IMMUNOLOGICAL CONSIDERATIONS FOR IMPROVED VACCINES

DEFINING FEATURES OF ANTIBODY-MEDIATED IMMUNITY AGAINST INFLUENZA VIRUS: IMPLICATIONS FOR OPTIMAL VACCINE DESIGN AND DELIVERY

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Title: Defining features of antibody-mediated immunity against influenza virus: implications for optimal vaccine design and delivery

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Lay Abstract

The COVID-19 pandemic and repeated influenza virus pandemics over the past century emphasize the need for improved vaccines. Current seasonal influenza vaccines are limited by the narrow specificity of induced responses and varied efficacy across populations and seasons. Given these shortcomings, this dissertation explores various aspects of the human antibody response to influenza vaccination. However, these findings can be readily applied to other pathogens of interest. This body of work expands our understanding of antibody functions beyond neutralization and explores the role of vaccine type on immune imprinting and implications for universal vaccine platform selection. Furthermore, we explored the immunogenicity of seasonal vaccines in a heart-failure patient cohort to support the observation that vaccination reduces cardiac events. Investigating these, and other, features of the immune response to influenza vaccination will help to progress more effective strategies to combat viral pathogens.

Abstract

Viral pathogens impose a significant burden on global public health. Given the increasing frequency of epidemics/pandemics, emphasis should be placed on improving current vaccines and developing next generation immunization strategies. The significant challenges observed in generating an efficacious seasonal influenza virus vaccine highlight the need for such advancements.

Influenza results in approximately 650,000 deaths annually, most of which occur in pediatric, elderly, and immunocompromised patient populations. Currently approved influenza vaccines primarily elicit antibodies targeting the highly variable head domain of the hemagglutinin (HA) protein. Acquisition of point mutations in this region render antibodies elicited by seasonal vaccines less effective in subsequent seasons. Furthermore, seasonal vaccines do not provide protection from a pandemic influenza strain. Immense research efforts have, therefore, focused on the generation of a 'universal' influenza vaccine.

Identifying optimal vaccine platforms is an ongoing challenge for influenza vaccine development. Vaccine delivery route and platform have been shown preferentially elicit different antibody isotypes. We demonstrated that stalk IgA antibodies elicit unique Fc-effector functions in neutrophils which may contribute to protection *in vivo.* As such, platforms which stimulate IgA should be considered for universal vaccines. Additionally, immune imprinting and pre-existing immunity to influenza impact both strain-specific and broadly-protective responses. In children, inactivated vaccines may skew immune responses to conserved regions of HA head shared by previously-encountered antigens. Induction of antibodies to conserved stalk epitopes is desired for

a universal vaccine. We observed varied induction of stalk antibodies across vaccine platforms. Finally, seasonal vaccines can be of particular benefit to high-risk populations, such as patients with cardiovascular conditions, by preventing potentially life-threating exacerbations of their underlying conditions. We showed that inactivated seasonal vaccines were immunogenic and effective in these patients. These findings strengthen the observation that vaccine mediated protection helps to reduce cardiac events in vaccinated individuals.

Together, this dissertation sheds light on factors affecting the elicitation and function of antibodies against influenza virus, which has implications for improving both seasonal and universal vaccine development. While the studies presented here focus on influenza virus, many of the findings may also be applied to other viral pathogens that cause respiratory infection, and are prone to antigenic drift, such as SARS-CoV-2.

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Table of Contents

List of All Abbreviations and Symbols

A-TIV	Adjuvanted trivalent inactivated vaccine
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
ADE	Antibody dependent enhancement
AIDS	Acquired immunodeficiency syndrome
ARDS	Acute respiratory distress syndrome
AV-IIV	Adjuvanted- inactivated influenza vaccine
BCR	B cell receptor
BMI	Body mass index
bnAbs	Broadly neutralizing antibodies
Cal/09	A/California/04/2009
CDC	Centres for Disease Control and Prevention
CGD	Chronic granulomatous disease
cHA	Chimeric hemagglutinin
CHIKV	Chikungunya virus
CI	Confidence interval
COBRA	Computationally optimized broadly reactive antigen
COVID-19	Coronavirus Disease 2019
cRCT	Cluster randomized control trial
CVD	Cardiovascular disease
DAMP	Damage associated molecular pattern
dIgA	Dimeric IgA
DMEM	Dulbecco modified eagle medium
DPI	Diphenyleneiodonium chloride
EBOV	Ebola virus
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked-immunosorbent assay
ER	Emergency Room
Fab	Fragment antigen binding
FBS	Fetal bovine serum
FcαR	Fc alpha receptor
FcR	Fc receptor
FcRγ	Common gamma chain
FcγR	Fc gamma receptor
GC	Germinal centre
GISRIS	Global Influenza Surveillance and Response System
GMT	Geometric mean titer
HA	Hemagglutinin

HAI	Hemagglutination inhibition assay
HF	Heart failure
HIV	Human immunodeficiency virus
hIVIG	Hyperimmune intravenous immunoglobulin
HK/14	A/Hong Kong/4801/2014 (H3N2)
HPAI	Highly pathogenic avian influenza virus
I-REV	Imprint regulated effect of vaccine
IAV	influenza A virus
IBV	influenza B virus
IC	Immune complex
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIV	Inactivated influenza vaccine
ITAM	Intracellular tyrosine activation motif
ITIM	Intracellular tyrosine inhibitory motif
IVVE	Influenza vaccine to reduce adverse vascular events in patients with heart failure
J-chain	Joining chain
kDa	Kilodalton
LAIV	Live-attenuated influenza vaccine
LRT	Lower respiratory tract
M1	Matrix 2
M2	Matrix 1
MBC	Memory B cell
MDCK	Madin Darby Canine Kidney cells
MERS	Middle east respiratory syndrome
MI	Myocardial infarction
Mich/15	A/Michigan/45/2015(H1N1)
mIgA	monomeric IgA
MMP9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
MVA	Modified vaccinia Ankara
MZ	Mozambique
NA	Neuraminidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAI	Neuraminidase inhibitor
NE	Neutrophil elastase

NEP	Nuclear export protein
NET	Neutrophil extracellular trap
NF-kB	Nuclear factor kappa-light-chain enhancer of activated B cells
NG	Nigeria
NIAID	National Institute of Allergy and Infectious Disease
NOX	NADPH oxidases
NP	Nucleoprotein
NS1	Non-structural protein 1
OAS	Original antigenic sin
oxLDL	Oxidized low density lipoprotein
PAD4	Protein arginine deiminase 4
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PH	Philippines
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol 12-myrisate 13-acetate
PMN	polymorphonuclear cell
PR8	A/Puerto Rico/8/1934 (H1N1)
PRR	Pattern recognition receptor
QIV	Quadrivalent inactivated vaccine
RA	Rheumatoid arthritis
RBCs	Red blood cells
RCT	Randomized control trial
rHA	Recombinant hemagglutinin
ROS	Reactive oxygen species
RR	Relative risk
RSV	Respiratory syncytial virus
RT	room temperature
SARS	Severe acute respiratory syndrome
SARS2	SARS-CoV-2
sIgA	Secretory IgA
Sing/16	A/Singapore/INFIMH-16-0019/2016 (H3N2)
SLE	Systemic lupus erythematosus
Sw/17	A/Switzerland/8060/2017 (H3N2)
TIV	Trivalent inactivated vaccine
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
Tx/12	A/Texas/50/2012 (H3N2)
UIV	Universal influenza vaccine
URT	Upper respiratory tract

VE	Vaccine efficacy
VH	Variable heavy region
Vic/11	A/Victoria/361/2011 (H3N2)
vRNP	Viral ribonucleoprotein
WHO	World health organization
ZA	Zambia

Declaration of Academic Achievement

Chapter 3: IgA potentiates NETosis in response to viral infection

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- M.S.M., C.E.M., and H.D.S conceived and designed the study
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- J.C.A performed phlebotomy and viral preparation
- K.E., M.Z., C.K., E.C., and D.M.E.B., provided key reagents
- H.D.S. performed the data analysis
- H.D.S. wrote the manuscript with input from C.E.M. and M.S.M.

Chapter 4: The nature of early childhood imprinting to influenza virus shapes subsequent antibody responses

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- H.D.S. analyzed the data
- P.S. identified samples and provided statistical support
- M.L. provided clinical samples
- H.D.S. wrote the manuscript with input from M.S.M.

Chapter 5: Immunogenicity of inactivated influenza vaccination in patients with heart failure: Secondary analysis of a multinational randomised, double-blind, placebo-controlled trial

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- M.L. provided clinical samples and analysis input
- M.L. and M.S.M. conceived the study and acquired funding

1.0- Chapter 1:Introduction

1.1 Preface: respiratory viral infections and human health

The generation and administration of effective vaccination remains one of the most impactful scientific developments in history[1,2]. The discovery and implementation of widespread vaccination has effectively eradicated many pathogens in certain areas of the world, and has substantially reduced mortality resulting from infection[1,2]. However, the 21st-century has been defined by the emergence of multiple epidemics/pandemics of viral origin[3]. Notably, severe acute respiratory syndrome (SARS) coronavirus in 2003, pH1N1 swine flu in 2009, Middle East respiratory syndrome coronavirus (MERS) in 2011, and the ongoing SARS-CoV-2 (SARS2) and human immunodeficiency (HIV) pandemics[3]. Additionally, regional, and recurring epidemics of West Nile, Zika and Ebola viruses have also been observed. The emergence and global impact of SARS-CoV-2 underscores the need for development of innovative strategies to combat viral infections[4,5]. Additionally, pathogens that have plagued humans for centuries, such as influenza virus, are still circulating and pose a significant threat to public health[6].

While respiratory viruses have obvious health consequences, the impacts of these viruses can be felt on societal economic fronts. One study estimated that in the United States alone, both direct healthcare related costs and indirect effects (ex. lost productivity), the annual economic burden of influenza is approximately $11.2 billion[7]. In contrast, the economic repercussions of COVID-19 (Coronavirus Disease 2019) are thought to be in the trillions[8]. The impact of public health measures implemented during the COVID-19 pandemic such as lockdowns and school closures have been linked to worsened childhood mental health[9]. In developing nations, the lack of access to schools has been particularly detrimental for young girls further exacerbating pre-existing inequalities. The

Malala Fund found that across African countries, over 20 million secondary-school aged-girls would not return to school when classrooms re-opened[10]. For both individual and global public health, strategies that can effectively combat viral infections prior to widespread outbreaks are urgently needed.

In summary, respiratory pathogens place massive burden on all facets of our society, and vaccines represent the best strategy for combating these infections. This dissertation sheds light on the role of vaccines and antibodies in preventing influenza virus infection and provides insight into strategies for improved seasonal and universal influenza vaccine design.

1.1.1 Symptoms and epidemiology of respiratory viral infections

In the Northern Hemisphere, respiratory viruses such as influenza and respiratory syncytial virus (RSV) circulate seasonally [11]. Increased infection rates are generally observed during the winter months, November to February, and are associated with reduced relative humidity, reduced temperatures, and behavioural changes[12,13]. Influenza virus generally presents with symptoms in the upper respiratory tract (URT) such as fever, sore throat, runny nose, fatigue, and cough. The virus can disseminate to the lower respiratory tract (LRT) with more severe cases resulting in secondary bacterial pneumonia[14]. Prior to the COVID-19 pandemic, the World Health Organization (WHO) estimated that influenza virus was associated with approximately 1 billion infections, and 290,000-600,000 deaths globally each year[15]. Public health measures associated with preventing the spread of SARS2 drastically reduced circulation of other respiratory pathogens[16,17]. The lifting of these measures in 2022 has resulted in a marked increase in acute respiratory virus infections.

1.2 Influenza Virus

1.2.1 Influenza virus virology

Influenza viruses are enveloped, single stranded, negative sense RNA viruses that belong to the Orthomyxoviridae family[14,18]. Influenza A virus (IAV) and influenza B virus (IBV) most commonly infect and cause respiratory symptoms in humans. Influenza C and D viruses have also been identified, but more commonly cause infections in pigs and cattle. IAV contains eight genomic segments, of which 4 and 6, encode for the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA)[14,18]. HA is a homotrimeric protein responsible for mediating viral attachment to sialic acid on host cells and is composed of two domains, the globular head, and the conserved stalk. NA is responsible for cleavage of sialic acids and mediating the release of mature virions. The matrix-2 protein (M2), which mediates acidification and subsequent uncoating of the viral particle, is also embedded in the lipid membrane. The additional segments of the genome encode for the viral RNA dependent RNA polymerase subunits (PB1, PB2, and PA), viral nucleoprotein (NP), matrix-1 proteins (M1), and the immunomodulatory factor non-structural protein 1 (NS1) and non-structural protein 2 also called nuclear export protein (NEP)[14,18].

1.2.2 Influenza virus lifecycle

Influenza viruses bind and enter host cells at mucosal membranes. Virions enter through receptor-mediated endocytosis after HA binds to sialic acids. Viruses with tropism for human cells bind to α-2,6-sialic acids, found in abundance in the human URT, while avian influenza viruses preferentially bind to α-2,3-sialic acids found within the avian gut and in human lower airways[19,20]. Following entry, the virus enters a host cell endosome which upon acidification, triggers fusion of

the endosomal and viral membranes through conformational changes in HA. The M2 channel acidifies the viral core releasing viral ribonucleoproteins (vRNPs) into the host cell cytoplasm[21]. Nuclear localization signals direct the vRNPs to the nucleus where transcription and replication occurs. To generate mRNAs required for viral protein production and the generation of new virions, the viral polymerase snatches 5' caps from host cell mRNA[14,18]. Translation of the new mRNAs then occurs in the cytoplasm, using host cell machinery. After protein synthesis, HA, NA and M2 are trafficked to the plasma membrane where the new vRNPs are packaged and mature virions bud off the cell surface. HA is generally cleaved by host proteases at the host respiratory mucosa, allowing virions to spread to new cells. Of note, influenza viruses with pandemic potential often contain a polybasic cleavage site allowing for more efficient dissemination, as HA0 can be cleaved into HA1 and HA2 by ubiquitous host proteases rather than those limited to the URT[22,23]

1.2.3 Influenza classification and nomenclature

Most viral typing is based on the HA and NA proteins. To-date, 18 HA and 11 NA subtypes have been identified[24]. Based on sequence homologies, IAV HA can be further subdivided into two distinct phylogenetic groups (Group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16) and Group 2 (H3, H4, H7, H10, H14, H15))[14,25,26]. Influenza B viruses (IBV), which also causes infections in humans, can be subdivided into two lineages, B/Yamagata/16/1988 (B/Yamagata)- or B/Victoria/2/1987 (B/Victoria)-like strains[27]. Although in recent years, no B/Yamagata isolates have been detected, after public health measures put in place for COVID-19 mitigated the spread of seasonal influenza viruses[28]. Currently, H1N1 and H3N2 viruses are endemic in the human population alongside viruses of both IBV lineages. As a result, seasonal vaccines contain the

dominant circulating strains of H1N1 and H3N2 IAV and one ,or both, IBV lineages as recommended by the WHO[29].

1.2.4 Antigenic diversity of influenza viruses

One of the major challenges in combating influenza viruses is the high level of antigenic diversity amongst the main surface proteins HA and NA. Influenza viruses are susceptible to antigenic drift, the main driver of annual reformulation of seasonal influenza vaccines[14]. The viral polymerase is error prone and imparts mutations in the genome that are then subject to selective pressures[30]. Mutations in the HA head domain that reduce antibody binding can confer a selective advantage.

The segmented nature of the genome also allows for antigenic shift. These drastic antigenic changes can result in the generation of pandemic influenza virus strains[31]. Co-infection with viruses from different host species can result in re-assortment events which generate virus strains with that express glycoproteins against which humans have little/no prior immunity[32].

1.2.5 Influenza virus transmission and disease pathogenesis

Influenza viruses have been demonstrated to spread through both contact and airborne transmission[33]. Human to human viral spread can also occur by direct contact of an infected individual to a naïve person or via fomites on a contaminated surface[34]. On average, the incubation period for influenza is 24-28 hours, and patients are most infectious one or two days before the onset of symptoms.

Severe IAV infection is usually characterized by lower airway involvement[35]. Infection of lower airway epithelial cells can cause significant disruption in gas exchange[35]. In extreme cases, patients

may develop acute respiratory distress syndrome (ARDS) and multi-organ failure[36,37]. A substantial portion of the pathology that occurs during influenza virus infection is mediated by aberrant immune host responses. Additionally, secondary bacterial infections and pneumonia are responsible for a significant portion of influenza related mortality. Post-mortem tissues collected during the 1918 Spanish flu have demonstrated that bacterial infiltration into the lungs likely contributed to the high death rates[38]. More recently, during the pH1N1 swine flu pandemic in 2009, bacterial lung infections were found in most fatal cases[39].

Aquatic birds are generally regarded as the reservoir of IAV as infections in these animals rarely results in symptomatic infection[40]. Low pathogenic avian influenza viruses spread through the fecal-water-oral route and commonly cross over to domestic poultry. Direct transmission of avian viruses to humans has been recorded, particularly in agricultural settings where prolonged contact with infected animals can occur[41]. However, it is generally thought that an intermediate species, such as swine, are required to allow adequate adaptation for humans. It has been demonstrated that very few mutations are required to alter receptor specificity. Additionally, recently isolated avian viruses (H9N2) have been observed to possess dual α-2,6, and α-2,3 specificity after acquisition of a Q226L mutation[42]. Receptor specificity, presence of a polybasic cleavage site, and ability to spread through airborne transmission are usually features indicative of a virus with pandemic potential in humans. However, mutations in other gene segments, such as NS1, can also increase pathogenesis in humans[43].

1.2.6 Influenza virus epidemiology

H1N1, H2N2, and H3N2 viruses have all caused pandemics in humans[44,45]. The 1918 Spanish flu is regarded as the deadliest pandemic in history, resulting in >40 million deaths[46]. The 1918 H1N1 virus circulated in humans until it was replaced by H2N2 in 1957 [32,47]. Although its origins are debated, an H1N1 that showed a high degree of similarity to H1N1 viruses that circulated prior to 1957 re-emerged in 1977, and H1 and H3 viruses have co-circulated in humans ever since[45]. Highly pathogenic avian influenza viruses (HPAIs) cause periodic spillover events in humans and sporadic outbreaks of H5 and H7 have occurred over the past two decades. Although these viruses have high mortality rates, there has not been widespread human to human transmission.

Children (<1) and the elderly (>65 years of age) are at high risk for complications following influenza virus infection. The developing neonatal immune system is, in part, responsible for the susceptibility of young children. In contrast, severe outcomes in elderly individuals may be the result of immunosenescence and co-morbitities such as high body mass index (BMI), obesity, pulmonary and cardiac conditions, etc. Pregnancy is also a risk-factor for complications from influenza virus infection.

1.2.7 Prevention and treatment of influenza virus infection

Numerous antiviral treatments and vaccines for combatting influenza virus are approved and in use. Three of the four approved drugs (Osteltamivir (Tamiflu), Zanamivir (Relenza), and Paramivir (Rapivab)) are neuraminidase inhibitors (NAIs) which competitively inhibit NA, preventing release of mature virions[48]. The fourth antiviral, Baloxavir Marboxyl (Xofluza), is an orally delivered cap-dependent endonuclease inhibitor that was approved for use by the FDA in 2018[49]. Unlike NAIs, baloxavir marboxyl interferes with viral RNA transcription. The most effective

means of preventing influenza virus infection is through administration of seasonal influenza vaccines. These vaccines have been developed across a number of platforms including inactivated influenza vaccines (IIV), live-attenuated influenza vaccines (LAIV), adjuvanted inactivated influenza vaccines (AV-IIV), and recombinant HA (rHA) vaccines.

1.2.7.1 Influenza antivirals

Antiviral drugs have been demonstrated to reduce the length and severity of influenza symptoms. However, these drugs are most effective when administered within 24-48 hours of symptoms onset[48]. Generally, antivirals are administered to hospitalized patients, patients with severe illness, or those at risk of developing severe complications. In high-risk settings such as healthcare environments or long-term care homes, antivirals can be taken prophylactically, however, this is not the done routinely to reduce the chance of resistance emerging[50].

1.2.7.2 Other treatments

Non-NAI treatment of Influenza infection has also been demonstrated to have benefit in a clinical setting. Hyperimmune intravenous immunoglobulin (hIVIG) is generated by pooling antibodies from multiple donors. IVIG has been demonstrated to have strong anti-inflammatory effects for other infectious and autoimmune conditions and acts through a variety of mechanisms. IVIG can provide protection through neutralization, saturation of FcγR - reducing aberrant signalling, inhibition of complement, and alteration of T cell responses[51]. IgG from IVIG has been found to be heavily sialidated which has been associated with less inflammatory responses[52]. In a ferret challenge model, IVIG generated prior to the 2009 pandemic reduced viral load and mortality from both H1N1 and H5N1[53].

A 2011 clinical trial examined the effect of treatment with hIVIG from patients recovered from a pH1N1 infection compared to standard IVIG[54]. They found that hIVIG resulted in reduced viral load and mortality. A subsequent multi-national RCT (randomized controlled trial) compared IVIG to placebo and observed no differences in outcomes of hospitalized patients[55]. Increasingly, evidence has shown that use of corticosteroids increases adverse outcomes in influenza infected patients[56]. In general, these strategies demonstrated the best result when an NAI was administered alongside the adjunctive therapy.

1.2.7.3 Seasonal influenza vaccines

Currently approved seasonal vaccines are designed to elicit neutralizing antibodies that prevent viral attachment. However, these antibodies are usually strain-specific and do not confer long-lasting immunity as the globular head domain undergoes antigenic drift. Seasonal vaccine efficacy various greatly, even in years with good antigenic match between the vaccine and circulating strains, efficacy can range from 40-60 %[57]. However, in years where mismatch has occurred efficacy can be as low as 0 %.

Mismatch between vaccine strains and circulating strains can be attributed, in part, to the lengthy timeline required for generation in embryonated chicken eggs, with the process generally beginning 8 months prior to the start of the influenza season. The WHO Global Influenza Surveillance and Response System (GISRS) evaluates global influenza isolates before recommending the vaccine strains for the Northern Hemisphere in February ahead of the next flu season, and September for the Southern Hemisphere[58]. The seed strains must either be egg (or cell

culture) adapted and verified for antigenicity relative to the reference strain which can take months prior to scaling up production and purification. Egg-based adaptations can also impart antigenic changes that lead to reduced vaccine effectiveness[59,60].

Conventionally, the generation of neutralizing antibodies with hemagglutination inhibition (HAI) activity are regarded as a strong correlate of protection. The HAI assay used to measure these responses is based on the ability of IAVs to bind sialic acid on chicken red blood cells (RBCs) resulting in agglutination[61–63]. A post-vaccination increase four-fold above baseline HAI titers is considered indicative of seroconversion and an absolute HAI response >1:40 is thought to be protective. Although, more recent studies have indicated that higher titers are likely required for protection, particularly in children[64].

1.2.7.3.1 IIVs (Trivalent and Quadrivalent)

The first formalin- inactivated monovalent influenza vaccines were administered in the 1940s[65]. However, by 1947 it became apparent that the vaccine was no longer providing protection from the circulating virus and multiple strains would need to be included in the vaccine[66]. Today's inactivated vaccines are composed of the dominant H1 and H3 strains, and one IBV strain from either lineage, in the case of trivalent inactivated vaccines (TIVs)[67]. Quadrivalent vaccines (QIVs) contain both a B/Victoria and a B/Yamagata strain. Standard dose IIVs contain 15 µg of HA/strain, as measured by the single radial immunodiffusion assay[67]. Interestingly, the NA content of seasonal vaccines is not directly assessed however, neuraminidase inhibiting antibodies are thought to play an important role in protection[68]. IIVs are generally administered intramuscularly, with most being approved for use in individuals >6 months of age. In immunologically naïve

children, two doses administered 4 weeks apart has been demonstrated to be required to generate protective responses[67].

IIVs can be split or subunit vaccines. Most vaccines are inactivated by either formaldehyde or beta-propiolactone and split with a detergent such as TritonX-100 or taurodeoxycholate[69]. Detergents work to dissociate the viral lipid envelope. Subunit vaccines undergo further purification steps to remove other viral proteins[70]. A test negative- study of adults >50 years old during 2008-2012 who presented to the emergency room (ER) with influenza infections. Split vaccines were observed to have 33.5% better relative effectiveness over subunit vaccines in older adults[71]. A European multi-centre case-controlled study found no statistical differences in vaccine effectiveness between split and subunit vaccines in the 2012-2013 season[72]. Differences in the cellular responses induced by IIVs may contribute to variable responses as internal viral protein content of the vaccines can vary based on formulation.

The majority of IIV vaccines are generated in embryonated chicken eggs. However, a cell culture vaccine Flucelvax (Seqirus) has also been approved[73]. Flucelvax Quad is a quadrivalent inactivated vaccine generated in Madin Darby Canine Kidney (MDCK) cells. Candidate vaccine viruses are propagated in MDCKs, inactivated with beta propriolactone and purified. While both effective, clinical trial findings have demonstrated increased relative effectiveness between cell culture and egg grown seasonal vaccines[74,75].

Special high-dose IIV vaccine formulations have been generated for use in those >65 years of age. Fluzone High Dose (Trivalent and Quadrivalent, Sanofi) is an inactivated, split-virion vaccine

contains 60 μg of each strains HA per dose[76]. A trial comparing standard and high dose TIVs in individuals 65+ found that the high dose formulation increased seroconversion, absolute HAI titers, and reduced laboratory confirmed influenza cases relative to the standard dose vaccine[76].

1.2.7.3.2 LAIV

In contrast to IIVs, LAIV is administered intranasally, circumventing the use of needles, and more closely mimicking a natural infection. The vaccine was generated through reverse genetics and serial passage of donor viruses at cold temperatures (25°C), such that the virus can only replicate at the cooler temperatures of the URT[77]. The HA and NA segments of the WHO recommended strain are combined with the six segments of the cold-adapted virus backbone (commonly A/ Ann Arbor/6/60 and B/ Ann Arbor/1/66) and then propagated in eggs[67]. Currently in Canada, the vaccine is recommended for use in individuals 2-17 years of age. LAIV has been shown to generate mucosal IgA, cross-reactive T cell responses, and systemic IgG in children which can persist for up to one year[78,79]. Vaccine effectiveness has been observed to be higher in children than adults, as T cells and local immunity in older individuals can clear the vaccine before mounting an effective vaccine response[80].

Systemic HAI titers, which have been shown to be a strong correlate of IIV protection, are less robust for LAIV. Nasal IgA and IFNγ titers have been suggested as strong predictors of LAIV protection[78,81]. The presence of alternate internal vaccine antigens in LAIV in contrast to subunit IIVs has been hypothesized to contribute to the heterosubtypic protection afforded by LAIV. This closely resembles a natural infection where cytotoxic T- cells against M and NP are generated.

Low LAIV vaccine effectiveness in the 2016-2017 season resulted in the vaccine advisory committees in the U.S. and Canada to recommend against use of LAIV in that season[82,83]. Following this, some hesitancy surrounding LAIV effectiveness in children has lingered. However, two trials in Western Canada spanning multiple Influenza seasons found that both LAIV and IIV were equally efficacious in child cohorts[84,85]. When investigating immunogenicity of LAIV and IIV in children during 2014-2015 seasons, our group has shown that both vaccines induced vaccine strain HAI titers however, consistent with the literature, only LAIV induced significant mucosal IgA titers[86].

1.2.7.3.3. AV-IIV

Inclusion of adjuvants in vaccines has been shown to increase immunogenicity and allow for dose sparing[87]. Enhanced seasonal vaccine formulations that contain adjuvants are likely to be beneficial for the young children and elderly population who respond poorly to standard dose vaccines. In Canada the MF59 adjuvanted vaccine, Fluad (Seqirus), is approved for use in adults over the age of 65[88]. Fluad Pediatric is also approved and contains only 7.5. µg of HA per strain[89]. The inclusion of adjuvants in seasonal influenza vaccines has been associated with increased humoral and cellular responses in elderly populations[90]. A retrospective observational analysis in high-risk older adults in the 2017-2018 and 2018-2019 influenza seasons found adjuvanted vaccines to be 7.1 (95% CI, 3.3-10.8) and 20.4 (95% CI, 16.2-24.4) times more effective than standard IIV[91]. Enhanced vaccine formulations also increased antibody avidity, polyfunctional T cells and stalk specific responses in elderly recipients further supporting the use of these vaccines in the highly susceptible population[92].

Although higher reactogenicity has been observed, AV-IIVs have also demonstrated superiority over standard IIVs in children[93]. In clinical trials, HAI titers against vaccine strains and mismatched strains were higher in children who received adjuvanted vaccine[94]. Greater expansion of vaccine specific CD4+ T cells was also observed[95]. In addition to magnitude, persistence of antibodies was also found to be greater in children who received AV-IIV[96]. Vaccinating children with either AV-IIV or QIV performed similarly in preventing community transmission however, adjuvanted vaccines were more effective in preventing RT-PCR confirmed infection in children[97].

Many pandemic H1N1 and H5N1 vaccines were developed with adjuvants to increase dose availability during times of high global demand. The inclusion of MF59 in an H5N1 vaccine resulted in higher seroconversion relative to unadjuvanted vaccine recipients and MF59 pH1N1 monovalent vaccines induced higher HAI and neutralizing antibody titers than those without adjuvant[98,99].

The increased immunogenicity of adjuvanted vaccines has been attributed to a variety of mechanisms. Increased production of inflammatory cytokines has been measured at the site of injection which can promote antigen uptake and presentation. Inclusion of MF59 and AS03 has also been shown to increase the breadth of responses following vaccination[100–102]. Universal vaccines are being tested both with and without the inclusion of adjuvants.

1.2.7.3.4 rHA vaccines

One of the primary challenges faced by seasonal influenza vaccines is long lead production time required for growth in embryonated chicken eggs. Sub-unit vaccines have been developed composed of recombinant HA protein generated using immortalized insect cell lines [67].

Flublok (Sanofi), is a quadrivalent recombinant HA vaccine that is currently licensed for use in people over the age of 18. This high dose formulation contains 45 μg of each vaccine antigen and has demonstrated efficacy against infection and superior immunogenicity than standard dose TIVs in older adults[103,104]. In children aged 6-59 months FluBlok was safe but less immunogenic than TIV[105]. In a small study comparing cellular responses in adults administered Fluzone (QIV, Split, egg), Flucelvax (QIV, Subunit, cells), or FluBlok, found that FluBlok induced greater HAI and HA specific $CD4^+$ T cells[106]. When compared to Fluzone high dose, the rHA vaccine still induced stronger T cell responses, which suggested that higher antigen dose was not mediating the effect. Differences in HA glycosylation by insect and mammalian cells may explain some of these observations. The absence of bulky glycans on conserved epitopes can increase accessibility and breadth of responses.

1.2.8 Challenges addressed by next generation influenza vaccines

Currently approved seasonal influenza vaccines represent the most effective strategy for preventing infection however, there remains much room for improvement[107]. The limited breadth and longevity of protection afforded by current vaccines remains their largest limitation. Generating immune responses against more conserved viral epitopes/ proteins will help to overcome some of these limitations. During the 2020-2021 season, 193.8 million doses of seasonal influenza vaccines were distributed in the United States alone, with an average cost per dose of

about $25 dollars[108,109]. This results in over $8 billion dollars being spent every year to revaccinate just the U.S. population.

Many of the challenges faced in the generation of more efficacious influenza vaccines are shared by other respiratory pathogens. For example, immune evasion by emerging variants of SARS2 has reduced vaccine effectiveness necessitating the development of bivalent boosters[110]. Thus, many of the challenges and considerations for generating next-generation influenza vaccines can be applied to other pathogens that undergo antigenic drift.

1.2.8.1 Understanding and harnessing immune history

Unlike other viral pathogens, for which vaccination or infection can confer lifelong immunity, humans are repeatedly exposed to influenza virus through both annual vaccination and infection[111]. These repeated exposures result in diverse immune histories and antibody repertoires which can influence the response to subsequent exposures. The first exposure to an influenza virus leaves an indelible imprint on the immune system, such that antibody titers against strains that circulated earliest in an individual's life remain the highest. This phenomenon was termed 'original antigenic sin' (OAS)[112]. These antibodies are maintained by 'back-boosting' events through exposure to drifted viruses[113].

The boosting of antibodies to historical strains can be detrimental when focused on non-neutralizing epitopes in the variable head domain. However, recent work has suggested that OAS responses can also generate cross-reactive antibodies[114]. Recall of immune responses against conserved epitopes is the goal of next-generation vaccines, in this way OAS may be beneficial.

Rational immunogen design and inclusion of adjuvants are being employed to stimulate memory responses against more desirable epitopes.

The influence of immune history becomes increasingly challenging with age. The impact of prior exposure on vaccination is extremely nuanced and has been associated with both increased protection from infection and interference with mounting effective vaccine responses. Understanding these intricacies is imperative in generation of more broadly protective vaccines.

1.2.8.2 Targeting conserved epitopes

Immunodominance refers to the preference of the immune system to display certain peptides, skewing antibody responses to dominant epitopes on antigens. For influenza viruses immunodominance can impact immune responses at various levels. At the protein level, despite M1 being the most abundant protein in viral particles, followed by NP, HA and NA in terms of quantity (M1 >NP >HA >NA), antibody responses are preferentially mounted against HA[115].

Immunodominance hierarchies also exist within the HA protein itself, with the HA stalk domain being immunosubdominant to the HA head. Accessibility of stalk-reactive B cells to the HA stalk on virions or the surface of infected cells, limiting activation, can in part explain the disparity. However, when responses to recombinant HA were assessed, increasing accessibility to stalk epitopes, significant increases in stalk antibodies were not observed. This suggests that other intrinsic factors such as host genetics, pre-existing immunity, and B cell receptor (BCR) affinity/ frequency may drive the establishment of these hierarchies. Specific epitopes within HA have also been found to be immunodominant. For H1 responses are generally focused on the antigenic sites Cb, Sb, Ca1, Ca2, and Sa[116,117] and on H3 viruses' antigenic sites A, B, C, D, E are preferred[118,119].

Observed germline restrictions also contribute to the induction of broadly reactive B cells. Similar immunoglobulin variable heavy (V_H) gene usage has been observed for broadly neutralizing antibodies against IAV, HIV and Hepatitis C virus. For example, the usage of the V_H1-69 gene has been observed to be a common feature across many infectious pathogens. V_H1-18, V_H6-1, V_H3-30 genes have also been observed to confer IAV stalk binding[120–122]. Germline targeting strategies with specific immunogens may offer another method of stimulating stalk responses.

The generation of broadly-neutralizing antibodies (bnAbs) may be further limited by the observation that these antibodies often display poly-reactivity, including to self-antigen[123–125]. B cells which express these antibodies may be removed during selection to maintain tolerance leaving a smaller pool of cells which can be stimulated to produce HA stalk antibodies. Recent work has demonstrated that polyreactivity is a feature of naïve B cells which go on to form the broadly-neutralizing MBC pool[126]. This suggests, that polyreactivity is a pre-requisite and not acquired through affinity maturation. Polyreactive antibodies were also observed to have increased flexibility and affinity which may contribute to the breadth of binding they exhibit. Next-generation vaccines will need to overcome immunodominance and target naïve B cell populations with desirable fates to stimulate responses against HA stalk epitopes.

1.2.8.3 New routes of administration

Numerous candidate antigens which generate broadly reactive responses have been identified. However, the ideal vaccine formulation and/ or route of administration of these immunogens has not been determined. Vaccine delivery via the mucosal route represents an attractive strategy for

combatting respiratory viral infections. Intranasal administration of LAIV has demonstrated efficacy in combating seasonal influenza[84]. In the context of COVID-19 vaccines, the elicitation of site-specific mucosal immunity has demonstrated benefit over conventional systemic immune responses[127]. Mucosal vaccines provide protection at the site of infection and can induce stronger tissue resident effector and memory responses than systemic vaccination. In addition to increasing the breadth of antibody responses, next-generation vaccines may also endeavor to enhance tissue specific and cellular responses. The use of mucosal delivery systems, mucosal targeted adjuvants or "prime-pull" vaccine regimens should be explored alongside broadly- reactive immunogens.

1.2.8.4 Special populations

Influenza infections disproportionately impact the very young and elderly. Immunosenesence can impair both the ability to combat infection and the generation of effective vaccine responses. Evidence from seasonal vaccine trials has demonstrated that not all vaccine platforms may preform equally in all age cohorts. For example, LAIV has demonstrated superiority in younger individuals with limited exposure histories. Similar differences may exist for UIV platforms. Recent work from our group found that sequential seasonal vaccination with IIV or LAIV increased stalk antibodies in children[81]. Interestingly, the magnitude of increase was inversely correlated with age. In adults stalk antibodies have been observed to naturally increase with age, likely due to repeated exposure to drifted viruses[113,128]. While the long-lived nature of stalk antibodies can contribute to protection, boosting stalk responses may get more difficult with age and exposure[125,129]. Following the 2009 pandemic it was observed that people previously vaccinated with a related 1976 H1N1 strain had pre-exisitng H1 stalk antibodies. However, they did not exhibit a significant boost in stalk antibodies following vaccination for 2009 pH1N1[130]. Extensive exposure histories likely

result in reactivation of memory responses to the immunodominant HA head domain. Results from an rHA vaccine study showed that despite the highest baseline of stalk antibodies in elderly individuals (65+), while the greatest induction occurred in middle aged participants 18-49)[128]. These findings support that different universal vaccine platforms may be required for different age cohorts to overcome issues such as pre-existing immunity.

1.2.8.5 Pandemic preparedness

One of the major challenges universal or next-generation vaccines must address is prophylactic protection from pandemic strains. Viruses are rapidly changing, and effective, prophylactic treatment methods represent the strongest means of protection against these threats. One of the early challenges in combating SARS-CoV-2 infection was the lack of effective therapeutics or prophylactic antiviral drugs. Similar delays in availability were observed during the 2009 Swine flu outbreak[131]. By the time the pandemic vaccine was deployed and administered, the largest wave of pH1N1 had already passed. Avian influenza viruses represent viruses with high pandemic potential, however current seasonal vaccines do not provide protection from these strains. Advancing vaccines against pandemic threats in the clinical pipeline would drastically improve our readiness for the next pandemic. For example, during the 2014-2015 outbreak of Ebola virus (EBOV), the VSV- EBOV vaccine was able to be rapidly deployed for use and was a contributing factor in controlling the outbreak[132]. However, this would not have been made possible without decades of prior work. Investment and investigation into strategies for emergent pathogens before an outbreak is our best defense in the face of new viral threats.

1.2.9 Next generation influenza vaccine strategies

Next generation or broadly-neutralizing influenza vaccines aim to provide long lasting, broad protection against all circulating and future strains of influenza virus[133]. Ideally, a UIV would provide protection from all group 1 and group 2 subtypes, as well as IBVs. The National Institute of Allergy and Infectious Disease (NIAID) established that that UIVs should be at least 75% effective in all age groups and provide protection for at least one year from all IAVs[133]. These vaccines will undoubtedly have different correlates of protection in comparison to conventional vaccines. While stalk antibody titers have been indicative of protection in animal models, they were only found to reduce viral shedding in a human challenge model[134,135]. Additionally, serological data from adults hospitalized during the 2015-2016 influenza season suggested that HA head antibodies were more effective in providing protection than stalk antibodies[136]. In contrast, a more recent study which examined household transmission of influenza virus did find stalk antibodies to be predictive of protection[137]. Stalk-based antibodies provide protection through engagement of Fc effector functions. The evaluation of functional responses may require the use, and standardization, of more informative assays such as antibody dependent cellular cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP), and multiplexed bead-based readouts[138]. Other facets of immunity such as T cell and mucosal responses should also be considered in next generation vaccine design.

1.2.9.1 HA stalk-based strategies

HA based strategies remain the most heavily investigated in the context of UIVs. Numerous strategies have demonstrated promise pre-clinically. One of the challenges in generating next-generation vaccines is diverting the immune response to immunosubdominant epitopes in the HA stem. To circumvent this, researchers have designed stabilized “headless HAs” which provided

homologous and heterologous protection in animal models[139,140]. The immunogenicity of HA stem only immunogens was observed to increase when administered in the context of self-assembling ferritin HA stem nanoparticles[141]. Stabilized H1 stem nanoparticles provided heterologous protection from H5 infection in mice. Refocusing the immune response on the HA stem has also been achieved by modification of glycans on the HA head domain. By introducing glycans at the dominant epitopes in the H1 head (Ca, Cb, Sa, Sb) higher titers of HA- stalk antibodies were induced[142]. Computationally optimized broadly reactive antigens (COBRA) have also been generated to increase the breadth of responses against HA. Using this method consensus sequences of HAs from different circulating and pandemic subtypes are generated and expressed on viral like particles (VLPs)[143].

The administration of chimeric HAs (cHA) is one of the most developed strategies along the clinical pipeline[144]. cHAs are composed of conserved HA stalk domains but express exotic HA heads, such as those from avian influenzas to which humans have no pre-existing immunity[145]. Sequentially exposing individuals to cHAs with the same stalk but repeatedly different heads directs memory responses against the stalk domain, as *de novo* head responses are required. Results from a Phase I trial demonstrated that this vaccination method was safe and immunogenic, with inactivated adjuvanted cHA vaccines inducing stronger responses after one dose than LAIV cHAs[146]. The IIV formulation was pursued in a phase I-II study, where priming with adjuvanted cH8/1N1 and boosting with cH5/1N1 demonstrated superior immunogenicity[147]. More clinical evidence is required to demonstrate superior efficacy of UIVs over conventional vaccines.

With the success of nucleotide-based vaccines during the COVID-19 pandemic, this technology is already being applied to both seasonal and pandemic influenza vaccines. A recent mRNA vaccine candidate composed of modified mRNA encoding for 20 different HAs, encompassing all IAV and IBV subtypes was recently shown to be protective in both mice and ferrets[148].

1.2.9.2 Other universal vaccine strategies

Other vaccine strategies have focus on targeting internal proteins, such as NP, or the M2 ion channel. Both targets are highly conserved across IAVs making them attractive immunogens for broad acting vaccines. These platforms have been demonstrated to provide protection through T cells or non-neutralizing, fc-activating antibodies[149]. Several M2 vaccines have been tested in pre-clinical and clinical settings. Independently, M2 is not strongly immunogenic so fusion proteins have been generated to increase immunogenicity. Fusion of M2 to the hepatitis B viral core and vaccination intraperitonially and intranasally, protected mice from homologous and heterologous infection[150]. Expressing fusions of M2 to flagellin (TLR5 agonist) was also shown to be protective in animal models[151–153].

Viral vectored and peptide vaccines aimed to enhance T cell mediated immunity have also been explored. A recent clinical trial assessing immunogenicity of a conserved peptide based M1-M2-NP vaccine demonstrated enhanced cellular immunity ($CD4^+$, $CD8^+$ T cells, and IFN-γ) in vaccinated participants[154]. A modified vaccinia Ankara (MVA) based vaccine expressing NP and M1 induced strong $CD8^+$T cell responses when assessed in Phase I trials[155]. While T cell-based strategies have been demonstrated to reduce severe disease, cellular immunity alone is likely

insufficient in preventing URT infection. Next-generation vaccines will likely need to induce both antibody and T- cell based immunity.

NA is also being considered as a target for UIVs. Current vaccines can elicit anti-NA response, although quantification of NA in vaccine preparations and subsequent immunogenicity are not regularly monitored. However, anti-NA antibodies elicited after infection have been observed to provide broad protection[156]. Within NA subtypes, less antigenic drift is observed across seasons[157]. Therefore, the breadth of protection afforded by influenza vaccines may be improved by inclusion of NA targeted approaches.

1.3 Fc-Effector functions

1.3.1 Antibody structure and function

Human antibodies are "Y" shaped, approximately 150 kDa (kilodalton) proteins that are composed of two heavy (50 kDa) and two light chains (25 kDa) linked by disulphide bonds[158]. Antibodies are analogous to BCRs however, the lack of a hydrophobic anchor sequence results in the secretion of the protein. The first domain of the antibody is known as the variable or antigen binding (Fab) region and confers the ability to bind specific antigen. The sequence of the constant domain determines antibody isotype[158]. In humans, antibodies can be found as IgM, IgD, IgG, IgA and IgE, with IgG being further broken down into the IgG1, IgG2, IgG3, and IgG4 subtypes. IgA, which is most abundant at mucosal surfaces, is expressed as IgA1 and IgA2 in humans. In addition to binding pathogens via the Fab, antibodies elicit protection through Fc-effector functions that are stimulated by interaction of the Fc segment of the antibody and cognate Fc receptors (FcR) on immune cells. Antibody isotype has been found to influence antibody effector functions.

1.3.2 FcR general description

Humans express a host of activating and inhibitory FcR. IgG antibodies bind to FcγR, this family of receptors includes activating receptors; FcγRI, FcγRIIa, and FcγRIIIa. These receptors contain intracellular tyrosine activation motifs (ITAMs) which become phosphorylated and initiate effector functions such as ADCP, ADCC, and cytokine release. Both FcγRI and FcγRIIIa require recruitment of the common FcRγ chain as they do not have intrinsic signalling motifs. FcγRIIb and FcγRIIIb are inhibitory receptors which containing intracellular tyrosine inhibitory motifs (ITIMs). These receptors also vary in their affinity for binding IgG, only FcγRI is a high affinity FcR. Due to its high affinity, FcγRI can become saturated by monomeric IgG. The remaining receptors require multivalent immune complexes to initiate efficient receptor crosslinking and signalling, with IgG1 and IgG3 displaying the highest affinity for activating receptors[159].

The previously discussed FcγR are known as type I receptors; however, immunoglobulins can also bind type II receptors such as DC-SIGN and CD23. These lower affinity receptors can induce anti-inflammatory responses to IgG binding[159]. Fc composition, such as subtype and glycan presence, can influence binding to FcR. Glycosylation at position A297 mediates binding to type I or II receptors. For example, sialydation alters Fc structure such that affinity for IgG is enhanced for type II receptors[160]. Lack of a fucose at this position increases binding to FcγRIIIa stimulating more inflammatory responses. Recent findings have demonstrated the importance of post-translational antibody modification on COVID-19 disease severity. The abundance of afucosylated IgG1 was shown to be associated with increased disease severity[161–163]. IgA also binds to an activating type I receptor, FcαR, which is present on myeloid cells[164].

1.3.2.1 Fc-FcR are required for protection by bnAbs *in vivo*

The engagement of FcR has been demonstrated to be essential for influenza broadly-neutralizing antibodies to provide protection *in vivo*. Murine studies showed that when an HA stalk binding monoclonal antibody was expressed as IgG2a, IgG1, or with a D265A mutation only mice administered the bnAb in the activating IgG2a backbone were protected[165]. For both IgG1 and D265A antibodies, FcR engagement and activation was abrogated, as IgG1 binds the mouse inhibitory FcγRIIB and D265A abolishes Fc-FcR binding. A follow-up study demonstrated that all non-HAI antibodies, including stalk-binding, non-neutralizing, and NAI antibodies require FcγR engagement to provide optimal protection[166]. Through cellular depletion experiments in mice, it was demonstrated that alveolar macrophages are the Fc-bearing cell required for Fc- FcγR mediated protection by bnAbs[167]. However, similar studies have not been performed for IgA isotype bnAbs. As a result, the contributions of IgA mediated effector functions in antibody mediated protection from influenza remain to be further studied.

1.4 Immunoglobulin A (IgA)

1.4.1 IgA biology

IgA is the most abundant immunoglobulin at mucosal sites and is found in numerous forms throughout the body. In serum, IgA is largely present in its monomeric form. In humans, monomeric IgA can be further classified into IgA1 and IgA2. IgA1 has a longer hinge region and is more abundant in serum (present at 9:1). Although the relative ratio of IgA1 and IgA2 differs throughout the body, IgA1 is found to predominate at the nasal mucosa (80-90%) and saliva (60%)[164]. The subtypes are more evenly distributed (50%) in the female genital tract and the large

intestine. Differences in neutrophil effector functions induced by IgA1 and IgA2 have recently been observed[168].

At mucosal surfaces, IgA is present as dimeric IgA (dIgA), two monomers linked by a joining chain (J-chain). After being transported to the apical side of the mucosal epithelium by the polymeric immunoglobulin receptor (pIgR) part of the receptor, the secretory component, remains bound to the antibody[164]. dIgA with the addition of the secretory component results in secretory IgA (sIgA). The role of IgA in immunity is often overlooked, as IgA deficiency is common. It is estimated that 1 in 500-1000 people have selective IgA deficiency, although most of these individuals do not have elevated susceptibility to infection[169].

1.4.2 IgA FcαR (CD89)

IgA binds to the FcαR on the surface of myeloid cells. The receptor is composed of two extracellular domains (EC1 and EC2), a transmembrane domain and short cytoplasmic tail. FcαR binds IgA at a 2:1 ratio at the interface of the EC1 and second region constant heavy chain. Additionally, the FcαR does not have intrinsic signalling domains and requires interaction with the common γ chain (FcRγ) which has intracellular tyrosine activation motifs (ITAMs) to initiate signalling. The FcRγ chain is also required for FcγRI and FcγRIIIa. Tyrosines of the FcRγ are phosphorylated by src family kinases. Downstream signalling results in activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) transcription factor that results in pro-inflammatory functions. Immune complexes containing IgA most effectively initiate receptor crosslinking and signal transduction, monomeric IgA alone can induce iTIM signalling.

Interestingly, mice do not express a functional FcαR homologue which has limited in vivo work elucidating the role of IgA mediated Fc-effector functions[170].

1.4.3 IgA fc-effector functions

Antibody isotype has been shown to influence the nature of neutrophil effector functions. IgG stimulates phagocytosis while IgA preferentially triggers NETosis[171,172]. In the context of stalk mediated IgG responses, it is known that two-points of contact are required to efficiently engage Fc-responses; the HA receptor to sialic acid on the host cell and the Fc of an antibody bound to HA binding to its cognate FcR on an innate immune cell[173]. Interestingly, IgA has been demonstrated to neutralize viruses relative more potently than IgG[174]. While, the presence of multimeric IgA at mucosal surfaces and increased avidity may in part explain the increase. C-terminal sialic acids on IgA molecules have been demonstrated to bind the HA receptor binding site[175]. This glycosylation can therefore account for the increased potency and antiviral activity exerted by non-neutralizing IgA. Recent work has also identified that only stalk epitope specific IgA antibodies engage FcαR[176]. IgA has been demonstrated to induce unique effector functions in neutrophils. Further, IgA1 and IgA2 induce different functional outcomes[168]. IgA2 elicits more inflammatory functions in neutrophils[168]. This has been attributed to differential glycosylation patterns on the IgA subtypes, with IgA1 being more heavily sialydated.

1.4.4 The role of IgA in influenza responses

Vaccination with LAIV has been demonstrated to disproportionately induce IgA antibodies relative to IIVs, particularly at the mucosa[86]. Recombinant IgA more efficiently prevented influenza infection in guinea pigs compared to IgG[177]. IgA has also been demonstrated to have

enhanced heterosubtypic protection relative to IgG[178,179]. In a human challenge model of infection reduced viral shedding from the nose was correlated with high levels of serum and mucosal IgA[180]. Current intramuscular vaccines elicit serum IgG to a greater magnitude. The elicitation of influenza specific IgA responses in the URT and LRT by IgA plasmablasts was found to be induced only following intranasal vaccination[181]. In conclusion, designing vaccine strategies which take advantage of the IgA response to influenza viruses should be considered.

1.5 Neutrophils

1.5.1 Neutrophil biology

Neutrophils or polymorphonuclear cells (PMNs) are the most abundant white blood cells in circulation and the first cells to respond to sites of infection and inflammation. These short-lived cells are characterized by a distinctive multi-lobed nuclei and cytoplasmic granules. The primary granules contain proteases such as neutrophil elastase (NE), myeloperoxidase (MPO), and cathepsin G. Other proteins such as lactoferrin, matrix metalloproteinase 9 (MMP9), and gelatinase can be found in secondary and tertiary granules. Neutrophils are generally regarded as professional phagocytic cells but also utilize the production of reactive oxygen species (ROS), degranulation, and the release of neutrophil extracellular traps (NETs) to clear pathogens[182,183]. These antimicrobial defenses are often stimulated by the detection of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) on the surface of the cell[184]. However, human neutrophils also express a full complement of FcR on through which they can interact with antibodies and perform Fc- effector functions such as ADCP and ADCC[138].

1.5.2 Neutrophil extracellular traps (NETs)

Neutrophils undergo a unique form of cell death called NETosis, which results in the formation of Neutrophil Extracellular Traps (NETs). NETs are composed of decondensed chromatin which is released outside of the cell and coated with antimicrobial enzymes previously stored with the neutrophilic granules. While these structures can cause immunopathology and inflammation, NETs are also able to trap and inactivate a variety of pathogens, as first identified by Brinkmann and Zychlinksy[182]. Most commonly, NETs have been associated with the inactivation of bacteria and fungi but have been demonstrated to inactivate viruses (ex. HIV-1, RSV, Chikungunya virus (CHIKV), IAV). The role of NETs in sterile inflammatory conditions such as autoimmune diseases (systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)), cancer, diabetes, cystic fibrosis, thrombosis, gallstone formation is now also beginning to be recognized[184].

NETosis is a regulated form of cell death which can occur in response to a variety of stimuli. Following detection of PAMPs and DAMPs, the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex (NOX) produces ROS which activates protein arginine deiminase 4 (PAD4). PAD4 mediates the citrullination of histones by changing arginine residues to citrulline, resulting in reduced positive charge and chromatin decondensation[185]. Generation of ROS was demonstrated to be essential for NET release, as neutrophils from individuals with chronic granulomatous disease (CGD) who have compromised NADPH activity do not undergo NETosis[186]. Decondensation is further promoted by NE and MPO which get released from granules and translocate to the nucleus[187]. NET release culminates with nuclear and plasma membrane disruption.

Yipp et al. recently identified that neutrophils may also be able to undergo a process known as 'vital' NETosis in response to bacterial infections[188]. In contrast to the classical 'suicidal' NETosis, vital NETosis occurs rapidly and without disruption of the plasma membrane. The cells, now 'anuclear cytoplasts' can continue to perform some functions such as phagocytosis and crawling[189]. There is some evidence that NETs are instead packaged and released via vesicles during vital NETosis[188].

1.5.3 Role of neutrophils in protections from influenza virus

Infiltration of neutrophils into the lung has been demonstrated to protect mice from influenza infection. Depletion of neutrophils *in vivo* resulted in higher viral titers and increased mortality, suggesting that neutrophils are required during the initial stages of infection[190–192]. In contrast, excessive neutrophil and macrophage lung infiltration has been attributed to the high mortality observed in the 1918 pandemic. Neutrophils also play important roles in mediating $CD8^+$ T cell responses against influenza infection[193]. Following infection, neutrophils leave behind a CXCL-12 trail which attracts T cells to the site of infection. The multifaceted role of neutrophil during viral infection further supports the complex nature of these cells.

1.5.4 Role of neutrophils in influenza infection pathogenesis

Neutrophil activation and recruitment have been associated with ARDS and secondary bacterial pneumonia, both commonly observed complications following influenza infection. In severe cases of influenza infection, high levels of plasma NETs or NET components were associated with worsened outcome[194]. In a murine model of infection, it was observed that increased neutrophil recruitment to the lung resulted in increased lung tissue damage[195]. Neutrophils mediate this

through the release of granular enzymes which can damage the alveolar epithelium leading to edema and vascular leakage. Additionally, viral infection may also impair the ability of neutrophils to combat bacterial infections. The bactericidal capacity of neutrophils was significantly reduced following uptake of viral particles, as oxidative burst was impaired[196]. Subsequent studies involving IAV and Staphylococcus aureus demonstrated that MPO release and granule-phagosome fusion was altered[197]. These findings suggest that NETs can contribute to adverse outcomes and increased disease severity following influenza infection.

1.5.5 NETs and other viruses

The impact of NETs on outcomes to other viral infections is also highly context dependent. Regarding protection, NETs have also been found to be protective in Chikungunya infection[198]. HIV-1 virions have also been shown to be trapped and inactivated by NETs in an MPO and α-defensin specific manner[199]. Although, neutropenia is often observed in patients who have advanced acquired immunodeficiency syndrome (AIDS) resulting in heightened susceptibility to bacterial infections. The role of NETs in respiratory syncytial virus (RSV) has further reinforced the circumstantial role of NETosis in infectious illness. *In vitro* assays demonstrated that RSV can be sequestered by NETs[200]. However, in children and calves NETs were observed to airway obstruction following infection. Similarly, increases in dsDNA following rhinovirus infections were shown to exacerbate type-2 inflammation in humans and mice[201]. Degrading NETs or inhibiting NE were demonstrated to alleviate immunopathology in a murine model. NETosis has also been observed to play a role in the pathogenesis of non-respiratory viral infections[202]. Activation of platelets during dengue virus infection releases extracellular vesicles which result in toll-like receptor 2 (TLR2) mediated NET formation. Blockage of this pathway resulted in

increased survival in a murine model[203]. Platelet activation of neutrophils and subsequent blockages in the liver were also observed following poxvirus infection[204].

NETs have also been implicated in the pathogenesis of COVID-19. The progression to ARDS, which has been previously associated with neutrophilic infiltration, was also observed following SARS2 infection. Elevated NET markers (MPO-DNA, and histone 3) were detected in the sera of infected patients[205,206]. NETs were also found in post-mortem lungs from patients who succumbed to SARS2 infection. Interestingly, SARS2 was observed to directly activate neutrophils[205]. These observational studies point to NETs contributing to COVID-19 pathogenesis however, whether NETs drive disease severity or result as a by-product of infection remained to be further investigated. In a study of critically ill COVID-19 patients, neutrophil function, including NET release, correlated with disease severity[207]. Humoral responses have been shown to influence neutrophil responses in COVID-19. IgA antibodies were shown to induce NETosis, and IgA-to-IgG ratios were elevated in severe disease, further suggesting the contribution of neutrophil activation in COVID-19 pathogenesis[208,209]. Furthermore, intravascular NETs have also been hypothesized to contribute to the formation of thrombi and may contribute to abnormal coagulopathy and multi-organ failure observed in severe COVID-19 infections[210]. In summary, the role of NETs during viral infections in multifaceted. While the release of NETs can provide effective prevention in slowing viral dissemination, overabundant NET formation can drive immunopathology.

1.6 The role of immune history on influenza immune responses

1.6.1 OAS and immune imprinting

Original antigenic sin (OAS) was first identified by Thomas Francis in the 1960s[112]. His early studies described that HAI titers remained highest against influenza strains an individual was exposed to earliest in life[211]. He hypothesized that these antibodies were boosted by exposures to closely related strains. These findings were also supported by work in animal models and other human samples[212,213]. Since the identification of this serological response, our understanding of the antigenic variability of influenza has increased. Antigenic imprinting builds off Francis' observation and specifically refers to the phenomenon of first exposure to influenza virus shaping subsequent immune responses. Mechanistically, the hierarchal nature of antibodies to influenza by OAS and following imprinting are supported by back-boosting events[113]. Where secondary exposure to related viruses boosts antibodies against conserved epitopes.

Additionally, OAS is not limited to influenza viruses. It has been observed following sequential Dengue infection[214]. More recently, OAS has been shown in the context of seasonal coronaviruses and SARS2[215]. Despite the negative connotation of 'sin', the role of OAS in immunity is highly context dependent. Both historical and more recent studies have demonstrated that OAS can be beneficial in some instances.

1.6.2 Mechanisms underlying OAS

Both cellular and humoral immunity have been implicated in the establishment of 'OAS' and imprinted immune responses. Strong evidence has implicated high titers of pre-existing antibodies in the establishment of OAS. Elevated antibodies against conserved but non-neutralizing epitopes may bind and sequester antigen (antigen clearance) before *de novo* response can be generated[216].

In doing so, responses against novel vaccine antigen or virus strains are blunted. This process is known as epitope masking.

Additionally, memory B cells (MBCs) from prior exposures may additionally drive OAS responses. MBCs specific to conserved portions of HA may have lower activation thresholds than naïve B cells and outcompete naïve B cells for help for T cells in the germinal centre skewing responses to more historical strains. However, recent work in mouse models has demonstrated that naïve B cells make up a significant portion of secondary germinal centres[217,218]. This suggests that serological contributions to OAS may be more profound. The Victora lab also recently demonstrated using molecular fate mapping that epitope masking by pre-existing antibodies, rather than MBC competition in germinal centres (GCs), interfere with naïve B cell activation[219].

$CD4^+$ T cells also play an imperative role in the antibody response to influenza virus as they are required for the generation of effective humoral responses. The Sant lab has demonstrated that individuals with repeat exposure to influenza have diminished $CD4^+$ T cells which may further impact antibody responses[220].

Various immunological features contribute to antibody hierarchies, however antigenic characteristics also influence these responses. Namely, the antigenic distance between two related viruses has been shown to result in positive or negative interference of historical antibodies against novel strains[221]. When the level of relatedness between the primary and secondary strains is high, significant back-boosting of antibodies occurs. This has a positive effect on protection when the

circulating virus is also antigenically similar. When circulating strains are highly dissimilar to the priming antigen antibodies raised following vaccination[221].

1.6.3 The role of OAS in protection from infection

There has been a variety of epidemiological evidence supporting the beneficial role of original antigenic sin-like responses in providing protection from secondary infections. During the 1918 pandemic lower mortality was observed in older adults, this has been attributed to prior exposure to H1N1[222,223]. Similarly, prior exposure to the 1918 H1N1 virus or closely related variants, provided immunological benefit during the 2009 swine flu outbreak. Mortality was lower in older individuals who had previous H1N1 exposure[224,225].

Imprinting has also been shown to provide heterologous protection, this occurs by stimulation of antibodies to the conserved HA stalk. Gostic et al. found that imprinting with seasonal HA provided protection from avian HAs of the same phylogenetic group. For example, early childhood exposure to group 1 HAs (H1, H2) reduced susceptibility to H5N1 infection and exposure to group 2 (H3) reduced infection to H7N9[114]. Similarly, stalk antibodies induced after vaccination with pandemic H5N1 or H7N9 vaccines were found to vary depending on early life H1 or H3 exposure, respectively[226,227].

1.6.4 The role of OAS in susceptibility to infection

Notably, unique excess mortality patterns were observed in the 1918 Spanish flu pandemic. Unlike other pandemics which increased mortality in the very young and the elderly, creating a ‘U-shaped’ mortality curve, a ‘W-shaped’ curve was seen in 1918. Excess mortality was observed in

middle- aged adults (20-40 years of age). One of the hypotheses proposed to explain this unusual increase is the presence of pre-existing immunity to another influenza subtype, likely the H3Nx virus which circulated in 1890[223]. More recently, imprinting may explain the high disease burden amongst middle aged adults in the 2013-2014 influenza season where the acquisition of a point mutation prevented binding of antibodies from earlier H1N1 exposures[228].

1.6.5 The impact of OAS on vaccine responses

A variety of factors contribute to the varied influenza vaccine effectiveness observed across seasons. Immune history of vaccinees undoubtedly plays a role in some of the discrepancies in protection between years, although OAS has been improperly implicated in the reduced effectiveness observed in repeat vaccinees[229]. A series of studies examining the effectiveness of repeat vaccination reported conflicting results. Hoskins et al. found no benefit of repeat vaccination, while a 5-year study of repeat vaccination with an inactivated vaccine supported repeated annual vaccination[230]. The antigenic distance hypothesis has since been presented to reconcile the differing results. Recently real-world evidence from three influenza seasons in Canada demonstrated that impact of prior vaccination varies on a season-to-season basis consistent with the antigenic distance hypothesis[231].

Additionally, imprinting on vaccine specific epitopes has been implicated in years with reduced vaccine effectiveness, particularly against A/H3N2. This was particularly apparent during the 2012-2013 season and 2016-2017 following the acquisition or absence of a key glycosylation site in egg-grown vaccine strains, respectively[59,60]. In 2012-2013 VE was observed to be low despite good antigenic match to circulating strains. Skowronski et al. identified that the vaccine strain

acquired a glycosylation site that was not present in circulating viruses. As a result, antibodies elicited by vaccines provided limited protection[59]. Similarly, in 2016-2017 circulating H3N2 3C.2a viruses had a new glycosylation site, egg-adapted vaccine strains did not. Antibodies against the egg-adapted strain were shown to poorly neutralize[60].

An imprint regulated effect of vaccine (I-REV) has recently been proposed due to observations in birth year cohort specific efficacy against contemporary H3N2 strains[232,233]. In 2018-2019, VE against the H3N2 component of the vaccine varied depending on age and by H3N2 clade.

1.6.6 The impact of vaccine formulation on OAS

One of the major outstanding questions regarding OAS is how the nature of first influenza exposure shape the formation of antigenic hierarchies. Recent work in mice has demonstrated that both infection and vaccination can induce OAS to varying degrees and the inclusion of adjuvants during either vaccination may be able to overcome OAS[234,235]. Similarly, vaccine formulations with a finite bolus of antigen may be more susceptible to OAS, in contrast platforms that replicate (LAIV) or that can evade pre-existing antibodies (mRNA) may more effectively overcomes OAS[236].

1.6.7 The role of immune history/ imprinting on development of next-generation vaccines

In the context of seasonal vaccination, OAS can be detrimental when memory responses are focused on conserved, non-neutralizing epitopes at the expense of generating *de novo* responses against protective epitopes. In contrast, OAS may be beneficial if elicited against conserved protective epitopes such as the HA stalk domain. Therefore, while strategies to overcome HA

head-based OAS should be explored in the context of seasonal vaccines, imparting memory on cross-protective epitopes will be essential for next-generation vaccines.

1.6.8 OAS and other antigenically plastic viruses

OAS responses have also been observed for other pathogens that cause repeated infections. Classically, OAS has been negatively associated with worsened outcomes to secondary Dengue exposure. There are four serotypes of Dengue, primary exposure generally results in acute febrile illness and confers life-long protection[214]. Secondary infection with a heterologous serotype can elicit non-neutralizing antibodies. In contrast to influenza viruses, dengue can replicate in FcR bearing cells, resulting in antibody dependent enhancement of disease (ADE) and dengue hemorrhagic fever[237]. As previously discussed, there is epidemiological evidence of OAS increasing susceptibility to influenza virus infection. However, FcγR bearing cells demonstrate susceptibility and not permissibility to influenza infection. Additionally, passive transfer of non-neutralizing monoclonal stalk antibodies does not result in enhancement of disease[167].

More recently, imprinting and OAS have also been demonstrated in SARS2 responses. SARS2 infection was shown to increase antibodies against seasonal coronaviruses[215]. Reduced OAS antibodies were observed following vaccination. Regarding successive SARS2 vaccination, antibody titers against the original Wuhan-1 strain have been observed to be higher following vaccination with a Bivalent booster against BA.4/BA.5[238]. Although, early studies examining primary vaccination regimens demonstrated that two-doses were required to generate sufficient neutralization titers[239,240]. This suggests that additional Omicron boosters may be sufficient to overcome this response. Early studies postulated that ADE may be contributing to the severity of

SARS2 infections however, no experimental evidence has supported this hypothesis[241]. Furthermore, while FcγR mediated uptake of SARS2 by monocytes has been shown, no active viral replication was observed[242].

1.7 Influenza infection in highly-susceptible populations

1.7.1 Cardiovascular disease (CVD)

Cardiovascular disease (CVD), including ischemic heart disease and stroke, was the leading cause of death globally in 2019, resulting in approximately 17.9 million deaths or 32% of all deaths worldwide[243]. CVD encompasses a variety of conditions including, heart attack, stroke, heart failure (HF), arrythmias, and heart valve failures. However, 85% of CVD related deaths result from heart attack or stroke[244]. Both conditions are related to atherosclerosis which is caused by plaque build-up in the arteries, leading to narrowing of blood vessels and reduced blood flow to the heart and other organs. If plaques become dislodged, blood flow to the heart and regions of the brain can become entirely restricted culminating in heart attack and stroke, respectively[245].

1.7.1.1 Heart failure

Heart failure refers to reduced efficiency in the ability to pump blood throughout the body. Commonly, heart failure is associated with other cardiovascular conditions (ex. hypertension) and patients often present with symptoms of dyspnea (shortness of breath), fatigue and peripheral edema (swelling in the legs)[246]. These symptoms are non-specific to HF and can be observed with other cardiac conditions making diagnosis challenging. HF generally impacts individuals over the age of 65, with higher prevalence in men than women[247]. The number of HF cases globally has been increasing, rising from 33.5 million in 1990 to 64.3 million in 2017[247]. In addition to heath

impact, HF causes substantial economic burden. In 2012 it was estimated that approximately $108 billion USD were spent on direct and indirect HF costs[248].

1.7.1.2 Myocardial infarction

Acute myocardial infarction (MI) or heart attack is caused by reduction in blood flow to the muscles of the heart resulting in irreversible muscle damage. The causes of MI are multifaceted, however commonly plaques occlude coronary blood flow resulting in thrombosis. High cholesterol and hypertension have also been associated with elevated risk of MI.

1.7.1.3 Ischemic stroke

Similarly, occluded blood vessels can result in reduced blood flow to cerebral tissues. Thromboembolisms are one of the many causes of reduced cerebellar blood flow. In 2020, the American Heart Association estimated that stroke was responsible for approximately 150,000 deaths in the United States[249].

1.7.2 Influenza and cardiovascular events

Influenza has been demonstrated to increase the risk of cardiovascular events and deaths. Epidemiological data has demonstrated that excess mortality from cardiac related illnesses increases following periods of influenza circulation[250,251]. Additionally, a 2016 time-course analysis examined CVD mortality in relation to emergency room visits for influenza-like illness. This study found strong associations with mortality from MI and influenza infection in the 14-days prior[252]. Using within person comparisons, Smeeth et al. examined individuals with first MI (n= n=20,486) and with stroke (n=19,063) who had recently received a common vaccination (ex.

influenza, pneumococcal, tetanus). They found no increased risk for cardiovascular event after vaccination however, risk was elevated for MI and stroke in the days following diagnosis with a respiratory virus[253].

A systematic review specifically examining the impact of acute influenza infection on MI compared findings across 39 studies[254]. Several studies found correlation between rates of influenza circulation and rates of CVD mortality on a population level. Observational studies looked at by the authors also found association between influenza infection and MI[254]. However, acute infection with a variety of bacterial and viral pathogens has been associated with increased cardiac events[255]. This suggests that this is not an influenza specific phenomenon, and that the systemic inflammation and/or dysregulated immune responses resulting from any infection may be able to trigger thrombotic events.

1.7.3 Pathophysiology of influenza and cardiac events

Epidemiological data supports a correlative link between influenza infection and cardiac events however the underlying mechanism remains to be determined. Two ideologies may explain cardiac dysfunction observed following influenza virus infection. The first posits that cardiac tissues may be directly infected, while other studies support that systemic lung inflammation may indirectly impact cardiac health. The relationship between influenza and cardiac disease is likely multifaceted as patients with worsened outcomes to infection often have multiple co-morbidities (ex. diabetes, high BMI, cholesterol, etc.).

A recent study in mice using a genetically modified influenza virus that was precluded from infecting cardiomyocytes, demonstrated that severe lung inflammation alone was not sufficient to cause cardiac pathology[256]. This suggests that direct effects are required however, these mice were not genetically predisposed to CVD, so the distal effects of acute inflammation may not be encompassed. Influenza has been demonstrated to cause direct cardiac abnormality. Influenza virus has been detected in post-mortem cardiac tissues of humans and non-human primates. Virus has also been found in the heart tissue of infected mice[257].

Other features of influenza infection may also exacerbate the pre-existing inflammatory environment of atherosclerosis. Atherosclerotic plaques are composed of a lipid core, inflammatory cells (macrophages, T cells, mast cells) necrotic tissue, cholesterol, cytokines, and a fibrous cap formed of collagen[258]. Endothelial permeability is altered during atherosclerosis such that there is increased infiltration of monocytes and lymphocytes. Low density lipoprotein is oxidized (oxLDL) and phagocytosed by macrophages which results in a foamy cell phenotype. As the disease progresses, and plaque size increases, atrial stenosis can occur, leading to reduced blood flow to surrounding tissues. Thrombotic events result when the plaque ruptures stimulating further inflammation, platelet activation, and the coagulation cascade. The resulting occlusion can block the vessel at the site of the plaque or at distal sites leading to MI or ischemic stroke[258].

Atherosclerosis is now recognized to be a chronic inflammatory condition. Cells within the plaque release a variety of cytokines and proinflammatory mediators and acute infection further contributes to systemic inflammation[258]. Additionally, influenza infection can result in thrombus formation and platelet activation. Physical vasculature changes because of infection (ex. blood

volume, blood pressure, vasodilation, etc.) and can put stress on the plaque resulting in rupture. Both direct and indirect effects of influenza infection as it relates to cardiac dysfunction are plausible.

1.7.4 Prevention of complications from influenza infections

Given the high rates of CVD and influenza like illness, prophylactic vaccination represents a means to prevent both infection and resulting cardiovascular dysregulation. Numerous clinical trials have aimed to assess the protective effects of seasonal vaccination on CVD with varying results. A recent systematic analysis of 6 RCTs, examining ~9000 patients, found that vaccination resulted in a 34% reduced risk of cardiovascular events[259]. Similarly, a Cochrane review compiling multiple RCTs where participants were divided into a vaccination and placebo group found that cardiovascular mortality was reduced in vaccine recipients[260]. Additionally, numerous observational studies have demonstrated that vaccination was effective in reducing MI[261,262]. Pooling data from both RCTs and observational studies, a 2021 systematic review found that vaccination reduced all-cause mortality, cardiovascular mortality, and adverse cardiac events. Although, they did not observe a significant effect on MI[263].

The recently completed **I**nfluenza **V**accine to reduce adverse **V**ascular **E**vents (IVVE) RCT demonstrated that seasonal influenza vaccination reduced cardiac outcomes in a heart failure cohort[264]. Prior to the completion of this trial, no placebo-controlled trials had been performed in heart failure patients. In summary, these findings support that influenza vaccination should continue to be recommended for individuals with underlying cardiac conditions.

2.0- Chapter 2: Rationale, Objectives and Overarching Hypothesis

2.1 Rationale

Despite the current global focus on managing SARS-CoV-2, influenza viruses remain a major threat to global public health. Influenza viruses have caused a pandemic approximately every 30 years for the last century and changing global patterns in climate and ecology increase the likelihood of future zoonotic spillover events[6,44]. Furthermore, the aging population has resulted in waning population-level immunity against prior pandemic subtypes (i.e. H2N2) that may represent a risk for re-emergence, per the "virus recycling" hypothesis[265]. Prevention of another global health crisis caused by a respiratory virus will only be prevented by improving vaccine breadth and durability, while investing in next-generation strategies that can be produced quickly and at scale.

Despite a mature understanding of mechanisms that drive broadly-neutralizing antibody production, and the pre-clinical and early clinical development of many universal influenza virus vaccine candidates, no universal influenza vaccines have been approved for use in humans. Systematic comparisons of vaccine platforms and delivery which elicit bnAbs have not yet been performed in sufficient depth. Seasonal vaccine data supports differences in the immune responses elicited by distinct routes-of -administration. Furthermore, recent phase I human clinical trial data assessing a chimeric HA strategy demonstrated formulation-specific differences in both breadth of immunity and magnitude of responses[146]. These results underscore that vaccine platform can drastically influence the quality of vaccine-elicited immunity.

Historically, most studies focused on broadly neutralizing antibody responses have limited their analyses to systemic IgG responses[165,167]. While Fc-FcγR interactions are required for protection mediated by IgG broadly-neutralizing antibodies *in vivo*, responses to vaccination are polyclonal and protection mediated by other antibody isotypes, namely IgA, also contribute to protection from influenza virus. Better understanding the non-neutralizing functions of IgA antibodies may therefore inform the design of more effective vaccines and monoclonal antibody-based therapeutics.

Numerous vaccine strategies have demonstrated potent immunogenicity and broad protection in preclinical animal models. However, this success has not yet been mirrored in human clinical trials. This disconnect may, in part, be driven by the complex immune histories to influenza virus that exist within the human population resulting from continuous exposure in influenza infections and vaccinations over the course of a lifetime[111]. Understanding how previous exposure influences vaccination responses is an essential consideration in the development of more effective vaccines.

In children, limited exposure history to influenza virus contributes to increased mortality and severe complications from infection. Childhood exposure to influenza virus is also disproportionately impactful due to the effects of "immune imprinting" – which shapes all subsequent immune responses to influenza virus later in life . Most studies of immune imprinting have been conducted in the context of infection. However, many children now first encounter influenza virus via childhood vaccination. Identifying seasonal and universal vaccine formulations that are best for use in children will greatly reduce the overall burden of influenza.

Individuals with underlying cardiovascular conditions represent an additional high-risk group for severe outcomes following influenza infection. The recent influenza vaccine in patients with heart failure to reduce adverse vascular events (IVVE) randomized control trial demonstrated that during peak influenza circulation, the influenza vaccine reduced all cause death, cardiovascular death, hospitalizations, and pneumonia[264]. While developing broadly neutralizing vaccines represents the future direction of influenza research, these strategies are still likely years from being realized. Better understanding how seasonal vaccines provide direct and in-direct protection in at-risk populations can further reduce the burden influenza viruses place on the healthcare system at present.

In summary, there remains clear gaps in our framework for understanding the ways in which vaccines optimally protect against influenza. An enhanced framework for understanding the impacts of vaccine route, formulation, and immune responses in the highest risk groups for severe disease are needed to reduce the burden of seasonal influenza, and better protect against future pandemics.

2.2 Objectives

1) Determine whether IgA antibodies induce FcαR specific effector functions (Chapter 3)
2) Define how first exposure to various seasonal influenza vaccine platforms impacts functional HA head domain and HA stalk domain-focused responses following subsequent exposures (Chapter 4)

3) Assess the immunogenicity of a seasonal influenza vaccine across multiple countries to measure immunogenicity and estimate vaccine effectiveness in patients who had a cardiovascular event within the study period (Chapter 5)

2.3 Overarching Hypothesis

We hypothesize that improvement of seasonal and next generation influenza virus vaccination strategies can be facilitated by considering the immunological repercussions of vaccination platforms/ routes of administration and understanding differences imparted on immunogenicity by immune history and in special populations such as the immunologically naïve and those with underlying cardiovascular health conditions.

3.0- Chapter 3: Investigating unique IgA Fc-effector functions in response to viral infections

Manuscript title: IgA potentiates NETosis in response to viral infection

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IgA potentiates NETosis in response to viral infection

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IgA is the second most abundant antibody present in circulation and is enriched at mucosal surfaces. As such, IgA plays a key role in protection against a variety of mucosal pathogens including viruses. In addition to neutralizing viruses directly, IgA can also stimulate Fc-dependent effector functions via engagement of Fc alpha receptors (Fc-αRI) expressed on the surface of certain immune effector cells. Neutrophils are the most abundant leukocyte, express Fc-αRI, and are often the first to respond to sites of injury and infection. Here, we describe a function for IgA–virus immune complexes (ICs) during viral infections. We show that IgA–virus ICs potentiate NETosis—the programmed cell-death pathway through which neutrophils release neutrophil extracellular traps (NETs). Mechanistically, IgA–virus ICs potentiated a suicidal NETosis pathway via engagement of Fc-αRI on neutrophils through a toll-like receptor–independent, NADPH oxidase complex–dependent pathway. NETs also were capable of trapping and inactivating viruses, consistent with an antiviral function.

NETosis | viruses | influenza | SARS-CoV-2 | neutrophils

IgA antibodies have pleiotropic roles in regulating the response to microbes. In the context of infection, IgA antibodies enriched at mucosal surfaces as secretory IgA (sIgA) are capable of neutralizing viruses in an "anti-inflammatory" manner, since these antibodies block infection but do not activate immune cells via Fc-receptor engagement. However, monomeric IgA (mIgA) antibodies, which are abundant in serum, are capable of engaging Fc receptors on the surface of immune cells to elicit effector functions (1).

Neutrophils are not only the most abundant leukocytes but are often the first to respond to sites of injury and infection (2). Human neutrophils express the Fc alpha receptor (Fc-αRI/CD89) and are capable of exerting a variety of effector functions including phagocytosis, respiratory burst, antibody-dependent cellular phagocytosis (ADCP), and NETosis (3, 4). Data regarding the protective versus pathogenic role of neutrophils during viral infection is nuanced and suggests context is critical in determining outcome. For example, while neutrophils are required for protection during the early stages of influenza A virus (IAV) infection, neutrophils also release reactive oxygen species (ROS), proteolytic enzymes, and a variety of inflammatory mediators that can damage lung tissues. As a result, excessive neutrophil infiltration has been associated with severe lung injury (5).

The generation of neutrophil extracellular traps (NETs) was first described by the Zychlinsky laboratory in 2004 as an antibacterial effector mechanism (6). NETs are produced via a specialized form of programmed cell death called NETosis and are composed primarily of decondensed chromatin studded with antimicrobial proteins. Extensive work by many laboratories has since demonstrated that NETs can have not only protective but also pathogenic consequences in infections and many other diseases (4). The understanding of how NETs influence viral infections continues to evolve. In the context of Chikungunya virus and poxvirus, NETs were capable of trapping viruses and controlling infection in a mouse model of disease (7, 8). Likewise, NETs have been shown to trap and inactivate HIV (9). However, NETs have also been described as exacerbating disease in the context of Dengue virus, rhinovirus, respiratory syncytial virus, influenza virus, and, most recently, SARS-CoV-2 infection (10–16). Thus, the overall impact of NETs during a viral infection must be interpreted carefully in conjunction with other infection parameters.

Recently, Fc-dependent effector functions have been shown to play a central role in the protection conferred by broadly neutralizing antibodies (bnAbs) that bind to the hemagglutinin (HA) stalk domain of IAV (17–19). However, these studies have only been performed in the context of monoclonal IgG antibodies. Elicitation of bnAbs is now the goal of several "universal" influenza-virus vaccine candidates including "chimeric" HA vaccines that were recently tested in a Phase-I clinical trial (20). Antibody-dependent cellular cytotoxicity (ADCC) may also augment protection mediated by HIV-neutralizing antibodies (21). However, despite the fact that both IAV and HIV are mucosal pathogens, almost nothing is known about the contribution of IgA-mediated Fc-dependent effector functions during infection. This is due, in large part, to the fact that mice do not express an Fc-αR homolog, which presents significant challenges for assessing the contributions of IgA to outcomes in vivo (1).

Significance

IgA antibodies are enriched at mucosal surfaces and play an important role in host defense against viral pathogens. In addition to neutralizing viruses directly, antibodies can also engage specific receptors (Fc receptors) expressed on various immune-cell subsets to stimulate antimicrobial activities. While IgG Fc-mediated effector functions are known to mediate important antiviral activities, if and how IgA-mediated Fc-effector functions influence viral infections remains poorly understood. Here, we show that IgA–virus immune complexes stimulate neutrophils to undergo NETosis, a specific type of programmed cell death that results in release of chromatin studded with antimicrobial effector proteins. These neutrophil extracellular traps trap and inactivate viruses but can also have pathogenic consequences when poorly regulated.

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Here, we show that IgA–virus immune complexes (ICs) potentiated NETosis through Fc-αRI signaling on neutrophils. This potentiation was not virus specific and could be observed for IAV, HIV, and SARS-CoV-2 spike-pseudotyped lentiviruses and extended to IgA ICs generated with antibodies/autoantigens from rheumatoid arthritis (RA) patients. In contrast to NETosis stimulated by viruses directly, IgA–virus ICs stimulated suicidal NETosis that was independent of toll-like receptor (TLR) signaling. Finally, viruses were trapped and inactivated in NETs, suggesting a protective role in vivo when properly regulated.

Results

IgA–IAV ICs Stimulate NETosis. Historically, antibodies have been thought to mediate protection against influenza viruses primarily by binding to the HA head domain and blocking interaction between the receptor binding site on HA and sialic acids on the surface of host cells. However, more recently, it has become clear that bnAbs that bind to the HA stalk domain mediate protection in vivo primarily by elicitation of Fc-dependent effector functions (17–19). Antigen-specific IgA antibodies have been shown to neutralize IAV, but relatively little is known about IgA-mediated Fc-dependent effector functions during IAV infection (22). Neutrophils are the most abundant leukocyte and are among the first to respond during IAV infection (23). Neutrophils also express FcαRI, and we have previously shown that IgA–IAV ICs stimulate ROS production in neutrophils; however, unlike IgG–influenza virus ICs, this could not be fully inhibited by cytochalasin D, indicating that IgA-mediated ROS production was not due to ADCP (24). To determine whether IgA was capable of potentiating NETosis upon binding IAV, neutrophils were exposed to antibody–IAV ICs composed of polyclonal (monomeric) IgA or IgG from the peripheral blood of donors previously vaccinated with seasonal influenza vaccines containing the A/California/04/2009 (Cal/09) H1N1 component. Phorbol 12-myrisate13-acetate (PMA), a potent inducer of NETosis, was used as a positive control (25). IgA–IAV ICs stimulated significantly higher levels of NETosis than antibodies or viruses alone, whereas IgG–IAV ICs did not induce NETosis above background levels (Fig. 1 *A* and *B*). A 2:1 ratio of IgA to virus reliably induced NETosis above background levels, while IgA-to-virus ratios of up to 10:1 did not

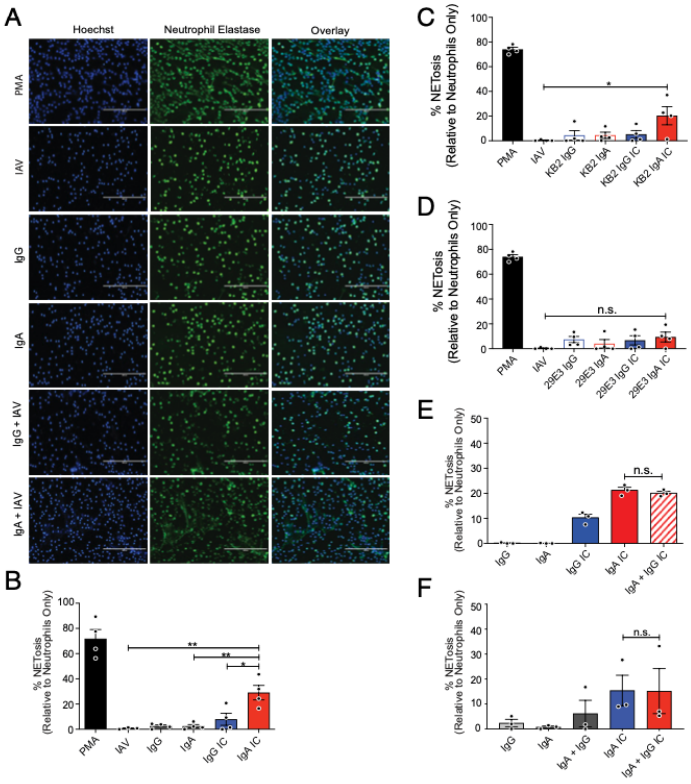

Fig. 1. IgA–IAV ICs potentiate NETosis. (*A–F*) Primary human neutrophils were isolated from the peripheral blood of healthy donors ($n = 3$ or 4) and stimulated with PMA, monoclonal or polyclonal IgG or IgA antibodies, or ICs for 3 h as shown. NETosis was assessed by immunofluorescence microscopy after costaining for DNA (Hoechst) and neutrophil elastase. (*A*) Representative images are shown (20×). (Scale bars, 200 μm.) (*B*) The percentage of cells that had undergone NETosis (defined by typically NET morphology and costaining of DAPI + neutrophil elastase) was quantified in a blinded manner from five fields in four independent experiments. (*C* and *D*) The assay was repeated using monoclonal antibodies, (*C*) KB2 and (*D*) 29E3, which bind the HA stalk and head domain of Cal/09, respectively. (*E* and *F*) To determine the phenotype of mixed IgG/IgA ICs, polyclonal IgG and IgA were mixed with Cal/09 at (*E*) a 1:1 ratio or (*F*) at the ratio naturally found in serum. For all experiments, percent NETosis was normalized to unstimulated neutrophils. Three or four independent neutrophil donors were used for each experiment. Means and SE (SEM) of independent experiments are shown. Statistical significance was determined using one-way ANOVA with Tukey post hoc test. $*P < 0.05$; $**P < 0.01$.

significantly further increase NETosis induction (*SI Appendix*, Fig. S1*A*).

In the context of IgG, bnAbs that bind to the stalk domain have been shown to potently elicit Fc-dependent effector functions, whereas antibodies that bind to the HA head domain and exhibit hemagglutination inhibiting activity do not. This is because HA stalk-binding bnAbs allow for two points of contact between target and effector cells (26, 27). To determine whether broadly neutralizing IgA–IAV ICs are primarily responsible for the induction of NETosis observed in the context of IAV-specific polyclonal IgA, we used a panel of previously described monoclonal antibodies that bind to neutralizing epitopes on either the HA head or stalk domains (28–30). The antibody KB2 binds to the HA stalk domain of H1 viruses, while 29E3 is specific to the HA head domain of Cal/09 (31). When human neutrophils were incubated with ICs containing an IAV–IgA1 stalk-binding antibody (KB2), significant induction of NETosis was observed following 3-h stimulation (Fig. 1*C*). In contrast, NETosis was not induced by IgG1–IAV ICs or by antibodies or viruses alone (Fig. 1*C*). We have previously shown that, under these conditions, IgG–IAV ICs potently induce neutrophil ADCP via FcγR signaling (24). As we had observed in the context of ADCP, all ICs generated with an HA head-binding antibody (29E3) failed to induce NETosis (Fig. 1*D*).

In blood, IgA cocirculates with other antibodies, including IgG, which signals through distinct FcRs (FcγRs) and can also induce NETosis (32). Mixed ICs composed of IgG/IgA–HIV have also been shown to act cooperatively to stimulate ADCC by monocytes (33). We therefore tested whether mixed ICs composed of IAV bound by IgA and IgG together would influence the magnitude of NETosis induction relative to IgA alone. When ICs were generated with a 1:1 ratio of IgG to IgA, the magnitude of NETosis induction was similar to IgA alone (Fig. 1*E*). In serum, IgG is significantly more abundant than IgA (~4:1 to 10:1). Thus, to recapitulate the physiological stoichiometry of IgG–IgA, we purified each immunoglobulin from serum of matched donors and then recombined them at their natural physiological ratio. Here again, the magnitude of NETosis observed in mixed IgG–IgA ICs was similar to IgA alone, indicating that IgG does not potentiate IgA-mediated NETosis, nor does it interfere with the ability of IgA to stimulate NETosis (Fig. 1*F*). Taken together, these results demonstrate that IgA–virus ICs stimulate neutrophils to undergo NETosis.

IgA-Mediated NETosis Is Not an IAV-Specific Phenomenon. NETs have been observed in the context of many other infections including those caused by COVID-19 and HIV. In these studies, viruses were presumed to stimulate NETosis directly (14, 16, 34, 35). We thus performed an experiment to test the amount of viruses needed to stimulate NETosis independent of FcαR signaling. Neutrophils were stimulated with increasing concentrations of purified lentiviruses pseudotyped with the SARS-CoV-2 spike protein. A significant elevation in NETosis was observed when neutrophils were exposed to 0.05 and 0.2 mg/mL purified virus (Fig. 2*A*). We then purified IgA from convalescence serum of a SARS-CoV-2–infected individual and a SARS-CoV-2–naïve individual, incubated them with substimulatory concentrations (0.0125 mg/mL) of spike-pseudotyped lentiviruses to allow for IC formation, and then incubated these mixtures with primary human neutrophils from healthy donors. As we observed in the context of IAV, IgA–virus IC generated with IgA purified from SARS-CoV-2 convalescence serum was capable of stimulating NETosis, whereas pseudovirus–IgA mixtures from naïve serum was not (Fig. 2*B*). These results confirm that IgA–virus ICs more potently stimulate NETosis when compared to viruses alone and that ICs are required for this potentiation, since IgA from seronegative individuals did not significantly induce NETosis when mixed with pseudotyped lentivirus.

We also incubated neutrophils with antibody–HIV ICs, which contained HIV-specific IgA isolated from the serum of HIV+ individuals. Following stimulation, a significant increase in NETosis was observed in cells treated with anti-HIV IgA-containing ICs (Fig. 2*C*). Background levels of NETosis were observed when cells were treated with either IgA or viruses alone. These findings demonstrate that IgA-induced NETosis likely happens in the context of many viral infections.

NETs have also been implicated in the pathogenesis of a variety of autoimmune conditions, including RA, in which they serve as a source of autoantigen (36, 37). Patients with autoimmune diseases commonly have autoantibodies against NET elements such as histones, DNA, and neutrophil elastase. Here, neutrophils were stimulated with Ab–autoantigen ICs, composed of IgA or IgG purified from the serum of RA patients or healthy donors, and recombinant citrullinated human fibrinogen, a common autoantigen in RA (38). Induction of NETosis was observed in neutrophils stimulated with IgA–citrullinated fibrinogen ICs from RA patients but not in those stimulated with IgG-containing ICs or ICs generated with antibodies from healthy donors (Fig. 2*D*). Together, these data demonstrate that potentiation of NETosis is a common property of virus–IgA ICs as well as ICs composed of IgA–autoantigens.

Induction of NETosis by IgA ICs Is Dependent on FcαRI and Independent of TLR Signaling. We next assessed whether sIgA purified from human saliva was capable of inducing NETosis. Whereas mIgA is found predominantly in circulation, sIgA is enriched at mucosal surfaces and is generally regarded as an anti-inflammatory antibody. sIgA from saliva and serum-derived mIgA was purified from matched vaccinated donors used to generate ICs with IAV. ICs containing sIgA did not potentiate NETosis, whereas serum-derived mIgA from the same donors was capable of eliciting NETosis, as we had observed previously (Fig. 3*A*). These results are consistent with previous studies that have demonstrated that the secretory component sterically blocks binding of sIgA to FcαRI (CD89) (39).

Given the observation that sIgA–IAV ICs failed to induce NETosis, we investigated whether mIgA-mediated NETosis was dependent on engagement of FcαRI (CD89). To this end, neutrophils were incubated with a blocking monoclonal anti-CD89 antibody prior to stimulation with IgG–virus or IgA–virus ICs. Blocking with anti-CD89 abrogated induction of NETosis following stimulation with IgA ICs (Fig. 3*B*). As expected, anti-CD89 treatment had no effect on PMA-induced NETosis (*SI Appendix*, Fig. S1*B*), confirming that engagement of FcαRI is required for IgA–virus IC-mediated induction of NETosis.

TLR8 activation has been shown to shift neutrophils from phagocytosis to NETosis in the context of IgG IC-mediated NETosis via FcγRIIA signaling (32). We thus set out to determine whether TLR signaling was required for IgA-mediated NETosis induction. TLR8 senses single-stranded RNA and is an important pattern-recognition receptor during RNA virus infection (40). Since IAV particles contain RNA, we elected to use a system free from TLR7/8 ligands. To this end, polystyrene beads (roughly equal in number to IAV particles used in previous experiments) were coated with protein L and polyclonal IgA. Protein L binds to the κ-light chain of antibodies, leaving the antibody Fc region capable of interacting with FcRs on the cell surface. Following stimulation, IgA–bead ICs induced significant NETosis relative to beads alone (Fig. 3*C*). This suggests that, unlike IgG IC-mediated NETosis, IgA IC-mediated NETosis is likely independent of TLR signaling.

Neutrophils are professional phagocytes, and ADCP is one of the many Fc-mediated effector functions that contribute to their defense against pathogens (24). To directly measure whether IgA ICs induced phagocytosis, fluorescent, protein L–coated polystyrene beads were complexed with IgA or IgG prior to incubation with neutrophils. After incubation with beads, cells were washed extensively to remove any beads that had not been phagocytosed.

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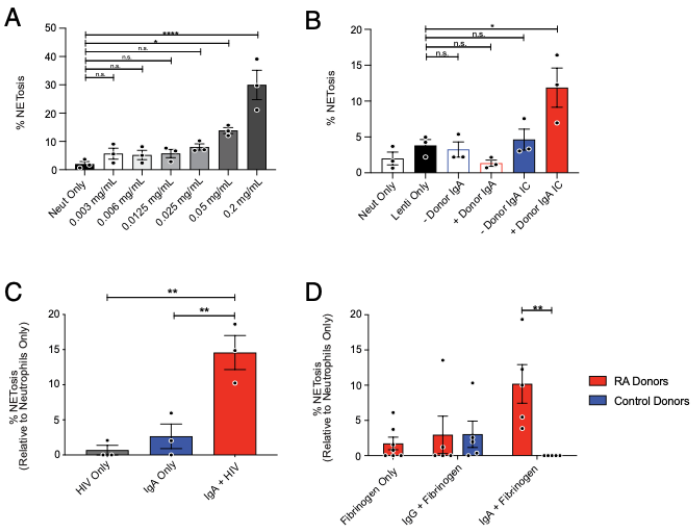

Fig. 2. Potentiation of NETosis by IgA ICs is not an IAV-specific phenomenon. (*A*) Purified COVID-19 spike-pseudotyped lentivirus was titrated onto primary human neutrophils from healthy donors ($n = 3$) and incubated for 3 h prior to staining for DNA (DAPI) and neutrophil elastase. (*B*) Polyclonal IgA was isolated from serum of a convalescent COVID-19 donor and from prepandemic donor serum (COVID-19 seronegative) and incubated with spike-pseudotyped lentivirus to form ICs prior to stimulation of neutrophils isolated from healthy donors ($n = 3$) for 3 h prior to staining for DNA (DAPI) and neutrophil elastase. (*C*) ICs were formed with IgA purified from serum of HIV-positive individuals ($n = 3$) and HIV-1 X4 gp120 (HxB2)-pseudotyped lentivirus. Neutrophils were stimulated for 3 h prior to staining for DNA (DAPI) and neutrophil elastase. (*D*) Cells were stimulated with ICs containing IgA purified from the serum of healthy donors ($n = 5$) or RA patients ($n = 5$) in complex with citrullinated fibrinogen. NETosis was quantified in a blinded manner from five fields per condition. Mean and SEM of independent experiments are shown. (*A–C*) *P* values were determined by one-way ANOVA with Tukey post hoc test. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. (*D*) *P* values were determined by *t* test with multiple comparisons. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

Significantly greater bead uptake was recorded for neutrophils that were exposed to the IgG-opsonized beads compared to those coated with IgA, which actually inhibited phagocytosis relative to protein L–coated control beads (Fig. 3*D*). This further demonstrates that endosomal TLR activation by viral pathogen-associated molecular patterns (PAMPs) are not required for the potentiation of NETosis by IgA–virus ICs. As further confirmation, instead of using soluble ICs, as had been done in previous experiments, ICs were immobilized on glass coverslips. Consistent with all experiments that had been performed using soluble ICs, significantly higher levels of NETosis were observed when neutrophils were incubated with immobilized IgA–virus ICs relative to immobilized IgG–virus-containing ICs (Fig. 3*E*). Combined, these data suggest that phagocytosis is not required for IgA IC-mediated stimulation of NETosis.

IgA ICs Stimulate NADPH Oxidase Complex–Dependent Suicidal NETosis. The most common and well-characterized type of NETosis is called "suicidal NETosis," which results in the death of the cell. More recently, other types of NETosis have been described, including "vital" NETosis (41). Suicidal NETosis requires ROS production and occurs between 1 and 3 h after stimulation, while vital NETosis does not require the generation of ROS and occurs between 5 and 60 min after stimulation (41). To determine whether IgA–virus IC–induced NETosis was vital or suicidal, we first performed a time-course experiment following stimulation with PMA, a well-characterized stimulant of suicidal/ROS-dependent NETosis, or IgA–IAV ICs for 0.5, 1.5, 3, or 6 h (Fig. 4*A*). A significant increase in NETosis was observed following incubation with IgA–IAV ICs for 3 h, consistent with suicidal NETosis. No further increases were observed after 6 h in either the IgG or IgA IC conditions. Unsurprisingly, PMA—a far more potent stimulant—significantly induced NETosis beginning at 1.5 h after stimulation (Fig. 4*A*). Conversely, to inhibit the production of ROS, a small-molecule inhibitor of the NADPH oxidase (NOX) complex, diphenyleneiodonium chloride (DPI), was preincubated with neutrophils prior to stimulation with IgA–IAV ICs. DPI completely inhibited NETosis induced by IgA ICs (Fig. 4*B*). Together, these observations demonstrate that IgA–virus ICs stimulate suicidal NET release in a NOX-dependent manner.

Virus Particles Are Trapped and Inactivated by NETs. In the context of bacterial infections, NETs exert antimicrobial activity by trapping and killing bacteria with antimicrobial effector proteins associated with NETs. We thus set out to determine whether NETs were similarly capable of trapping and inactivating viruses. Neutrophils were either left unstimulated or were treated with PMA to induce suicidal NETosis (viruses containing ICs were not used to avoid the confounding issue of having viruses present during induction of NETosis). IAV was then incubated in wells of stimulated or unstimulated neutrophils, and unbound virus was washed away. Using immunofluorescence microscopy, we observed that IAV particles become trapped in NETs induced following stimulation with PMA (Fig. 4*C*). Using ImageJ software, we quantified GFP pixel density and normalized this to the number of cells (and/or NETs) per field. Consistent with the stark visual contrast observed in the images, significantly more virus was associated with PMA-stimulated neutrophils that had undergone NETosis than unstimulated neutrophils (Fig. 4*D*).

To test whether IAV was inactivated after being trapped in NETs, we used an mNeon reporter virus (42). IAV-mNeon was incubated with unstimulated neutrophils, PMA-stimulated neutrophils that had undergone NETosis, or PMA-stimulated neutrophils treated with DNase to digest NETs. DNase digestion specifically

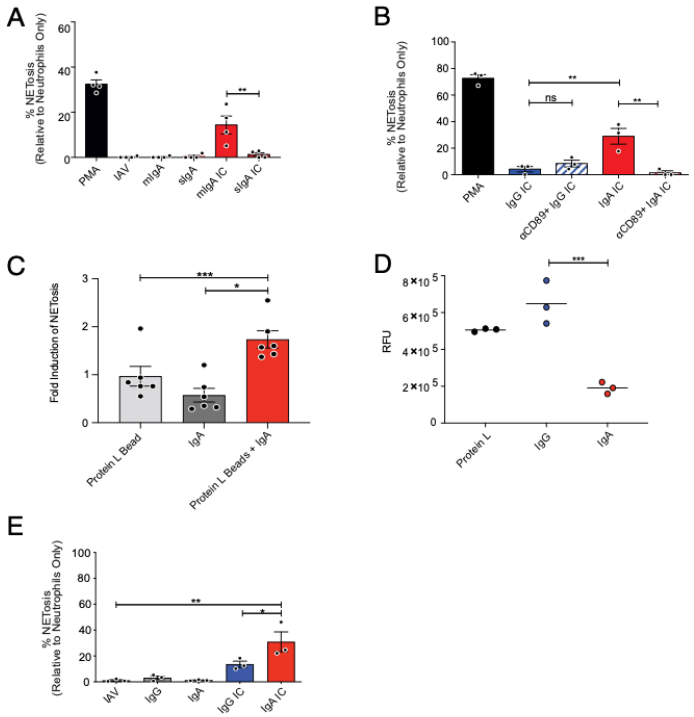

Fig. 3. IgA ICs induce NETosis via FcαRI engagement independently of TLR signaling and phagocytosis. (*A*) Primary human neutrophils were stimulated for 3 h with antibody–IAV ICs generated from matched salivary IgA and serum IgA of healthy IAV-exposed donors ($n = 4$). (*B*) Primary human neutrophils were incubated with an anti-CD89 (FcαRI) antibody prior to stimulation with IgG–IAV or IgA–IAV ICs ($n = 3$) (*C*) Primary human neutrophils were stimulated with polyclonal IgA, polystyrene beads coated with protein L, or polystyrene beads coated with protein L and IgA. For all experiments, NETosis was assessed by immunofluorescence microscopy analysis of cells costained for DNA (DAPI) and neutrophil elastase. NETosis in stimulated conditions was normalized to untreated cells ($n = 6$). (*D*) Fluorescent polystyrene beads were coated with protein L followed by either polyclonal IgG or IgA. Human neutrophils were isolated and incubated with the beads at a 500-beads-per-cell ratio. After washing, phagocytosis of beads was measured using a SpectraMax i3 plate reader (Molecular Devices) ($n = 3$). (*E*) Purified Cal/09 was immobilized on glass coverslips prior to the addition of IgG or IgA. Primary human neutrophils were added to wells for 3 h before being fixed and stained for quantification ($n = 3$). Mean and SEM of independent experiments are shown. Statistical significance was evaluated by one-way ANOVA and Tukey post hoc test. *$P < 0.05$; **$P < 0.01$, *** $P < 0.001$.

allowed us to test whether being trapped in an NET was necessary for inactivation or whether factors released by neutrophils during NETosis were alone sufficient to inactivate IAV (*SI Appendix*, Fig. S2 and Fig. 4*E*). After 3 or 6 h incubation, viral media was collected from all wells incubated on Madin Darby Canine Kidney cells (MDCKs) to quantify the remaining infectious virus. Incubation of virus with PMA-stimulated neutrophils that had undergone NETosis significantly reduced infectivity after 3 and 6 h incubation. Interestingly, digestion of NETs produced by PMA-simulated cells with DNase prior to the addition of the virus had no significant impact on infectivity, suggesting that physical contact with NETs is required for inactivation and that soluble factors released during the process of NETosis alone are not sufficient to mediated inactivation (Fig. 4*E*). Taken together, these data demonstrate that viruses can be trapped and inactivated by NETs.

Discussion

NETosis has been most extensively studied as an antipathogen immune response in the context of bacterial infections (43). However, accumulating evidence suggests that NETs have antiviral activity but can also contribute to the pathogenesis of viral disease in certain circumstances (8–11, 44, 45). While pathogens like viruses and bacteria can trigger NETosis directly as an innate immune mechanism, there is also an important intersection of neutrophils/NETs and the adaptive immune response, since neutrophils express Fc receptors capable of recognizing both soluble ICs and antibody-bound cells. Here, we show that IgA significantly lowers the amount of virus required to trigger NETosis.

Immobilized IgG ICs have been reported to stimulate NETosis via FcγRIIA. Soluble ICs were primarily phagocytosed but could be shifted to stimulate NETosis upon TLR7/8 activation, which resulted in furin-mediated cleavage and shedding of the FcγRIIA N terminus—inhibiting further phagocytosis (32). We observed that IgA ICs did not simulate phagocytosis but rather preferentially induced NETosis even in the absence of TLR activation. While IgG ICs could stimulate NETosis, the induction of NETosis was notably more pronounced upon stimulation of neutrophils with IgA ICs.

In the context of IAV, bnAbs that bind to the conserved HA stalk domain have become a major focus for the development of "universal" influenza virus vaccines and monoclonal antibody prophylactics/therapeutics. Although bnAbs are relatively weak neutralizers of IAV, they confer protection in vivo via potent induction of Fc-dependent effector functions (17–19, 22, 24, 26, 27).

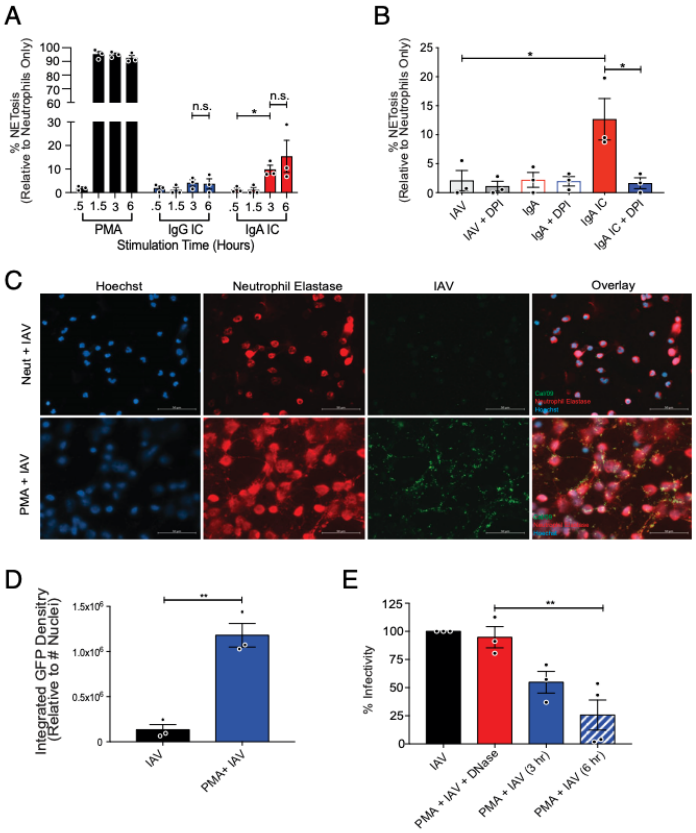

Fig. 4. Influenza virus particles are trapped and inactivated by NETs released via suicidal NETosis. (*A*) Primary human neutrophils were stimulated with PMA, IgG–IAV, or IgA–IAV ICs for 0.5, 1.5, 3, or 6 h prior to fixation and staining for DNA (DAPI) and neutrophil elastase to quantify NETosis. (*B*) Primary human neutrophils were incubated with DPI, a NOX inhibitor, prior to 3 h of simulation with IgA–IAV ICs ($n = 3$). (*C*) Neutrophils were stimulated with PMA for 90 min before the addition 10^5 PFU per well of IAV. The virus was incubated with the NETs for 3 h and then fixed and stained with anti-hemagglutinin antibodies (6F12), DNA (Hoechst), and neutrophil elastase. Immunofluorescence microscopy was used to measure the colocalization of viral particles (green) with NETs composed of DNA (blue) coated with neutrophil elastase (red) ($n = 3$) (*D*) Quantification of the raw integrated GFP density was measured using ImageJ and normalized to the number of cells per field. (*E*) Neutrophils were stimulated with PMA prior to the addition of an IAV expressing an mNeon reporter. The virus was incubated on intact NETs or NETs that had been digested with DNase ($n = 3$). The contents of wells were collected, and MDCK cells were infected for 8 h to measure residual infectivity. Mean and ± SEM are shown. Statistical significance was evaluated using one-way ANOVA with Tukey post hoc test. $*P < 0.05$; $**P < 0.01$.

The ability of bnAbs to elicit potent effector functions (relative to conventional neutralizing antibodies that bind to the HA head domain) relies on a unique reciprocal contact model, whereby Fc receptors of immune-effector cells bind to the Fc domain of bnAbs bound to HA on target cells, while HA expressed on target cells, in turn, binds to sialic acid residues of the effector cell (26, 27). However, almost everything that is known about the function of bnAbs has been studied in the context of IgG. Of the other immunoglobulin isotypes, IgA plays a particularly important role in protection against mucosal viruses. Indeed, local IgA responses correlate with protection offered by live-attenuated influenza virus vaccines (46–48). A recent Phase-I trial of a chimeric HA universal vaccine candidate reported potent induction of IgA bnAbs after vaccination, further highlighting the urgent need to understand how antibodies of this isotype contribute to protection (20, 49). Here, we show that, consistent with prior studies, bnAbs are primarily responsible for the induction of FcαRI-dependent NETosis, likely because these antibodies also promote the reciprocal binding events between IgA–FcαRI and HA–sialic acid (50).

Importantly, the ability of IgA ICs to potentiate NETosis was widespread across several different viruses including IAV, lentiviruses pseudotyped with the SARS-CoV-2 S protein, and HIV. Indeed, this phenomenon could also be recapitulated with IgA-coated beads and extended beyond the context of infectious diseases to ICs composed of IgA from RA patients in complex with citrullinated fibrinogen—a common RA autoantigen. Our findings support previous work from the van Egmond laboratory demonstrating that IgA ICs isolated from synovial fluid of RA patients also induce NETosis (36). Previous work by our group has shown that, upon exposure to IgG–IAV ICs, neutrophils undergo ADCP and potently induce ROS. Inhibition of phagocytosis with cytochalasin D almost completely abolished ROS

induction by IgG–IAV ICs. In contrast, IgA–IAV ICs were able to stimulate ROS even when phagocytosis was inhibited (24). Those observations are in line with the data presented herein showing that IgA–ICs induced neutrophils to undergo ROS-dependent suicidal NETosis in a phagocytosis-independent manner.

In serum, IgA is present at concentration of ~82 to 624 mg/dL, whereas IgG is found at ~694 to 1,803 mg/dL (51). The antibody distribution in tissues has been estimated to be 4 to 16% of the plasma concentration (52). Thus, the concentrations of antibodies that we have used in this study (50 to 100 μg/mL or 5 to 10 mg/dL) were chosen to reflect these physiologically relevant conditions. In serum, IgA1 is the dominant subclass, existing at a ratio of roughly 9:1 when compared to IgA2. We therefore used monoclonal IgA1 antibodies in our studies However, IgA2 antibodies were recently shown to induce more potent inflammatory signaling through FcαRI than IgA1 antibodies, perhaps due to differences in their glycosylation profiles (53). Thus, in contexts wherein IgA2 is enriched, such as in mucosal tissues or in the context of certain autoimmune diseases, the potentiation of NETosis may be even more potent.

In the context of HIV, mixed IgG/IgA–HIV ICs generated using the gp41-specific bnAb 2F5 cooperatively triggered ADCC of HIV-infected cells by monocytes but did not act cooperatively to induce ADCP (33, 54). Likewise, we observed no cooperativity in the induction of NETosis when IgA and IgG were combined at a 1:1 ratio or at physiological ratios. These results suggest that signaling downstream of FcγRs and FcαRI leads to distinct effector outcomes in monocytes and neutrophils.

While ICs composed of serum-derived IgA and monomeric monoclonal IgA could both potentiate NETosis, sIgA purified from saliva could not. This is consistent with prior studies that have demonstrated that the secretory component sterically interferes with binding to FcαRI and suggests that IgA-stimulated NETosis is unlikely to occur in the airways in which sIgA is enriched, but instead would be expected to take place primarily in tissues and vasculature (55).

The data presented here demonstrate that NETs can both trap and inactivate viruses. This suggests that they may have a protective antiviral function. NETs are decorated with antimicrobial proteins including myeloperoxidase, and cationic peptides like α-defensin and cathelicidins that are known to inactivate viral particles (56, 57). We speculate that, in individuals who lack virus-specific IgA, the high concentrations of virus needed to stimulate NETosis might exacerbate inflammation and potentiate disease as has been observed for those with COVID-19 (14–16, 35). However, for individuals with preexisting immunity—such as that conferred by vaccines—low levels of IgA-induced NETosis might help to trap and inactivate viruses early in infection, thereby limiting virus spread and progression to severe disease.

In summary, we report an antiviral effector function mediated by virus–IgA ICs. The mechanism through which virus–IgA ICs stimulate NETosis is distinct from—and considerably more potent than—virus alone. Since mice do not express an FcαR, it will be important to develop alternative models for in vivo studies to determine when IgA–virus IC-mediated NETosis may be protective and when it may exacerbate disease.

Materials and Methods

Human Serum and Blood Samples. Human blood samples used to isolate serum antibodies were obtained with permission from consenting male and female IAV-vaccinated donors, SARS-CoV-2 infected donors, HIV-positive individuals, and RA patients. Human blood for neutrophil isolations was collected with permission from consenting healthy male and female donors. All protocols involving human samples were approved by the Hamilton Integrated Research Ethics Board and the Western Research Ethics Board. Blood was collected into ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Vacutainer).

Neutrophil Isolation. Neutrophils were isolated from the peripheral blood of healthy male and female donors by density gradient centrifugation as described previously (24). Briefly, 3 mL room-temperature (RT) Histopaque 1119 (Sigma-Aldrich) was added to a 15-mL falcon tube followed by gentle addition of 3 mL Histopaque 1077 (Sigma- Aldrich). A total of 6 mL blood was layered on top, and samples were centrifuged at 930 × *g* for 30 min at RT with no deceleration in an Allegra X-12R centrifuge (Beckman Coulter). The neutrophil layer was collected between the Histopaque layers and diluted in 4 °C polymorphonuclear cell (PMN) buffer (0.5% bovine serum albumin [BSA], 0.3 mM EDTA in Hank's balanced salt solution [Sigma-Aldrich]) to a total volume of 50 mL. PMNs were then centrifuged at 450 × *g* for 5 min at RT. The supernatant was discarded, and the cell pellet was resuspended by flicking the tube. To lyse red blood cells, 3 mL ammonium-chloride-potassium lysis buffer (8.3 g/L NH_4Cl, 1 g/L $KHCO_3$, and 0.05 mM EDTA, in sterile distilled H_2O) was added to the PMNs and incubated for 3 min with agitation every 30 s. The PMNs were diluted in 30 mL PMN buffer and centrifuged at 450 × *g* for 5 min at RT followed by one additional wash.

Antibody Purification. Heat-inactivated human serum was diluted 1:10 in phosphate-buffered saline (PBS) and applied to a gravity polypropylene flow column (Qiagen) containing 1 mL Protein G-Sepharose resin (Invitrogen) to purify IgG. Flow through sera was then applied to a gravity flow column containing 1 mL Peptide M-Sepharose resin (InvivoGen) to purify IgA. Columns were washed with two column volumes of PBS. IgG and IgA were eluted with 0.1 M glycine-HCl buffer (pH 2.7) into 2 M Tris-HCl neutralizing buffer (pH 10). Antibodies were concentrated and resuspended in PBS using 30 kDa cutoff Macrosep Advanced Centrifugal Devices (Pall Corporation). To purify monoclonal antibodies, clarified cell-culture supernatants were applied directly to Protein G-Sepharose columns prior to washing and elution.

Monoclonal Antibodies. The variable light and heavy chain sequences of KB2 and 29E3 antibodies (30, 58) were cloned into pFuse vectors (pFUSE-CHIg-hG1, pFUSEss-CHIg hA1, and pFUSE2ss-CLIg-hK, Invivogen). KB2 binds to the stalk domain of H1 viruses, while the 29E3 antibody is specific to the head domain of Cal/09. HEK293T cells were cotransfected with pFUSE plasmids according to the manufacturer's recommendations, and antibodies were subsequently purified from supernatants using Protein G-Sepharose columns as described (see *Antibody Purification*).

Cells and Viruses. MDCK cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific). At 100% confluency, MDCK cells were infected for 1 h with Cal/09 H1N1 (kind gift of Peter Palese, Icahn School of Medicine at Mount Sinai, New York, NY) in 1× minimum essential medium (MEM, Sigma-Aldrich) supplemented with 2 mM L-glutamine, 0.24% sodium bicarbonate, 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], MEM amino acids solution (Sigma-Aldrich), MEM vitamins solution (Sigma-Aldrich), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific), and 0.42% BSA (Sigma-Aldrich). Cells were then washed with PBS, and media were replaced. Cells were left for 72 h, and supernatant was collected. A/Puerto Rico/8/1934/H1N1-mNeon (PR8-mNeon), which was a kind gift from the laboratory of Nicholas Heaton (Duke University, Durham, NC) (42), was propagated in 10-d-old embryonated chicken eggs as per standard protocols (59).

Influenza Virus Purification. Clarified supernatants from IAV-infected MDCK cells were layered on top of 8 mL 20% sucrose (Bioshop) in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) inside Ultra-Clear Ultracentrifuge tubes (Beckman Coulter). Samples were spun at 76,650 × *g* for 2 h at 4 °C inside a SW 32i rotor using an Optima L-90K Ultracentrifuge (Beckman Coulter). Purified viruses were quantified using a bicinchoninic acid assay (BCA) Protein Assay Kit (Pierce Biotechnology) according to the manufacturer's instructions and by hemagglutination assay.

Psuedotyped Lentivirus Production. HIV-1 X4 gp120-pseudotyped lentiviruses were prepared described previously (60). Briefly, HEK293T cells were cultured in DMEM supplemented with 10% FBS, L-glutamine, and 100 U/mL penicillin-streptomycin and maintained in 5% CO_2 at 37 °C. Briefly, 5 × 10^5 cells were seeded onto 6-well plates 1 d prior to transfection. Cells were cotransfected on the next day at 70 to 80% confluency with pLenti-CMV-GFP-Puro (1.5 μg) along with $pEnv_{Hx8}$ (0.5 μg) and psPAX2 (1 μg) plasmids. The medium was changed 24 h posttransfection. The supernatant was then harvested, filtered with 0.22-μm filters (Millipore), and titered as described previously (60). The virus was stored at −80 °C until use.

SARS-CoV-2 S protein–pseudotyped lentiviruses were produced as described by Crawford et al. (61), and the following reagents were obtained through the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), National Institute of Allergy and Infectious Diseases (NIAID), NIH: SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike-Pseduotyped Lentiviral Kit, NR-52948. In brief, HEK293T cells seeded in 15-cm dishes at 1.1×10^7 cells/mL in 15 mL standard DMEM. A total of 16 to 24 h postseeding, cells were cotransfected with HDM-nCoV-Spike-IDTopt-ALAYT, pHAGE-CMV-Luc2-IRES-ZsGreen-W (BEI catalog number NR-52516), HDM-Hgpm2 (BEI catalog number NR-52517) HDM-tat1b (BEI catalog number NR-52518), and pRC-CMV-Rev1b (BEI catalog NR-52519). A total of 18 to 24 h posttransfection, the media were replaced with full DMEM. A total of 60 h posttransfection, the supernatant was collected and filtered with a 0.45-μm filter and stored at −80° C. For purification, 40 mL supernatant was concentrated by spinning at 19,400 rpm for 2 h in an SW 32 Ti rotor using a Beckman Coulter Optima L-90K ultracentrifuge. The resulting pellet was resuspended in 400 μl Hank's Balanced Salt Solution (HBSS) followed by 15 min of continuous vortex at RT. The protein concentration was confirmed by BCA.

Coating Polystyrene Microspheres with Protein L and a Polyclonal Antibody. Fluorescent carboxylate microspheres of 0.5 μm (Polysciences) were coated with Protein L (Thermo Fisher Scientific) followed by polyclonal IgA or IgG. The polystyrene microspheres were first washed with 1× PBS and centrifuged at 13,523 × *g*. The PBS wash was repeated, and then the microspheres were incubated at RT with 750 μg Protein L for 4 h with gentle mixing. Following another PBS wash, 300 μg polyclonal IgA was added to the microspheres and left to incubate at RT with gentle mixing overnight. Following this incubation, the microspheres were centrifuged at 13,523 × *g* for 10 min, and the resulting pellet was resuspended in 1 mL PBS for 30 min with gentle mixing at RT. Following the final incubation, the microspheres were centrifuged at 13,523 × *g* for 5 min and resuspended in 500 μL PBS.

Neutrophil Stimulation with Soluble ICs. Glass coverslips of 15 mm were placed inside wells of a sterile 24-well plate, and 4.0×10^5 PMNs were added to each well and allowed to settle for 1 h. For IAV–polyclonal Ab stimulations, mixtures of 25 μg Cal/09 (2^{10} hemaglutinin units (HAU)) and 50 μg/mL polyclonal IgG or IgA antibodies were incubated for 30 min at RT before addition to PMNs. ICs containing monoclonal HA stalk (KB2) or head-binding (29E3) antibodies were generated at a 2:1 ratio of antibody to virus (100 μg/mL and 50 μg/mL, respectively) and allowed to incubate for 30 min at RT prior to stimulation of PMNs. To test the ability of ICs generated with sIgA to stimulate NETosis, matched sIgA and serum IgA were purified from the saliva of four healthy donors using peptide M columns. ICs containing 100 μg per well of serum-derived monomeric IgA or sIgA and 50 μg per well of Cal/09 were allowed to form by incubation at RT 30 min. HIV-specific ICs were generated by purifying IgA from the serum of three HIV-1–positive donors. ICs were formed by incubating 100 μg/mL polyclonal IgA and 50 μg/mL HIV-1 gp120-pseudotyped lentiviruses for 30 min at RT. SARS-CoV-2 ICs were generated using antibodies purified from the convalescent sera of an individual who had been infected with SARS-CoV-2. ICs were formed by incubating 100 μg/mL polyclonal IgA and 12.5 μg/mL pseudotyped spike lentiviruses for 30 min at RT. For RA samples, ICs were formed by incubating 50 μg per well of citrullinated human fibrinogen (Cayman Chemicals) with 100 μg per well of polyclonal IgA or IgG for 30 min at RT. Stimulation of IgA-coated beads was performed by incubating neutrophils with 5.0×10^8 beads. Antibodies/viruses/beads/ICs were then incubated with PMNs for 3 h at 37 °C before being fixed with 3.7% paraformaldehyde (PFA) (Pierce Protein Biology) prior to staining and imaging.

Immobilized IC Assay. Purified viruses were plated on 15-mm sterile coverslips in a 24-well plate at 2 μg/mL and incubated at 37 °C for 18 h. Wells were washed twice with PBS, and 250 μg of either polyclonal IgA or IgG was added for 30 min at 37 °C. Wells were washed twice with PBS prior to the addition of 4.0×10^5 PMNs per well. PMNs were incubated for 3 h at 37 °C, fixed, and stained (see *Fluorescence Microscopy and Quantification of NETosis*).

FcαRI Blockade. Glass coverslips of 15 mm were placed inside wells of a sterile 12-well plate, and 4.0×10^5 PMNs were added to each well in a total volume of 500 μL and allowed to settle for 1 h. To block Fc-αRI, 20 μg/mL mouse anti-human CD89 antibody (AbD Serotec) was added to neutrophils for 20 min at 4 °C. PMNs were then stimulated with various conditions for 3 h at 37 °C before being fixed with 3.7% PFA and stored at 4 °C until staining.

Fluorescence Microscopy and Quantification of NETosis. Cells were fixed with 3.7% PFA (Pierce Protein Biology) at 4 °C, washed in PBS three times, and then permeabilized using 0.5% Triton X-100 (Thermo Fisher Scientific) in phosphate-buffered saline, 0.1% Tween (PBS-T). Fixed and permeabilized cells were then blocked for 30 min at RT in blocking buffer (10% FBS in PBS-T). Cells were incubated with primary rabbit anti-neutrophil elastase antibody (Abcam) at a 1:100 dilution for 1 h at RT. Coverslips were washed with PBS three times and then incubated with Alexa Fluor 488-conjugated donkey anti-rabbit antibody (Molecular Probes) diluted as per the manufacturer's recommendation (two drops per milliliter) for 1 h at RT, protected from light. Coverslips were then washed with PBS three times. A total of 1 μg/mL Hoechst 33342, trihydrochloride, trihydrate (Life Technologies) was incubated for 5 min at RT, protected from light. Cells were washed with PBS three times, and coverslips were mounted onto glass slides in EverBrite Mounting Medium (Biotium). Cells were imaged using an EVOS FL microscope (Life Technologies). Five random fields per condition were captured at 20× magnification. NETosis was quantified by counting cells that had decondensed chromatin colocalized with neutrophil elastase. Percent NETosis was expressed as the number of cells that had undergone NETosis/the number of total cells.

Influenza Viral-Particle Trapping and Inactivation in NETs. Sterilized glass coverslips were placed in a 24 well plate, and neutrophils at 4.0×10^5 cells per well were allowed to settle for 1 h prior to stimulation. Neutrophils were stimulated with PMA for 3 h at 37 °C, 5% CO_2. Cal/09 at 10^5 PFU/mL was then allowed to settle on the preformed NETs for 3 h at 37 °C; following this, incubation cells were fixed with 3.7% PFA. Staining was performed as previously described (see *Fluorescence Microscopy and Quantification of NETosis*). Primary antibodies used included the following: primary rabbit anti-neutrophil elastase antibodies (Abcam, 1:100 dilution) and 6F12 generated from in-house hybridomas at 1 μg/mL. Secondary antibodies included the following: Alexa Fluor 488 conjugated donkey anti-mouse antibodies (Molecular Probes, 1:4,000) and Alexa Flour 594 donkey anti-rabbit (Molecular Probes, 1:4,000). Coverslips were incubated with 1 μg/mL Hoechst 33342, trihydrochloride, trihydrate (Life Technologies) to probe for DNA. Cells were visualized and imaged using GFP (Ex 470 nm/Em 525 nm) and DAPI (Ex 360 nm/Em 447 nm), with Texas Red (Ex 585/Em 624) color cubes in the EVOS FL microscope (Life Technologies). To evaluate inactivation, 10^5 PFU/mL of PR8-mNeon was incubated with PMA-stimulated neutrophils for 3 to 6 h. A total of 25 units per milliliter of DNaseI (Thermo Fisher Scientific) were added to PMA-stimulated neutrophils and were allowed to incubate for 90 min to digest NETs. Samples were collected and stored at −80° C until further use. Prior to virus quantification, MDCK cells were seeded in 24-well plates and used when 90% confluent. The sample was diluted 1:10 in 1× MEM (Sigma-Aldrich), supplemented with 2 mM L-glutamine, 0.24% sodium bicarbonate, 20 mM Hepes, MEM amino acids solution (Sigma-Aldrich), MEM vitamins solution (Sigma-Aldrich), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific), and 0.42% BSA (Sigma-Aldrich), before being added to cells. After 1 h, this was replaced with DMEM containing 10% FBS (Gibco), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. The number of fluorescent cells was assessed 12 h postinfection. Cells were fixed with PFA and incubated with 1 μg/mL Hoechst 33342, trihydrochloride, trihydrate (Life Technologies). Five fields per condition were taken on the EVOS FL microscope, and percent infectivity was determined as the number of infected cells/the total number of cells.

Phagocytosis Assay of Polyclonal Antibody–Coated Microspheres. This protocol was performed as previously described (24). Briefly, fluorescent carboxylate microspheres of 0.5 μm (Polysciences) were coated with protein L and polyclonal IgA or IgG and were incubated with neutrophils at a 500:1 ratio at 37 °C for 15 min with gentle mixing. This was followed by centrifugation at 930 × *g* for 10 min. Cells were washed twice with PBS before being plated in a 96-well plate. Fluorescence was measured with the SpectraMax i3 plate reader at 526 nm (Molecular Devices).

NOX Assay. Neutrophils were purified as described above (see *Neutrophil Isolation)* and allowed to settle on glass coverslips for 1 h at 37 °C. While settling, neutrophils were incubated with 20 μm DPI (Sigma-Aldrich), a neutrophil NADPH oxidase inhibitor. Neutrophils were then stimulated with IgA–IAV ICs or PMA (0.1 mg/mL, Sigma-Aldrich) as a positive control, and DPI was maintained in the media. Cells were then fixed with 3.7% PFA and stored at 4 °C until staining and imaging.

Statistics. Graphs and statistical analyses were generated using Graphpad Prism version 9 (GraphPad Software). $P < 0.05$ was considered to be significant across all experiments.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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1. J. E. Bakema, M. van Egmond, The human immunoglobulin A Fc receptor FcαRI: A multifaceted regulator of mucosal immunity. *Mucosal Immunol.* **4**, 612–624 (2011).
2. E. Kolaczkowska, P. Kubes, Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).
3. R. C. Monteiro, J. G. J. Van De Winkel, IgA Fc receptors. *Annu. Rev. Immunol.* **21**, 177–204 (2003).
4. V. Papayannopoulos, Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* **18**, 134–147 (2018).
5. J. V. Camp, C. B. Jonsson, A role for neutrophils in viral respiratory disease. *Front. Immunol.* **8**, 550 (2017).
6. V. Brinkmann *et al.*, Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535 (2004).
7. C. H. Hiroki *et al.*, Neutrophil extracellular traps effectively control acute Chikungunya virus infection. *Front. Immunol.* **10**, 3108 (2020).
8. C. N. Jenne *et al.*, Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. *Cell Host Microbe* **13**, 169–180 (2013).
9. T. Saitoh *et al.*, Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* **12**, 109–116 (2012).
10. P. S. Sung, T. F. Huang, S. L. Hsieh, Extracellular vesicles from CLEC2-activated platelets enhance dengue virus-induced lethality via CLEC5A/TLR2. *Nat. Commun.* **10**, 2402 (2019).
11. B. Cortjens *et al.*, Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. *J. Pathol.* **238**, 401–411 (2016).
12. M. Toussaint *et al.*, Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nat. Med.* **23**, 681–691 (2017).
13. T. Narasaraju *et al.*, Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am. J. Pathol.* **179**, 199–210 (2011).
14. C. Radermecker *et al.*, Neutrophil extracellular traps infiltrate the lung airway, interstitial, and vascular compartments in severe COVID-19. *J. Exp. Med.* **217**, e20201012 (2020).
15. E. A. Middleton *et al.*, Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. *Blood* **136**, 1169–1179 (2020).
16. B. J. Barnes *et al.*, Targeting potential drivers of COVID-19: Neutrophil extracellular traps. *J. Exp. Med.* **217**, e20200652 (2020).
17. D. J. DiLillo, P. Palese, P. C. Wilson, J. V. Ravetch, Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *J. Clin. Invest.* **126**, 605–610 (2016).
18. D. J. DiLillo, G. S. Tan, P. Palese, J. V. Ravetch, Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat. Med.* **20**, 143–151 (2014).
19. W. He *et al.*, Alveolar macrophages are critical for broadly-reactive antibody-mediated protection against influenza A virus in mice. *Nat. Commun.* **8**, 846 (2017).
20. D. I. Bernstein *et al.*, Immunogenicity of chimeric haemagglutinin-based, universal influenza virus vaccine candidates: Interim results of a randomised, placebo-controlled, phase 1 clinical trial. *Lancet Infect. Dis.* **20**, 80–91 (2019).
21. D. N. Forthal, A. Finzi, Antibody-dependent cellular cytotoxicity in HIV infection. *AIDS* **32**, 2439–2451 (2018).
22. W. He *et al.*, Broadly-neutralizing anti-influenza virus antibodies: Enhancement of neutralizing potency in polyclonal mixtures and IgA backbones. *J. Virol.* **89**, 3610–3618 (2015).
23. M. D. Tate *et al.*, Neutrophils ameliorate lung injury and the development of severe disease during influenza infection. *J. Immunol.* **183**, 7441–7450 (2009).
24. C. E. Mullarkey *et al.*, Broadly neutralizing hemagglutinin stalk-specific antibodies induce potent phagocytosis of immune complexes by neutrophils in an Fc-dependent manner. *mBio* **7**, e01624-16 (2016).
25. T. A. Fuchs *et al.*, Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **176**, 231–241 (2007).
26. W. He *et al.*, Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 11931–11936 (2016).
27. P. E. Leon *et al.*, Optimal activation of Fc-mediated effector functions by influenza virus hemagglutinin antibodies requires two points of contact. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E5944–E5951 (2016).
28. G. S. Tan *et al.*, A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. *J. Virol.* **86**, 6179–6188 (2012).
29. G. S. Tan *et al.*, Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. *J. Virol.* **88**, 13580–13592 (2014).
30. N. S. Heaton *et al.*, In vivo bioluminescent imaging of influenza a virus infection and characterization of novel cross-protective monoclonal antibodies. *J. Virol.* **87**, 8272–8281 (2013).
31. R. Hai *et al.*, Influenza viruses expressing chimeric hemagglutinins: Globular head and stalk domains derived from different subtypes. *J. Virol.* **86**, 5774–5781 (2012).
32. C. Lood, S. Arve, J. Ledbetter, K. B. Elkon, TLR7/8 activation in neutrophils impairs immune complex phagocytosis through shedding of FcgRIIA. *J. Exp. Med.* **214**, 2103–2119 (2017).
33. M. Duchemin, D. Tudor, A. Cottignies-Calamarte, M. Bomsel, Antibody-dependent cellular phagocytosis of HIV-1-Infected cells is efficiently triggered by IgA targeting HIV-1 envelope subunit gp41. *Front. Immunol.* **11**, 1141 (2020).
34. F. P. Veras *et al.*, SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J. Exp. Med.* **217**, e20201129 (2020).
35. Y. Zuo *et al.*, Neutrophil extracellular traps in COVID-19. *JCI Insight* **5**, e138999 (2020).
36. E. Aleyd, M. Al, C. W. Tuk, C. J. van der Laken, M. van Egmond, IgA complexes in plasma and synovial fluid of patients with rheumatoid arthritis induce neutrophil extracellular traps via FcαRI. *J. Immunol.* **197**, 4552–4559 (2016).
37. H. L. Wright, R. J. Moots, S. W. Edwards, The multifactorial role of neutrophils in rheumatoid arthritis. *Nat. Rev. Rheumatol.* **10**, 593–601 (2014).
38. J. A. Hill, J. Al-Bishri, D. D. Gladman, E. Cairns, D. A. Bell, Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *J. Rheumatol.* **33**, 2115–2119 (2006).
39. A. B. Herr, E. R. Ballister, P. J. Bjorkman, Insights into IgA-mediated immune responses from the crystal structures of human FcalphaRI and its complex with IgA1-Fc. *Nature* **423**, 614–620 (2003).
40. F. Heil *et al.*, Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**, 1526–1529 (2004).
41. B. G. Yipp, P. Kubes, NETosis: How vital is it? *Blood* **122**, 2784–2794 (2013).
42. A. T. Harding, B. E. Heaton, R. E. Dumm, N. S. Heaton, Rationally designed influenza virus vaccines that are antigenically stable during growth in eggs. *mBio* **8**, e00669-17 (2017).
43. V. Papayannopoulos, A. Zychlinsky, NETs: A new strategy for using old weapons. *Trends Immunol.* **30**, 513–521 (2009).
44. L. Zhu *et al.*, High level of neutrophil extracellular traps correlates with poor prognosis of severe influenza A infection. *J. Infect. Dis.* **217**, 428–437 (2018).
45. C. N. Jenne, P. Kubes, Virus-induced NETs–critical component of host defense or pathogenic mediator? *PLoS Pathog.* **11**, e1004546 (2015).
46. C. S. Ambrose, X. Wu, T. Jones, R. M. Mallory, The role of nasal IgA in children vaccinated with live attenuated influenza vaccine. *Vaccine* **30**, 6794–6801 (2012).
47. D. F. Hoft *et al.*, Comparisons of the humoral and cellular immune responses induced by live attenuated influenza vaccine and inactivated influenza vaccine in adults. *Clin. Vaccine Immunol.* **24**, e00414-16 (2017).
48. J. C. Ang *et al.*, Comparative immunogenicity of the 2014-2015 Northern hemisphere trivalent IIV and LAIV against influenza A viruses in children. *Vaccines (Basel)* **7**, 87 (2019).
49. R. Nachbagauer *et al.*, A chimeric hemagglutinin-based universal influenza virus vaccine approach induces broad and long-lasting immunity in a randomized, placebo-controlled phase I trial. *Nat. Med.* **27**, 106–114 (2020).
50. A. W. Freyn *et al.*, Influenza hemagglutinin-specific IgA Fc-effector functionality is restricted to stalk epitopes. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2018102118 (2021).
51. A. Gonzalez-Quintela *et al.*, Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin. Exp. Immunol.* **151**, 42–50 (2008).
52. D. K. Shah, A. M. Betts, Antibody biodistribution coefficients: Inferring tissue concentrations of monoclonal antibodies based on the plasma concentrations in several preclinical species and human. *mAbs* **5**, 297–305 (2013).
53. U. Steffen *et al.*, IgA subclasses have different effector functions associated with distinct glycosylation profiles. *Nat. Commun.* **11**, 120 (2020).
54. M. Duchemin, M. Khamassi, L. Xu, D. Tudor, M. Bomsel, IgA targeting human immunodeficiency virus-1 envelope gp41 triggers antibody-dependent cellular cytotoxicity cross-clade and cooperates with gp41-specific IgG to increase cell lysis. *Front. Immunol.* **9**, 244 (2018).
55. E. Aleyd, M. H. Heineke, M. van Egmond, The era of the immunoglobulin A Fc receptor FcαRI; its function and potential as target in disease. *Immunol. Rev.* **268**, 123–138 (2015).
56. G. Schönrich, M. J. Raftery, Neutrophil extracellular traps go viral. *Front. Immunol.* **7**, 366 (2016).
57. E. Gwyer Findlay, S. M. Currie, D. J. Davidson, Cationic host defence peptides: Potential as antiviral therapeutics. *BioDrugs* **27**, 479–493 (2013).
58. B. Manicassamy *et al.*, Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines. *PLoS Pathog.* **6**, e1000745 (2010).
59. WHO, Manual for the laboratory diagnosis and virological surveillance of influenza. (2011). https://apps.who.int/iris/handle/10665/44518. Accessed 4 March 2015.
60. M. A. Zahoor, S. Philip, H. Zhi, C.-Z. Giam, NF-κB inhibition facilitates the establishment of cell lines that chronically produce human T-lymphotropic virus type 1 viral particles. *J. Virol.* **88**, 3496–3504 (2014).
61. K. H. D. Crawford *et al.*, Protocol and reagents for pseudotyping lentiviral particles with SARS-CoV-2 spike protein for neutralization assays. *Viruses* **12**, 13–15 (2020).

4.0- Chapter 4: Exploring the effects of seasonal influenza vaccine formulation on imprinting and functional antibody responses in a pediatric cohort

Manuscript title: The nature of early childhood imprinting to influenza virus shapes subsequent antibody responses

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The nature of early childhood imprinting to influenza virus shapes subsequent antibody responses

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INTRODUCTION

Children represent a high-risk population for hospitalization and severe outcomes following influenza virus infection. Globally, ~ 90 million cases of influenza occur annually in children under the age of 5[1]. Early childhood exposure to influenza virus has been demonstrated to indelibly influence subsequent responses, with antibody titers remaining highest against virus strains children are infected with earliest in life[2–7] Originally termed, 'Original Antigenic Sin' (OAS), this hierarchy is maintained following repeated exposure to antigenically related strains[2]. OAS-like responses can be deleterious when antibodies against conserved non-neutralizing epitopes are boosted in favor of de novo antibodies against novel immunizing/circulating strains. However, back-boosting of antibodies against conserved epitopes is a natural feature of immune memory and has been demonstrated to be a key component of secondary immune responses to influenza virus[8]. The immunological 'imprint' left by early-life exposure has since been demonstrated to provide homo- and hetero-subtypic protection against later life infections[9].

Vaccination currently represents the most effective means of providing protection against influenza virus. With widespread rollout of influenza vaccination campaigns, many children now experience their first exposure to influenza in the context of vaccination. The impacts of vaccination versus infection on immunological priming/imprinting are not well understood. Further, multiple seasonal vaccine formulations are approved for use in almost all populations. While imperfect, evidence from murine models suggests that priming in the context of vaccination results in imprinting, albeit to a lesser degree than infection[10,11]. The specific vaccine formulation administered as the priming exposure also had significant impact on the degree of imprinting that was imparted.

In Canada and the US, all children >6 months of age are recommended to receive seasonal influenza vaccination. Children >6 months are eligible to receive trivalent inactivated vaccines (TIV) and quadrivalent inactivated vaccines (QIV), which are delivered intramuscularly. Intranasally delivered live-attenuated influenza vaccines (LAIV) are also approved for use in individuals between the ages of 2-49, although these tend to be used most frequently in children, since effectiveness declines with age and increased exposure to influenza virus[12–14]. LAIV is composed of a cold-adapted virus capable of replicating in the upper respiratory tract to more closely mimic natural infection[12] Special formulations, such as those containing adjuvants (ex. MF59 or AS03) are commonly used for at risk populations such as the elderly to promote dose sparing and overcome immunosenescence[15]. An adjuvanted trivalent influenza vaccines (A-TIV) which include MF59 (Fluad Pediatric) has also been approved for use in children[16].While many vaccine formulations have demonstrated efficacy in children, they vary in the specific qualities of the immune responses that they elicit. Furthermore, little is known about how vaccine formulation influences immunes responses following repeated vaccination.

Current seasonal influenza vaccines do not provide protection from pandemic strains of influenza virus and require annual reformulation to remain effective against seasonal strains. These limitations of seasonal vaccines have catalyzed major global efforts to develop 'universal' influenza vaccine (UIV) strategies, several of which aim to elicit antibodies against the conserved HA stalk domain[17]. Many immunogens capable of inducing broad protection have been investigated. However, almost all studies of broadly-neutralizing antibodies (bnAbs) in humans have been conducted in adult cohorts. There is very limited data concerning the induction of bnAbs

in young children, and whether vaccine platforms differentially impact the elicitation of these antibodies[18].

Understanding how early-life imprinting shapes immune histories to influenza viruses is imperative to both designing effective 'universal' influenza vaccines and improving seasonal vaccines for high-risk populations. Therefore, we examined how early life exposure to various seasonal influenza vaccine formulations shaped subsequent responses using a subset of samples from two randomized controlled trials (RCTs) to assess relative efficacy of TIV versus LAIV and ATIV versus QIV[19,20]. We demonstrated that TIV induced stronger anamnestic/recall antibody responses in children than LAIV. In addition, we showed that priming with ATIV avoided preferential responses against the HA head domain of previously-encountered strains and elicited strong and durable HA stalk-binding antibody responses. These data shed important new light on the impact of vaccine formulation in imprinting of children, and the differential ability of these vaccines to generate antibodies to the variable head domain or highly-conserved stalk domain of HA.

RESULTS

Study Participants

Serum samples were analyzed from two previously-described cRCT cohorts[19,20]. The first cohort consisted of matched pre and post vaccination serum from 74 cRCT participants (median age 4.0, Table 1), who had received either a trivalent inactivated influenza vaccine (TIV, n=35) or a live-attenuated influenza vaccine (LAIV, n=39) in each of the three consecutive study years[19].The second cohort consisted of matched pre- and post-vaccination serum from 45 participants (median

age 3.0, Table 1), who received either an adjuvanted trivalent inactivated vaccine (ATIV, n=18) or a quadrivalent inactivated vaccine (QIV, n=27). For both trials, participants received the same vaccine formulation across all three years of the study.

Effects of TIV or LAIV on HAI recall responses

To determine the impact of vaccine formulation on establishment of antibody hierarchies, HAIs were performed against the H1 and H3 vaccine antigens (Table 2) using the pre- and post-vaccination serum from children sequentially vaccinated from 2012-2014. Across the three seasons of the first cRCT, the WHO H1N1 vaccine strain, A/California/07/2009(H1N1)-like virus (Cal/09), remained consistent (Figure 2A). Conventionally, an HAI titer of 1:40, or a four-fold increase from baseline, has been used to determine seroconversion, though, significantly higher titers are likely required for protection in children[21]. Both TIV and LAIV induced low absolute Cal/09 HAI titers in children (Figure 2B). No significant increases in post vaccination HAI titers were observed and few participants met the criteria for seroconversion (4-fold increase from baseline, HAI $\geq$40); TIV Y1: (6/35, 2/35), Y2: (3/35, 6/35), Y3: (0/35, 1/35), LAIV Y1: (1/39, 1/39), Y2 (1/39, 2/39), Y3 (3/39, 5/39). The lack of seroconversion suggests poor Cal/09 immunogenicity in this largely naïve cohort (only 1/35 TIV participants had detectable Cal/09 HAI titers at baseline and 0/39 in the LAIV group). There were no significant differences in vaccine responses to the Cal/09 H1N1 component of the vaccine following successive vaccination as measured by HAI (Figure 2C).

HAIs were also performed against the recommended H3N2 vaccine strains, which changed in the final year of the trial. In contrast to H1 responses, TIV induced significant increases in Vic/11 HAI

titers post vaccination in all three years of the trial (Figure 2D). While significant post-vaccination increases in absolute Vic/11 HAI titer were not observed for LAIV recipients, 87.17% (34/39) of participants had HAI ≥40 in year 1 of the study. Similarly, in year 2, 82% (32/39) participants were observed to have post vaccination Vic/11 HAI titers ≥40. The strongest Vic/11 HAI responses were observed in the first year of the study (Figure 2E).

In the 2014-2015 season (trial year 3), the H3 strain was changed to A/Texas/50/2012(H3N2) (Tx/12). To compare the magnitude of the antibody response to the previous H3N2 vaccine component (Vic/11) to the new TX/12 strain , HAIs were performed against both viruses using serum collected in year 3 (Figure 2F). When absolute HAI titers against Tx/12 were assessed, a significant increase was observed following LAIV vaccination (GMT, 7.20 vs 8.03, p=0.0484), but not after TIV (GMT, 8.53 vs. 8.20). Interestingly, despite the vaccine no longer containing Vic/11 in year 3, a significant increase in absolute Vic/11 HAI antibodies was observed following vaccination with TIV containing Tx/12 as the H3 antigen (GMT, 224 vs 374, p=0.001), but not after LAIV vaccination. When comparing the magnitude of Vic/11 and Tx/12 HAI antibody induction, there was a significant elevation in the Log2 fold change of Vic/11 responses in TIV vaccinees compared to Tx/12 (0.742 versus -0.057, p=0.0009). The magnitude of the response against the antecedent H3, Vic/11, was also greater in TIV participants compared to LAIV (0.742 versus 0.184, p=0.032) (Figure 2G). Thus, TIV promoted back-boosting of antibodies to shared epitopes between the related HA proteins to a greater extent than LAIV.

To quantify whether generation of de novo antibody production to more recent strains was impaired, we generated an ‘OAS’ index which compares the fold change in titer against the

previous and the current vaccine strain. If the fold-change was observed to be greater against the previous strain (OAS ratio >1), responses were largely anamnestic. If the ratio was =1, the response was balanced, and if the ratio was <1 (the magnitude of increase was greater against more contemporary strains) a *de novo* response was favored. Following the H3 antigen change, more TIV recipients were observed to have anamnestic responses, 21/35 (60.0%), than their LAIV counterparts 10/38 (26.32%) (Figure 2H). Conversely, a greater proportion of LAIV vaccinees generated *de novo* responses relative to TIV ((12/38 (31.58%) vs 4/35 (11.43%)) (Figure 2H) . Despite maintaining a high degree of sequence similarity, three mutations in key antigenic sites exist between Vic/11 and Tx/12 (Figure 2I). These results suggest that LAIV is better able to stimulate *de novo* responses to novel antigenic epitopes in young children.

Effects of TIV or LAIV on HA stalk antibody ADCC responses

To assess the effect of TIV and LAIV on induction of functional bnAb that bind to the HA stalk domain, antibody-dependent cellular cytotoxicity (ADCC) assays were performed on cells infected with either a cH5/1 (Group 1 HA stalk) or cH4/3 (Group 2 HA stalk) virus (Figure 3A). These viruses express chimeric HA molecules with head domains against which children are naïve, but with H1 or H3-dervied stalk domains. The magnitude of the ADCC response against the group 1 HA stalk domain (cH5/1) was greatest for both vaccines in year 1 of the trial (Figure 3B). For LAIV recipients the fold change in group 1 ADCC inducing antibodies was significantly greater in the first year of the trial relative to the third (Log2 Ratio 0.77 versus -0.28, $p < 0.0001$) (Figure 3B). No significant differences were observed between TIV and LAIV in year 1 for group 1 or group 2 antibodies.

Our group has previously shown that Group 1 HA stalk antibodies increase in response to sequential seasonal vaccination[18]. Consistent with these findings, mean pre-vaccination ADCC antibody titers increased with time for both vaccine groups. However, the magnitude of induction decreased with each successive vaccination. Significant post-vaccination increases in ADCC-inducing antibodies were observed following vaccination with TIV or LAIV in year 1 (GMT, TIV;2.80 versus 4.31, p=0.001 and LAIV;1.44 versus 2.46, p=0.001) and year 2 of the study (GMT, TIV 3.24 versus 4.07, p=0.022 and LAIV 1.67 versus 2.08, p=0.0147) (Figure 3C, 3D). Neither formulation induced significant group 1 ADCC responses in the third year of study (Figure 3E). These results suggest that in the context of vaccination, early exposures result in the largest induction of group 1 stalk antibodies. In the cRCT from which these samples were acquirecd, the group 1 vaccine antigen (Cal/09) remained the same across study seasons. Thus, this finding is consistent with previous literature demonstrating that repeated exposure to the same antigen focuses antibody responses on HA head epitopes[22–24].

We also evaluated group 2 stalk responses following administration of LAIV and TIV. As for group 1 HA stalk antibody responses, LAIV and TIV recipients generated the strongest ADCC antibody responses following the first year of vaccination (Figure 3F). These findings suggest that despite the immunodominance of the HA head domain, children with relatively naïve immune histories can generate relatively strong bnAb responses following seasonal vaccination. This induction is then dampened following repeated vaccination to antigenically similar strains as immune responses are redirected against the HA head. Notably, when comparing induction of ADCC mediated by group 2 stalk antibodies, a significant increase in GMT post vaccination was only observed following LAIV in the first year of the study (GMT 3.17 versus 7.40, P<0.0001)

(Figure 3G). In contrast, in all three years of the study vaccination with TIV induced significant elevation in GMTs of group 2 ADCC antibodies (Figure 3G, 3H, 3I). This suggests that in addition to strong HA head-based imprinting, TIV can also induced strong anamnestic responses against conserved epitopes in the HA stalk.

Effects of ATIV or QIV on HAI responses

We next used pre- and post-vaccination serum collected from sequentially vaccinated children in the 2016-2019 influenza seasons with either QIV or ATIV. During this trial the H1 vaccine antigen changed from Cal/09 to Mich/15 in the 2017-2018 season and the H3 strain change from HK/14 to Sing/16 in 2018/2019 (Figure 4A). No striking differences in GMT HAI titers against Cal/09 were observed when comparing QIV and ATIV and overall responses were modest (Figure 4B, C. Both formulations significantly boosted HAI antibodies directed against Mich/15 when that strain was introduced in year 2 (2017-2018) (Figure 4D-F).

HK/14 was also weakly immunogenic in this population, with no significant increases in HK/14 HAI GMTs observed over successive years (Figure 4G, H). However, significant boosting of HAI titers for the year 3 antigen, Sing/16, were elevated in both QIV and ATIV (QIV; 53.63 to 132.76, p=0.0001, ATIV; 72.93 to 291.75, p=0.0107) (Figure 4I, 4J). When expressed as an OAS ratio, QIV recipients were observed to have a more anamnestic response (4/26, 15.3%) (Figure 4K). The lack of significant back boosting in ATIV recipients suggests that seasonal vaccine formulations with increased antigen availability may be less susceptible to focusing of HA head-based responses.

Effects of ATIV or QIV on HA stalk-antibody ADCC responses

We then went on to investigate the impact of ATIV or QIV on functional bnAb responses using ADCC assays. Both vaccines induced significant responses to the group 1 HA stalk (CH5/1) in all years of the trial (Figure 5A, C, D). In year 1, the magnitude of induction of ADCC-inducing stalk-antibodies was higher in the ATIV group than the QIV group (Figure 5A).However, there were no differences in group 1 ADCC inducing antibodies when comparing ATIV and QIV in subsequent trial years, suggesting that first exposure to adjuvanted vaccine significantly enhances bnAb production.

In the context of group 2 HA ADCC responses (as measured by cH4/3), both QIV and ATIV stimulated significant responses in each year of the study, and no differences in magnitude of response was observed when comparing the two vaccine platforms (Figure 5E-H). These results suggest both vaccine platforms efficiently induce group 2 ADCC stimulating HA stalk antibodies. The GMT post-vaccination titer of group 2 ADCC antibodies increased over time for both cohorts (Figure 5F-H). Taken together, these results show that repeated vaccination with both seasonal vaccine platforms were able to boost bnAb responses despite no major antigenic shifts in vaccine components.

Impact of pre-existing immunity on subsequent HA head and stalk responses

Although the cohort selected for our analyses were study naïve and unlikely to have been previously-vaccinated, several participants had HAI titers $\geq$40 in the first year, suggesting that they had experienced an infection as their priming event. In trial year 1, titers of $\geq$40 were observed against Vic/11 in 29/35 (82.85%) TIV and 13/39 (33.33%) of LAIV recipients. It has been

previously established that high titers of circulating antibodies at the time of vaccination are associated with reduced vaccine responses[25,26]. Additionally, these pre-existing antibodies can mask epitopes on the boosting immunogen and are thought to be a driving mechanism of OAS. In line with these findings, we observed that TIV recipients who had <40 HAI titer against Vic/11 prior to vaccination in the first year of the trial trended towards having stronger HAI antibody responses post-vaccination relative to participants with previous exposure at the start of the study. Similarly, children in the LAIV cohort who had HAI titer <40 in year 1 exhibited a greater increase in HAI antibody induction post vaccination (Log2 Ratio of 3.32 vs. 0.23, $p<0.0001$) (Figure 6A). This effect was lost following successive vaccination (Figure 6B, 6C) and pre-exposure status did not have any effect on the magnitude of the vaccine-induced HAI response against Tx/12 in either vaccine recipient group (Figure 6D). Thus, higher pre-existing antibodies reduced the magnitude of the vaccine response independent of vaccine type. Low numbers of participants were observed to have pre-existing titers in year 1 pre-vaccination samples for the H1 or H3 strains during the second trial. Thus, we were unable to run a similar analysis comparing ATIV vs. QIV.

Interestingly, for both TIV and LAIV, children who were imprinted by vaccination (no pre-existing antibody group) exhibited stronger anamnestic responses when ratios of HAI titers against Vic/11 and Tx/12 were compared (Figure 6E). Vaccine formulation did seem to impact the ability of children to generate ADCC-inducing HA stalk antibodies. In naïve children, LAIV was able to induce strong induction of group 2 HA stalk antibodies following administration of one seasonal vaccine dose (Figure 6G). However, in children who were seropositive for H3 virus, no increase in stalk antibodies was observed (Figure 6F, 6G). In contrast, TIV was able to effectively induce HA stalk antibodies in previously infected individuals (Figure 6F, 6G). It is possible that strong

local immunity at the site of vaccination (respiratory tract) in these children limited vaccine replication, impairing the immune response.

DISCUSSION

While the impact of OAS on IAV immunity is context dependent, a large body of evidence from both experimental and clinical studies demonstrate that the first exposure to influenza ("imprinting") profoundly influences subsequent immune responses[4]. Our understanding of germinal centre (GC) and B cell mechanisms has matured significantly since the concept of OAS was first described. While immunologically naïve children are at heightened risk for severe complications following IAV infection, there are now many approved seasonal vaccine formulations for this vulnerable group. Rational selection of the most appropriate vaccine type with which to "imprint" young children represents a unique opportunity to prime the immune system in a way that optimizes downstream immune responses against influenza virus, potentially for life.

To-date, there has been a paucity of comparative studies examining the impact of priming vaccine platform on subsequent immune responses against influenza virus in children. Here, we performed two sub analyses on antibody responses against the HA head and stalk in a subset of children whose first receipt of influenza vaccination was in the context of two cluster randomized controlled trials. These original trials examined the relative efficacy of LAIV (FluMist) vs. TIV (Vaxigrip) across the 2012-2014 influenza seasons in Canada, and efficacy of ATIV (Fluad Pediatric) vs. QIV (Fluzone) across 2016-2018[19,20]. These trials presented a unique opportunity to interrogate the impact of vaccine type on imprinting, antibody specificity, and establishment of antibody

hierarchies (or "OAS-like responses) against the HA head and stalk domains using a very well-defined clinical cohort. The specific features of these cohorts that made them ideally suited to address these questions were 1) the fact that these pediatric populations were largely unvaccinated prior to initiation of these studies, and 2) the longitudinal nature of these studies spanned strain changes in the vaccines, which provided an opportunity to compare *de novo* vs. anamnestic responses.

For the comparison of TIV and LAIV, the H1 antigen (Cal/09) remained constant throughout the trial and was observed to be weakly immunogenic irrespective of vaccine platform. In contrast, when looking at serological Vic/11 (H3) titers, TIV recipients exhibited a greater induction of HAI antibodies post-vaccination in trial years where Vic/11 was the administered vaccine strain. In 2014, the H3 component of the vaccine was switched to TX/12, which contains 4 amino acid changes, 3 of which fall in key antigenic sites. Of particular interest is the antigenic change in immunodominant site B at S198P[27]. Changes in this region are often associated with significant alteration in immunogenicity. Notably, the 2014-2015 influenza season in Canada and the United States was characterized by exceedingly poor vaccine efficacy (VE) as a result of strain mismatch against circulating strains[28–30]. Interestingly, we found that antibodies against the historical Vic/11 antigen were significantly boosted by vaccination with TX/12 , particularly in TIV recipients.

Elegant work by Skowronski et al. found that repeat vaccination beginning in 2012-2013 leading up to the 2014-2015 season amplified the poor VE against H3N2 [28]. These findings clearly highlight the importance of understanding prior vaccination history on downstream infection outcomes. While our study was not sufficiently powered to assess the differential impacts of

priming with various vaccine formulations on vaccine effectiveness, we did observe greater anamnestic responses in sequentially vaccinated TIV recipients relative to LAIV recipients following antigenic substitution to TX/12 in the 2014-2015 season. In alignment with the antigenic distance hypothesis[31] and the previously discussed observations by Skowronski et al., our results suggest that imprinting with TIV could have been particularly detrimental to vaccine effectiveness in that season. While children vaccinated with LAIV in the 2014-2015 season would still have been subjected to vaccine mismatch, negative interference from prior vaccines may have been minimized.

Inclusion of adjuvants or increased antigen doses in seasonal vaccines have demonstrated enhanced immunogenicity in high-risk populations like the young and elderly[15]. However, whether these specialized vaccines differentially prime the immune response remains unknown. In our study we compared the effects of priming with Fluad Pediatric, a trivalent adjuvanted (MF59) vaccine, and Fluzone, a quadrivalent vaccine. The H1 vaccine strain was changed from Cal/09 to Mich/15 in the 2017-2018 season (study year 2). This change was motivated by an observation by the Hensley laboratory, that imprinted antibody responses against seasonal H1N1 virus in middle aged adults was resulting in diminished antibody responses against the Cal/09 HA[32,33]. Both ATIV and QIV induced significant increases in HAI titers against Mich/15 despite the H1 strain switch, no significant differences in the OAS indices between A-TIV and QIV were observed in years two or three (data not shown), presumably because this strain change did not have a major impact on antigenicity for children. However, anamnestic HAI responses were lower in children who received sequential A-TIV vaccinations, which is consistent with studies in murine models that

have shown that adjuvanted vaccine formulations can OAS responses focused on epitopes in the HA head domain[10].

Despite common misconceptions surrounding the negative impacts of OAS, strong epidemiological evidence suggests that the role of early life imprinting can provide protection from homo- and heterosubtypic infections[9]. Most stalk bnAbs do not exhibit protection through conventional HAI or neutralization mechanisms. Instead, they act at primarily through interactions with FcR on immune cells [34–37]. Thus, prior studies examining only HAI antibodies after sequential exposure ignore the contribution of cross-reactive HA stalk antibodies (which tend not have HAI activity). Therefore, we elected to perform ADCC assays to measure the induction of stalk antibodies in children following vaccination with various seasonal vaccine platforms. Interestingly, the magnitude of ADCC antibody induction varied by vaccine platform. This was particularly evident in the first trial, wherein children who were seronegative at the time of vaccination and received LAIV induced significant ADCC antibodies post-vaccination however, vaccination of children who had pre-existing antibodies did not mount significant ADCC responses to either group 1 or group 2 chimeric viruses in any year of the trial. In contrast, children with no prior immunity did not mount ADCC responses following TIV vaccination. However, in children who had >40 HAI titer in year 1 significant stalk antibodies were induced. This suggests that there may be platform and age specific differences that should be considered when developing a UIV. Perhaps UIV strategies for children will require priming with seasonal vaccines or a two-dose regimen to induce strong stalk specific responses. Furthermore, in older children who are not immunologically naïve, a TIV formulation may be desired.

High pre-existing antibodies have been negatively correlated with effective vaccine responses. In line with these findings, we demonstrate that children with no pre-existing antibodies against the relevant H3 strains at the outset of the TIV vs LAIV trial experienced a more profound boost in HAI titers following vaccination. This is in comparison to children who had titers >40 in year one. Surprisingly, the children who had no prior exposure to H3, irrespective of vaccine platform, displayed higher anamnestic responses. This result is in contrast with murine data suggesting that infection imparted stronger OAS-like responses[11]. Clearing of vaccine antigen by circulating antibody and memory cells are thought to create competition for T cell help, skewing the response towards historical antigen.

These findings are likely to have implications for other antigenically diverse pathogens, such as SARS-CoV-2, for which there are multiple vaccine platforms, and a need to update vaccine antigens on a regular basis. The evolutionary pressure exerted on IAV and SARS-CoV-2 by widespread population level immunity will continue to drive selection of immune evasive strains/variants and re-infection in previously exposed individuals. In the context of SARS2, the impacts of priming with the various vaccine platforms (ex. mRNA, viral vectored vaccines, etc) or infection with variants on immunogenicity and induction of cross-reactive immunity are only beginning to be understood. Understanding how immune histories shape vaccine responses, will allow us to better design seasonal and broadly-protective vaccines.

Taken together, these findings provide important new insight into the impact of vaccine platform on immunological imprinting. We show that while all vaccines tested in the original cRCTs demonstrated efficacy, they showed differential propensities to back-boost functional antibodies

against the variable HA head domain (as measured by HAI) and the HA stalk. TIV tended to induce more anamnestic responses, while LAIV and ATIV elicited stronger d*e novo* responses against novel epitopes on vaccine-matched strains. Importantly, both TIV and ATIV boosted ADCC inducing antibodies that bound to the HA stalk following repeated vaccination, suggesting that these platforms may be better suited for immunogen delivery in the context of a universal influenza virus vaccine for children. The naivety of the childhood immune system represents a unique window to prime the immune response against influenza virus in order and optimize protection from both seasonal and possible future pandemic viruses.

LIMITATIONS

Much debate surrounds the ultimate impact of OAS-like responses on seasonal vaccine efficacy. Our study was not powered to assess the impact of original antigenic sin on vaccine mediated protection. Therefore, it remains possible that responses classified as 'anamnestic' by our OAS index do not reduce overall protection. Indeed, elegant work by Linderman and Hensley identified that OAS antibodies raised during secondary exposure are largely cross reactive[8].

Recent evidence from the Sant group has demonstrated the importance of the cellular immune compartment, namely pre-existing CD4+ T cells, in shaping immune responses to vaccination[38]. The lack of prior T cell immunity to influenza virus in children has been postulated explain, in part, severe outcomes in this age group following exposure. In our study, only serum was available for analysis. However, examining T cell responses following first exposure, be it vaccination or infection, in a similarly naïve cohort will be imperative in understanding how to optimally shape the immune response.

METHODS

Study setting and participant recruitment. This study represents a sub analysis of participants who had participated in two randomized clinical trials (RCT) assessing that impact of seasonal influenza vaccine formulation on community-level protection in Hutterite colonies in Canada (ClinicalTrials.gov: NCT01653015 and ClinicalTrials.gov: NCT02871206)[19,20]. Samples were randomly selected from the youngest children in these cohorts (See Table S1) who had received a vaccination in each of the three years included in the study. Prior to these studies, vaccination of Hutterite children in these communities for seasonal influenza was extremely uncommon, and so these vaccines would have almost certainly represented the first exposure of these children to vaccination. For comparison of LAIV and TIV 74 pre- and post-vaccination samples were identified from matched participants in the 2012-13, 2013-14, and 2014-15 influenza seasons. LAIV vaccinees received 0.2 mL of LAIV (FluMist, MedImmune) intranasally, while children in the TIV received 0.5 mL intramuscularly (Vaxigrip, Sanofi Pasteur). Influenza A virus vaccine strains included in each season are shown in Table 2. For comparison of ATIV and QIV, 45 pre- and post- vaccination sera were selected for matched participants in the 2016-17, 2017-18, and 2018-19 influenza seasons. The ATIV and QIV vaccinees received either trivalent MF59 adjuvanted vaccine (Fluad Pediatric, Seqirus) via 0.25 mL intramuscular dose or a 0.5 mL dose of the quadrivalent formulation (Fluzone, Sanofi Pasteur) intramuscularly. The following influenza strains were used in in the vaccines administered across the study A/California/7/2009(H1N1)- , A/Michigan/45/2015(H1N1)-, A/Hong Kong/4801/2014 (H3N2)-, A/Singapore/INFIMH-16-0019/2016 (H3N2)-, B/Brisbane/60/2008-like virus, B/Phuket/3073/2013 –, and B/Colorado/06/2017-like viruses (Table 2). For both trials samples were collected on day 0 pre

vaccination and day 28 post vaccination. No differences in demographic characteristics of the participants were observed between vaccine cohorts within each study. All study procedures were approved by the McMaster University Research Ethics Board

Sample collection and processing. Participant blood was collected as previously described[18]. Briefly, participant blood (5mL) was collected into serum separator tubes (BD) by venipuncture at the trial site and centrifuged at 1300 x g for 15 minutes. All samples were store at -80°C prior to analysis. Serum was heat inactivated at 56°C for 30 minutes prior to ADCC assays. Serum inactivation was performed with a combination of 56°C heat and trypsin-heat-periodate treatment prior to HAI.

Cells. Madin Darby Canine Kidney (MDCK) and A549 cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco), 2 mM L- glutamine and 100 U/mL penicillin-streptomycin (Thermo Fisher).

Viruses. Confluent MDCK cells were infected for one hour with either A/California/04/2009 H1N1 (kind gift of Dr. Peter Palese, Icahn School of Medicine at Mount Sinai, New York, NY), A/Hong Kong/4801/2014 (H3N2), or A/Texas/50/2012 (H3N2) in 1x minimum essential medium (MEM, Sigma Aldrich) supplemented with 2 mM L-glutamine, 0.24% sodium bicarbonate, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MEM amino acids solution (Sigma Aldrich), MEM vitamins solution (Sigma Aldrich), 100 U/mL penicillin-streptomycin (Thermo Fisher), and 0.42% bovine serum albumin (Sigma Aldrich). Cells were then washed with

PBS and media was replaced. Cells were left for 72 hours, or until 90% cytopathic effects were observed, at which point virus was collected from supernatant.

Chimeric HA expressing viruses, cH5/1 N3 and cH4/3 N2 (kind gift of Dr. Peter Palese and Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, New York, NY) were propagated in 11- day- old embryonated chicken eggs as per standard protocols[39]. The cH5/1N3 virus expresses cH5/1 HA, a fusion of the A/Vietnam/1203/04 H5 head domain with the A/Puerto Rico/8/34 H1 stalk domain[40]. The cH4/3N2 virus expresses the H4 head of A/duck/Czech/1956(H4N6) and the H3 stalk of A/Perth/16/2009(H3N2), as well as the NA from A/Victoria/2011(H3N2)[41]. The seasonal vaccine strains, A/Singapore/INFIMH-16-0019/2016 (H3N2), A/Michigan/45/2015 (H1N1), and A/Victoria/361/2011 (H3N2) were also propagated using the embryonated egg method.

Hemagglutination inhibition (HAI) assay. HAI assay was performed according to standard as described previously[39,42]. Briefly, serum was inactivated using trypsin-heat-periodate treatment prior to HAI assay. From 20µl serum starting amount, samples were treated with 0.5 times starting volume of 8mg/ml TPCK-trypsin (Sigma-Aldrich) and incubated at 56°C for 30 minutes. Samples were cooled to RT and 3 volumes of 11mM potassium periodate solution (Sigma-Aldrich) was added. After 15 minutes of incubation at RT, 3 volumes of 1% glycerol-PBS solution was added and incubated for another 15 minutes at RT. Lastly, 2.5 volumes of 0.85% PBS was added to the sample. Virus and serum were incubated for 30 minutes at RT. Chicken red blood cells were then added to the wells and plates were incubated at 4°C for 45 minutes prior to reading. HAI titers were defined as the highest serum dilution factor with HAI activity (where the button of

erythrocytes showed a 'drip'); samples with no detectable HAI activity were assigned as half the limit of detection of the assay, "5".

Antibody dependent cellular cytotoxicity (ADCC) assay. A549 cells were plated in opaque white 96-well plates (Corning Costar) at $2.5x10^4$ cells per well. The following day, cells were washed with RPMI, and infected for 18 hours with either a group one chimeric virus (cH5/1), MOI 5, or group 2 chimeric virus (cH4/3), at MOI 3. Prior to performing the assay, all serum samples were heat inactivated at 56°C for 30 minutes. Serum samples were serially diluted in RPMI supplemented with 4% low-IgG FBS and incubated with the infected cell monolayer for 1 hour. Following incubation, engineered Jurkat effector cells expressing the human FcgRIIIa (CD16), and a luciferase reporter gene under the expression of a nuclear factor activated T cells (NFAT) element (Promega) were added at $7.5x10^4$cells/well. After a 6-hour incubation at 37°C, 75µl/ well of Bio-Glo luciferase substrate (Promega) was added, and luminescence was read on the Spectramax I3 (Molecular Devices, San Jose, CA, USA).

Generation of HA molecular model. The crystal structures of A/Calfornia/04/2009 (H1N1) (PDB 5GJS), A/Victoria/361/2011 (PDB 4O5N), A/Michigan/15/2014(H3N2) (PDB 6BKP) were downloaded from the Protein Data Bank and used as molecular models. No structures for A/Hong Kong/4801/2014 or A/Singapore/INFIMH-16-0019/2016 were deposited on PDB, a related H3N2 clade 3c2.A strain, A/Michigan/15/2014(H3N2), was used instead. Key antigenic sites as defined by Caton et al., Brownlee and Fodor, and Jackson et al. and amino acid differences between strains were depicted using the open UCSF Chimera software[43–47].

Statistical analysis

All statistical analyses were performed in GraphPad Prism version 9.1.1

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AUTHOR CONTRIBUTIONS

Conceptualization- M.S.M and M.L. Access to patient samples- M.L. Investigation and analysis- H.D.S. , A.Z. , J.C.A. , M.S.M. Writing- H.D.S. and M.S.M. Reviewing and editing manuscript- H.D.S., A.Z., J.C.A., P.S., M.L., and M.S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

References

1. Ruf, B. R. & Knuf, M. The burden of seasonal and pandemic influenza in infants and children. *Eur. J. Pediatr.* **173**, 265–276 (2014).
2. Francis, T. On the Doctrine of Original Antigenic Sin. *Proc. Am. Philos. Soc.* **104**, 572–578 (1960).
3. Davenport, B. Y. F. M., Hennessy, A. V & Francis, T. Antigenic Variants, Antibody Content and Immunity. (1953).
4. Zhang, A., Stacey, H. D., Mullarkey, C. E. & Miller, M. S. Original Antigenic Sin: How First Exposure Shapes Lifelong Anti–Influenza Virus Immune Responses. *J. Immunol.* **202**, 335–340 (2019).
5. Fazekas de St Groth & Webster, R. G. Disquisitions of Original Antigenic Sin. I. Evidence in man. *J. Exp. Med.* **124**, 331–45 (1966).
6. Davenport, B. Y. F. M. & Hennessy, A. V. A serologic recapitulation of past experiences with Influenza A; antibody response to a monovalent vaccine. (1956).
7. Yewdell, J. W. & Santos, J. J. S. Original antigenic sin: How original? how sinful? *Cold Spring Harb. Perspect. Med.* **11**, 1–16 (2021).
8. Linderman, S. L. & Hensley, S. E. Antibodies with 'Original Antigenic Sin' Properties Are Valuable Components of Secondary Immune Responses to Influenza Viruses. *PLOS Pathog.* **12**, e1005806 (2016).
9. Gostic, K. M., Ambrose, M., Worobey, M. & Lloyd-Smith, J. O. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science* **354**, 722–726 (2016).

10. Kim, J. H., Davis, W. G., Sambhara, S. & Jacob, J. Strategies to alleviate original antigenic sin responses to influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13751–6 (2012).

11. Kim, J. H., Skountzou, I., Compans, R. & Jacob, J. Original antigenic sin responses to influenza viruses. *J. Immunol.* **183**, 3294–301 (2009).

12. Mohn, K. G. I., Smith, I., Sjursen, H. & Cox, R. J. Immune responses after live attenuated influenza vaccination. *Hum. Vaccines Immunother.* **14**, 571–578 (2018).

13. Belshe, R. B. *et al.* Live Attenuated versus Inactivated Influenza Vaccine in Infants and Young Children. *N. Engl. J. Med.* **356**, 685–696 (2007).

14. He, X.-S. *et al.* Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J. Virol.* **80**, 11756–11766 (2006).

15. Li, A. P. Y. *et al.* Immunogenicity of standard, high-dose, MF59-adjuvanted, and recombinant-HA seasonal influenza vaccination in older adults. *npj Vaccines* **6**, 1–12 (2021).

16. Cruz-Valdez, A. *et al.* MF59-adjuvanted influenza vaccine (FLUAD®) elicits higher immune responses than a non-adjuvanted influenza vaccine (Fluzone®): A randomized, multicenter, Phase III pediatric trial in Mexico. *Hum. Vaccines Immunother.* **14**, 386–395 (2018).

17. Krammer, F. & Palese, P. Advances in the development of influenza virus vaccines. *Nat. Rev. Drug Discov.* **14**, 167–182 (2015).

18. Yegorov, S. *et al.* Inactivated and live-attenuated seasonal influenza vaccines boost broadly neutralizing antibodies in children. *Cell Reports Med.* **3**, 100509 (2022).

19. Loeb, M. *et al.* Live attenuated versus inactivated influenza vaccinein hutterite children a

cluster randomized blinded trial. *Ann. Intern. Med.* **165**, 617–624 (2016).

20. Loeb, M. *et al.* Adjuvanted trivalent influenza vaccine versus quadrivalent inactivated influenza vaccine in Hutterite Children: A randomized clinical trial. *Vaccine* **39**, 6843–6851 (2021).
21. Black, S. *et al.* Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza Vaccines in children. *Pediatr. Infect. Dis. J.* **30**, 1081–1085 (2011).
22. Krammer, F. The human antibody response to influenza A virus infection and vaccination. *Nat. Rev. Immunol.* (2019) doi:10.1038/s41577-019-0143-6.
23. Miller, M. S. *et al.* Neutralizing Antibodies Against Previously Encountered Influenza Virus Strains Increase over Time: A Longitudinal Analysis. *Sci. Transl. Med.* **5**, 198ra107 (2013).
24. Ellebedy, A. H. *et al.* Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 13133–8 (2014).
25. Andrews, S. F. *et al.* Immune history profoundly affects broadly protective B cell responses to influenza. *Sci. Transl. Med.* **7**, 316ra192-316ra192 (2015).
26. Miller, M. S. & Palese, P. Peering into the crystal ball: Influenza pandemics and vaccine efficacy. *Cell* **157**, (2014).
27. Wu, N. C. *et al.* Major antigenic site B of human influenza H3N2 viruses has an evolving local fitness landscape. *Nat. Commun.* **11**, (2020).
28. Skowronski, D. M. *et al.* A perfect storm: Impact of genomic variation and serial vaccination on low influenza vaccine effectiveness during the 2014-2015 season. *Clin. Infect. Dis.* **63**, 21–32 (2016).

29. Zimmerman, R. K. *et al.* 2014–2015 Influenza Vaccine Effectiveness in the United States by Vaccine Type. *Clin. Infect. Dis.* **63**, 1564–1573 (2016).

30. Chambers, B. S., Parkhouse, K., Ross, T. M., Alby, K. & Hensley, S. E. Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014–2015 Influenza Season. *Cell Rep.* **12**, 1–6 (2015).

31. Smith, D. J., Forrest, S., Ackley, D. H. & Perelson, A. S. Variable efficacy of repeated annual influenza vaccination. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14001–6 (1999).

32. Gao, J. *et al.* Antigenic drift of the influenza A(H1N1)pdm09 virus neuraminidase results in reduced effectiveness of A/California/7/2009 (H1N1pdm09)-specific antibodies. *MBio* **10**, 1–17 (2019).

33. Linderman, S. L. *et al.* Potential antigenic explanation for atypical H1N1 infections among middle-aged adults during the 2013–2014 influenza season. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 15798–15803 (2014).

34. Dilillo, D. J., Tan, G. S., Palese, P. & Ravetch, J. V. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat. Med.* **20**, 143–51 (2014).

35. He, W. *et al.* Alveolar macrophages are critical for broadly-reactive antibody-mediated protection against influenza A virus in mice. *Nat. Commun.* **8**, 1–13 (2017).

36. He, W. *et al.* Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus. *Proc. Natl. Acad. Sci.* **113**, 11931–11936 (2016).

37. Stacey, H. D. *et al.* IgA potentiates NETosis in response to viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–9 (2021).

38. Richards, K. A. *et al.* Evidence That Blunted CD4 T-Cell Responses Underlie Deficient Protective Antibody Responses to Influenza Vaccines in Repeatedly Vaccinated Human Subjects. *J. Infect. Dis.* **222**, 273–277 (2020).

39. Who. Manual for the laboratory diagnosis and virological surveillance of influenza. *World Heal. Organ. 2011* 153 (2011).

40. Hai, R. *et al.* Influenza Viruses Expressing Chimeric Hemagglutinins: Globular Head and Stalk Domains Derived from Different Subtypes. *J. Virol.* **86**, 5774–5781 (2012).

41. Rajendran, M. *et al.* An immuno-assay to quantify influenza virus hemagglutinin with correctly folded stalk domains in vaccine preparations. *PLoS One* **13**, e0194830 (2018).

42. Miller, M. S. *et al.* 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. *J. Infect. Dis.* **207**, 98–105 (2013).

43. Caton, A. J., Brownlee, G. G., Yewdell, J. W. & Gerhard, W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**, 417–427 (1982).

44. Brownlee, G. G. & Fodor, E. The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. *Philos. Trans. R. Soc. B Biol. Sci.* **356**, 1871–1876 (2001).

45. Jackson, D. C., Murray, J. M., White, D. O. & Gerhard, W. U. Enumeration of antigenic sites of influenza virus hemagglutinin. *Infect. Immun.* **37**, 912–918 (1982).

46. Broecker, F. *et al.* Immunodominance of Antigenic Site B in the Hemagglutinin of the Current H3N2 Influenza Virus in Humans and Mice. *J. Virol.* **92**, 1–13 (2018).

47. Pettersen, E. F. *et al.* UCSF Chimera?A visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).

Figure Captions :

Figure 1-Study flow charts. **(A)** Depiction of participants for trial comparing the LAIV and TIV across the 2012-2013 to 2014-2015 influenza seasons. **(B)** Depiction of participants for the trial comparing AV-IIV and IIV across the 2016-2017 to 2018-2019 influenza seasons. RCT: Randomized Control Trial; LAIV: live attenuated influenza vaccine; TIV: trivalent inactivated influenza vaccine; ATIV: adjuvanted trivalent inactivated influenza vaccine; QIV: quadrivalent inactivated influenza vaccine; bnAbs: broadly-neutralizing antibodies.

Figure 2- TIV skewed responses towards historical vaccine strains following a vaccine antigen change in 2014. (A) H1 and H3 vaccine antigens between years 1-3 of the RCT comparing LAIV and TIV. **(B)** Serum HAI activity against Cal/09 virus assessed pre and post vaccination in all three years of the trial. **(C)** represents log2 transformed ratios (Post Vaccination/ Pre Vaccination) of the HAI titers. **(D)** Serum HAI activity against Vic/11 assessed pre and post vaccination in all three years of the trial. **(E)** represents log2 transformed ratios (Post Vaccination/ Pre Vaccination) of the HAI titers **(F)**Serum HAI activity against Tx/12 assessed pre and post vaccination in the final year of the trial and **(G)** represents the corresponding year 3 log2 transformed ratios (Post Vaccination/ Pre Vaccination) of the HAI titers to Vic/11 and Tx/12 . **(H)** OAS indexes based on fold change of HAI antibodies against current and historical H3 vaccine antigens. **(I)** Map of antigenic site changes between Vic/11 and Tx/12 mapped onto the HA structure of Vic/1**1 (**PDB 4O5N).**B-D** Dots and brackets represent the geometric mean titer and 95% CIs, statistical significance of matched pre- and post- vaccination titers was assessed using a paired t-test. **E-G** Dots and brackets represent the mean and 95% CIs, differences between vaccine groups and years was assessed by ordinary one-way ANOVA with Tukey's multiple comparisons.

Figure 3- Induction of ADCC inducing antibodies following seasonal LAIV or TIV vaccination. (A) A monolayer of A549s was infected with either cH5/1N3 (Group 1) or cH4/3N2 (Group 2) chimeric virus for 18 hours. Serial dilutions of pre and post serum from each clinical trial year were incubated on the infected cells for 1 hours. Jurkat reporter cells were then added expressing a luciferase gene, were then added, and incubated for 6 hours prior to the addition of luciferase substrate. Images made with BioRender. **(B)** Log2 transformation of post vaccination/ pre vaccination ratio of induction of group 1 ADCC inducing antibodies at a 1:10 serum dilution. **(C-E)** Matched donor pre- and post- vaccination induction of group 1, ADCC inducing antibodies. Where fold induction is taken as a measure of signal over plate background. **(F)** Log2 transformation of post vaccination/ pre vaccination ratio of induction of group 2 ADCC inducing antibodies at a 1:10 serum dilution. **(G-I)** Matched donor pre- and post- vaccination induction of group 2, ADCC inducing antibodies.

Figure 4- H1 and H3 vaccine strain HAI titers for Hutterite children followed throughout the ATIV vs. QIV trial. (A) H1 and H3 vaccine antigens between years 1-3 of the RCT comparing ATIV and QIV.**(B)** Serum HAI activity against Cal/09 virus assessed pre and post vaccination in all three years of the trial**(C)** Log2 transformed ratios (Post Vaccination/ Pre Vaccination) of Cal09 HAI titers.**(D)** Serum HAI activity against Mich/15 assessed pre and post vaccination in years 2 and 3. For figures B-D dots and brackets represent the mean and 95% CI, p –values. **(E)** Log2 transformed ratios (Post Vaccination/ Pre Vaccination) Mich/15 HAI titers. **(F)** Map of antigenic site changes between Cal/09 and Mich/15 mapped onto the HA structure of Cal/09 **(**PDB 5GJS). **G)** Serum HAI titers against HK/14. **(H)** Log2 transformed ratios (Post Vaccination/ Pre Vaccination) HK/14 HAI titers.**(I)** Serum HAI titers against Sing/16. **(J)** Log2 transformed ratios (Post Vaccination/ Pre Vaccination) Sing/16 HAI titers. **(K)** OAS indexes based on fold change of HAI antibodies against current and historical H3 vaccine antigens**. (L)** Map of antigenic site changes between HK/14 and Sing/16 mapped onto the HA structure of A/Michigan/2015 (H3N2), a related A/H3N2 clade 3c2.A strain.

Figure 5- Induction of ADCC inducing antibodies following administration of seasonal ATIV or QIV. (A) Log2 transformation of post vaccination/ pre vaccination ratio of induction of group 1 ADCC inducing antibodies at a 1:10 serum dilution. **(C-E)** Matched donor pre- and post-vaccination induction of group 1, ADCC inducing antibodies. Where fold induction is taken as a measure of signal over plate background. **(E)** Log2 transformation of post vaccination/ pre vaccination ratio of induction of group 2 ADCC inducing antibodies at a 1:10 serum dilution. **(F-H)** Matched donor pre- and post- vaccination induction of group 2, ADCC inducing antibodies.

Figure 6- Prior influenza infections impacts HA head and stalk responses following seasonal vaccination. For all analyses participants with an H3 HAI titer of ≥40 in the pre-vaccination sample of trial Year 1 were classified to the 'pre-existing' immunity group and presumed to be primed on infection. **(A-C)** represent log2 transformed ratios (Post Vaccination/ Pre Vaccination) of the HAI titers against Vic/11. **(D)** represent log2 transformed ratios (Post Vaccination/ Pre Vaccination) of the HAI titers against Tx/12. **(E)** OAS indexes based on fold change of HAI antibodies against current and historical H3 vaccine antigens, stratified by exposure history in trial year 1. **(F)** Group 1 ADCC responses broken down by priming on infection (pre-existing) or priming on vaccination (no pre-existing) **(G)** Group 2 ADCC responses broken down by priming on infection (pre-existing) or priming on vaccination (no pre-existing). **(F+G)** Bars are representative of mean, 95% CI. Significance was determined by paired T-test with Holm Sidak method.

Trial I	**All (74)**	**LAIV Vaccinees (39)**	**TIV Vaccinees (35)**	**P- Value**
Median age, years [IQR]	4.0 [4.0, 5.0]	4.0 [3.0, 5.0]	4.0 [4.0, 5.0]	0.1214
Male gender, n(%)	47 (63.51%)	21 (53.84%)	26 (74.2%)	0.0916
Trial II	**All (45)**	**ATIV Vaccinees (18)**	**QIV Vaccinees (27)**	**P- Value**
Median age, years [IQR]	3.0 [2.0,4.0]	3.0 [2.75, 4.0]	3.0 [2.0,4.0]	>0.999
Male gender, n(%)	23 (51.11%)	8 (44.44%)	15 (55.56%)	0.5499

Table 1- Demographic characteristics of participants in year 1 of enrollment for trial 1 (LAIV vs. TIV) and trial 2 (ATIV and QIV). Data is n (n/N %) or n [IQR]. IQR= Interquartile Range. Age and gender differences were assessed using the two-sided Mann- Whitney U and Fisher's exact t-tests respectively.

		H1 Strain	H3 Strain	IBV Strain
LAIV/ TIV Trial	**2012-2013**	A/California/07/2009(H1N1)-like virus	A/Victoria/361/2011(H3N2)-like virus	B/Wisconsin/01/2010-like virus
	2013-2014	A/California/07/2009(H1N1)-like virus	A/Victoria/361/2011(H3N2)-like virus	B/Massachusetts/02/2012-like virus
	2014-2015	A/California/7/2009(H1N1)-like virus	A/Texas/50/2012(H3N2)-like virus	B/Massachusetts/02/2012-like virus
ATIV/ QIV Trial	**2016-2017**	A/California/7/2009(H1N1)-like virus	A/Hong Kong/4801/2014 (H3N2)-like virus	B/Brisbane/60/2008-like virus
	2017-2018	A/Michigan/45/2015(H1N1)-like virus	A/Hong Kong/4801/2014 (H3N2)-like virus	B/Brisbane/60/2008-like virus B/Phuket/3073/2013 -like virus (for quadrivalent vaccine)
	2018-2019	A/Michigan/45/2015(H1N1) pdm09-like virus	A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus	B/Colorado/06/2017-like virus B/Phuket/3073/2013 -like virus (for quadrivalent vaccine)

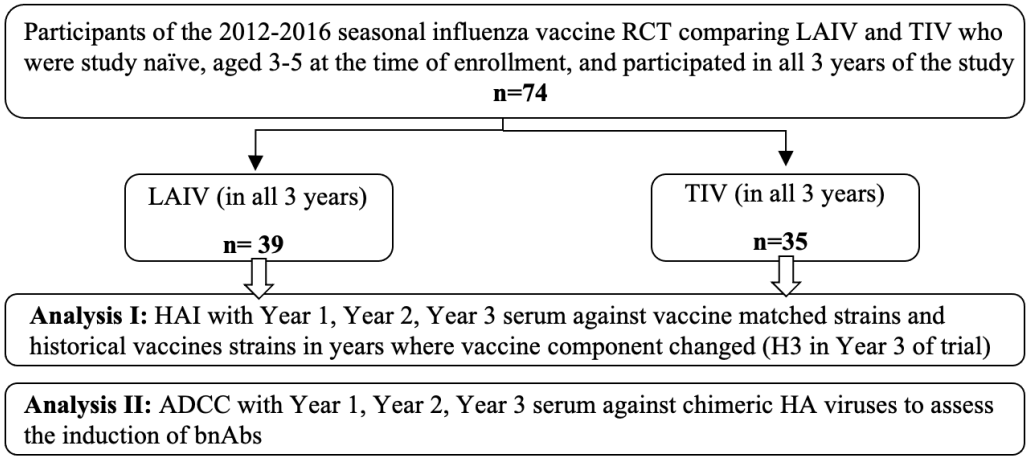
Table 2- WHO recommended vaccine strains for seasonal influenza vaccines across study years.

		Subtype	Dominant Circulating Strain
LAIV/ TIV Trial	**2012-2013**	H3N2	A/Victoria/361/2011(H3N2)-like virus
	2013-2014	H1N1	A/California/07/2009(H1N1)-like virus
	2014-2015	H3N2	A/Switzerland/9715293/2013 (H3N2)
AV-IIV/ IIV Trial	**2016-2017**	H3N2	A/Hong Kong/4801/2014 (H3N2)-like virus
	2017-2018	H3N2	A/Hong Kong/4801/2014 (H3N2)-like virus
	2018-2019	H1N1	A/Michigan/45/2015(H1N1)

Table 3- Dominant circulating influenza strains across study period, as reported by the Canadian FluWatch Surveillance program.

Figure 1 – Study flow charts

A



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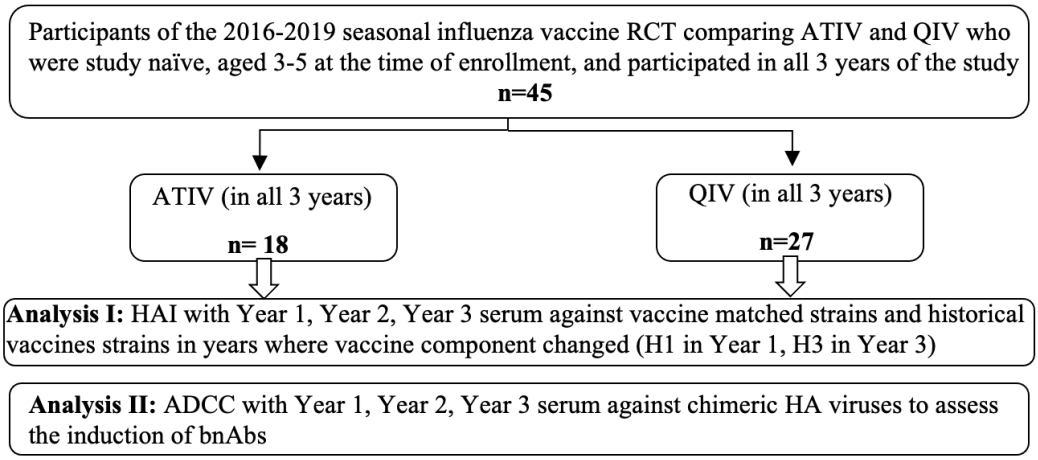


Figure 2- TIV skewed responses towards historical vaccine strains following a vaccine antigen change in 2014

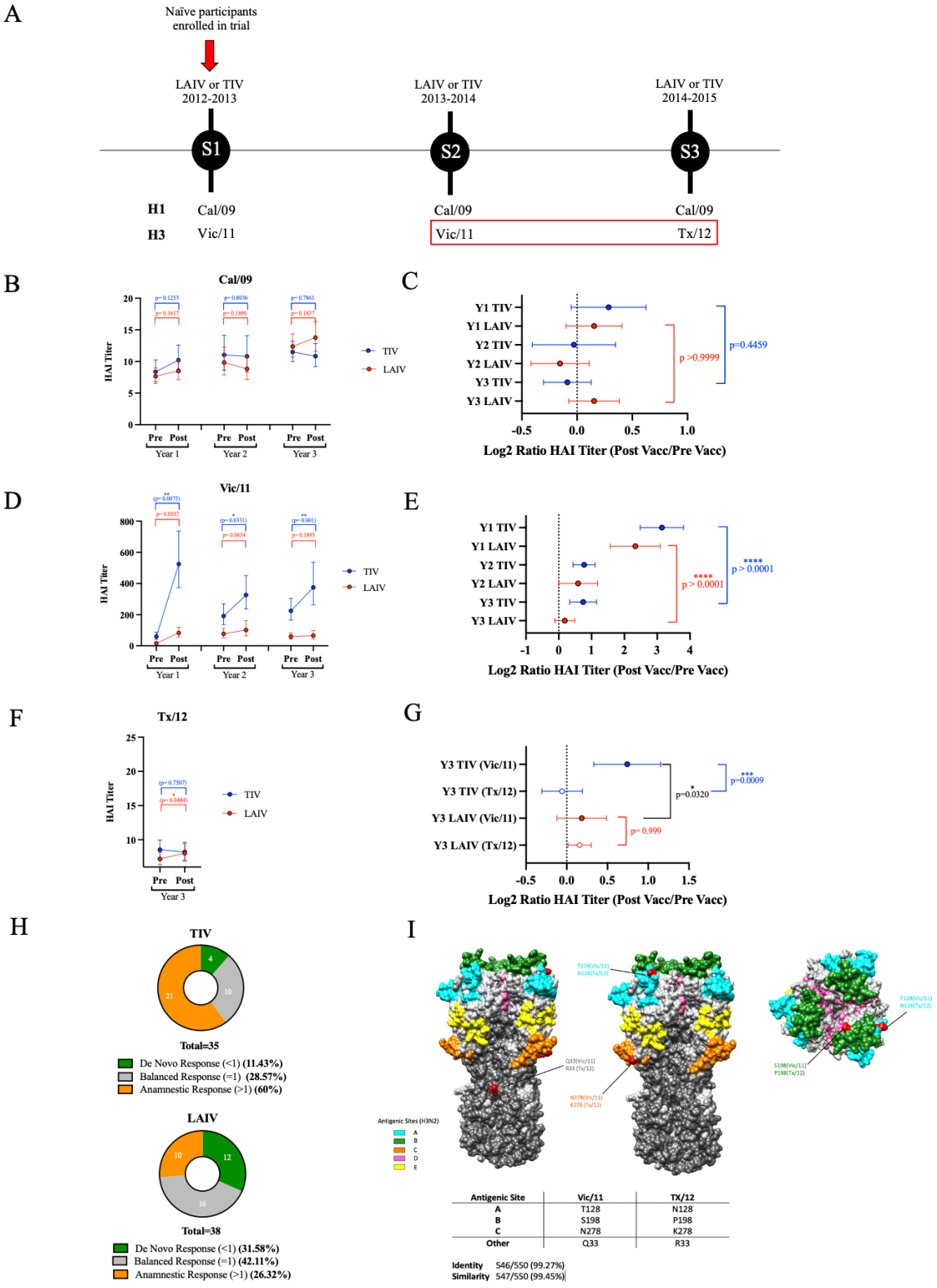


Figure 3- Induction of ADCC inducing antibodies following seasonal LAIV or TIV

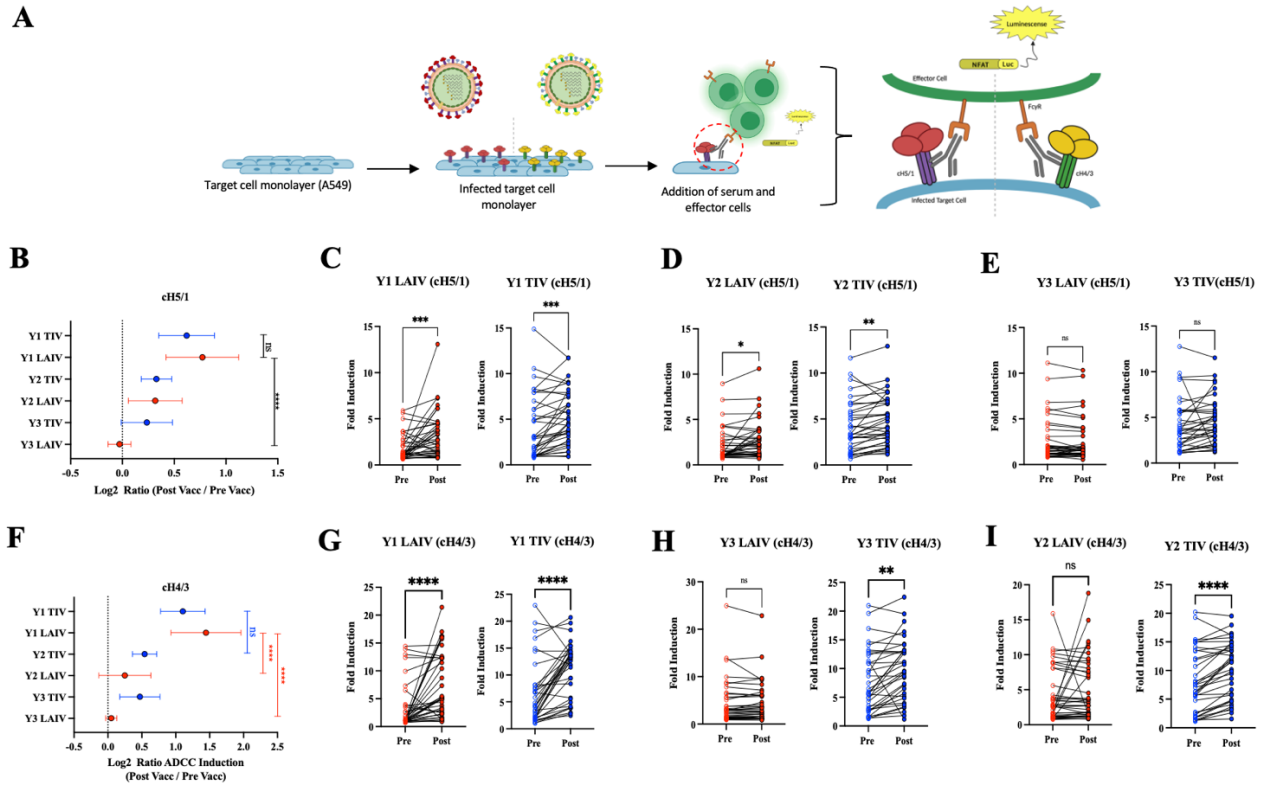


Figure 4- H1 and H3 vaccine strain HAI titers for Hutterite children followed throughout the ATIV vs QIV trial.

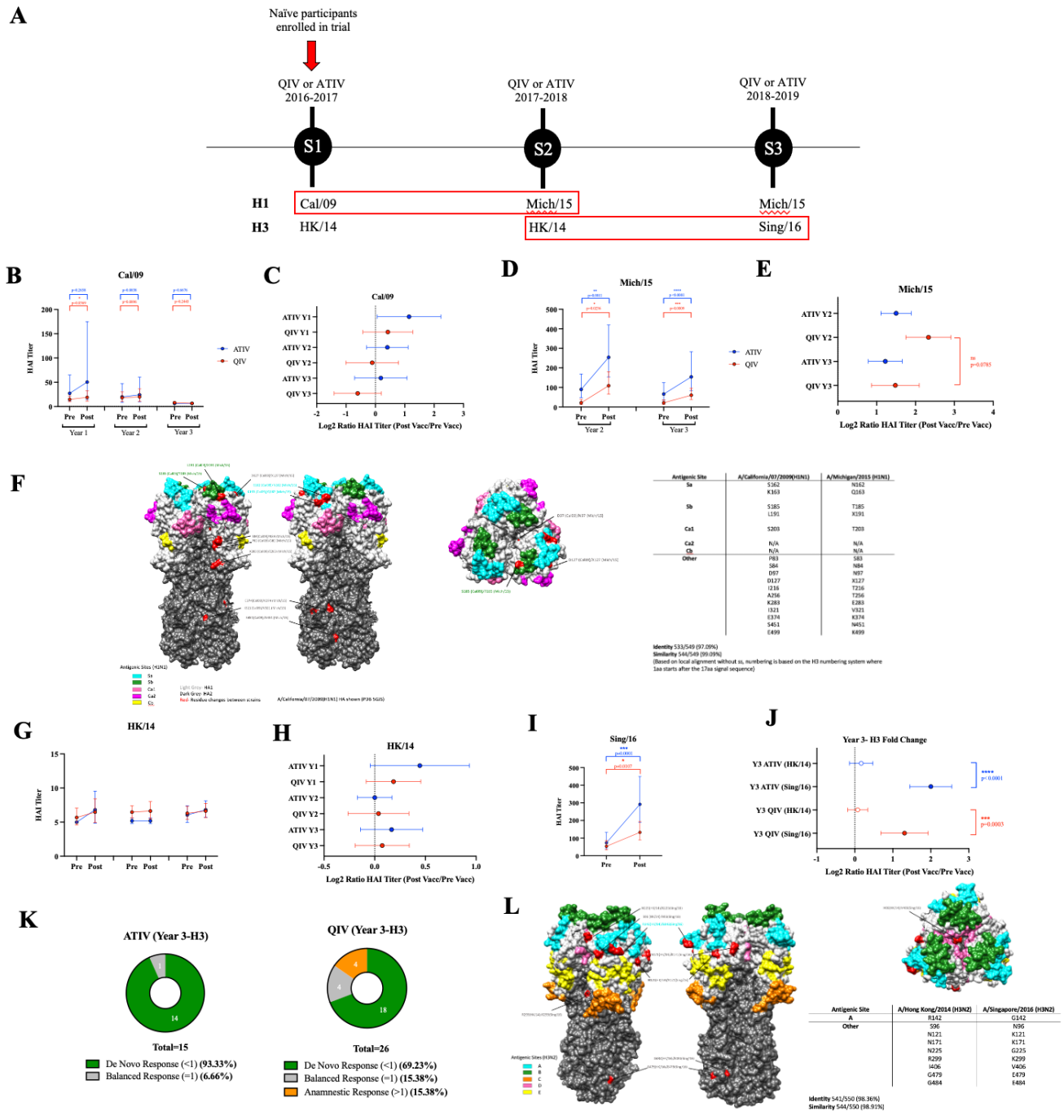


Figure 5- Induction of ADCC inducing antibodies following administration of seasonal ATIV or QIV

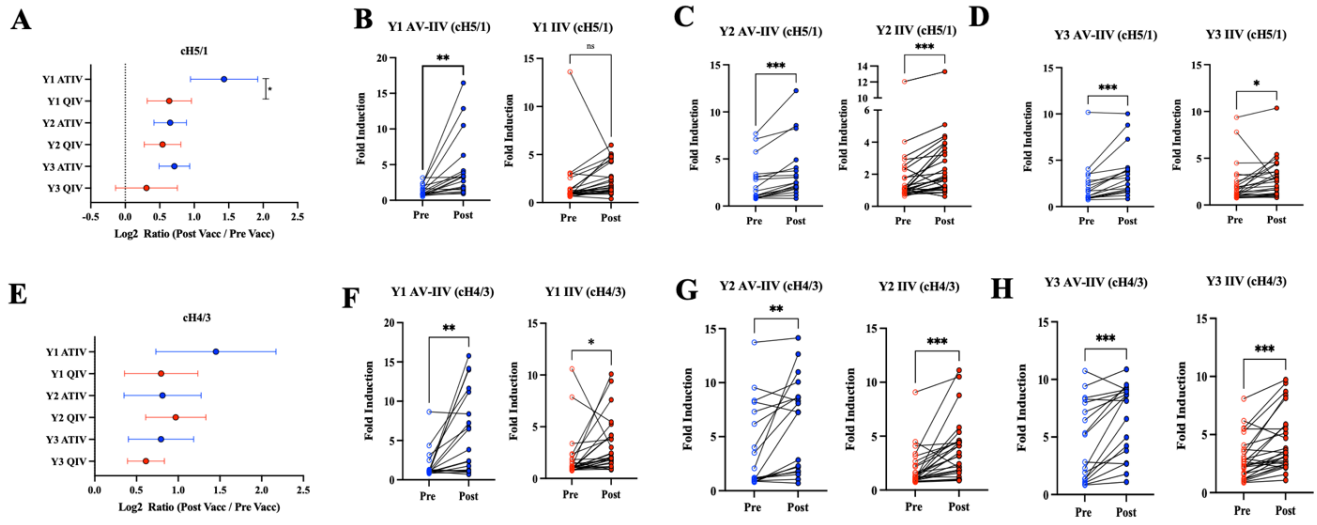
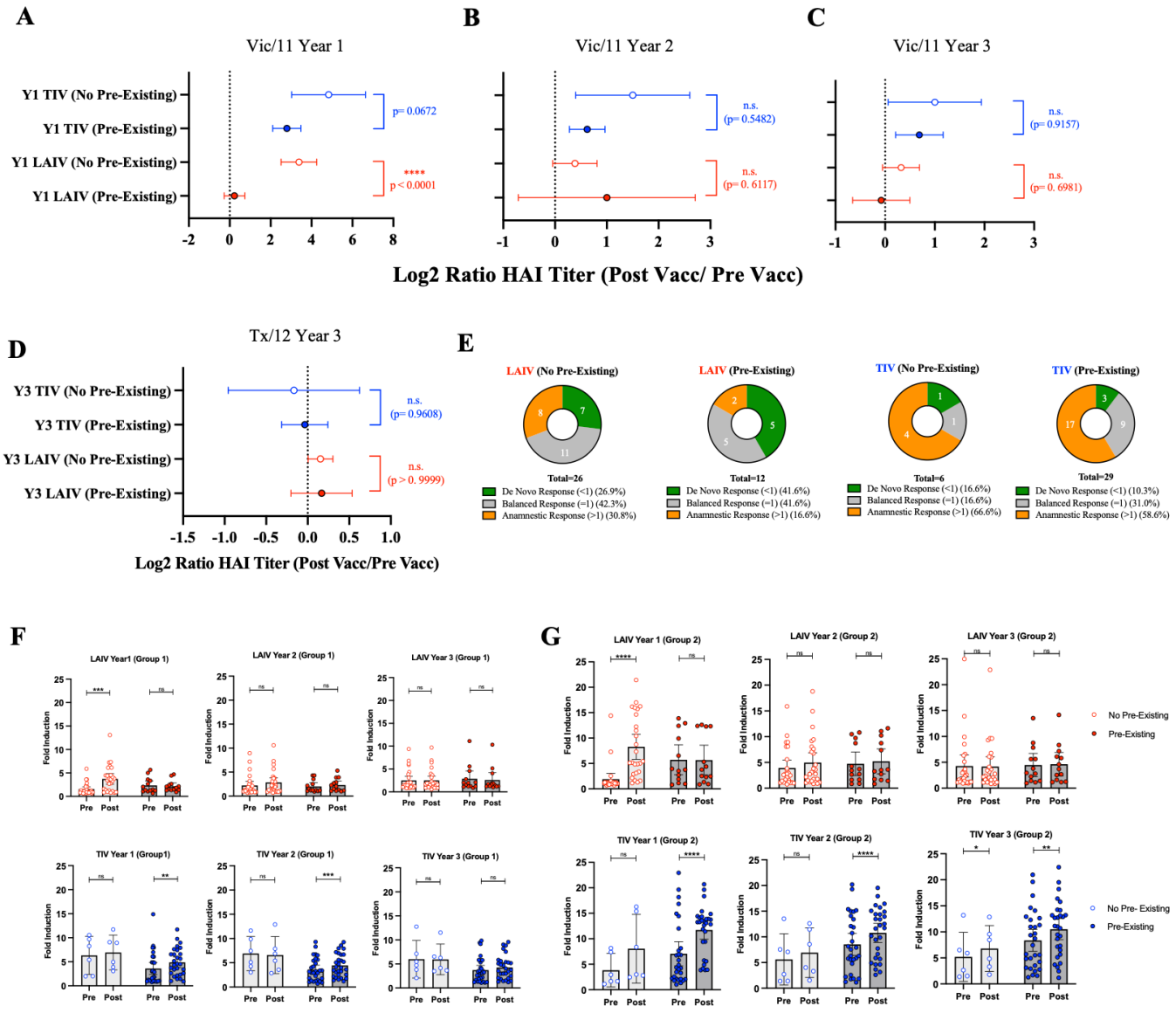


Figure 6- Prior influenza infection impacts HA head and stalk responses following seasonal vaccination.



5.0- Chapter 5: Determining the immunogenicity and efficacy of an inactivated seasonal vaccine in patients with heart failure

Manuscript title: Immunogenicity of inactivated influenza vaccination in patients with heart failure: Secondary analysis of a multinational randomised, double-blind, placebo-controlled trial

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Immunogenicity of inactivated influenza vaccination in patients with heart failure: Secondary analysis of a multinational randomised, double-blind, placebo-controlled trial

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Key Words: heart failure, cardiovascular, influenza virus, inactivated influenza vaccine, antibodies, vaccine efficacy, hemagglutination inhibition, randomized control trial, vaccination

ABSTRACT

Influenza infection has been shown to increase the risk of cardiovascular complications and related death. The **I**nfluenza **v**accine to reduce adverse **v**ascular **e**vents in patients with heart failure (IVVE) trial demonstrated that seasonal influenza vaccination reduced cardiovascular events during periods of peak influenza circulation. However, the immunogenicity and efficacy of the inactivated influenza vaccine (IIV) in this high-risk cohort had not been evaluated. Here, we measured vaccine immunogenicity in a subset of IVVE participants. Samples for this analysis were collected at baseline (T1), ~4 weeks post-vaccination (T2), and ~6 months post-vaccination (T3) in the Philippines, Mozambique, Nigeria and Zambia during the 2017-2018, 2018-2019 and 2019-2020 influenza seasons. The seasonal vaccine induced significantly higher HAI titers in the vaccinated cohort when compared to placebo. Vaccine efficacy was estimated by characterizing serological evidence of infection as a 4-fold or 2-fold increase in titers between T3/T2. Using a 4-fold cut-off, VE against any influenza A subtype was estimated at 91.5% (50.3-98.6) in 2017, 72.29% (30.0-89.24) in 2018, and 86.2% (63.2- 94.9) in 2019. Interestingly, vaccinees who had a cardiovascular event following vaccination exhibited blunted vaccine responses relative to vaccinees who did not have a cardiac event.

INTRODUCTION

Influenza infections and cardiovascular disease are among the leading causes of morbidity and mortality globally[1,2]. Influenza virus infections are known to increase the risk of cardiovascular events and death[3–6] while seasonal vaccination has been reported to reduce some cardiovascular outcomes[7–10]. As such, vaccination against influenza virus in high-risk patients living with, or at risk of developing, cardiovascular disease should be a public health priority. Further, improving our understanding of how these high-risk patient populations respond to current seasonal vaccines may help to prevent severe outcomes and deaths.

The recently published **I**nfluenza **v**accine to reduce adverse **v**ascular **e**vents in patients with heart failure (IVVE) placebo-controlled trial[10,11]was a multinational, randomized, double-blind study to test whether seasonal influenza vaccination reduced cardiovascular events in a heart failure patient cohort. That study found that during peak influenza circulation there was a significant reduction in a composite outcome of cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke. Secondary outcomes such as all-cause death, cardiovascular death, and pneumonia were also significantly reduced in the vaccinated cohort.

We therefore set out to explore the immunological basis for this protection by measuring immunogenicity of the seasonal influenza vaccine in a subset of the study cohort, and by estimating vaccine efficacy using serological surrogates of infection. Our data show that the inactivated influenza vaccine was both immunogenic and efficacious against influenza A virus subtypes. These findings suggest that in the IVVE trial, vaccine-mediated protection against cardiovascular outcomes was directly associated with protective vaccine responses that prevented influenza virus

infection. Interestingly, vaccinated participants who had a cardiac event appeared to have blunted vaccine responses, particularly against the H1N1 component, relative to participants who did not experience cardiovascular events. Taken together, these data reinforce the benefits of vaccinating individuals with underlying cardiovascular conditions to protect against subsequent influenza-associated cardiovascular complications.

RESULTS

Study participants

Immunogenicity of the inactivated seasonal influenza vaccine (IIV) was investigated using a subset of 673 participants from across all study sites (Figure 1, Table 1), 345 of whom received IIV and 328 in the placebo group. The demographic characteristics of participants in the vaccinated and placebo groups were similar (Table 1). Females were over-represented in the study cohort, making up 71.6% of the vaccine group and 71.0% of the placebo group.

In the 2017-2018 influenza season, serological data was only obtained from the Philippines (PH) (n=149 total, n=80 IIV and n=69 placebo). For the 2018-2019 season, samples from PH (n= 133), Mozambique (MZ) (n=150), and Nigeria (NG) (n=40) were used. For the 2019-2020 season samples from all four study sites, including Zambia (ZA) were available (Supplemental Table 1).

Serum H1N1 HAI responses to IIV across multiple influenza seasons

HAI titers are conventionally used as a correlate of protection for seasonal influenza vaccines[12,13]. To assess vaccine responses in this cohort, sera was collected from participants at baseline (T1), ~14 days post-vaccination (T2), and at the end-of-season or ~6 months post vaccination (T3)

(Figure 2). We evaluated serum HAI titers against both the H1 and H3 vaccine antigens, pre- and post-vaccination in all countries where samples were available (Figure 3). During the 2017-2018 season there was a 9.11 (95% CI: 6.47-12.48) geometric mean fold increase (GMFR,Table 2) in H1N1 HAI titers in the vaccinated cohort (GMT 13.78 pre-vaccination compared to 125.2 post-vaccination). The post-vaccination HAI titers in vaccinees was also significantly elevated compared to the placebo group, as expected (Table 2). Seroconversion is conventionally defined as a 4-fold increase in titer from baseline levels. In the vaccine group 59/80 (73.75%) participants met this threshold following vaccination in the 2017-2018 season. The rate of seroconversion was also statistically different from that observed in the placebo group ($p < 0.0001$) (Table 2).

In the 2018-2019 season, a strong vaccine response against the H1N1 component was also observed. The mean $\log_{10}$ (standard deviation) H1N1 HAI titers increased significantly post-vaccination from 1.48 (0.52) to 1.93 (0.48) ($p < 0.0001$) (Table 2). The post vaccination H1 titers were also showed significant increase relative to the placebo group ($p < 0.0001$). The GMTs (95% CI) in the vaccine recipients rose from 30.77 (25.60-36.97) to 86.13 (72.78-101.9), a GMFR of 2.80 (95% CI: 2.32-3.36) (Table 2). Relative to the previous season, H1N1 seroconversion declined to 34.91% (59/169). However, strong evidence has demonstrated that high levels of pre-existing antibody, such as those raised from prior vaccination, can blunt *de novo* antibody synthesis.

Finally, immunogenicity against H1N1 was also significant in the 2019-2020 season. In the vaccinees, HAI GMTs increased from 45.12 (95% CI: 38.68-52.64) to 98.79 (95%: 86.32- 113.1)

following vaccination (Table 2). This represents an GMFR of 2.189 (95% CI: 1.90-2.51) (p <0.0001). The H1N1 seroconversion rate in the vaccine group was 30.91% (64/207) (Table 2).

Similar results were observed when each country was analyzed individually by year. Mean log_{10} H1N1 HAI titers were significantly elevated post vaccination in all countries and years, apart from NG in 2018 and 2019 (Supplemental Table 2). These findings demonstrate that across all years assessed in this secondary serological analysis of participants from the IVVE trial, seasonal influenza vaccines induced strong responses against the H1N1 antigen in this high-risk cohort.

Serum H3N2 HAI responses to IIV across multiple influenza seasons

Current seasonal IIVs contain both the dominant circulating H1N1 and H3N2 IAV strains. Thus, we next evaluated the pre- and post- vaccination HAI titers against the H3N2 antigens in the 2017, 2018 and 2019 seasonal vaccines (Figure 2). In the 2017-2018 season, post-vaccination HAI GMTs in the vaccine group were increased from 16.96 (15.41-18.67) to 18.85 (17.17-20.69) but were not significantly different from baseline (p=0.4743) (Table 3). Seroconversion against the H3N2 antigen was very low, with only 1.25% (1/80) of the participants who received vaccine exhibiting at least a 4-fold increase in titers (Table 3).

Vaccination in 2018 resulted in a significant increase in H3N2 HAI titers, from a mean log_{10} titer of 1.31 (0.41) at baseline to 1.61 (0.45) (p <0.0001) following vaccination (Table 3). Post-vaccination titers in the vaccine group were significantly higher than the placebo group (p <0.0001). The rate of seroconversion between the first two time was also significantly higher in

the vaccine group (29.58 %, 50/169) compared to the placebo group, where only 6.49% (10/154) seroconverted, likely due to infection ($p<0.0001$) (Table 3).

In 2019, there was a 1.56 (1.41-1.72) increase in the GMFR of H3N2 HAI titers in the vaccine group following vaccination (Table 3). The GMTs remained stable in the placebo group, 21.83 (20.07-23.74) pre-vaccination to 23.58 (21.43-25.96) post-vaccination. The H3N2 post-vaccination titers were significantly different between the vaccinated and placebo groups ($p<0.0001$) (Table 3).

When H3N2 immunogenicity was assessed in each country by year, there was a significant elevation in mean $\log_{10}$ H3N2 HAI titers in PH and MZ in 2018, as well as PH and ZA in 2019 (Supplemental Table 3). Interestingly, there was a significant difference in the seroconversion against H1N1 and H3N2 within the vaccine group in both 2017 ($p <0.0001$) and 2019 ($p= 0.0002$) (Supplemental Table 4). In 2017, 73.75% (59/80) of vaccinated participants seroconverted to the H1N1 antigen. In contrast only 1.25% (1/80) seroconverted to the H3N2 strain (Supplemental Table 4). Similarly, in 2019, (30.91%) 64/207 of vaccinees seroconverted to H1N1 following vaccination, whereas only 14.97% (31/207) seroconverted to H3N2 (Supplemental Table 4).

Seasonal vaccine effectiveness against Influenza A Virus across multiple seasons

Influenza vaccine efficacy (VE) is known to vary substantially from season-to-season. Efficacy generally ranges from 40-60 %, although in season with antigenic mismatch, efficacy has been observed as low as 10 %[14]. Evaluation of VE in RCTs is often determined using PCR-confirmation of infection, however, 4-fold rise in antibodies in paired samples can also be used as a serological

surrogate of infection[15,16]. Here, we defined a ≥4-fold increase between post-vaccination and end of season (T3/T2) paired samples as serological evidence of infection. Vaccine efficacy was then calculated as (1- Relative Risk(RR)) x 100%. In the current study VE was not adjusted for age, sex, etc. In 2017-2018, the unadjusted VE (95% CI) estimate against any influenza A subtype was 91.5% (50.3-98.6). In 2018-2019, VE was 72.29% (30.0-89.24) and in 2019-2020, the VE was estimated at 86.2% (63.2-94.9) (Table 4).

Other studies have demonstrated that using serological confirmation of infection in an IIV cohort can result in an overestimation of VE. This is hypothesized to result from participants reaching an 'antibody ceiling' following vaccination, such that titers are not significantly boosted even after infection[16]. Thus, for population level estimates of attack rate, a 2-fold increase in antibody titers was suggested to more accurately estimate rates of infection[17]. We therefore also estimated VE in our cohort using a 2-fold increase between T3/T2 as the cut-off. Using this method IIV still demonstrated efficacy in all seasons against both influenza A subtypes (2017: 28.29 (-16.0-55.8), 2018: 51.32 (28.04-67.36), 2019: 71.31 (59.11-80.13)) (Table 5). However, wide confidence intervals were observed in 2017 which may suggest variability between participants.

VE was also assessed against each influenza A subtype individually. Using the 4-fold seroconversion threshold, VE against H1N1 in 2017-2018 was 90.6% (44.3-98.5), in 2018-2019 85.22% (8.19-97.65), and 78.45% (13.0-94.5) in 2019-2020 (Table 6). The estimated VE against H3N2 in 2017 was estimated at 100 (-2.248-100), though the wide range of confidence interval and low H3N2 infection rate makes this estimate unreliable (Table 6). In 2018 the VE was 64.53%

(-4.2-88.02%) against H3N2 and 89.8% (61.4-97.3) in 2019 (Table 6). The same analysis was also completed with a 2-fold cut-off (Supplemental Table 5).

In the placebo group during the 2017-2018 season, most infections were caused by H1N1, 12.86% (9/70) relative to H3N2 infection 1.43% (1/70) (p=0.0173) (Supplemental Table 6). This observation aligns with FluNet data, which shows that that majority of influenza A specimens collected in PH were H1N1 from July 2017- September 2017[18]. In 2018-2019 and 2019-2020 the number of infections caused by H1N1 or H3N2 were not significantly different in the placebo group (Supplemental Table 6). For the 2018 seasons this is consistent with surveillance data, which demonstrated waves of H1N1 and H3N2 in MZ and NG. Although, A/H1N1 dominated in PH. In 2019 there was a non-significant difference (p=0.0756) in H3N2 infections (19/191) relative to H1N1 (9/191) (Supplemental Table 6). In 2/4 countries surveyed, MZ and NG, H3N2 was the dominant IAV strain. During the 2019 season PH experienced an IBV dominant year and in NG H1N1 predominated[10].

Impact of vaccine immunogenicity on cardiovascular events

We next sought to investigate whether there were differences in vaccine immunogenicity in participants who were vaccinated but had a cardiac outcome, compared to individuals who did not. Individuals who had an event between their T2 and T3 blood draw were included in this analysis. Seven participants in the vaccine group and four in the placebo group had a 'cardiac outcome' defined as, hospitalization for heart failure, stroke, myocardial infarction, all cause death, or pneumonia. When looking at responses against H1N1, seroconversion rates (T2/T1) against the vaccine antigen in vaccinated individuals who had a cardiovascular event were similar to those

observed for the whole cohort (4/7, 57.14%, data not shown). However, the overall magnitude of the post-vaccine HAI titers in vaccinated participants who had an event (GMT (95% CI), 40.00 (16.16-99.03)) was reduced relative to vaccinated, non-event participants (GMT (95% CI), 100.1 (90.37-110.9)) (Figure 4A). The absolute titer of H1N1 HAI titers at the T3 time point was also significantly lower in patients who had an event (GMT (95% CI), 14.86 (6.57-33.59)) and was similar to the H1 T3 titers of placebo participants who did not have a cardiac event ((GMT (95% CI), 14.64 (13.11-16.36)) (Figure 4A).

The overall magnitude of H3N2 HAI titers was lower than those against H1N1 in both the placebo and vaccinated groups. No differences in H3N2 HAI titers were observed across groups irrespective of event status (Figure 4B).

Factors associated with serological antibody responses to seasonal influenza vaccination

The impact of factors such as age, sex, and pre-existing HAI titers on both absolute post-vaccination titers and fold change in HAI titers was assessed using linear regression. Neither age nor sex were associated with vaccine responses (Table 7). Pre-existing absolute HAI titers were significantly associated with post-vaccination HAI titers both H1N1 and H3N2, in all years (Table 7). In line with existing literature, pre-existing HAI titers demonstrated a significant negative association with fold change in antibodies against both H1N1 and H3N2 vaccine antigen across all years of the study (Table 7). These findings suggest that individuals with high titers of circulating antibody at baseline generate higher absolute titers following vaccination. However, high pre-existing antibodies can blunt the magnitude of the post-vaccination response.

Along these lines, mean $\log_{10}$ T2 H1N1 or H3N2 HAI titers were negatively correlated with $\log_{10}$ fold change in T3/T2 antibodies across all years of the study (Table 8). Increased titers between vaccination and the 6-month follow-up are indicative of infection. This observation agrees with longstanding evidence that high absolute HAI titers following vaccination are protective against infection[19].

DISCUSSION

Patients with cardiovascular disease represent a high-risk group for developing severe outcomes following influenza virus infection. In the recently completed IVVE trial, seasonal vaccination was demonstrated to reduce the risk of several cardiac events, including cardiovascular death, non-fatal myocardial infarction, and stroke[10]. However, direct evidence of vaccine efficacy in this cohort was not assessed. We therefore sought to evaluate the immunogenicity of IIV in this study cohort to further establish the direct role of vaccine mediated protection from influenza infection in reducing cardiac events in vaccinated participants. We also assessed whether immunogenicity and efficacy of seasonal influenza vaccine differed across countries, or in individuals who suffered a cardiac event compared to those who did not.

Using HAI to assess immunogenicity, H1N1 antibodies increased significantly in the vaccine group post-vaccination during all three seasons. The overall magnitude of H3N2 HAI responses were lower in vaccinees compared to H1N1 titers. In 2017 no meaningful increase in H3N2 antibodies was observed following vaccination. However, in 2018-2019 and 2019-2020 significant increases were observed in H3N2 HAI titers post-vaccination.

The vaccine was efficacious in all seasons when protection was assessed against either influenza A subtype. This result was consistent regardless of defining serological evidence of infection as a $\geq$4-fold increase or $\geq$2-fold increase in HAI titers between T2 and T3. These findings support the conclusions of the primary IVVE trial and suggest that vaccine mediated protection limited influenza virus infections in the vaccinated group resulting in a reduction in cardiac events.

We were interested in evaluating whether the response to vaccination might differ in participants who had a cardiovascular event when compared to those who did not. Rates of seroconversion following vaccination did not seem to be impaired. However, the GMT of HAI antibodies in participants who had a cardiovascular event was reduced post-vaccination and significantly different from patients who did not have an event at the 6-month timepoint. This effect was particularly apparent for H1N1 HAI titers.

An HAI titer of 1:40 is classically regarded as a correlate of protection against influenza infection, although more recent studies suggest that higher levels more accurately predict protection[20]. One study demonstrated that 1:110 may more be a more accurate titer for 50% protection[20]. The vaccinated participants who had a cardiac event just met this threshold post-vaccination, and fell below it by the 6 month timepoint (T3), likely leaving them more susceptible to infection. It is unclear whether these modest HAI titers were a result of some underlying condition, a result of their exposure history (lower baseline antibody titers), or simply stochastic variation in the overall magnitude of their vaccine response. However, these results suggest that low antibody titers to seasonal influenza viruses is a risk factor for cardiac events in individuals with a history of cardiovascular disease.

The modest immunogenicity of the H3N2 component of the seasonal vaccine and relatively low efficacy of protection against that subtype in our study corresponds with the plethora of real-world data demonstrating considerable challenges in mounting efficacious vaccine responses against contemporary H3N2 strains[21]. The reduced efficacy against H3N2 strains may be due, in part, from the immense antigenic diversity of recent H3N2 clades[22]. Co-circulation of multiple subclades in the same region can impact H3N2 vaccine efficacy. Indeed, antigenic mismatch in the 2019 H3N2 dominated season was observed in Mozambique and Zambia during our study[10]. Reduced VE against H3N2 in recent years has also been attributed to antigenic imprinting, namely changes in antigenic site B in contemporary H3N2 strains relative to those that circulated historically[23–25]. Given that the mean age of our vaccinated cohort was 52.25, these individuals likely had early life exposure to viral strains with S159. The H3N2 vaccine strains used in this study belong to the 3C2.a clade which has Y at position 159. Egg-based production methods have also been hypothesized to reduce the antigenicity of H3N2 vaccine antigens by imparting egg-adaptions[26]. Although vaccines responses were lower against H3N2 compared to H1N1, the vaccine remained efficacious in our analysis.

Prevention of PCR-confirmed infection is the gold standard for evaluating VE in influenza vaccine RCTs. However, serological surrogates of infection have also been used to estimate VE[16]. A recent comparison of efficacy endpoints in IIV and LAIV recipients demonstrated that using serological endpoints in vaccinated individuals can result in an overestimated VE[16]. This is hypothesized to result from an ‘antibody ceiling’, where high post-vaccination titers blunt re-seroconversion following viral infection. In placebo and LAIV recipients, PCR and serological infection aligned

more closely. However, only 23% of IIV participants with PCR confirmed infection showed serological evidence of infection[16]. Similarly, using a 4-fold increase in HAI titers to estimate infection in our study resulted in VE estimates that were well above the CDC estimates of effectiveness for the same seasons[14]. It is common for VE of seasonal influenza vaccines to range from 10-60%. For population level analyses, a 2-fold increase in titers has been demonstrated to reasonably estimate attack rates[17]. This has been hypothesized to help account for patient-to-patient variations in factors like length of time from infection. To address this dichotomy, we also assessed VE in our cohort using a $\geq$-2-fold change between T2 and T3 as the cut-off. In our study, both 2- and 4-fold cut-off values suggested efficacy of the vaccine, though VE estimates using a 2-fold cut-off are more in line with reported values.

Interestingly, the IVVE study reported that the number of first co-primary events in the Philippines was higher in the vaccinated group (7.2%) relative to the placebo (5.6%), despite the fact that overall, a significant reduction in cardiac outcomes in the vaccinated group was observed. In this report, 2017 data reflects samples from the Philippines alone. When VE was analyzed using a 2-fold increase in titers between T2 and T3, we observed that 2017 had the lowest VE against both influenza A subtypes (28.29 (-16.0 - 55.8)). Additionally, when VE was assessed against H3N2, 2017 was the only year in which no benefit of the vaccine was observed (-6.7 (-110.9-45.6)). The poor efficacy of the vaccine in the Philippines in 2017 may in part explain why cardiac events were not reduced in the vaccinated group for this location.

Given the observation that vaccinated patients who had cardiac events tended to have lower HAI titers, it would be interesting to explore in the future whether there are underlying risk factors (e.g.,

genetics, drugs used to treat cardiovascular disease, etc.) in a subset of individuals with cardiovascular disease that lead to impaired vaccine mediated responses. It may also be useful to consider whether "enhanced" vaccine formulations (i.e., high dose or adjuvanted) might benefit these individuals[27].

In conclusion, we demonstrate that IIV was immunogenic and efficacious in high-risk individuals with underlying cardiovascular disease. In agreement with the original IVVE trial, our results reinforce that seasonal influenza vaccination reduces the risk of cardiovascular events in this high-risk cohort by preventing infection. Regular seasonal vaccination in patients with underlying cardiovascular conditions should therefore be strongly recommended.

METHODS

Study design and participants

In this secondary analysis we assessed 673 participants who were enrolled in the IVVE randomized control trial (ClinicalTrials.gov: NCT02762851) between 2017 and 2019 (Supplemental Table 1). The original IVVE trial compared whether seasonal influenza vaccination reduced death and cardiovascular events in a heart failure cohort. Randomisation, masking, and establishment of outcomes are described in the in the main trial manuscript[11]. Briefly, 5129 participants from ten countries were randomly assigned to the vaccine (n=2560) or placebo groups (n=2560). Vaccinees received one dose intramuscularly of a standard dose inactivated influenza vaccine (Sanofi Pasteur- VAXIGRIP) or saline intramuscularly if in the placebo branch. Participants in the vaccine group received IIV with the WHO recommended strains relevant to the hemisphere of the study site. Across all years and sites included in this secondary analysis the H1N1 vaccine strain was

A/Michigan/45/2015(H1N1)- like virus. The H3N2 vaccine component changed across seasons. In 2017-2018 the H3N2 vaccine component was A/Hong Kong/4801/2014 (H3N2), in 2018-2019 A/Singapore/2016 (H3N2), and in 2019-2020 A/ Switzerland/8060/2017 (H3N2). The first outcome investigated was a composite of cardiovascular death, non-fatal myocardial infarction, non-fatal stroke and hospitalization for heart failure. Secondary outcomes included individual events as well as all cause death, pneumonia and all cause hospitalization. Events were assessed every 6 months during the whole study period and periods of peak influenza circulation. For the current event analysis, cardiac events were stratified to those occurring between T2 and T3. A cardiac event was any one of: hospitalization for heart failure, myocardial infarction, pneumonia or all-cause death.

Sample collection

Blood samples were drawn from a subset of each treatment group at baseline (T1), ~4-weeks post vaccination (T2), and ~6 months post vaccination (T3). For the current study serological samples from 2 sites in PH, 1 site in ZA, 1 in MZ, and 1 in NG were used, totalling 673 participants. Serological samples were obtained following vaccination in 2017-2018, 2018-2019 and 2019-2020.

Cells

Madin Darby Canine Kidney (MDCK) were grown in Earles minimum essential medium (Earles MEM) containing 10% fetal bovine serum (FBS) (Gibco), 2 mM L- glutamine, 100 U/mL penicillin-streptomycin (Thermo Fisher), 1mM hepes (Sigma Aldrich), and sodium pyruvate (Sigma Aldrich).

Viruses

A/Singapore/2016 (H3N2) and A/Michigan/45/2015(H1N1)- were propagated in 11- day- old embryonated chicken eggs as per standard protocols[28]. A/Hong Kong/4801/2014 (H3N2) and A/ Switzerland/8060/2017 (H3N2) were propagated in MDCK cells. Briefly, at 100% confluency MDCK cells were infected for one hour at MOI 0.01, in 1x minimum essential medium (MEM, Sigma Aldrich) supplemented with 2 mM L-glutamine, 0.24% sodium bicarbonate, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MEM amino acids solution (Sigma Aldrich), MEM vitamins solution (Sigma Aldrich), 100 U/mL penicillin-streptomycin (Thermo Fisher), 0.42% bovine serum albumin (Sigma Aldrich), and 0.5 mg/ml trypsin (Sigma Aldrich). Media was replaced with media containing 0.5 mg/ml trypsin (Sigma Aldrich). Cells were left for 72 hours, or until significant cytopathic effects were observed, supernatant was then collected.

Hemagglutination inhibition assay

HAI assay was performed according to standard WHO procedure and as described previously[28,29]. Briefly, serum was inactivated using trypsin-heat-periodate treatment prior to HAI assay. From 20µl serum starting amount, samples were treated with 0.5 times starting volume of 8mg/ml TPCK-trypsin (Sigma-Aldrich) and incubated at 56°C for 30 minutes. Samples were cooled to RT and 3 volumes of 11mM potassium periodate solution (Sigma-Aldrich) was added. After 15 minutes of incubation at RT, 3 volumes of 1% glycerol-PBS solution was added and incubated for another 15 minutes at RT. Lastly, 2.5 volumes of 0.85% PBS was added to the sample. Virus and serum were incubated for 30 minutes at RT. Chicken red blood cells were then added to the wells and plates were incubated at 4°C for 45 minutes prior to reading. HAI titers were defined as the

highest serum dilution factor with HAI activity; samples with no detectable HAI activity were assigned as half the limit of detection of the assay, "5".

Statistical Analysis

We used X^2 with Fisher exact test to compare categorical data. Linear regression, excluding variable interactions was performed in SPSS. ANOVA with Tukey's multiple comparisons was used to compare log transformed HAI titers. A p-value <0.05 indicates statistical significance. Vaccine efficacy was estimated by first determining the RR (95% CI) of infection by $X^{2,}$ with Fisher exact test. VE was then calculated as (1-RR) x100, estimates were not adjusted for age or gender. All statistics were performed with either IBM SPSS Software version 29.0 or GraphPad version 9.5.0. Data visualization was performed by GraphPad version 9.5.0.

Contributors

HDS, SY, ML and MSM conceived the study. ML and MSM were responsible for funding acquisition. HDS and JCA performed the experiments. PS and SY performed data curation. HDS and SY performed the data analysis. HDS wrote the original draft of the manuscript and all authors helped to critically review and edit for final submission.

Declaration of Competing Interests

The authors declare no competing interests.

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References

1. WHO | Influenza (Seasonal). *WHO* http://www.who.int/mediacentre/factsheets/fs211/en/ (2018).
2. WHO- The Top 10 Causes of Death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (2020).
3. Nguyen, J. L. *et al.* Seasonal influenza infections and cardiovascular disease mortality. *JAMA Cardiol.* **1**, 274–281 (2016).
4. Smeeth, L. *et al.* Risk of Myocardial Infarction and Stroke after Acute Infection or Vaccination. *N. Engl. J. Med.* **351**, 2611–2618 (2004).
5. Warren-Gash, C., Smeeth, L. & Hayward, A. C. Influenza as a trigger for acute myocardial infarction or death from cardiovascular disease: a systematic review. *Lancet Infect. Dis.* **9**, 601–610 (2009).
6. Kwong, J. *et al.* Acute Myocardial Infarction after Laboratory-Confirmed Influenza Infection. *N. Engl. J. Med.* 345–353 (2018) doi:10.1056/NEJMoa1702090.
7. Behrouzi, B., Bhatt, D. L., Cannon, C. P., Vardeny, O. & Lee, D. S. Association of Influenza Vaccination With Cardiovascular Risk. **5**, 1–9 (2022).
8. Clar, C. *et al.* Influenza vaccines for preventing cardiovascular disease (Review). *Cohrane Rev.* (2015) doi:10.1002/14651858.CD005050.pub3.www.cochranelibrary.com.
9. Yedlapati, S. *et al.* Effects of Influenza Vaccine on Mortality and Cardiovascular Outcomes in Patients With Cardiovascular Disease : A Systematic. (2021) doi:10.1161/JAHA.120.019636.
10. Loeb, P. M. *et al.* Influenza vaccine to reduce adverse vascular events in patients with

heart failure : a multinational randomised ,. *Lancet Glob. Heal.* **10**, e1835–e1844.

11. Loeb, M. *et al.* Randomized controlled trial of influenza vaccine in patients with heart failure to reduce adverse vascular events (IVVE): Rationale and design. *Am. Heart J.* **212**, 36–44.
12. Trombetta, C. M. & Montomoli, E. Influenza immunology evaluation and correlates of protection: a focus on vaccines. *Expert Rev. Vaccines* **15**, 967–976 (2016).
13. Ng, S. *et al.* Novel correlates of protection against pandemic H1N1 influenza A virus infection. *Nat. Med.* **25**, 962–967 (2019).
14. CDC. CDC Seasonal Flu Vaccine Effectiveness Studies. https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm.
15. Weinberg, G. A. *et al.* Superiority of Reverse-Transcription Polymerase Chain Reaction to Conventional Viral Culture in the Diagnosis of Acute Respiratory Tract Infections in Children. *J. Infect. Dis.* **189**, 706–710 (2004).
16. Petrie, J. G., Ohmit, S. E., Johnson, E., Cross, R. T. & Monto, A. S. Efficacy studies of influenza vaccines: Effect of end points used and characteristics of vaccine failures. *J. Infect. Dis.* **203**, 1309–1315 (2011).
17. Cauchemez, S. *et al.* Influenza Infection Rates, Measurement Errors and the Interpretation of Paired Serology. *PLoS Pathog.* **8**, (2012).
18. WHO- Influenza Laboratory Surveillance Information Virus Detections by Subtype Reported to FluNet. https://app.powerbi.com/view?r=eyJrIjoiZTkyODcyOTEtZjA5YS00ZmI0LWFkZGUtODIxNGI5OTE3YjM0IiwidCI6ImY2MTBjMGI3LWJkMjQtNGIzOS04MTBiLTNkYzI4MGFmYjU5MCIsImMiOjh9.

19. Andrews, S. F. *et al.* High Preexisting Serological Antibody Levels Correlate with Diversification of the Influenza Vaccine Response. *J. Virol.* **89**, 3308–3317 (2015).

20. Black, S. *et al.* Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza Vaccines in children. *Pediatr. Infect. Dis. J.* **30**, 1081–1085 (2011).

21. Russell, K. *et al.* Influenza vaccine effectiveness in older adults compared with younger adults over five seasons. *Vaccine* **36**, 1272–1278 (2018).

22. Bedford, T. *et al.* Integrating influenza antigenic dynamics with molecular evolution. *Elife* **3**, (2014).

23. Kissling, E. *et al.* Low 2018/19 vaccine effectiveness against influenza A(H3N2) among 15–64-year-olds in Europe: Exploration by birth cohort. *Eurosurveillance* **24**, 1–12 (2019).

24. Zost, S. J. *et al.* Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 12578–12583 (2017).

25. Gouma, S. *et al.* Middle-aged individuals may be in a perpetual state of H3N2 influenza virus susceptibility. *Nat. Commun.* **11**, 1–8 (2020).

26. Lin, Y. *et al.* The characteristics and antigenic properties of recently emerged subclade 3C.3a and 3C.2a human influenza A(H3N2) viruses passaged in MDCK cells. *Influenza Other Respi. Viruses* **11**, 263–274 (2017).

27. Li, A. P. Y. *et al.* Immunogenicity of standard, high-dose, MF59-adjuvanted, and recombinant-HA seasonal influenza vaccination in older adults. *npj Vaccines* **6**, 1–12 (2021).

28. Who. Manual for the laboratory diagnosis and virological surveillance of influenza. *World*

Heal. Organ. 2011 153 (2011).

29. Miller, M. S. *et al.* 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. *J. Infect. Dis.* **207**, 98–105 (2013).

Figure Legends:

Figure 1- Secondary analysis study profile. A schematic breakdown of participant numbers by intervention group, year, and country.

Figure 2- Timeline of serum collection for participants. Blood was drawn from participants in the vaccine and placebo group at three timepoints; T1- baseline, T2- approximately 14 days post-vaccination, and T3- approximately 6 months after vaccination which corresponded to the end of the influenza season.

Figure 3- H1N1 and H3N2 HAI titers by study year, across all included study sites. A, C, E. Bar graphs representing the GMTs of H1N1 HAI antibodies of individuals in the placebo and vaccine group pre (T1) and post- vaccination (T2) in 2017-2018 (A), 2018-2019 (C), and 2019-2020 (E). **B, D, F.** Bar graphs representing the GMTs of H3N2 HAI antibodies of individuals in the placebo and vaccine group pre (T1) and post- vaccination (T2) in 2017-2018 (A), 2018-2019 (C), and 2019-2020 (E). Bars reflect GMTs with 95% CIs. GMT= geometric mean titer.

Figure 4- Magnitude of HAI titers in participants who had cardiac event versus those who did not. A. Bar graph representing the GMTs of H1N1 HAI antibodies of individuals in the placebo and vaccine group who did or did not have a cardiac event within the 6-month period post vaccination. **B.** Bar graph representing the GMTs of H3N2 HAI antibodies of individuals in the placebo and vaccine group who did or did not have a cardiac event within the 6-month period post vaccination**. A+ B**. Participants who had one of all cause death, non-fatal myocardial infarction, non-fatal stoke, pneumonia between T2 and T3 were included as an 'event'. Bars represent GMTs with 95% CI of HAI titers across all study years and countries. GMT= geometric mean titer.

	All (n=673)	Influenza Vaccine (n=345)	Placebo (n=328)
All Regions			
Mean age, years	52.64 (15.9)	52.25 (15.15)	53.05 (16.67)
Sex			
Female	480 (71.3%)	247 (71.6%)	233 (71%)
Male	193 (28.7%)	98 (28.4%)	95 (29%)
Mozambique	**(n=164)**	**(n=83)**	**(n=81)**
Mean age, years	44.17 (16.45)	45.43 (16.55)	42.88 (16.36)
Sex			
Female	120 (73.2%)	63 (75.9%)	57 (70.4%)
Male	44 (26.8%)	20 (24.1%)	24 (29.6%)
Philippines	**(n=275)**	**(n=146)**	**(n=129)**
Mean age, years	57.48 (12.08)	56.70 (11.35)	58.36 (12.85)
Sex			
Female	192 (69.8%)	100 (68.5%)	92 (71.3%)
Male	83 (30.2%)	46 (31.5%)	37 (28.7%)
Zambia	**(n=150)**	**(n=75)**	**(n=75)**
Mean age, years	56.09 (15.14)	54.08 (14.55)	58.09 (15.55)
Sex			
Female	111 (74.0%)	58 (77.3%)	53 (70.7%)
Male	39 (26.0%)	17 (22.7%)	22 (29.3%)
Nigeria	**(n=84)**	**(n=41)**	**(n=43)**
Mean age, years	47.15 (18.72)	46.80 (18.65)	47.49 (19.00)
Sex			
Female	57 (67.6%)	26 (63.4%)	31 (72.1%)
Male	27 (32.1%)	15 (36.6%)	12 (27.9%)

Table 1- Baseline participant characteristics in all study sites, independent of year. Data is mean (SD) or n/N (%).

	H1 Titer Influenza Vaccine			H1 Titer Placebo		
	T1	T2	GMFR (95% CI) or p value	T1	T2	GMFR (95% CI) p value
2017 HAI Titer						
GMT (95% CI)	13.78 (10.99-17.28)	125.2 (93.01-168.6)	9.11 (6.47-12.81)	12.23 (9.51-15.71)	15.16 (11.50-19.98)	1.24 (1.02-1.52)
Mean of log titer (SD)	1.139 (0.44)	2.098 (0.58)	<0.0001	1.087 (0.45)	1.181 (0.50)	0.6919
≥4-fold increase		59/80 (73.75%)			5/69(7.24%)	<0.0001
2018 HAI Titer						
GMT (95% CI)	30.77 (25.60-36.97)	86.13 (72.78-101.9)	2.80 (2.32-3.36)	12.30 (10.54-14.36)	12.81 (10.84-15.13)	1.04 (0.95-1.13)
Mean of log titer (SD)	1.48 (0.52)	1.93 (0.48)	<0.0001	1.09 (0.42)	1.10 (0.45)	0.9881
≥4-fold increase		59/169 (34.91%)			6/154 (3.89%)	<0.0001
2019 HAI Titer						
GMT (95% CI)	45.12(38.68-52.64)	98.79 (86.32-113.1)	2.189 (1.90-2.51)	14.33 (12.37-16.62)	14.43 (12.40-16.79)	1.007 (0.93-1.08)
Mean of log titer (SD)	1.65(0.48)	1.99(0.42)	<0.0001	1.15(0.46)	1.15 (0.47)	>0.9999
≥4-fold increase		64/207 (30.91%)			5/206 (2.41%)	<0.0001

Table 2- H1N1 HAI titers and vaccine seroconversion in all study years. Data is GMTs (95% CI) of HAI antibodies, mean of log transformed titers (SD), and n/N(%) with a 4-fold increase in T2/T1. A p-value <0.05 was significant. GMT= geometric mean titer, GMFR= geometric mean titer fold ratio, SD= standard deviation.

	H3 Titer Influenza Vaccine			H3 Titer Placebo		
	T1	T2	GMFR (95% CI) or p value	T1	T2	GMFR (95% CI) or p value
2017 HAI Titer						
GMT	16.96 (15.41-18.67)	18.85 (17.17-20.69)	1.119 (1.01-1.24)	17.91 (15.78-20.32)	19.22 (17.12-21.58)	1.07 (0.93-1.22)
Mean of log titer (SD)	1.23 (0.18)	1.27 (0.18)	0.4743	1.25 (0.22)	1.28 (0.21)	0.8069
≥4-fold increase		1/80 (1.25%)			2/69 (2.89%)	0.5964
2018 HAI Titer						
GMT	20.58 (17.83-23.76)	40.83 (34.77-47.94)	1.984 (1.706-2.307)	14.27 (12.63-16.13)	15.76 (13.78-18.01)	1.104 (1.002-1.217)
Mean of log titer (SD)	1.31 (0.41)	1.61 (0.45)	<0.0001	1.15 (0.33)	1.19 (0.36)	0.7775
≥4-fold increase		50/169 (29.58%)			10/154 (6.49%)	<0.0001
2019 HAI Titer						
GMT	28.33 (25.90-30.99)	44.23 (39.87-49.06)	1.56 (1.41-1.72)	21.83 (20.07-23.74)	23.58 (21.43-25.96)	1.080 (0.998-1.17)
Mean of log titer (SD)	1.45 (0.28)	1.64 (0.32)	<0.0001	1.33 (0.26)	1.37 (0.30)	0.6574
≥4-fold increase		31/207 (14.97%)			8/206 (3.88%)	0.0001

Table 3- H3N2 HAI titers and vaccine seroconversion in all study years. Data is GMTs (95% CI) of HAI antibodies, mean of log transformed titers (SD), and n/N(%) with a 4-fold increase in T2/T1. A p-value <0.05 was significant. GMT= geometric mean titer, GMFR= geometric mean titer fold ratio, SD= standard deviation.

	Any Infection Influenza Vaccine	Any Infection Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥4-fold increase	1/82 (1.21%)	10/70 (14.29%)	0.0028	0.0740 (0.006-0.458)	0.085 (0.014-0.497)	91.5 (50.3-98.6)
2018						
≥4-fold increase	5/150 (3.33%)	16/133 (12.0%)	0.0061	0.2522 (0.09931-0.7)	0.2771 (0.1076-0.7)	72.29 (30.0-89.24)
2019						
≥4-fold increase	4/197 (2.03%)	28/191 (14.66%)	<0.0001	0.120 (0.044-0.346)	0.138 (0.051-0.368)	86.2 (63.2-94.9)

Table 4- Vaccine efficacy estimates against both A/H1N1 and A/H3N2 subtypes, using 4-fold change in T3/T2 antibodies as serological evidence of infection. Data is n/N (%), OR (95% CI), RR (95% CI), and VE= (1-RR)x100. OR= odds ratio, RR= relative risk, VE= vaccine efficacy.

	Any Infection Influenza Vaccine	Any Infection Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥2-fold increase	21/82 (25.60%)	25/70 (35.71%)	0.2157	0.6197 (0.303-1.236)	0.7171 (0.442-1.16)	28.29 (-16.0-55.8)
2018						
≥2-fold increase	28/150 (18.67%)	51/133 (38.34%)	0.0003	0.3690 (0.2195-0.6385)	0.4868 (0.3264-0.7196)	51.32 (28.04-67.36)
2019						
≥2-fold increase	29/197 (14.72%)	98/191 (51.30%)	<0.0001	0.1638 (0.0994-0.2671)	0.2869 (0.1987-0.4089)	71.31 (59.11-80.13)

Table 5 - Vaccine efficacy estimates against both A/H1N1 and A/H3N2 subtypes, using 2-fold change in T3/T2 antibodies as serological evidence of infection. Data is n/N (%), OR (95% CI), RR (95% CI), and VE= (1-RR)x100. OR= odds ratio, RR= relative risk, VE= vaccine efficacy.

	H1 Influenza Vaccine	H1 Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥4-fold increase	1/82 (1.21%)	9/70 (12.86%)	0.0059	0.083 (0.007-0.543)	0.094 (0.015-0.557)	90.6 (44.3-98.5)
2018						
≥4-fold increase	1/150 (0.67%)	6/133 (4.51%)	0.0541	0.142 (0.01230-0.884)	0.1478 (0.0235-0.9181)	85.22 (8.19- 97.65)
2019						
≥4-fold increase	2/197 (1.02%)	9/191 (4.71%)	0.0335	0.2074 (0.044-0.8068)	0.2155 (0.052-0.870)	78.45 (13.0-94.8)

	H3 Influenza Vaccine	H3 Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥4-fold increase	0/82 (0%)	1/70 (1.43%)	0.4605	0.000 (0.000-7.7683)	0.000 (0.000-3.248)	100 (-2.248-100)
2018						
≥4-fold increase	4/150 (2.67%)	10/133 (7.52%)	0.0966	0.3370 (0.1144-1.026)	0.3547 (0.1198-1.042)	64.53 (-4.2-88.02%)
2019						
≥4-fold increase	2/197 (1.02%)	19/191 (9.95%)	<0.0001	0.093 (0.212-0.364)	0.102 (0.027-0.386)	89.8 (61.4-97.3)

Table 6- Vaccine efficacy estimates against either A/H1N1 and A/H3N2 subtypes, using 4-fold change in T3/T2 antibodies as serological evidence of infection. Data is n/N (%), OR (95% CI), RR (95% CI), and VE= (1-RR)x100. OR= odds ratio, RR= relative risk, VE= vaccine efficacy.

	T2 H1 HAI Titer		T2/T1 H1 HAI Fold Change	
	B (95% CI)	p-value	B (95% CI)	p-value
2017				
Age	-0.008 (-0.020-0.004)			0.207
Sex (vs Female)	-0.274 (-0.565-0.018)			0.066
T1 H1 Titer	0.308 (0.016-0.601)	0.039	-0.692 (-0.984—0.399)	<0.001
2018				
Age	-0.003 (-0.008-0.002)			0.186
Sex (vs Female)	0.00 (-0.144-.144)			0.997
T1 H1 Titer	0.380 (0.246-0.514)	<0.001	-0.620 (-0.754-0.486)	<0.001
2019				
Age	0.00(-0.004-0.003)			0.824
Sex (vs Female)	-0.020 (-0.133-0.094)			0.735
T1 H1 Titer	0.468 (0.362-0.574)	<0.001	-0.532 (-0.638- (-)0.426)	<0.001

	T2 H3 HAI Titer		T2/T1 H3 HAI Fold Change	
	B (95% CI)	p-value	B (95% CI)	p-value
2017				
Age	-0.002 (-0.005-0.002)			0.319
Sex (vs Female)	-0.017 (-0.104-0.071)			0.703
T1 H3 Titer	0.424 (0.216-0.632)	<0.001	-0.576 (-0.784- (-)0.368)	<0.001
2018				
Age	-0.002 (-0.005-0.002)			0.319
Sex (vs Female)	-0.017 (-0.104-0.071)			0.703
T1 H3 Titer	0.424 (0.216-0.632)	<0.001	-0.576 (-0.784-(-)0.0368)	<0.001
2019				
Age	0.002 (0.00-0.005)			0.094
Sex (vs Female)	-0.055 (-0.144-0.034)			0.226
T1 H3 Titer	0.547 (0.407-0.687)	<0.001	-0.453 (-0.593-(-)0.313)	<0.001

Table 7- Factors associated with vaccine mediated protection. Data is β coeffient (95% CI), p-values >0.05 were considered significant.

	Log T3/T2 HAI Titer	
	Influenza Vaccine	**Placebo**
Log T2 HAI Titer		
2017		
H1N1	-0.546 (<0.001)	-0.188 (0.119)
H3N2	-0.428 (<0.001)	-0.508 (<0.001)
2018		
H1N1	-0.393 (<0.001)	-0.300 (<0.001)
H3N2	-0.414 (<0.001)	-0.206 (0.017)
2019		
H1N1	-0.321 (<0.001)	-0.249 (<0.001)
H3N2	-0.598 (<0.001)	-0.422 (<0.001)

Table 8 – Factors correlated with serological evidence of infection. Data is Pearson co-efficient with (2-tailed sig).

Figure 1- Secondary analysis study profile

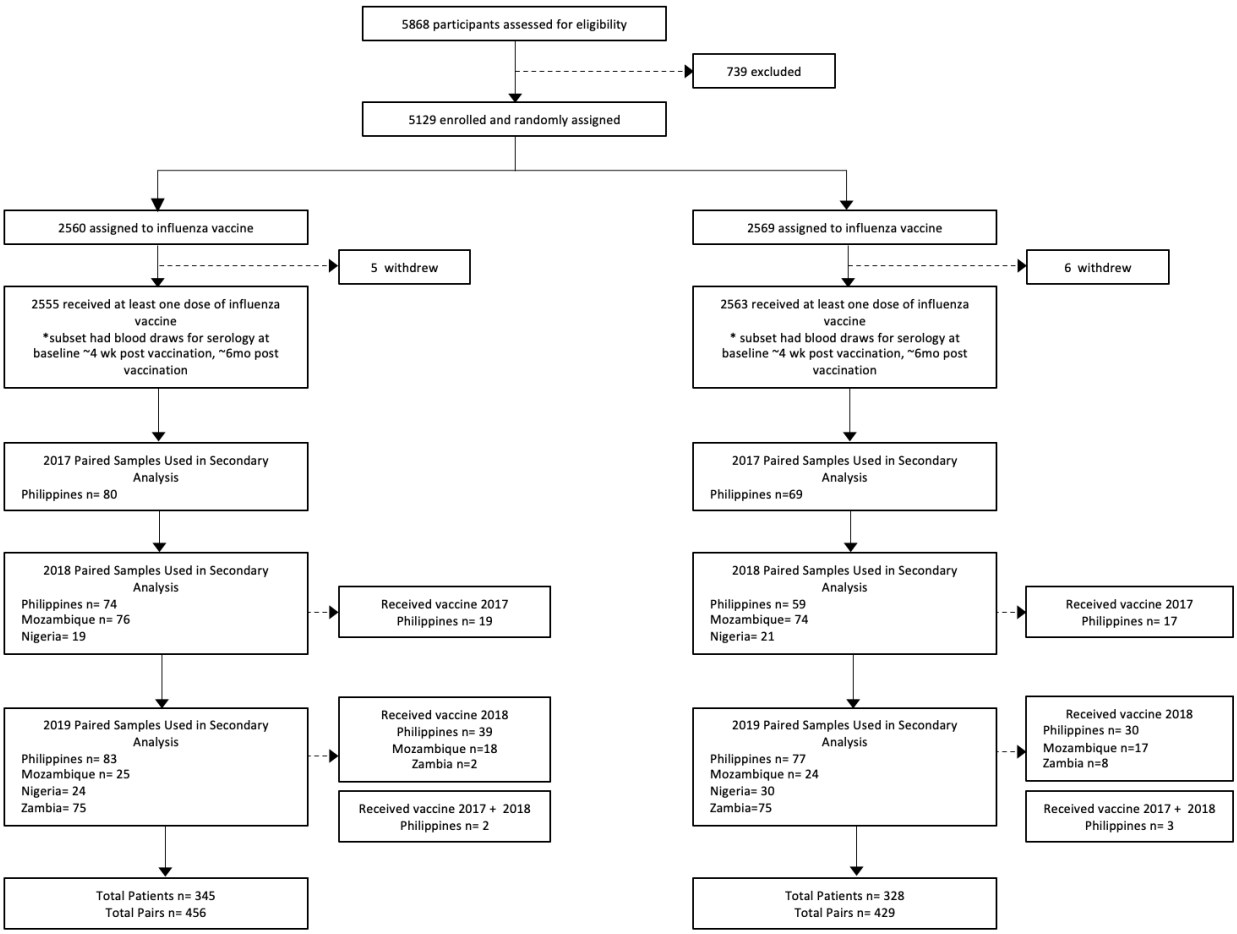


Figure 2- Timeline of serum collection timepoints for participants

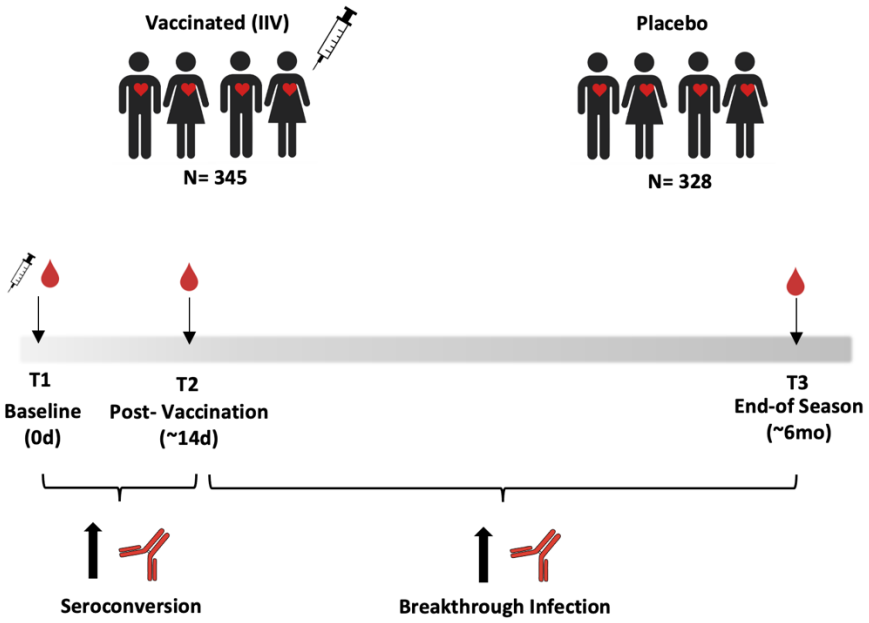


Figure 3- H1N1 and H3N2 HAI titers by study year, across all included study sites

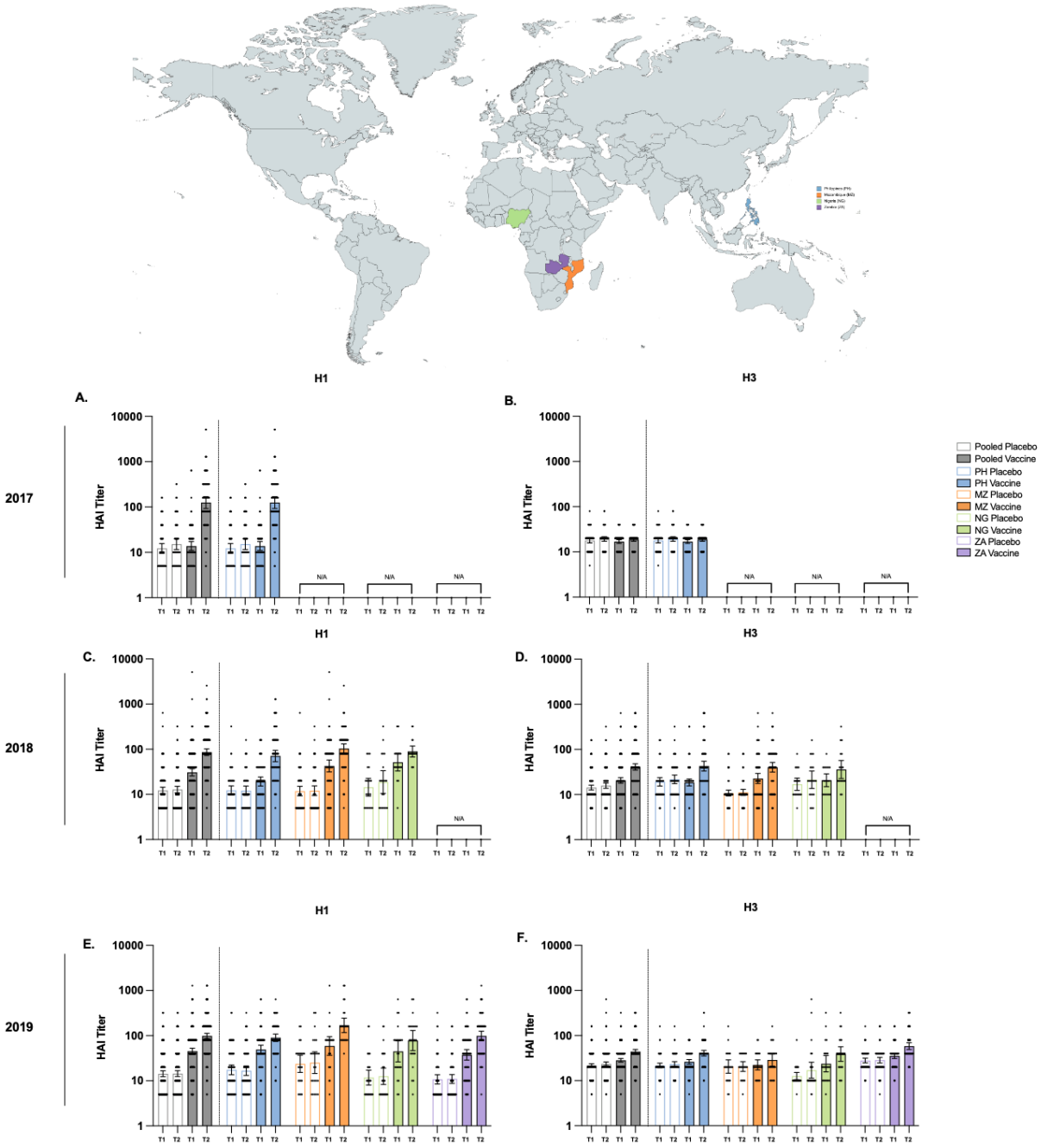
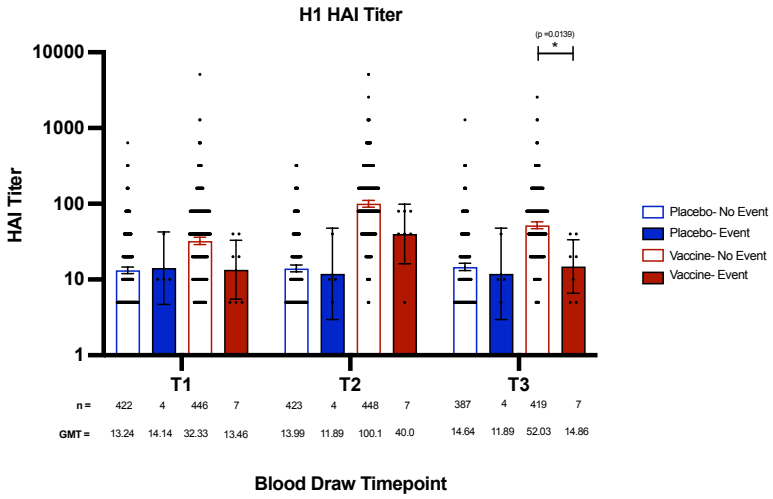
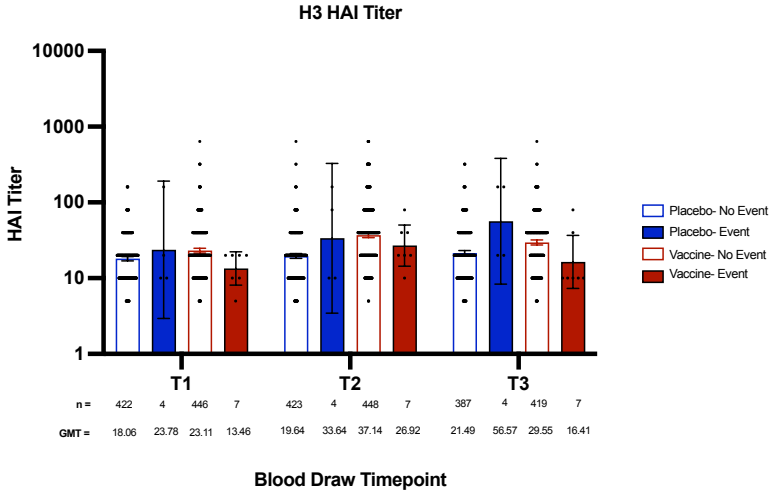


Figure 4- Magnitude of HAI titers in participants who had cardiac event versus participants who did not

A-



B-



	All	Influenza Vaccine	Placebo
2017			
Philippines	n=149	n=80	n=69
Mean age, years	58.17 (11.49)	57.79(10.83)	58.62(12.2)
Sex			
Female	110 (73.8%)	59 (73.8%)	51 (73.9%)
Male	39 (26.2%)	21 (26.3%)	18 (26.1%)
2018			
Philippines	n=133	n=74	n=59
Mean age, years	56.81 (12.48)	55.82(11.67)	58.05(13.43)
Sex			
Female	81 (60.9%)	42 (56.8%)	39 (66.1%)
Male	52 (39.1%)	32 (43.2%)	20 (33.9%)
Mozambique	n=150	n=76	n=74
Mean age, years	44.82(16.31)	46.08 (16.63)	43.53(15.98)
Sex			
Female	107 (71.3%)	56 (73.7%)	51 (68.9%)
Male	43 (28.7%)	20 (26.3%)	23 (31.1%)
Nigeria	n=40	n=19	n=21
Mean age, years	39.08 (13.19)	39.11 (12.7)	39.05 (13.93)
Sex			
Female	29 (72.5%)	13 (68.4%)	16 (76.2%)
Male	11 (27.5%)	6 (31.6%)	5 (23.8%)
2019			
Philippines	n=160	N=83	n=77
Mean age, years	57.63 (11.68)	56.08 (12.20)	59.29 (10.92)
Sex			
Female	117 (73.1%)	59(71.1%)	58 (75.3%)
Male	43 (26.9%)	24 (28.9%)	19 (24.7%)
Mozambique	n=49	n=25	n=24
Mean age, years	38.96 (15.89)	40 (14.9)	37.88 (17.09)
Sex			
Female	41 (83.7%)	21 (84%)	20 (83.3%)
Male	8 (16.3%)	4 (16%)	4 (16.7%)
Nigeria	n=54	n=24	n=30
Mean age, years	52.28 (19.45)	51.58 (20.69)	52.83 (18.73)
Sex			
Female	35 (64.8%)	15 (62.5%)	20 (66.7%)
Male	19 (35.2%)	9 (37.5%)	10 (33.3%)
Zambia	n=150	n=75	n=75
Mean age, years	56.09 (15.14)	54.08 (14.55)	58.09 (15.55)
Sex			
Female	111 (74%)	58 (77.3%)	53 (70.7%)
Male	39 (36%)	17 (22.7%)	22 (29.3%)

Supplemental Table 1- Baseline participant characteristics in all study sites and years. Data is mean (SD) or n/N (%).

	H1 Titer Influenza Vaccine			H1 Titer Placebo		
	T1	T2	GMFR or p value	T1	T2	GMFR or p value
2017 HAI Titer						
Philippines						
GMT (95% CI)	13.78 (10.99-17.28)	125.2 (93.01-168.6)	9.11 (6.47-12.81)	12.23 (9.51-15.71)	15.16 (11.50-19.98)	1.24 (1.02-1.52)
Mean of log titer (SD)	1.139 (0.44)	2.098 (0.58)	<0.0001	1.08 (0.45)	1.18 (0.5)	0.6919
≥4-fold increase		59/80 (73.75%)			5/69(7.24%)	<0.0001
2018 HAI Titer						
Philippines						
GMT (95% CI)	19.26 (15.33-24.21)	70.83 (53.17-94.35)	3.677 (2.71-4.97)	12.21 (9.67-15.41)	12.07 (9.52-15.29)	0.988 (0.869-1.123)
Mean of log titer (SD)	1.28 (0.42)	1.85 (0.53)	<0.0001	1.08 (0.38)	1.08 (0.39)	>0.9999
≥4-fold increase		37/74 (50%)			3/59 (5.08%)	<0.0001
Mozambique						
GMT (95% CI)	42.64 (31.56-57.60)	103.3 (81.16-131.4)	2.422 (1.85-3.16)	11.84 (9.32-15.03)	12.06 (9.40-15.46)	1.019 (0.936-1.108)
Mean of log titer (SD)	1.63 (0.57)	2.01(0.45)	<0.0001	1.07(0.44)	1.08(0.46)	0.9996
≥4-fold increase		19/76 (25%)			0/74 (0%)	<0.0001
Nigeria						
GMT (95% CI)	51.64 (32.98-80.85)	89.25 (67.54-117.9)	1.728 (1.225-2.440)	14.38 (9.13-22.62)	18.72 (10.47-33.47)	1.302 (0.803-2.112)
Mean of log titer (SD)	1.71(0.40)	1.95 (0.25)	0.3260	1.15(0.43)	1.27 (0.55)	0.8217
≥4-fold increase		3/19 (15.78%)			3/21 (14.28%)	>0.9999
2019 HAI Titer						
Philippines						
GMT (95% CI)	49.29 (39.45-61.58)	89.92 (74.67-108.3)	1.824 (1.555-2.141)	17.47 (13.7-22.29)	16.70 (13.11-21.29)	0.9560 (0.8679-1.053)
Mean of log titer (SD)	1.69 (0.44)	1.95 (0.36)	0.0008	1.24 (0.46)	1.22 (0.46)	0.9925
≥4-fold increase		21/83 (25.3%)			1/77 (1.29%)	<0.0001
Mozambique						
GMT (95% CI)	58.97 (36.67-94.83)	169.1 (117.0-244.5)	2.868 (1.734-4.744)	23.78 (15.28-37.01)	25.20 (14.53-43.69)	1.059 (0.8188-1.371)
Mean of log titer (SD)	1.77 (0.49)	2.22 (0.38)	0.006	1.37 (0.45)	1.4 (0.56)	0.9979
≥4-fold increase		9/25 (36%)			2/24 (8.33%)	0.0374
Nigeria						
GMT (95% CI)	44.90 (26.25-76.79)	77.72 (46.48-130.0)	1.731 (1.039-2.884)	11.76 (8.018-17.23)	12.60 (8.31-19.09)	1.072 (0.7612-1.509)
Mean of log titer (SD)	1.65 (0.55)	1.89 (0.52)	0.3540	1.07 (0.44)	1.1 (0.48)	0.9955
≥4-fold increase		6/24 (25%)			2/30 (6.66%)	0.1195
Zambia						
GMT (95% CI)	37.49 (28.65-49.06)	98.95 (78.15-125.3)	2.639 (2.038-3.417)	10.77 (8.50-13.62)	10.97 (8.74-13.75)	1.019 (0.9460-1.097)
Mean of log titer (SD)	1.57 (0.50)	1.99 (0.44)	<0.0001	1.03 (0.44)	1.04 (0.42)	0.9996
≥4-fold increase		28/75 (37.33%)			0/75 (0%)	<0.0001

Supplemental Table 2- H1N1 HAI titers and vaccine seroconversion in all study years and sites. Data is GMTs (95% CI) of HAI antibodies, mean of log transformed titers (SD), and n/N(%) with a 4-fold increase in T2/T1. A p-value <0.05 was significant. GMT= geometric mean titer, GMFR= geometric mean titer fold ratio, SD= standard deviation.

	H3 Titer Influenza Vaccine			H3 Titer Placebo		
	T1	T2	GMFC or p value	T1	T2	GMFC or p value
2017 HAI Titer						
Philippines						
GMT (95% CI)	16.96 (15.41-18.67)	18.85 (17.17-20.69)	1.119 (1.01-1.24)	17.91 (15.78-20.32)	19.22 (17.12-21.58)	1.07 (0.93-1.22)
Mean of log titer (SD)	1.23 (0.18)	1.27 (0.18)	0.4743	1.25 (0.22)	1.28 (0.21)	0.8069
≥4-fold increase		1/80(1.25%)			2/69 (2.89%)	0.5964
2018 HAI Titer						
Philippines						
GMT (95% CI)	18.56 (15.51-22.20)	42.71 (33.28-54.82)	2.302 (1.834-2.888)	19.8 (15.40-23.65)	21.71 (17.47-26.99)	1.138 (0.981-1.32)
Mean of log titer (SD)	1.26 (0.33)	1.63 (0.46)	<0.0001	1.28 (0.35)	1.33 (0.36)	0.8602
≥4-fold increase		25/74 (33.7%)			3/59 (5.08%)	<0.0001
Mozambique						
GMT (95% CI)	22.72 (17.53-29.46)	40.37(31.60-51.56)	1.776 (1.415-2.230)	10.78 (9.28-12.51)	11.19 (9.6-13.04)	1.038 (0.933-1.154)
Mean of log titer (SD)	1.35 (0.49)	1.60 (0.46)	0.0007	1.03 (0.28)	1.04 (0.28)	0.9945
≥4-fold increase		21/76 (27.63%)			2/74 (2.70%)	<0.0001
Nigeria						
GMT (95% CI)	20.74 (15.00-28.69)	35.85 (22.57-56.95)	1.728 (1.056-2.829)	16.86 (12.39-23.21)	21.36 (13.69-33.34)	1.260 (0.778-2.040)
Mean of log titer (SD)	1.31 (0.29)	1.55 (0.41)	0.1924	1.22 (0.29)	1.33 (0.42)	0.8083
≥4-fold increase		4/19 (21.05%)			5/21 (23.8%)	>0.9999
2019 HAI Titer						
Philippines						
GMT (95% CI)	26.13 (23.17-29.46)	40.67 (35.37-46.78)	1.557 (1.355-1.789)	21.69 (19.24-24.44)	23.31 (20.64-26.32)	1.075 (0.9930-1.163)
Mean of log titer (SD)	1.41 (0.23)	1.60 (0.27)	<0.0001	1.33 (0.22)	1.36 (0.23)	0.8593
≥4-fold increase		9/83 (10.84%)			2/77 (2.59%)	0.0584
Mozambique						
GMT (95% CI)	22.35 (17.28-28.89)	28.68 (21.03-39.12)	1.283 (1.091-1.510)	20.59 (14.66-28.91)	20.59 (16.01-26.47)	1.000 (0.795-1.257)
Mean of log titer (SD)	1.34 (0.27)	1.45 (0.32)	0.5889	1.31 (0.34)	1.31 (0.25)	>0.9999
≥4-fold increase		0/25 (0%)			0/24 (0%)	>0.9999
Nigeria						
GMT (95% CI)	23.78 (15.69-36.06)	38.86 (26.82-56.32)	1.634 (1.116-2.391)	12.60 (10.48-15.15)	17.01 (11.34-25.53)	1.350 (0.9211-1.980)
Mean of log titer (SD)	1.37 (0.42)	1.59 (0.38)	0.2252	1.1 (0.21)	1.23 (0.47)	0.5558
≥4-fold increase		6/24 (25%)			3/30 (10%)	0.1646
Zambia						
GMT (95% CI)	35.47 (31.00-40.58)	58.43 (49.17-69.43)	1.647 (1.367-1.985)	27.89 (24.78-31.40)	28.42 (24.59-32.83)	1.019 (0.9034-1.149)
Mean of log titer (SD)	1.55 (0.25)	1.76 (0.32)	<0.0001	1.44 (0.22)	1.45 (0.27)	0.9979
≥4-fold increase		16/75 (21.33%)			3/75 (4%)	0.0058

Supplemental Table 3- H3N2 HAI titers and vaccine seroconversion in all study years and sites. Data is GMTs (95% CI) of HAI antibodies, mean of log transformed titers (SD), and n/N(%) with a 4-fold increase in T2/T1. A p-value <0.05 was significant. GMT= geometric mean titer, GMFR= geometric mean titer fold ratio, SD= standard deviation.

	H1 Influenza Vaccine	H3 Influenza Vaccine	
	T2/ T1	T2/T1	p value
2017			
≥4-fold increase	59/80 (73.75%)	1/80 (1.25%)	<0.0001
2018			
≥4-fold increase	59/ 169 (34.91%)	50/169 (29.58%)	0.3519
2019			
≥4-fold increase	64/207 (30.91%)	31/207 (14.97%)	0.0002

	H1 Placebo	H3 Placebo	
	T2/ T1	T2/T1	p value
2017			
≥4-fold increase	5/69 (7.24%)	2/69 (2.89%)	0.4411
2018			
≥4-fold increase	6/154 (3.89%)	10/154 (6.49%)	0.4422
2019			
≥4-fold increase	5/206 (2.41%)	8/ 206 (3.88%)	0.5748

Supplemental Table 4- Comparison of seroconversion rates to A/H1N1 and A/H3N2 antigen by year within groups. Data is n/N (%), p-values >0.05 were significant.

	H1 Influenza Vaccine	H1 Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥2-fold increase	6/82 (7.31%)	13/70 (18.57)	0.0486	0.3462 (0.125-0.9429)	0.3940 (0.1617-0.9469)	60.6 (5.31-83.83)
2018						
≥2-fold increase	8/150 (5.33%)	19/133 (14.29%)	0.0141	0.3380 (0.1509-0.8065)	0.3733 (0.1716-0.8047)	62.67 (19.53-82.84)
2019						
≥2-fold increase	10/197 (5.08%)	28/191 (14.67%)	0.0018	0.3113 (0.1531-0.6391)	0.3463 (0.1746-0.6807)	65.37 (31.93-82.54)

	H3 Influenza Vaccine	H3 Placebo				
	T3/ T2	T3/T2	p value	OR (95% CI)	RR	VE=(1-RR) x100 (95% CI)
2017						
≥2-fold increase	15/82 (18.29%)	12/70 (17.14%)	>0.9999	1.082 (0.4768-2.471)	1.067 (0.544-2.109)	-6.7(-1.1-45.6)
2018						
≥2-fold increase	20/150 (13.33%)	32/133 (24.06%)	0.0217	0.4856 (0.2567-0.9050)	0.5542 (0.3343-0.9135)	44.58 (8.65-66.57)
2019						
≥2-fold increase	19/197 (9.64%)	70 /191 (36.65%)	<0.0001	0.1845 (0.1068-0.3198)	0.2632 (0.1648-0.4149)	73.68 (58.51-83.52)

Supplemental Table 5- Vaccine efficacy estimates against either A/H1N1 and A/H3N2 subtypes, using 2-fold change in T3/T2 antibodies as serological evidence of infection. Data is n/N (%), OR (95% CI), RR (95% CI), and VE= (1-RR)x100. OR= odds ratio, RR= relative risk, VE= vaccine efficacy.

	H1 Influenza Vaccine	H3 Influenza Vaccine	
	T3/ T2	T3/T2	p value
2017			
≥4-fold increase	1/82 (1.22%)	0/82 (0%)	>0.9999
2018			
≥4-fold increase	1/150 (0.67%)	4/150 (2.67%)	0.3708
2019			
≥4-fold increase	2/ 197 (1.02)	2/197 (1.02%)	>0.9999

	H1 Placebo	H3 Placebo	
	T3/ T2	T3/T2	p value
2017			
≥4-fold increase	9/70 (12.86%)	1/70 (1.43%)	0.0173
2018			
≥4-fold increase	6/133 (4.51%)	10/ 133 (7.52%)	0.4402
2019			
≥4-fold increase	9/191 (4.71%)	19/191 (9.94%)	0.0756

Supplemental Table 6- Comparison of breakthrough infections by influenza subtype and intervention group. Data is n/N (%), a p-value >0.05 was considered significant.

			H1 Strain	H3 Strain	IBV Strain
2017-2018	**SH2017 VAXIGRIP® TIV**	**Philippines**	A/Michigan/45/2015 (H1N1)pdm09-like virus	A/Hong Kong/4801/2014 (H3N2)-like virus	B/Brisbane/60/2008-like virus
2018-2019	**SH2018 VAXIGRIP® TIV**	**Philippines Mozambique Nigeria**	A/Michigan/45/2015 (H1N1)pdm09-like virus	A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus	B/Phuket/3073/2013-like virus
	NH2018 VAXIGRIP® TIV	**Mozambique Nigeria**	an A/Michigan/45/2015 (H1N1)pdm09-like virus	A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus	B/Colorado/06/2017-like virus (B/Victoria/2/87 lineage)
2019-2020	**SH2019 VAXIGRIP® TIV**	**Philippines Mozambique Nigeria Zambia**	A/Michigan/45/2015 (H1N1)pdm09-like virus	A/Switzerland/8060/2017 (H3N2)-like virus	B/Colorado/06/2017-like virus (B/Victoria/2/87 lineage)

Supplemental Table 7- WHO recommended vaccine strains for 2017,2018 and 2019.

6.0- Chapter 6: Discussion

6.1 Improving current vaccines

Despite reductions in circulation during the COVID-19 pandemic, influenza virus infections still pose a significant threat to public health[16]. The rates of infection in younger cohorts during the COVID-19 pandemic has resulted in increased burdens on hospitals as influenza, RSV, and other respiratory viruses have rebounded with the lifting of public health mitigation measures[266]. Older adults remain at significant risk of morbidity and mortality from influenza infection, as do those with underlying health conditions such as cardiovascular disease. While there has been a major emphasis on development of a 'universal' vaccines over the past decade, improving seasonal vaccines and optimization of vaccination recommendations, particularly in high-risk cohorts, represents as accessible and short-term strategy to combat influenza virus more effectively. This dissertation aims to enhance our understanding of the benefits and limitations of influenza vaccines, especially in children and high-risk patients.

6.1.1 Leveraging mucosal antibody responses

Currently approved seasonal vaccines already utilise a variety of vaccine platforms and routes of administration, which vary in both the types and magnitude of antibody responses induced. In Chapter 3, we demonstrate that IgA antibodies, which are more readily elicited by mucosal vaccines such as LAIV, induce a unique Fc-dependent effector function in neutrophils. We observed that IgA in complex with a variety of viruses, as well as auto-antigen, induces release of NETs via NETosis[172]. IgA immune complexes induced NETosis more potently than IgG immune complexes. Furthermore, we showed that these NETs can trap and inactivate influenza viruses, consistent with previous observations in the context of bacterial and fungal microbes[182]. The

mechanisms underlying the differences between the downstream effects of FcγR and FcαR engagement have yet to be elucidated. Altered signalling pathways after FcR binding may in part explain these differences[267]. Alternatively, different Fc region modifications (i.e. glycosylation) have also been hypothesized to contribute to the functional differences between IgA and IgG[175].

In the context of influenza virus, NETosis seems to be driven primarly by bnAbs that bind to the HA stalk domain, which has implications for universal influenza vaccine design. Our group has recently demonstrated that both seasonal IIVs and LAIVs are capable of inducing bnAbs that bind to the HA stalk[81]. Additionally, these results build on previous observations from our group which identified that bnAb neutralization potency was enhanced by IgA[174]. HA stalk-binding IgA induced following seasonal vaccination may therefore contribute to protection from seasonal vaccines and should be evaluated when investigating vaccine immunogenicity.

Given these results, the relative contribution of IgA to protection *in vivo* should be more carefully evaluated. Now a suitable murine model expressing the human FcαR is available and distinguishing the protective versus pathogenic effects of IgA mediated NETosis following infection is possible[268].

6.1.2 Understanding vaccine response in pediatric cohorts

Young children are at significant risk for hospitalization and severe complications following influenza virus infection. In Canada, during the three influenza seasons prior to COVID-19, hospitalizations among children aged 0-4 were eclipsed only by those in adults age 65+[269]. In addition to increased risk of morbidity, influenza exposure in early life has been shown to influence

subsequent immune responses to infection and vaccination[111]. While both TIV, LAIV and A-TIV are approved for use in children, and have demonstrated immunogenicity and efficacy, little is known about how immune imprinting with different vaccine formulations shape antibody hierarchies. The results presented in Chapter 4 suggest that primary exposure with different vaccine formulations may impact strain specific recall responses and the induction broadly-neutralizing HA stalk antibodies.

The concept antibody hierarchies against historical strains of influenza has been widely recognized for decades. Thomas Francis Jr. coined the term, 'original antigenic sin' in the 1960s[112]. However, there are currently no widely accepted assays or methods for quantification of 'OAS' responses. Classically, reduced antibody titers against a contemporary strain relative to a historical strain, as measured by binding assay (ELISA) or HAI have been used. Recent work has demonstrated that OAS-like antibodies with reduced binding *in vitro* can still provide protection *in vivo*[270]. However, the beneficial or detrimental impacts of OAS are surely context-dependent. Building on the results of Chapter 4 it would be of great interest to directly investigate the impact of OAS-like responses induced by different vaccine formulations on efficacy over multiple seasons – a question that we were not sufficiently powered to address.

It is important to note that we were limited to serological assessment of OAS in the current study and other groups have demonstrated that $CD4^+$ T cells contribute to the establishment of OAS[220]. Future studies that explore the impact of vaccine type/formulation on T cell responses would also be important and of considerable interest. Establishing the underlying immunological mechanisms that drive OAS-like responses, and the ways in which vaccines impact those responses, may

facilitate more rationale design of vaccines to leverage the beneficial features of OAS, while minimizing those that are detrimental.

6.1.3 Understanding vaccine responses in high-risk cohorts

Influenza virus infections have been shown to exacerbate cardiovascular illness, resulting in increased cardiac events, such as myocardial infarction and death[254]. As such, the ability of seasonal influenza vaccines to provide protection against infection in high-risk cardiovascular patients should be carefully evaluated. The recently completed IVVE clinical trial demonstrated that seasonal influenza vaccines reduce cardiovascular events during periods of peak influenza circulation[264]. In Chapter 5, we show in a secondary analysis using a subset of IVVE samples, that the inactivated influenza vaccine was immunogenic. Based on serological surrogates of infection, seasonal vaccination was also efficacious against both influenza A subtypes. These findings provide further evidence that seasonal vaccines are effective in patients with heart failure and support the observation that they limit cardiovascular events by reducing infection.

The results from Chapter 4 and 5 demonstrate that investigating the impact of seasonal vaccine types/formulations in high-risk cohorts, such as the immunologically naïve or those with underlying risk factors, can yield important implications for making recommendations for effective vaccination strategies in these populations.

6.1.4 Utilizing sero-epidemiological evidence to evaluate vaccine efficacy

Given the modest real-world effectiveness of seasonal influenza vaccines, ensuring that vaccine efficacy is accurately estimated during clinical trials is essential. Laboratory confirmed-influenza

is regarded as the gold-standard for evaluation of VE. However, given scale, cost, and feasibility, PCR testing is not always available. Using alternative methods, such as serological evidence of infection and symptom reporting can also yield reasonably accurate estimates of efficacy[271]. In Chapter 5 we show that serological evidence of infection can be used to estimate VE. PCR and/or serological- endpoints in conjunction with self-reported symptoms would help to distinguish between mild, asymptomatic illness and severe illness more clearly. In the IVVE study, for example, is not entirely clear whether the severity of infection is associated cardiac outcomes or if the even mild influenza infection is sufficient to trigger cardiac outcomes.

Although the VEs reported in Chapter 5 were higher than those observed by the CDC during the same seasons, the agreement between our serological ascertainment of efficacy during the secondary analysis and the vaccine's effect against cardiovascular outcomes in the main trial suggests that this method can be used in the absence of laboratory confirmed infection[272].

6.2 Advancing next-generation vaccines and therapeutics

Current seasonal vaccines are subject to costly annual reformulation and are not protective against pandemic strains of influenza virus. The induction of long-lived, broadly protective responses, in contrast to strain-specific antibodies, is the goal of next-generation vaccines. At present, the National Institute for Allergy and Infectious Disease (NIAID) criteria for a universal vaccine includes protection against all group 1 and 2 influenza viruses, with IBV being a secondary target[133]. Iterative improvements on current seasonal vaccine breadth to be subtype specific (all strains in a given subtype) or multi-subtype (multiple subtypes in one group) may be realized before a true universal vaccine is developed.

The development of stalk-binding monoclonal antibody therapies may also benefit treatment of patient populations for whom vaccination is not an option. Antibody cocktails of different specificities and isotypes, analogous to a polyclonal antibody response following vaccination, may be beneficial as both prophylactic and therapeutic treatments. Further investigation into the mechanisms of IgA mediated protection, such as those in Chapter 3, will be required to optimize these biologics.

6.2.1 Harnessing immune imprinting

The induction of anamnestic antibody responses against non-neutralizing epitopes may be detrimental to seasonal vaccines responses. In contrast, the induction of memory responses against conserved epitopes is the goal of many universal vaccine strategies. Early childhood infection has been demonstrated to provide within group, heterosubtypic protection from later infection[114]. However, when priming with a vaccine, the impact of having multiple HA antigens present simultaneously during imprinting is not clear. Selection of a universal vaccine platform for optimal use in children will be essential, as rationally imprinting the immune system may facilitate optimal generation of long-lived broadly protective responses. In Chapter 4 we show that seasonal LAIV, IIV and A-TIV induced group 1 and group 2 HA stalk antibodies following vaccination. This induction was highest upon primary vaccination but diminished in following years. This is analogous to what is observed for immunodominant responses against the HA head domain following repeat seasonal vaccination[130]. While we were fortunate to have access to sequential and paired samples, we were unable to assess the longevity of the bnAb responses in children imprinted on seasonal vaccines beyond 3 years. Further, vaccine was received in each of the years studied.

The durability of stalk responses elicited by a universal vaccine is a critically important feature. Prior work from our group suggests that bnAb responses are long-lived, although these findings were in an adult population and these titers may, in part, be sustained by repeated exposures to influenza[113]. As such, the durability of stalk antibodies induced in childhood requires additional investigation.

6.2.2 Considerations for vaccine formulation

Immunogens that stimulate broadly-protective antibody responses have been widely explored in both pre-clinical and early clinical studies. Given that seasonal vaccine platforms induce varied responses in different cohorts, developing a one-size fits all universal vaccine will be challenging. However, there has been limited comparison of broadly-reactive immunogens in the context of different vaccine platforms across age groups. A recent Phase I clinical trial results showed that chimeric HA antigens had unique immunogenicity profiles when delivered as IIV, A-IIV, or LAIV[146]. It is likely that specialized vaccine platforms may be required to induce a significant boost in bnAbs in older populations with naturally high pre-existing bnAbs.

Similar platform specific differences will likely be observed in pediatric cohorts. We demonstrated in Chapter 4, using a reporter ADCC assay, that both intramuscularly delivered IIV and mucosally administered LAIV induced significant HA stalk antibodies following primary vaccination. However, similar to the blunting of HAI antibodies that occurs following sequential LAIV administration, repeated LAIV recipients were observed to have reduced ADCC responses as well. LAIV also showed reduced ADCC induction in children who had been primed by prior infection.

Route of vaccine delivery will also undoubtedly play a role in the realization of a universal vaccine. In the context of COVID-19, the inability of current intramuscular vaccines to elicit sterilizing systemic immunity and declining vaccine effectiveness against emerging variants has resulted in increased interest in mucosal vaccination. Recently, increased titers of RBD and spike-specific mucosal IgA were shown to correlate with protection from omicron breakthrough infections[273,274]. While current intramuscular vaccines induce these antibodies at low levels, these findings suggest that specific targeting of these responses may improve vaccine mediated protection.

Recent work from our group in the context of a COVID-19 adenovirus vectored vaccine, also clearly showed a route-dependent effect of vaccine immunogenicity and protection in murine models. In our study, both candidate vaccines were more immunogenic when delivered intranasally, compared to intramuscular administration. Additionally, the intranasally delivered vaccine was able to provide protection against both ancestral strain and variants of SARS2[127].

The findings of Chapter 3 further underscore the unique properties of mucosal antibodies. Universal vaccine platforms that aim to elicit stalk binding IgA antibodies should also be considered. Emphasis will also need be placed on standardized functional assays to quantify IgA mediated Fc effector functions in order to accurately quantify the contribution of IgA in universal vaccine mediated protection.

In summary, the challenges facing improved influenza vaccines are multifaceted. Increasing the breadth of vaccine mediate responses will undoubtedly reduce costs associated with annual reformulation and considerably advance pandemic preparedness. In the interim, improving strain-

specific seasonals vaccines will reduce the burden of influenza infections on global public health. By considering multiple facets of the immune response to influenza virus, more effective vaccines can be realized.

7.0- References

1. Nabel, G. J. Designing Tomorrow's Vaccines. *N. Engl. J. Med.* **368**, 551–560 (2013).
2. Pollard, A. J. & Bijker, E. M. A guide to vaccinology: from basic principles to new developments. *Nat. Rev. Immunol.* **21**, 83–100 (2021).
3. Meganck, R. M. & Baric, R. S. Developing therapeutic approaches for twenty-first-century emerging infectious viral diseases. *Nat. Med.* **27**, 401–410 (2021).
4. Rappuoli, R. *et al.* Vaccinology in the post−COVID-19 era. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–7 (2021).
5. De Gregorio, E. & Rappuoli, R. From empiricism to rational design: A personal perspective of the evolution of vaccine development. *Nat. Rev. Immunol.* **14**, 505–514 (2014).
6. Baker, R. E. *et al.* Infectious disease in an era of global change. *Nat. Rev. Microbiol.* **20**, 193–205 (2022).
7. Putri, W. C. W. S., Muscatello, D. J., Stockwell, M. S. & Newall, A. T. Economic burden of seasonal influenza in the United States. *Vaccine* **36**, 3960–3966 (2018).
8. Cutler, D. M. & Summers, L. H. The COVID-19 Pandemic and the $16 Trillion Virus. *JAMA* **324**, 1495 (2020).
9. Cost, K. T. *et al.* Mostly worse, occasionally better: impact of COVID-19 pandemic on the mental health of Canadian children and adolescents. *Eur. Child Adolesc. Psychiatry* **31**, 671–684 (2022).
10. Malala Fund-COVID Initiative. https://covid.malala.org/.
11. Hawkes, M. T. *et al.* Seasonality of Respiratory Viruses at Northern Latitudes. *JAMA Netw. Open* **4**, e2124650 (2021).

12. Lowen, A. C., Mubareka, S., Steel, J. & Palese, P. Influenza Virus Transmission Is Dependent on Relative Humidity and Temperature. *PLoS Pathog.* **3**, e151 (2007).

13. Lowen, A. C. & Steel, J. Roles of Humidity and Temperature in Shaping Influenza Seasonality. *J. Virol.* **88**, 7692–7695 (2014).

14. Krammer, F. *et al.* Influenza. *Nat. Rev. Dis. Prim.* **4**, 1–21 (2018).

15. WHO | Influenza (Seasonal). *WHO* http://www.who.int/mediacentre/factsheets/fs211/en/ (2018).

16. Chow, E. J., Uyeki, T. M. & Chu, H. Y. The effects of the COVID-19 pandemic on community respiratory virus activity. *Nat. Rev. Microbiol.* **0123456789**, (2022).

17. Zhang, A. *et al.* The Collapse of Infectious Disease Diagnoses Commonly Due to Communicable Respiratory Pathogens During the Coronavirus Disease 2019 Pandemic: A Time Series and Hierarchical Clustering Analysis. *Open Forum Infect. Dis.* **9**, (2022).

18. Palese, P. & Shaw, M. Orthomyxoviridae: the viruses and their replication. in *Fields virology* (eds. Knipe, D. & Howley, P.) (Williams & Wilkins, 2007).

19. Rogers, G. N. & Paulson, J. C. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361–373 (1983).

20. Stevens, J. *et al.* Glycan Microarray Analysis of the Hemagglutinins from Modern and Pandemic Influenza Viruses Reveals Different Receptor Specificities. *J. Mol. Biol.* **355**, 1143–1155 (2006).

21. Wang, C., Lamb, R. A. & Pinto, L. H. Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue. *Biophys. J.* **69**, 1363–1371 (1995).

22. Senne, D. A. *et al.* Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: Amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Dis.* **40**, 425–437 (1996).

23. Horimoto, T., Nakayama, K., Smeekens, S. P. & Kawaoka, Y. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. *J. Virol.* **68**, 6074–6078 (1994).

24. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179 (1992).

25. Tong, S. *et al.* A distinct lineage of influenza A virus from bats. *Proc. Natl. Acad. Sci.* **109**, 4269–4274 (2012).

26. Tong, S. *et al.* New World Bats Harbor Diverse Influenza A Viruses. *PLoS Pathog.* **9**, e1003657 (2013).

27. Rota, P. A. *et al.* Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59–68 (1990).

28. Koutsakos, M., Wheatley, A. K., Laurie, K., Kent, S. J. & Rockman, S. Influenza lineage extinction during the COVID-19 pandemic? *Nat. Rev. Microbiol.* **19**, 741–742 (2021).

29. WHO. WHO recommendations on the composition of influenza virus vaccines. https://www.who.int/influenza/vaccines/virus/recommendations/en/.

30. Chen, R. & Holmes, E. C. Avian Influenza Virus Exhibits Rapid Evolutionary Dynamics. *Mol. Biol. Evol.* **23**, 2336–2341 (2006).

31. White, M. C. & Lowen, A. C. Implications of segment mismatch for influenza A virus evolution. *J. Gen. Virol.* **99**, 3–16 (2018).

32. Kawaoka, Y., Krauss, S. & Webster, R. G. Avian-to-human transmission of the PB1 gene

of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603–4608 (1989).

33. Tellier, R. Aerosol transmission of influenza A virus: a review of new studies. *J. R. Soc. Interface* **6**, (2009).

34. Bean, B. *et al.* Survival of Influenza Viruses on Environmental Surfaces. *J. Infect. Dis.* **146**, 47–51 (1982).

35. Sanders, C. J. *et al.* Compromised respiratory function in lethal influenza infection is characterized by the depletion of type I alveolar epithelial cells beyond threshold levels. *Am. J. Physiol. Cell. Mol. Physiol.* **304**, L481–L488 (2013).

36. Habib, A., Jain, A., Singh, B. & Jamshed, N. H1N1 influenza presenting as severe acute pancreatitis and multiorgan dysfunction. *Am. J. Emerg. Med.* **34**, 1911.e1-1911.e2 (2016).

37. Kalil, A. C. & Thomas, P. G. Influenza virus-related critical illness: pathophysiology and epidemiology. *Crit. Care* **23**, 258 (2019).

38. Morens, D. M., Taubenberger, J. K. & Fauci, A. S. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. *J. Infect. Dis.* **198**, 962–970 (2008).

39. MacIntyre, C. R. *et al.* The role of pneumonia and secondary bacterial infection in fatal and serious outcomes of pandemic influenza a(H1N1)pdm09. *BMC Infect. Dis.* **18**, 637 (2018).

40. Yoon, S.-W., Webby, R. J. & Webster, R. G. Evolution and Ecology of Influenza A Viruses. in 359–375 (2014). doi:10.1007/82_2014_396.

41. Claas, E. C. *et al.* Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **351**, 472–477 (1998).

42. Matrosovich, M. N., Krauss, S. & Webster, R. G. H9N2 Influenza A Viruses from Poultry

in Asia Have Human Virus-like Receptor Specificity. *Virology* **281**, 156–162 (2001).

43. Heui Seo, S., Hoffmann, E. & Webster, R. G. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nat. Med.* **8**, 950–954 (2002).

44. Horimoto, T. & Kawaoka, Y. Influenza: Lessons from past pandemics, warnings from current incidents. *Nat. Rev. Microbiol.* **3**, 591–600 (2005).

45. Monto, A. S. & Fukuda, K. Lessons From Influenza Pandemics of the Last 100 Years. *Clin. Infect. Dis.* (2019) doi:10.1093/cid/ciz803.

46. Johnson, N. P. A. S. & Mueller, J. Updating the accounts: global mortality of the 1918-1920 'Spanish' influenza pandemic. *Bull. Hist. Med.* **76**, 105–115 (2002).

47. Scholtissek, C., Rohde, W., Von Hoyningen, V. & Rott, R. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**, 13–20 (1978).

48. Gaitonde, D. Y., Moore, F. C. & Morgan, M. K. Influenza: Diagnosis and treatment. *Am. Fam. Physician* **100**, 751–758 (2019).

49. Ikematsu, H. *et al.* Baloxavir Marboxil for Prophylaxis against Influenza in Household Contacts. *N. Engl. J. Med.* **383**, 309–320 (2020).

50. Uyeki, T. M. *et al.* Clinical Practice Guidelines by the Infectious Diseases Society of America: 2018 Update on Diagnosis, Treatment, Chemoprophylaxis, and Instiutional Outbreak Management of Seasonal Influenza. *Clin. Infect. Dis.* **68**, 1790–1790 (2019).

51. Nimmerjahn, F. & Ravetch, J. V. The antiinflammatory activity of IgG: the intravenous IgG paradox. *J. Exp. Med.* **204**, 11–15 (2007).

52. Anthony, R. M., Wermeling, F., Karlsson, M. C. I. & Ravetch, J. V. Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc. Natl. Acad. Sci.* **105**, 19571–19578 (2008).

53. Rockman, S. *et al.* Intravenous Immunoglobulin Protects Against Severe Pandemic Influenza Infection. *EBioMedicine* **19**, 119–127 (2017).

54. Hung, I. F. N. *et al.* Hyperimmune IV Immunoglobulin Treatment. *Chest* **144**, 464–473 (2013).

55. Davey, R. T. *et al.* Anti-influenza hyperimmune intravenous immunoglobulin for adults with influenza A or B infection (FLU-IVIG): a double-blind, randomised, placebo-controlled trial. *Lancet Respir. Med.* **7**, 951–963 (2019).

56. Lee, N. *et al.* Viral Loads and Duration of Viral Shedding in Adult Patients Hospitalized with Influenza. *J. Infect. Dis.* **200**, 492–500 (2009).

57. Osterholm, M. T., Kelley, N. S., Sommer, A. & Belongia, E. A. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. *Lancet Infect. Dis.* **12**, 36–44 (2012).

58. Gerdil, C. The annual production cycle for influenza vaccine. *Vaccine* **21**, 1776–1779 (2003).

59. Skowronski, D. M., Janjua, N. Z., Serres, G. De, Sabaiduc, S. & Eshaghi, A. Low 2012 – 13 Influenza Vaccine Effectiveness Associated with Mutation in the Egg-Adapted H3N2 Vaccine Strain Not Antigenic Drift in Circulating Viruses. *PLoS One* **9**, (2014).

60. Zost, S. J. *et al.* Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proc. Natl. Acad. Sci.* **114**, 12578–12583 (2017).

61. Hirst, G. K. The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus. *Science (80-.).* **94**, 22–23 (1941).

62. Hirst, G. K. The Quantitative Determination of Influenza Virus and Antibodies by Means

of Red Cell Agglutination. *J. Exp. Med.* **75**, 49–64 (1942).

63. Who. Manual for the laboratory diagnosis and virological surveillance of influenza. *World Heal. Organ. 2011* 153 (2011).

64. Black, S. *et al.* Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza Vaccines in children. *Pediatr. Infect. Dis. J.* **30**, 1081–1085 (2011).

65. Hirst, G. K., Rickard, E. R., Whitman, L. & Horsfall, F. L. Antibody Response of Human Beings Following Vaccination with Influenza Viruses. *J. Exp. Med.* **75**, 495–511 (1942).

66. Salk, J. E. & Suriano, P. C. Importance of Antigenic Composition of Influenza Virus Vaccine in Protecting against the Natural Disease. *Am. J. Public Heal. Nations Heal.* **39**, 345–355 (1949).

67. Wong, S. S. & Webby, R. J. Traditional and new influenza vaccines. *Clin. Microbiol. Rev.* **26**, 476–492 (2013).

68. Krammer, F. *et al.* NAction! How Can Neuraminidase-Based Immunity Contribute to Better Influenza Virus Vaccines? *MBio* **9**, (2018).

69. Duxbury, A. E., Hampson, A. W. & Sievers, J. G. M. Antibody Response in Humans to Deoxycholate-Treated Influenza Virus Vaccine. *J. Immunol.* **101**, 62–67 (1968).

70. Bachmayer, H., Liehl, E. & Schmidt, G. Preparation and properties of a novel influenza subunit vaccine. *Postgrad. Med. J.* **52**, 360–367 (1976).

71. Talbot, H. K. *et al.* Clinical Effectiveness of Split-Virion Versus Subunit Trivalent Influenza Vaccines in Older Adults. *Clin. Infect. Dis.* **60**, 1170–1175 (2015).

72. Kissling, E. *et al.* Influenza vaccine effectiveness estimates in Europe in a season with three influenza type/subtypes circulating: the I-MOVE multicentre case–control study, influenza season 2012/13. *Eurosurveillance* **19**, (2014).

73. Lamb, Y. N. Cell-Based Quadrivalent Inactivated Influenza Virus Vaccine (Flucelvax® Tetra/Flucelvax Quadrivalent®): A Review in the Prevention of Influenza. *Drugs* **79**, 1337–1348 (2019).

74. Frey, S. *et al.* Clinical Efficacy of Cell Culture–Derived and Egg-Derived Inactivated Subunit Influenza Vaccines in Healthy Adults. *Clin. Infect. Dis.* **51**, 997–1004 (2010).

75. Dawood, F. S. *et al.* Comparison of the Immunogenicity of Cell Culture-Based and Recombinant Quadrivalent Influenza Vaccines to Conventional Egg-Based Quadrivalent Influenza Vaccines Among Healthcare Personnel Aged 18–64 Years: A Randomized Open-Label Trial. *Clin. Infect. Dis.* **73**, 1973–1981 (2021).

76. DiazGranados, C. A. *et al.* Efficacy of High-Dose versus Standard-Dose Influenza Vaccine in Older Adults. *N. Engl. J. Med.* **371**, 635–645 (2014).

77. Maassab, H. F. Adaptation and Growth Characteristics of Influenza Virus at 25° C. *Nature* **213**, 612–614 (1967).

78. Wright, P. F. *et al.* Correlates of Immunity to Influenza as Determined by Challenge of Children with Live, Attenuated Influenza Vaccine. *Open Forum Infect. Dis.* **3**, (2016).

79. Hoft, D. F. *et al.* Live and Inactivated Influenza Vaccines Induce Similar Humoral Responses, but Only Live Vaccines Induce Diverse T-Cell Responses in Young Children. *J. Infect. Dis.* **204**, 845–853 (2011).

80. He, X.-S. *et al.* Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J. Virol.* **80**, 11756–11766 (2006).

81. Yegorov, S. *et al.* Inactivated and live-attenuated seasonal influenza vaccines boost broadly neutralizing antibodies in children. *Cell Reports Med.* **3**, 100509 (2022).

82. (NACI), N. A. C. on I. Canadian Immunization Guide Chapter on Influenza and Statement

on Seasonal Influenza Vaccine for 2016-2017. https://www.canada.ca/en/public-health/services/immunization/national-advisory-committee-on-immunization-naci/canadian-immunization-guide-chapter-on-influenza-statement-on-seasonal-influenza-vaccine-2016-2017-addemdum-laiv-use-children-adolescents.html.

83. CDC. ACIP votes down use of LAIV for 2016-2017 flu season. https://www.cdc.gov/media/releases/2016/s0622-laiv-flu.html.

84. Loeb, M. *et al.* Live attenuated versus inactivated influenza vaccinein hutterite children a cluster randomized blinded trial. *Ann. Intern. Med.* **165**, 617–624 (2016).

85. Buchan, S. A. *et al.* Effectiveness of Live Attenuated vs Inactivated Influenza Vaccines in Children During the 2012-2013 Through 2015-2016 Influenza Seasons in Alberta, Canada. *JAMA Pediatr.* **172**, e181514 (2018).

86. Ang, J. C. *et al.* Comparative Immunogenicity of the 2014–2015 Northern Hemisphere Trivalent IIV and LAIV against Influenza A Viruses in Children. *Vaccines* **7**, 87 (2019).

87. Wilkins, A. L. *et al.* AS03- and MF59-Adjuvanted Influenza Vaccines in Children. *Front. Immunol.* **8**, (2017).

88. Sequirus. Fluad- Influenza Vaccine for Seniors 65. https://www.fluad.ca/patient/en/index.html.

89. NACI. An advisory committee statement (ACS). National Advisory Committee on Immunization (NACI). Literature Review on Pediatric Fluad Influenza Vaccine Use in Children 6-72 Months of Age. *Can. Commun. Dis. Rep.* **28**, 1–12 (2015).

90. Frey, S. E. *et al.* Comparison of the safety and immunogenicity of an MF59®-adjuvanted with a non-adjuvanted seasonal influenza vaccine in elderly subjects. *Vaccine* **32**, 5027–5034 (2014).

91. Boikos, C. *et al.* Effectiveness of the Adjuvanted Influenza Vaccine in Older Adults at High Risk of Influenza Complications. *Vaccines* **9**, 862 (2021).

92. Li, A. P. Y. *et al.* Immunogenicity of standard, high-dose, MF59-adjuvanted, and recombinant-HA seasonal influenza vaccination in older adults. *npj Vaccines* **6**, 1–12 (2021).

93. Vesikari, T., Groth, N., Karvonen, A., Borkowski, A. & Pellegrini, M. MF59®-adjuvanted influenza vaccine (FLUAD®) in children: Safety and immunogenicity following a second year seasonal vaccination. *Vaccine* **27**, 6291–6295 (2009).

94. Vesikari, T. *et al.* Enhanced Immunogenicity of Seasonal Influenza Vaccines in Young Children Using MF59 Adjuvant. *Pediatr. Infect. Dis. J.* **28**, 563–571 (2009).

95. Zedda, L. *et al.* Dissecting the Immune Response to MF59-adjuvanted and Nonadjuvanted Seasonal Influenza Vaccines in Children Less Than Three Years of Age. *Pediatr. Infect. Dis. J.* **34**, 73–78 (2015).

96. Nolan, T. *et al.* Enhanced and persistent antibody response against homologous and heterologous strains elicited by a MF59®-adjuvanted influenza vaccine in infants and young children. *Vaccine* **32**, 6146–6156 (2014).

97. Loeb, M. *et al.* Adjuvanted trivalent influenza vaccine versus quadrivalent inactivated influenza vaccine in Hutterite Children: A randomized clinical trial. *Vaccine* **39**, 6843–6851 (2021).

98. Bernstein, D. I. *et al.* Effects of Adjuvants on the Safety and Immunogenicity of an Avian Influenza H5N1 Vaccine in Adults. *J. Infect. Dis.* **197**, 667–675 (2008).

99. Clark, T. W. *et al.* Trial of 2009 Influenza A (H1N1) Monovalent MF59-Adjuvanted Vaccine. *N. Engl. J. Med.* **361**, 2424–2435 (2009).

100. Khurana, S. *et al.* MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. *Sci. Transl. Med.* **3**, 1–10 (2011).

101. Goll, J. B. *et al.* The antibody landscapes following AS03 and MF59 adjuvanted H5N1 vaccination. *npj Vaccines* **7**, (2022).

102. Khurana, S. *et al.* Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. *Sci. Transl. Med.* **2**, 1–8 (2010).

103. Treanor, J. J. *et al.* Protective efficacy of a trivalent recombinant hemagglutinin protein vaccine (FluBlok®) against influenza in healthy adults: A randomized, placebo-controlled trial. *Vaccine* **29**, 7733–7739 (2011).

104. Baxter, R. *et al.* Evaluation of the safety, reactogenicity and immunogenicity of FluBlok® trivalent recombinant baculovirus-expressed hemagglutinin influenza vaccine administered intramuscularly to healthy adults 50–64 years of age☆. *Vaccine* **29**, 2272–2278 (2011).

105. King, J. C. *et al.* Evaluation of the safety, reactogenicity and immunogenicity of FluBlok® trivalent recombinant baculovirus-expressed hemagglutinin influenza vaccine administered intramuscularly to healthy children aged 6–59 months. *Vaccine* **27**, 6589–6594 (2009).

106. Richards, K. A. *et al.* Recombinant HA-based vaccine outperforms split and subunit vaccines in elicitation of influenza-specific CD4 T cells and CD4 T cell-dependent antibody responses in humans. *npj Vaccines* **5**, 77 (2020).

107. Sparrow, E. *et al.* Global production capacity of seasonal and pandemic influenza vaccines in 2019. *Vaccine* **39**, 512–520 (2021).

108. 2020-2021 Seasonal Influenza Vaccine Total Doses Distributed (CDC).

https://www.cdc.gov/flu/prevent/vaccinesupply-2020.htm.

109. CDC Vaccine Price List.

110. Andrews, N. *et al.* Covid-19 Vaccine Effectiveness against the Omicron (B.1.1.529) Variant. *N. Engl. J. Med.* 1–15 (2022) doi:10.1056/nejmoa2119451.

111. Zhang, A., Stacey, H. D., Mullarkey, C. E. & Miller, M. S. Original Antigenic Sin: How First Exposure Shapes Lifelong Anti–Influenza Virus Immune Responses. *J. Immunol.* **202**, 335–340 (2019).

112. Francis, T. On the Doctrine of Original Antigenic Sin. *Proc. Am. Philos. Soc.* **104**, 572–578 (1960).

113. Miller, M. S. *et al.* Neutralizing antibodies against previously encountered influenza virus strains increase over time: A longitudinal analysis. *Sci. Transl. Med.* **5**, (2013).

114. Gostic, K. M., Ambrose, M., Worobey, M. & Lloyd-Smith, J. O. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science* **354**, 722–726 (2016).

115. Angeletti, D. & Yewdell, J. W. Understanding and Manipulating Viral Immunity : Antibody Immunodominance Enters Center Stage. *Trends Immunol.* 1–13 (2018) doi:10.1016/j.it.2018.04.008.

116. Brownlee, G. G. & Fodor, E. The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. *Philos. Trans. R. Soc. B Biol. Sci.* **356**, 1871–1876 (2001).

117. Caton, A. J., Brownlee, G. G., Yewdell, J. W. & Gerhard, W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**, 417–427 (1982).

118. Broecker, F. *et al.* Immunodominance of Antigenic Site B in the Hemagglutinin of the

Current H3N2 Influenza Virus in Humans and Mice. *J. Virol.* **92**, 1–13 (2018).

119. Jackson, D. C., Murray, J. M., White, D. O. & Gerhard, W. U. Enumeration of antigenic sites of influenza virus hemagglutinin. *Infect. Immun.* **37**, 912–918 (1982).

120. Lingwood, D. *et al.* Structural and genetic basis for development of broadly neutralizing influenza antibodies. *Nature* **489**, 566–570 (2012).

121. Wheatley, A. K. *et al.* H5N1 Vaccine–Elicited Memory B Cells Are Genetically Constrained by the IGHV Locus in the Recognition of a Neutralizing Epitope in the Hemagglutinin Stem. *J. Immunol.* **195**, 602–610 (2015).

122. Joyce, M. G. *et al.* Vaccine-Induced Antibodies that Neutralize Group 1 and Group 2 Influenza A Viruses. *Cell* **166**, 609–623 (2016).

123. Bajic, G. *et al.* Autoreactivity profiles of influenza hemagglutinin broadly neutralizing antibodies. *Sci. Rep.* **9**, 3492 (2019).

124. Khurana, S., Hahn, M., Klenow, L. & Golding, H. Autoreactivity of Broadly Neutralizing Influenza Human Antibodies to Human Tissues and Human Proteins. *Viruses* **12**, 1140 (2020).

125. Andrews, S. F. *et al.* Immune history profoundly affects broadly protective B cell responses to influenza. *Sci. Transl. Med.* **7**, (2015).

126. Guthmiller, J. J. *et al.* Polyreactive Broadly Neutralizing B cells Are Selected to Provide Defense against Pandemic Threat Influenza Viruses. *Immunity* **53**, 1230-1244.e5 (2020).

127. Afkhami, S. *et al.* Respiratory mucosal delivery of next-generation COVID-19 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2. *Cell* **185**, 896-915.e19 (2022).

128. Nachbagauer, R., Choi, A., Izikson, R., Cox, M. M. & Palese, P. Age Dependence and

Isotype Specificity of Influenza Virus Hemagglutinin Stalk-Reactive Antibodies in Humans. *MBio* **7**, 1–10 (2016).

129. Henry, C. *et al.* Influenza Virus Vaccination Elicits Poorly Adapted B Cell Responses in Elderly Individuals. *Cell Host Microbe* **25**, 357-366.e6 (2019).
130. Miller, M. S. *et al.* 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. *J. Infect. Dis.* **207**, 98–105 (2013).
131. Vincent Rananiello. Pandemic Influenza Vaccine was Too Late in 2009. https://www.virology.ws/2010/12/09/pandemic-influenza-vaccine-was-too-late-in-2009/.
132. Plummer, F. A. & Jones, S. M. The Story of Canada's Ebola Vaccine. *CMAJ* **189**, E1326–E1327 (2017).
133. Erbelding, E. J. *et al.* A Universal Influenza Vaccine: The Strategic Plan for the National Institute of Allergy and Infectious Diseases. *J. Infect. Dis.* **218**, 347–354 (2018).
134. Memoli, M. J. *et al.* Influenza A Reinfection in Sequential Human Challenge: Implications for Protective Immunity and "Universal" Vaccine Development. *Clin. Infect. Dis.* **70**, 748–753 (2020).
135. Park, J.-K. *et al.* Evaluation of Preexisting Anti-Hemagglutinin Stalk Antibody as a Correlate of Protection in a Healthy Volunteer Challenge with Influenza A/H1N1pdm Virus. *MBio* **9**, (2018).
136. Christensen, S. R. *et al.* Assessing the Protective Potential of H1N1 Influenza Virus Hemagglutinin Head and Stalk Antibodies in Humans. *J. Virol.* **93**, (2019).
137. Ng, S. *et al.* Novel correlates of protection against pandemic H1N1 influenza A virus infection. *Nat. Med.* **25**, 962–967 (2019).
138. Zhang, A. *et al.* Beyond neutralization: Fc-dependent antibody effector functions in

SARS-CoV-2 infection. *Nat. Rev. Immunol.* (2022) doi:10.1038/s41577-022-00813-1.

139. Valkenburg, S. A. *et al.* Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Sci. Rep.* **6**, 22666 (2016).

140. Impagliazzo, A. *et al.* A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science (80-.).* **349**, 1301–1306 (2015).

141. Kanekiyo, M. *et al.* Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* **499**, 102–106 (2013).

142. Eggink, D., Goff, P. H. & Palese, P. Guiding the Immune Response against Influenza Virus Hemagglutinin toward the Conserved Stalk Domain by Hyperglycosylation of the Globular Head Domain. *J. Virol.* **88**, 699–704 (2014).

143. Carter, D. M. *et al.* Design and Characterization of a Computationally Optimized Broadly Reactive Hemagglutinin Vaccine for H1N1 Influenza Viruses. *J. Virol.* **90**, 4720–4734 (2016).

144. Krammer, F. & Palese, P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr. Opin. Virol.* **3**, 521–530 (2013).

145. Hai, R. *et al.* Influenza Viruses Expressing Chimeric Hemagglutinins: Globular Head and Stalk Domains Derived from Different Subtypes. *J. Virol.* **86**, 5774–5781 (2012).

146. Nachbagauer, R. *et al.* A chimeric hemagglutinin-based universal influenza virus vaccine approach induces broad and long-lasting immunity in a randomized, placebo-controlled phase I trial. *Nat. Med.* **27**, 106–114 (2021).

147. Folschweiller, N. *et al.* Reactogenicity, safety, and immunogenicity of chimeric haemagglutinin influenza split-virion vaccines, adjuvanted with AS01 or AS03 or non-adjuvanted: a phase 1–2 randomised controlled trial. *Lancet Infect. Dis.* **22**, 1062–1075

(2022).

148. Arevalo, C. P. *et al.* A multivalent nucleoside-modified mRNA vaccine against all known influenza virus subtypes. *Science (80-.).* **378**, 899–904 (2022).

149. El Bakkouri, K. *et al.* Universal Vaccine Based on Ectodomain of Matrix Protein 2 of Influenza A: Fc Receptors and Alveolar Macrophages Mediate Protection. *J. Immunol.* **186**, 1022–1031 (2011).

150. Neirynck, S. *et al.* A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat. Med.* **5**, 1157–1163 (1999).

151. Kim, M.-C. *et al.* Virus-like Particles Containing Multiple M2 Extracellular Domains Confer Improved Cross-protection Against Various Subtypes of Influenza Virus. *Mol. Ther.* **21**, 485–492 (2013).

152. De Filette, M. *et al.* An Influenza A Vaccine Based on Tetrameric Ectodomain of Matrix Protein 2. *J. Biol. Chem.* **283**, 11382–11387 (2008).

153. Huleatt, J. W. *et al.* Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* **26**, 201–214 (2008).

154. Pleguezuelos, O. *et al.* Immunogenicity, Safety, and Efficacy of a Standalone Universal Influenza Vaccine, FLU-v, in Healthy Adults. *Ann. Intern. Med.* **172**, 453 (2020).

155. Berthoud, T. K. *et al.* Potent CD8+ T-Cell Immunogenicity in Humans of a Novel Heterosubtypic Influenza A Vaccine, MVA-NP+M1. *Clin. Infect. Dis.* **52**, 1–7 (2011).

156. Chen, Y.-Q. *et al.* Influenza Infection in Humans Induces Broadly Cross-Reactive and Protective Neuraminidase-Reactive Antibodies. *Cell* **173**, 417-429.e10 (2018).

157. Sandbulte, M. R. *et al.* Discordant antigenic drift of neuraminidase and hemagglutinin in

H1N1 and H3N2 influenza viruses. *Proc. Natl. Acad. Sci.* **108**, 20748–20753 (2011).

158. Hsu, D. C. *Janeways Immunobiology, 7th Edition*. *Shock* vol. 29 (2008).

159. Bournazos, S., Wang, T. T., Dahan, R., Maamary, J. & Ravetch, J. V. Signaling by antibodies: Recent progress. *Annu. Rev. Immunol.* **35**, 285–311 (2017).

160. Wang, T. T. *et al.* Anti-HA Glycoforms Drive B Cell Affinity Selection and Determine Influenza Vaccine Efficacy. *Cell* **162**, 160–169 (2015).

161. Larsen, M. D. *et al.* Afucosylated IgG characterizes enveloped viral responses and correlates with COVID-19 severity. *Science (80-.).* **371**, (2021).

162. Chakraborty, S. *et al.* Proinflammatory IgG Fc structures in patients with severe COVID-19. *Nat. Immunol.* **22**, 67–73 (2021).

163. Chakraborty, S. *et al.* Early non-neutralizing, afucosylated antibody responses are associated with COVID-19 severity. *Sci. Transl. Med.* **14**, (2022).

164. Woof, J. M. & Ken, M. A. The function of immunoglobulin A in immunity. *J. Pathol.* **208**, 270–282 (2006).

165. Dilillo, D. J., Tan, G. S., Palese, P. & Ravetch, J. V. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat. Med.* **20**, 143–51 (2014).

166. DiLillo, D. J., Palese, P., Wilson, P. C. & Ravetch, J. V. Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *J. Clin. Invest.* (2016) doi:10.1172/JCI84428.

167. He, W. *et al.* Alveolar macrophages are critical for broadly-reactive antibody-mediated protection against influenza A virus in mice. *Nat. Commun.* **8**, 1–13 (2017).

168. Steffen, U. *et al.* IgA subclasses have different effector functions associated with distinct

glycosylation profiles. *Nat. Commun.* **11**, (2020).

169. Yel, L. Selective IgA Deficiency. *J. Clin. Immunol.* **30**, 10–16 (2010).

170. Aleyd, E., Heineke, M. H. & Egmond, M. Van. The era of the immunoglobulin A Fc receptor Fc a RI ; its function and potential as target in disease. *Immunol. Rev.* **268**, 123–138 (2015).

171. Lood, C., Arve, S., Ledbetter, J. & Elkon, K. B. TLR7 / 8 activation in neutrophils impairs immune complex phagocytosis through shedding of FcgRIIA. *J. Exp. Med.* 1–17 (2017).

172. Stacey, H. D. *et al.* IgA potentiates NETosis in response to viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–9 (2021).

173. Leon, P. E. *et al.* Optimal activation of Fc-mediated effector functions by influenza virus hemagglutinin antibodies requires two points of contact. *Proc. Natl. Acad. Sci. U. S. A.* **113**, (2016).

174. He, W. *et al.* Broadly-Neutralizing Anti-Influenza Virus Antibodies: Enhancement of Neutralizing Potency in Polyclonal Mixtures and IgA Backbones. *J. Virol.* (2015) doi:10.1128/JVI.03099-14.

175. Maurer, M. A. *et al.* Glycosylation of Human IgA Directly Inhibits Influenza A and Other Sialic-Acid-Binding Viruses. *Cell Rep.* **23**, 90–99 (2018).

176. Freyn, A. W. *et al.* Influenza hemagglutinin-specific IgA Fc-effector functionality is restricted to stalk epitopes. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–11 (2021).

177. Seibert, C. W. *et al.* Recombinant IgA Is Sufficient To Prevent Influenza Virus Transmission in Guinea Pigs. *J. Virol.* **87**, 7793–7804 (2013).

178. Muramatsu, M. *et al.* Comparison of Antiviral Activity between IgA and IgG Specific to Influenza Virus Hemagglutinin: Increased Potential of IgA for Heterosubtypic Immunity.

PLoS One **9**, e85582 (2014).

179. Okuya, K. *et al.* Potential Role of Nonneutralizing IgA Antibodies in Cross-Protective Immunity against Influenza A Viruses of Multiple Hemagglutinin Subtypes. *J. Virol.* **94**, (2020).

180. Gould, V. M. W. *et al.* Nasal IgA Provides Protection against Human Influenza Challenge in Volunteers with Low Serum Influenza Antibody Titre. *Front. Microbiol.* **8**, (2017).

181. Oh, J. E. *et al.* Intranasal priming induces local lung-resident B cell populations that secrete protective mucosal antiviral IgA. *Sci. Immunol.* **6**, (2021).

182. Brinkmann, V. *et al.* Neutrophil Extracellular Traps Kill Bacteria. *Science (80-.).* **303**, 1532–1535 (2004).

183. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).

184. Jorch, S. K. & Kubes, P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat. Rev. Gastroenterol. Hepatol.* **23**, 279–287 (2017).

185. Rohrbach, A. S., Slade, D. J., Thompson, P. R. & Mowen, K. A. Activation of PAD4 in NET formation. *Front. Immunol.* **3**, (2012).

186. Fuchs, T. A. *et al.* Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **176**, 231–241 (2007).

187. Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A Myeloperoxidase-Containing Complex Regulates Neutrophil Elastase Release and Actin Dynamics during NETosis. *Cell Rep.* **8**, 883–896 (2014).

188. Yipp, B. G. *et al.* Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* **18**, 1386–1393 (2012).

189. Yipp, B. G. & Kubes, P. Review Article NETosis : how vital is it ? **122**, 2784–2795 (2017).

190. Tate, M. D. *et al.* Neutrophils Ameliorate Lung Injury and the Development of Severe Disease during Influenza Infection. *J. Immunol.* **183**, 7441–7450 (2009).

191. Tate, M. D. *et al.* The Role of Neutrophils during Mild and Severe Influenza Virus Infections of Mice. *PLoS One* **6**, e17618 (2011).

192. Tate, M. D., Brooks, A. G. & Reading, P. C. The role of neutrophils in the upper and lower respiratory tract during influenza virus infection of mice. *Respir. Res.* **9**, 57 (2008).

193. Lim, K. *et al.* Neutrophil trails guide influenza- specific CD8 + T cells in the airways. *Science (80-.).* **349**, (2015).

194. Zhu, L. *et al.* High Level of Neutrophil Extracellular Traps Correlates With Poor Prognosis of Severe Influenza A Infection. *J. Infect. Dis.* **217**, 428–437 (2018).

195. Narasaraju, T. *et al.* Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am. J. Pathol.* **179**, 199–210 (2011).

196. Abramson, J. S., Mills, E. L., Giebink, G. S. & Quie, P. G. Depression of monocyte and polymorphonuclear leukocyte oxidative metabolism and bactericidal capacity by influenza A virus. *Infect. Immun.* **35**, 350–355 (1982).

197. Abramson, J. S. *et al.* Inhibition of neutrophil lysosome-phagosome fusion associated with influenza virus infection in vitro. Role in depressed bactericidal activity. *J. Clin. Invest.* **69**, 1393–1397 (1982).

198. Hiroki, C. H. *et al.* Neutrophil Extracellular Traps Effectively Control Acute Chikungunya Virus Infection. *Front. Immunol.* **10**, 3108 (2020).

199. Saitoh, T. *et al.* Neutrophil extracellular traps mediate a host defense response to human

immunodeficiency virus-1. *Cell Host Microbe* **12**, 109–116 (2012).

200. Cortjens, B. *et al.* Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. *J. Pathol.* **238**, 401–411 (2016).

201. Toussaint, M. *et al.* Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nat. Med.* **23**, 681–691 (2017).

202. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nature Reviews Immunology* vol. 18 134–147 (2018).

203. Sung, P. S., Huang, T. F. & Hsieh, S. L. Extracellular vesicles from CLEC2-activated platelets enhance dengue virus-induced lethality via CLEC5A/TLR2. *Nat. Commun.* **10**, (2019).

204. Jenne, C. N. *et al.* Neutrophils Recruited to Sites of Infection Protect from Virus Challenge by Releasing Neutrophil Extracellular Traps. *Cell Host Microbe* **13**, 169–180 (2013).

205. Veras, F. P. *et al.* SARS-CoV-2 triggered neutrophil extracellular traps (NETs) mediate COVID-19 pathology. *J. Exp. Med.* **217**, (2020).

206. Zuo, Y. *et al.* Neutrophil extracellular traps in COVID-19. *JCI Insight* **5**, (2020).

207. Masso-Silva, J. A. *et al.* Increased Peripheral Blood Neutrophil Activation Phenotypes and Neutrophil Extracellular Trap Formation in Critically Ill Coronavirus Disease 2019 (COVID-19) Patients: A Case Series and Review of the Literature. *Clin. Infect. Dis.* **74**, 479–489 (2022).

208. Bartsch, Y. C. *et al.* Humoral signatures of protective and pathological SARS-CoV-2 infection in children. *Nat. Med.* **27**, 454–462 (2021).

209. LaSalle, T. J. *et al.* Longitudinal characterization of circulating neutrophils uncovers

phenotypes associated with severity in hospitalized COVID-19 patients. *Cell Reports Med.* **3**, 100779 (2022).

210. Barnes, B. J. *et al.* Targeting potential drivers of COVID-19: Neutrophil extracellular traps. *J. Exp. Med.* **217**, 1–7 (2020).

211. Fazekas de St Groth & Webster, R. G. Disquisitions of Original Antigenic Sin. I. Evidence in man. *J. Exp. Med.* **124**, 331–45 (1966).

212. Jensen, K. E., Davenport, F. M., Hennessy, A. V & Francis, T. Characterization of influenza antibodies by serum absorption. *J. Exp. Med.* **104**, 199–209 (1956).

213. Davenport, F. M., Hennessy, A. V & Francis, T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. *J. Exp. Med.* **98**, 641–56 (1953).

214. Rothman, A. L. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat. Rev. Immunol.* **11**, 532–543 (2011).

215. Anderson, E. M. *et al.* SARS-CoV-2 infections elicit higher levels of original antigenic sin antibodies compared with SARS-CoV-2 mRNA vaccinations. *Cell Rep.* **41**, 111496 (2022).

216. Zarnitsyna, V. I. *et al.* Masking of antigenic epitopes by antibodies shapes the humoral immune response to influenza. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **370**, 20140248 (2015).

217. Viant, C. *et al.* Antibody Affinity Shapes the Choice between Memory and Germinal Center B Cell Fates. *Cell* **183**, 1298-1311.e11 (2020).

218. Mesin, L. *et al.* Restricted Clonality and Limited Germinal Center Reentry Characterize Memory B Cell Reactivation by Boosting. *Cell* **180**, 92-106.e11 (2020).

219. Schiepers, A. *et al.* Molecular fate-mapping of serum antibody responses to repeat immunization. *Nature* (2023) doi:10.1038/s41586-023-05715-3.

220. Richards, K. A. *et al.* Evidence That Blunted CD4 T-Cell Responses Underlie Deficient Protective Antibody Responses to Influenza Vaccines in Repeatedly Vaccinated Human Subjects. *J. Infect. Dis.* **222**, 273–277 (2020).

221. Smith, D. J., Forrest, S., Ackley, D. H. & Perelson, A. S. Variable efficacy of repeated annual influenza vaccination. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14001–6 (1999).

222. Luk, J., Gross, P. & Thompson, W. W. Observations on Mortality during the 1918 Influenza Pandemic. *Clin. Infect. Dis.* **33**, 1375–1378 (2001).

223. Gagnon, A. *et al.* Age-Specific Mortality During the 1918 Influenza Pandemic: Unravelling the Mystery of High Young Adult Mortality. *PLoS One* **8**, e69586 (2013).

224. Simonsen, L. *et al.* Global mortality estimates for the 2009 Influenza Pandemic from the GLaMOR project: a modeling study. *PLoS Med.* **10**, e1001558 (2013).

225. Nguyen, A. M. & Noymer, A. Influenza mortality in the United States, 2009 pandemic: burden, timing and age distribution. *PLoS One* **8**, e64198 (2013).

226. Ellebedy, A. H. *et al.* Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 13133–8 (2014).

227. Henry Dunand, C. J. *et al.* Both Neutralizing and Non-Neutralizing Human H7N9 Influenza Vaccine-Induced Monoclonal Antibodies Confer Protection. *Cell Host Microbe* **19**, 800–813 (2016).

228. Linderman, S. L. *et al.* Potential antigenic explanation for atypical H1N1 infections among middle-aged adults during the 2013–2014 influenza season. *Proc. Natl. Acad. Sci.*

U. S. A. **111**, 15798–15803 (2014).

229. Monto, A. S., Malosh, R. E., Petrie, J. G. & Martin, E. T. The Doctrine of Original Antigenic Sin: Separating Good From Evil. *J. Infect. Dis.* **215**, 1782–1788 (2017).

230. Keitel WA, Cate TR, Couch RB, Huggins LL & Hess KR. Efficacy of repeated annual immunization with inactivated influenza virus vaccines over a five year period. *Vaccine* **15**, 1114–1122 (1997).

231. Skowronski, D. M. *et al.* Serial vaccination and the antigenic distance hypothesis: Effects on influenza vaccine effectiveness during A(H3N2) epidemics in Canada, 2010-2011 to 2014-2015. *J. Infect. Dis.* **215**, 1059–1069 (2017).

232. Kissling, E. *et al.* Low 2018/19 vaccine effectiveness against influenza A(H3N2) among 15–64-year-olds in Europe: Exploration by birth cohort. *Eurosurveillance* **24**, 1–12 (2019).

233. Skowronski, D. M. *et al.* Paradoxical clade- And age-specific vaccine effectiveness during the 2018/19 influenza A(H3N2) epidemic in Canada: Potential imprint-regulated effect of vaccine (I-REV). *Eurosurveillance* **24**, (2019).

234. Kim, J. H., Skountzou, I., Compans, R. & Jacob, J. Original antigenic sin responses to influenza viruses. *J. Immunol.* **183**, 3294–301 (2009).

235. Kim, J. H., Davis, W. G., Sambhara, S. & Jacob, J. Strategies to alleviate original antigenic sin responses to influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13751–6 (2012).

236. Yewdell, J. W. & Santos, J. J. S. Original antigenic sin: How original? how sinful? *Cold Spring Harb. Perspect. Med.* **11**, 1–16 (2021).

237. Kilks, S. Antibody-Enhanced Infection of Monocytes as the Pathogenetic Mechanism for

Severe Dengue Illness. *AIDS Res. Hum. Retroviruses* **6**, 993–998 (1990).

238. Reynolds, C. J. *et al.* Immune boosting by B.1.1.529 (Omicron) depends on previous SARS-CoV-2 exposure. *Science (80-.).* **377**, (2022).

239. Tauzin, A. *et al.* A single dose of the SARS-CoV-2 vaccine BNT162b2 elicits Fc-mediated antibody effector functions and T cell responses. *Cell Host Microbe* **29**, 1137-1150.e6 (2021).

240. Tauzin, A. *et al.* Strong humoral immune responses against SARS-CoV-2 Spike after BNT162b2 mRNA vaccination with a 16-week interval between doses. *Cell Host Microbe* **30**, 97-109.e5 (2022).

241. Liu, L. *et al.* Anti-spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection. *JCI insight* **4**, (2019).

242. Junqueira, C. *et al.* FcγR-mediated SARS-CoV-2 infection of monocytes activates inflammation. *Nature* **606**, 576–584 (2022).

243. WHO- The Top 10 Causes of Death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (2020).

244. WHO- Cardiovascular Diseases. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

245. Corrales-Medina, V. F., Madjid, M. & Musher, D. M. Role of acute infection in triggering acute coronary syndromes. *Lancet Infect. Dis.* **10**, 83–92 (2010).

246. McMurray, J. & Pfeffer, M. Heart Failure. *Lancet (Seminar)* **365**, 1877–1889 (2005).

247. Bragazzi, N. L. *et al.* Burden of heart failure and underlying causes in 195 countries and territories from 1990 to 2017. *Eur. J. Prev. Cardiol.* **28**, 1682–1690 (2021).

248. Cook, C., Cole, G., Asaria, P., Jabbour, R. & Francis, D. P. The annual global economic

burden of heart failure. *Int. J. Cardiol.* **171**, 368–376 (2014).

249. Feske, S. K. Ischemic Stroke. *Am. J. Med.* **134**, 1457–1464 (2021).

250. Housworth, J. & Langmuir, A. D. Excess Mortality from Epidemic Influenza, 1957-1966. *Am. J. Epidemiol.* **100**, 40–48 (1974).

251. Collins, S. D. Excess Mortality from Causes Other than Influenza and Pneumonia during Influenza Epidemics. *Public Heal. Reports* **47**, 2159 (1932).

252. Nguyen, J. L. *et al.* Seasonal influenza infections and cardiovascular disease mortality. *JAMA Cardiol.* **1**, 274–281 (2016).

253. Smeeth, L. *et al.* Risk of Myocardial Infarction and Stroke after Acute Infection or Vaccination. *N. Engl. J. Med.* **351**, 2611–2618 (2004).

254. Warren-Gash, C., Smeeth, L. & Hayward, A. C. Influenza as a trigger for acute myocardial infarction or death from cardiovascular disease: a systematic review. *Lancet Infect. Dis.* **9**, 601–610 (2009).

255. Alber, D. G., Powell, K. L., Vallance, P., Goodwin, D. A. & Grahame-Clarke, C. Herpesvirus Infection Accelerates Atherosclerosis in the Apolipoprotein E–Deficient Mouse. *Circulation* **102**, 779–785 (2000).

256. Kenney, A. D. *et al.* Influenza virus replication in cardiomyocytes drives heart dysfunction and fibrosis. *Sci. Adv.* **8**, 1–10 (2022).

257. Fislová, T. *et al.* Multiorgan distribution of human influenza A virus strains observed in a mouse model. *Arch. Virol.* **154**, 409–419 (2009).

258. Hansson, G. K. & Hermansson, A. The immune system in atherosclerosis. *Nat. Immunol.* **12**, 204–212 (2011).

259. Behrouzi, B., Bhatt, D. L., Cannon, C. P., Vardeny, O. & Lee, D. S. Association of

Influenza Vaccination With Cardiovascular Risk. **5**, 1–9 (2022).

260. Clar, C. *et al.* Influenza vaccines for preventing cardiovascular disease (Review). *Cohrane Rev.* (2015) doi:10.1002/14651858.CD005050.pub3.www.cochranelibrary.com.

261. MacIntyre, C. R. *et al.* Ischaemic heart disease, influenza and influenza vaccination: a prospective case control study. *Heart* **99**, 1843–1848 (2013).

262. Siriwardena, A. N., Gwini, S. M. & Coupland, C. A. C. Influenza vaccination, pneumococcal vaccination and risk of acute myocardial infarction: matched case-control study. *Can. Med. Assoc. J.* **182**, 1617–1623 (2010).

263. Yedlapati, S. *et al.* Effects of Influenza Vaccine on Mortality and Cardiovascular Outcomes in Patients With Cardiovascular Disease : A Systematic. (2021) doi:10.1161/JAHA.120.019636.

264. Loeb, P. M. *et al.* Influenza vaccine to reduce adverse vascular events in patients with heart failure : a multinational randomised ,. *Lancet Glob. Heal.* **10**, e1835–e1844.

265. Dowdle, W. R. Influenza Pandemic Periodicity, Virus Recycling, and the Art of Risk Assessment. *Emerg. Infect. Dis.* **12**, 34–39 (2006).

266. Ontario, P. H. COVID-19 and Other Respiratory Illnesses in Pediatric Populations. 1–35 (2022).

267. Brandsma, A. M. *et al.* Potent Fc Receptor Signaling by IgA Leads to Superior Killing of Cancer Cells by Neutrophils Compared to IgG. *Front. Immunol.* **10**, (2019).

268. Koernig, S. *et al.* Topical application of human-derived Ig isotypes for the control of acute respiratory infection evaluated in a human CD89-expressing mouse model. *Mucosal Immunol.* **12**, 1013–1024 (2019).

269. 2018-19 Influenza Season (Aug 26 , 2018 to Aug 24 , 2019) Overall Summary Influenza

/ Influenza-like Illness (ILI) Activity (geographic spread) Laboratory-Confirmed Influenza Detections. **13**, (2019).

270. Linderman, S. L. & Hensley, S. E. Antibodies with 'Original Antigenic Sin' Properties Are Valuable Components of Secondary Immune Responses to Influenza Viruses. *PLOS Pathog.* **12**, e1005806 (2016).

271. Petrie, J. G., Ohmit, S. E., Johnson, E., Cross, R. T. & Monto, A. S. Efficacy studies of influenza vaccines: Effect of end points used and characteristics of vaccine failures. *J. Infect. Dis.* **203**, 1309–1315 (2011).

272. CDC. CDC Seasonal Flu Vaccine Effectiveness Studies. https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm.

273. Havervall, S. *et al.* Anti-Spike Mucosal IgA Protection against SARS-CoV-2 Omicron Infection. *N. Engl. J. Med.* **387**, 1333–1336 (2022).

274. Zuo, F., Marcotte, H., Hammarstrom, L. & Pan-Hammarstrom, Q. Mucosal IgA against SARS-CoV-2 Omicron Infection. *N. Engl. J. Med.* **387**, e55 (2022).