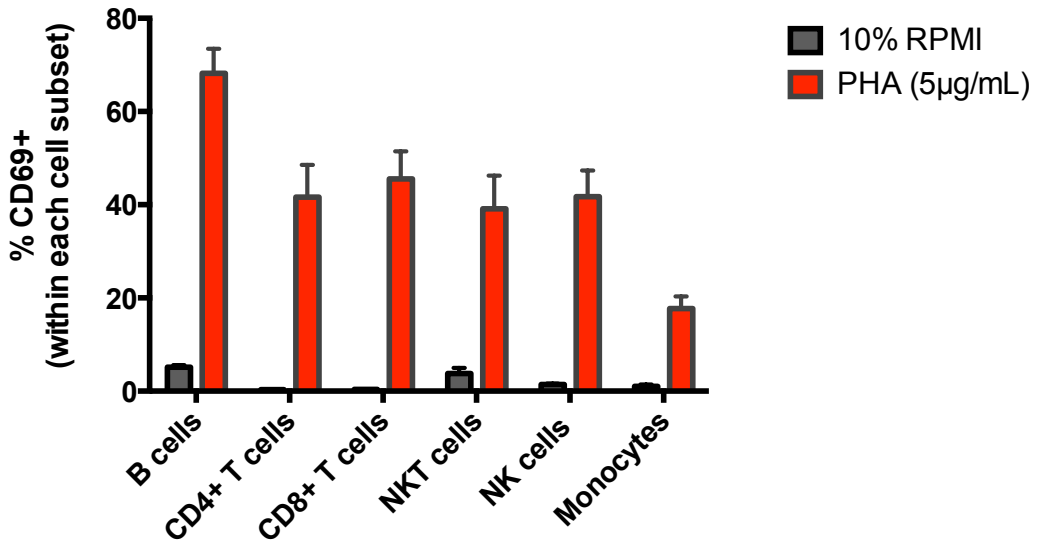
Supplemental figure 2.4. Flow cytometry to quantify PBMC clustering. PBMC were incubated +/- H1-VLP for 30 min at 37°C. Viability and clustering were measured by flow cytomtery. (A) Representative dot plots demonstrating the use of forward scatter properties (cell size) to distinguish single cells from clustered cells. Decrease in single cells is accompanied by an increase in cell clusters. (B) Total cell viability was measured following stimulation with increasing concentrations of the H1-VLP. Stimulation with the H1-VLP does not affect cell viability. Error bars represent the SEM, n=4.



Supplemental figure 2.5. Actin localization following stimulation with the H1-VLP. $5x10^5$ PBMC were incubated with 5 µg/mL H1-VLP for 30min at 37°C. Cells were attached to poly-L-lysine coated glass slides by cytospin and fixed with 4% PFA. VLP were labeled overnight (mouse anti-HA) and detected by secondary antibody conjugated to AlexaFluor405 (Biolegend). F-actin was labeled using Alexa Fluor 488 phalloidin (Thermo Fisher). White arrows indicate actin polarization towards VLP-rich foci. Images were acquired using a Zeiss LSM780 laser scanning confocal microscope (100X objective).



Supplemental figure 2.6. Stimulation with the H5-VLP does not elicit B cell activation. $1x10^6$ PBMC were stimulated with H5-VLP (2µg/mL or 5µg/mL) for 6h (37°C) and CD69 was detected by flow cytometry. Data are presented as the proportion of B cells that are $CD69^+$. Error bars represent the SEM, n=6. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. (*n.s. P>0.05*).



Supplemental figure 2.7. CD69 is expressed by all PBMC subsets following stimulation with 5µg/mL PHA. $1x10^6$ PBMC were stimulated with PHA (5µg/mL) for 6h (37°C) and CD69 was detected by flow cytometry. Data are presented as the proportion of $CD69^+$ cells within each cell sub-population. Error bars represent the SEM, n=8.

PREFACE TO CHAPTER 3

In **chapter 2**, we described the interactions of VLPs bearing avian (H5) and mammalian (H1) hemagglutinins (HA) with subsets of human immune cells. We demonstrated that the patterns of interaction were strongly influenced by the differing sialic acid (SA) preferences of H5 and H1 and that these initial binding events strongly influenced downstream activation. These findings suggested that HA binding properties may play a previously unappreciated role in the context of influenza vaccine responses and motivated us to investigate the impact of HA-SA interactions *in vivo*. To accomplish this, we generated a novel 'non-binding' H1-VLP vaccine using a well-characterized HA mutation (Y98F) that prevents binding to SA without impacting antigenicity. The following chapter describes the humoral and cellular responses elicited by the 'non-binding' H1-VLP ($H1_{Y98F}$-VLP) and the wild-type H1-VLP ($H1_{WT}$-VLP). In addition, we compared the efficacy of each vaccine by evaluating viral clearance and pulmonary inflammation following challenge in vaccinated mice. These proof-of-principle studies highlight the profound impact of the HA-SA interaction on the immunogenicity and efficacy of an H1-VLP in mice.

This chapter was adapted from the following manuscript: Elimination of receptor binding by influenza hemagglutinin improves vaccine-induced immunty. **Hendin, H.E.** *et al.* (under review at *npj Vaccines*).

CHAPTER 3

Elimination of receptor binding by influenza hemagglutinin improves vaccine-induced immunity

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*Manuscript under review at *npj Vaccines*

3.1 SUMMARY

Influenza vaccines are limited by variable immunogenicity and rapidly waning protection. Hendin *et al*. demonstrate that preventing hemagglutinin in vaccines from binding to host sialic acids improves both the quality and durability of the antibody response and enhances protection.

3.2 ABSTRACT

The binding of influenza hemagglutinin (HA) to sialic acid (SA) receptors plays a well-defined role in shaping infection but the impact of such binding on vaccine responses has not yet been explored. We generated a virus-like particle (VLP) vaccine bearing the HA of H1N1 A/California/07/09 that is unable to bind to its α2,6-linked SA (H1$_{Y98F}$-VLP) receptor and compared its immunogenicity and efficacy to a wild-type H1-VLP (H1$_{WT}$-VLP) in mice. The H1$_{Y98F}$-VLP elicited significantly stronger and more durable antibody responses (hemagglutination inhibition and microneutralization titers) and greater avidity maturation, likely attributable to improved germinal center formation. H1$_{Y98F}$-VLP also resulted in a robust population of IL-2^{+}TNFα^{+}IFNγ^{-} CD4^{+} T cells that correlated with antibody responses. Compared to H1$_{WT}$-VLP vaccination, mice immunized with H1$_{Y98F}$-VLP had 2.3-log lower lung viral loads and significantly lower pulmonary inflammatory cytokine levels 5 days post-challenge. These findings suggest that abrogation of HA-SA interactions may be a promising strategy to improve the quality and durability of influenza vaccine-induced humoral responses.

3.3 INTRODUTION

Vaccination is widely recognized as the most effective way to prevent influenza infection and to reduce the societal and economic burden of seasonal influenza epidemics. However, influenza vaccines have remained largely unchanged since their introduction in the mid-1940s[1,2] despite significant year-to-year and strain-to-strain variation in efficacy[3,4]. This inconsistency raises concerns surrounding pandemic preparedness and highlights the need for novel and more reliable approaches to influenza vaccination.

Influenza hemagglutinin (HA) is a trimeric glycoprotein on the surface of all influenza viruses that initiates infection by binding to sialic acid (SA) receptors on the surface of respiratory epithelial cells[5]. Antibodies that bind to the receptor binding domain (RBD) of HA can block this initial interaction with the host cell and are the basis for the most widely used correlate of protection for influenza infection[6,7]. Thus, HA proteins are therefore the major and, in some cases, the only antigens in all commercial influenza vaccines[8]. The binding properties of influenza HA are strain-specific and have been extensively studied in the context of disease severity and transmissibility[5,9-11]. However, the implications of HA binding properties on influenza vaccine immunogenicity and efficacy have not been investigated despite the presence of SA receptors on the surface of cells throughout the body[12-14].

We previously demonstrated that the strain-specific binding properties of influenza HA influence the pattern of interaction of HA-bearing virus-like particles (VLP) with human peripheral blood mononuclear cells (PBMC) *in vitro*[15]. These interactions were driven by differential expression of α2,3- and α2,6-linked SA on human PBMC and strongly influenced downstream innate immune activation[15], raising the possibility that the binding properties of HA may be important modulators of influenza vaccine immunogenicity.

In the current work, we sought to determine whether HA-SA interactions influence vaccine responses *in vivo.* We generated a novel plant-based HA-VLP vaccine bearing H1 (A/California/07/2009) that was unable to bind SA and compared its immunogenicity and efficacy to the wild-type (WT) H1-VLP using a murine model. Mutation of H1 binding was achieved by a single amino acid substitution from tyrosine to phenylalanine at residue 98 (Y98F, H3 numbering), which prevents HA-SA interactions by eliminating the hydroxyl group required for hydrogen bonding with SA. This mutation was originally described by Martín *et al.* using H3 (A/Aichi/2/68)[16] but has subsequently been shown to prevent SA binding of many influenza A HAs, including H1 (A/California/07/2009), due to its position within a conserved region of the RBD[17]. This mutation does not affect the integrity of the RBD or HA folding[17,18] making the H1$_{Y98F}$-VLP a good candidate for studying the possible impact of HA-SA interactions on vaccine responses. Herein, we demonstrate for the first time that elimination of HA-SA interactions significantly improves both the immunogenicity and efficacy of a plant-based H1-VLP vaccine.

3.4 RESULTS

3.4.1 Generation and validation of H1$_{Y98F}$-VLP

Virus-like particles (VLP) composed of a plant-lipid bilayer studded with the WT H1 (H1$_{WT}$-VLP) or Y98F H1 (H1$_{Y98F}$-VLP) were expressed in *Nicotiana benthamiana* as previously described using a 2X35S/CPMV160/NOS expression system[19-21] (**figure 3.1a**). Expression of HA in crude leaf digests was confirmed by western blot (**figure 3.1b**). Following purification, VLP preparations were separated by gel electrophoresis and visualized using Coomassie G-250 staining to evaluate the protein composition and purity (**figure 3.1c)**. HA was predominantly expressed in its uncleaved form (HA0), however, faint bands corresponding to cleaved HA (HA1 and HA2) and HA dimers were observed. The purity of the HA-VLP products was determined by analysis of the densitometry profile of each protein band and was comparable between formulations (~95%). VLP size (~100nm) and morphology were consistent with previous reports[20,22] and were unaffected by the Y98F mutation (**figure 3.1d**).

Due to multi-valent HA expression, H1$_{WT}$-VLPs are capable of mediating hemagglutination by binding to SA on the surface of red blood cells (RBC). Introduction of the Y98F mutation prevented hemagglutination indicating that SA binding is greatly reduced or absent (**figure 3.1e**). Furthermore, H1$_{Y98F}$-VLP failed to agglutinate human peripheral blood mononuclear cells (PBMC) and dramatically reduced polyclonal B cell activation that occurs following binding of the H1$_{WT}$-VLP[15,23] (**supp. figure 3.1**). This was the first indication that reduced binding was able to modulate downstream immune responses. To confirm that the Y98F mutation inhibits HA-SA interactions on a molecular level, we measured binding of H1$_{WT}$- and H1$_{Y98F}$-VLP to immobilized SA by surface plasmon resonance (SPR). The slope of the association curve was 1.66 resonance units per second (RU/s) for H1$_{WT}$-VLP and 0.3 RU/s for H1$_{Y98F}$-VLP (**figure 3.1f**). Importantly, H1$_{Y98F}$-VLP was tested at a much higher concentration than H1$_{WT}$-VLP (44x). When the slopes were adjusted for HA content, a 99.6% reduction in binding was attributable to the Y98F mutation (H1 0.59 RU/s; Y98F H1 0.002 RU/s) (**figure 3.1g**). Taken together, these data suggested that H1$_{Y98F}$-VLP was an appropriate tool to evaluate the role of HA-SA interactions in the context of influenza vaccine responses.

3.4.2 H1$_{Y98F}$ -VLP elicits stronger and more durable humoral responses

To establish whether HA-SA interactions influence the humoral immune response to vaccination in mice, we measured the development of H1-specific antibodies in sera following vaccination with 3µg of H1$_{WT}$-VLP or H1$_{Y98F}$-VLP or an equivalent volume of PBS (placebo). Total H1-specifc IgG was measured by ELISA and functional antibodies were measured using the hemagglutination inhibition (HI) assay to measure antibodies that block the binding of live virus to avian RBCs[24] and the microneutralization (MN) assay to measure antibodies that prevent infection of Madin-Darby Canine Kidney (MDCK) cells[25]. Antibodies were measured at 21 days post-vaccination (dpv) to characterize pre-challenge humoral responses and on a monthly basis (1-3m, 7m) to evaluate the kinetics and durability of the antibody responses.

Immunization with H1$_{WT}$-VLP or H1$_{Y98F}$-VLP resulted in comparable H1-specific IgG titers by ELISA at all time points (**figure 3.2a**) but there were marked differences in antibody functionality. Most notably, vaccination with H1$_{Y98F}$-VLP resulted in significantly higher HI and MN titers at 21dpv (p=0.002 and p<0.001, respectively) (**figure 3.2b-c**). Titers increased until 3 months post-vaccination (mpv) in both groups but were consistently higher among mice vaccinated with H1$_{Y98F}$-VLP. Furthermore, H1$_{Y98F}$-VLP resulted in improved durability of HI titers, which declined dramatically between 3 and 7mpv in mice vaccinated with H1$_{WT}$-VLP. As a result, HI titers were >4-fold higher among mice vaccinated with H1$_{Y98F}$-VLP at 7mpv (p=0.029) (**figure 3.2b**). MN titers were better maintained in the H1$_{WT}$-VLP group at 7mpv but still declined in 4/8 animals and remained significantly lower than mice vaccinated with H1$_{Y98F}$-VLP (p=0.029) (**figure 3.2c**). H1-specific antibodies were not detected in the placebo group.

Vaccination with H1$_{Y98F}$-VLP also resulted in better IgG avidity maturation. Although H1-specific IgG titers plateaued around 2mpv, IgG avidity significantly increased between 2 and 3mpv in mice vaccinated with H1$_{Y98F}$-VLP (p=0.03). As a result, IgG avidity was ~3-fold higher in the H1$_{Y98F}$-VLP group at 3mpv and this difference was maintained until 7mpv (p=0.014) (**figure 3.2d**). No avidity maturation occurred beyond 2mpv in mice vaccinated with H1$_{WT}$-VLP.

Sustained production of high avidity IgG is thought to be mediated by plasma cells (PC) residing in the bone marrow (BM)[26]. To evaluate whether these cells contribute to the improved durability of antibody responses in mice vaccinated with H1$_{Y98F}$-VLP, we quantified H1-specific IgG-producing BM PC at 7mpv by enzyme-linked immune absorbent spot (ELISpot) assay. Few

H1-specific BM PC were detected in the placebo group (2±1 PC/1x10^6 cells). H1-specific BM PC were detected in both vaccine groups and the frequency was higher among mice vaccinated with H1$_{Y98F}$-VLP, although this difference did not reach statistical significance (**figure 3.2e**). Interestingly, the frequency of H1-specific BM PC seemed to correspond with the durability of antibody responses: MN titers declined between 3 and 7mpv in all mice with <10 PC/1x10^6 BM cells and were maintained in animals with ≥10 PC/1x10^6 BM cells regardless of vaccine group (**figure 3.2e**). Furthermore, the frequency of H1-specific BM PC significantly correlated with both HI (r_s=0.604, p=0.015) and MN titers (r_s=0.657, p=0.007) at 7mpv (**figure 3.2f**).

Although adults typically receive a single dose of inactivated influenza vaccine each year, children who are immunologically naïve to influenza require two doses for an adequate immune response[27]. Because laboratory mice are immunologically naive, we evaluated the humoral responses in mice given 2 doses of H1$_{WT}$-VLP or H1$_{Y98F}$-VLP administered 21d apart. By 28d post-boost, HI titers were significantly higher among mice vaccinated with H1$_{Y98F}$-VLP (p=0.02) (**supp. figure 3.2a**) and a similar trend was observed in MN titers (**supp. figure 3.2b**). While no significant differences were observed regarding specific IgG titers (**supp. figure 3.2c**) immunization with 2 doses of H1$_{Y98F}$-VLP resulted in significantly higher IgG avidity (**supp. figure 3.2d**, p=0.03 at 4M and 6M urea) and H1-specific IgG-producing PC in the BM by 28d post-boost (p=0.02) (**supp. figure 3.2e**). Once again, the frequency of BM PC was strongly correlated with HI titers, MN titers, and IgG avidity in both vaccine groups (**supp. figure 3.2f**). Improved humoral responses were also observed among mice vaccinated with two doses of a plant-based non-binding H1$_{Y98F}$-VLP based on the H1 A/Idaho/07/2018 sequence (**supp. figure 3.3a-c**) and cell culture-based H1$_{Y98F}$ trimers based on the A/Brisbane/02/2018 sequence (**supp. figure 3.4**), suggesting that HA-SA interactions broadly impact humoral responses to H1.

3.4.3 H1$_{Y98F}$-VLP improves germinal center reactions

The germinal center (GC) is central to the development of high avidity antibodies and long-lived PC. To determine whether differences in the GC account for improved antibody responses to H1$_{Y98F}$-VLP, we evaluated GC kinetics in the draining popliteal lymph node (pLN) following footpad injection of H1$_{WT}$- or H1$_{Y98F}$-VLP. The frequencies of GC B cells ($CD19^+GL7^+Fas^+$) and T_{FH} cells ($CD3^+CD4^+CXCR5^+PD\text{-}1^+$) were evaluated by flow cytometry at 3d intervals 7-19dpv

(see **supp. figure 3.5** for full gating strategy). Both VLPs resulted in similar frequencies of GC B cells at all time points and peaked at 13dpv (**figure 3.3a**). However, striking differences in T_{FH} frequencies and kinetics were observed (**figure 3.3b**). H1$_{Y98F}$-VLP resulted in more rapid induction of T_{FH} cells that were maintained until 19dpv. In contrast, the H1$_{WT}$-VLP resulted in a gradual expansion of T_{FH} cells until 13dpv followed by a rapid decline. As a result, the frequency of T_{FH} cells was significantly higher among H1$_{Y98F}$-VLP-vaccinated mice in early and late GCs (7dpv p=0.017; 19dpv p=0.03). No increase in GC B cells or T_{FH} cells was observed at any time point among control mice injected with PBS (**supp. figure 3.6**).

Given the importance of T_{FH} cells in avidity maturation of GC B cells, we next sought to determine whether vaccination with H1$_{Y98F}$-VLP results in improved recognition of the H1 antigen among GC B cells. Antigen-specific GC B cells were distinguished based on their ability to bind H1$_{Y98F}$-VLP *in vitro* and were detected using fluorescently labeled anti-H1. This strategy allows for reliable detection of cognate B cells while avoiding SA-mediated interactions with non-cognate cells. H1-specific GC B cells were quantified prior to vaccination and at intervals corresponding to early (7dpv), peak (13dpv) and late (19dpv) GC evolution (**figure 3.3c-d**). Prior to vaccination GC B cells were rare in all groups (<1% of B cells) and did not bind the H1$_{Y98F}$-VLP probe (**figure 3.3c**). Following vaccination, H1-specific GC B cells were readily detected in both vaccine groups, however, H1$_{Y98F}$-VLP resulted in an increased frequency of these cells within the GC at all time points. This effect was most pronounced at 19dpv when the frequency of H1-specific B cells in the GC was ~30% higher among mice vaccinated with H1$_{Y98F}$-VLP compared to H1$_{WT}$-VLP (p=0.011) (**figure 3.3d**). Among non-GC B cells ($CD19^+GL7^-Fas^-$), the frequency of H1-specific cells was similar between vaccine groups and was comparable to pre-vaccination levels (**supp. figure 3.7**). Taken together, H1$_{Y98F}$-VLP resulted in an enhanced GC reaction with improved maintenance of T_{FH} cells and expansion of H1-specific B cells within the GC.

3.4.4 $CD4^+$ T cell response to H1$_{Y98F}$-VLP correlates with antibody responses

Antigen-specific $CD4^+$ T cells in the spleen and BM were quantified by flow cytometry following vaccination with H1$_{WT}$-VLP or H1$_{Y98F}$-VLP. Freshly isolated cells were stimulated with H1$_{WT}$-VLP (18h) or a pool of 131 overlapping peptides (15aa) spanning the H1 sequence (6h) and responding cells were characterized as antigen-experienced ($CD44^+$) $CD4^+$ T cells expressing

IL-2, IFNγ and/or TNFα (see **supp. figure 3.8** for full gating strategy). The frequencies of H1-specific $CD4^+$ T cells observed following stimulation with either the $H1_{WT}$-VLP or the H1 peptide pool were highly correlated (r_s=0.5344, p<0.0001) and had similar cytokine signatures (**supp. figure 3.9**). However, the magnitude of the response was greater upon stimulation with the $H1_{WT}$-VLP, allowing for more reliable identification of rare populations. Thus, functional signatures were evaluated in cells stimulated with $H1_{WT}$-VLP.

A single 3μg dose of $H1_{WT}$-VLP or $H1_{Y98F}$-VLP elicited significant populations of H1-specific $CD4^+$ T cells in the spleen compared to placebo at 28d post-vaccination (p=0.01 and p=0.009, respectively). There were no differences in the magnitude or functional signatures between vaccine groups (**figure 3.4a**). Both vaccines elicited significant populations of IL-$2^+TNF\alpha^+IFN\gamma^-$ $CD4^+$ T cells ($H1_{WT}$ p=0.009; $H1_{Y98F}$ p=0.01) and IFNγ single-positive cells compared to the placebo group ($H1_{WT}$ p=0.02; $H1_{Y98F}$ p=0.002). In contrast, vaccination with two 0.5μg doses 21d apart resulted in resulted in marked differences in $CD4^+$ T cell signatures between vaccine groups (**figure 3.4b**). In the spleen, the IL-$2^+TNF\alpha^+IFN\gamma^-$ population continued to account for the majority of polyfunctional cells. However, this population was significantly larger in mice vaccinated with $H1_{Y98F}$-VLP (p=0.01). Conversely, $IFN\gamma^+$ cells were more prevalent among H1-specific $CD4^+$ T cells elicited by $H1_{WT}$-VLP. It should be noted that the frequency of $IFN\gamma^+$ cells was low in both groups despite substantial $IFN\gamma^+$ populations elicited by a single 3μg dose of either vaccine. This discrepancy likely reflects the lower antigen dose in the two-dose regimen, as the magnitude of the $CD4^+$ T cell response to a plant-based $H1_{WT}$-VLP is dose dependent in humans[28]. As expected, $IFN\gamma^+$ and polyfunctional populations were markedly increased in mice that received two 3μg doses, but functional signatures remained unchanged (**supp. figure 3.10**). Furthermore, similar signatures were observed following vaccination with $H1_{WT}$- and $H1_{Y98F}$-VLPs targeting H1 A/Idaho/07/2018 suggesting that HA-SA interactions broadly influence H1-specific $CD4^+$ T cell responses (**supp. figure 3.3d-e**).

Antigen-specific $CD4^+$ T cells were also measured in the BM, which serves as a major reservoir for long-term maintenance of memory $CD4^+$ T cells following vaccination[29]. Immune cells were isolated from bilateral femurs 28d post-boost and evaluated in parallel with splenocytes. Overall, cytokine signatures in the BM resembled those observed in the spleen, however, only the $H1_{Y98F}$-VLP resulted in a significant increase in the frequency of H1-specific $CD4^+$ T cells

compared to the placebo group (p=0.03) (**figure 3.4c**). The frequency of H1-specific $CD4^+$ T cells in the BM strongly correlated with the frequency of responding cells in the spleen in mice vaccinated with $H1_{Y98F}$-VLP (r_s=1.00, p=0.0167) but not $H1_{WT}$-VLP (r_s=-0.1, p=0.95), suggesting that a defect in recruitment contributes to the low frequency of H1-specific $CD4^+$ T cells in the BM of $H1_{WT}$-VLP-vaccinated mice (**figure 3.4d**). Similar to the spleen, the response to Y98F H1-VLP was dominated by IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells and IFNγ-expressing cells were virtually absent. Conversely, nearly 30% of BM $CD4^+$ T cells elicited by $H1_{WT}$-VLP were IFNγ^+ and IL-2^+TNFα^+IFNγ^- cells were underrepresented in the BM compared to the spleen (24 vs. 32% of responding cells) (**figure 3.4c**).

Given that $CD4^+$ T cells with the IL-2^+TNFα^+IFNγ^- phenotype have been shown to correlate with antibody responses to a number of vaccine antigens[30,31], we sought to determine whether expansion of this population correlated with improved humoral responses among mice vaccinated with $H1_{Y98F}$-VLP. In the spleen, the frequency of IL-2^+TNFα^+IFNγ^- cells correlated with IgG avidity maturation in mice vaccinated with $H1_{Y98F}$-VLP (r_s=0.6809, p=0.0355) but not $H1_{WT}$ -VLP (r_s=0.07976, p=0.8355) (**figure 3.4e**). However, in the BM, this T cell population was strongly correlated with HI titers in both vaccine groups (r_s=0.7957, p=0.008), suggesting that enhanced recruitment of IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells to the BM may contribute to enhanced humoral immune responses in mice vaccinated with $H1_{Y98F}$-VLP (**figure 3.4f**).

3.4.5 $H1_{Y98F}$-VLP results in reduced viral load and pulmonary inflammation following homologous challenge

Mice were challenged with $1.6x10^3$ times the median tissue culture infectious dose ($TCID_{50}$) of H1N1 (A/California/07/09) 28 days post-vaccination with a single dose of 3µg $H1_{WT}$- or $H1_{Y98F}$-VLP or an equivalent volume of PBS. Infection resulted in substantial weight loss (17.3±1.3% at d5) and 64.3% mortality in the placebo group. All mice vaccinated with $H1_{WT}$-VLP or $H1_{Y98F}$-VLP survived and there was no significant difference in post-infection weight loss (4-6% at d5) (**figure 3.5a**). However, significant differences in the rate of viral clearance and pulmonary inflammation were observed.

To evaluate viral clearance, lungs were collected at 3d post-infection (dpi) or 5 dpi and the $TCID_{50}$ of lung homogenates was determined (**figure 3.5b**). Vaccination with $H1_{Y98F}$-VLP, but not

H1$_{WT}$-VLP, resulted in a decrease in the viral titer compared to placebo at 3dpi (p<0.001). In both groups, viral titers were inversely correlated with HI and MN titers (HI r_s=-0.695, p=0.001; MN r_s=-0.7067, p=0.001). By 5dpi, viral titers in mice vaccinated with H1$_{Y98F}$-VLP were nearly 2-log lower than mice vaccinated with H1$_{WT}$-VLP (p=0.03) and there was no significant difference between the placebo and H1$_{WT}$-VLP-vaccinated mice (p=0.31).

Lung homogenates were evaluated by multiplex ELISA (Quansys) to quantify several cytokines (TNFα, IFNγ, IL-1α, IL-1β and IL-6) and chemokines (MCP-1, MIP-1α and RANTES) implicated in influenza-mediated acute lung injury (**figure 3.5c-d**)[32,33]. Baseline levels were established using lung homogenates from mock-infected mice. Overall, vaccination with either H1$_{WT}$-VLP or H1$_{Y98F}$-VLP resulted in reduced cytokines and chemokines compared to the placebo group at 3dpi, but there were no significant differences between the vaccinated groups. However, by 5dpi there was a marked divergence in pulmonary inflammation and all cytokine/chemokine levels except for TNFα were significantly lower in the H1$_{Y98F}$-VLP vaccinated mice compared to the H1$_{WT}$-VLP group. Strikingly, mice vaccinated with H1$_{WT}$-VLP exhibited a significant increase in IFNγ compared to 3dpi (p=0.005) with levels nearing the placebo group (1570±315 and 1887±367pg/mL, respectively). IFNγ levels correlated with the viral titer (r_s=-0.7050, p=0.001) in both vaccinated groups and remained near the baseline in mice that received H1$_{Y98F}$-VLP (172±46pg/mL). IL-1β, MIP-1α and RANTES levels followed similar trends. Consistent with these findings, histopathological evaluation of lungs collected at 4dpi revealed that mice vaccinated with H1$_{Y98F}$-VLP had less pulmonary inflammation compared to H1$_{WT}$-VLP-vaccinated mice and more closely resembled mock-infected animals (**figure 3.5e**). Together, these findings suggest that while both vaccines protected from lethal influenza infection, mice vaccinated with H1$_{Y98F}$-VLP were better able to control and clear the virus and exhibited reduced influenza-associated lung inflammation.

3.5 DISCUSSION

Vaccines are the most effective means of preventing influenza-associated morbidity and mortality, however, their effectiveness is often limited by variable immunogenicity and rapidly waning protection[34,35]. We have demonstrated for the first time that the binding properties of

influenza HA influence vaccine responses and that ablation of HA-SA interactions can improve the quality and durability of immune responses in mice. Non-binding H1 mutants were generated using the previously described Y98F mutation, which prevents SA binding without affecting antibody recognition of the globular head or receptor binding domain (RBD) of HA[17]. Although historically used as probes to identify HA-specific B cells[17,23,36] and more recently in studies targeting the generation of stem-specific antibodies[37,38], the Y98F mutation was not thought to influence immunogenicity. By directly comparing $H1_{WT}$ and $H1_{Y98F}$ antigens in vaccination and challenge models, we have demonstrated that HA-SA binding can have profound impact on influenza vaccine responses.

Antibodies that mediate hemagglutination inhibition (HI) have long been shown to provide protection and are widely considered to be a good predictor of vaccine efficacy. Thus, the induction of high HI titers is important for licensure of influenza vaccines in many jurisdictions[39-41]. Microneutralization (MN) titers also correlate with protection and provide useful insight into the functional neutralizing capacity of vaccine-induced antibodies[42]. In this study we demonstrated that ablation of HA-SA binding has broad impact on the humoral response to vaccination. Consistent with previous reports, we observed a strong correlation between HI and MN titers[42] and pre-infection antibody were inversely correlated with pulmonary peak viral load (3 dpi) in both vaccine groups, suggesting that these antibodies play a direct role in controlling infection. While viral titers do not always correlate with morbidity[43,44], the strong inverse correlations between viral titers and pulmonary inflammatory cytokines and infiltrates suggest that the viral load is a good reflection of disease severity in this model. The improved functional antibody response elicited by $H1_{Y98F}$-VLP is therefore likely to have played an important role in reducing the severity of infection.

Our data suggest that eliminating HA-SA binding may also improve the durability of the antibody response to influenza vaccines. Vaccine-induced HI titers typically decline rapidly post-vaccination[45-48], consistent with our observations in mice that received $H1_{WT}$-VLP. In sharp contrast, HI and MN titers in mice vaccinated with $H1_{Y98F}$-VLP were maintained at peak levels until 7mpv. While the mechanisms underlying this observation are under further investigation, improved specificity and longevity of the GC reaction and an increase in HA-specific PC in the BM likely play an important role. BM PC can persist for decades following antigen exposure in humans and are thought to mediate long-term maintenance of serum antibody titers following

vaccination[18,49]. Our finding that increased frequencies of BM PC correlate with HI and MN titers at 7mpv suggests that this population is central to the durability of humoral response elicited by H1$_{Y98F}$-VLP. Weisel *et al.* have shown that long-lived PC in the BM arise from late GC B cells (18-20d) after a prolonged period of somatic hypermutation and avidity maturation[50]. The increased HA-specific BM PC we found in the H1$_{Y98F}$-VLP group is therefore likely a reflection of the increased HA-specific B cells and T_{FH} cells in the GC of these animals at 19dpv.

Although influenza vaccine developers have focused primarily on humoral responses, antigen-specific T cells undoubtedly play important roles in protection and are often more broadly reactive than antibodies[51]. $CD4^+$ T cells support both B cell affinity maturation and $CD8^+$ T cell responses and can mediate protection in a murine vaccination/challenge model in the absence of neutralizing antibodies[52]. Previous studies have demonstrated that plant-based H1$_{WT}$-VLPs elicit strong $CD4^+$ T cell responses in both mice and humans with increased $CD4^+$ T cell polyfunctionality compared to inactivated vaccines[53-57]. The overall pattern of $CD4^+$ T cell responses in this study were consistent with previous reports and were not compromised in the absence of HA-SA interactions. However, there were no differences between vaccine groups after a single dose, suggesting that enhanced viral clearance in this model was driven primarily by the better humoral response to the H1$_{Y98F}$-VLP vaccine. Interestingly, two doses of H1$_{Y98F}$-VLP resulted in significant expansion of the IL-2^+TNFα^+IFNγ^- population, which was strongly correlated with humoral responses. Others have demonstrated that this population is comprised of Th1 cells and a population of primed but uncommitted T helper cells (Thpp) with high proliferative and differentiation potential[58]. Thpp cells are frequently generated in primary responses to antigens including influenza[58], thus, it is not surprising that both vaccines elicited similar frequencies of IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells after a single dose. However, subsequent exposure to influenza HA typically results in expression of IFNγ[58,59]. Expansion of the IFNγ^- population in the spleen upon boosting with H1$_{Y98F}$-VLP but not the H1$_{WT}$-VLP is reminiscent of the $CD4^+$ T cell responses to protein vaccines such as hepatitis B, diphtheria and tetanus, that are dominated by Thpp cells and elicit robust and durable antibody responses[30,31,60]. To our knowledge, Thpp cells have not been described in the BM and it is unknown whether this population is a common feature of other protein vaccines. However, BM CD4+ T cells are a major reservoir for long-lasting immunity and are known to provide efficient help to B cells[29,61]. Thus, enhanced homing of $CD4^+$ T cells to the

BM may contribute to the enhanced durability of the responses we observed following H1$_{Y98F}$-VLP vaccination.

Beyond the possibility that Y98F HAs behave similarly to traditional protein vaccines, the mechanisms underlying their improved immunogenicity are not yet clear. However, a likely contributor is better trafficking to the draining lymph node (LN) in the absence of SA binding. Terminal α2,3 and α2,6 SAs are widely distributed throughout the body, including on skeletal muscle cells[12,62,63]. Thus, the H1$_{WT}$-VLP and recombinant WT HAs may be more likely to be sequestered at the site of injection or en route to the draining LN due to HA-SA interactions. In support of this hypothesis, previous work has demonstrated that while eGFP-labelled H5-VLP can be detected in the popliteal LN 10 minutes after footpad injection[64], a substantial eGFP signal remains at the site of injection for up to 24h. Soluble eGFP was not retained in the footpad, suggesting that SA binding may contribute to injection site retention[65]. Although depot-type adjuvants that 'trickle' antigen into the draining LN can enhance affinity maturation through prolonged antigen availability in the GC[66], the rapid decline in T_{FH} cells suggests that this is not occurring in mice vaccinated with H1$_{WT}$-VLP. It is possible that H1$_{WT}$-VLPs retained at the injection site are degraded prior to delivery to the draining LN. Thus, retention of H1$_{WT}$ antigen at the site of injection may reduce the concentration of antigen delivered to draining LN without providing sustained antigen presentation. Ongoing investigations aim to characterize the impact of SA binding on trafficking of the H1 antigen to the draining lymph node and handling by antigen presenting cells.

In addition to these mechanistic studies, we are also evaluating the generalizability of these findings with respect to different influenza strain HAs and different production platforms. The fact that a H1$_{Y98F}$-VLP targeting an antigenically distinct H1 (A/Idaho/07/2018) has similar immune effects suggests that eliminating HA-SA interactions may be a promising strategy to improve immunogenicity of vaccines against current and emerging strains of influenza. Confirmatory studies with additional non-binding VLPs (eg: H3N2 and B strains) will be required to evaluate the broad generalizability of this approach. The fact that improved humoral responses were observed with soluble H1$_{Y98F}$ trimers (A/Brisbane/02/2018) suggests that this strategy may confer similar benefits across recombinant vaccine platforms. Although egg-based propagation of influenza A strains bearing Y98F HA has been described[16], mutations that restore HA binding may limit the viability of this approach in platforms requiring the growth of live virus[67]. Finally, since

receptor-binding proteins are often targets of choice for viral vaccines and many of the receptors for these viruses have wide tissue distribution, our observations raise the possibility that manipulation of non-cognate binding interactions may have application beyond influenza vaccines. For example, SA binding is a common feature of many viruses and vaccine antigens including the hemagglutinin-neuraminidase protein of mumps virus[68], the VP8* domain of bovine-human reassortant rotavirus vaccine strains[69], and SARS-CoV-2 spike protein[70].

Taken together, we have demonstrated that altering the binding of HA to its SA receptor can have profound impact on influenza vaccine immunogenicity and efficacy in mice. Elimination of the HA-SA interaction may be a simple, effective and readily implemented strategy to improve both the quality and durability of influenza vaccine responses. Although there is still much to learn mechanistically, if these findings are confirmed in human studies, the use of non-binding HAs may make an important contribution to the development of next-generation influenza vaccines.

3.6 MATERIALS AND METHODS

3.6.1 H1$_{WT}$ and H1$_{Y98F}$ expression cassettes

The sequences encoding mature wild-type (WT) and Y98F HA0 A/California/07/2009 fused to alfalfa PDI secretion signal peptide (PDISP) were cloned into 2X35S/CPMV160/NOS expression system using PCR-based methods. To generate the H1$_{WT}$ expression cassette, the PDISP-A/California/07/2009 coding sequence was amplified using primers IF-CPMV(fl5'UTR)_SpPDI.c (5'-TCGTGCTTCGGCACCAGTACAATGGCGAAAAACGT-TGCGATTTTCGGCT-3') and IF-H1cTMCT.S1-4r (5'-ACTAAAGAAAATAGGCCTTTAAATACATATTCTACACTGTAG-AGAC-3'). To generate the H1$_{Y98F}$ expression cassette, the PDISP-A/California/07/2009 coding sequence with the mutated Y98F amino acid (H3 numbering) was amplified first using primers IF-CPMV(fl5'UTR)_SpPDI.c and H1_Cal(Y91F).r (5'-AAATCTCCTGGGAAACACGTTCCATT-GTCTGAACTAGGTGTT-TCCACAA-3'), and second using primers H1_Cal(Y91F).c (5'-AGACAATGGAACGTGT-TTCCCAGGAGATTTCATCGATTATGAGGAGCTA-3') and IF-H1cTMCT.S14r. The PCR products from both amplifications were mixed and used as a template for amplification using primers IF-CPMV(fl5'UTR)_SpPDI.c and IF-H1cTMCT.S14r. The final amplification products were assembled into the pCAMBIA binary plasmid containing

2X35S/CPMV 160/NOS and linearized by digestion with *Sac*II and *Stu*I restriction enzymes using the In-Fusion cloning system (Clontech, Mountain View, CA).

3.6.2 Protein expression and VLP purification

Virus-like particles (VLPs) were produced by transient transfection of *Nicotiana benthamiana* plants with *Agrobacterium tumefaciens* carrying $H1_{WT}$ or $H1_{Y98F}$ expression cassettes. Briefly, *N. benthamiana* plants (41–44 days old) were vacuum infiltrated in batches and the aerial parts of the plants were harvested and frozen (-80°C) after 7 days of incubation. To extract and purify VLPs, frozen plant leaves were homogenized in 1L of extraction buffer [50 mM Tris, 500 mM NaCl (pH 7.4) with 0.04% (w/v) $Na_2S_2O_5$]/kg biomass. The homogenate was pressed through a 400 µm nylon filter and the fluid was retained. Filtrates were clarified by centrifugation 5000x*g* and filtration (1.2µm glass fiber, 3M Zeta Plus, 0.45-0.2µm filter) and then concentrated by centrifugation (75000x*g*, 20min). VLPs were further concentrated and purified by ultracentrifugation over an iodixanol density gradient (120000x*g*, 2h). VLP-rich fractions were pooled and dialyzed against 50 mM $NaPO_4$, 65 mM NaCl, 0.005% Tween 80 (pH 6.0). This clarified extract was captured on a Poros HS column (Thermo Scientific) equilibrated in 50 mM $NaPO_4$, 1M NaCl, 0.005% Tween 80. After washing with 25mM Tris, 0.005% Tween 80 (pH 8.0), the VLPs were eluted with 50 mM $NaPO_4$, 700 mM NaCl, 0.005% Tween 80 (pH 6.0). Purified VLPs were dialyzed against formulation buffer (100mM $NaKPO_4$, 150mM NaCl, 0.01% Tween 80 (pH 7.4)) and passed through a 0.22µm filter for sterilization. Protein concentrations were determined using Pierce™ micro BCA protein assay kit according to the manufacturer's instructions.

3.6.3 Gel electrophoresis and immunoblot analysis

To confirm HA expression, 2g of biomass were homogenized in 4mL extraction buffer with 1% phenylmethylsulfonyl fluoride (PMSF). Homogenates were clarified by centrifugation (10000x*g*, 10min) and the crude extracts (25µL/sample) were separated a Criterion XT 4-12% Bis-Tris gel (Bio-Rad) under reducing conditions and then transferred onto a PVDF membrane. Successful transfer was confirmed using ponceau red staining followed by de-staining with water. Membranes were blocked overnight (4°C) with 5% skim milk in TBST (tris-buffered saline, 0.1% Tween 20)

and then incubated with rabbit polyclonal anti-H1 (Cat. No. IT-003-SW, Immune Technology) diluted 1:500 in TBST+2% skim milk for 1h at room temperature (RT). Membranes were then incubated for 1h (RT) with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat. No. IT-200-01, Immune Technology) diluted 1:20,000 in TBST+2% skim milk. Bands were developed using Super Signal West Pico chemiluminescent substrate (Thermo Fischer) and detected on X-ray films. For VLP composition and purity analysis, purified VLP products (5µg/sample) were separated on a 4-12% Bis-Tris gel as described above followed by staining with biosafe Coomassie G-250 (Bio-Rad). Gels were imaged using ChemiDoc™ XRS+ system (Bio-Rad).

3.6.4 Transmission electron microscopy (TEM)

VLP samples were diluted to 100µg/mL in PBS and 5µL of each were placed on 200 copper grids (Agar Scientific) for 45s. Grids were washed 2x (1min each) with 5µL distilled water followed by two incubations with 1.5% uranyl acetate. Excess fluid was removed and samples were left to air dry. Grids were imaged on a Tecnai G2 Spirit Twin 120 kV Cryo-TEM (FEI) equipped with a Gatan Ultrascan 4000 CCD camera model 895 (Gatan).

3.6.5 Surface plasmon resonance (SPR)

Binding of VLP to biotinylated α-2,6 sialic acid glycans (6'-sialyl(LacNAc)-PEG-biotin) was quantified by SPR using a Biacore™ 8K system (Cytiva, formerly GE Healthcare Life Sciences). Biotinylated synthetic glycan (Sussex Research Laboratories Inc.) was immobilized to a Series S sensor chip SA at a minimum target of 400 resonance units (RU) in the test flow cells. VLPs were diluted in HBS-EP+ Buffer (assay running buffer) and injected at a flow rate of 50 µL/min (120s contact time) at 4°C. H1$_{WT}$-VLP was diluted 150x and 100x (2.5 and 4 µg/mL) and H1$_{Y98F}$-VLP was diluted 10x and 5x (88 and 176 µg/mL). The standard curve included 8 dilutions ranging from 10 µg/mL to 0.08 µg/mL. The flow was initially directed over a mock surface to which no protein is bound, followed by the biotinylated glycan. Response from the protein surface is corrected for the response from the mock surface (surface reference). Data analysis was performed using Biacore™ 8K Insight Evaluation Software (version 2.0.15.12933) using concentration analysis mode (fitting function linear analysis). The slope of the association

curve was measured for each VLP and normalized for HA content. Relative binding was calculated based on the adjusted slopes, with the H1$_{Y98F}$-VLP assigned a value of 100%.

3.6.6 Vaccination and sample collection

All animal procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University. Female Balb/c mice (8-10 weeks old, Charles River Laboratories) were immunized by injection into the quadricep muscle with VLP formulations containing 0.5-3µg HA (50µL total in PBS). Mice were vaccinated on day 0 and boosted on day 21 (when indicated). To evaluate humoral and cell-mediated immune responses mice were euthanized on day 28 (one-dose) or day 49 (28d post-boost) by CO_2 asphyxiation. A small group of mice were maintained for 7 months to evaluate long-term immunity and sera were collected monthly. Blood was collected from the left lateral saphenous vein before each vaccination and by cardiac puncture at study endpoints. Sera were obtained by centrifugation of blood in microtainer serum separator tubes (Beckton Dickinson) (8000x*g*, 10min) and stored at -20°C until further analysis. Spleens and bilateral femurs were harvested and splenocytes and bone marrow immune cells were isolated as previously described[71,72]. To evaluate GC responses mice were vaccinated in the right hind limb footpad with 0.5µg VLP (30µL total in PBS). Draining popliteal LN were collected at indicated time points and digested with collagenase D (1mg/mL) and DNaseI (10µg/mL) (40 min, 37°C, shaking at 220 RPM) prior to mechanical dissociation as described for spleens.

3.6.7 Antibody titer measurement

Neutralizing antibodies were evaluated by hemagglutination inhibition (HI) assay and microneutralization (MN) assay as previously described[25,73]. Titers are reported as the reciprocal of the highest dilution to inhibit hemagglutination (HI) or cytopathic effects (MN). Samples below the limit of detection (<10) were assigned a value of 5 for statistical analysis. Total HA-specific IgG was quantified by enzyme-linked immunosorbent assay (ELISA) as previously described[56] with the following modifications: plates were coated with 2µg/mL recombinant HA (Immune Technologies) or HA$_{WT}$-VLP (Medicago Inc.) and HA-specific IgG was detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Southern Biotech) diluted 1:20000 in blocking

buffer. To evaluate the avidity of HA-specific IgG, wells containing bound antibody were incubated with urea (0M-8M) for 15min and re-blocked for 1h prior to detection. Avidity index (AI) = [IgG titer 2-8M urea/IgG titer 0M urea].

3.6.8 ELISpot

HA-specific IgG ASC were quantified by ELISpot (Mouse IgG ELISpot^{BASIC}, Mabtech). Sterile PVDF membrane plates (Millipore) were coated with Anti-IgG capture antibody and blocked according to the manufacturer's guidelines. To quantify *in vivo* activated ASCs, wells were seeded with 250,000 (bone marrow) or 500,000 (splenocyte) freshly-isolated cells and incubated at 37°C, 5% CO_2 for 16-24h. HA-specific ASCs were detected according to the manufacturer's guidelines using 1µg/mL biotinylated HA (immune tech, biotinylated using Sulfo-NHS-LC-Biotin). To evaluate memory ASCs, freshly isolated cells were polyclonally activated with 0.5µg/mL R848 and 2.5ng/mL recombinant mouse IL-2 ($1.5x10^6$ cells/mL in 24-well plates) for 72h (37°C, 5% CO_2). Activated cells were re-counted and the assay was carried out as described above.

3.6.9 Flow cytometry

To identify total GC B cells and T_{FH} cells, freshly isolated cells from the popliteal LN ($1x10^6$ cells/200µL) were washed 1x with PBS in a 96-well round-bottom plate (350x*g*, 7min, 4°C) and labeled with Fixable Viability Dye eFluor 780 (eBioscience) (20min, 4°C). Cells were washed 3x followed by incubation with Fc Block (BD Biosciences) for 15min at 4°C. Samples were incubated for an additional 30min upon addition of the surface cocktail containing the following anti-mouse antibodies: anti-CD3 Alexa Fluor 700 (17A2, Biolegend), anti-CD19 PE-CF594 (1D3, BD), anti-Fas PE (15A7, Thermo Fisher), anti-GL7 FITC or Alexa Fluor 647 (GL7, Biolegend), anti-PD-1 Alexa Fluor 647 (29F.1A12, Biolegend), anti-CXCR5 biotin (2G8, BD Biosciences). Cells were washed 2x followed by incubation with streptavidin BV421 (BD Biosciences) for 40min at 4°C. Cells were washed 3x and fixed for 30min (Fix/Perm solution, BD Biosciences) prior to acquisition. To quantify H1-specific GC B cells, cell suspensions were incubated with 1µg/mL Y98F H1-VLP for 1h prior to surface staining and VLP-bound cells were detected using anti-H1 FITC (Immune Technologies) using the above panel omitting anti-CXCR5 and anti-PD-1.

To identify antigen-specific $CD4^+$ T cells, freshly isolated splenocyte or bone marrow immune cell suspensions ($1x10^6$/200µL in a 96-well U-bottom plate) were stimulated with 10% cRPMI (negative control), 2-2.5µg/mL homologous HA_{WT}-VLP (18h), or a pool of overlapping peptides (15aa) spanning the HA sequence (6h, BEI resources) (37°C, 5% CO_2). Golgi Stop and Golgi Plug (BD Biosciences) were added 5h before the end of the stimulation according to the manufacturer's instructions. Cells were washed 2x with PBS (320x*g*, 8min, 4°C) and labeled with Fixable Viability Dye eFluor 780 (eBioscience) (20min, 4°C). Cells were washed 3x followed by incubation with Fc Block (BD Biosciences) for 15min at 4°C. Samples were incubated for an additional 30 min upon addition of the surface cocktail containing the following antibodies: anti-CD3 FITC (145-2C11, eBioscience), anti-CD4 V500 (RM4-5, BD Biosciences) anti-CD8 PerCP-Cy5.5 (53-6.7, BD Biosciences), anti-CD44 BUV395 (IM7, BD Biosciences) and anti-CD62L BUV373 (MEL-14, BD Biosciences). Cells were washed 3x and fixed (Fix/Perm solution, BD Biosciences) overnight. For detection of intracellular cytokines, fixed cells were washed 3x in perm/wash buffer (BD Biosciences) followed by intracellular staining with the following antibodies (30min, 4°C): anti-IL-2 APC (JES6-5H4, Biolegend), anti-IFNγ PE (XMG1.2, BD Biosciences) and anti-TNFα eFluor450 (MP6-XT22, Invitrogen). Cells were washed 3x in perm/wash buffer and then resuspended in PBS for acquisition.

All flow cytometry was conducted using a BD LSRFortessa or BD LSRFortessa X20 cell analyzer. Data was analyzed using FlowJo software (Treestar, Ashland).

3.6.10 Challenge

Mice were challenged with $1.6x10^3$ times the median tissue culture infectious dose ($TCID_{50}$) of H1N1 A/California/07/09 (National Microbiology Laboratory, Public Health Agency of Canada) diluted in HyClone SFM4MegaVir (Cytiva) supplemented with 10µg/mL gentamicin (Gibco Life Technologies), 100,000 U/mL penicillin G (Sigma) and 20µg/mL glutamine (Wisent). Mice were anesthetized using isoflurane and infected by intranasal instillation (25µL/nare). Mice were monitored for weight loss for 12 days post-infection and were euthanized if they lost ≥20% of their pre-infection weight (humane end-point). A subset of mice in each group was sacrificed 3-5d post-infection (dpi) and lungs were harvested for evaluation of viral load and inflammation.

3.6.11 Preparation of lung homogenates

Lungs were individually homogenized in an equal amount (wt/wt) of complete MegaVir (see above) using micro-tube homogenizer for 3 min. Solid tissue was removed by centrifugation (14,000x*g*, 5 min, 4°C) and supernatants were stored at -80°C until further analysis.

3.6.12 Lung viral load

Infectious virus titers in the supernatant of lung homogenates were determined by $TCID_{50}$ as previously described[56]. Briefly, lung homogenates were serially diluted (1:10) in complete MegaVir media (see above) supplemented with TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (2mg/mL, Sigma) and applied to MDCK monolayers for 1h (37°C, 5% CO_2). Diluted homogenates were then removed from the cells and replaced with fresh MegaVir-trypsin. Cells were incubated for 4 days (37°C, 5% CO_2) and then evaluated for CPE. The $TCID_{50}$ was calculated using the Karber method[74].

3.6.13 Multiplex ELISA

Pulmonary cytokines in lung homogenates obtained from infected and non-infected mice were measured using the Q-Plex™ mouse cytokine screen (16-plex, Quansys Bio) according to the manufacturer's instructions.

3.6.14 Histology

Lungs were collected and fixed as previously described[75]. Briefly, whole lungs were inflated with 10% formalin (Fisher Scientific) and then fixed for ≥24h in 10% formalin. Fixed lungs were embedded in paraffin (Leica), cut into 4µm sections, and applied to glass slides. Sections were stained with hematoxylin and eosin (H&E) as previously described[56]. Images were obtained using a Zeiss Primo Star light microscope equipped with an AxioCam ERc5s (Zeiss) camera.

3.7 AUTHOR CONTRIBUTIONS

HEH designed and performed all experiments and prepared the manuscript with guidance from BJW, SP, M-AD, and NL. P-OL generated VLP expression cassettes and provided biomass for VLP purification by HEH. PS coordinated analytic testing of VLP reagents and studies involving H1-Idaho VLPs. JMG assisted with germinal center experiments. BJW and M-AD were responsible for research supervision and strategy.

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3.10 FIGURES AND LEGENDS

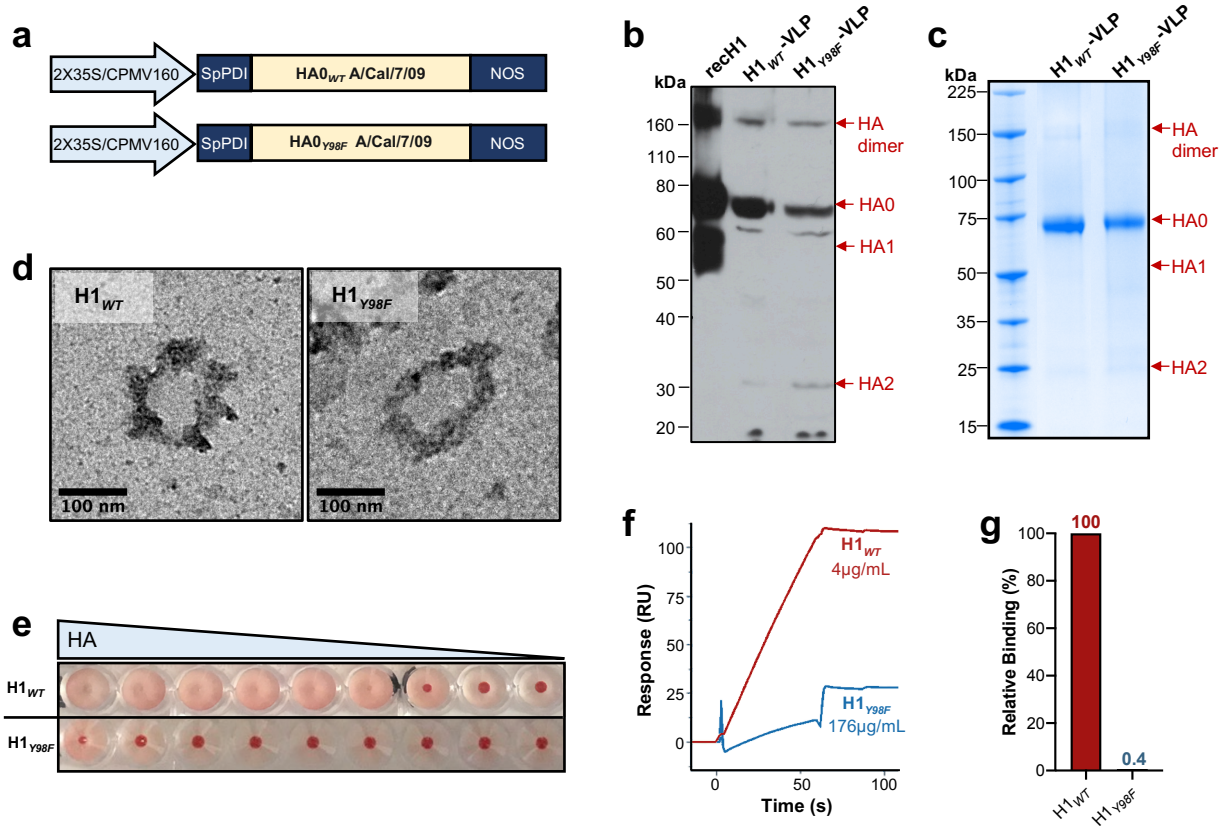


Figure 3.1. Y98F mutation abrogates SA binding without affecting HA expression or VLP structure. Wild-type (WT) H1 ($H1_{WT}$) and Y98F H1 ($H1_{Y98F}$) were expressed in *Nicotiana benthamiana*. (a) Representation of the $H1_{WT}$ (top) and $H1_{Y98F}$ (bottom) expression cassettes. 2X35S/CPMV160 promoter, double 35S promoter fused to the 5' UTR of a cowpea mosaic virus (CPMV) expression enhancer; SpPDI, signal peptide from alfalfa protein disulfide isomerase; NOS, nopaline synthase terminator signal. (b) Expression of HA was confirmed by SDS-PAGE of crude leaf extracts followed by immunoblot analysis. Commercially available recombinant H1 (recH1, 1µg, Immune Technologies) was included as a positive control. 1º ab: rabbit polyclonal anti-H1 1:500 (Cat. No. IT-003-SW, Immune Technology); 2º ab: horseradish peroxidase-conjugated goat anti-rabbit IgG 1:20000 (Cat. No. IT-200-01, Immune Technology). (c) VLP composition and purity were evaluated by SDS-PAGE of purified leaf digests followed by

Coomassie G-250 staining. (d) Representative TEM images show the similar size and morphology of H1$_{WT}$- and H1$_{Y98F}$-VLP. Images were acquired using a Tecnai G2 Spirit transmission electron microscope. (e) Sialic acid (SA) binding was evaluated based on hemagglutination of turkey red blood cells following incubation (30 min) with serial 2-fold dilutions of H1$_{WT}$- and H1$_{Y98F}$-VLP (starting at 1:150 and 1:10, respectively). SA binding was further quantified by SPR. (f) SPR sensorgram showing the binding response of H1$_{WT}$- (red) and H1$_{Y98F}$-VLP (blue) to α-2,6 SA captured on a streptavidin-coated chip surface. (g) Relative binding of H1$_{Y98F}$-VLP when adjusted for HA content.

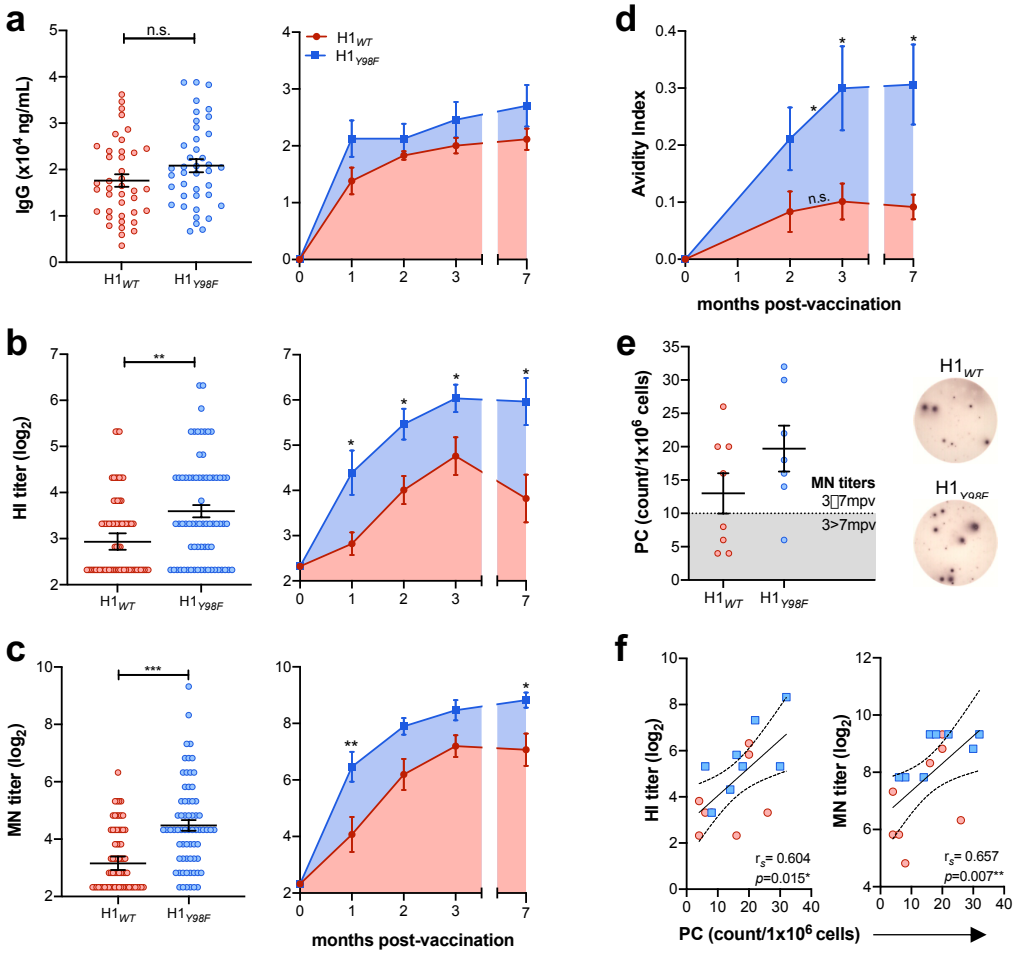


Figure 3.2. H1$_{Y98F}$-VLP elicits a more robust and durable antibody response. Mice were vaccinated (IM) with H1$_{WT}$- or H1$_{Y98F}$-VLP (3µg/dose). (a-c) sera were collected at d21 (left panel) or on a monthly basis (right panel) to measure (a) total H1-specific IgG by ELISA, (b) hemagglutination inhibition titers and (c) microneutralization titers. (d) Avidity indices of sera obtained 2-7mpv following incubation with 8M urea. (e) H1-specific IgG-producing plasma cells (PC) in the bone marrow measured by ELISpot. Representative wells from each group are shown on the right. (f) Spearman's rank correlation technique was applied to evaluate the relationship between the frequency of PC and HI titers (left) and MN titers (right). At d21, N=40-70/group and data are pooled from six independent experiments. For long-term studies, N=7-8/group. Statistical significance between vaccine groups was determined by Mann-Whitney test. Statistical significance between time points within the same group was determined by two-way ANOVA with the Geisser-Greenhouse correction and Sidak's multiple comparisons (*p<0.033, **p<0.01, ***p<0.001).

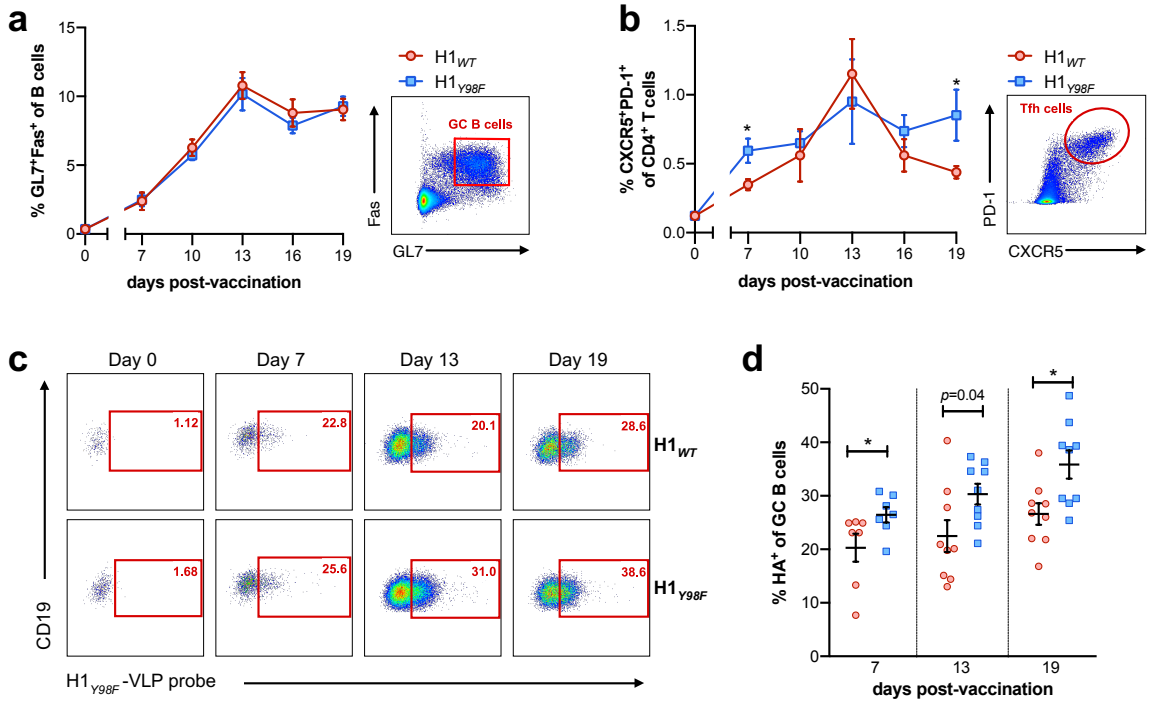


Figure 3.3. H1$_{Y98F}$-VLP promotes enhanced germinal center selection. Mice were immunized with 0.5µg H1$_{WT}$- or H1$_{Y98F}$-VLP in the right hind-limb footpad and popliteal lymph nodes (pLN) were harvested at indicated time points. The mean frequencies (±SEM) of (a) CD19$^+$Fas$^+$GL7$^+$ GC B cells and (b) CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$ T_{FH} cells were determined by flow cytometry. Representative plots showing GC B cell and T_{FH} gating are shown on the right. To identify H1-specific GC B cells, freshly isolated cells were incubated with 1µg/mL H1$_{Y98F}$-VLP (30min, 4^{o}C) and cognate GC B cells were detected following staining with anti-H1 FITC. (c) Representative plots and (d) mean frequency (±SEM) of HA$^+$ cells among GC B cells. Data are pooled from 3 independent experiments, n=7-13/group at each time point. Statistical significance between groups at each time point was determined by Mann-Whitney test (*p<0.033).

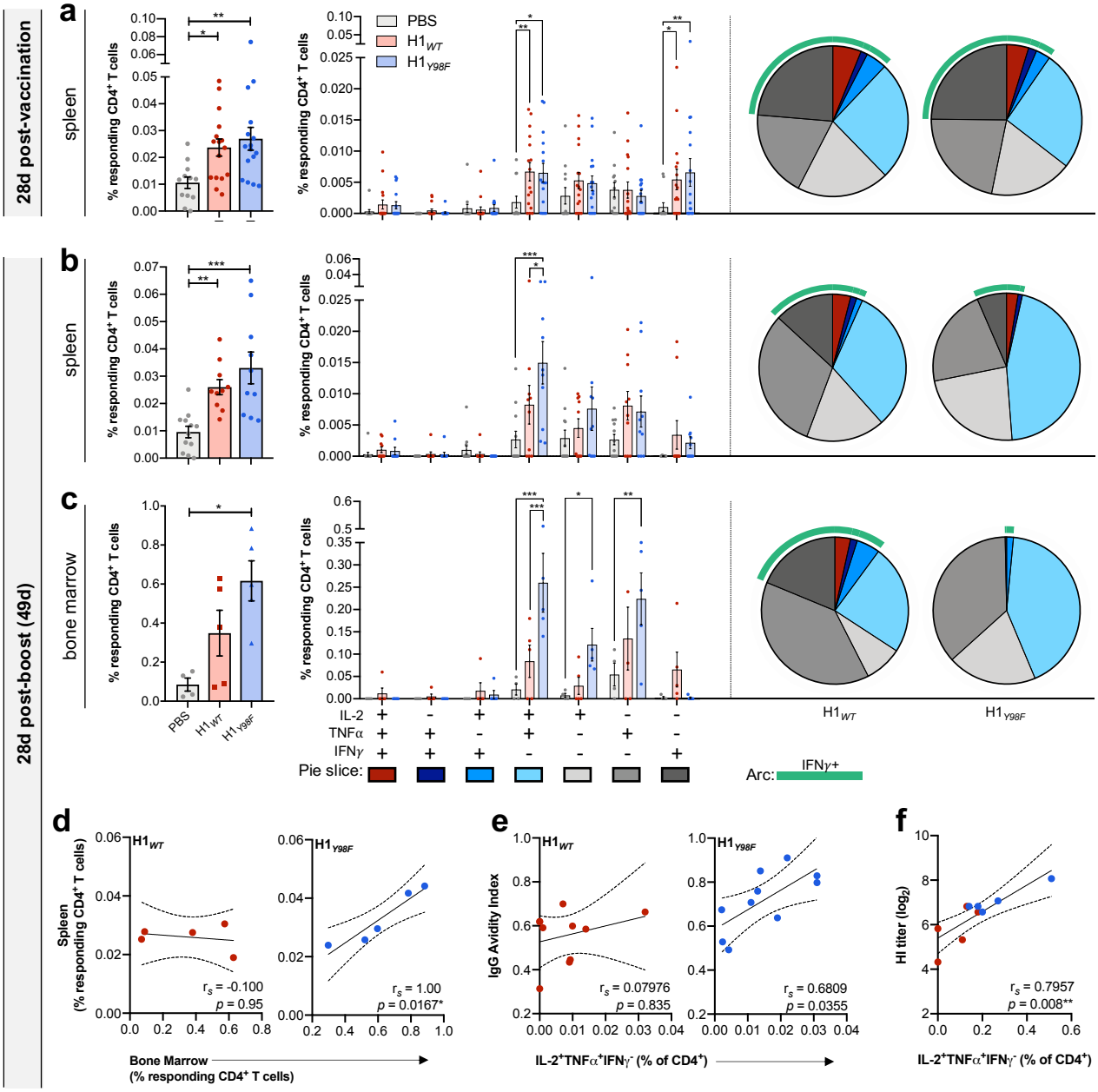


Figure 3.4. H1$_{Y98F}$-VLP elicits robust CD4$^+$ T cell responses with enhanced recruitment of antigen-specific CD4$^+$ T cells to the bone marrow. Splenocytes and BM immune cells were stimulated for 18h with 2.5µg/mL H1$_{WT}$-VLP. Flow cytometry was used to quantify H1-specific CD4$^+$ T cells in (a) splenocytes isolated at 28d post-vaccination (3µg) and in (b) splenocytes and (c) BM immune cells at 28d post-boost (0.5µg/dose). (a-c) The left panel shows the frequency of CD4$^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. The right panel shows the individual cytokine signatures for each mouse obtained by Boolean analysis. Background values

obtained from non-stimulated samples were subtracted from values obtained following stimulation with $H1_{WT}$-VLP. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001). Spearman's rank correlation technique was applied to evaluate the relationship between (d) the frequency of H1-specific $CD4^+$ T cells in the spleen and BM, (e) the frequency of IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells in the spleen and IgG avidity index (4M urea) and (f) the frequency of IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells in the BM and HAI titer.

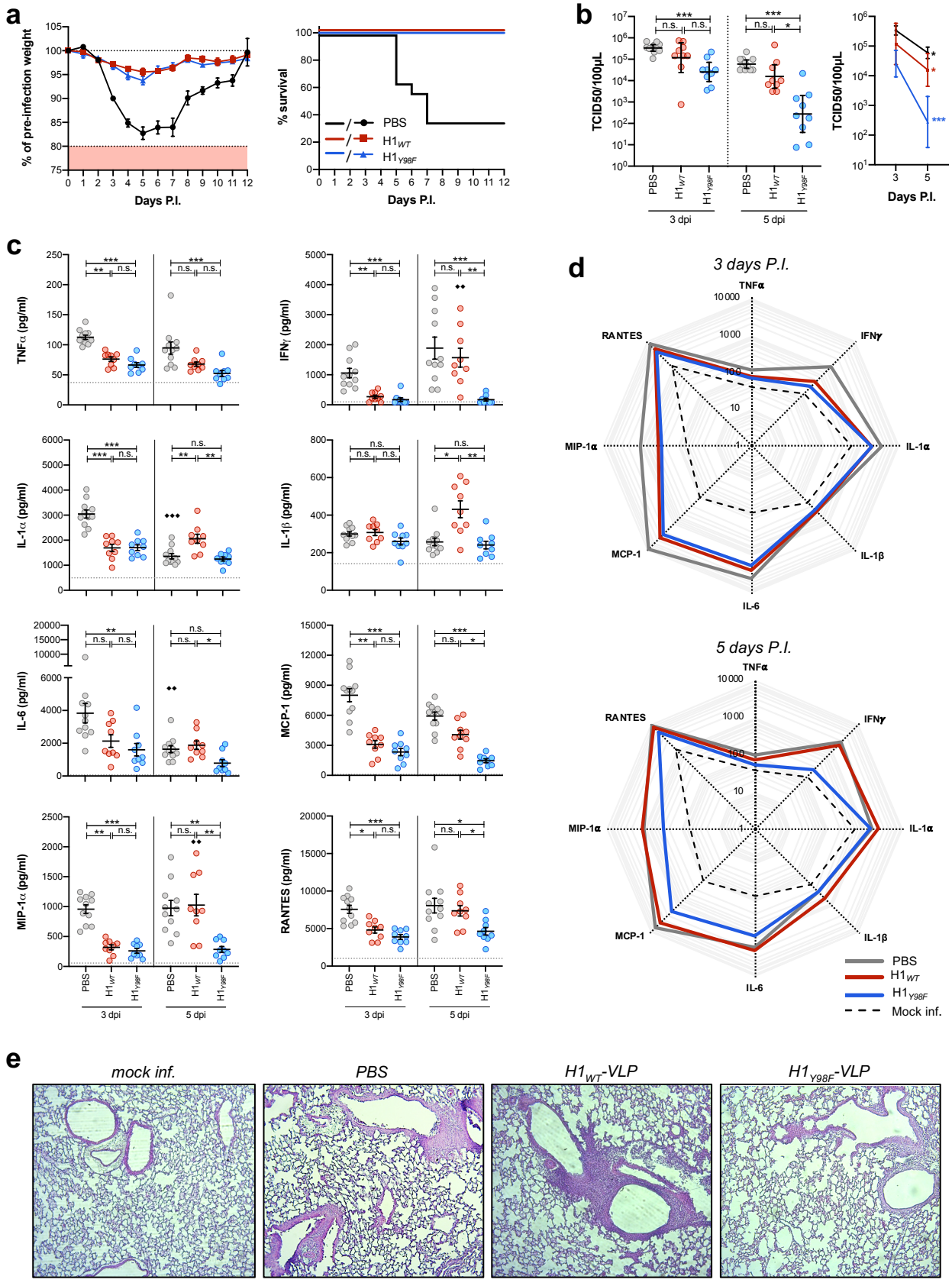
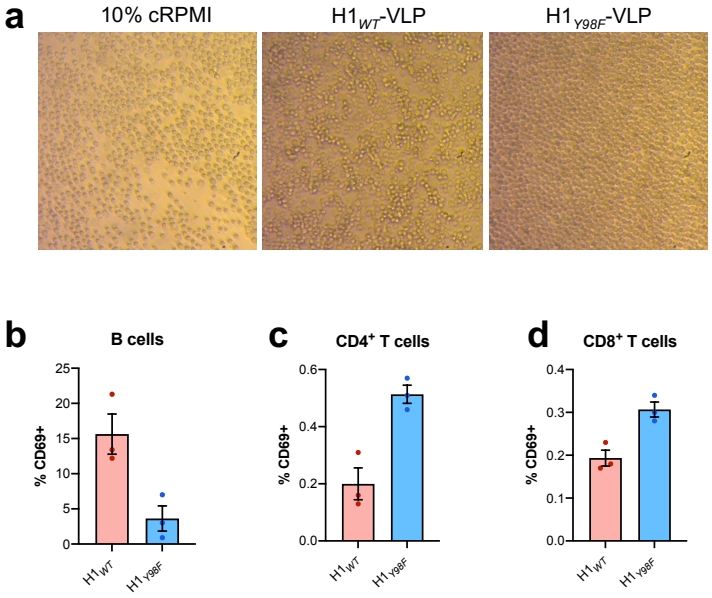


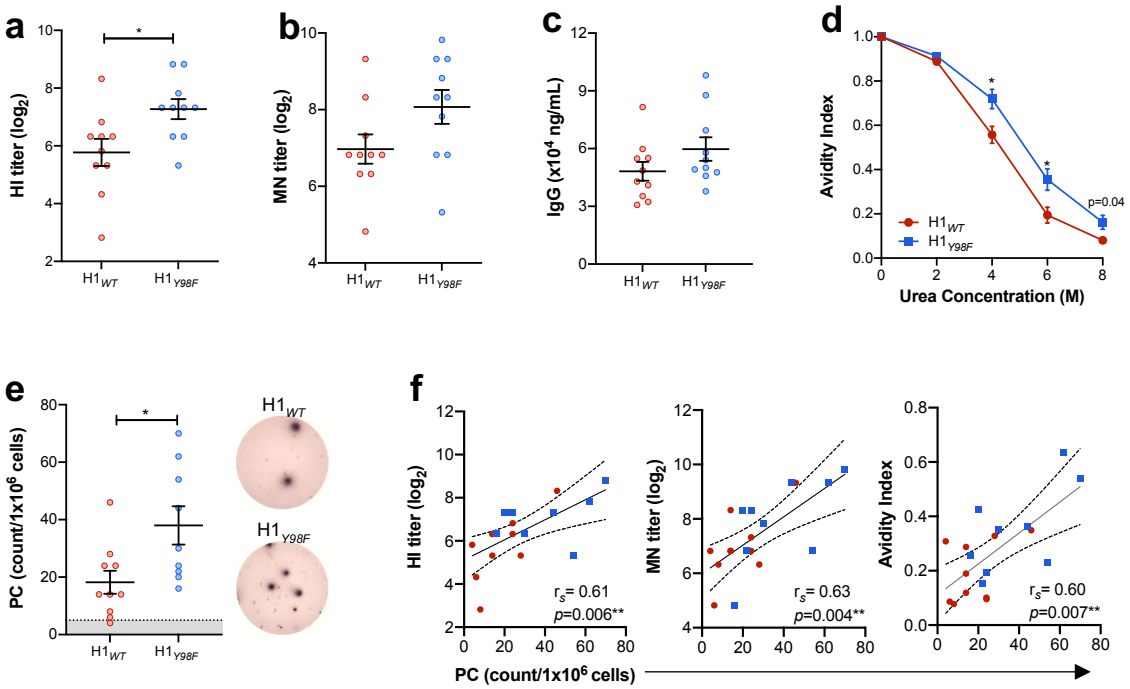
Figure 3.5. (legend on the following page)

Figure 3.5. H1$_{Y98F}$-VLP improves viral clearance and reduces pulmonary inflammation upon lethal challenge. Female Balb/c mice were challenged with $1.6x10^3$ $TCID_{50}$ of H1N1 (A/California/07/09) 28 days post-vaccination with 3µg H1$_{WT}$- or H1$_{Y98F}$-VLP or an equivalent volume of PBS. (a) weight loss (left) and survival (right) were monitored for 12dpi (*n*=12-14/group). Mice weighing <80% of their pre-challenge weight were euthanized. A subset of challenged mice (*n*=9/group) were euthanized at 3dpi and 5dpi for evaluation of the viral load and pulmonary inflammation. (b) Viral titers in the supernatant of lung homogenates calculated using the Karber method and reported as $TCID_{50}$/100µL supernatant (GMT ± 95% C.I.). (c) Concentrations of cytokines and chemokines in the supernatant of lung homogenates measured by multiplex ELISA (mean ± SEM). The horizontal line represents the mean of mock-infected mice as a baseline. (d) Radar plots showing the cytokine profiles of mock-infected and infected lungs at 3dpi and 5dpi. (e) H&E stains of lungs collected 4dpi (10X magnification). Statistical significance for (b) and (c) were determined by Kruskal-Wallis test with Dunn's multiple comparisons. Comparisons between groups at the same time point are represented by * (*p<0.033, **p<0.01, ***p<0.001) and comparisons within the same group over time are represented by ♦(♦♦p<0.01, ♦♦♦p<0.001).

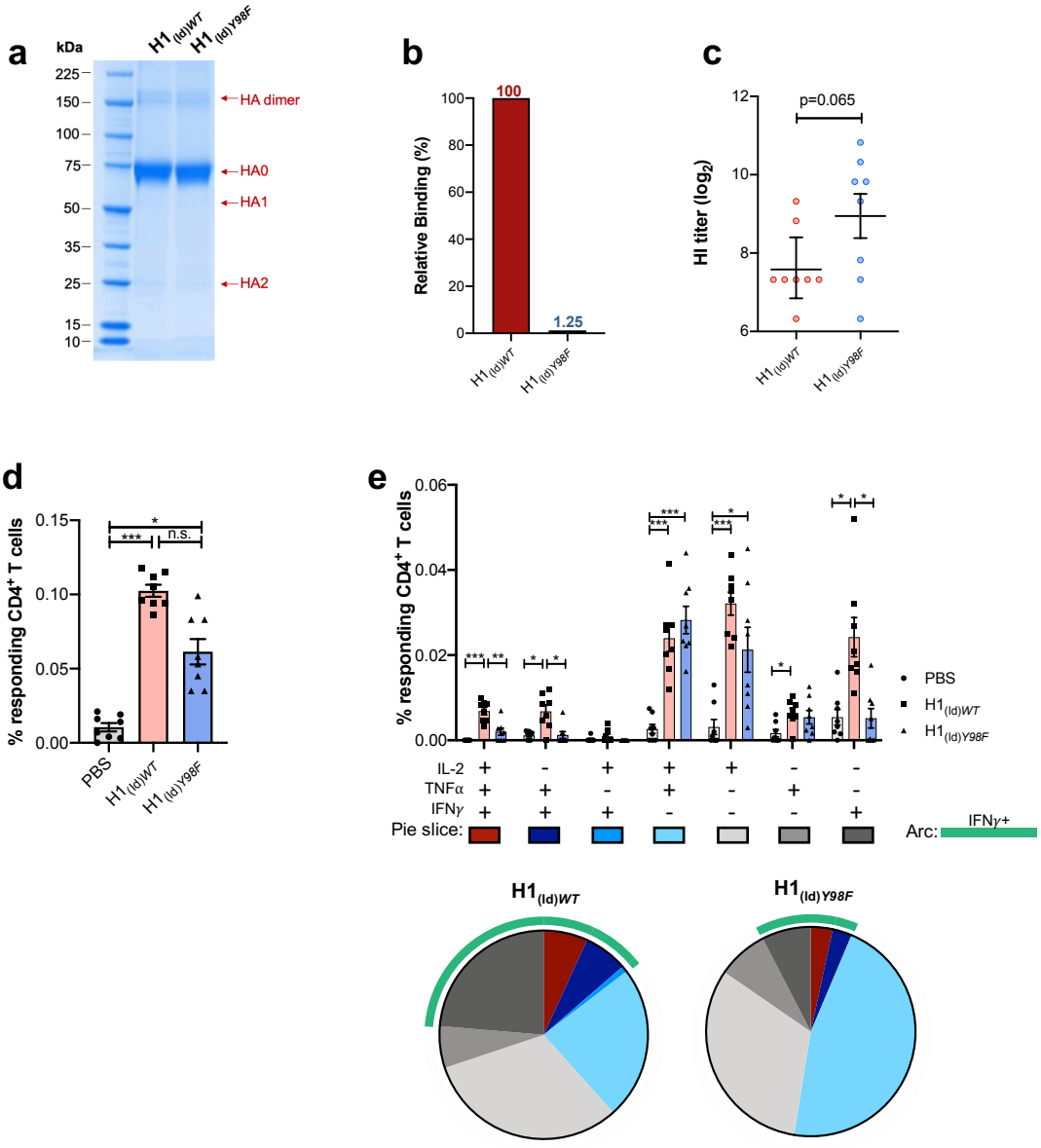
3.11 SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental figure 3.1. Elimination of SA binding prevents PBMC clustering and impacts downstream cell activation. (a) 1x10^6 Human PBMC were co-incubated with H1$_{WT}$- or H1$_{Y98F}$-VLP (5µg/mL) for 30min (37°C, 5%CO_2). Cells were visualized using a Zeiss Primovert inverted microscope equipped with an AxioCam ERc5s camera (10X). To evaluate activation 1x10^6 Human PBMC were co-incubated with H1$_{WT}$- or H1$_{Y98F}$-VLP (1µg/mL) for 6h (37°C, 5%CO_2) and CD69 expression was measured in (b) B cells, (c) CD4$^+$ T cells, and (d) CD8$^+$ T cells by flow cytometry. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with VLP.

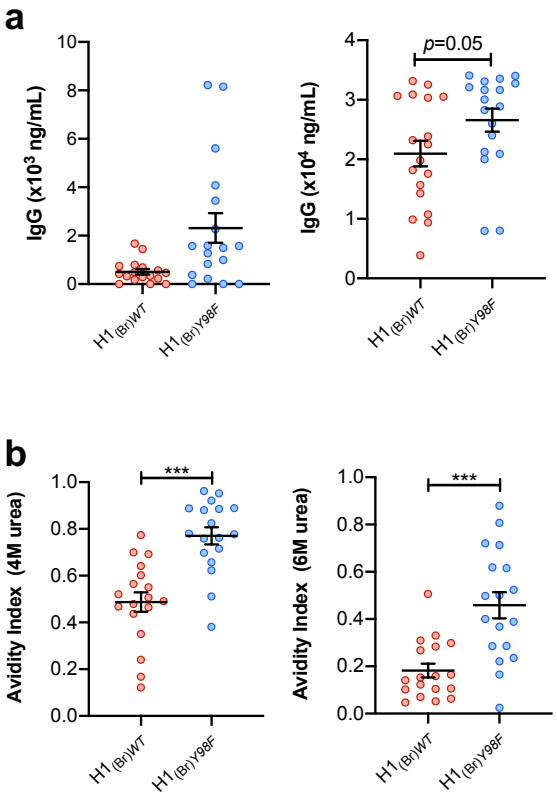


Supplemental figure 3.2. Improved humoral immunity elicited by H1$_{Y98F}$-VLP is maintained post-boost. Female Balb/c mice were vaccinated with 0.5µg H1$_{WT}$- or H1$_{Y98F}$-VLP on days 0 and 21. Mice were euthanized 28d post-boost and sera were collected to evaluate (a) Total H1-specific IgG titers by ELISA, (b) HI titers, (c) MN titers, and (d) IgG avidity indices. Immune cells were also isolated from the BM to evaluate (e) the frequency of H1-specific IgG producing PC. (f) Spearman's rank correlation technique was applied to evaluate the relationship between the frequency of PC and HI titers, MN titers, and IgG avidity indices following incubation with 6M Urea. For (a-c) and (e) statistical significance was evaluated by Mann-Whitney test and for (d) statistical significance was evaluated by Mann-Whitney test at each concentration of Urea with Holm-Sidak's multiple comparisons test (*p<0.033).

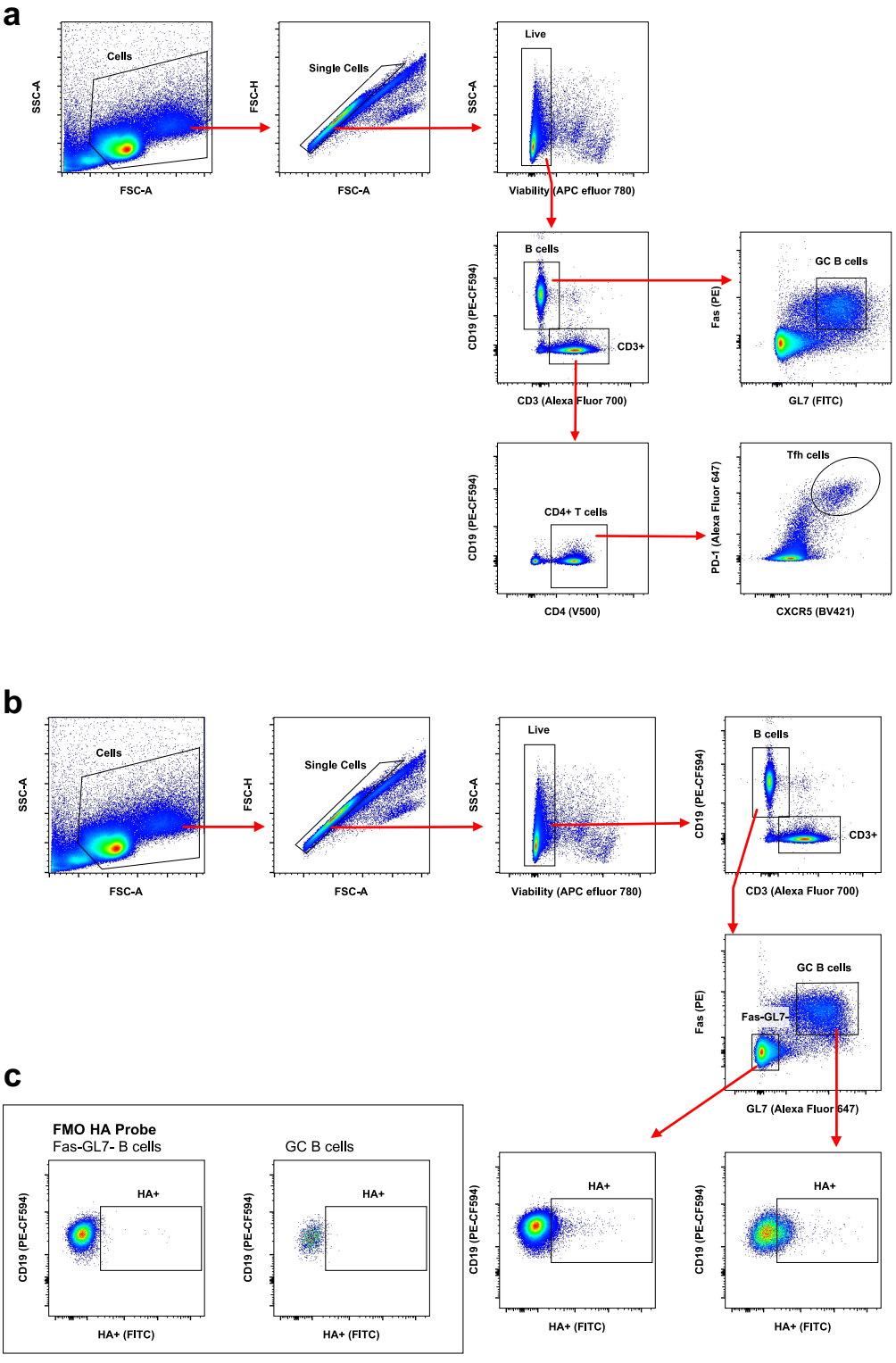


Supplemental figure 3.3. Plant-based H1$_{Y98F}$-VLP targeting H1 A/Idaho/07/2018 elicits similar patterns of immunity to H1$_{Y98F}$-VLP targeting H1 A/California/07/2009. Wild-type (WT) and Y98F H1-VLPs targeting A/Idaho/07/2018 (H1$_{(Id)WT}$- and H1$_{(Id)Y98F}$-VLPs, respectively) were expressed and purified using the same methods as the H1 A/California/07/2009 VLPs. (a) VLP composition and purity were evaluated by SDS-PAGE of purified leaf digests followed by Coomassie G-250 staining. (b) SA binding was quantified by SPR and data represent relative binding of VLPs to α-2,6 SA captured on a streptavidin-coated chip surface (adjusted for HA content). Female Balb/c mice were vaccinated (IM) with 2 doses (1µg each) of H1$_{(Id)WT}$-VLP or

$H1_{(Id)Y98F}$-VLP 21d apart and were euthanized 28d post-boost. (c) HI titers were increased in mice vaccinated with $H1_{(Id)Y98F}$-VLP but narrowly failed to achieve statistical significance determined by Mann-Whitney test. Splenocytes were restimulated with 2.5μg/mL $H1_{(Id)WT}$-VLP to measure $H1_{(Id)}$-specific $CD4^+$ T cells. (d) Both VLPs elicited a significant population of $CD44^+$ $CD4^+$ T cells expressing at least one of IL-2, TNFα or IFNγ. (e) Individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with $H1_{(Id)WT}$-VLP. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).

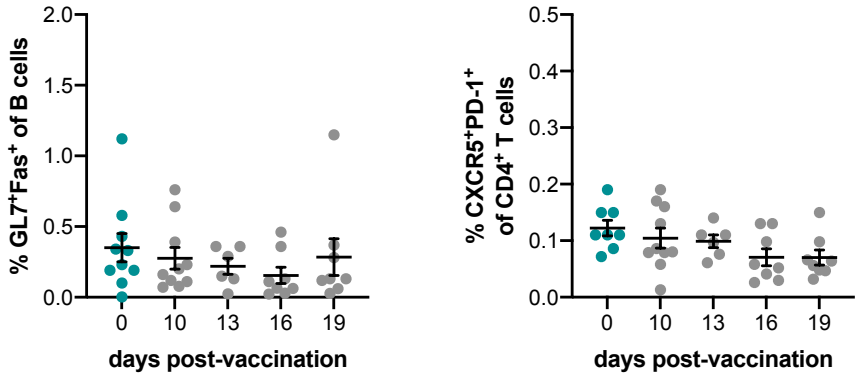


Supplemental figure 3.4. Cell-culture derived recombinant H1$_{Y98F}$ trimers targeting H1 A/Brisbane/02/2018 elicit similar patterns of humoral immunity to H1$_{Y98F}$-VLP targeting H1 A/California/07/2009. Recombinant wild-type (WT) and Y98F H1 A/Brisbane/02/2018 trimers (H1$_{(Br)WT}$ and H1$_{(Br)Y98F}$, respectively) were purchased from eEnzyme (cat. No. IA-H1-B18WP and IA-H1-B18WPm, respectively). H1$_{(Br)}$ is considered to be antigenically similar to H1$_{(Id)}$ by the WHO and only differs by 3 amino acids in the stem region (WHO vaccine recommendations 2019). Thus, the Y98F mutation was assumed to ablate binding similar to H1$_{(Id)Y98F}$. Female Balb/c mice were vaccinated (IM) with 2 doses (0.5µg each) of recombinant H1$_{(Br)WT}$ or H1$_{(Br)Y98F}$ 21d apart. (a) H1$_{(Br)}$-specific IgG was measured by ELISA on d21 (pre-boost, left panel) and 21d post-boost (right panel). (b) At 21d post-boost, IgG avidity was evaluated by ELISA following incubation with 4M urea (left) or 6M urea (right). Statistical significance was determined by Mann-Whitney test (***p<0.001).

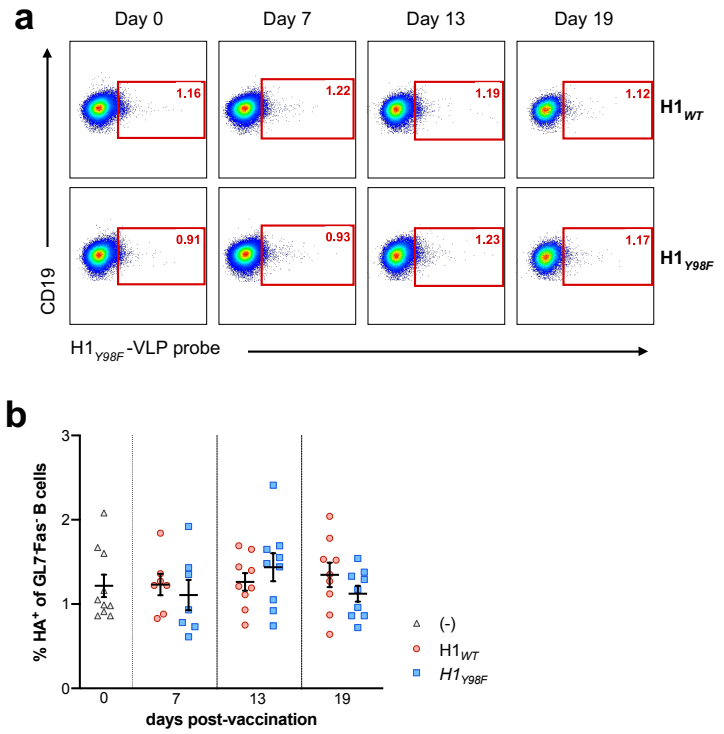


Supplemental figure 3.5. (legend on the following page)

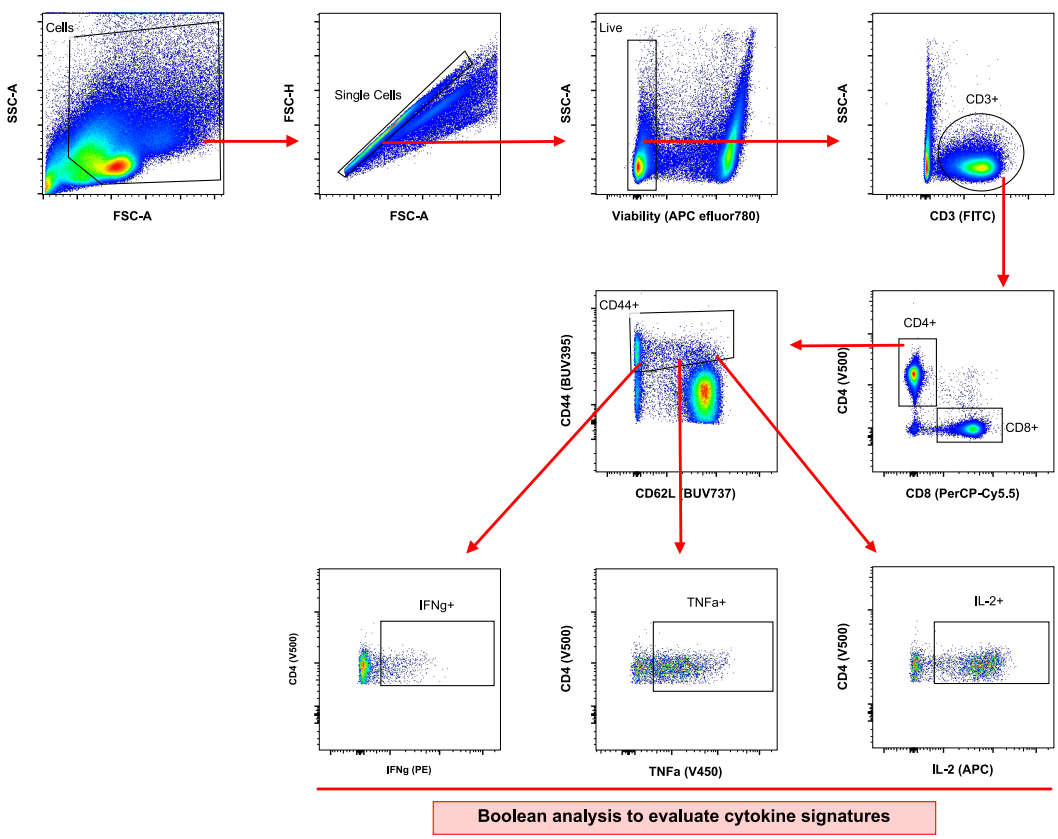
Supplemental figure 3.5. Flow cytometry gating strategy to identify germinal center B cells and T_{FH} cells. In all analyses cells were first gated to exclude debris, cell clusters and dead cells. (a) Gating strategy to identify total GC B cells and T_{FH} cells in the draining popliteal LN following footpad injection. GC B cells are $CD19^{+}Fas^{+}GL7^{+}$ and T_{FH} cells are $CD3^{+}CD4^{+}CXCR5^{+}PD\text{-}1^{+}$. (b) Gating strategy to identify HA-specific B cells among GC B cells and non-GC B cells ($Fas^{-}GL7^{-}$). Prior to staining, cells were incubated with 1μg/mL $H1_{Y98F}$-VLP as a probe to identify antigen-specific cells. B cells that bound to the probe were identified via staining with anti-H1 FITC. (c) HA+ cells were negligible when the $H1_{Y98F}$-VLP probe was not added prior to staining.



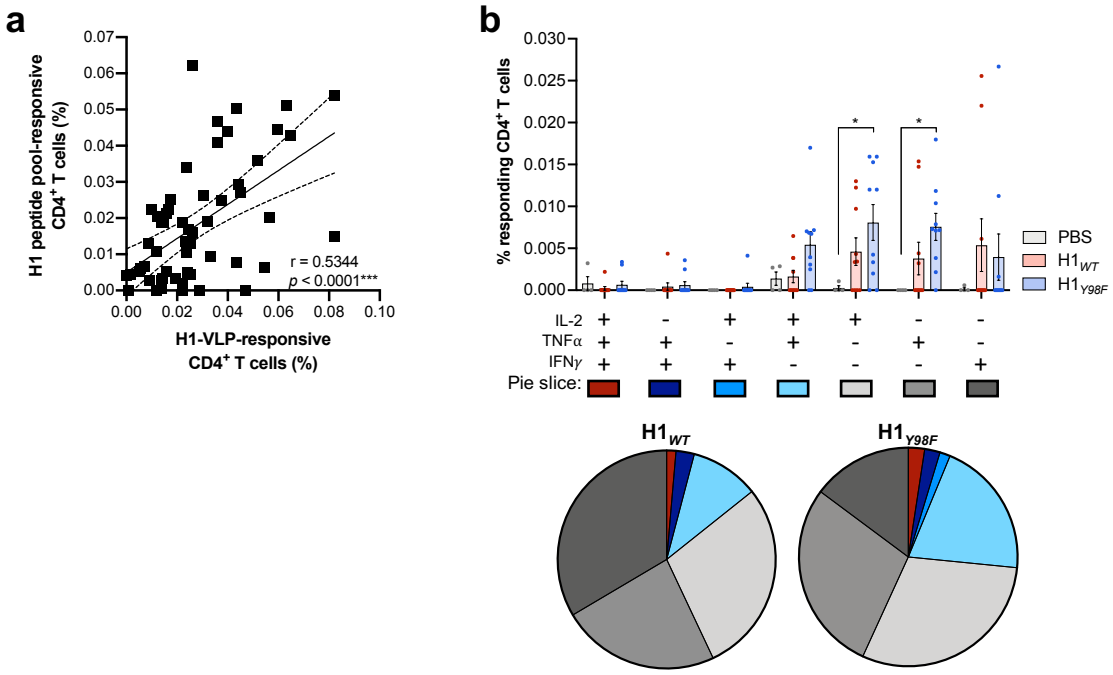
Supplemental figure 3.6. Footpad injection of PBS does not elicit a GC response in the draining popliteal LN. Mice were injected in the right hind limb footpad with 50µL PBS and draining pLN were collected at indicated time points following injection. Frequencies of (a) $CD19^+GL7^+Fas^+$ GC B cells and (b) $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ T_{FH} cells were determined by flow cytometry (mean±SEM). There was no increase in either population compared to pLN collected from non-vaccinated mice (D0, blue).



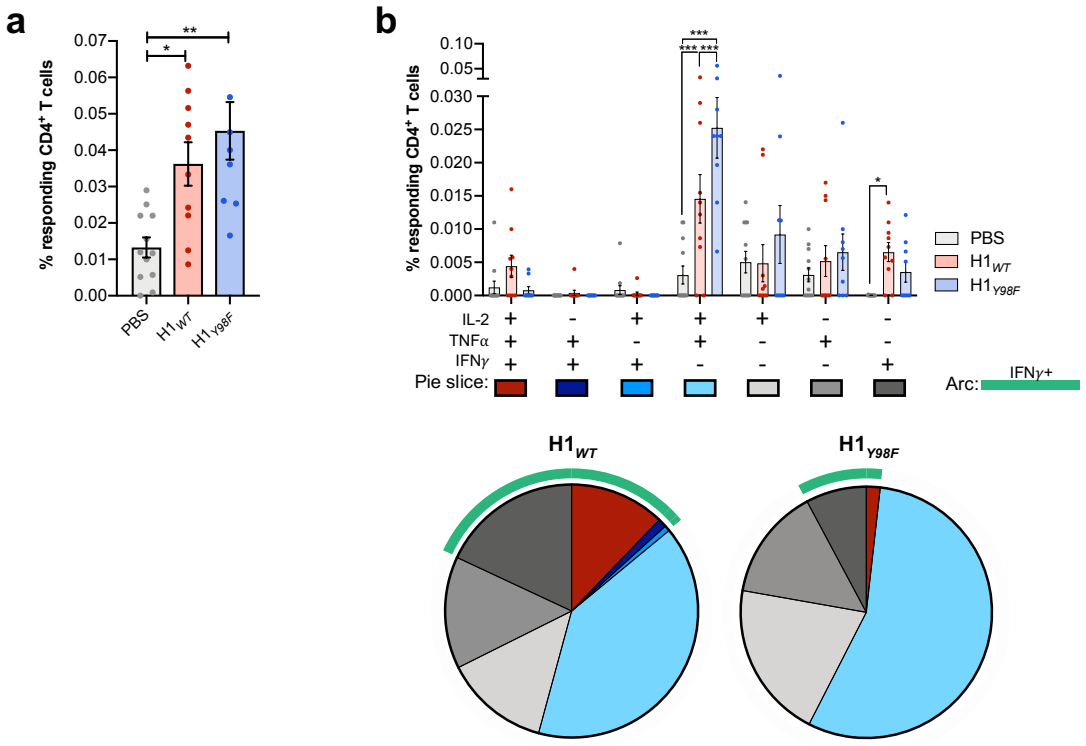
Supplemental figure 3.7. HA-specific B cells outside of the GC remain near baseline levels following vaccination. Mice were immunized with 0.5µg H1$_{WT}$- or H1$_{Y98F}$-VLP in the right hind-limb footpad and popliteal lymph nodes (pLN) were harvested at indicated time points. (a) Representative plots and (b) mean frequency (±SEM) of HA+ cells among Fas^{-}GL7^{-} B cells.



Supplemental figure 3.8. Flow cytometry gating strategy to identify antigen-specific CD4$^+$ T cells. Cells were first gated to exclude debris, cell clusters, and dead cells. Antigen experienced CD4$^+$ T cells were distinguished based on expression of CD44. H1-specific CD4$^+$ T cells were identified based on expression of IFNγ, TNFα, and IL-2 following stimulation with H1$_{WT}$-VLP or H1 peptide pool. Boolean analysis was used to evaluate cytokine signatures.



Supplemental figure 3.9. Stimulation of splenocytes with H1$_{WT}$-VLP and H1 peptide pool results in similar patterns of cytokine expression. Mice were vaccinated with 2 doses (21d apart) with 0.5µg H1$_{WT}$- or H1$_{Y98F}$-VLP. Mice were euthanized 28d post-boost and freshly isolated splenocytes were stimulated with 2.5µg/mL H1$_{WT}$-VLP or a pool of 131 overlapping peptides (15aa) spanning the entire H1 A/California/07/2009 sequence. The frequency of antigen-experienced (CD44$^+$) CD4$^+$ T cells expressing at least one of IL-2, TNFα or IFNγ was determined by flow cytometry. (a) Spearman's rank correlation comparing the frequency of responding antigen-experienced CD4$^+$ T cells following stimulation with H1$_{WT}$-VLP or H1 peptide pool. (b) Individual cytokine signatures for each mouse determined by Boolean analysis of responding CD4$^+$ T cells. The frequency of responding cells in non-stimulated samples were subtracted from frequency of responding cells following stimulation with H1 peptide pool (2.5µg/mL). Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons (*p<0.033, **p<0.01, ***p<0.001)



Supplemental figure 3.10. Increased antigen dose increases the magnitude of the CD4$^+$ T cell response but does not affect cytokine signatures. Mice were vaccinated twice with 3µg H1$_{WT}$- or H1$_{Y98F}$-VLP 21d apart. Spleens were harvested 28d post-boost and splenocytes were stimulated for 18h with 2.5µg/mL H1$_{WT}$-VLP. Antigen-specific CD4$^+$ T cells were quantified by flow cytometry. (a) Frequency of CD4$^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. (b) Individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with H1$_{WT}$-VLP. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).

PREFACE TO CHAPTER 4

In **chapter 3** we demonstrated that abrogating HA-SA interactions significantly improves the immunogenicity and efficacy of a plant-based H1-VLP. Vaccination of mice with the 'non-binding' H1$_{Y98F}$-VLP resulted in a marked improvement in antibody titers, and durability as well as viral clearance and pulmonary inflammation following challenge. This novel approach highlighted the importance of considering HA binding properties in the rational design of next-generation influenza vaccines. To evaluate the generalizability of these findings, we next sought to determine whether this approach could be used to improve the immunogenicity of VLP vaccines targeting highly pathogenic strains of avian influenza, which tend to elicit poor immune responses compared to seasonal vaccines. To accomplish this, we generated additional 'non-binding' VLPs targeting H7N9 (A/Shanghai/02/2013) and H5N1 (A/Indonesia/05/2005). These modified VLPs were designed based on the well-known Y98F HA mutation, similar to our approach with the 'non-binding' H1-VLP. The following chapter describes the impact of reduced HA-SA interactions on the humoral and cellular immune responses elicited by these vaccines compared to their wild-type counterparts.

CHAPTER 4

Reduced sialic acid binding by influenza hemagglutinin improves the immunogenicity of vaccines targeting highly pathogenic strains of avian influenza

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4.1 ABSTRACT

Highly pathogenic avian influenza (HPAI) viruses pose a major threat to human health and vaccines targeting these strains tend to be poorly immunogenic compared to seasonal influenza vaccines. Influenza viruses gain entry into respiratory epithelial cells using their hemagglutinin (HA) proteins that bind to sialic acids (SA) on host cell surface glycoproteins. These SAs are widely distributed in different tissues such as skeletal muscle, where most vaccines are administered, and on immune cells. Our previous work suggested that non-cognate HA-SA binding at the site of injection or on immune cell subsets has the potential to influence vaccine responses. These studies demonstrated that abrogating the HA-SA interaction greatly improved the immunogenicity of several seasonal H1N1 candidate vaccines. In this work, we generated virus-like particle (VLP) vaccines bearing the HA of H7N9 A/Shanghai/02/2013 or H5N1 A/Indonesia/05/2005 with reduced capacity to bind to SA ($H7_{Y98F}$-VLP and $H5_{Y98F}$-VLP) and compared their immunogenicity to wild-type (WT) VLPs ($H7_{WT}$-VLP and $H5_{WT}$-VLP) in mice. The $H7_{Y98F}$-VLP elicited significantly stronger antibody responses than the $H7_{WT}$-VLP, including increased hemagglutination inhibition titers and improved IgG avidity maturation. In contrast, $H5_{Y98F}$- and $H5_{WT}$-VLPs elicited similar antibody responses. However, compared to WT VLPs, both $H7_{Y98F}$- and $H5_{Y98F}$-VLPs resulted in significantly more robust populations of HA-specific plasma cells in the bone marrow, which are thought to be associated with long-lasting immunity. These findings suggest that the introduction of mutations that reduce or eliminate HA-SA interactions may be a promising strategy to improve the immunogenicity of vaccines targeting HPAI viruses.

4.2 INTRODUCTION

Wild waterfowl are the primary zoonotic reservoir for influenza viruses and carry a wide range of influenza subtypes that do not typically circulate in humans[1]. Most avian influenza viruses are of low pathogenicity in both birds and humans. However, acquisition of a polybasic cleavage site and other mutations can give rise to highly pathogenic avian influenza (HPAI) strains that can replicate systemically in birds[2,3]. HPAI viruses with H5 and H7 hemagglutinin (HA) subtypes have recently been responsible for devastating outbreaks in domestic poultry[4] and have occasionally

spilled-over into humans with high mortality[5,6]. To date, human-to-human transmission of these strains has been rare due to the limited capacity of HPAI viruses to replicate in the upper airways[7]. However, the potential for further adaptive mutations that promote upper airway replication represents a tremendous threat for human health[8-10]. Furthermore, vaccines targeting HPAI viruses tend to be poorly immunogenic compared to seasonal influenza vaccines[11,12], raising significant concerns about pandemic preparedness. Thus, new strategies are required to generate more immunogenic avian influenza vaccines.

We previously demonstrated that the non-cognate binding properties of influenza HA can significantly impact immune responses to influenza virus-like particle (VLP) vaccines both *in vitro*[13] and *in vivo*[14] and may be important to consider in the development of better influenza vaccines. *In vitro*, we demonstrated that H1- and H5-VLPs exhibit distinct patterns of interactions with human immune cells, that are driven by their receptor preferences and markedly impact downstream immune responses[13]. Consistent with these findings, Xu *et al*. have shown that H5N1 and H7N9 virus-like particles (VLPs) capable of binding to mammalian α(2,6)-linked SA are more immunogenic in mice than wild-type (WT) VLPs that predominantly bind to α(2,3)-linked SA[15]. Taken together, these findings suggest that either binding of WT avian HAs to α(2,3)-linked SA or their inability to bind to α(2,6)-linked SA – or both – may interfere with their immunogenicity. However, we recently discovered that complete ablation of HA binding to α(2,6)-linked SA can significantly improve the immunogenicity and efficacy of candidate H1-VLP vaccines in mice[14], raising the possibility that elimination of SA binding by avian influenza HAs may confer similar benefits.

In the current work, we sought to determine whether preventing interactions between HA and α(2,3)-linked SA improves the relatively poor immunogenicity of plant-based virus-like particle (VLP) vaccines for avian influenza[12,16]. Similar to our previous studies examining a 'non-binding' H1-VLP[14], candidate VLP vaccines bearing H7 (A/Shanghai/02/2013) and H5 (A/Indonesia/05/2005) were generated by incorporating a Y98F mutation (H3 numbering) in the HA proteins. This approach has been shown to prevent SA binding without impacting antigenicity and is thought to be broadly applicable to most influenza A viruses[17,18]. Herein we demonstrate for the first time that preventing HA-SA interactions is a promising strategy to improve the immunogenicity of VLP vaccines targeting HPAI viruses.

4.3 RESULTS

4.3.1 Generation and validation of H7$_{Y98F}$-VLP

Virus-like particles (VLP) composed of a plant-lipid bilayer studded with the WT H7 (H7$_{WT}$-VLP) or Y98F H7 (H7$_{Y98F}$-VLP) were expressed in *Nicotiana benthamiana* as previously described using a 2X35S/CPMV160/NOS expression system[19-21] (**figure 4.1a).** VLPs were then purified from frozen biomass. To evaluate protein composition and purity, VLP preparations were separated by gel electrophoresis and visualized using Coomassie G-250 staining (**figure 4.1b**). Identification of HA bands was confirmed by immunoblot analysis (**figure 4.1c**). HA was predominantly expressed in its uncleaved form (HA0) and monomers, dimers, and trimers were observed. The purity of the HA-VLP products was determined by analysis of the densitometry profile of each protein band and was comparable between formulations (95-98%).

Although the Y98F mutation in HA is thought to eliminate SA binding by most influenza A viruses, its impact on the binding properties of H7 A/Shanghai/02/2013 has not previously been evaluated. To determine whether the Y98F mutation prevents SA binding by H7-VLPs, we examined the ability of H7$_{WT}$- and H7$_{Y98F}$-VLPs to hemagglutinate turkey red blood cells (RBC). With multiple active HA trimers on their surface, H7$_{WT}$-VLPs hemagglutinate the RBC by binding to SA on the cell surface and had a hemagglutination titer of 153,600. The Y98F mutation did not completely prevent binding, however, the hemagglutination titer of the H7$_{Y98F}$-VLP was reduced to 1,200 (**figure 4.1d**). When adjusted for HA content, the hemagglutination titer was reduced by 98.8%. Thus, the H7$_{Y98F}$-VLP is an appropriate tool for evaluating the role of HA-SA interactions in vaccine responses despite the capacity to bind SA at low levels.

4.3.2 Y98F mutation of H7 improves humoral response

To determine whether the Y98F mutation has any impact on the immunogenicity of an H7-VLP, we evaluated antibody responses in mice vaccinated with two doses of H7$_{WT}$- or H7$_{Y98F}$-VLPs (3µg/dose) or an equivalent volume of PBS administered 8 weeks (w) apart. Total H7-specific IgG titers and IgG avidity were measured by ELISA and functional antibodies were evaluated using the hemagglutination inhibition (HI) assay. The HI assay typically measures the ability of antibodies to block the binding of live virus to avian RBCs, however, the H7$_{WT}$-VLP was used as a surrogate for live virus due to the high pathogenicity of H7N9 strains. Antibodies were

measured 2w, 4w, and 8w post-prime and 5w post-boost (13w total) to evaluate the quality and kinetics of the humoral responses.

Similar to our observations with the H1$_{Y98F}$-VLP[14], both H7$_{WT}$- and H7$_{Y98F}$-VLPs resulted in comparable H7-specific IgG titers by ELISA at all time points (**figure 4.2a**) but antibody functionality and avidity maturation were markedly improved in the animals that received H7$_{Y98F}$-VLP (**figure 4.2b-c**). HI titers were significantly higher in the H7$_{Y98F}$-VLP group by 4 weeks post-vaccination (wpv) (p=0.002) and this difference was maintained following administration of the second dose (p=0.02) (**figure 4.2b**). To determine whether H7$_{Y98F}$-VLP also results in improved avidity maturation we utilized a modified ELISA that incorporates a brief incubation with urea to dissociate weakly bound antibodies. Avidity is presented as an index of IgG that remained bound following urea exposure (IgG titer 8M urea/0M urea). Avidity indices were similar in both groups at 4wpv, however, the H7$_{Y98F}$-VLP group had improved avidity maturation at later time points. Compared to the H7$_{WT}$-VLP, the H7$_{Y98F}$-VLP resulted in significantly higher IgG avidity by 8wpv (p=0.007) and differences were maintained post-boost (p=0.001) (**figure 4.2c**). H7-specific antibodies were not detected in the placebo group.

While the trends observed in this study were remarkably similar to those observed in mice vaccinated with H1$_{WT}$- and H1$_{Y98F}$-VLPs, it is important to acknowledge that the vaccine dosing schedule was quite different compared to our previous studies and other studies examining the immunogenicity of plant-based VLPs[14]. Typically, two doses are administered 3-4w apart, however, the second dose in this study was only administered 8wpv due to restrictions at the onset of the Covid-19 pandemic. To ensure that the responses we observed were not influenced by delaying the second dose, we also evaluated humoral immune responses in a subset of mice vaccinated with two doses of H7$_{WT}$- or H7$_{Y98F}$-VLPs administered 3w apart. Antibody titers and avidity were measured 3w post-prime and 4w post-boost (**supp. figure 4.1**). Overall, HI titers and IgG avidity were considerably lower when the second dose was administered at 3w compared to 8w after the primary vaccination. However, trends between vaccine groups were maintained regardless of the dosing schedule (**supp. figure 4.1b-c**). Taken together, these results suggest that reducing HA-SA interactions is an effective strategy to improve the humoral immune response to an H7-VLP.

4.3.3 H7$_{Y98F}$-VLP results in increased H7-specific bone marrow plasma cells

Memory B cells (MBC) and bone marrow (BM) plasma cells (PC) are important mediators of lasting protection following vaccination[22,23]. To determine whether these populations contributed to the improved humoral responses elicited by H7$_{Y98F}$-VLP, mice were euthanized 5w post-boost to evaluate the frequency of H7-specific IgG-secreting MBC in the spleen and PC in the BM. Consistent with our previous studies examining the H1$_{Y98F}$-VLP[14], there were no differences in the frequency of splenic MBCs by 5w post-boost (**figure 4.2d**). However, BM PC were significantly higher at 5w in the H7$_{Y98F}$-VLP group compared to the animals that received H7$_{WT}$-VLP (p=0.03) (**figure 4.2e**) and the frequency of this population was correlated with HI titers (r_s=0.7109, p=0.0004) (**figure 4.2f**). Similar trends in BM PCs and their correlation with HI titers were observed among mice vaccinated with two doses administered 3w apart although the frequency of BM PC was lower in both groups compared to the delayed boost (**supp. figure 4.1e-f**). Interestingly, splenic MBC were also higher in H7$_{Y98F}$-VLP-vaccinated mice with the shorter dosing schedule, which did not occur following a delayed boost or in previous studies examining the H1$_{Y98F}$-VLP[14] (**supp. figure 4.1d**). Although we did not evaluate the durability of the H7-specific antibody responses in these studies, BM PC are known to play an important role in the sustained production of high avidity IgG[23] and are associated with improved maintenance of functional antibody titers following vaccination with H1$_{Y98F}$-VLP[14]. Thus, these data suggest that reduction of interactions between HA and α2,3-linked SA may also improve the durability of humoral responses to vaccination, however, further evaluation is required to confirm this hypothesis.

4.3.4 H7$_{WT}$- and H7$_{Y98F}$-VLPs elicit robust CD4$^+$ T cell responses

Antigen-specific CD4$^+$ T cells were quantified by flow cytometry following vaccination with two doses of H7$_{WT}$-VLP or H7$_{Y98F}$-VLP or an equivalent volume of PBS administered 8w apart. Five weeks post-boost, the spleen and right hind limb femur and tibia were harvested and freshly isolated immune cells were re-stimulated with H7$_{WT}$-VLP (2μg/mL) for 20h. Responding cells were characterized as antigen-experienced (CD44$^+$) CD4$^+$ T cells expressing IL-2, IFNγ and/or TNFα (see **supp. figure 4.2** for full gating strategy).

Vaccination with either $H7_{WT}$- or $H7_{Y98F}$-VLP resulted in significant increases in H7-specific $CD4^+$ T cells in the spleen compared to the PBS control group ($H7_{WT}$ p=0.002; $H7_{Y98F}$ p<0.001), however, the magnitude of the response was similar between vaccine groups (**figure 4.3a**). Boolean analysis of responding cells revealed that both vaccines elicited significant populations of IL-2^+TNFα^+IFNγ^- $CD4^+$ T cells (p<0.001) and IL-2 single-positive $CD4^+$ T cells ($H7_{WT}$ p=0.02; $H7_{Y98F}$ p<0.001) compared to the PBS control group. However, the $H7_{Y98F}$-VLP resulted in significantly more IL-2 single-positive cells than the $H7_{WT}$-VLP (p<0.001). In contrast, the $H7_{WT}$-VLP resulted in an overall higher proportion of $CD4^+$ T cells expressing IFNγ compared to mice vaccinated with $H7_{Y98F}$-VLP, but this difference was not statistically significant. Similar trends were observed when vaccines were administered 3w apart (**supp. figure 4.3a**).

Antigen-specific $CD4^+$ T cell populations were also evaluated in the BM, which are thought to be an important source of long-term memory following vaccination[24]. Overall, trends were quite similar to those observed in the spleen (**figure 4.3b**). Both $H7_{WT}$- or $H7_{Y98F}$-VLP resulted in a significant increase in H7-specifc $CD4^+$ T cells in the BM compared to the PBS control group ($H7_{WT}$ p=0.003; $H7_{Y98F}$ p=0.002) and there was marked expansion of IL-2^+IFNγ^- populations. Similar trends were observed when vaccines were administered 3w apart, however, there were fewer polyfunctional $CD4^+$ T cells in the BM in both groups when the second dose was administered at this time (**supp figure 4.3b**), suggesting that delaying the second dose may improve the quality of the memory $CD4^+$ T cell response.

4.3.5 Reduced SA binding improves some aspects of H5-VLP immunogenicity

Highly pathogenic avian influenza (HPAI) strains can arise from low pathogenicity strains for both H5 and H7 HA subtypes[3]. To determine whether reduced HA-SA interactions can also improve the immunogenicity of a VLP targeting an H5 subtype HPAI virus, we generated an HA-VLP bearing H5 from H5N1 A/Indonesia/05/2005 with the Y98F mutation ($H5_{Y98F}$-VLP) and compared its immunogenicity to a wild-type H5-VLP ($H5_{WT}$-VLP). H5-VLPs were produced and purified using the same techniques as $H7_{WT}$- and $H7_{Y98F}$-VLPs and final VLP formulations had a purity of 94-95% (**supp. figure 4.4**). The $H5_{WT}$-VLP readily hemagglutinated turkey RBC with a hemagglutination titer of 76,800. The hemagglutination titer of the $H5_{Y98F}$-VLP was below the

limit of detection, suggesting that the Y98F mutation successfully abrogated α2,3-linked SA binding.

Mice were vaccinated with two doses of H5$_{WT}$- or H5$_{Y98F}$-VLP administered 8w apart, and sera were collected 5w post-boost to evaluate humoral immune responses. In contrast to the H7$_{Y98F}$-VLP and previous observations with H1$_{Y98F}$-VLP[14], the H5$_{Y98F}$-VLP had no impact on the H5-specific IgG titer, HI titer, or IgG avidity compared to H5$_{WT}$-VLP (**figure 4.4a-c**). Similar results were obtained when the two vaccine doses were administered 3w apart, but antibody titers and avidity were notably lower when the second dose was administered earlier (**supp. figure 4.5**). Interestingly, mice vaccinated with H5$_{Y98F}$-VLP had increased H5-specific PC in the BM when boosted at either 8w (**figure 4.4d**) or 3w (**supp. figure 4.5**), suggesting that the H5$_{Y98F}$-VLP may still confer improved durability of immunity. Furthermore, the CD4$^+$ T cell response in mice vaccinated with H5$_{Y98F}$-VLP was biased towards IL-2$^+$IFNγ$^-$ populations (**figure 4.4e, supp. figure 4.5**), similar to the H7$_{Y98F}$-VLP and H1$_{Y98F}$-VLP[14]. Taken together, these data suggest that the H5$_{Y98F}$-VLP behaves similarly to the other 'non-binding' VLPs, despite this not being reflected in the serum antibody outcomes assessed. Functional assays such as the serum neutralization as well as challenge studies, which could not be performed due to lack of access to a BSL3 facility, will be useful in confirming whether antibody responses are improved by eliminating HA-SA interactions for both the H7$_{Y98F}$- and H5$_{Y98F}$-VLP vaccines.

4.4 DISCUSSION

The relatively poor immunogenicity of most avian influenza vaccines compared to seasonal vaccines is well documented and raises concerns surrounding pandemic preparedness[11,12]. We have demonstrated for the first time that reducing interactions between HA and α2,3-linked SA can significantly improve the humoral immune responses elicited by plant-based H7-VLP and H5-VLP vaccines. SA binding mutants were generated by incorporating a Y98F mutation (H3 numbering) into the target HA sequence, which limits interactions with SA without impacting the antigenicity of the HA globular head or receptor binding domain[17,18]. We have previously shown that this strategy shows promise when applied to both VLP and recombinant HAs targeting H1N1 strains that bind to α2,6-linked SA[14], prompting us to investigate whether this approach may confer

similar benefits for vaccines targeting HPAI strains that preferentially use α2,3-linked SA as a receptor.

The impact of the Y98F mutation on SA binding is well documented and is thought to be broadly applicable to influenza A HAs due to its conserved position in the receptor binding pocket[17,18]. Consistent with a previous report by Whittle *et al.*[17], this mutation resulted in nearly a complete loss in hemagglutination by the H5-VLP. However, to our knowledge, the binding properties of H7$_{Y98F}$ have not been described and we were surprised to find that some hemagglutinating capacity was retained. Nonetheless, the humoral immune responses to the reduced-binding H7$_{Y98F}$-VLP closely mirrored trends observed with the H1$_{Y98F}$-VLP[14], suggesting that complete ablation of SA binding is not essential for improved immunogenicity. It is possible that a reduction in binding affinity, which is thought to occur in Y98F HA mutants due to reduced depth of SA binding pocket, is sufficient to support the improved humoral response. The SA binding affinity of H7$_{Y98F}$-VLP was not measured in this study beyond its agglutination of avian erythrocytes but efforts to better-characterize its SA binding capacity and affinity using surface plasmon resonance are ongoing.

Although vaccine efficacy could not be assessed in the current study for lack of access to a BSL3 facility, the patterns of cellular and humoral immunity elicited by the H7$_{Y98F}$-VLP were very similar to our previous observations with H1$_{Y98F}$-VLP, which conferred superior protection in mice[14]. The H7$_{Y98F}$-VLP elicited significantly higher HI titers than the H7$_{WT}$-VLP, which are the most well-defined correlate of protection for influenza[25-27] and significantly correlated with improved outcomes following challenge in mice vaccinated with H1$_{Y98F}$-VLP[14]. Thus, it seems likely that mice vaccinated with H7$_{Y98F}$-VLP are better protected from challenge, however, further experimentation is required to confirm this hypothesis. In contrast, we did not detect any improvement in HI titers among mice vaccinated with H5$_{Y98F}$-VLPs, despite some evidence that humoral responses were improved (e.g. increased H5-specific PCs in the BM) and CD4$^+$ T cell responses that closely mirrored the H7$_{Y98F}$- and H1$_{Y98F}$-VLPs[14]. The mechanism underlying this difference between the two VLP vaccines targeting the avian strains is not yet clear. One possibility is that the HI assay itself lacks the sensitivity to detect differences as this assay is known to have low sensitivity for H5-specific antibodies[28,29]. The sensitivity of the HI assay for H5 strains can be improved using horse RBC instead of turkey RBC[29] and a re-evaluation of the HI response in these animals using this more sensitive technique is on-going. It is also possible that the glycosylation

pattern of the H5 globular head limits the impact of the Y98F mutation on vaccine responses. Glycans surrounding the receptor binding domain (RBD) of HA are known to reduce the affinity of HA-SA interactions[30]. Thus, the presence of two glycans directly surrounding the RBD of H5[31], but not H1 or H7[31], may reduce the impact of the Y98F mutation in the H5-VLP. These glycans may also act as a 'shield' to prevent antibody recognition of the immunodominant regions on the globular head that are detected using the HI assay[31]. Lastly, it is possible that the high antigen dose used to vaccinate mice in these studies could have masked an effect that could be seen at lower doses. These possibilities highlight the need for further analysis of the humoral response to the H5$_{WT}$- and H5$_{Y98F}$-VLPs to understand the potential generalizability of the non-binding strategy to improve vaccine responses.

Another important consideration in the design of next-generation vaccines for avian influenza is improving the durability of immune responses. Others have demonstrated that antibody titers decline rapidly following vaccination with monovalent inactivated H5N1 and H7N9 vaccines, even when combined with an adjuvant[32,33]. Although we did not assess the durability of immune responses in the studies presented herein, both the H7$_{Y98F}$- and H5$_{Y98F}$-VLPs resulted in a significant increase in HA-specific PC in the BM, which are thought to mediate long-term maintenance of serum antibody titers following vaccination[34]. These results are consistent with our previous findings in mice vaccinated with H1$_{Y98F}$-VLP, in which the frequency of BM PC was associated with improved maintenance of neutralizing antibody titers[14]. Furthermore, the H5$_{Y98F}$-VLP also resulted in an increase in HA-specific CD4$^+$ T cells in the BM when vaccine doses were administered 8 weeks apart. This population is thought to confer long-lasting immunity[24,35] and this finding further suggests that the ablation of HA-SA interactions may improve the durability of immunity elicited by the H5-VLP. Similar trends were not observed among mice vaccinated with H7$_{Y98F}$-VLP, however, the frequency of HA-specific CD4$^+$ T cells in the BM was quite high in H7$_{Y98F}$- and H7$_{WT}$-VLP-vaccinated mice and cytokine signatures were consistent with studies examining the H5- and H1-VLPs[14]. Based on these findings, it seems likely that the H7$_{Y98F}$- and H5$_{Y98F}$-VLPs result in more durable immune responses but additional long-term studies are required to confirm this hypothesis.

In addition to assessing the impact of HA-SA interactions on immune responses, restrictions associated with the coronavirus pandemic caused us to inadvertently evaluate the impact of delaying the second vaccine dose. Importantly, our findings were highly reproducible

with similar trends regardless of whether mice were boosted 3w or 8w after the primary dose. However, delaying the second dose resulted in substantially higher antibody titers and avidity, increased memory B cells in the spleen and increased bone marrow plasma cells in all vaccine groups. Similar effects have also been observed in a number of vaccines when the second dose is delayed, including an adenovirus-vectored Covid-19 vaccine[36,37], an attenuated vaccinia virus vaccine[38], and a chimeric tuberculosis vaccine[39]. Thus, it may be advantageous to delay the second dose of VLP vaccines targeting H5N1 and H7N9 if sufficient short-term protection is elicited by the first vaccine dose. If successful, this strategy may also be implemented to improve responses to current influenza vaccines in young children who receive 2 doses until next-generation vaccines are available.

Taken together, we have demonstrated that reducing the binding of HA to its SA receptor is a promising strategy to improve the immunogenicity of plant-based VLP vaccines targeting avian influenza strains. These findings highlight the breadth of the 'non-binding' approach that we have previously described with both VLP and recombinant vaccines targeting H1N1[14], although further investigation is required to determine whether the H7$_{Y98F}$- and H5$_{Y98F}$-VLPs confer similarly enhanced antibody durability and protection. Of course, it remains to be demonstrated that this simple approach will also improve immune responses to seasonal and avian strain influenza vaccines in humans and these studies are in development. The demonstration of a positive effect of using non-binding antigens could contribute to the development of better next-generation influenza vaccines across multiple production platforms.

4.5 MATERIALS AND METHODS

4.5.1 HA expression cassettes

The sequences encoding mature wild-type (WT) and Y98F HA0 A/Shanghai/02/2013 (H7N9) of A/Indonesia/05/2005 (H5N1) fused to alfalfa PDI secretion signal peptide (PDISP) were cloned into 2X35S/CPMV160/NOS expression system using PCR-based methods. Sequences of the primers indicated below can be found in **Table 1.** To generate the H7$_{WT}$ expression cassette, the PDISP-A/Shanghai/02/2013 coding sequence was amplified using primers IF-CPMV(fl5'UTR)_SpPDI.c and IF-H7Shang.r. To generate the H7$_{Y98F}$ expression cassette, the

PDISP-A/Shanghai/02/2013 coding sequence with the mutated Y98F amino acid (H3 numbering) was amplified first using primers IF-CPMV(fl5'UTR)_SpPDI.c and H7Shang(Y88F).r, and second using primers H7Shang(Y88F).c and IF-H7Shang.r. The PCR products from both amplifications were mixed and used as a template for amplification using primers IF-CPMV(fl5'UTR)_SpPDI.c and IF-H7Shang.r. To generate the $H5_{WT}$ expression cassette, the PDISP-A/Indonesia/05/2005 conding sequence was amplified using primers IF-CPMV(fl5'UTR)_SpPDI.c and IF-H5ITMCT.sl-4r. To generate the $H5_{Y98F}$ expression cassette, the PDISP-A/Indonesia/05/2005 coding sequence with the mutated Y98F amino acid (H3 numbering) was amplified first using primers IF-CPMV(fl5'UTR)_SpPDI.c and H5Indo(Y91F).r, and second using primers H5Indo(Y91F).c and IF-H5ITMCT.sl-4r. The PCR products from both amplifications were mixed and used as a template for amplification using primers IF-CPMV(fl5'UTR)_SpPDI.c and IF-H5ITMCT.sl-4r. The final amplification products were assembled into the pCAMBIA binary plasmid containing 2X35S/CPMV160/NOS and linearized by digestion with *Sac*II and *Stu*I restriction enzymes using the In-Fusion cloning system (Clontech, Mountain View, CA).

4.5.2 Protein expression and VLP purification

Virus-like particles (VLPs) were produced by transient transfection of *Nicotiana benthamiana* plants with *Agrobacerium tumefaciens* carrying $H7_{WT}$, $H7_{Y98F}$, $H5_{WT}$ or $H5_{Y98F}$ expression cassettes. *N. benthamiana* plants (41–44 days old) were vacuum infiltrated in batches and the aerial parts of the plants were harvested and frozen (-80°C) after 7 days of incubation. $H7_{WT}$- and $H7_{Y98F}$-VLPs were extracted and purified exactly as previously described. $H5_{WT}$ and $H5_{Y98F}$ were extracted and purified as previously described with the following modification: once captured on the Poros HS column (Thermo Scientific), samples were washed with 5mM $NaPO_4$, 50mM Bis-Tris, 0.005% Tween 80 (pH 6.0) instead of 25mM Tris, 0.005% Tween 80 (pH 8.0). Final formulations were dialyzed against formulation buffer (100mM $NaKPO_4$, 150mM NaCl, 0.01% Tween 80 (pH 7.4)) and passed through a 0.22µm filter for sterilization. Protein concentrations were determined using Pierce™ micro BCA protein assay kit according to the manufacturer's instructions.

4.5.3 Gel electrophoresis and immunoblot analysis

To confirm HA expression, purified VLP products (1µg/sample) were separated a Criterion XT 4-12% Bis-Tris gel (Bio-Rad) under reducing conditions and then transferred onto a PVDF membrane. Successful transfer was confirmed using ponceau red staining followed by de-staining with water. Membranes were blocked overnight (4°C) with 5% skim milk in TBST (tris-buffered saline, 0.1% Tween 20) and then incubated with rabbit polyclonal anti-H7 (Cat. No. IT-003-008, Immune Technology) or rabbit polyclonal anti-H5 (Cat No. IT-003-005I, Immune Technology) diluted 1:1500 in TBST+2% skim milk for 1h at room temperature (RT). Membranes were then incubated for 1h (RT) with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat. No. IT-200-01, Immune Technology) diluted 1:20,000 in TBST+2% skim milk. Bands were developed using Super Signal West Pico chemiluminescent substrate (Thermo Fischer) and detected on X-ray films. For VLP composition and purity analysis, purified VLP products (5µg/sample) were separated on a 4-12% Bis-Tris gel as described above followed by staining with biosafe Coomassie G-250 (Bio-Rad). Gels were imaged using ChemiDoc^{TM} XRS+ system (Bio-Rad).

4.5.4 Hemagglutination assay

VLPs were serially two-fold diluted in PBS (beginning at 1:300) and incubated for 3h with an equivalent volume of 0.25% turkey red blood cells (RBC) diluted in PBS. Hemagglutination was confirmed by the absence of a RBC pellet following incubation. The hemagglutination titer is the reciprocal of the highest dilution where hemagglutination occurred.

4.5.5 Vaccination and sample collection

All animal procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University. Female Balb/c mice (8-10 weeks old, Charles River Laboratories) were immunized by injection into the quadricep muscle with 3µg VLP (50µL total in PBS). Mice were vaccinated on day 0 and boosted 8 weeks later. To evaluate humoral and cell-mediated immune responses mice were euthanized 5 weeks post-boost by CO_2 asphyxiation. Blood was collected from the left lateral saphenous vein before each vaccination, on weeks 2 and 4 following the first dose, and by cardiac puncture at study endpoints. Sera were obtained by centrifugation of blood in microtainer serum separator tubes

(Beckton Dickinson) (8000x*g*, 10min) and stored at -20°C until further analysis. Spleens and bilateral femurs were harvested and splenocytes and bone marrow immune cells were isolated as previously described[40,41].

4.5.6 Quantification of HA-specific IgG and avidity

Total HA-specific IgG was quantified by enzyme-linked immunosorbent assay (ELISA) as previously described. To evaluate the avidity of HA-specific IgG, wells containing bound antibody were incubated with urea (0M-8M) for 15min and re-blocked for 1h prior to detection. Avidity index (AI) = [IgG titer 2-10M urea/IgG titer 0M urea].

4.5.7 Hemagglutination inhibition (HI) assay

The hemagglutination inhibition (HI) assay was carried out as previously described[42,43]. However, given the high pathogenicity of H5N1 and H7N9 strains, HI assays were carried out using H5$_{WT}$-VLP or H7$_{WT}$-VLP instead of live virus as previously described[16,44]. Titers are reported as the reciprocal of the highest dilution to inhibit hemagglutination. Samples below the limit of detection (<10) were assigned a value of 5 for statistical analysis.

4.5.8 IgG ELISpot

HA-specific IgG-producing ASCs were quantified by ELISpot (Mouse IgG ELISpot^{BASIC}, Mabtech). Sterile PVDF membrane plates (Millipore) were coated with Anti-IgG capture antibody and blocked according to the manufacturer's guidelines. To quantify *in vivo* activated ASCs, wells were seeded with 250,000 (bone marrow) or 500,000 (splenocyte) freshly-isolated cells and incubated at 37°C, 5% CO_2 for 24h. HA-specific ASCs were detected according to the manufacturer's guidelines using 1µg/mL biotinylated H5 or H7 (immune tech, biotinylated using Sulfo-NHS-LC-Biotin). To evaluate memory ASCs, freshly isolated cells were polyclonally activated with 0.5µg/mL R848 and 2.5ng/mL recombinant mouse IL-2 ($1.5x10^6$ cells/mL in 12-well plates) for 48h (37°C, 5% CO_2). Activated cells were re-counted and the assay was carried out as described above.

4.5.9 Flow cytometry

To identify antigen-specific $CD4^+$ T cells, freshly isolated splenocyte or bone marrow immune cell suspensions ($1x10^6$/200µL in a 96-well U-bottom plate) were stimulated with 10% cRPMI (negative control) or 2µg/mL homologous HA_{WT}-VLP (18h) (37°C, 5% CO_2). Golgi Stop and Golgi Plug (BD Biosciences) were added 5h before the end of the stimulation according to the manufacturer's instructions. Cells were washed 2x with PBS (320x*g*, 8min, 4°C) and labeled with Fixable Viability Dye eFluor 780 (eBioscience) (20min, 4°C). Cells stained exactly as described previously with the following antibodies: anti-CD3 FITC (145-2C11, eBioscience), anti-CD4 V500 (RM4-5, BD Biosciences) anti-CD8 PerCP-Cy5.5 (53-6.7, BD Biosciences), anti-CD44 BUV395 (IM7, BD Biosciences) anti-CD62L BUV373 (MEL-14, BD Biosciences) anti-IL-2 APC (JES6-5H4, Biolegend), anti-IFNγ PE (XMG1.2, BD Biosciences) and anti-TNFα eFluor450 (MP6-XT22, Invitrogen). All flow cytometry was conducted using a BD LSRFortessa X20 cell analyzer. Data was analyzed using FlowJo software (Treestar, Ashland).

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4.8 TABLE

Table 1. Primers used to prepare VLP constructs

Primer Identifier	Sequence
IF-CPMV(fl5'UTR)_SpPDI.c	TCGTGCTTCGGCACCAGTACAATGG CGAAAAACGTTGCGATTTTCGGCT
IF-H7Shang.r	ACTAAAGAAAATAGGCCTTTATATA CAAATAGTGCACCGCATGTTTCCAT
H7Shang(Y88F).r	ACGAATTTCCCAGGGAAACAGACA TCACTTCCTTCTCGCCTCTCSSTSST
H7Shang(Y88F).c	AGGAAGTGATGTCTGTTTCCCTGGG AAATTCGTGAATGAAGAAGCTCTGA
IF-H5ITMCT.sl-4r	ACTAAAGAAAATAGGCCTTTAAATG CAAATTCTGCATTGTAACGATCCAT
H5Indo(Y91F).r	GAAACTCCCTGGGAAACAGAGGTCATTG GTTGGATTGGCCTTCTCCACTATGTAAGA
H5Indo(Y91F).c	AACCAATGACCTCTGTTTCCCAGGG AGTTTCAACGACTATGAAGAACTGAA

4.9 FIGURES AND LEGENDS

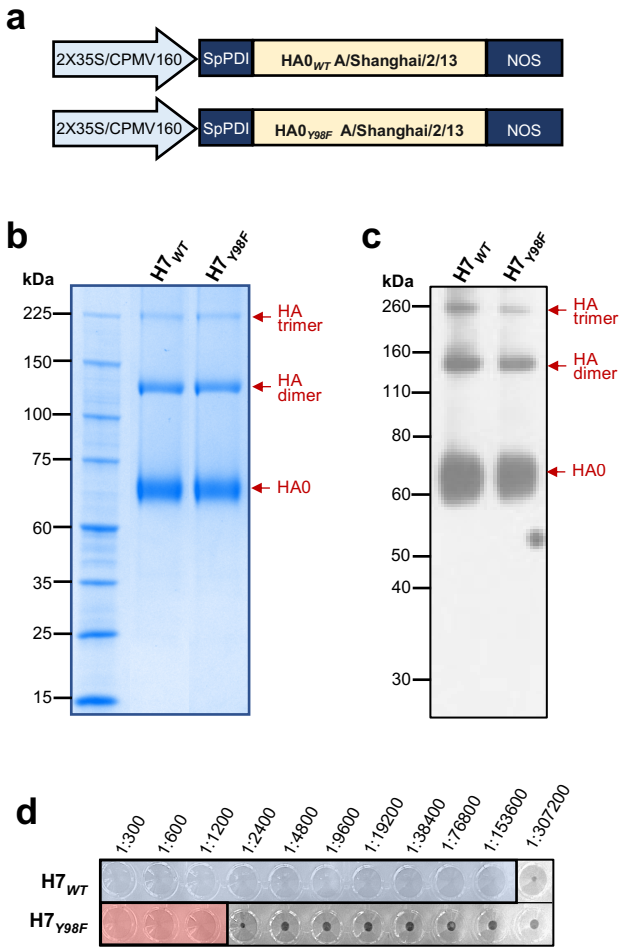


Figure 4.1. Y98F mutation of H7 A/Shanghai/02/2013 markedly reduces SA binding without impacting VLP expression in plants. Wild-type (WT) H7 ($H7_{WT}$) and Y98F H7 ($H7_{Y98F}$) were expressed in *Nicotiana benthamiana*. (a) Representation of the $H7_{WT}$ (top) and $H7_{Y98F}$ (bottom) expression cassettes. 2X35S/CPMV160 promoter, double 35S promoter fused to the 5' UTR of a cowpea mosaic virus (CPMV) expression enhancer; SpPDI, signal peptide from alfalfa protein disulfide isomerase; NOS, nopaline synthase terminator signal. (b) VLP composition and purity were evaluated by SDS-PAGE of purified leaf digests (5µg/well) followed by Coomassie G-250 staining. (c) Expression of HA was confirmed by SDS-PAGE of purified leaf digests (1µg/well) followed by immunoblot analysis. 1º ab: rabbit polyclonal anti-H7 1:1500 (Cat. No. IT-003-008, Immune Technology); 2º ab: horseradish peroxidase-conjugated goat anti-rabbit IgG 1:20000 (Cat. No. IT-200-01, Immune Technology). (d) Sialic acid (SA) binding was evaluated based on hemagglutination of turkey red blood cells following incubation (30 min) with serial 2-fold dilutions of $H7_{WT}$- and $H7_{Y98F}$-VLP (starting at 1:300). Horizontal bars represent the HA titer for each VLP formulation (the highest dilution where hemagglutination occurs).

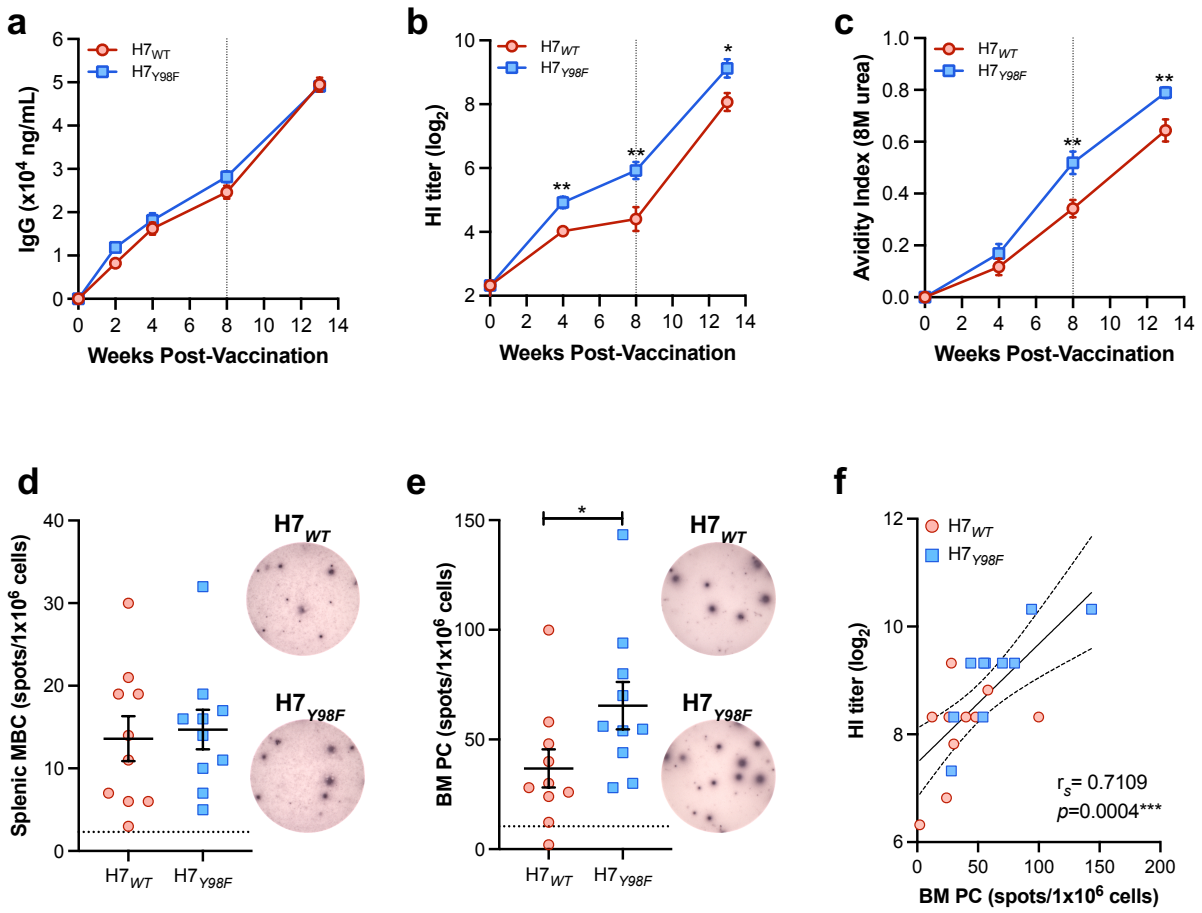


Figure 4.2. H7$_{Y98F}$-VLP elicits a more robust humoral immune response than H7$_{WT}$-VLP. Mice were vaccinated (IM) with two doses, 8 weeks apart, of H7$_{WT}$- or H7$_{Y98F}$-VLP (3µg/dose, N=10/group). (a-c) Sera were collected at weeks 2, 4, 8 and 13 to measure (a) total H7-specific IgG by ELISA, (b) hemagglutination inhibition (HI) titers, and (c) IgG avidity indices, which represent the ratio of IgG that remains bound following 15min incubation with 8M Urea. Administration of the second dose is indicated by vertical dashed lines. Mice were euthanized 5 weeks post-boost (week 13) to quantify H7-specific IgG-producing memory B cells (MBC) and plasma cells (PC) by ELISpot. (d) To identify MBC, splenocytes were stimulated with R848 and recombinant mouse IL-2 for 48h prior to probing for H7-specific antibody secreting cells. (e) Bone marrow immune cells isolated from the right hind-limb femur and tibia were probed for H7-specific PC. In (d) and (e) the frequency of H7-specific MBC and PC in PBS-vaccinated mice is indicated by a dashed line (mean, N=10) and representative wells from each group are shown on the right. (f) Spearman's rank correlation technique was applied to evaluate the relationship between the frequency of BM PC and HI titers in in H7$_{WT}$- and H7$_{Y98F}$-VLP vaccinated mice. Statistical significance between vaccine groups was determined by Mann-Whitney test (*p<0.033, **p<0.01, ***p<0.001).

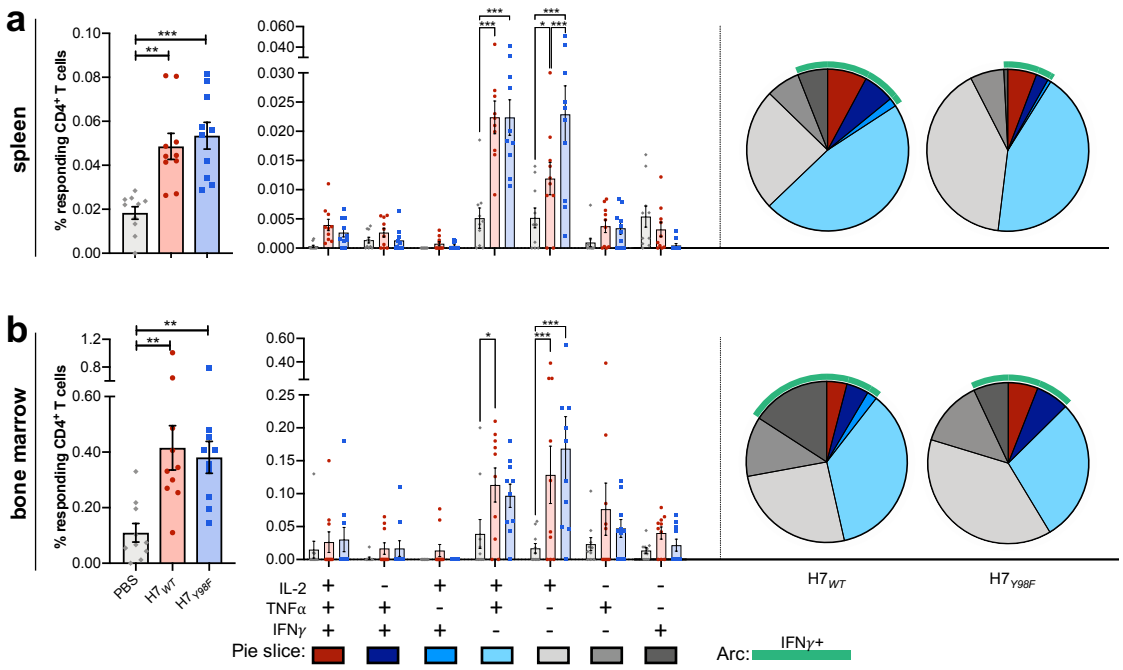


Figure 4.3 H7$_{WT}$- and H7$_{Y98F}$-VLPs elicit robust CD4$^+$ T cell responses. Female Balb/c mice were vaccinated with two doses of H7$_{WT}$- or H7$_{Y98F}$-VLP (3µg/dose) administered 8w apart. Five weeks post-boost splenocytes and BM immune cells were harvested and stimulated for 20h with 2µg/mL H7$_{WT}$-VLP. Flow cytometry was used to quantify H7-specific CD4$^+$ T cells in (a) splenocytes and (b) BM immune cells. The left panel shows the frequency of CD4$^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. The right panel shows the individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with H7$_{WT}$-VLP. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).

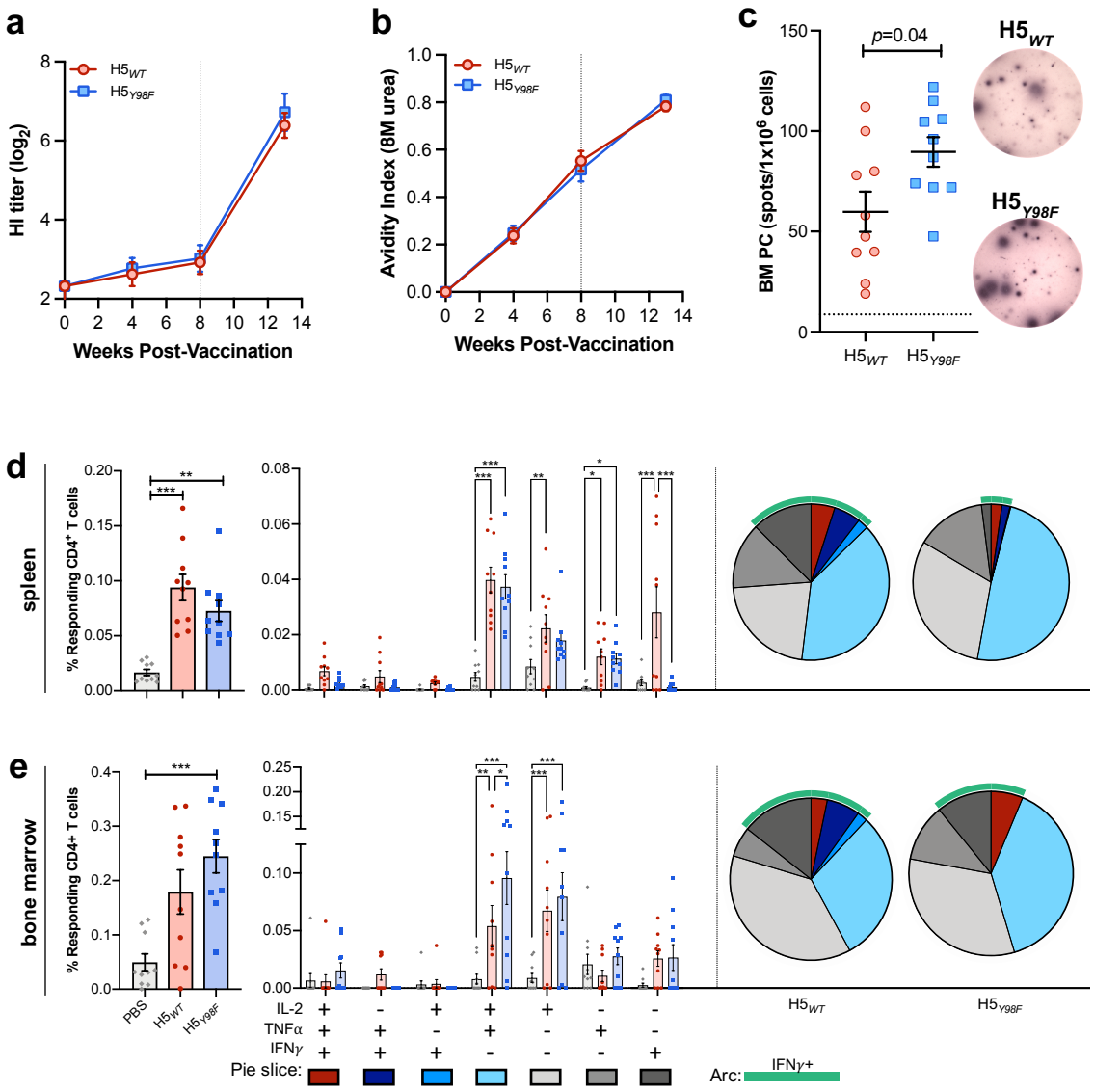
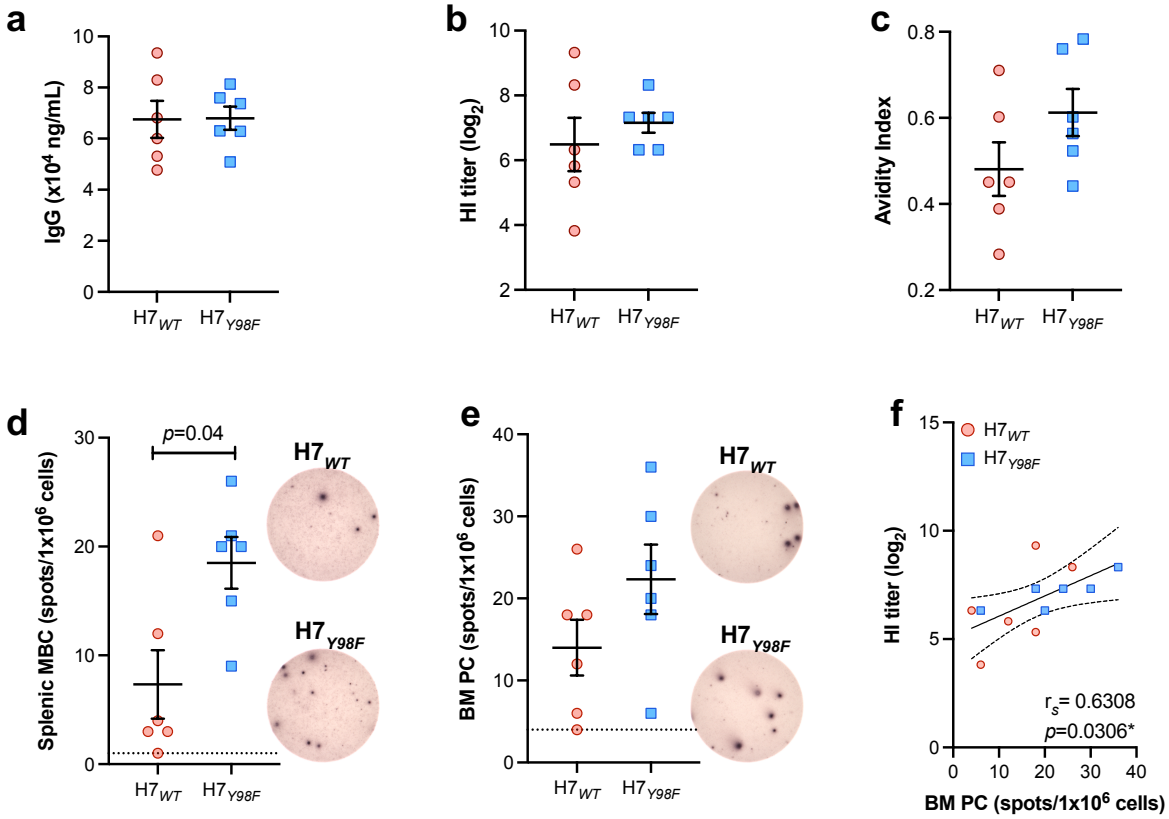


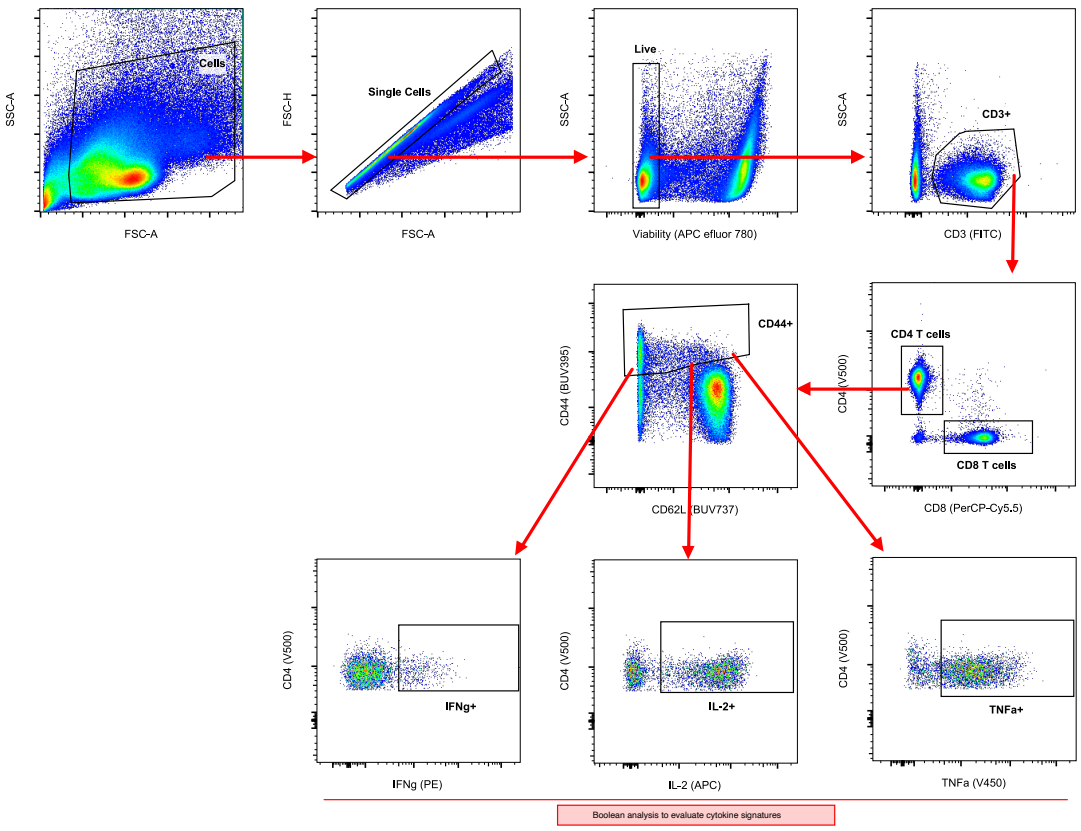
Figure 4.4. H5$_{Y98F}$-VLP elicits similar populations of CD4$^+$ T cells and BM PC to H7$_{Y98F}$-VLP without improving antibody titers. Mice were vaccinated (IM) with two doses, 8 weeks apart, of H5$_{WT}$- or H5$_{Y98F}$-VLP (3µg/dose, N=10/group). (a-c) Sera were collected at weeks 2, 4, 8 and 13 to measure (a) hemagglutination inhibition (HI) titers and (b) IgG avidity indices, which represent the ratio of IgG that remains bound following 15min incubation with 8M Urea. Administration of the second dose is indicated by vertical dashed lines. Mice were euthanized 5 weeks post-boost (week 13) to quantify (c) H5-specific IgG-producing plasma cells (PC) in the bone marrow by ELISpot. The frequency of H5-specific PC in PBS-vaccinated mice is indicated by a dashed line (mean, N=10). (d) Splenocytes and (e) BM immune cells were stimulated for 20h

with 2μg/mL $H5_{WT}$-VLP and H5-specific $CD4^+$ T cells were detected by flow cytometry. The left panel of (d) and (e) shows the frequency of $CD4^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. The right panel shows the individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with $H7_{WT}$-VLP. For (a-c) statistical significance between vaccine groups was determined by Mann-Whitney test. For (d) and (e) statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).

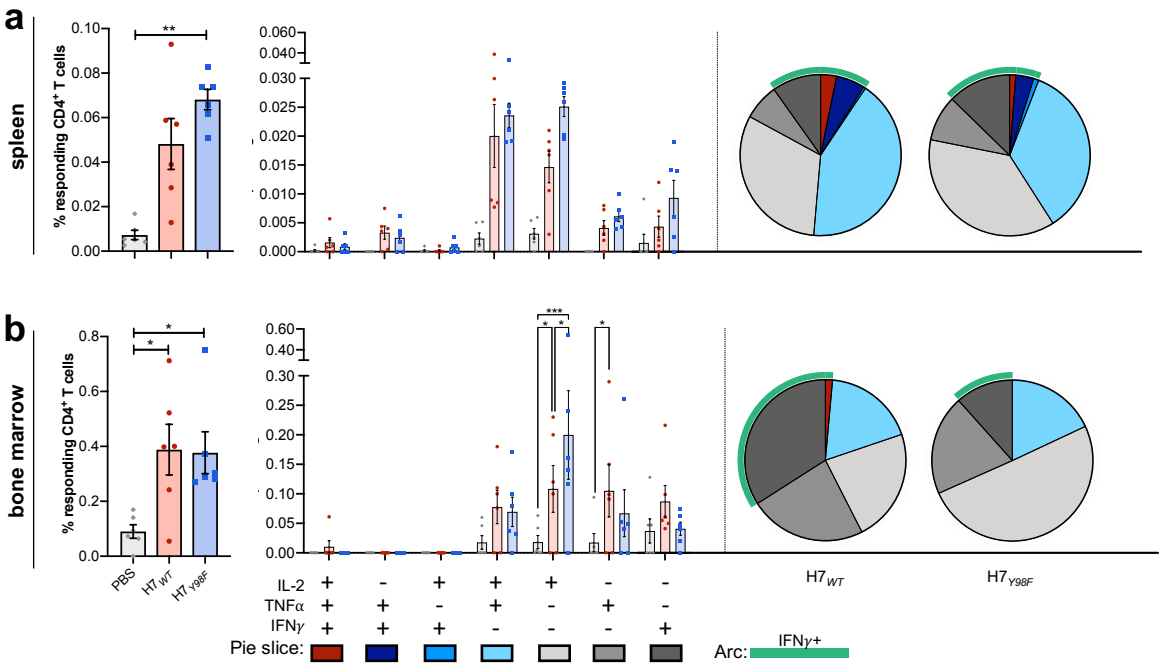
4.10 SUPPLEMENTAL FIGURES AND LEGENDS



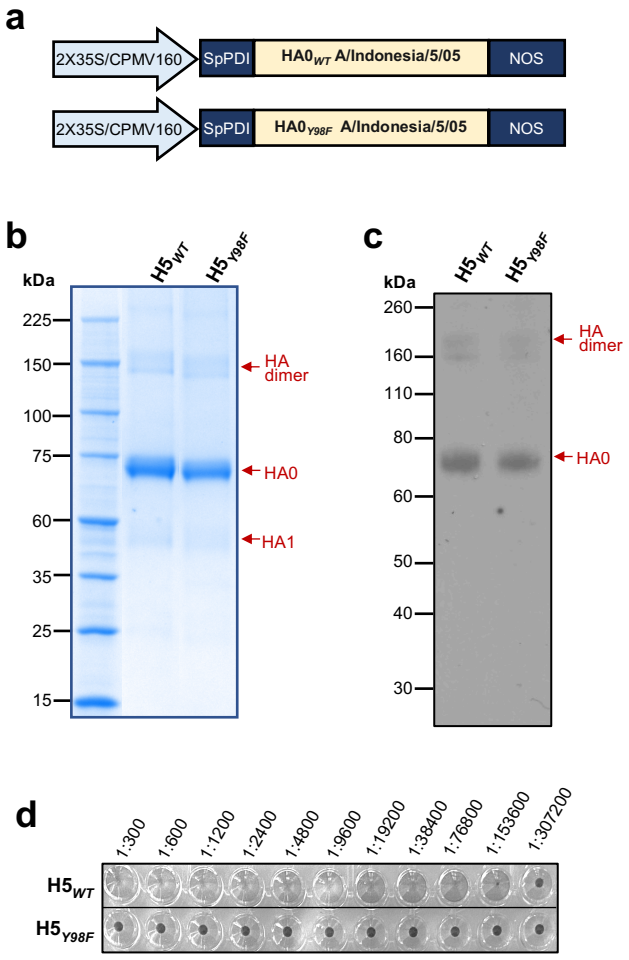
Supplemental figure 4.1. H7$_{Y98F}$-VLP results in enhanced humoral immune responses when doses are administered 3 weeks apart. Mice were vaccinated (IM) with two doses, 3 weeks apart, of H7$_{WT}$- or H7$_{Y98F}$-VLP (3µg/dose, N=6/group). (a-c) Sera were collected 4w post-boost to measure (a) total H7-specific IgG by ELISA, (b) hemagglutination inhibition (HI) titers, and (c) IgG avidity indices, which represent the ratio of IgG that remains bound following 15min incubation with 6M Urea. Mice were euthanized 4 weeks post-boost to quantify H7-specific IgG-producing memory B cells (MBC) and plasma cells (PC) by ELISpot. (d) To identify MBC, splenocytes were stimulated with R848 and recombinant mouse IL-2 for 48h prior to probing for H7-specific antibody secreting cells. (e) Bone marrow immune cells isolated from the right hind-limb femur and tibia were probed for H7-specific PC. In (d) and (e) the frequency of H7-specific MBC and PC in PBS-vaccinated mice is indicated by a dashed line (mean, N=6) and representative wells from each group are shown on the right. (f) Spearman's rank correlation technique was applied to evaluate the relationship between the frequency of BM PC and HI titers in in H7$_{WT}$- and H7$_{Y98F}$-VLP vaccinated mice. Statistical significance between vaccine groups was determined by Mann-Whitney test.



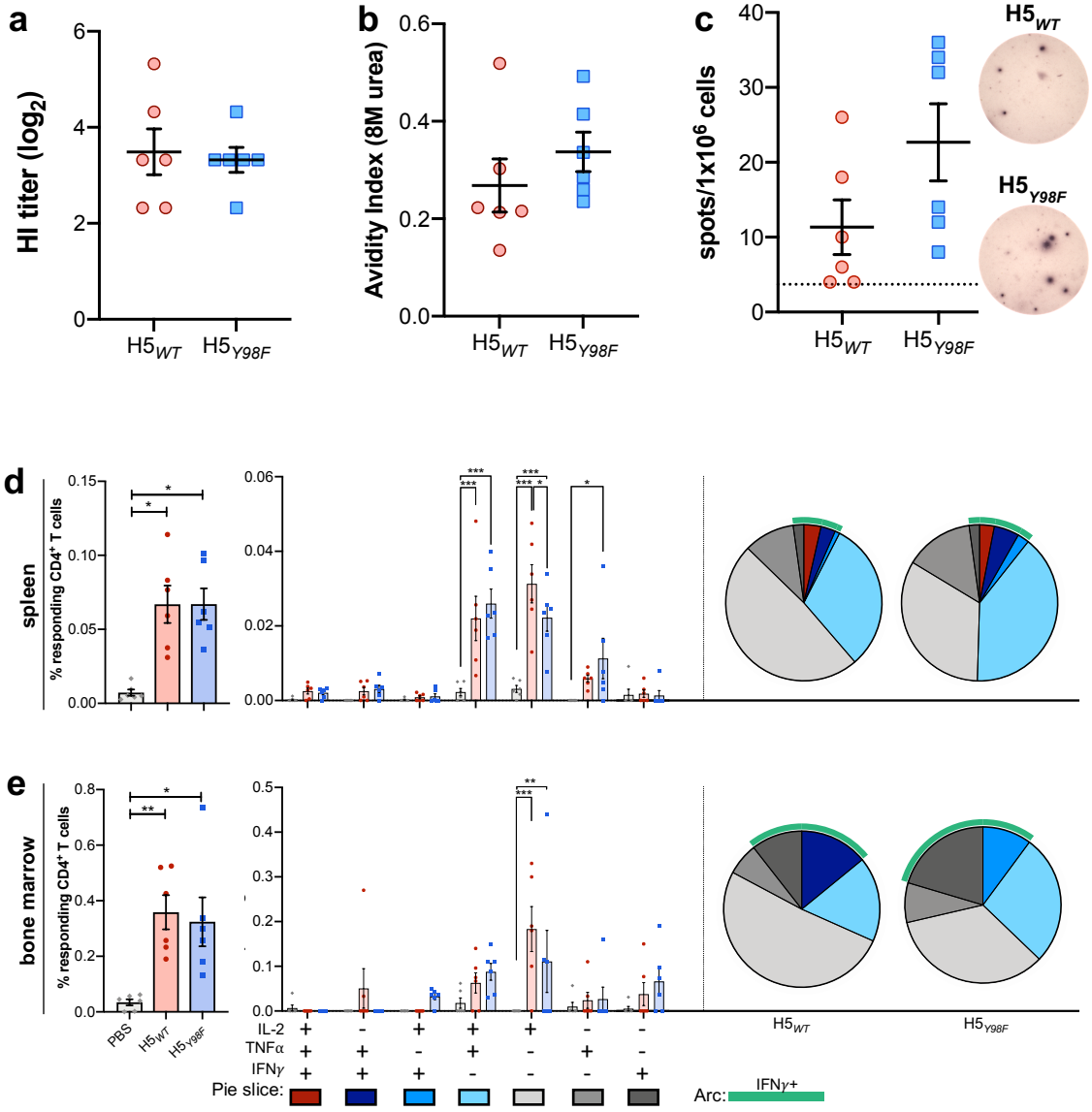
Supplemental Figure 4.2. Flow cytometry gating strategy to identify antigen-specific CD4$^+$ T cells. Cells were first gated to exclude debris, cell clusters, and dead cells. Antigen experienced CD4$^+$ T cells were distinguished based on expression of CD44. HA-specific CD4$^+$ T cells were identified based on expression of IFNγ, TNFα, and IL-2 following stimulation with H1$_{WT}$-VLP or H1 peptide pool. Boolean analysis was used to evaluate cytokine signatures.



Supplemental Figure 4.3 H7$_{WT}$- and H7$_{Y98F}$-VLPs elicit robust CD4$^+$ T cell responses when doses are administered 3w apart. Female Balb/c mice were vaccinated with two doses of H7$_{WT}$- or H7$_{Y98F}$-VLP (3µg/dose) administered 3w apart. Four weeks post-boost splenocytes and BM immune cells were harvested and stimulated for 20h with 2µg/mL H7$_{WT}$-VLP. Flow cytometry was used to quantify H7-specific CD4$^+$ T cells in (a) splenocytes and (b) BM immune cells. The left panel shows the frequency of CD4$^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. The right panel shows the individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with H7$_{WT}$-VLP. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).



Supplemental Figure 4.4. Y98F mutation of H5 A/Indonesia/05/2005 markedly reduces SA binding without impacting VLP expression in plants. Wild-type (WT) H5 ($H5_{WT}$) and Y98F H5 ($H5_{Y98F}$) were expressed in *Nicotiana benthamiana*. (a) Representation of the $H5_{WT}$ (top) and $H5_{Y98F}$ (bottom) expression cassettes. 2X35S/CPMV160 promoter, double 35S promoter fused to the 5' UTR of a cowpea mosaic virus (CPMV) expression enhancer; SpPDI, signal peptide from alfalfa protein disulfide isomerase; NOS, nopaline synthase terminator signal. (b) VLP composition and purity were evaluated by SDS-PAGE of purified leaf digests (5µg/well) followed by Coomassie G-250 staining. (c) Expression of HA was confirmed by SDS-PAGE of purified leaf digests (1µg/well) followed by immunoblot analysis. 1º ab: rabbit polyclonal anti-H5 1:1500 (Cat. No. IT-003-005I, Immune Technology); 2º ab: horseradish peroxidase-conjugated goat anti-rabbit IgG 1:20000 (Cat. No. IT-200-01, Immune Technology). (d) Sialic acid (SA) binding was evaluated based on hemagglutination of turkey red blood cells following incubation (30 min) with serial 2-fold dilutions of $H5_{WT}$- and $H5_{Y98F}$-VLP (starting at 1:300).



Supplemental Figure 4.5. Cellular and humoral immune responses to H5$_{WT}$- and H5$_{Y98F}$-VLPs. Mice were vaccinated (IM) with two doses, 3 weeks apart, of H5$_{WT}$- or H5$_{Y98F}$-VLP (3µg/dose, N=6/group). (a-c) Sera were collected 4 weeks post-boost to measure (a) hemagglutination inhibition (HI) titers and (b) IgG avidity indices, which represent the ratio of IgG that remains bound following 15min incubation with 8M Urea. Mice were euthanized 4 weeks post-boost (week 13) to quantify (c) H5-specific IgG-producing plasma cells (PC) in the bone marrow by ELISpot. The frequency of H5-specific PC in PBS-vaccinated mice is indicated by a dashed line (mean, N=6). (d) Splenocytes and (e) BM immune cells were stimulated for 20h with 2µg/mL H5$_{WT}$-VLP and H5-specific CD4$^+$ T cells were detected by flow cytometry. The left panel

of (d) and (e) shows the frequency of $CD4^+$ T cells expressing CD44 and at least one of IL-2, TNFα or IFNγ. The right panel shows the individual cytokine signatures for each mouse obtained by Boolean analysis. Background values obtained from non-stimulated samples were subtracted from values obtained following stimulation with $H7_{WT}$-VLP. For (a-c) statistical significance between vaccine groups was determined by Mann-Whitney test. For (d) and (e) statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparisons (total response) or two-way ANOVA with Tukey's multiple comparisons (cytokine signatures) (*p<0.033, **p<0.01, ***p<0.001).

CHAPTER 5

General Discussion

Vaccines are widely considered to be one of the greatest achievements in modern medicine and are the first line of defense against influenza. However, current influenza vaccines have several limitations including production challenges, variable immunogenicity, and efficacy that can wane rapidly after administration[1-3]. Thus, influenza remains a considerable public health threat and improved vaccine approaches are needed to reduce the societal impact of influenza epidemics and pandemics. In recent years, several technologies that significantly improve the speed, scalability and reliability of influenza vaccine production have emerged. Among them, recombinant technologies including the plant-based VLPs used throughout this thesis have shown great promise[4]. However, these vaccines still suffer from variable immunogenicity similar to traditional egg-based vaccines. This variability can be particularly pronounced with the avian influenza vaccines[5,6]. These similarities suggest that strain-specific features of HA proteins directly impact vaccine immunogenicity and understanding this relationship is an active area of investigation[7-9]. Our approach to investigate HA binding properties in the context of vaccination was initiated by the serendipitous finding that plant-based VLPs bearing different HA molecules can non-specifically bind to SA on human immune cells. The central goal of this thesis work was to evaluate the impact of these non-cognate HA-SA interactions on influenza vaccine immunogenicity and efficacy, and in so doing, we discovered a promising strategy with the potential to improve both the quality and durability of influenza vaccine responses.

5.1 MAIN FINDINGS

Early experimental work aimed to determine whether differences in the innate immune response to VLPs targeting avian and seasonal influenza contribute to the relatively poor immunogenicity of avian vaccines. We initially focused on evaluating the activation of human PBMC subsets following *in vitro* stimulation with H1- or H5-VLP and found that the H1-VLP

resulted in more robust activation of B cells consistent with enhanced antibody responses *in vivo*. These studies also led to the unanticipated observation that the H1-VLP 'agglutinate' PBMCs and prompted us to investigate the relationship between SA binding and PBMC activation. In **Chapter 2** we describe the distinct patterns of interactions of H1- and H5-VLPs with various immune cell subsets driven by the differential distribution of α2,6- and α2,3-linked SA on these cells. Most notably, exclusive expression of α2,6-linked SA on the surface of human B cells[10] resulted in high levels of binding by H1-VLP and limited binding by H5-VLP. Furthermore, binding of H1-VLPs to B cells led to their activation, likely through BCR crosslinking as previously described[11]. This was later confirmed by the marked reduction in B cell activation upon stimulation with the 'non-binding' H1$_{Y98F}$-VLP in **Chapter 3**. Based on these *in vitro* findings we hypothesized that differences in early immune cell interactions caused by the strain-specific but non-cognate binding preferences of different HA molecules can contribute to variable immunogenicity of influenza vaccines.

In **Chapter 3** and **Chapter 4**, we focused on determining whether HA-SA interactions influence VLP immunogenicity *in vivo* in mice. As a proof of principle, we designed an H1-VLP bearing H1 (A/California/07/2009) that does not bind to SA and compared its immunogenicity and efficacy to wild-type H1-VLP (H1$_{WT}$-VLP). This 'non-binding' mutant was generated using the well-described Y98F mutation (H1$_{Y98F}$-VLP), which inhibits binding to SA without affecting antigenicity[12,13]. We demonstrated that introduction of the Y98F mutation does not influence plant-based H1-VLP size or morphology but successfully inhibits interactions with SA. In mice, this novel vaccine resulted in markedly improved humoral immune responses and protection from homologous challenge. Key features of the humoral response elicited by the H1$_{Y98F}$-VLP included increased functional antibody (MN and HI) titers, improved antibody durability, and improved IgG avidity maturation compared to mice vaccinated with H1$_{WT}$-VLP. The mechanisms underlying these responses are not yet clear, however, increased frequencies of HA-specific germinal center (GC) B cells and increased longevity of the GC reaction in mice vaccinated with H1$_{Y98F}$-VLP likely contributes. We also demonstrated that functional antibody titers strongly correlated with reduced pulmonary viral load and inflammation, suggesting that the improved humoral immune responses elicited by H1$_{Y98F}$-VLP contribute to improved protection in these mice. Next, we sought to determine whether the benefits of non-binding status could be extended to VLPs bearing the HA protein of highly pathogenic avian influenza (HPAI) viruses, which tend to be poorly

immunogenic. In **Chapter 4**, we demonstrated that the Y98F HA mutation also improves the immunogenicity of VLPs bearing H5 (A/Indonesia/05/2005) and H7 (A/Shanghai/02/2013). H7$_{Y98F}$-VLP elicited improved antibody responses compared to H7$_{WT}$-VLP including increased HI titers and avidity maturation. Furthermore, vaccination with H7$_{Y98F}$- or H5$_{Y98F}$-VLP resulted in increased frequencies of HA-specific plasma cells in the bone marrow, which are thought to be long-lived. Although we could not evaluate protection due to the containment requirements for working with avian influenza viruses, parallels in the humoral and cellular responses between H7$_{Y98F}$-, H5$_{Y98F}$- and H1$_{Y98F}$-VLPs suggest that this approach may also improve durability of responses and protection from HPAI viruses. Taken together, we have demonstrated that eliminating HA-SA interactions is a promising strategy to improve the quality and durability of both seasonal and poorly immunogenic avian influenza vaccines.

5.2 FUTURE PERSPECTIVES

We have demonstrated that 'non-binding' HA-VLP vaccines targeting H1N1, H7N9, and H5N1 can improve the quality and durability of responses. However, whether this approach confers similar benefits among vaccines targeting other strains of influenza and different vaccine platforms remains unknown. Furthermore, the possible impact of non-cognate HA-SA interactions on vaccine-induced responses in humans has yet to be determined and may be influenced by differences in SA distribution and immune history. Future work will focus on evaluating the generalizability of the 'non-binding' approach and its potential for use in developing next-generation seasonal and pandemic influenza vaccines.

5.2.1 Generating 'non-binding' VLPS targeting additional HAs

This thesis describes the benefits of eliminating HA-SA interactions on the immunogenicity of VLPs bearing H1 A/California/07/2009, H5 A/Indonesia/05/2005, and H7 A/Shanghai/02/2013 in mice. Limited data from mice vaccinated with VLPs bearing H1 A/Idaho/02/2019 suggests that this approach may also be useful for improving the immunogenicity of more recently circulating strains. However, similar evaluations using 'non-binding' VLPs targeting H3 and influenza B virus (IBV) strains have been hindered by the lack of known

mutations to eliminate SA binding. Given that the efficacy of influenza vaccines tends to be lower for H3N2 strains[2,14] and that plant-based HA-VLPs elicit relatively modest antibody titers against IBV strains[15], evaluating the potential benefits of inhibiting SA binding in these strains is highly desirable. Thus, generating and validating novel H3 and IBV HA binding mutants in collaboration with Medicago Inc. is a major focus of ongoing work.

The binding mutants we have tested to date were generated using the well-described Y98F mutation, which prevents SA binding without impacting antigenicity. Substitution of tyrosine for phenylalanine removes a terminal hydroxyl group at amino acid 98, which is required to form two hydrogen bonds with SA[16,17]. The Y98F mutation also eliminates another hydrogen bond with H183, which reduces the depth of penetration into the SA binding pocket and limits SA binding avidity[16,17]. Although several other residues are known to be implicated in binding to SA, the Y98F mutation has been shown to be sufficient to eliminate binding in H1 and H5 and is widely thought to be broadly applicable to influenza A viruses (IAV)[12,13]. However, we and others[16] have demonstrated that the Y98F mutation is not always sufficient to eliminate SA binding by H3 HAs and its effects are strain-dependent. For example, the Y98F mutation results in substantial loss of SA binding by H3 A/Aichi/2/68 (20-fold reduction)[16] but has little impact on SA binding by H3 A/Kansas/14/2017 (**figure 5.1a**). Thus, ongoing efforts aim to identify novel mutants that reliably eliminate SA binding by H3 HAs. To this end, Medicago Inc. has generated a series of H3-VLP candidates targeting H3 A/Kansas/14/2017 with mutations at sites known to interact with SA (S136D/N, S137N, D190G/K, R222W, and S228N/Q±Y98F). Mutation at each of these sites resulted in nearly complete ablation of hemagglutination and several were sufficient to eliminate binding even without the Y98F mutation (**figure 5.1a-b**). Ongoing work will focus on evaluating the antigenicity of these non- or reduced-binding mutants to ensure that the immune responses elicited by vaccination will recognize the wild-type virus.

Although the Y98F mutation is often effective at eliminating binding by IAV, this mutation does not appear to work for IBV. In fact, IBV HAs naturally have a phenylalanine residue at position 95 (B/HK/73 numbering), which is equivalent to position 98 in IAV (H3 numbering)[18]. This results in reduced avidity of binding compared to IAV, however, binding still occurs through interactions with several other conserved residues within the binding pocket[18,19]. A handful of studies examining IBV HA-specific B cells have utilized a T139G HA mutant as a presumed 'non-binding' HA probe for B/Phuket/3073/2013 since this residue is positioned within the SA binding

pocket and is predicted to form two hydrogen bonds with SA[13,20]. However, the binding properties of T139G HA were not evaluated in these studies. In our hands, T139A mutation of the B/Phuket/3073/2013 HA did not reduce SA binding (**figure 5.2a**). This difference is unlikely to be caused by substitution with alanine instead of glycine, since these amino acids are quite similar and lack the terminal hydroxyl group required for hydrogen bonding with SA. Thus, considerable efforts have been put towards discovering mutations that prevent binding to of IBV HA to SA. Similar to their approach with the H3-VLPs, Medicago Inc. has generated a series of novel VLP candidates mutated at sites known to interact with SA (G138A, S140A, S142A, D195G, L203A/W). Each of these mutants results in substantial loss of HA binding in B-VLPs targeting Victoria and Yamagata lineage strains and efforts to evaluate the antigenicity and immunogenicity of these mutants are ongoing (**figure 5.2a-b**). Optimization of these mutants in addition to the 'non-binding' H3-VLPs described above will allow us to formulate a seasonal quadrivalent 'non-binding' influenza vaccine for clinical testing.

5.2.2 Considerations for use in humans

The 'non-binding' approach to influenza vaccines has shown great promise in the murine model. However, several factors may influence the success of this approach in humans. The following sections outline the possible benefits and challenges associated with transferring our findings to the clinical setting.

5.2.2.1 Sialic acid distribution

Sialic acids (SA) are widely expressed as the terminal sequence of glycoproteins and glycolipids of most mammalian cells[21]. In skeletal muscle, both mice and humans express high levels of α2,3- and α2,6-linked SA[22-24]. Thus, the potential impact of muscle sequestration on the immunogenicity of 'non-binding' VLPs is likely similar between mice and humans. However, the distribution of α2,3- and α2,6-linked SA on the various immune cell subsets is species-specific and may influence the immune response to HA binding mutants in humans. The most notable difference is that human B cells predominantly express α2,6-linked SA on their surface whereas murine B cells express both α2,3- and α2,6-linked SA at high levels[10,25]. In **Chapter 2**, we demonstrated that binding of VLPs to SA on immune cells can powerfully influence downstream

innate activation and that the absence of α2,3-linked SA on B cells precludes their activation by an H5-VLP. It remains to be determined whether the improved immunogenicity of H1$_{Y98F}$-, H5$_{Y98F}$- or H7$_{Y98F}$-VLPs in mice is attributable to a lack of non-cognate interactions with B cells. However, it is important to consider that avian type HAs bind to murine but not human B cells due to differences in α2,3-linked SA expression. Thus, the Y98F mutation may have a greater impact on H7- and H5-VLP immunogenicity in mice than humans if changes in HA-B cell interactions are an important aspect of improved responses. In contrast, the interactions between mammalian type HAs and lymphocytes are likely quite similar between mice and humans because α2,6-linked SA are ubiquitously expressed in both species[10,25,26]. Further investigation will be required to determine the exact mechanisms underlying improved immunogenicity of 'non-binding' HA-VLPs and to establish whether differences in SA expression in various tissues (eg: skeletal muscle) and immune cell subsets influence the success of this approach in humans.

5.2.2.2 Impact of pre-existing immunity

The vast majority of individuals receiving influenza vaccines have been exposed to a variety of influenza strains through previous vaccination and natural infection. Estimates for annual exposure to influenza viruses vary widely but generally fall between 10-25% in adults[27]. The studies described in this thesis were therefore limited by the fact that the mice were naïve for influenza. Given the complex relationship between immune history and influenza vaccine responses[28], it will be important to determine whether or not pre-existing immunity influences the impact of using 'non-binding' HA-VLPs in human vaccines.

The impact of immune history on influenza vaccine responses has been an area of active investigation since the late 1950s when Thomas Francis first described the phenomenon of 'original antigenic sin'[29]. Although more recent models have suggested that a combination of early life exposures and recently encountered strains have a greater impact than the 'original' exposure, the potential impact of pre-existing immunity is undisputed[28,30,31]. Whether the benefits of the 'non-binding' HA-VLPs will be maintained in influenza-experienced individuals remains to be determined. However, a recent study by Mesin *et al.* found that post-boost GCs are primarily composed of clonally diverse naïve B cells, suggesting that memory responses from prior influenza exposures may have limited impact on germinal center (GC) reactions following vaccination[32].

Although this phenomenon limits further diversification and affinity maturation of existing memory B cells (MBC), it suggests that pre-existing MBC are unlikely to interfere with the improved GC responses elicited by the HA binding mutants. Furthermore, our finding that the benefits of non-binding HA trimers and VLPs were maintained upon administration of a second vaccine dose suggests that this strategy may be beneficial even in the context of pre-existing immunity. Examining the impact of prior influenza exposure on the response to HA binding mutants is a focus of ongoing work and will be an important consideration in designing and evaluating clinical studies.

5.2.2.3 Breadth of protection

Conventional influenza vaccines are well known to elicit narrow and short-lived protection[3,33]. In the context of a rapidly evolving virus, these challenges can significantly hinder vaccine efficacy and there is a strong push to develop more broadly protective influenza vaccines that can confer long-term immunity[34]. One of the greatest limitations of our 'non-binding' VLP vaccine candidates is that they limit the breadth of the antibody response compared to their wild-type counterparts. We recently found that vaccination of mice with H1$_{WT}$-VLP resulted in modest levels of IgG that cross-reacted with H5 (A/Indonesia/05/2005) but very little H5-specific IgG was detected in mice vaccinated with H1$_{Y98F}$-VLP (**figure 5.3**). This is not terribly surprising given that affinity maturation and prolonged GC reactions tend to limit clonal diversity[35,36]. However, we were encouraged to find that both vaccines resulted in IgG with a high degree of cross-reactivity to a drifted pdm09-like H1 (A/Brisbane/02/2018) (**figure 5.3**). The fact that improved avidity maturation in response to H1$_{Y98F}$-VLP does not impact recognition of a drifted HA suggests that the reduced breadth may not limit efficacy against either drifted or modestly mis-matched HAs. Combined with the improved durability of responses elicited by the H1$_{Y98F}$-VLP, the use of 'non-binding' HAs in vaccines may represent a beneficial and easily implemented strategy until a 'universal' or more broadly protective influenza vaccine is developed.

5.2.2.4 Balancing cellular and humoral immune responses

Although humoral immune responses are the primary correlate of protection considered for influenza vaccine licensure, there is a growing appreciation that cellular immunity can play an

important role in protection[37-39]. Medicago's plant-based HA-VLP vaccines are known to elicit strong HA-specific $CD4^+$ T cell responses including high levels of polyfunctional cells (expressing two or more of IL-2, IFNγ and TNFα), which are thought to contribute to superior protection[40-42]. We were encouraged to find that the frequencies of total responding and polyfunctional $CD4^+$ T cells were not impacted by the loss of HA-SA interactions with any of the VLPs tested. However, 'non-binding' HAs consistently resulted in reduced frequencies of $CD4^+$ T cell populations expressing IFNγ. The implications of this finding have yet to be determined. On one hand, mice vaccinated with $H1_{Y98F}$-VLP were better protected from infection than mice vaccinated with $H1_{WT}$-VLP despite markedly reduced $IFN\gamma^+$ $CD4^+$ T cells. This is consistent with prior reports that IFNγ is dispensable for protection from influenza[43,44]. However, others have demonstrated that in the absence of neutralizing antibodies, influenza-specific $IFN\gamma^+$ $CD4^+$ T cells can reduce the severity of infection in mice and humans[37,45]. Given that influenza-specific $CD4^+$ T cells are more broadly reactive than antibodies[46], this population may be an important source of cross-protection in the event of a vaccine mis-match when neutralizing antibodies are reduced or absent.

A major focus of ongoing work will be to assess the individual roles of HA-specific antibodies and $CD4^+$ T cells in mediating protection following vaccination with wild-type and 'non-binding' HA-VLPs. This will be accomplished by challenging mice with homologous or heterologous strains of influenza following adoptive transfer of T cells or passive transfer of antibodies from vaccinated mice. Furthermore, investigations are ongoing to develop a strategy to increase IFNγ production by HA-specific $CD4^+$ T cells without compromising the improved humoral responses elicited by 'non-binding' HA-VLPs. We are currently evaluating the efficacy of a 'mix-and-match' strategy whereby mice are either vaccinated twice with a mix of WT and 'non-binding' VLPs or are primed with WT VLP and boosted with 'non-binding' VLP (and vice-versa) in hopes of achieving a more balanced humoral and cellular immune response.

5.2.3 Potential applications beyond plant-based influenza vaccines

The introduction of mutations that prevent HA-SA interactions has shown great promise in improving the immunogenicity and efficacy of plant-based VLP vaccines for influenza. However, it is unknown whether this approach will confer similar benefits among vaccines produced using different production platforms and vaccines targeting other SA-binding viruses.

The following sections will discuss potential applications for the "non-binding" approach beyond plant-based HA-VLPs and important considerations for their success.

5.2.3.1 Influenza vaccine production platforms

Introduction of mutations that prevent HA-SA interactions is a simple and effective strategy that can be readily implemented in a number of existing influenza vaccine production platforms. Our finding that recombinant H1$_{Y98F}$ trimers resulted in increased antibody titers and avidity suggests that this approach may confer benefits across all recombinant vaccine production platforms, which account for a large proportion of new and emerging influenza vaccine technologies[47,48]. Furthermore, nucleic acid vaccines have garnered much attention in the wake of COVID-19 vaccine successes and candidate mRNA vaccines encoding influenza HAs have shown promise in pre-clinical development[49]. It remains to be determined whether 'non-binding' HAs expressed *in vivo* will confer similar benefits to injected recombinant proteins, however, this could readily be investigated given the flexibility of nucleic acid-based platforms.

In contrast, the 'non-binding' approach cannot be directly transferred to vaccines that rely on the growth of live virus such as traditional egg-based and cell-culture based live-attenuated and inactivated vaccines. Although viral entry and replication can occur in the absence of SA binding[50-52], studies using a reverse engineered H3N2 virus bearing Y98F HA found that mutations that restored HA binding frequently occurred as a result of selective pressure[16]. Nonetheless, it is possible that HA-SA interactions by wild-type vaccine strains can be prevented by saturating the receptor binding domain (RBD) of HA with a soluble ligand prior to vaccination. One such example is the use of DNA aptamers that bind to the RBD and prevent SA binding. These short oligonucleotides have been shown to block entry of a wide range of seasonal and avian influenza strains both *in vitro* and *in vivo*[53-56]. Other small molecules reported to block SA binding by HA include *N*-cyclohexyltaurine and soluble sialylactose conjugates[57,58]. However, additional investigations will be required to determine whether such ligands impact immune recognition of the HA globular head domain.

5.2.3.2 Other viruses that bind sialic acid

Although the receptor binding properties of influenza are the most well-defined, a broad

spectrum of viral antigens are known to bind to SA including many found in currently licensed vaccines. For example, the mumps hemagglutinin-neuraminidase (HN) protein primarily binds to α2,3-sialylated trisaccharides[59,60] and is the major target of most neutralizing antibodies following natural infection and vaccination with the measles, mumps, and rubella (MMR) vaccine[61,62]. Interestingly, mumps is the only SA-binding virus of the three vaccine components and is also the least effective component of the MMR vaccine[63]. A study examining the antibody response to the MMR vaccine found that the mumps component elicited the lowest avidity antibodies and resulted in the greatest decline in antibody titers and avidity over time[64]. This pattern closely mirrors responses to influenza vaccines and suggests that elimination of HN-SA interactions may improve the quality of the humoral response to mumps vaccination. Kubota *et al.* found that a Y369A mutation dramatically reduced HN binding without impacting protein conformation[59], suggesting that this mutant may be a good candidate for assessing the impact of SA binding on mumps vaccine immunogenicity. Other vaccine antigens that are known to bind SA include the VP8* domain of bovine-human reassortant rotavirus vaccine strains (RotaTeq) and the SARS-CoV-2 spike protein, which bind to α2,6-linked and α2,3-linked SA, respectively[65,66]. Although both viruses are also capable of binding to other receptors, the ability to bind SA may impact vaccine responses. Amino acids thought to be implicated in the SA binding of these viruses have been identified[66,67], however, further investigation is required to screen binding mutant candidates and evaluate the impact of SA binding on vaccine immunogenicity and efficacy. In addition to current vaccine antigens, binding to SA is also a common feature of many human and veterinary pathogens for which vaccines are not yet available, including reoviruses, some adenoviruses, some noroviruses, enteroviruses, and human parainfluenza viruses[68,69]. Thus, SA binding properties may be an important consideration for ongoing and future development of a number of viral vaccines.

5.3 CONCLUDING REMARKS

In this work, we explored the possibility that the binding properties of influenza HA impact immune responses to plant-based VLP vaccines targeting influenza. Initial studies demonstrated that the differential binding preferences of H1- and H5-VLPs dramatically impact their interactions with immune cells and downstream innate immune responses *in vitro*. These studies suggested that the ability to bind α2,6-linked SA was associated with stronger humoral immune responses; a

hypothesis that was later confirmed by Xu *et al.* with their demonstration that H5N1 and H7N9 VLP vaccines capable of binding to α2,6-linked SA were more immunogenic in mice[70]. However, we provide the first evidence that SA binding irrespective of receptor type hinders the immune response to influenza VLP vaccination in mice and that abrogation of SA binding is a promising strategy to improve the immunogenicity vaccines targeting both seasonal and avian influenza strains. Considerable work is still required to characterize the mechanisms of improved immunogenicity and the efficacy of 'non-binding' HAs in humans, however, this approach appears to represent a promising strategy in the development of more effective, next-generation influenza vaccines.

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5.4 FIGURES AND FIGURE LEGENDS

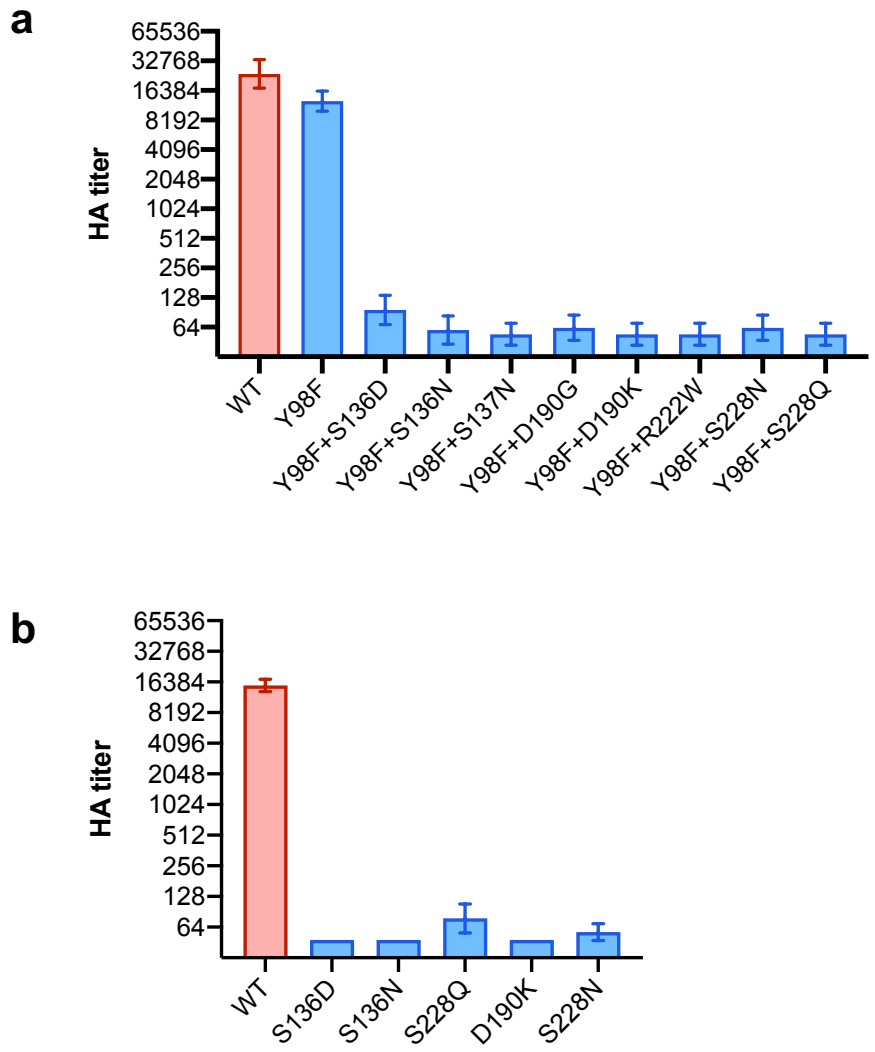

Figure 5.1. Candidates for the development of a 'non-binding' H3-VLP. VLPs bearing H3 A/Kansas/14/2017 with the point mutations indicated in each graph were produced in *Nicotiana benthamiana* plants. (a) the Y98F mutation alone did not prevent hemagglutination of turkey erythrocytes by an H3-VLP. However, the Y98F mutation in combination with mutations of other sites known to interact with SA resulted in nearly complete loss of hemagglutination. (b) Several of the mutations tested were also capable of eliminating hemagglutination in the absence of the Y98F mutation. Successful binding mutants will be further screened to hopefully identify a candidate that has little-to-no impact on HA antigenicity.

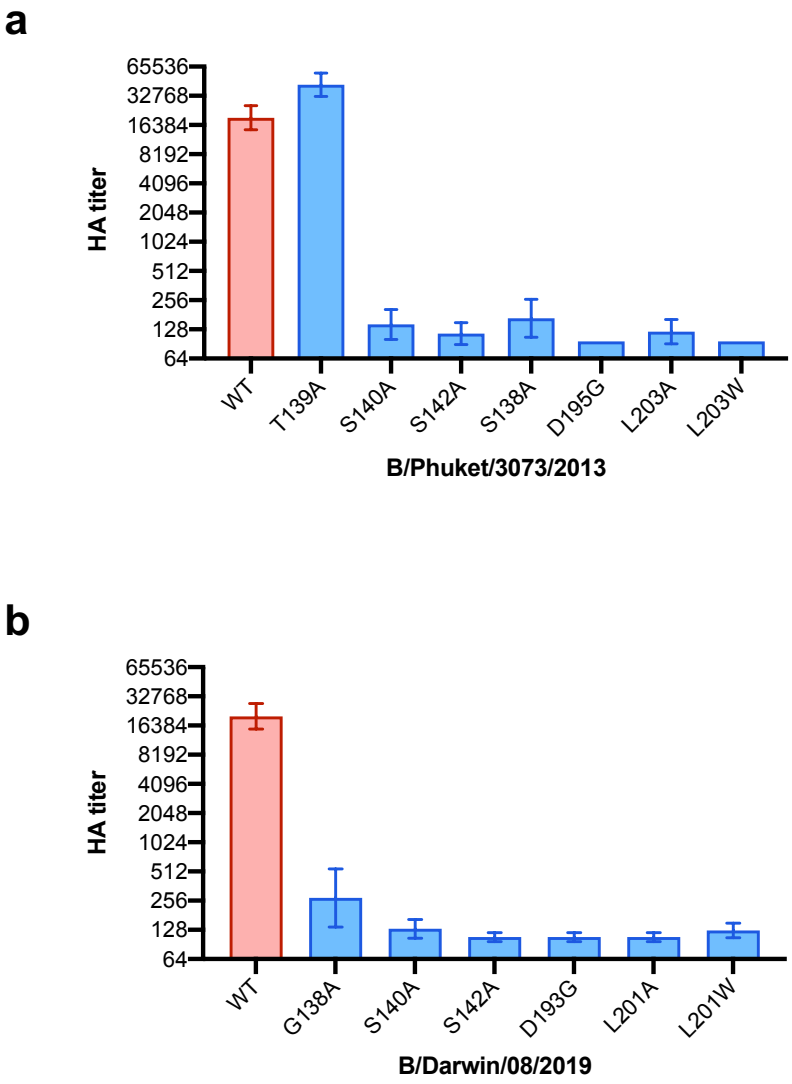

Figure 5.2. Candidates for the development of a 'non-binding' B-VLP. VLPs bearing IBV HAs from (a) B/Phuket/3073/2013 (B/Yamagata lineage) and (b) B/Darwin/20/2019 (B/Victoria lineage) with the point mutations indicated in each graph were produced in *Nicotiana benthamiana* plants. The previously described mutation at amino acid 139 had no impact on the binding of B/Phuket/3073 HA, however, several other point mutations markedly reduced hemagglutination in both IBV lineages. These mutants will be further screened to identify the candidate that has the least impact on HA antigenicity prior to assessing immunogenicity.

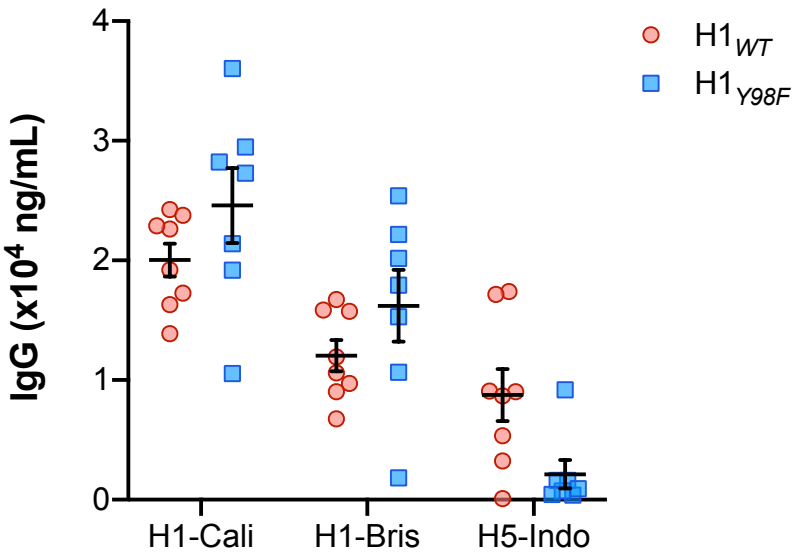

Figure 5.3. Cross-reactivity of IgG elicited by H1$_{WT}$- and H1$_{Y98F}$-VLPs. Female Balb/c mice were vaccinated with 3µg H1$_{WT}$-VLP or H1$_{Y98F}$-VLP targeting H1 A/California/07/2009 (H1-Cali). Sera were collected 12 weeks post-vaccination and IgG specific for H1 A/California/07/2009 (H1-Cali), H1 A/Brisbane/02/2018 (H1-Bris), and H5 A/Indonesia/05/2005 (H5-Indo) were measured by ELISA. There was considerable cross-reactivity between the vaccine strain and the more recently circulating H1-Bris strain in both vaccine groups. However, cross-reactivity towards a more distantly related group 1 HA (H5-Indo) was markedly reduced among mice vaccinated with H1$_{Y98F}$-VLP compared to those vaccinated with H1$_{WT}$VLP.