Humoral and cellular immune responses after pandemic and seasonal influenza vaccination in humans

Sarah Larteley Lartey Jalloh

Thesis for the degree of Philosophiae Doctor (PhD)
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Gye Nyame,

"*Action without thought is empty.*

Thought without action is blind"

Dr. Kwame Nkrumah (1909-1972)

I dedicate this work to my husband Alusine Alan and

my children Vanessa, Florence and Melanie

Scientific environment

The doctoral project was performed at the Influenza Centre, Department of Clinical Sciences, as part of the Ph.D. program at the Faculty of Medicine, University of Bergen. The research was completed between 2016 and 2022. We conducted two vaccine clinical trials, the first was in collaboration with the Bergen Clinical Vaccine Centre at Haukeland University Hospital. The second vaccine clinical trial was conducted in collaboration with the Pediatric Clinical Trial Unit and the Ear, Nose, and Throat clinic at Haukeland University Hospital. The studies were financed by the Influenza Centre, the Faculty of Medicine, University of Bergen, Norway, the Bergen Clinical Vaccine Consortium and EU IMI115672 FLUCOP project. The Influenza Centre is also funded by the European Union (EU IMI Inno4vac 101007799, INCENTIVE H2020 874866, Vaccelerate H2020 101037867 and Univax 601738), the Ministry of Health and Care Services, Norway, the Norwegian Research Council Globvac (284930), Helse-Vest (F-11628), the K.G. Jebsen Centre for Influenza Vaccine Research, the Trond Mohn Stiftelse (TMS2020TMT05), EU Nanomedicines Flunanoair (ERA-NETet EuroNanoMed2 i JTC2016).

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List of publications in this thesis

I. **Lartey S**, Pathirana RD, Zhou F, Jul-Larsen Å, Montomoli E, Wood J, Cox RJ. Single dose vaccination of the AS03-adjuvanted A(H1N1)pdm09 monovalent vaccine in health care workers elicits homologous and cross-reactive cellular and humoral responses to H1N1 strains. Human Vaccines & Immunotherapeutics. 2015 Jul; 11 (7): 1654-1662. doi:10.1080/21645515.1048939

II. Trieu MC, Zhou F, **Lartey S**, Sridhar S, Mjaaland S, Cox RJ. Augmented $CD4^+$ T-cell and humoral responses after repeated annual influenza vaccination with the same vaccine component A/H1N1pdm09 over 5 years. Nature Partner Journal Vaccines, 2018 Aug 14; 3: 37. doi: 10.1038/s41541-018-0069-1

III. **Lartey S,** Zhou F, Brokstad KA, Mohn KG, Slettevoll SA, Pathirana RD, Cox RJ. Live attenuated influenza vaccine induces tonsillar follicular T helper cell responses that correlate with antibody induction. The Journal of Infectious Diseases. 2020 Jan 1; 221(1): 21-32. doi: 10.1093/infdis/jiz321

List of publications not incuded in this thesis

1. Hequet D, Pascual M, **Lartey S**, Pathirana RD, Breholt G, Hoschler K, Hullin R, Meylan P, Cox RJ, Oriol M. Humoral, T-cell and B-cell immune responses to seasonal influenza vaccinein solid organ transplant recipients receiving anti-T-cell therapies. Vaccine. 2016 Jun 30; 34(31): 3576-83. doi: 10.1016/j.vaccine.2016.05.021

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4. Trieu M-C, Zhou F, **Lartey S**, Jul-Larsen Å, Mjaaland S, Sridhar S, Cox RJ. Long-term Maintenance of the Influenza-Specific Cross-Reactive Memory CD4+ T-Cell Responses. Following Repeated Annual Influenza Vaccination. The Journal of Infectious Diseases. 2017 Mar 1; 215(5): 740-749. doi 10.1093/infdis/jiw619

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Summary

Vaccination is the most effective prophylaxis against epidemic and pandemic influenza. Annual seasonal influenza vaccination is recommended for high-risk groups such as younger children <5 years old and occupational workers such as frontline healthcare workers (HCWs). In 2009, a novel A/H1N1 influenza virus emerged causing the first pandemic of the 21st century. The AS03-adjuvanted inactivated monovalent A/H1N1 pandemic vaccine was rapidly deployed prior to the peak of pandemic in Bergen. HCWs were prioritized for the first round of the vaccination, during the autum of 2009 to maintain the integrity of the healthcare system. The A(H1N1)pdm09 strain was subsequently included in seasonal trivalent inactivated influenza vaccines (TIVs) from 2010/2011 until 2016/2017. The trivalent Live Attenuated Influenza Vaccine (LAIV) was licensed for seasonal use in Europe in 2012 and it is recommended for children 2-17 years old.

We conducted two vaccine clinical trials using licensed influenza vaccines. In the first clinical trial we evaluated both the immediate and durable humoral and cellular immune responses in HCWs vaccinated with the AS03-adjuvanted pandemic vaccine and subsequent annual seasonal TIVs. In the second clinical trial we investigated in depth the follicular helper T (T_{FH}) cell and antibody responses elicited by LAIV in children and adults. We reported that the pandemic vaccine induced rapid homologous and cross-reactive T cell, B cell and antibody responses against the A(H1N1)pdm09 strain and pre-2009 seasonal influenza A/H1N1 strains. We observed that the baseline A(H1N1)pdm09-specific immune responses significantly increased from 2009 to 2013 and were maintained at high levels after 3–4 repeated vaccinations. Collectively our data from the HCW study provide the immunological evidence for continuing annual influenza vaccination policy in adults. Furthermore, we demonstrated that LAIV induced significant increase in influenza specific systemic and local antibody responses against the three vaccine strains tested as early as day 14 post-vaccination. We also showed that LAIV elicited potent and rapid influenza specific T_{FH} cell responses in children. Our LAIV results provide valuable insights into the immunogenicity of LAIV in different age groups with variable levels of pre-existing immunity.

Abbreviations

ACIP	Advisory Committee on Immunization Practices
ADCC	Antibody-dependent cell cytotoxicity
AIDS	Acquired immune deficiency syndrome
AECs	Airways epithelial cells
APC	Antigen presenting cell
AMs	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
ASC	Antibody secreting cells
AS03	Adjuvant System 3
ASA	Acetyl salicylic acid
ASC	Antibody-secreting cell
Bcl-6	B cell lymphoma-6
BEI Resources	Biodefense and Emerging Infections Research Resources
CHMP	Committee for Medicinal Products for Human Use
T CM cell	T Central memory cell
CO_2	Carbon dioxide
Cy3	Cyanine dye 3
CCR7	C-C-chemokine receptor 7
CDC	Centers for Disease Control and Prevention
CHMP	Committee for Medicinal Products
CMR1	Chromosomal maintenance 1
COBRA	Computationally Optimized Broadly Reactive Antigen
CTL	Cytotoxic T lymphocytes
COBRA	Computationally Optimized Broadly Reactive HA Antigens
COVID-19	Coronavirus Disease 19
cRNP	Complementary ribonucleoprotein
CrCl3	Chromium chloride
CXCR5	C-X-C-chemokine receptor 5
DZ	Dark zone
DC	Dendritic cell
DISC	Death-inducing signaling complex

DLN	Draining lymph nodes
DMSO	Dimethyl sulfoxide
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immune absorbent spot
EMA	Europe Medicines Agency
ENT	Ear, Nose, and Throat
FBS	Fetal bovine serum
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FluoroSpot	Fluorescent enzyme-linked immunospot
GC	Germinal Center
GISRS	Global Influenza Surveillance and Response System
GLaMOR	Global Influenza Mortality project
GMT	Geometric mean hemagglutination-inhibition titer
GSK	GlaxoSmithKline
H1N1pdm09	H1N1 strain caused influenza pandemic in 2009
HA	Hemagglutinin
HAI	Hemagglutination inhibition assay
HAU	Hemagglutinin units
HCW	Healthcare worker
HEF	Hemagglutinin-esterase fusion
HI	Hemagglutination inhibition
H.I	Heterosubtypic immunity
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICOS	Inducible co-stimulator
ICS	Intracellular cytokine-staining
IFN	Interferon
Ig	Immunoglobulin
IV	Influenza virus

IIV	Inactivated influenza vaccine
IHC	Immunohistochemistry
IL	Interleukin
IBV	Influenza B viruses
IRR	International Reagent Resources
ISG	Interferon stimulated genes
LAIV	Live-attenuated influenza vaccine
LFA	Lateral flow assay
LRT	Lower respiratory tract
LZ	Light zone
M1/M2	Matrix 1 or 2 protein
M2e	Matrix protein 2 ectodomain
MAbs	Monoclonal antibodies
MBC	Memory B cell
MDCK cell	Madin-Darby canine kidney cell
MHC	Major histocompatibility complex
MN	Microneutralization
NA	Neuraminidase
NAAT	Nucleic acid amplification test
NEP	Nuclear Export Protein
NK	Natural killer
NIBSC	National Institute for Biological Standards and Control
NIC	National Influenza Centre
NIPH	Norwegian Institute of Public Health
NOD	Nucleotide-binding oligomerization domain like receptors
NP	Nucleoprotein
NS1/NS2	Nonstructural protein 1or 2
PA	Polymerase acidic
OPD	Ortho-Phenylenediamine dihydrochloride substrate
PAMP	Pathogen-associated molecular pattern
PB1/PB2	Polymerase basic protein 1 or 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PKR	Protein-Kinase R
PRR	Pattern recognition receptor
RBS	Receptor binding site
RDE	Receptor-destroying enzyme
RIDT	Rapid influenza diagnostic tests
RNA	Ribonucleic acid
RIG-1	Retinoid acid inducible gene-1
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe Acute Respiratory Corona Virus
SRH	Singer radial hemolysis
TCR	T cell receptor
T_{FH} cell	T follicular helper cell
T_{H} cell	T helper cell
TLR	Toll-like receptors
TEM cell	T effector memory cell
TMNC	Tonsillar mononuclear cells
TNF	Tumor necrosis factor
TIV	Trivalent inactivated influenza vaccines
TRBC	Turkey red blood cells
TRIM	Tripartite motif
T_{REG} cell	T regulatory cell
URT	Upper respiratory tract
VE	Vaccine effectiveness
VN	Virus neutralization
SPSS	Statistical Package for the Social Sciences
vRNP	Viral ribonucleoprotein
WIV	Whole inactivated virus vaccines
WHO	World Health Organization

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1. INTRODUCTION

1.1 Influenza epidemiology

Influenza is a major global health concern because of its rapid evolution and the threat to public health, with seasonal epidemics and less frequent pandemic resulting in significant health and socioeconomic burden [1]. The name influenza originated from 15th century Italian language meaning "influence", which was believed to be caused by the influence of astrological movements during the winter [2]. In the Northern Hemisphere, influenza generally circulates during the winter months in temperate climate regions including north American and Europe between October to April, when low temperatures favor transmission. In the Southern Hemisphere, influenza circulates between May to September. In the tropical regions influenza outbreaks occur throughout the year with high transmission and peak activities during the rainy season [3]. The primary mode of influenza transmission is through inhalation of large or small airborne droplets containing infectious viral particles. These airborne droplets are expelled during sneezing and coughing from infected individuals. The incubation period of influenza is 1-2 days and infected individuals are infectious prior to symptom onset, but also for up to ten days in younger children. Younger children and immunosuppressed individuals have higher viral loads and may shed the virus for longer periods [4, 5]. Influenza affects people of all ages; however, it is the most common cause of acute respiratory illness in the elderly ≥ 65 years of age, infants, and children <5 years old, immunocompromised individuals and pregnant women. Younger children are the main transmitters of influenza virus in the community [6].

1.2 Burden of influenza disease

The world health organization (WHO) recommends estimation of the national and the global burden of influenza disease to document the overall impact of influenza on various aspects of public health, and thus strengthening national and global influenza pandemic preparedness [7]. Furthermore, influenza is associated with considerable socioeconomic burden on families, healthcare services and society.

the socioeconomic burden of influenza includes hospitalization, visit to physician, absenteeism from school and work either due to personal illness or caring for a sick child. Estimating the burden of influenza is important to inform policymakers about the overall magnitude of the economic costs of the influenza disease to prioritize allocation of resources for influenza prevention and control efforts. The estimated economic burden of seasonal influenza ranges between $95-$479 million in Norway [8, 9]. WHO estimated the annual seasonal influenza epidemics to be responsible for approximately 3-5 million cases of severe influenza illness and 250-500 000 influenza associated mortality worldwide, with highest annual excess mortality rate occurring in the elderly ≥ 65 years old. In Norway, approximately 5000 people are hospitalized and 900 people die each year as the result of influenza illness [3, 10, 11].

The annual influenza attack rate is estimated to be 5-10% in adults and 20-30 % in preschool children but may be higher among children in daycare [12]. A recent meta-analysis found that influenza is associated with 14% hospitalization among all adults with the elderly contributing to most of the hospitalizations [13]. The global influenza mortality project (GLAMOR) reported that 67% of influenza related deaths occur in the elderly ≥ 65 years old in high income countries with the highest percent of deaths found in Europe (85%). Whereas in the low-income countries the highest percent of influenza related death in sub-Saharan Africa occur in adults <65 years old [14]. Studies have demonstrated that influenza associated deaths were highest in the elderly during the seasons when influenza A/H3N2 strain was the dominant circulating virus, followed by seasons when influenza B or influenza A/H1N1 were dominant [14-16]. Additionally, Paget *et al.* found that influenza associated deaths among the <65 age group coincided with circulation of the influenza A(H1N1)pdm09 strain [14]. Infants and children <5 years of age are at great risk for influenza-associated hospitalizations and deaths worldwide compared to other respiratory diseases. The annual burden of influenza illness in children <5 years old was estimated to be approximately 800 000 hospitalizations and 15 000 deaths globally [17]. A recent study found that an average of 1013 children were hospitalized with influenza in Norway during the 2018-2019

influenza season with the highest rate of hospitalization among infant <6 months old and the majority (75%) of the children did not have pre-existing risk conditions [18].

1.3 Current influenza virus circulation

Since the emergence of SARS-CoV-2 virus and the global pandemic, low level of influenza activity was reported during the 2020-2021 influenza season, compared to previous influenza seasons. The drop in influenza cases was observed in March of 2020, when drastic mitigation infection control measures were implemented as a response to the COVID-19 pandemic and influenza activity has since remained low. Globally, approximately 3 million respiratory virus samples were collected between November 2019 to the end of December 2020 by National Influenzas Centers (NIC), of these 19% (600 000) were positive for influenza, furthermore it was estimated that 99% of these samples were collected between early November 2019 to April 2020 [19]. A similar trend in influenza activity was reported by the Norwegian Institute of Public Health (NIPH) during the 2019-2020 influenza season, compared to previous seasons there was no or low numbers of influenza cases in Norway. The low numbers of influenza like-illness, influenza hospitalization and low Intensive Care Unit (ICU) admission made it impossible to estimate influenza associated excess mortality during the 2020-2021 season both in Norway and globally [10, 19]

1.4 Influenza virus

1.4.1 First influenza virus isolation

Influenza is an acute infection, which is caused by an enveloped single-stranded negative-sense RNA virus that belongs to the family of *orthomyxoviridae.* Four types of influenza viruses have been isolated and characterized within the *orthomyxoviridae* family, these include influenza A, B, C and D viruses [20]. Following the devastating 1918 influenza pandemic “the Spanish flu”, an intensive quest to identify the causative agent responsible for the pandemic was carried out. In 1933 the first human influenza A virus was isolated in ferrets and this was followed by the isolation of influenza B and C viruses in 1940 and 1947 respectively [2, 21]. Among the four influenza types, three are known to infect humans (influenza A, B and C), while the novel influenza D

is mainly known to infect cattle, however other small ruminates are susceptible to IDV infection [22] (Figure 1.1). Influenza A and B viruses are responsible for the annual influenza epidemics and can cause morbidity and mortality in otherwise healthy humans, whereas influenza C viruses infects and causes illness in the upper respiratory tract with mild clinical symptoms in man [23].

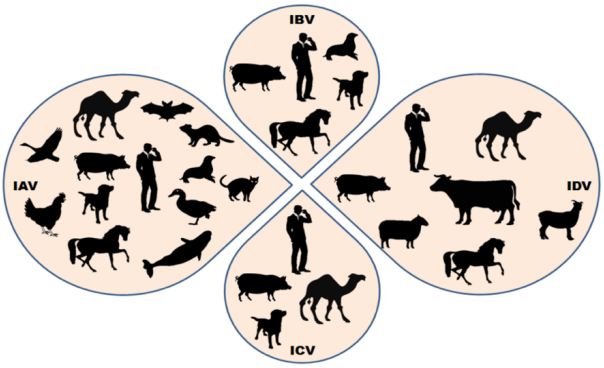

Figure 1.1 The host range of influenza viruses

Influenza A viruses has a wide host range with aquatic birds being the main natural reservoir. Influenza A, B and C viruses are known to infect humans. The primary reservoir of influenza D viruses are cattle, however, it remains controversial whether influenza D viruses infect humans, as the virus was found in people exposed to cattle, but the virus has not yet been isolated [22]. The figure is preprinted with permission from [22]

1.4.2 Influenza taxonomy

The two major surface glycoprotein hemagglutinin (HA) and neuraminidase (NA) play crucial roles in the pathogenesis of the influenza A and B viruses. Influenza C and D lack NA and only have one distinct surface glycoprotein called hemagglutinin-esterase fusion (HEF), which functions as both a receptor binding and a receptor destroying protein [24]. The WHO established a standard guideline for the nomenclature of influenza viruses in 1980, consisting of the influenza type (A, B, C or D), host of origin (if not isolated from humans), geographical region of origin, isolation number, year of isolation, and for influenza A viruses only, HA and NA subtype, described by letter and number, H1 to H18 and N1 to N11. The influenza virus that caused the 2009 influenza pandemic is for example denoted as A/California/04/2009 (H1N1) for

influenza type A, first isolated in California, isolation number 04, year of isolation 2009 and influenza subtype H1N1 [25].

1.4.3 Influenza A and B viruses

Influenza A viruses are major public health concern as they are the only influenza type known so far to have the potential to cause an influenza pandemic. They were the causative agents of all the pandemics recorded to date (A/H1N1, A/H2N2 and AH3N2 subtypes). Influenza A viruses are further divided into different subtypes based on their surface glycoproteins HA and NA. Where 18 HAs and 11 NAs have been identified and classified, which can give rise to 198 different influenza A subtypes. Influenza A viruses infect a wide range of mammals including humans; however, the main natural animal reservoir are aquatic birds, possessing majority of HAs and NAs (Figure 1.1) [2, 3]. Currently two influenza A subtypes A(H1N1) and A(H3N2) circulate in man and are responsible for the global annual influenza epidemics [26]. Only avian influenza A viruses can cause zoonotic infection in human, the highly pathogenic avian influenza viruses (HPAIV) including A/H5N1 subtype are of great public concern with the potential to cause an influenza pandemic. The first human case of A/H5N1 virus was reported in 1997 and has since then sporadically infected humans, causing severe disease and high mortality. Most human A/H5N1 cases (~75%) were reported in young adults and children [27]. As of 30th December 2021, there have been 863 cases and 456 deaths attributed to A/H5N1 infection in man globally [28].

Humans are the primary reservoir for IBV; however, they have also been identified in seals and horses (Figure 1.1) [26, 29]. Influenza B viruses infects both the lower and upper respiratory tract causing mild to severe influenza like illness. As opposed to IAV, IBV contain only one HA and one NA subtype and are therefore not categorized into subtypes, but rather classified into two distinct evolutionary lineages namely the B/Yamagata and B/Victoria lineages. Influenza B viruses have a narrower host range, due to their lack of subtype diversity and gene reassortment, hence they have little pandemic potential as they do not undergo antigenic shift. Individuals previously exposed to IBV may have less severe illness compared to naïve unexposed individuals, as new variants of IBV rarely occur [30].

1.4.4 Viral structure

Influenza is an enveloped virus composed of a host-cell derived lipid membrane; the viral particle size is approximately 80-120 nm in diameter (Figure 1.2). The two major viral surface glycoproteins; HA and NA and the less abundant matrix protein 2 (M2) ion channel protrude from the membrane. The interior of the membrane is lined with the structural protein matrix 1 (M1), which provides a scaffold that helps to determine the shape of the virion. Inside the virion are the eight RNA genome segments, each segment is made up of the viral ribonucleoprotein (vRNP) complex, which consists of the single-stranded negative-sense viral RNA (vRNA) with sizes ranging from 890 to 2341 nucleotides in length, the three RNA polymerase subunits (Polymerase basic 2 (PB2), PB1 and Polymerase acid (PA)) and the nucleoprotein (NP) [20, 31] The eight vRNAs encode 10 viral proteins (external proteins; HA, NA, M2 and internal proteins M1, PA, PB1, PB, NP and the nonstructural proteins (NS1/NS2)), The genome structure and genetic makeup of influenza A and B viruses are similar and differ from that of influenza C and D viruses (ICV/IDV). ICV and IDV have seven RNA genome segments, which encode seven viral proteins [32-34].

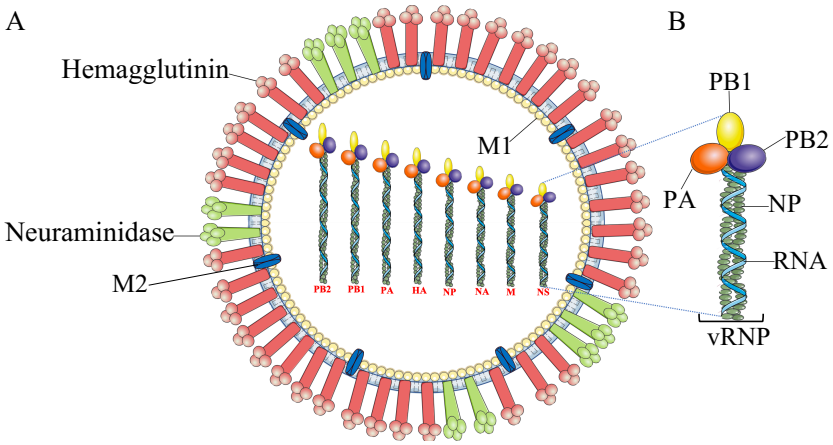

Figure 1.2 Schematic illustration of the influenza virus and the ribonucleoprotein

(A) Influenza is spherical (or filamentous) in shape with host cell derived envelope. The two major surface glycoproteins HA, NA and the ion channel M2 are embedded in the viral envelope, whereas the M1 protein line the interior of the virion forming a proteinaceous shell under the envelope. Eight RNA segments make up the viral genome, each segment consists of the viral RNA wrapped around the nucleoprotein (NP) oligomers in complex with three polymerase subunits (PA, PB1 and PB2). **(B)** The three polymerase subunits, the viral RNA and NP oligomers form the viral RNP complex. Each segment encodes one viral protein, apart from segments 7 (M1/M2 proteins) and 8 (NS1/NS2 proteins) that encode two viral proteins by alternative splicing. The illustration was made with PowerPoint using artwork from smart servier medical art [35]

1.4.5 Hemagglutinin

Hemagglutinin is the most abundant influenza virus surface glycoprotein that enables viral entry into the host cell. It is more abundant than the NA glycoprotein outnumbering it by 5-fold. The structure of the HA protein was first described by Wilson *et al*. in 1981; it is a glycosylated type 1 integral membrane protein. The HA homotrimer consists of a globular head domain which is anchored to the membrane and the stem/stalk domain protruding into the virion [36]. Influenza A virus have 18 HA subtypes, which are further divided into two subgroups based on their stem/stalk structure. Group 1 HAs include H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18, whereas group 2 HAs include H3, H4, H7, H10, H14 and H15 [37]. HA is produced as a single fused-polypeptide precursor HA0, which is proteolytically cleaved to generate mature disulphide-bonded HA1 and HA2 functional subunits, resulting in the activation of the HA glycoprotein. Conserved residues of the HA protein are found in the receptor binding site (RBS) located in the HA1 subunit and also in the stalk domain, which forms part of HA1 the whole HA2 subunits [38] The HA structure and the different HA subtypes are illustrated in Figure 1.3.

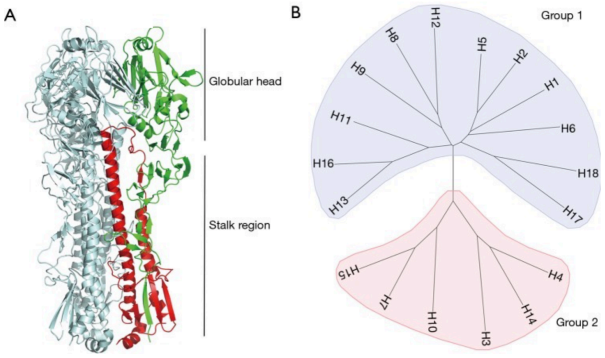

Figure 1.3 Structure of HA and the phylogenetic tree of influenza A HAs

(A) The HA glycoprotein is a homotrimer consisting of a globular head domain (HA1 subunit) and a stalk domain (HA2 subunit), each monomer is shown in different color (red, green and light blue). **(B)** A phylogenetic tree of the two HA subgroups. Group 1 HAs (blue) include H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18 and group 2 HAs (red) included H3, H4, H7, H10, H14 and H15. The figure is adapted with permission from [39]

Studies on antigenic mapping and sequence analysis revealed five major antigenic binding sites located in the globular head domain for H1 HAs (Sa, Sb, Ca1, Ca2, and

Cb) [40], five major antigen sites for H3 HAs (Site A-E) and four antigen site for influenza B HA (Loop120, 140, 150 and 190) [38, 41, 42] (Figure 1.4). These antigenic sites are targets for neutralizing antibodies, constantly evolving through antigenic drift to escape the host pre-existing immunity. Several key amino acid residues in the RBS determines the HA receptor-specificity and are conserved among influenza A and B viruses [43].

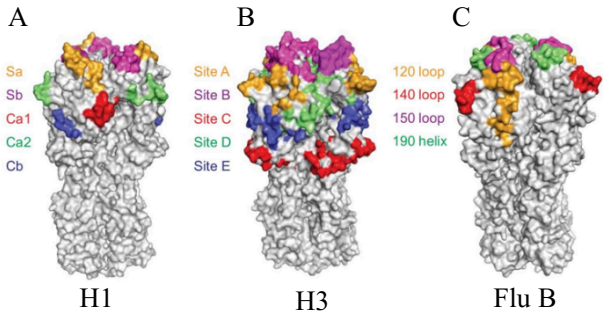

Figure 1.4 Illustration of the antigenic binding sites in influenza H1, H3 and B HAs

(A) Antigenic binding sites in HI HA RBC (Sa, Sb, Ca1, Ca2 and Cb), **(B)** H3 HA RBC (site A-E), **(C)** influenza B HA RBD (loop120, 140, 150 and 190) respectively. The figure is adapted with permission from [42]

Avian influenza viruses have HA receptor binding specificity for the α2,3-linked sialic acid, whereas human adapted influenza viruses preferentially bind to the α2,6-linked sialic acid receptor. A switch in avian HA receptor specificity from α2,3-linked to α2,6-linked sialic acid is required for inter human transmission of avian influenza virus, which may ultimately result in an influenza pandemic [38, 44, 45]. Swine epithelia cells expresses both sialic acid receptor types (α2,3-linked to α2,6-linked) and may explain why they can serve as a mixing vessel for emergence of novel pandemic viruses. Furthermore, the A/H5N1 virus preferentially binds to α2,3-linked sialic acid receptors expressed on some epithelial cells in the lower respiratory tract. This may be the reason why A/H5N1 viruses exhibit limited transmission in humans [46, 47].

1.4.6 Neuraminidase

The second major viral surface glycoprotein neuraminidase (NA) is a type II integral membrane glycoprotein, which is embedded into the viral envelope as a homotetramer consisting of four identical catalytic head domains with sialidase enzymatic activity, along with the stalk, transmembrane and cytosolic tail domains [48]. Like the grouping

of HA subtypes, 9 NAs subtypes are categorized into two distinct groups: group 1 contains N1, N4, N5 and N8 subtypes, while group 2 contains N2, N3, N6, N7 and N9 subtypes. All the 9 NA subtypes are found in waterfowl influenza A viruses and only two NA subtypes are found in human adapted influenza A viruses (N1 and N2) [49]. Furthermore, N10 and N11 are bat NA-like proteins, while influenza B viruses possess one NA protein. The main function of NA is the catalytic cleavage of terminal sialic acids from sialylated glycoproteins on host cell surface or on the surface of newly formed virions enabling the release of new progeny [49, 50]. Hemagglutinin molecules on the newly formed virions readily bind to the sialic acid receptors on the host cell surface near the budding site, the virus particles may aggregate on the host cell surface in the absence of NA sialidase enzymatic activity [51].

1.4.7 Influenza virus life cycle

Influenza viruses attach to sialic acid residues on receptors expressed by respiratory epithelial cells through the HA protein. Efficient attachment of HA to the host cell surface receptor is aided by NA, as opposing cooperating actions of both HA and NA facilitates the rolling of virus particles over sialylated receptor surfaces until the virion encounters a receptor with an active endocytosis site, subsequently mediating viral entry [52, 53]. Upon HA binding the virus enters by receptor-mediated endocytosis. Since the avidity of a single HA for sialic acid is low, multivalent receptor interactions of multiple HA molecules are required to trigger the internalization process, which is achieved when the viral particle is filamentous as opposed to spherical in shape [33, 54]. Acidification of the endosomes is mediated by the proton channel M2, which allows influx of protons into the virion interior [55]. At the lower pH of 5.0, a confirmation changes of the HA protein result in the viral and endosomal membrane fusion. A fusion pore is formed through which the vRNPs are released into the cytoplasm and subsequently transported into the nucleus [56]. Influenza viruses are unlike other RNA viruses in that transcription and replication of their genome occurs in the host cell nucleus. This is crucial because the transcription of vRNA is a primer dependent process which requires host cell 5`cap RNA. Furthermore, cellular spliceosome is required to generate spliced transcripts of M and NS genes that encode the M2 and the

nuclear export protein (NEP) proteins from segment 7 and 8, respectively [55]. The viral RNA is transcribed into viral mRNA, which is exported out of the nucleus into the cytoplasm, where translation of viral proteins is carried out by cellular translation machinery [20, 31]. The newly synthesized viral RNA polymerases and NP monomers associate with the complementary RNA (cRNA) to form cRNP, which acts as a template for the synthesis of additional vRNAs, followed by recruitment of more PB1, PB2, PA and NP to form new vRNP complex. The synthesized vRNPs are exported out of the nucleus (mediated by NEP) and trafficked toward the cell membrane, where they accumulate together with viral proteins and are packed into progeny virions [57]. M2 is involved in bud neck formation and scission of the membrane disconnecting the progeny virion from the plasma membrane [58]. Finally, NA facilitates the release of the virus by removing local sialic acid residues, thus preventing HA binding to the cell surface. The virus replication cycle is illustrated in Figure 1.5.

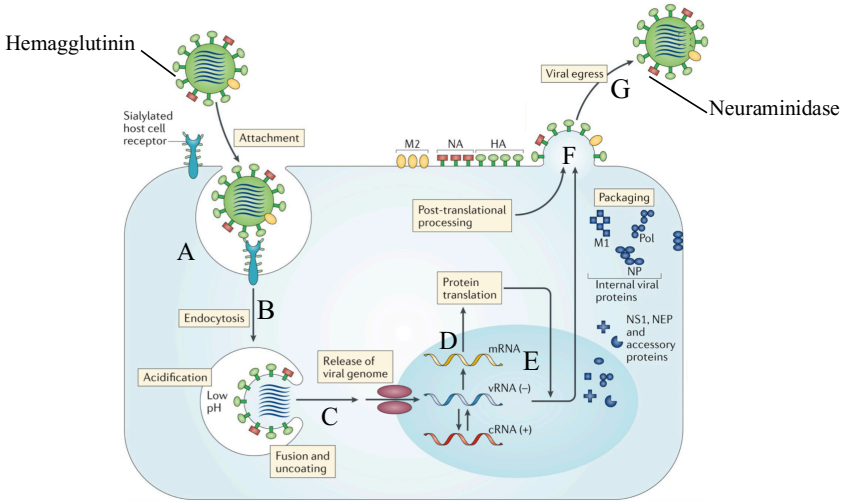

Figure 1.5 Influenza virus replication cycle

(A) The virus enters the host cell by receptor-mediated endocytosis. **(B)** Low pH in the endosome induces confirmational change of the HA protein mediating viral-endosomal membrane fusion and subsequently release of the vRNP complexes into the cytoplasm. **(C)** The vRNP complex is transported to the nucleus where replication and transcription take place. **(D)** The viral mRNAs are exported to the cytoplasm for the translation of viral proteins. **(E)** The polymerase proteins (PB1, PB2 and PA) required for replication are transported back into the nucleus, where they associate with cRNA for the synthesis of new vRNAs. **(F)** The newly synthesized vRNPs are exported out of the nuclear and accumulates at the apical of the plasma membrane, where the vRNPs and viral proteins are assembled into progeny virion **(G)** At the final stage of the replication cycle NA mediates viral egress. The figure is adapted with permission from [59] .

1.5 Antigenic Drift

The influenza virus is a constantly evolving pathogen, owing to antigenic changes that occurs in its surface glycoproteins HA and NA. It acquires the ability to evade the pre-existing host immunity and continues to survive in a population with substantial prior exposure history [3]. It utilizes two mechanisms to escape pre-existing immunity that arises from natural infection or vaccination: antigenic drift and antigenic shift. Antigenic drift is a process whereby minor changes are introduced in crucial epitopes in the viral surface glycoproteins (HA and NA), through accumulation of spontaneous point mutations in the HA and NA gene segments [26, 60]. Antigenic drift occurs in all influenza types albeit at lower rate in influenza B and C viruses. The conserved HA stalk domain, the NA protein, and the internal proteins of influenza A viruses acquire point mutation through antigenic drift , but at a slower rate compared to the HA protein [61]. Amino acid substitutions that occur at major antigenic sites such as the HA RBS, were found to be crucial in changing the virus antigenicity. Thus, allowing the virus to evade the host immune defense, gain fitness advantage and emerge as a novel epidemic strain, replacing the circulating strain. Antigenic drift and waning host immunity are the two main factors why annual influenza epidemic occur and necessitate the reviewing and updating of annual influenza vaccine strain compositions [2, 38, 47].

1.6 Antigenic Shift

The second mechanism that drastically alters the antigenicity of influenza A viruses is referred to as antigenic shift and occurs because of genetic reassortment of the influenza viral RNA genome [3]. This introduces novel HA and NA from for example zoonotic influenza A reservoir into the circulating human viruses, which can result in a novel influenza virus. Since the virus genome consists of 8 RNA segments, co-infection (which rarely occurs) of a host cell with avian, swine or human influenza A viruses can give rise to progeny viruses containing exchanged gene segments from the original parental viral strains [26, 60]. To date antigenic shift is only reported to occur in influenza A viruses and is often associated with influenza pandemics [47]. Antigenic shift has resulted in six influenza pandemics in 1889, 1898, 1918, 1957, 1968 and 2009 [62].

1.7 Influenza Pandemics

An influenza pandemic is described as an outbreak of a novel influenza virus that spreads rapidly worldwide infecting a large percentage of the human population with significantly high morbidity and mortality. The 1918 influenza pandemic was the most severe pandemic in human history referred to as the "Great influenza pandemic" [63, 64]. Influenza pandemics in the 20th and 21st centuries are depicted in (Figure 1.6).

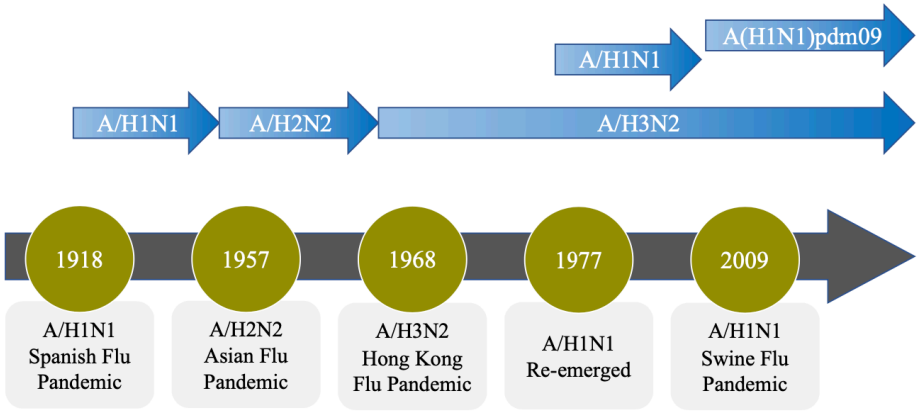

Figure 1.6 The timeline of previous human influenza pandemics

Depicting the years and influenza A subtypes. Historically six influenza pandemics had occurred, two in the 19th century (1889 and 1898 not shown here), three in the 20th century (1918, 1957 and 1968) and one in the 21st century (2009). The 1977 global influenza epidemic is not considered as an influenza pandemic but is included in the figure since it is relevant for this thesis. The figure was made using PowerPoint inspired by [65]

1.7.1 Influenza pandemics in the 20th century

Three influenza pandemics have occurred in the 20th century: the 1918 A/H1N1 "Spanish Flu" pandemic, the 1957 A/H2N2 "Asian Flu" pandemic and the 1968 A/H3N2 "Hong Kong Flu" pandemic. The 1918-1919 influenza pandemic was deemed the mother of all influenza pandemics as it was the deadliest pandemic in human history [64, 66]. It was estimated that approximately 500 million people accounting for 1/3 of the world's population were infected and staggering 50-100 million deaths were recorded globally [66, 67]. The high mortality associated with the 1918 pandemic was due to the incidence of secondary bacterial infections, which resulted in severe bacterial

pneumonia along with the lack of antiviral/antimicrobial drugs at the time and the end of the first world war [62]. A key feature of this pandemic was the unusually high infection and mortality rate in healthy young adults (20-45 years of age) with a W-shaped mortality curve compared to seasonal influenza epidemic with disproportionally high mortality rates in younger children and the elderly having a U-shaped mortality curve [68]. By 1920, the 1918 pandemic A/H1N1 virus began circulating as a seasonal virus and continued to circulate until 1957, when it was replaced and outcompeted by the novel A/H2N2 virus responsible for the 1957 Asian influenza pandemic. A decade later a reassortant A/H3N2 virus strain emerged completely replacing the A/H2N2 virus and caused the Hong Kong influenza pandemic in 1968. In contrast to the devastating 1918-19 pandemic, these two latter influenza pandemics were milder and exhibited similar patterns of morbidity and mortality without excess mortality in young adults. It was estimated that between 1-4 million deaths could be attributed to each pandemic globally. Following the Hong Kong pandemic in 1968, the A/H3N2 virus became endemic and continues to circulate to date as seasonal A/H3N2 virus [69].

The A/H1N1 virus reemerged in 1977 and caused a global influenza epidemic termed the "Russia flu" mostly in young adults <25 years of age. This age distribution was attributed to the fact that persons >25 years old may have either experienced the 1918 pandemic or encountered the A/H1N1 virus prior to its disappearance in 1957 [64, 70]. The 1977 Russia influenza epidemic has never been considered a true pandemic, as the virus that caused it was not novel but was a descendant of the 1918 A/H1N1 virus that was antigenically similar to the A/H1N1 virus circulating in 1957 [71]. The 1977 A/H1N1 virus has co-circulated with the seasonal A/H3N2 viruses and drifted subtypes caused seasonal influenza epidemics until the emergence of the novel swine origin A/H1N1 virus in 2009.

1.7.2 Influenza pandemic in the 21st century

Prior to the A/H1N1 pandemic in 2009, the influenza community worldwide was preparing (with stockpiled antivirals and pre-pandemic vaccine development) for the

spread and possible pandemic from the HPAI A/H5N1 and the AH7N9 avian viruses. These viruses have caused sporadic zoonosis with severe disease in human and in poultry in Asia. Hence, the emergence of a novel A/H1N1 virus was unexpected [47]. A novel influenza A/H1N1 virus of swine origin emerged causing the first influenza pandemic of the 21st century, it was first detected in the spring of 2009 in California and then in Mexico. The virus quickly spread globally and on June 11th the WHO signaled the pandemic phase 6 alert level, officially declaring the start of the swine influenza pandemic, after nearly 30,000 confirmed cases had been reported in 74 countries [72]. Antigenic characterization of the novel A(H1N1)pdm09 virus, revealed that it was a triples reassortant virus possessing unique combination of genes originating from avian, human and swine influenza viruses. Furthermore, it was antigenically distinct from previously circulating seasonal A/H1N1 strains [73-75]. Initially there were fears that the novel A(H1N1)pdm09 virus would cause a pandemic of similar magnitude to that of 1918 pandemic, when severe diseases and 80% mortality were reported among immunologically naïve healthy young adults [76, 77]. It was a relief when a later report found that the A(H1N1)pdm09 virus generally caused relatively mild disease comparable to seasonal influenza epidemics. The estimated suspected cases were between 700 million to 1.4 billion and approximately 148-250 000 deaths were reported in mainly persons <65 years of age worldwide [78].

The second pandemic in the 21st century is the ongoing COVID-19 pandemic, which is caused by the SARS-CoV-2 virus. Although SARS-CoV-2 is not an influenza virus is it nevertheless a respiratory pathogen with similar clinical manifestations as influenza. The knowledge acquired, lessons learnt from pass influenza pandemics/epidemics and WHO´s national and global pandemic preparedness have paved the way for rapid response to mitigate the COVID-19 pandemic. For example, the technology used to produce the COVID-19 mRNA vaccines, was developed decades ago to produce mRNA vaccines for cancer immunotherapy and for prophylaxis against infectious pathogens including influenza viruses [79, 80].

1.8 Pathogenesis

Influenza infection in the upper respiratory tract may trigger infected epithelial cell death (necrosis) following viral replication as a means of spreading the newly formed progeny virions [81]. Viral replication can also occur in other parts of the respiratory tract leading to superficial necrosis of tracheobronchitis and pulmonary parenchymal [82]. Uncontrolled necrosis of alveolar epithelial cells may compromise gas exchange during respiration [83]. The extension of viral infection to the lower respiratory tract can lead to the progression and development of severe pneumonia, which is frequently associated with secondary or concomitant bacterial pneumonia [82]. Severe influenza leading to excessive infiltration of innate immune cells like neutrophils or macrophages into the lung may results in lung epithelial damage, promoting bacteria invasion of the damaged lung. Secondary bacterial infection with for example *Streptococcus pneumonia* following severe influenza often led to excessive mortality [84]. This is more prevalent and problematic during influenza pandemics, as >90% and >50% of people who died during the A/H1N1 pandemic in 1918 and 2009, respectively, were due to secondary bacterial pneumonia [85]. Influenza-induced pneumonia is one of the major causes of death in children under 5 years old in middle to low-income countries [86].

Recruitment of large numbers of immune cells like cytolytic T lymphocytes (CTLs) to infection site may hyper-activate epithelial cells deaths and consequently irreversible lung tissue damage, which was shown to correlate with the severity of influenza induced acute respiratory syndrome (ARDS) [87, 88]. Furthermore, increased secretion of pro-inflammatory cytokines by infected cells and host immune cells along with insufficient control of anti-inflammatory responses may lead to severe cytokine storm, causing severe influenza immunopathology including pulmonary edema, ARDS, and influenza related death [89, 90]. Zoonotic infection with A/H5N1 and A/H7N9 were shown to cause massive inflammatory cytokine storm in the lungs, leading to severe toxic shock and multi-organ failure with 80% mortality rate [91].

1.9 Immunity to influenza virus

1.9.1 Innate immunity

The airways epithelial cells (AECs) of the upper respiratory tract are the primary site of influenza virus encounter and functions as the first immune barrier to safeguard the host against severe illness [92]. During viral replication, the host innate immune system detects viral infection through recognition of distinct pathogen-associated molecular pattern (PAMPs). PAMPs include various form of viral RNA species like double or single-stranded RNA and RNA with 5`-triphosphate. These viral RNA structures are sensed by the host cell as foreign and stimulate the innate immune response when they are recognized by pattern recognition receptors (PRRs) expressed on AECs and various innate immune cells such as macrophages, and neutrophils [93-95]. Three families of PRRs are involved in the innate immune responses against influenza, these includes Toll-like receptors (TLRs), retinoid acid inducible gene-1 (RIG-1)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Recognition of single-stranded viral RNA (ss-vRNA) by TLR7 in the endosome trigger the production of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α). Viral RNA with 5´triplephospate presents in the cytoplasm are recognized by RIG-1, which then translocate to the nucleus where they activate transcription of type I and type III interferons: IFN-α/IFN-β and IFN-λ1, λ2 and λ3, respectively [96, 97]. NLRs respond to cytosolic ss-vRNA and forms inflammasome complex. Then trigger a cascade of caspase and pro-inflammatory cytokines activation, which result in pyrolysis of infected cells [98, 99].

Innate leukocytes like dendritic cells (DCs) are recruited to the site of infection by chemokines secreted by the infected epithelial cells during viral replication [100]. DCs and macrophages are professional antigen presenting cells (APCs) of the innate immune system, they either ingest virus infected cells or are directly infected with influenza viruses. The DCs processed the ingested viral antigens then migrate from the site of infection to the draining lymph nodes (DLN), where they present the viral antigen to $CD4^+$, $CD8^+$ T cells and B cells [101, 102]. The effector functions of DCs bridges the innate immune system to the adaptive immune system [103].

1.9.2 Adaptive immunity

As previously described the innate immune response serves as the first line of defense, it is activated instantly following recognition of an influenza virus, its main role is to limit viral replication and further spread to the lower respiratory tract (LRT) [104]. When the innate immunity is ineffective in eliminating or controlling the influenza virus infection, the adaptive (acquired) immunity is then activated. After primary exposure to the influenza virus, it takes approximately five days for the adaptive immune system to be activated [105], it is specific as opposed to the innate immunity for effectively viral clearance and capable of generating immunological memory [106]. Adaptive immunity is crucial for the recruitment and activation of effector T and B cells. Both the humoral (antibodies) and cellular (T and B cells) responses of the adaptive immune system are involved in orchestrating protective immunity against influenza [107, 108].

Humoral Immunity

The humoral immunity is an antibody mediated immunity. Influenza vaccination or infection induce the production of influenza-specific antibodies that target viral proteins, especially the viral surface proteins HA, NA and M2 [104] (Figure 1.7). These antibodies are produced by terminally differentiated B cells, antibody secreting plasma cells. There are five types of antibodies/immunoglobulins (Ig), IgA, IgD, IgE, IgG and IgM, three of these are importing for humoral immunity against influenza, IgM, IgA, and IgG. IgA is the major neutralizing antibodies on mucosal surface limiting influenza virus entry [105]. IgM and IgD are co-expressed on B cells and are the primary antibodies secreted during acute influenza infection. IgM was shown to provide long-term protective immune response against influenza infection in mice model, while the function of IgD is unknown [104, 109]. The IgG (IgG1, IgG2, IgG3 and IgG4) account for approximately 75% of serum antibodies. IgG1 and IgG3 are important for anti-influenza immunity as they both have high affinity for the Fc-γ receptor, mediating antibody-dependent cell mediated cytotoxicity (ADCC), they are also involved in direct inhibition of virus replication [59]. IgE is implicated in immune response against parasites and is associated with allergic reaction [104]

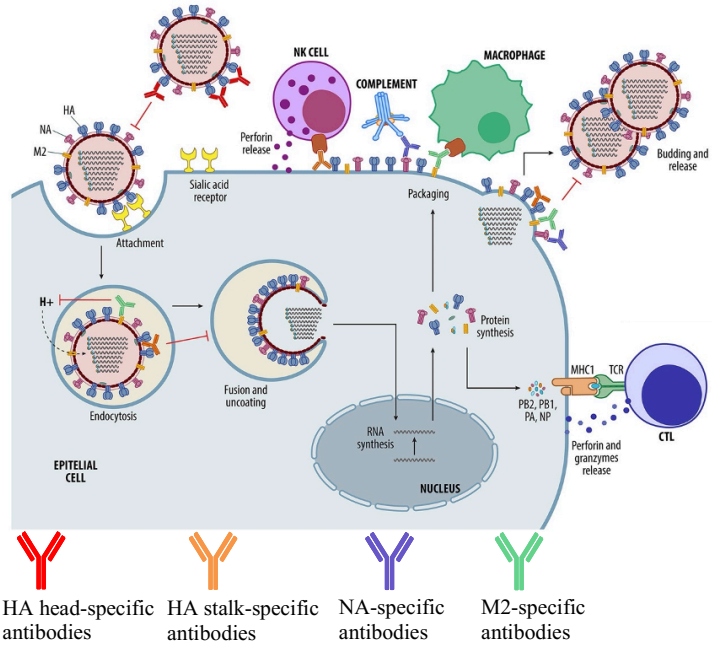

Figure 1.7 Antibodies and CTL mediated immune responses against influenza

HA-head specific antibodies (red) bind to the HA globular head domain inhibiting viral attachment to the sialic acid receptor (yellow) on the host cell. These antibodies typically bind the receptor binding pocket of HA [59]. HA stalk-specific antibodies (orange) inhibits viral-endosomal membrane fusion by locking the HA in a pre-fusion conformation and inhibit the uncoating and the release of viral RNA genome. HA-stalk-specific antibodies are bound by natural killer (NK) cells and can mediate ADCC. Additionally, some HA stalk-specific antibodies may prevent virion egress, blocks NA access to sialic acid by steric hindrance [110, 111]. NA inhibiting antibodies (purple) prevent progeny virion budding and egress, thus limiting virus spread, they can also activate complement. M2-specif antibodies (green) block M2 mediated acidification of the endosome and therefore inhibit vRNP release. They also prevent virion budding and mediated Fc-opsonization by macrophages. Viral internal protein presented by the major histocompatibility complex (MHC) class I on target cells are reorganized by cytotoxic T lymphocytes (CTLs), which then release cytotoxic granules containing granzymes and perforin leading to lysis of target cells [112]. The figure is modified with permission from [112]

Humoral immunity to influenza infection and vaccination

The breadth and durability of the antibody responses elicited by natural influenza infection and influenza vaccination are quite different. Natural infection elicits broadly reactive and long-lived antibody responses, whereas influenza vaccines induce a narrower and short-lived antibody response [113]. HA-specific antibodies are neutralizing and mostly target the immunodominant HA globular head, HA head-specific antibodies are strain specific and have a narrower binding range [111]. Antibodies are directed towards the conserved immune-subdominant HA stalk domain induced after influenza infection and vaccination although at a lower magnitude than

HA head -specific antibodies due to limited access to the stalk domain embedded in the viral membrane. The HA stalk domain is highly conserved among different influenza viruses, thus antibodies that target the stalk domain have a wider binding range and are broadly cross-reactive binding across different influenza subtypes [114]. HA stalk-specific antibodies were reported to induce neutrophile phagocytosis of immune complexes and can cross-react H2 and H5 viruses with pandemic potential [115, 116]. HA-stalk-specific antibodies were recently shown to provide protection from lower respiratory infection and prevent influenza disease progression [117]. NA is immuno-subdominant compared to HA when presented to the immune system, however NA as an antigen alone in the absence of HA is highly immunogenic [118]. NA-specific antibodies are more broadly reactive against diverse influenza strains [119] and were shown to reduce the severity of the influenza disease, reduce viral shedding and load in the lung [120]. Furthermore, NA-specific antibodies were shown to correlate with functional NA inhibition titres in children, adults, and the elderly [119, 121].

A key goal of seasonal and pandemic vaccines is to elicit appropriate immunological responses that ultimately result in reducing viral replication and provide immune protection against severe illness and death. Inactivated influenza vaccines mainly elicit systemic antibodies that can be detected 2-6 days after vaccination and rarely induces mucosal antibody response. Additionally, IIV induces non-neutralizing antibodies targeting NA, which can mediate ADCC activity [59, 113]. Live attenuated vaccine administered intranasally elicit immune response that mimic natural infection, resulting in broadly cross-reactive antibody responses, which may persist longer than IIV induced antibodies [122]. LAIV induces mucosal IgA and serum IgG antibody responses especially in children [123]. Furthermore, LAIV was reported to induce cross-reactive T cell and B cell immune responses in children [124-126].

Influenza specific-antibodies play curial role in virus neutralization and limit viral replication during the acute phase of influenza infection and the presence of HA-specific antibodies in serum correlate with immune protection [104, 127]. However, antibodies on their own are incapable of viral clearance, which is task carried out by immune cells involved in cell-mediated immunity.

Cellular Immunity

The immune cells involved in orchestrating cellular immunity include DCs, $CD8^+$ and $CD4^+$ T and B cells [104]. In the DLN, DCs load processed viral antigen onto the major histocompatibility complex (MHC) class II or I, which then displays and present the viral antigen onto T cell receptor (TCR) on naïve $CD4^+$ or $CD8^+$ T cells respectively. This triggers the initiation of the adaptive immune response [104].

Activated $CD8^+$ T cells undergo several rounds of clonal expansion and differentiate into influenza specific effector $CD8^+$ T cells or CTLs [105]. Influenza-specific effector $CD8^+$ T cells control influenza infection by secreting pro-inflammatory cytokines like IFN-γ, Interleukin-2 (IL-2) and TNF-α [128]. Viral infected cells secret chemokines, that attract CTLs to the site of viral infection, infiltrated CTLs mediate destruction and optimal clearance of virus-infected cells, by producing cytotoxic granules that contain perforin and granzymes. Perforin binds to target cells and form pores in the cell membrane, through which granzymes diffuses and induce apoptosis [105]. Following successful viral clearance, influenza-specific memory $CD8^+$ T cells are generated. Upon reencounter with the same viral antigen, these influenza -specific memory $CD8^+$ T cells are recalled, and they can quickly differentiate into effector $CD8^+$ T cells promptly clearing the viral infection, preventing further viral spread to the LRT [129].

Naïve $CD4^+$ T cells located in the lymph nodes are activated by specialized antigen-bearing APCs [130, 131]. Activated $CD4^+$ T cells differentiate into effector T helper (T_H) cells like T_H1, T_H2, T_H17 cells, regulatory T (Treg) cells and follicular helper T (T_{FH}) cells (Figure 1.8) [132, 133]. Influenza infection or vaccination induce influenza specific $CD4^+$ T cell response that contribute to protective immunity against influenza, with Th1 cells characterized by the secretions of IFN-γ, TNF-α and IL-2 cytokines dominating the $CD4^+$ T cell responses [134]. IFN-γ secreted by Th1 cells can activated macrophages that enhances the efficiency of anti-viral immunity [104]. Influenza-specific effector $CD4^+$ T cells can carry out cytotoxic elimination of infected cells, although they are not as potent as CTLs. The general acknowledged effector functions of $CD4^+$ T cells are to provide help to $CD8^+$ T cells for cytolytic destruction and clearance of infected cells and promotion of optimal memory $CD8^+$ T cell responses.

Additionally, CD4+ T cells support the activation and differentiation of B cells, which subsequently results in the production of high affinity class- switched antibodies [135, 136].

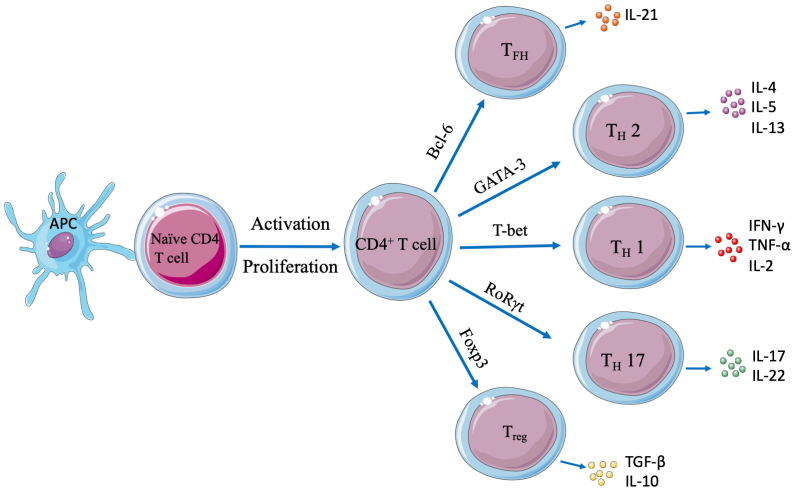

Figure 1.8 The differentiation of CD4+ T cells into different effector subsets

Naïve CD4 T cells are activated by APCs. Activated CD4+ T cells differentiate into distinct CD4+ T cell subsets like effector helper T (T_H) cells T_H1, T_H2 and T_H17, Treg and T_{FH} cells, Different transcription factors direct CD4+ T cell differentiate into lineages, e.g., the transcription factor T-bet direct CD4+ T cell differentiation towards T_H1 lineage expressing the cytokines IFN-γ, IL-2 and TNF-α [137]. Whereas the transcription factor B cell lymphoma-6 (Bcl-6) direct CD4+ T cell differentiation towards T_{FH} cell lineage. T_{FH} cell highly express the cytokine IL-21. This illustration was made in PowerPoint using artwork from smart servier medical art [35]

The humoral immune response against influenza is dependent on the interaction between T and B cells to generate high affinity isotype switched antibodies, which are critical for neutralizing the influenza virus limiting virus spread and provide immune memory for long term protection against influenza reinfection. The induction of antibody producing plasma cells and the generation of memory B cells (MBC) require the help of specialized population of CD4+ T cells called follicular helper T (T_{FH}) cells. These CD4+ T cell subsets were discovered in human tonsillar sample and were shown to play a pivotal role in providing survival and differentiation signals to B cells (Figure 1.9). Additionally, they are important during germinal center reaction and the generation of CD4+ T cell-dependent B-cell humoral immunity [130, 138, 139].

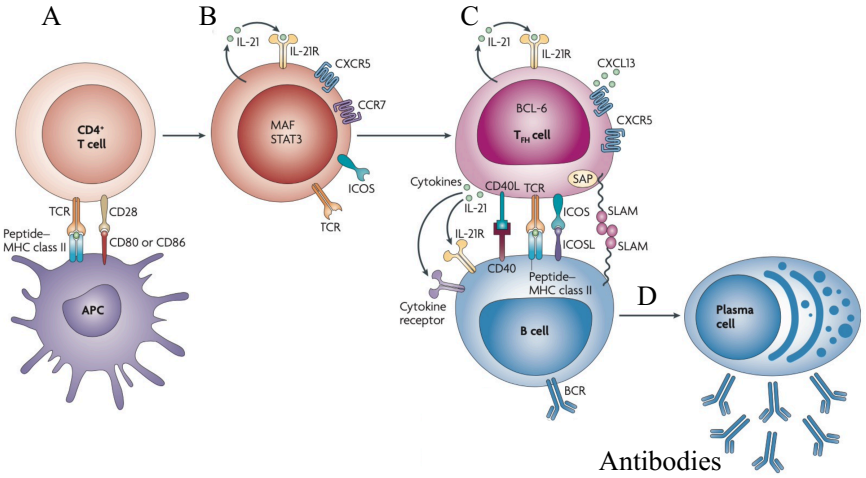

Figure 1.9 Activation of CD4⁺ T cell and T_{FH} cell proving B cell help

(A) Naïve CD4⁺ T cells located in T cell zone are activated by APC through interaction of the costimulatory molecule CD28 and TCR on CD4 T cell with CD80/86 and antigen-MHC class II complex express on APC, respectively. **(B)** Activated CD4⁺ T cells expresses the B cell follicle homing marker C-X-C-chemokine receptor 5 (CXCR5, CXCL13 is the ligand for CXCR5), secrete the cytokine IL-21 and the inducible co-stimulator (ICOS). Up-regulated IL-21 production by activated CD4⁺ T cells, trigger the expression of T_{FH} cell lineage-specific transcriptional factor B cell lymphoma-6 (Bcl-6) [140]. **(C)** Primed T_{FH} cells highly express effector molecules crucial for their function and development. These effector molecules include co-stimulatory molecules such as ICOS, CD40 ligand (CD40L), programmed cell death 1 (PD1 not shown here) the cytokine IL-21 and the adaptor protein SLAM-associated protein (SAP) [141]. Bcl-6 is a master transcriptional repressor critical for directing T_{FH} cell differentiation [142]. Bcl-6 up-regulates the expression CXCR5 and suppresses the expression of T cell zone homing marker C-C-chemokine receptor 7 (CCR7) and other TH cells transcriptional factors like T-bet, thus promoting CD4⁺ T cell differentiation towards T_{FH} cell lineage. ICOS express by T_{FH} cell positively regulate B cell differentiation, CD40L stimulated B proliferation, whereas SAP interaction with signaling lymphocytic activation molecule (SLAM) is crucial for the antigen-dependent T cell-B cell interaction [143]. **(D)** Activated B cells differentiate into several subsets like antibody producing plasma cells. The figure was adapted with permission from [144]

Activated T_{FH} cells enriched in secondary lymphoid organs can be defined by the expression of CXCR5 and are categorized into distinct phenotypes based on their surface receptor [141]. Early T_{FH} cells ($CXCR5^{low}$ $ICOS^{low}$) located in the T-B cell border, provide help to naïve and memory B cells in the initial phase of B cell activation [130]. Two distinct T_{FH} cell subsets are identified in human tonsils 1) pre-T_{FH} cells ($CXCR5^{+}Bcl\text{-}6^{+}ICOS^{+}PD\text{-}1^{+}CD40L^{+}$) are located within the B cell follicles and highly express IL-21 [145, 146], 2) GC tonsillar T_{FH} cells ($CXCR5^{++}Bcl\text{-}6^{++}ICOS^{++}$ $PD\text{-}1^{++}CD40L^{++}CD57^{+}$) are located in the GC [147]. Another T_{FH} cell subset found in the peripheral blood are circulating T_{FH} cells [148]. Finally, a subset of T_{FH} cells expressing FOXP3 are regulatory T_{FH} cells (T_{FR}), located at the T-B cell border and within the B cell follicles, T_{FR} cells suppresses follicular B cell responses [149].

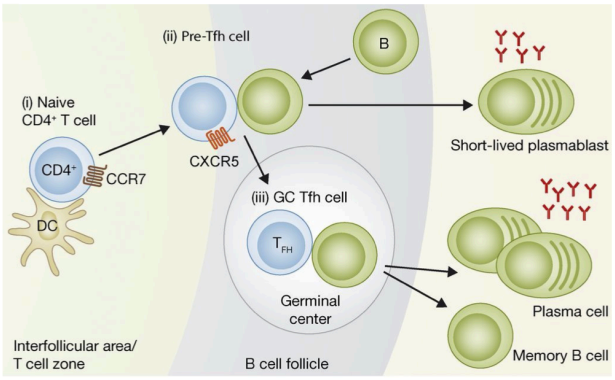

Figure 1.10 CD4$^+$ T cell activation of B cells and differentiation of B cells

(I) Naïve CD4 T cell is activated by DC in the T cell zone. **(II)** Interaction of T cells with B cell at the T-B border results in the differentiation of B cell into short-lived antibody producing plasma blast**. (III)** Following this initial step, the GC is generated. In the GC, interaction of antigen-specific T_{FH} cells with high-affinity B cell results in differentiation of B cell into long-lived class switched plasma cells that migrate to the bone marrow, providing long-lasting serological memory and maintenance of serum antibody levels [130]. A fraction of activated B cells differentiates into MBCs that circulate in the periphery providing immune surveillance [150]. The figure is adapted with permission from [130].

Following influenza infection or vaccination, naïve B cells can be activated with the same influenza antigen that primed the CD4$^+$ T cells. Primed antigen-specific B cells interact with T_{FH} cells at the border between T cell zone B cell follicle. The association of T_{FH} cell with antigen-specific B cell is a reciprocal interaction, as B cell is required for fully committing CD4$^+$ T cell differentiation to T_{FH} cell, likewise B cells depend on IL-21 signals from T_{FH} cells to proliferate and differentiate [131]. Co-stimulatory molecules like ICOS and CD40L are crucial for B cell development, as mutations of ICOS and CD40L genes results in defective humoral immune response [131, 151, 152]. T_{FH} cells promote B cells proliferation and differentiation into several subsets with distinct fates, these include short-lived antibody secreting cells (ASC) or plasma-blasts, long-lived plasma cells and MBC (Figure 1.10). Influenza infection or vaccination induce ASC response, which provide the first wave of influenza-specific serum antibodies in the periphery, peaking approximately 7 days post infection or vaccination [138]. Upon secondary infection or reencounter to similar viral antigen, MBC rapidly differentiate into ASC secreting high affinity antibodies [150]. Other B cell subsets in the GC proliferate and after several rounds of somatic hyper-mutations yield heterogeneous B cell clones with diverse B cell receptor repertoires with varying affinity to the influenza virus.

1.10 Clinical Features

Influenza viruses cause respiratory disease in human with the clinical manifestations range from mild asymptomatic to severe illness, over 50% of the infections are asymptomatic [89]. Influenza illness is characterized by an acute onset of systemic followed by respiratory symptoms including fever, headache, chills, sweat, prostration, malaise, myalgia, sore throat, nasal congestion or nasal discharge and a dry persistent cough [4, 153]. During the influenza season, fever and dry cough are the best predictors of influenza illness. Uncomplicated influenza is often presented as a mild upper respiratory tract illness with fever (temperature between 38℃ to 40℃), which may persist up to 5 days. Younger children may develop high fever with febrile seizures, whereas the elderly may present loss of appetite, fatigue, and confusion without fever. A dry cough is the most frequent respiratory symptom and can be accompanied by chest pain or burning sensation in the chest. Gastrointestinal symptoms such as abdominal, vomiting, and diarrhea are more common in children than adults [12].

Seasonal influenza epidemic is usually self-limiting, however people in the high-risk group including the elderly >65 years, institutionalized adults, younger children <5years old, pregnant women, the immunocompromised, individuals with chronic conditions like asthma, hematological disorders, neurological disorders, metabolic disorders, congenital heart disorders can develop severe influenza or die [133]. Acute respiratory distress syndrome (ARDS), Guillain-Barré syndrome, Reye syndrome, hemophagocytic syndrome, primary viral pneumonia and secondary bacterial pneumonia are some common complications of influenza associated with severe influenza [4]. In infants and younger children (for e.g.) common complication of influenza illness includes sepsis, acute otitis media, pneumonia, and bronchiolitis [12]

1.11 Influenza Diagnosis

During a period of increased influenza activity, testing for influenza virus is not required for making clinical diagnosis of outpatients with suspected influenza. Early diagnosis of influenza infection is however critical in clinical settings for rapid decision making such as initiation of antiviral treatment to reduce morbidity and mortality in

hospitalized patient. Influenza virus testing is recommended for all hospitalized patients with suspected severe influenza complications such as exacerbation of a chronic disease and concomitant pneumonia [154, 155]. Currently available diagnostic tests for detection of influenza infection include molecular diagnostic approaches, like nucleic acid amplification test (NAAT) with reverse transcription polymerase chain reaction (RT-PCR) being the most frequently used, rapid antigen tests, viral culture, or serological tests [155].

1.11.1 Molecular Diagnostic Testing

NAAT influenza diagnosis tests are based on polymerase chain reaction (PCR) for the detection of influenza viral genetic material. RT-PCR is the most common NAAT approach used worldwide for influenza diagnosis and is considered the gold standard for the detection of influenza viral RNA in respiratory samples with very high sensitivity and specificity [156]. However, RT-PCR require sophisticated equipment for sample processing and trained personnel for interpretation of the results. Current testing includes multiplex RT-PRC for rapid detection of large numbers of respiratory virus infection [157]. The extensive expansion of molecular testing capacity during the COVID-19 pandemic, may have implications for future laboratory diagnosis.

1.11.2 Rapid influenza diagnostic tests

There are several commercially available rapid influenza diagnosis tests used to detect influenza viral nucleoprotein antigens from both influenza A and B virus infections. Rapid influenza diagnostic tests (RIDTs) are near patient or point-of-care diagnostic tests providing results within 15-30 minutes with estimated sensitivity 50-70%. However, RIDTs do not distinguish between influenza A subtypes and high numbers of viral particles need to be present in the respiratory specimen for accurate detection [158, 159].

Several diagnostics approached have been developed in recent years. Two such methods are the rapid molecular assay and lateral flow assay (LFA), these are currently widely used during the ongoing COVID-19 pandemic as rapid point-of care diagnostic test. LFA is used to detect influenzas or SARS-CoV-2 antigens. However, a nucleic

acid version is available: NLFA, which can be used to test PCR products. Whereas a rapid molecular assay detects the presence of viral RNA in biological samples, both assays yield results within 5-30 mins. The estimated sensitivity and specificity of LFA for influenza diagnosis were 84 and 97% respectively and the sensitivity of rapid molecular test for influenza diagnosis ranges between 66 to100%. Overall, these two assays are inexpensive, easy to perform and have proven high sensitivity for fast detection of influenza and SARS-CoV-2 infections [156, 160, 161]. A bio-chemiluminescent sialidase assay using luciferin derivatized substrate for the detection of NA enzymatic activity was recently reported as an alternative influenza diagnosis test. This assay can be performed rapidly (15 minutes) with 82% sensitivity and 95% specificity [162]

1.12 Treatment

Influenza vaccination remains the most cost-effective prophylaxis measures to combat influenza and reduce the risk of severe influenza illness. However, the efficacy of current influenza vaccines is variable and particularly low if antigenic drift results in mismatch between the circulating virus strain and the vaccine strain. Drift in the circulating viral strain does not affect the effectiveness of antiviral drugs, as they recognize and target highly conserved viral epitopes [163]. The European and US Centers for Disease Control and Prevention (ECDC/CDC) recommend antiviral therapy for hospitalized patients with severe or progressive influenza illness and patients at high risk of influenza complication [164, 165]. In otherwise healthy adults and children with suspected influenza, for best outcome it is recommended to initiate treatment as soon as possible or within 24 hours of symptom onset and not later than 48 hours of symptom onset [155]. Early treatment of acute uncomplicated influenza with antiviral reduces symptom duration by one day[166, 167]

1.1.1 Antiviral drugs

Currently four antivirals are Food and Drug Administration (FDA) approved and recommended for influenza therapy: The NA inhibitors (NAI) oral administered

oseltamivir, inhaled zanamivir, intravenously administered peramivir, and the cap-dependent endonuclease inhibitor baloxavir marboxil, all of which target both influenza A and B viruses [4] (Table 1.1). Oseltamivir, zanamivir and baloxavir marboxil are approved in Europe and the United State, peramivir is approved in the US, China, Japan, South Korea, and the only NAI not yet approved in Europe [168]. The mechanism of action of all NAI is to competitively block the active site of NA and thus inhibit the enzymatic activity, by so doing prevent the release and spread of progeny virions [168, 169]. Baloxavir marboxil bind to the PA endonuclease domain and inhibits cellular mRNA cap cleavage [170]. A single amino acid substitution in the viral NA confers resistance and reduced sensitivity to oseltamivir, but this mutation was mostly reported in younger children and in immunocompromised individuals with prolonged virus shedding. Most oseltamivir resistance strains are found among A/H1N1 subtypes [168]. Resistance to zanamivir is rare and seasonal influenza A and B strains are sensitive to it, zanamivir is therefore recommended for people resistant to oseltamivir [4, 168].

Table 1.1 Recommendations and contraindications for use of antiviral drugs

Antivirals	Recommendations	Contraindications
Oseltamivir (Tamiflu®)	Acute uncomplicated influenza in patients ≥2 weeks old. Hospitalized patients, outpatients with severe complicated or progressive influenza and pregnant women. Chemoprophylaxis of influenza in patients ≥1 year old	
Zanamivir (Relenza™)	Acute uncomplicated influenza in patients ≥7 years old and pregnant women. Chemoprophylaxis of influenza in patients ≥5 years old	Patients with underlying airway disease including asthma and patients with severe influenza
Peramivir (Rapivab®)	Acute uncomplicated influenza in patients ≥6 months old	
Baloxavir marboxil (Xofluza®)	Treatment of acute uncomplicated influenza in patients ≥12 years old.	Severely immunocompromised patients' outpatients with complicated or progressive illness, hospitalized patients and pregnant women

1.13 Vaccines

Influenza vaccination is the primary prophylactic measure for controlling seasonal influenza illness and is administered before the influenza season. Currently there are three commercially available influenza vaccines, the inactivated influenza vaccines injected intramuscularly, the intranasal live attenuated influenza vaccines and the recombinant hemagglutinin (rHA) subunits vaccines [171] (Figure 1.11).

1.13.1 Influenza surveillance and vaccine strain selection

Vaccine production is a year-round initiative that requires extensive surveillance and accurate prediction of the circulating influenza strains. The influenza strains to be included in the seasonal vaccines are selected in February for the Northern hemisphere and takes approximately 6-8 months, before the first approved vaccines are available for delivery in September [172]. The first step in vaccine manufacturing begins with identification and acquisition of the vaccine strains, a task coordinated by the WHO initiative Global Influenza Surveillance and Response System (GISRS). Hundreds of national influenza surveillances centers routinely monitor and conduct risk assessment of the common circulating influenza strains or drifted strains. WHO and collaborating partners issues two separate recommendations for the influenza strains to be included in the seasonal vaccines; in February for the Northern hemisphere and in September for the Southern hemisphere vaccines [173]. Three influenza strains, two influenza A strains A/H1N1, A/H3N2 and one influenza B lineage (Victoria or Yamagata lineage) are included in the trivalent vaccines. As of 2013 influenza vaccine manufacturers switched from TIVs to quadrivalent vaccines (QIVs), which contains an additional influenza B lineage. Influenza vaccine must fulfill sets of vaccine immunogenicity criteria (Table 1.2) defined by the Committee for Medicinal Products for Human Use (CHMP) to obtain marketing licensure.

Table 1.2 CHMP criteria for influenza vaccines marketing licensure

Seroconversion rate	Seroprotection rate	Mean fold change
>40%	70%	>2.5

Seroconversion is defined as ≥ fold increase in HI titers. Seroprotection rate is defined as the proportion of subjects having HI titers ≥40 and SRH zone area ≥25mm^2. Whereas mean fold change is expressed as the geometric mean ration between pre- and post-vaccination [174].

1.13.2 Types of seasonal influenza vaccines

The commercially available influenza vaccines are produced by private sector manufacturers, all using different manufacturing technologies. Egg-based vaccine manufacturing process remains the most extensively used method for manufacturing influenza vaccines, accounting for approximately 1.5 billion (80%) of the global influenza vaccine doses [175, 176]. There are, however, several limitations to egg-based vaccine manufacturing process, such as acquisition of enough embryonated hen eggs for vaccine manufacturing to meet the global supply [177]. Circulating influenza virus strains may drift from the vaccine strains, this may result in reduce vaccine effectiveness [176] Therefore, it is essential to utilize alternative production technologies to produce influenza vaccines rapidly and increase the global vaccine supply, particularly during a pandemic [178]. Cell-based vaccine production provide an alternative technology for manufacturing influenza vaccines faster and on a large scale in mammalian cell lines [177]. Cell-based vaccine manufacturing process overcomes the problem of egg-grown adaptations. The first cell-based influenza vaccine *Flucelvax* was FDA approved in 2012 [179].

Table 1.3 Annual influenza vaccine recommendations and contraindications

Vaccines	Recommendations	Contraindications
IIV4	High risk group: Elderly ≥ 65 years old, younger children ≥ 6 months old, pregnant women, immunocompromised individuals, individuals with chronic medical conditions and healthcare workers	Persons with history of severe allergy or anaphylactic reactions to any of the vaccine component
LAIV4	Children aged 2-17 years old (Europe/UAS) Also, for adults up to 49 years old (USA) One dose for all > 9 years old Two doses for children ≤ 9 years old	Asthmatic children, pregnant women, immunocompromised individuals
rHA	All individuals 18-49 years old	

IIV4: Quadrivalent inactivated influenza vaccine. **LAIV4:** Quadrivalent live attenuated influenza vaccine

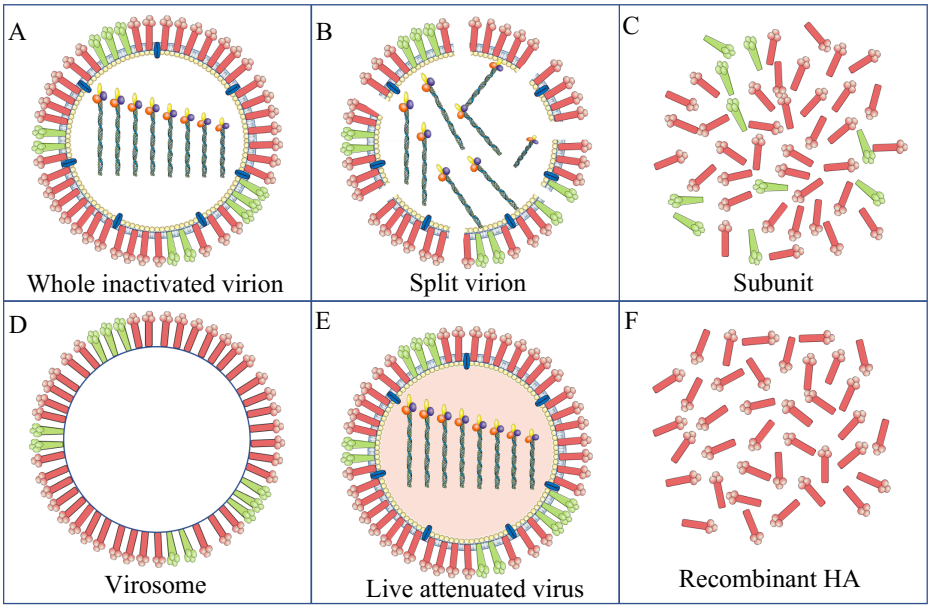

Figure 1.11 Overview of licensed influenza vaccines

(A) Whole virion IIVs containing whole virions inactivated with alkylating reagents. **(B)** Split IIVs contains activated and chemically disrupted viral components. **(C)** The subunit IIVs contains chemically disrupted viral and purified viral surface glycoproteins (HA and NA). **(D)** Virosome IIVs contain inactivated influenza virus envelope consisting of viral HA and NA **(E)** The nasal spray LAIV contains live attenuated (weaken) influenza virus. (**F**) Recombinant HA vaccine are manufactured using advanced recombinant technology and a baculovirus expression vector system and contain rHA protein). The illustration was made in PowerPoint using artwork from smart servier medical art [35]

1.13.3 Inactivated influenza vaccines

Annual immunization with inactivated influenza vaccine has been widely used to combat influenza virus in different populations, IIVs have shown good safety profile and are recommended especially for subjects in high-risk groups such as younger children age <5 years old [180]. Four types of IIVs are currently licensed, these are whole virus, split virus, subunit and virosome IIVs (Figure 1.11 A-D). Whole inactivated virus vaccines contain whole inactivated virion deprived on virulence and transmissibility (Figure 1.11 A). They are highly reactogenic and thus are currently not in used in Europe and North America, but are still in the market in low-income countries, due to easy production procedure [113]. The split virion vaccines are generated from whole influenza virus and inactivated by treatment with ether or other detergents to split the viral components and contains viral membrane carrying HA and

NA glycoproteins. (Figure 1.11 B). The subunit vaccine formulation is manufactured based on the split virion vaccine, the viral RNA and internal viral protein are then removed, and viral HA and NA proteins are further purified and concentrated (Figure 1.11 C). Virosomes vaccines contain inactivated influenza virus envelopes consisting of only HA and NA (Figure 1.11 D). All IIV formulation are standardized to contain 15 µg HA per influenza strain [59, 113, 180]. It is recommended to vaccinate the elderly either with adjuvated vaccine or vaccines with high HA content, *FluAd*® by Seqirus is a MF59-adjuvanted subunit influenza vaccine intended for use in the elderly with weak immune system [181]. *Fluzone-high dose (Fluzon-HD)* is a trivalent inactivated split virus vaccine containing high dose HA antigen (60 µg) of each influenza strain and recommended for vaccinating the elderly ≥65 years [182].

1.13.4 Live attenuated influenza vaccine

The live attenuated influenza vaccine is administered intranasally. LAIV has been licensed for use in the United States from 2003 as trivalent or currently quadrivalent *FluMist* (MedImmune). In Europe, LAIV is licensed since 2012 for used in children from 2-17 years of age [183]. LAIV is an egg-based vaccine, which is manufactured based on the backbone of cold-adapted A/Ann Arbor or A/Leningrad influenza donor strains. Seasonal LAIV contain six internal RNA segments of a cold-adapted strain and the recommended HA and NA of wild-type circulating influenza strains (Figure 1.11E). LAIV replicates (weakly) at the upper respiratory tract to induce immune response, making LAIV a strong inducer of mucosal IgA antibodies, which was shown to be associated with protection from influenza illness in younger children and adults [184, 185]. Furthermore, LAIV induces multifaceted immunity including systemic serum antibody responses and cell-mediated immunity [183, 185, 186] (Figure 1.12 depict LAIV induced immune responses). The immune responses elicited by LAIV are more superior in children under five years of age compared to IIV and studies have reported up to 80% LAIV effectiveness against influenza B and matched A/H3N2 virus infection in children compared to 40% vaccine efficacy in adults [187]. Due to the challenges in sampling and assaying mucosal antibodies, there are currently no known established quantitative correlates of protection for LAIV [187]. Therefore in order to

meet the CHMP criteria (Table 1.2) for vaccine marketing authorization, CHMP requires animal challenge studies for licensing seasonal LAIV [174].

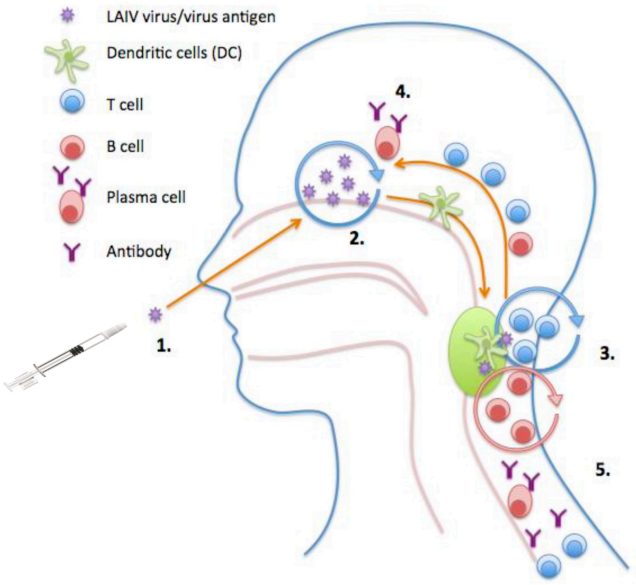

Figure 1.12 The mechanism of LAIV induced immune responses

(1) LAIV is administered intranasally, **(2)** The attenuated infectious viruses in LAIV replicate in the upper respiratory tract**. (3)** The viral particles are transported to the tonsils by dendritic cells (DC, green), DC ingest, process and present virus antigens to T cells (blue), which are then activated. **(4)** LAIV induced T_H1 or T_{FH} cells activates B cells (red), which differentiate into antibody producing plasma cells increasing antibody levels. **(5)** Following clearance of the virus infection, memory B and T cells are generated proving immunological memory [188]. The figure is kindly provided by Professor Karl Albert Brokstad, UIB, Norway

1.13.5 Recombinant HA vaccine

Recombinant HA (rHA) vaccine is produced using a third alternative vaccine manufacturing technology and contain only rHA protein (Figure 1.11 F) [189]. The first FDA approved rHA vaccine *Flublok* was licensed in the USA since 2013. It remains the only recombinant HA-based influenza vaccine available for the prevention of influenza in adults 18-49 years old [175]. Currently quadrivalent Flublok is commercially available consisting of high dose rHA protein (45 µg) per influenza strain (180 µg HA per vaccine dose) compared to the 15 µg HA content (60 µg HA per vaccine dose) in conventional QIVs. The immune response elicited by Flublok is HA specific and is shown to provide protection against confirmed influenza in older adults [190]

1.13.6 Adjuvants

Adjuvants are added to vaccines to enhance the host immune response. While non-adjuvanted influenza vaccines induce moderate to weak immune response, adjuvanted vaccines elicits higher magnitude, broader and long-lasting immunity [191]. Adjuvants can be use in dose sparing to increase the number of vaccine doses produced per egg such as in the adjuvant system 3 (AS03) pandemic vaccine contain 3.75 µg HA compared to 15µg HA content in standard dose vaccines [192]. This is particularly important during a pandemic when the global demand for pandemic vaccine is greatest [181]. Studies have shown that adjuvants greatly enhanced both cross-reactive memory B cell and strains-specific naïve B cell responses after A/H5N1 vaccination in adults. Additionally, adjuvanted A/H5N1 vaccine was demonstrated to induce broadly neutralizing antibodies and NA inhibiting antibodies, which correlated with protection [193, 194]. Pre-pandemic AS03-adjuvanted A/H5N1 subunit vaccines was FDA approved in 2013 and is currently part of the national stockpile in the United State. AS03-adjunvated A/H5N1 vaccines were shown to promote antibody diversity, affinity maturation and broadly cross-reactive immune responses in adults and the elderly [192, 195]

1.13.7 Influenza pandemic vaccines

To mitigate the health and social consequences of a pandemic, vaccination with a safe and efficacious pandemic vaccine is the best option by which the spread of the pandemic virus can be slowed down and reduced the disease severity [196]. The first influenza pandemic vaccine was developed during the 1957 Asian flu pandemic and subsequently in 1968 during the Hong Kong flu pandemic. During the recent influenza pandemic in 2009, most people were immunologically naïve to the A(H1N1)pdm09 virus, rendering seasonal influenza vaccines ineffective. Within 3 months after the emergence of the novel A(H1N1)pdm09 virus, several pandemic vaccines were developed according to the vaccine manufactures license including nonadjuvanted and adjuvanted vaccines. The AS03-adjuvanted Pandemrix was licensed for use in Europe, AS03-adjuvanted Arepanrix was licensed for use in North American and MF59-adjuvanted Focetria was licensed for use globally [197].

AS03-adjuvanted monovalent A(H1N1) pdm09 vaccine

The AS03-adjuvanted A/H1N1 pandemic vaccine (Pandemrix GlaxoSmithKline, Belgium) was licensed by the European Medicines Agency (EMA) for subjects aged 18-60 years, however this age range was extended by CHMP to >60 years old. Pandemrix is a monovalent split virion inactivated influenza vaccine with the final formulation containing 3.75 μg HA of A/California/07/2009-like (H1N1) strain per 0.5 ml dose and adjuvanted with the proprietary oil-in water emulsion AS03 [198]. Clinical trials evaluating the effectiveness of the pandemic vaccine, found that one dose was sufficient to induce protective immunity in the general population, however two doses may be required to immunize younger children and immunocompromised individuals [199, 200]. The WHO Strategic Adversary Group of Experts (SAGE) on immunization recommended prioritizing vaccination of frontline healthcare workers during the pandemic to reduce absenteeism and minimize transmission of A(H1N1)pdm09 virus to vulnerable patients. Norway was one of the first countries in Europe to acquire the pandemic vaccine and began mass vaccination of its general population. In October 2009, as part of Norway's mass vaccination campaign, HCW at Haukeland University Hospital were among the first prioritized to receive the AS03-adjuvanted A(H1N1)pdm09 vaccine prior to the peak of the pandemic.

1.13.8 Universal influenza vaccine and novel vaccine technologies

To address the limitations of current influenza vaccines, several approaches are under investigation into developing universal influenza vaccine that can induce broadly, cross-reactive, and long-lasting protection against divergent influenza strains. A universal vaccine could eliminate the need for annual reformation of influenza vaccines and will as well provide immunity in the public (herd immunity) in case of the emergence of novel pandemic strain. There are several criteria a universal vaccine is required to meet; the vaccine must confer cross-reactive protection against both group 1 and group 2 influenza viruses. It should be at least 80% effective against influenza infection in all age groups, furthermore the vaccine should induce durable protection over multiple influenza seasons. Moreover, it is important to establish new correlates of protection, as the current acceptable correlates of protection against influenza relies

on hemagglutination inhibition (HI) antibody titers. The HI assay only measure antibodies targeting the HA head domain and not antibody specific for the conserved viral antigens, which are targets for universal influenza vaccines [201]. In recent years multiple approaches and advances in vaccine platforms are under investigation with the aim of developing universal influenza vaccines. These include identification and targeting conserved viral antigens/epitopes such as epitopes residing in the HA-stalk region, M2 ectodomain and development of novel vaccination technologies including adenoviral vector-based, nanoparticle-based, DNA/RNA-based approaches. NA-based vaccines and addition of adjuvants to boost immune response have been investigated [201].

HA-based universal influenza vaccine

Upon the discovery of broadly reactive neutralizing antibodies targeting the conserved HA-stalk domain multiple universal influenza vaccine production strategies including chimeric HA, headless HA, Mosaic HA, and Computationally Optimized Broadly Reactive HA Antigens (COBRAs) are being tested in various lab worldwide [202, 203].

Chimeric HAs (cHAs) constructs are generated by replacing the HA head domain with an irrelevant exotic avian HA head domain onto the HA-stalk of a wild type circulating influenza strain. Chimeric HAs vaccines are being tested clinical trial and reported to induce cellular and humoral responses in animal models [204]. The safety and immunogenicity of cHA-based vaccine was tested in a randomized, placebo-controlled phase I clinical trial. Enrolled participants were prime with either chimeric LAIV vaccines and boosted with AS03-adjuvanted chimeric IIV vaccine. This sequentially prime and boost vaccination regime was reported to be safe and conferred durable and heterosubtypic immunity against the HA-stalk domain of divers' influenza viruses [205].

NA-based and M2 ectodomain-based universal influenza vaccine

The NA glycoprotein drifts at slower rate compared to the HA glycoprotein [206]. However, NA content in current seasonal influenza vaccines is not standardized. A recent study showed that NA-specific monoclonal antibodies (mAbs) are broadly cross-reactive and can provide protection against challenge with group 1and 2 NAs in

mice [207]. Madsen *et al*. reported that human mAbs from different influenza B viruses inhibited NA enzymatic activities and protected mice against influenza B virus challenge [208]. NA inhibition antibody titer is reorganized as independent correlates of immune protection [209], making NA an attractive universal influenza vaccine candidate. The M2 ectodomain (M2e) has been investigated as a potential candidate for a universal influenza vaccine [210]. Studies have shown that antibodies targeting the M2e can be protective in preclinical animal models [211, 212]. Several M2e-based universal vaccines have completed phase I clinical trials and are reported to induce broadly protective antibody and T-cell responses in healthy adults [213, 214].

Novel vaccine technologies

In a pandemic, rapid vaccine manufacturer is of utmost importance and vaccine platforms which can be speedily modified are important in controlling infection. Nanoparticle-based vaccine, adenovirus- based vaccine, viral vector-based vaccine and nucleic acid-based vaccine are among the multiple novel universal vaccine approaches being tested [215]. Approaches to utilize RNA as an influenza and cancer vaccines have been researched for decades and this has fortunately paved the way for the rapid development of the novel COVID-19 vaccine licensed in 2021. The COVID-19 pandemic has led to a paradigm shift in vaccinology with hundreds of millions of people vaccinated with the adenovirus vector-based or mRNA vaccines, which have proved highly effective.

1.13.9 Correlates of immune protection

A correlate of protection (COP) is defined as an immune response that is responsible for and statistically correlated with protection [216]. A good vaccination-induced correlate of protection is vital when evaluating vaccine immunogenicity or protective efficacy [217].

The HI assay has been used for decade and considered as the golden standard for measuring protection against influenza. It is often used when evaluating vaccine immunogenicity before vaccine licensure. An HI titer of ≥ 40 is a surrogate correlate of protection, which is associated with 50% protection against influenza disease in

adults [127]. However, parameters such as vaccine type, age and health status of the vaccinee may influence the measured correlate [216]. An HI titer of 40 was shown to not be predictive of immune protection in children as higher HI titers of ≥110 or ≥330 were shown to provide 50% or 80% protection in children respectively [218]. Furthermore, the HI assay was shown to underestimate LAIV induced protection [219]

Thus, establishment of novel immune correlates of protection are required. NA inhibiting (NI) and HA stalk-specific antibodies are among the potential immune correlates of protection currently being evaluated, both of which were identified as independent correction of protection against influenza [209, 220]. A study by Gould *et al*. reported that nasal IgA provided protection against human influenza challenge [221]. Furthermore, nasal IgA levels were found to be a strong correlate of LAIV induced protection in a placebo-controlled study conducted in children [222]. Non neutralizing antibodies that mediate ADCC, complement fixation and antibodies targeting internal viral proteins such as M1 and NP are currently being evaluated as markers for COP [223].

T cells have also been reported as potential markers of COP. Influenza-specific $CD4^+$ and $CD8^+$ T cells were reported to provide protective immunity against influenza infection in children [224]. Wilkinson *et al.* show that the frequency of baseline cross-reactive $CD4^+$ T cells inversely correlated with viral shedding [225]. Studies measuring IFN-γ producing T cells found that ≥20 IFN-γ spot forming unit (SFU) per million (10^6) peripheral blood mononuclear cells (PBMCs) was associated with reduced nasal viral shedding in community cases. Whereases, ≥100 IFN-γ SFU per 10^6 PBMC provided protection against influenza illness in children, which was suggested as a COP for T cell immunity [124, 226]. Influenza-specific CTLs were shown to provide protection against influenza infection. A study by Sridhar *et al.* demonstrated that higher frequency of $CD8^+$ T cell correlated with less severe influenza illness and furthermore, $CD8^+$ T cells recognizing conserved viral epitopes correlated with cross-protection against influenza [227]. However, the establishment of $CD8^+$ T cell immunity as correlate of protection is complicated due to immunological imprint of each person [92, 228].

2. AIMS OF THE THESIS

- The aim of the study in **paper I** was to investigate in detail the early homologous and cross-reactive immune responses after the AS03-adjuvanted A(H1N1)pdm09 vaccination in healthcare workers.

- In **paper II** we aimed to examine how priming with the A(H1N1)pdm09 vaccine followed by repeated annual vaccination impacted the durability of cellular and humoral immune responses.

- In **paper III** we aimed to investigate in depth the kinetics and magnitude of influenza specific tonsillar T_{FH} cell and antibody responses in children and adults after LAIV vaccination.

3. MATERIAL AND METHODS

At the peak of the influenza pandemic in 2009, Norway started a mass vaccination campaign and frontline HCWs were prioritized for the first round of vaccination with the pandemic vaccine. This was a mitigation action to protect the HCWs and the vulnerable patients they cared for and to maintain the integrity of the healthcare system. When the first COVID-19 vaccines were approved and deployed in 2021, similar approach was implemented.

3.1 Study design

3.1.1 Pandemic and seasonal vaccine clinical trial: Papers I & II

The studies included in **papers I and II** were part of a large single arm vaccine clinical trial conducted during the influenza pandemic in 2009 at the Influenza Centre in collaboration with Haukeland University Hospital, (Bergen, Norway). The study was an open label 5-years extension study, all healthcare workers were invited to participate in the study to evaluate the safety and immunogenicity of the AS03-adjuvanted monovalent A(H1N1)pdm09 vaccine. In **paper I,** we reported the homologous humoral and cellular immune responses against the A(H1N1)pdm09 strain and cross-reactivity to pre-pandemic A/H1N1 strains dating back to 1977 in HCWs after the pandemic vaccination. In **paper II**, where we evaluated the kinetics and longevity of A(H1N1)pdm09-specific antibody, B cell and $CD4^+$ T cell responses after repeated annual influenza vaccination. The clinical trial was approved by the Regional Ethics Committee (Regional Committee for Medical Research Ethics, Western Norway (REK Vest-2012/1772)) and the Norwegian Medicines Control Agency and registered in the European Clinical Trials Database (EudraCT 2009-016456-43), and National Institute for Health Database Clinical trials.gov (NCT01003288). The study was conducted at the Influenza Centre, Department of Clinical Science, University of Bergen in collaboration with the Bergen Clinical Vaccine Centre at Haukeland University Hospital. These two centers were involved in participant recruitment, vaccine administration, blood sample collection and handling, and vaccine adverse event. All laboratory work was performed at the Influenza Centre

and data analyses and interpretation of the results was conducted by our research group at the Influenza Centre. AS03-adjuvated A(H1N1)pdm09 vaccine clinical trial complied with the ethical standards of the Helsinki Declaration and was conducted under good clinical practice.

The inclusion criteria were HCWs aged 19-70 years old who provided written and signed informed consent, understood and complied with the study protocol, completed the adverse event form and attended the scheduled visits for the kinetic study. The exclusion criteria for the clinical trial were HCWs with a history of anaphylaxis or severe allergy reactions to any vaccines or hypersensitivity to any of the vaccine components and HCWs with a temperature of >38˚C in the preceding 72 hours or an acute respiratory infection up to 7 days prior to immunization. Subjects who had PCR-confirmed infection with the A(H1N1)pdm09 virus were not prioritized for vaccination and were excluded.

3.1.2 The healthcare workers study population: Papers I & II

We chose to recruit HCWs as our study population since they are an important population with good vaccination records and influenza exposure history, however this study population is not directly comparable to the general population. Nevertheless, the study population is considered representative of other healthcare personnel. Two-hundred and thirty-seven healthy HCWs were enrolled and received a single dose of the AS03-adjuvanted monovalent A/H1N1 pandemic vaccine between October 2009 and March 2010 and were followed up until December 2014 (Figure 3.1). The study was initially planned for two years follow-up but was extended for 3 more years, since the A(H1N1) pdm09 strain remained the same as the A/H1N1 component in the seasonal vaccines. Healthcare workers who chose to be vaccinated and provided blood samples in the four subsequent influenza seasons (2010/11 until 2013/14 seasons) were included in **paper II** (Figure 3.1). This five-year longitudinal study design was expensive, resource demanding, and time consuming. However, our research group had the expertise and infrastructures in place prior to 2009 when we conducted a similar phase 1 clinical trial with the adjuvanted A/H5N1 vaccine in healthy adults and

therefore were able to manage a study of this magnitude. Importantly, this study provided us with an invaluable biobank that can be used to assess the long-term dynamic of influenza vaccine immunogenicity (seasonal or pandemic) in a fixed study cohort over 5-year period.

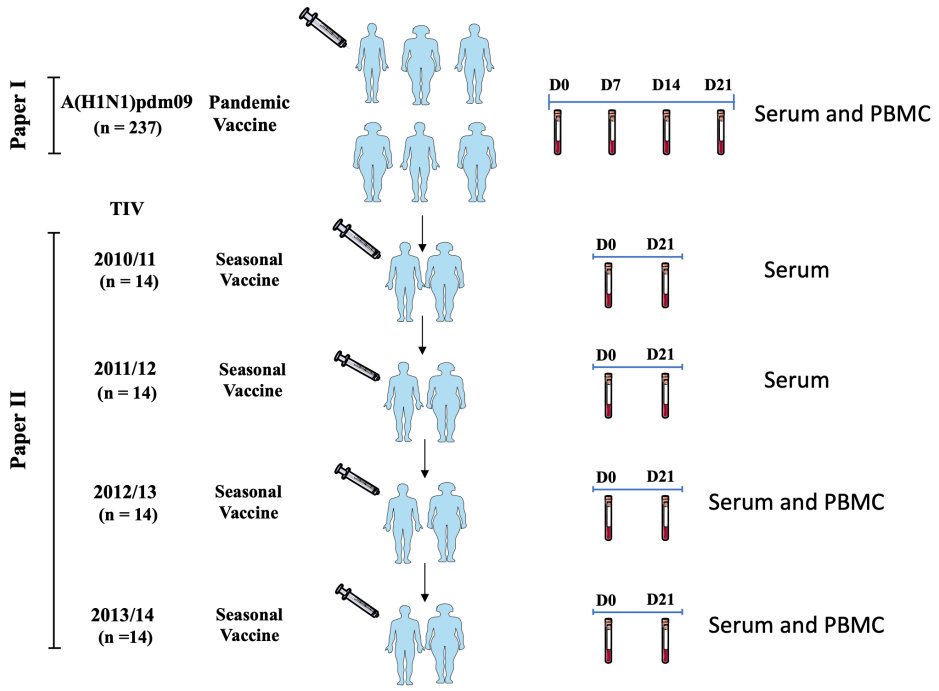

Figure 3.1 Overview of the study design: Papers I and II

Healthcare workers (n = 237) recruited during the 2009 influenza pandemic, were vaccinated with a single dose of the AS03-adjuvanted A(H1N1) pdm09 vaccine. Blood samples were collected pre-vaccination (D0) and at days 7, 14 and 21 post-vaccinations, were included in paper I. Fourteen HCWs subsequently vaccinated with seasonal TIV (2010-2013) provide blood samples pre (D0) and 21 days post vaccination were included in paper II. This figures was made in PowerPoint using artwork from smart servier medical art [35]

3.1.3 Live attenuated influenza vaccine clinical trial: Paper III

The Live Attenuated Influenza Vaccine was licensed for use in children 2-17 years old in Europe since 2012. At the time the clinical trial was conducted LAIV was not licensed in Norway and was thus imported from the UK and Finland solely for purpose

of the clinical trial. The trial was a collaborative work between the Influenza Centre, the Pediatric Clinical Trial Unit and the Ear, Nose, and Throat (ENT) clinic at Haukeland University Hospital, (Bergen, Norway). The clinical trial was approved by the Regional Ethical Committee of Western-Norway (REK West 2012/1088), the Norwegian Medicines Control Agency and registered in the European Clinical Trials Database (EUDRACT 2012-002848-24) and National Institute for Health Database; (NCT01866540). Adults and children over 12 years of ages that fulfilled the inclusion criteria were informed in detail regarding the study protocol and signed an informed consent. For children under 12 years, both their guardians signed the consent form. To ensure the safety of the vaccine, all subjects and/or their guardians had to fill out an adverse event form. The inclusion criteria were healthy children 3-17 years old and adults ≥ 18 years old with no symptoms of influenza-like illness 7 days prior to vaccination. Childbearing age females with a negative pregnancy test and subjects with mild or moderate asthma were included. Children and adults were excluded if they have fever (38°C) within the last 72 hours before vaccination, immunocompromised individuals or had immunocompromised family members, were pregnant, individuals with a history of anaphylaxis or severe allergic reaction to all vaccines or hypersensitivity to any of the vaccine components, had severe unstable asthma, patients on acetyl salicylic acid (ASA) or immunosuppressant. The LAIV clinical trial complied with the ethical standards of the Helsinki Declaration and was conducted under good clinical practice.

3.1.4 LAIV study population

The study population (n = 68) included in **paper III** were children (n = 37) and adults (n = 31) recruited from ENT at Haukeland University Hospital during the 2013-2014 influenza season. Adults and children registered for elective tonsillectomy were sent a letter of invitation to participate in the study. If tonsillectomy was required after consultation with a doctor at the ENT, the child's parents or the adults were then asked if they were willing to join the study and be vaccinated prior to tonsillectomy. The volunteers were made aware that this involved returning to the hospital for vaccination and follow-up blood and saliva sampling. The number of subjects include in the study

were the maximum number we could recruit by asking all eligible children and adults referred to the ENT clinic in the time-period of the influenza vaccination season. We did not include high risk subjects to minimize the risk and complexity of the trial, thus only healthy subjects were included in the study after a thorough clinical anamnesis and medical examination. The study participants were representative in terms of gender and age distribution for patients scheduled for elective tonsillectomy. Although they were operated due to chronic tonsillitis, tonsillar hypertrophy, or both, they were otherwise healthy. The median age of the children and adults were four years (age range 3-17 years) and 21 years (age range 18-59 years), respectively. Each participant provided blood, tonsils, and saliva samples pre-vaccination (Day 0), on the day of tonsillectomy after the first and second vaccinations and up to one year after vaccination with LAIV (Figure 3.2). The vaccinees were randomized into three subgroups based on the day of scheduled tonsillectomy: group 1 were vaccinated 2-5 days, group 2 were vaccinated 6-9 days, whereas group 3 were vaccinated 10-12 days prior to tonsillectomy after the first vaccine dose. We also recruited 6 age and gender-matched unvaccinated controls who provided tonsils on the day of tonsillectomy. The tonsils collected from these controls were used to measure background T_{FH} cell immune responses. We conducted a one year follow up study to investigate the kinetics and longevity of LAIV-induce immune response in children and adults. Importantly, we were one of the few labs worldwide with the unique opportunity of obtaining tonsillar tissue from both children and adults after LAIV, making our study unique.

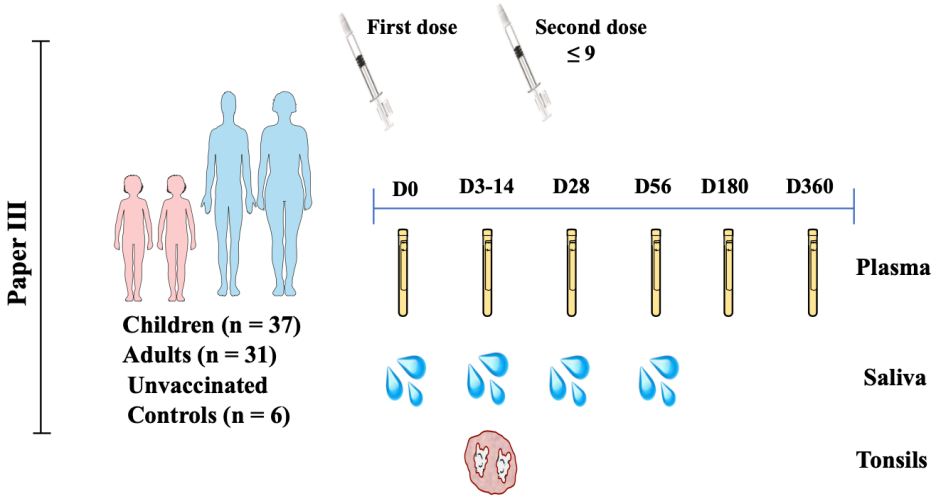

Figure 3.2 Overview of the study design: Paper III

Children (n =37) and adults (n = 31) were vaccinated with LAIV, and they provided blood, saliva, and tonsillar samples pre-vaccination on day 0 (D0) and up to one-year post-vaccination. All adults and children > 9 years old received a single dose of LAIV on D0, while children ≤ 9 years old received a second LAIV dose at four weeks interval on D28. Six age matched unvaccinated controls were also recruited and they provide blood and tonsillar samples. This figures was made in PowerPoint using artwork from smart servier medical art [35]

3.2 Vaccines used in the clinical trials

Table 3.1 An overview of the pandemic and seasonal influenza vaccine virus components

AS03-adjuvanted A(H1N1)pdm09 vaccine and seasonal trivalent inactivated influenza vaccines Papers I and II			
Influenza Seasons	A/H1N1 Strains	A/H3N2 Strains	Influenza B Strains
2009	A/California/07/2019		
2010/2011	A/California/07/2019	A/Perth/16/2009	B/Brisbane/60/2008
2011/2012	A/California/07/2019	A/Perth/16/2009	B/Brisbane/60/2008
2012/2013	A/California/07/2019	A/Victoria/361/2011	B/Wisconsin/1/2010
2013/2014	A/California/07/2019	A/Victoria/361/2011	B/Massachusetts/2/2012
Trivalent Live Attenuated Influenza Vaccine (LAIV3): Paper III			
2013/2014	A/California/07/2019	A/Texas/50/2012	B/Massachusetts/2/2012

3.2.1 Paper I

The pandemic vaccine was a low dose monovalent split virus vaccine adjuvanted with the proprietary adjuvant AS03, which was produced under Good Manufacturing Practice (GMP) by GlaxoSmithKline (GSK, Belgium). The vaccine was supplied in multi-dose vials and formulated at the point of administration to contain 3.75 μg HA of A/California/07/2009-like virus (H1N1) (Table 3.1), squalene, DL-α-tocopherol and polysorbate 8 per 0.5 ml dose. The vaccine was licensed by the European Medicines Agency's on the 24th of September 2009 for two doses and on the 19th of November 2009 as a single dose for most of the population, thus all the HCWs in this study received only one dose of the vaccine.

3.2.2 Paper II

The trivalent seasonal IIVs used during the 2010-11 to 2013-14 influenza seasons were either subunit vaccine Influvac® manufactured by Abbott Laboratories or split-virus vaccine Vaxigrip® manufactured by Sanofi Pasteur. The seasonal vaccines contained influenza A/H1N1, A/H3N2 and influenza B strains that were recommended for use in the Northern hemisphere and contained 15μg HA per strain. The A/H1N1 component was the A/California/07/2009 (H1N1)-like strain throughout the whole study period, whereas the A/H3N2 and influenza B components changed between seasons (See Table 3.1).

3.2.3 Paper III

The seasonal LAIV used was Fluenz® (AstraZeneca), which contained $10^{7.0 \pm 0.5}$ fluorescent focus units (FFU) of the three live attenuated influenza A(H1N1)pdm09, A/H3N2 and the influenza B strains (Table 3.1). The vaccine was administered according to the manufacture's recommendation, intranasally with 0.2 ml dose per nostril. Children >9 years old and all adults received a single dose, whereas children ≤ 9 years received a second dose of the vaccine at a 4-week interval. All the vaccinees in the study were vaccinated during the influenza season from October 2013 to February 2014.

3.3 Biological sampling

3.3.1 Blood samples

The blood samples for the HCW clinical trial were collected by the study nurses at each hospital department, whereas for the LAIV clinical trial blood samples were collected at the children pediatric unit. Each enrolled subject was allocated a unique identification number and data on baseline demographics and influenza vaccination history were collected. After enrollment HCWs (n = 237) provided blood samples pre-vaccination on (D0) and on 7-, 14- and 21-days post-pandemic vaccination. Sera were separated, aliquoted and stored at -80°C until used in the serological assays HI and SRH **paper I.** We collected blood samples in cell preparations tubes (CTP ™ BD, USA) pre-post-pandemic vaccination (Figure 3.1). Freshly separated PBMCs were used for the ASC ELISpot assay (n = 39) and intracellular cytokine staining (ICS) (n = 8 and 18). The remaining PBMCs were cryopreserved at -150°C in freezing medium containing 90% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO). These were prioritized for use in the MBC ELISpot (n = 27) and IFN-γ ELISpot (n = 9) assays included in **paper I.**

During the 2010-11 to 2013-14 influenza seasons HCWs annually vaccinated provided blood samples pre-vaccination (D0) and 21 days after each vaccination with TIVs. These were used in the HI assay (**paper II)**. During the last two seasons (2012/13 and 2013/14) HCWs (n=14) who were repeatedly vaccinated every year provided additional peripheral blood samples pre-vaccination and on day 21 post-vaccination (Figure 3.1). The PBMCs were isolated according to the manufacturer instructions, cryopreserved until used in the IFN-γ ELISpot, IFN-γ/IL-2 FluoroSpot, MBC assays and ICS included in **paper II**.

Children (n = 37) and adults (n = 31) vaccinated with LAIV provided peripheral blood samples pre-vaccination (D0), on the day of tonsillectomy (3-14 days), 28, 56, 180 and 360 days after vaccination (Figure 3.2). PBMCs and plasma samples were separated by density gradient centrifugation, the plasma samples were aliquoted and stored at -80°C until used in the HI and enzyme-linked immunosorbent assay (ELISA) included in **paper III**. The isolated PBMCs were cryopreserved at -150°C.

3.3.2 Tonsils

Tonsils were collected at the time of tonsillectomy in phosphate buffered saline (PBS) (Figure 3.2). One of the two tonsils from each participant was cut into 4-6 µm thick sections, mounted on super-frost plus microscope slides (Thermo Fisher Scientific) and immediately fixed in formaldehyde, paraffin-embedded and stored at 4°C. Tonsillar mononuclear cells (TMNCs) were isolated from the other tonsil by ficoll gradient centrifugation, following mechanical disruption of the connective tissue and cryopreserved until use. The tonsillar sections were used in the immunohistochemistry (IHC), while the TMNCs were used in surface staining and ICS for the characterization of $CD4^+$ T cell subset and influenza specific T_{FH} cell responses included in **paper III**.

3.3.3 Saliva

Saliva samples were collected using absorbing swabs (OraSure®, USA) pre-vaccination at day 0 and 28, 56 days after vaccination (Figure 3.2) from the lower buccal mucosa for two minutes. The swabs were centrifuged and then stored at -80°C until use in the ELISA for quantification of mucosal IgA responses (**paper III)**

3.4 Methodology

Table 3.2 An overview of the assays performed in the three papers

Serological assays	**Paper I**	**Paper II**	**Paper III**
HI	X	X	X
SRH	X		
ELISA			X
B cell assays			
ASC ELISpot	X		
MBC ELISpot	X	X	
T cell assays			
IFN-γ ELISpot	X	X	
ICS	X	X	X
IFN-γ/IL-2 FluoroSpot		X	
IHC			X

3.4.1 Serological assays

Hemagglutination inhibition assay

The HI assay was used to detect influenza-specific antibodies in serum/plasma samples and was performed as previously described [229]. Sera (HCWs, **papers I and II**) or plasma (children and adults, **paper III**) samples were treated with receptor-destroying enzyme (RDE: Denka Seiken co Ltd, Cat no. 370013) in 1:4 volume ratio and incubated overnight at 37°C. The RDE was inactivated by incubating the samples at 56°C for 30 minutes. The HI assay was carried out by performing two-fold serial dilution (1:10 starting dilution) of RDE treated sera/plasma samples in PBS across a V-bottom 96-well plate and incubated with 8 hemagglutinin units (HAU) of virus at room temperature (RT) for one hour. Fifty microlitres of 0.7% turkey red blood cells (TRBCs) was added and incubated at RT for 30 minutes. The HI titer was expressed as the reciprocate of the highest sera/plasma dilution that inhibited 50% hemagglutination. All the samples were tested in duplicates, and the geometric mean (GMT) HI titer was calculated. HI titers < 10 were assigned a value of 5 for calculation purposes. WHO influenza reagent resources, the National Institute for Biological Standards and Control (NIBSC, UK) and the International Reagent Resources (IRR, USA), provided all the influenza virus, the TRBCs, positive and negative reference sera used in the HI assay. The viruses used are listed in Table 3.3 and an illustration of the HI assay and plate readout are depicted in Figure 3.3.

Table 3.3 An overview of the influenza viruses used in the HI and SRH assays

Influenza virus name	Subtype	Paper
A/California/7/2009*	H1N1	I, II and III
A/Brisbane/59/1997	H1N1	I
A/New Caledonia/20/1999*	H1N1	I
A/Texas/36/1991*	H1N1	I
A/Taiwan/1/1986	H1N1	I
A/Brazil/11/1978	H1N1	I
A/USSR/90/1977	H1N1	I
A/Texas/50/2012	H3N2	III
B/B/Massachusetts/2/2012	B	III

*Viruses used in the SRH assay

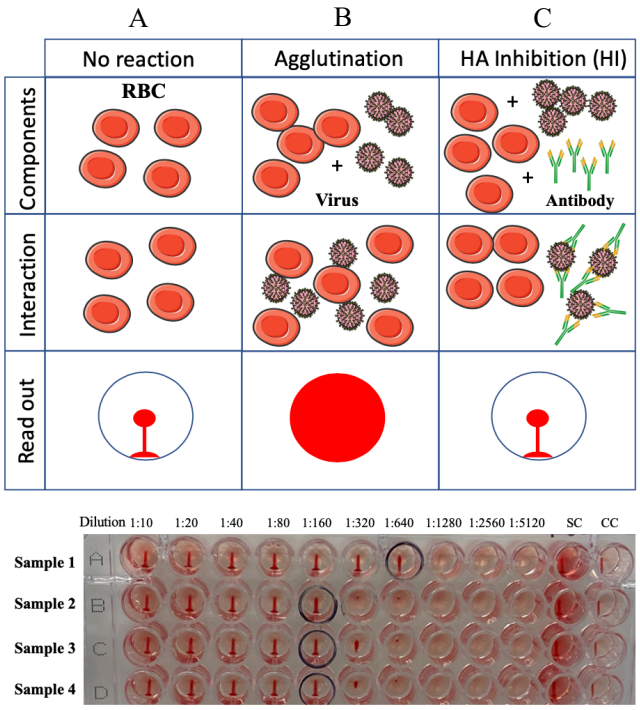

Figure 3.3 Illustration of the hemagglutination inhibition assay

(A) Red blood cells (RBC) in the absence of influenza virus will sink to the bottom of the well**. (B)** Upon addition of the influenza viruses, the viruses will bind to and agglutinate RBC into a lattice. **(C)** The HI assay is based on the ability of HA-specific antibodies present in serum/plasma to block the virus from binding to the RBC, thus inhibiting hemagglutination and the RBC sinks to the bottom of the well. A HI plate readout is depicted in the lower panel, with one sample per row and serum dilution across the plate (A1-A10) including serum control (SC: serum + RBC) and cell control (CC: RBC alone). This illustration was made in PowerPoint using artwork from smart servier medical art [35]

Single radial hemolysis assay

The SRH assay is used to detect influenza specific antibodies in serum, it was conducted at the University of Siena, Italy and performed as previously described [230]. Briefly, diluted influenza antigens (Table 3.3) were incubated with blood suspension at 4°C. A chromium chloride ($CrCl_3$) solution was added, mixed with guinea pig complement in 1.5 % agarose solution containing 0.1% sodium azide and allowed to solidify in a SRH plates. Wells were punctured in the agarose gel and heat-inactivated sera from HCWs, and controls were added before the plate was incubated overnight in a humid box. The hemolysis zone areas were read in millimeters (mm) with a calibrated viewer. A SRH titer of ≥ 25 mm^2 is associated with 50% protection against influenza.

Enzyme-linked immunosorbent assay

The concentration of IgG, IgA and IgM in plasma samples and local IgA levels in saliva were measured in children and adult using ELISA and performed as previously described [231]. Nunc Maxisorp plates (eBioscience) were coated with influenza antigens (2 μg/ml) and capture Ig antibodies (1μg/ml) (Coating antigens and antibodies are listed Supplementary Table 1 and 2) overnight at 4°C. The plates were blocked with PBS supplemented with 20% New Calf serum (NCS, Biochrom). Plasma/saliva and Ig standard antibodies were serial diluted (5-fold) in PBS/NCS, added to the plates and incubated for two hours at RT. Thereafter, appropriately diluted biotin-conjugated Ig detection antibodies (Supplementary Table 2) were added and incubation for 1.5 hours at RT, followed by the addition of extravidin-peroxidase (Sigma E-2886) diluted in PBS/NCS and allowed to react for 45min. The *ortho*-Phenylenediamine dihydrochloride (OPD, Sigma P-841, 10mg) substrate was diluted to contain 0.05M phosphate-citrate buffer and hydrogen peroxide (30%, Merck). The substrate was added to the plates and incubated at RT for 10 minutes. The reaction was then stopped with 1M sulfuric acid, and the absorbance was measured immediately at 492 nm using ELISA plate reader (Synergy hybrid reader: Biotek). The endpoint titers were calculated using the mean of the black plus three standard deviations as a cut off.

3.4.2 Methodological considerations for the serological assays

The HI assay is a long-established and the most frequently used serological assay, considered as the "Gold Standard" for assessing the presence of influenza-specific antibodies in serum following influenza infection or vaccination. In all the three-papers included in this thesis we used the HI assay to assess antibody responses targeting the immunodominant HA globular head domain. An HI titer of ≥ 40 correlates with 50% protection against clinical influenza disease in healthy young adults [127]. The HI assay is easy to perform and inexpensive, but it has limitations like low sensitivity in detecting antibodies to influenza A/H5N1, influenza B and current non-agglutinating A/H3N2 strains (A/H3N2 strains from 2014-2018) [232-234]. The HI assay underestimated the antibody responses induced by LAIV. As HI antibodies were not

boosted in primed children and adults after vaccination with LAIV, although they had increased in T cell responses (**paper III**), therefore HI is not an appropriate assay for determining vaccine immunogenicity for LAIV. The lack of standardized HI protocols also leads to high degree of inter-laboratory variability [235]. Earlie studies and a recent report by Waldock *et al.* demonstrated that inter-lab variability can be reduce when the HI protocol is harmonized, and standard biological reagents are used [236-239]. The HI results presented in this thesis were performed with standard operating procedure (SOP) and all the samples were run on one day in duplicates. Thus, we can conclude that our HI data may be within the acceptable range. The SRH assay is a more sensitive for detecting antibodies to avian and influenza B viruses (as it can distinguish between more closely related influenza strains) than the HI assay, The advantages of the SRH assay are that small quantities of virus and low serum volume are required to measure complement-mediated hemolysis of red blood cells induced by antibody-viral antigen complexes. The SRH assay may have lower specificity for detecting HA-specific antibodies, as it cannot distinguish between antibodies targeting the HA protein from those antibodies against the internal viral proteins [240].

ELISA is widely used for the detection of influenza-specific antibody responses after influenza infection or vaccination. It is sensitive for quantifying different Ig classes (IgA, IgG and IgM) of antibodies in serum, saliva and nasal wash and therefore not restricted to serum antibodies like the HI and SRH assays. The reagents (antigens, captured and conjugated Ig antibodies) used for ELISA can be standardized, thus the ELSA assay is robust compared to HI. ELISA can be performed using purified virus antigen or recombinant viral proteins (improving the specificity for ELISA), thus overcoming the need of using whole viruses like in the HI assay. The rational of using these assays to measure antibody responses was that large numbers of biological samples can be analyzed simultaneously in a short time frame, and they correlate well for influenza A viruses [241]. A combination of these assays (HI, SRH and ELISA) can improve the sensitivity when evaluating influenza vaccine immunogenicity.

The serological assays conducted in the studies reported here measures vaccine induced HA head-specific antibody responses. HA head-specific antibodies are strain specific

and have a narrower binding range [111]. We did not, however, assess antibodies targeting the immune-subdominant HA-stalk domain and the NA protein. These antibodies target the more conserved viral epitopes and were shown to be independent correlates of protection against influenza and are attractive targets for universal influenza vaccine [242, 243]. Both HA head-specific and stalk-specific antibodies can be quantified using the ELISA assay with appropriate proteins, whereas the enzyme-linked lectin assay (ELLA) is used for the assessment of NA inhibiting antibody responses [59]. Additionally, influenza vaccination was reported to induce functional neutralizing and non-neutralizing antibody responses which are implicated in protective immunity against influenza [244-246]. The virus or microneutralization (VN/MN) assay is a routinely used serological assay for the detection of infection or vaccination induced functional neutralizing antibody responses preventing *in vitro* infection. The ADDC assay is used to detect influenza-specific non-neutralizing antibodies with Fc-effector activities. These non-conventional assays were not routinely used in our lab during these studies, but have been used to investigate the spectrum of antibodies induced after vaccination and reported by others in our group [247-249]

3.4.3 Cellular assays

Antibody secreting cell ELISpot assay

Influenza-specific IgG, IgA, and IgM secreting ASC responses against the homologous A(H1N1)pdm09 vaccine strain and two pre-pandemic A/H1N1 strains were quantified using the ASC ELISpot assay and performed as previously described [231]. ELISpot plates (Millipore, Merck, USA) were coated with split influenza virus antigen (2 µg/ml) and capture antibody (1µg/ml) overnight at 4°C (Antigens used are listed Supplementary Table 1). Appropriate number (1-5 x 10^6) of freshly isolated pre- and post-vaccination PBMCs were resuspended in B cell medium (Roswell Park Memorial Institute (RPMI) 1640 medium Penicillin, Streptomycin, Amphotericin (PSA), FBS, 1 M HEPES buffer, 2-mercaptoethanol) and cultured in the coated plates undisturbed overnight. Following overnight incubation, biotin conjugated IgG, IgA and IgM antibodies diluted in PBS/T were added and incubated at RT for two hours. Extravidin

peroxidase diluted 1:1000 was added and incubated for one hour at room temperature. A substrate containing (2.5.4.2 Substrate 9-amino 3-ethyl carbazole (AEC) tablet dissolved in 2.5ml of dimethylformamide, acetate buffer and 30% hydrogen peroxide) was added to the plates and incubated for 30 minutes at in the dark. The reaction was stopped when the spots were visible by washing the plates thoroughly under running tap water, the plate was then allowed to air dry. Each spot represented an individual ASC (Figure 3.4), and they were counted using the ELISpot plate reader/counted (Autoimmune Diagnostical-AID, GmbH, Germany).

Memory B cell ELISpot assay

The memory B cell ELISpot assay was used to enumerate influenza-specific MBC response against the A(H1N1)pdm09 virus in papers **I and II** and against six pre-2009 seasonal A/H1N1 viruses in **paper I** as previously described [250]. Briefly, PBMCs (500,000) were cultured with a mixture of mitogens consisting of pokeweed mitogen extract (Sigma-Aldrich), 5'-cytosine-phosphate-guanine-3' oligonucleotide (ODN) (phosphothioated 24-mer, sequence 5'-tcgtcgttttgtcgttttgtcgtt-3') (InvivoGen) (3 μg/ml), and Pansorbin cells for *in vivo* polyclonal B-cell activation for 6 days. For negative control PBMCs were cultured in medium alone without mitogen stimulation. ELISpot plates were coated, blocked and the proliferated B cells were cultured in the coated plates as described above for the ASC ELISpot. After overnight incubation diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Southern Biotech, USA) was added and incubated for two hours at RT. The plates were developed using the 3,3',5,5'-tetramethybenzidine (TMB) solution (TMB-H, Moss, USA) that generated blue spots at the sites of the peroxidase activity. The reaction was stopped after 10 minutes by washing the plates under running tap water. The plates were air dried and stored in a dark place before analyzing in an automated ELISpot plate reader (Autoimmun Diagnostika-AID, GmbH, Germany. The MBC ELISpot assay readout is illustrated in Figure 3.4. The coating split influenza antigens are listed in Supplementary Table 1.

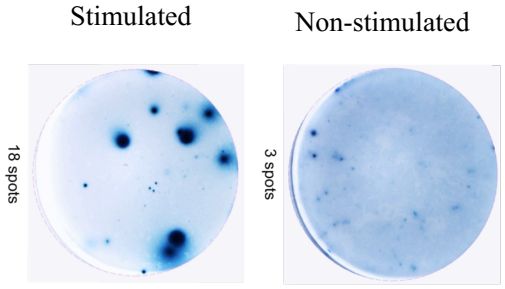

Figure 3.4 ASC or MBC ELISpot plate readout

The frequencies of influenza-specific ASCs/MBCs were calculated after quality assessment of the plates and the number of cells counted in uncoated control wells (non-stimulated = 3 spots) were subtracted from the number of ASC/MBC spots counted in wells coated with the split influenza virus antigen (Stimulated = 18 spots). Each blue-coloured spot represents IgA, IgG and IgM secreting ASC or IgG^+ MBC counted by with ELISpot plate reader.

IFN-γ ELISpot assay

The IFN-γ ELISpot assay was used to enumerate IFN-γ secreting $CD4^+$ T cell responses against the homologous A(H1N1)pdm09 strain and cross-reactivity to pre-pandemic A(H1N1) strains in **paper I**. In **paper II** we investigated the long-term impact of repeated vaccination of A(H1N1)pdm09-specific IFN-γ $CD4^+$ T cell response. The assay was performed in pre-coated plates with IFN-γ capture antibodies according to the manufacturer's instructions (MabTech AB, Sweden) [251]. Briefly cryopreserved PBMCs were thawed and seeded (4.0×10^5) in RPMI-1640 medium supplemented with 10% FCS and stimulated with influenza virus split antigens (2.5 μg/ml) (Supplementary Table 1) and medium only as background negative control or CD3-2 (1:1000 dilution) as positive control. Plates were incubated overnight undisturbed at 37°C /5% CO_2. After overnight stimulation the plates were develop by adding a detection antibody (7-B6-1biotiin) 1μg/ml diluted in PBS/FCS (0.5 %) and incubated at room temperature for 2 hours. Thereafter streptavidin alkaline phosphate-coupled detection antibody diluted in PBS/FCS (0.5 %) was added and incubated for one hour at RT. A filtered substrate solution (BCIP/NBT-plus MabTech) was added and the reaction was stopped when distinct spots emerged by washing the plates under

running tap water. The plates were analyzed and counted using an immunoassay (Autoimmun Diagnostika-AID, GmbH, Germany). Each spot represented an influenza antigen-specific $CD4^+$ T cell secreting IFN-γ, the background was subtracted from stimulation responses specific for the coated antigens.

IFN-γ-IL-2 FluoroSpot assay

The influenza-specific IFN-γ/IL-2-secreting $CD4^+$ T cells were quantified in the FluoroSpot assay, according to the manufacturer's instructions (MABTECH, Sweden) [252]. Cryopreserved PBMCs (400,000 cells) were thawed and cultured in IFN-γ and IL-2-specific capture antibodies pre-coated with the split A(H1N1)pdm09 antigen (2.5 μg/ml**,** supplementary table 1) or peptide pools (2 μg/ml) overnight at 37°C/5% CO_2. A positive (stimulated with anti-CD3, MABTECH, Sweden) and a negative control (non-stimulated) were included on each plate. After incubation, the plates were developed by adding a mixture of fluorophore-labeled IFN-γ/IL-2-specific detection antibodies (for dual staining). To amplify the signal a fluorescence enhancer solution was added. The fluorescence solution was then emptied, and the plate was left to dry in the dark cabinet to limit light exposure. The air-dried plate was then analyzed using automated fluorescence reader (Autoimmun Diagnostika-AID, GmbH, Germany). The fluorescence reader is fitted with different filters for fluorescein isothiocyanate (FITC) for IFN-γ and cyanine dye 3 (Cy3) for IL-2. One spot represented one cytokine-secreting cell. The two-colored spots are double cytokine IFN-γIL-2-secreting cells, identified by analysis of co-positioned spots in a computerized overlay of the single-colored images. The A(H1N1)pdm09 virus internal protein M1, NP, or PB1 peptide pools were obtained from BEI Resources. Conserved internal or external influenza specific CD8 and CD4 peptide pools were chemically synthesized and obtained from Mimotopes (Australia). These were used to assess the cross-reactive T cells. All the samples were run in duplicate, background responses (non-stimulated control) were subtracted from stimulation responses and the average spots of each sample were calculated.

Flow cytometry

In **paper I** $CD4^+$ T cell responses were assessed after the pandemic vaccine in HCWs using pre and post vaccination fresh PBMCs. In **paper II** we used pre- and post-vaccination cryopreserved PBMCs from 14 HCWs. In **paper III** we evaluated the T_{FH} cell response after LAIV and $CD4^+$ T cell phenotyping in children, adults, and controls using cryopreserved pre- and post-vaccination TMNCs samples. For ICS one million PBMCs were cultured with 2.5 μg/ml split influenza antigens (Supplementary Table 1) in complete RPMI-1640 medium supplemented with 10% FBS together with anti-CD28 (CD28.2), anti-CD49d (9F10) antibodies overnight at 37°C/5% CO_2. Monensin (GolgiStop™, BD USA) and brefeldin A (GolgiPlug™, BD USA) were added to block the cytokines within the cell cytoplasm upon stimulation. An unstimulated control and a positive control (stimulated with a mixture of phorbol-myristate acetate 25 ng/ml and ionomycin 250 ng/ml) were included for each sample. After overnight incubation the cells were then stained with LIVE/DEAD® fixable dead cell stain kit (ThermoFisher Scientific) antibodies to exclude dead cells from the analysis. Prior to surface staining the cells were blocked with pooled human serum to prevent non-specific binding, and then surface stained with fluorescent or biotin conjugated antibodies, followed by fixing and permeabilization of the cells before stained for intracellular cytokines IFN-γ, IL-2 and TNF-α.

For $CD4^+$ T cell phenotyping thawed and overnight rested TMNCs were cultured in RPMI-1640 medium supplemented with 10% FBS, then surface stained. To detect the transcriptional factor Bcl-6, the surface-stained cells were fixed and permeabilized with FoxP3/Transcription factor staining buffer set (eBioscience), followed by staining with anti-Bcl6 fluorescence conjugated antibody. The cells (PBMCs/TMNCs) were acquired on LSRFortessa flow cytometer (BD Biosciences, Becton Dickinson, USA) and the data were analysed in FlowJo (version 10 for Mac OS X Tree Star, USA). The gating strategy for the ICS assay is illustrated in supplementary Figure 1, the fluorochrome-conjugated antibodies used in the surface staining and ICS assays are shown in Supplementary Table 3.

Immunohistochemistry

The paraffin-embedded tonsillar sections were heated-treated for 20 minutes in retrieval solution (Dako, Glostrup, Denmark), then blocked using protein block for 10–20 minutes. Primary antibodies were added onto the section and incubated at RT for one hour. Furthermore, a HRP conjugated antibody was added and incubated at RT for 30 minutes. Thereafter the sections were incubated with a chromogen, 3,3'-diaminobenzidine (DAB) for 10 minutes treated 3-5 minutes in hematoxylin. The sections were dehydrated in a series of increasing ethanol concentrations before mounting on microscope cover glass (VWR International, Radnor, US). Prior to double staining, the section was incubated in double-staining block solution before adding a primary antibody. A second conjugated antibody was added to the sections and incubated for 10 minutes. Then a chromogen in permanent red substrate buffer diluted (1:100) was added and incubated for 10 to 30 minutes. The sections were then embodied in Hematoxylin for 4 minutes, followed by staining and digitalized using an OpticLab H850 histology scanner (Plustek, Taipei, Taiwan). The scanned sections were then analysed using Macnification v. 2.0 (Orbicule Inc., Leuven, Belgium). All the conjugated antibodies used in the IHC are listed in Supplementary Table 3.

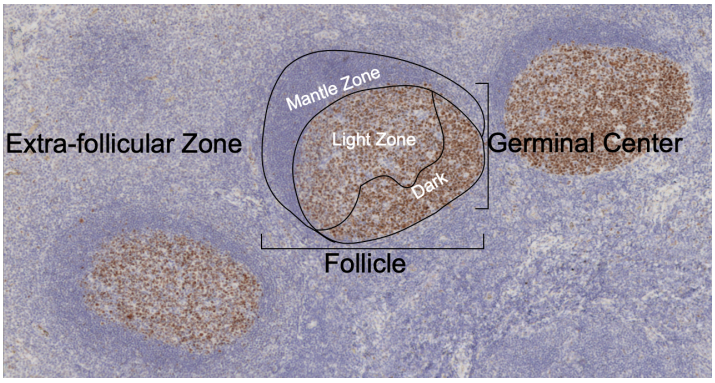

Figure 3.5 Representative of an immunohistochemistry section of a human tonsil

The three brown areas depict threes germinal centers in tonsils. The GCs are specialized microstructures within B cell follicles of secondary lymphoid organs (SLOs) such as tonsils in response to influenza virus infection or vaccination. Mature GC consist of two distinct compartments, the light zone (LZ) and the dark zone (DZ), within the vicinity of the L and D zone is the mantle zone and together these make up the B cell follicle. Somatic hype-mutation of the genes encoding B cell receptors occurs in the DZ, whereas affinity maturation and immunoglobulin isotypes class switch occurs in the LZ [253, 254]. T_{FH} cells distribution in the whole GC are marked with Bcl-6 antibody and stained with DAB (brown). The blue cells are counterstained with the nuclear stained hematoxylin and represents the extra-follicular zone enriched with T cells.

3.4.4 Methological considerations for the cellular assays

T cell responses are mostly directed against conserved epitopes of viral surface proteins (e.g., HA-stalk or NA) and internal viral proteins (e.g., NP). T cell responses can be assessed by functional assays including ELISpot assay and ICS [255]. In **papers I and II** we utilized both the IFN-γ ELISpot and the ICS to assess influenza specific Th1 responses. IFN-γ ELISpot is a highly sensitive assay for the detection of a single T cell secreting a particular cytokine (mainly IFN-γ or IL-2), which yields reproducible results (due to established harmonized ELISpot protocols and thus is less impacted by inter laboratory variabilities), relatively low number of cells are required for this assay and can thus be performed in parallel with other assays [256]. However, the ELISpot assay preferentially detects effector T cells due to the short overnight stimulation time, which results in underestimation of the magnitude of influenza-specific T cells, such as memory T cells. Memory T cells require several days of stimulation and may produce different cytokines than IFN-γ. IFN-γ/IL2 FluoroSpot assay is a modified version of the IFN-γ ELISpot assay and is capable of detecting a single T cell secreting two cytokines simultaneously [257]. The FluoroSpot assay is as sensitive as traditional ELISpot assays, however, it offers improved visualization of T cells spots by fluorophores instead of enzyme/substate combination and discriminate between single and double-cytokine secreting cells comparable to the ICS assay [258, 259]. ICS is a flow cytometry approach used for detecting antigen-specific T cell secreting multiple cytokines at a single cell level or from an entire T cell population and very useful for T cell phenotyping [255]. In **papers I and II** ICS was used to quantify the frequencies of single cytokine producing and multifunctional $CD4^+$ T cells. ICS is, however, a resource-intensive procedure, requiring larger number of cells (1×10^6) and more complicated to perform than the ELISpot assays. Since we collected small volume of peripheral blood 8-16 ml especially from younger children in the LAIV study, we could not perform ICS using PBMCs. Therefore, in **paper III**, T cell phenotyping and influenza specific T_{FH} cell responses after LAIV was assessed in TMNCs using flow cytometry. We could also have performed the multiplex cytokine assay, which is highly sensitive and allow simultaneous detection of numerous cytokines in a single sample

allowing differentiation of Th1 and Th2 cells requiring low blood volume [260, 261]. The multiplex cytokine assay has been used in our earlier study [262].

Recognizing the limitations and advantages of the different assays presented in this thesis and since no single assay can be used to assess all paraments in influenza vaccine immunity. We used the most appropriate methods to address the aims of each paper.

3.5 Statistical analysis

In **paper I** we used the non-parametric Kruskal-Wallis test to analyze the differences in the IFN-γ, ASC and MBC ELISpot responses. The Spearman correlation coefficient analysis was used to determine the correlations between pre-vaccination MBC responses against the A(H1N1)pdm09 virus and seasonal A/H1N1 influenza strains. The differences between the ICS responses at days 0 and 21 were determined by the student t test and a partial permutation test using SPICE version 5.1 software as described [263]. In **paper II,** the T-cell responses assessed in the FluoroSpot, or ICS assays and the HI titers were log-transformed, then the statistical analysis were performed using the nonparametric repeated-measure Friedman test, followed by the Dunn-Bonferroni post-hoc test. The retrospective data for IFN-γ-secreting T cells, cytokine profile and MBCs in 2009 were compared to the prospective data in 2012 or 2013 using the nonparametric. In **paper III** elevations of median fluorescence intensity from flow cytometry, HI titres, and Ig concentrations from ELISA were Ln transformed before the statistical tests. Sidak's multiple comparisons or multiple *t* tests with desired false-discovery rate of 1% were performed in a two-way analysis of variance. Non-parametric Spearman correlations were tested, and linear fitting curves were plotted when Spearman p <10. Fisher's exact analysis was performed with 2 × 2 contingency table. All statistical analyses were performed in SPSS-Statistics version-24 and in Prism version-7(GraphPad software, La Jolla, CA, USA). Adjusted p values < 0.05 was considered statistically significant.

The last literature search was performed in March 2022

4. SUMMARY OF RESULTS

4.1 Paper I: Pandemic vaccination

During the 2009 influenza pandemic, we conducted a clinical trial to evaluate the safety and immunogenicity of the AS03-adjuvanted A(H1N1)pdm09 monovalent vaccine. We recruited and vaccinated HCWs with a single dose of the AS03-adjuvanted A(H1N1)pdm09 vaccine. The objectives of the study included in paper I was to evaluate the homologous and cross-reactive immune responses against the novel A/H1N1 pandemic strain and six prototype A/H1N1 vaccine strains dating back to 1977 in vaccinated HCWs. The study participants provided serum and PBMCs pre-vaccination on day 0 and on 7-, 14- and 21-days post-vaccination.

4.1.1 Humoral responses after pandemic vaccination

The serological assays HI and SRH were used to measure A(H1N1)pdm09 antibody responses at all four timepoints after vaccination. Prior to vaccination, 13,5% of the subjects had an HI titer above the protective threshold of ≥ 40, and 24% of the HCWs had SRH titers of $\geq$ 25mm^2 against the homologous A(H1N1) pdm09 strain. Vaccination with the AS03-adjuvanted A(H1N1)pdm09 vaccine rapidly boosted the A(H1N1)pdm09 specific antibody responses, with 78-84% of the HCWs having sera protective antibody response one week after vaccination. A peak in antibody response was observed two weeks post-vaccination, when all the HCW had antibody titers above the protective threshold. When we examined the cross-reactive antibody responses against the six pre-pandemic A/H1N1 strains, we observed that 26% -56% of the HCWs had pre-vaccination antibody titers against all the pre-pandemic strains, with the highest pre-existing antibody titers detected against the A/H1N1strains circulating just prior to 2009 (A/Brisbane/2007 (29%), A/New Caledonia/1999 (31%) and A/Texas/1991 (56%)). Vaccination with AS03-adjuvanted pandemic vaccine significantly boosted the cross-reactive antibody responses, when 68%-94% of the HCWs had cross-reactive sera-protective antibody titers two weeks post-vaccination. The SRH assay correlated with the HI assay, as 92%-100% of the HCWs had SRH titers of $\geq$ 25 mm^2 two weeks post vaccination.

4.1.2 B cell responses after pandemic vaccination

B-cell responses were assessed in PBMCs using MBC and ASC ELISpot assays. Since the ASC responses were shown to peak on day 7 post-vaccine, we only examine the influenza-specific ASC response at this timepoint. An increase in IgG, IgA and IgM ASC responses were detected against the A(H1N1)pdm09 vaccine strain, with IgG significantly dominating the ASC response compared to IgA and IgM ($P < 0.001$). A similar trend in cross-reactive IgG ASC response was detected against the two-pre-pandemic seasonal A/H1N1 strains tested. The MBC response was evaluated against the homologous A(H1N1)pdm09 strain and six pre-pandemic seasonal A/H1N1 strains. Low frequencies of IgG MBC responses were detected pre-vaccination against all A/H1N1 strains tested. The frequencies of MBC responses were significantly boosted at 14 days post-vaccination against A(H1N1)pdm09 and the seasonal A/H1N1 strain circulating prior to 2009 (A/Brisbane/59/07). No significant boost in the frequencies of IgG MBC responses were detected against the other five seasonal A/H1N1 strains, although a trend of increase was observed.

4.1.3 T-Cell responses after pandemic vaccination

IFN-γ ELISpot was used to quantify the frequency of $CD4^+$ T cells producing the IFN-γ cytokine, whereas ICS was conducted to quantify the frequencies of single or multifunctional $CD4^+$ T-cells producing Th1 cytokines (IFN-γ, lL-2 and TNF-α) pre- and post-vaccination against the A(H1N1)pdm09 vaccine strain and pre-pandemic seasonal A/H1N1 strains. High frequencies of pre-existing IFN-γ secreting $CD4^+$ T cells were found against the pre-pandemic strains compared to the homologues A(H1N1) pdm09 strain. After vaccination, we observed a trend of increase in IFN-γ^+ $CD4^+$ T cell responses against all strains tested, but no significant increase was observed. Importantly we detected significant increases in multifunctional $CD4^+$ T cells against all the A/H1N1 strains tested.

4.2 Paper II: Repeated vaccination

In **paper II** we expanded our HCW study to investigate the impact of repeated annual influenza vaccination. HCWs were vaccinated with the AS03-adjuvanted pandemic vaccine in 2009 and subsequent annual vaccination with TIV. The seasonal TIV from 2010/2011 to 2012/2013 seasons contained the A(H1N1)pdm09 virus as the A/H1N1 component and different A/H3N2 and influenza B strains. The humoral and cellular immune responses were assessed using the HI, MBC, IFN-γ ELISpot, FluoroSpot assays and ICS. The study participants provided pre (Day 0) and post (D21) vaccination blood samples each year. Additional serum and PBMCs were collected in the last two seasons (2012/2013 and 2013/2014) for the B and T cell assays.

4.2.1 Humoral responses after repeated vaccination

We evaluated the A(H1N1)pdm09-specific antibody responses using the HI assay in paired serum samples. In **paper I** we showed that a single dose of the pandemic vaccine significantly boosted the HI titers at 21 days post-vaccination in all HCWs against A(H1N1)pdm09 strain. In **paper II** after repeated annual vaccination, the pre-vaccination HI titer gradually increased each season from 2009 to 2012 (from 28% to 86% in HCWs having HI tires ≥40) and was maintained between 2012 to 2013. Vaccination with TIV significantly boosted the HI titers after each seasonal vaccination from 2010/2011 to 2013/2014.

4.2.2 B and T cellular responses after repeated vaccination

As shown in **paper I**, the frequencies of A(H1N1)pdm09 specific MBCs were boosted two weeks after the pandemic vaccination. In **paper II**, we observed significantly higher frequency of baseline A(H1N1)pdm09 specific MBCs in 2012 and 2013 compared to 2009, but no increase was detected after each seasonal vaccination. Our findings show that repeated annual vaccination resulted in gradual increase of pre-vaccination A(H1N1)pdm09 specific antibody and MBC responses.

We further explored the long-term impact of repeated annual influenza vaccination on T cells immunity. We performed IFN-γ ELISpot to quantify the frequency of

A(H1N1)pdm09 specific $CD4^+$ T cells in HCWs vaccinated with the pandemic vaccine in 2009, compared to after repeated vaccination with TIV in 2012 and 2013. Our findings showed that the baseline A(H1N1)pdm09 specific IFN-γ $CD4^+$ T cells significantly increased after 3–4 repeated vaccinations compared to HCWs who were only vaccinated in 2009. The prevalence of baseline IFN-γ secreting $CD4^+$ T cells were maintained at higher levels throughout 2012 and 2013. Annual vaccination with TIV, however did not boost the $CD4^+$ T cell responses after each seasonal vaccination. Furthermore, we enumerated the frequency of IFN-γ or IL-2 single or double cytokine producing $CD4^+$ T cells against split A(H1N1)pdm09 virus and three internal viral proteins (PB1, NP and M1) using the FluoroSpot assay. We found that single IFN-γ or IL-2 and double IFN-γ/IL-2 secreting $CD4^+$ T cells were maintained at high levels against the split A(H1N1)pdm09 virus, but no significant boost was observed after vaccination in 2012 and 2013. A maintenance of the magnitude of IFN-γ producing $CD4^+$ T cells was observed against all three internal viral proteins, whereas a decline in IL-2 producing $CD4^+$ T cells were detected against the NP and M1 proteins.

We assessed the quality of the A(H1N1)pdm09 specific $CD4^+$ T cells after TIV vaccination using ICS. We found significant boost in the magnitude of multifunctional $CD4^+$ T cell responses in 2012 and 2013, showing maintenance of the quality of $CD4^+$ T responses after repeated vaccination. When assessing memory $CD4^+$ T cell subsets, we found significant boost in long-lived central memory (CM) $CD4^+$ T cells secreting IFNγ and IFNγ^+TNF-α^+ double cytokine secreting CM and effector memory (EM) $CD4^+$ T cell responses after vaccination in 2012 and 2013. We further evaluated the magnitude of influenza specific cross-reactive IFN-γ/IL-2-secreting $CD4^+$ T cells recognizing conserved CD4 external and internal epitopes after vaccination in 2012 and 2013. The frequencies of cross-reactive IFN-γ or IL-2 secreting $CD4^+$ T cells were maintained against CD4 specific external epitopes. Maintenance of cross-reactive IFN-γ or IFN-γ/ IL-2 doble cytokine secreting $CD4^+$ T specific for CD4 internal epitopes were detected after vaccination throughout the two seasons, whereas a declined in IL-2 secreting $CD4^+$ T cells was observed from 2012 to 2013.

4.3 Paper III: LAIV

In **paper III** we investigated in detail influenza specific T_{FH} cell responses by immunohistochemistry (IHC), flow cytometry and humoral antibody responses by HI and ELISA.

4.3.1 Influenza-specific antibody responses after LAIV

LAIV elicited significantly higher HI antibody responses two weeks after the first LAIV dose, which continued to increase after the second dose. The antibody responses were maintained above the 50% protective threshold up to a year against all three antigens in children. In adults, however, a significant increase in antibody response was only observed against the influenza B strain. Similar to the HI responses, LAIV elicited high levels of systemic influenza-specific IgG responses against all the three viruses and the responses were maintained up to one-year post-vaccination in children. A trend of increase in IgA and IgM were also observed the against A/H3N2 and influenza B viruses respectively two weeks post-vaccination in children. Adults had high pre-existing antibody levels, but no increase were detected after LAIV. We next measured local salivary IgA response up to day 56 post-vaccination. Our results showed that LAIV induced significant increase in local IgA levels against the influenza B virus in children. In general, we detected high salivary IgA levels in adults compared to children, but no increase was detected after LAIV most likely due the prevalence of high pre-existing local IgA levels.

4.3.2 T cell subsets and influenza specific T_{FH} cell responses after LAIV

We performed IHC using stained tonsillar sections from vaccinees and controls to measure the ICOS signal within the follicle. We observed high ICOS expression ($P < 0.001$) in the vaccinated subjects compared to the controls, children in general had higher ICOS signals compared to the adults. We characterized different subsets of $CD4^+$ T cells by flow cytometry and identify $CD4^+$ T cell $CXCR5^+CD57^+$ subsets in the tonsils highly expressing the T_{FH} cell markers such as ICOS, Bcl-6. We further investigated whether LAIV induced influenza specific T_{FH} cell response. TMNCs from controls and vaccinated subjects were stimulated with the vaccine A(H1N1)pdm09,

A/H3N2 and influenza B split virus antigen and the presence of T_{FH} cells expressing ICOS was analyzed. Our results showed that LAIV induced significant increase in influenza specific T_{FH} cell responses in children against all three viral antigens as early as 3 days post-vaccination. In the adults we observed significant increase in T_{FH} cell responses against all three viral antigens on day 7 post-vaccination.

4.3.3 T_{FH} response correlated with antibody responses after LAIV

We further evaluated the relationship between LAIV induced T_{FH} cell and antibody responses. We observed an increase in pre-existing salivary IgA levels with increasing age, which inversely correlated with the T_{FH} cell responses. Interestingly we found a positive correlation between the influenza specific T_{FH} cell response and fold-change of systemic IgG response after LAIV.

Overall, our data shows that LAIV induced influenza specific T_{FH} cell response in both children and adults and elicits long-term antibody responses, especially in children. We also suggest that local pre-existing IgA levels in the upper respiratory tract may hamper replication of the attenuated virus in LAIV in primed individuals, whiles systemic IgG responses correlated with LAIV induced T_{FH} responses.

5. DISCUSSION

The novel A(H1N1)pdm09 virus infection was reported to induce rapid boost in cross-reactive antibody responses. Studies have shown that adjuvants such as the oil-in water emulsion AS03 and MF59 adjuvants can enhance the breath (cross-reactive) and magnitude of antibody responses after influenza vaccination [195, 264-266]. Understanding the B cells responsible for the rapid antibody induction is of great importance. Influenza vaccination or infection results in activation of naïve or memory B cells, which proliferates and differentiates into antibody secreting cells/plasma-blast increasing serum antibody levels.

Rapid induction of antibodies and B cell responses

We observed a peak in the ASC responses on day 7 post-vaccination against the homologues A/H1N1 pandemic strain and cross-reactive ASCs responses against two pre-pandemic seasonal A/H1N1 strains (A/Texas/91 and A/New Caledonia/99), which is in line with previous studies [267, 268]. Overall, in **paper I** we detected significantly higher frequencies of A(H1N1) pdm09-specific and cross-reactive IgG secreting ASCs responses compared to IgA and IgM secreting ASC responses against all three A/H1N1 strains tested, which agrees with the study reported by Li *et al.* [269]. These ASCs produce functional antibodies, which may provide early protection upon viral infection [270]. Furthermore, low numbers of IgM ASC response were detected using split antigens targeting the novel HA epitopes after the A/H1N1 pandemic vaccination, this is to be expected of a novel virus with divergent globular head domain. Early ASC responses following influenza vaccination are derived from recall MBC subsets [269].

We have previously reported that a group of low responding HCWs who failed to maintain their HI antibody titers three months post-pandemic vaccination, had significantly higher proportion of HA stalk antibodies, which were maintained above baseline compared to the decline in HA head-specific antibodies. However, revaccination with a booster dose of the AS03-adjuvanted vaccine significant elevated both the HA head and HA-stalk specific antibody responses [247]. The first dose of the

A(H1N1)pdm09 vaccine induced a recall HA-stalk-specific IgG responses, whereas revaccination boosted mostly HA head-specific IgG response, in line with the findings of Ellebedy *et al*. [193]. Furthermore, the quality of HA-stalk-specific antibodies were superior to the quality of HA head-specific antibodies, indicative of the induction of HA stalk antibodies with extensive affinity maturation or high frequency somatic hyper-mutation (SHM) following the AS03-adjuvanted A(H1N1)pdm09 vaccination. The rapid and potently induction of A/H1N1 cross-reactive antibody responses and high frequency IgG secreting ASCs as well as low frequency of IgM ASC responses we observed, were all consistent with recall memory B cell responses, rather than primary response to the pandemic vaccine [269]. A recall response is characterized by activation of MBCs, which were generated by previous infection or vaccination [271]. In **paper I**, we detected high levels of pre-vaccination IgG-specific MBCs against all the influenza A/H1N1 strains tested similarly to Li *et al*. [269]. The frequencies of IgG-specific MBCs increased and peaked two weeks after a single dose of the AS03-adjuvanted vaccine. The observed peak in IgG MBC response was consistent with that reported by previous (influenza) and current (SARS-CoV-2) studies, that showed that A(H1N1) pdm09, and SARS-CoV-2-specific IgG MBC frequencies peaked two weeks after vaccination [265, 272].

Maintenance of memory B cell responses after repeated vaccination

The frequencies of pre-vaccination IgG MBCs were significantly higher in 2012 and 2013 than in 2009, when we assessed the longevity of the A(H1N1)pdm09 specific MBC responses after 4 repeated annual vaccination. In **paper I**, we showed that a single dose of the pandemic vaccine in 2009 significantly boosted the frequency of A(H1N1)pdm09 specific IgG MBCs (mean = 410 IgG MBC/1 x 10^6 PBMCs, $p < 0.031$), however, in **paper II** after repeated vaccination we observed maintenance of high frequencies of IgG MBCs responses, but no boost was detected following repeated seasonal vaccinations ($P<0.061$). The maintenance rather the boost in IgG MBC responses after repeated annual vaccination, was due to high prevalence of pre-existing MBCs and consistent with recent SARS-CoV2 mRNA vaccine study [272]. Goel *et al*. reported that after the first SARS-CoV-2 mRNA vaccination a significant increase in

spike and RBD-specific MBC responses were detected in naïve individuals, after the second vaccine dose, the MBC responses were further boosted. In contrast, SARS-CoV-2 recovered individuals had high frequencies of pre-existing MBCs and significant increase in spike and RBD-specific MBCs were detected after first vaccination, however following the second vaccine dose no further increase in spike- and RBD-specific MBC responses were detected [272].

Homologous and cross-reactive IFN-γ^+ $CD4^+$ T cell responses

Having established that a single dose of the AS03-adjuvanted A/H1N1 pandemic vaccine and subsequent repeated annual vaccinations with TIVs elicited potent, cross-reactive, and durable B cell responses. We observed that prior to vaccination low frequencies of IFN-γ secreting $CD4^+$ T cells responses (mean = 18 IFN-γ SFU/1 x 10^6 PBMCs) were detected against the homologous A/H1N1 pandemic strain, which was comparable to previous report [273]. The AS03-adjuvanted pandemic vaccine induced both homologous and cross-reactive A/H1N1-specific IFN-γ $CD4^+$ T cell responses as early as 7 days post-vaccination and further boosted at day 21 post-vaccination albeit not significant against all viruses tested. This finding is line with a study by Faenzi *et al*, where they found that a single dose of a MF59-adjuvanted A(H1N1)pdm09 recruited pre-existing memory immune cell, which resulted in rapid induction of $CD4^+$ T cells responses against the A(H1N1)pdm09 strain and cross-relativity to seasonal A/H1N1 strains [274].

Maintenance of pre-existing IFN-γ^+ $CD4^+$ T cell responses

In **paper II** we detected significantly higher frequencies of pre-vaccination A(H1N1)pdm09-specific IFN-γ $CD4^+$ T cell responses in 2012 and 2013 influenza season, compared to the pre-vaccination IFN-γ^+ $CD4^+$ T cell responses detected prior to the pandemic vaccination in 2009. Following subsequent repeated annual vaccinations from 2010 to 2013 with the seasonal TIV, no further boost in IFN-γ^+ $CD4^+$ T cell responses were detected in either 2012 or 2013. This observation was due to the maintenance of higher magnitude of pre-existing IFN-γ secreting $CD4^+$ T cells and is supported by Rosendahl Huber *et al*. They showed that vaccination with a single dose

of MF59-adjuvanted pandemic vaccine induced a significant increase in the frequency of IFN-γ^+ $CD4^+$ T cells. Like our finding neither a second booster dose with the adjuvanted pandemic vaccine nor subsequent TIV immunization further boosted A(H1N1)pmd09-specific IFN-γ responses [275]. Interestedly, Rosendahl Huber *et al.* showed a significant increase in IFN-γ $CD4^+$ T cell response following a single dose of the MF59-adjuvated pandemic vaccine, whereas we detected a trend of increase in IFN-γ^+ $CD4^+$ T cell response, which was not significant. The statistical significance observed in Rosendahl Huber *et al.* study could probably be explained by the number of study participants or antigens used for stimulation. Whereas they had more than 300 subjects and stimulated with whole influenza virus in their study, we had 9 subjects and stimulated with split influenza antigen in our study. The high frequencies of pre-existing A(H1N1)pdm09-specific IFN-γ secreting $CD4^+$ T cells observed in 2012 and 2013 indicated strong induction of cross-reactive memory $CD4^+$ T cells after the adjuvanted A/H1N1 pandemic vaccination in 2009.

Boosting and maintenance of homologous and cross-reactive multifunctional $CD4^+$ T cells responses

In **paper I**, in contrast to the high frequency of pre-existing IFN-γ^+ $CD4^+$ T cells against the seasonal A/H1N1 strains, significantly lower frequencies of pre-vaccination IFN-γ^+ $CD4^+$ T cells were detected against the vaccine A(H1N1)pdm09 strain (reflecting the ELISpot results). We found low frequencies of pre-vaccination IL-2 and TNF-α $CD4^+$ T cells against all three A/H1N1 strains. The pandemic vaccine significantly boosted $CD4^+$ T cells secreting all three Th1 cytokines (IFN-γ, IL-2, TNF-α) against all three A/H1N1 strains tested on day 21 post-vaccination. These findings are supported by a study reported by Roman *et al*, which showed that the AS03-adjuvant in the vaccine induced higher frequencies of $CD4^+$ T cells specific for A(H1N1)pdm09 split antigen, which was further boosted after the second dose compared to unadjuvanted vaccine with a similar HA content [199]. Importantly the pandemic vaccine enhanced the quality of the cytokine secreting $CD4^+$ T cell responses, as the frequencies of multifunctional cytokine producing $CD4^+$ T cells were elevated against all three A/H1N1 strains. Multifunctional cytokine producing influenza specific $CD4^+$ T cells derived from the memory T cell compartment were shown to provide superior immune

protection compared to single cytokine producing $CD4^+$ T cells [276]. In **paper I** we showed that there was high prevalence of cross-relative multifunctional memory $CD4^+$ T cells against the pre-2009 seasonal A/H1N1 strains prior to vaccination, which were further booster after the pandemic vaccination. Whereas in **paper II** we demonstrated that after vaccination with the pandemic vaccine in 2009, memory $CD4^+$ T cells were induced against the homologous A(H1N1)pdm09 strain. Importantly subsequent annual vaccination with TIVs over 5 influenza seasons resulted in persistently high $CD4^+$ T cell responses against the A(H1N1)pdm09 strain. These observed responses are multifunctional with predominantly IFN-γ secretion and a low level of IL-2^+ $CD4^+$ T cells. Following each seasonal vaccinations, we observed an increase in multi-cytokine secretion, and decreased single cytokine secreting cells after 3−4 repeated vaccinations. The ratio of IFN-γ to IL-2-secreting T cells, showed that the A(H1N1)pdm09-specific $CD4^+$ T cells remained IL-2 enriched in 2009 but changed towards predominantly IFN-γ secretion in 2012 and 2013. However, whether this IFN-γ dominance in cross-reactive $CD4^+$ T cells indicates a greater potential for help and/or other functions will need to be elucidated further.

Boost of multifunctional memory CD4+ T cell responses

Influenza-specific memory T cells generated following natural infection and vaccination (mainly with adjuvanted vaccines) are cross-reactive to multiple influenza strains and may provide long-term protection with a lifespan of approximately 10 years [277, 278]. Since we have demonstrated that the AS03-adjuvanted pandemic vaccine induced generation of memory $CD4^+$ T cells, we further assessed the cytokine profile of memory $CD4^+$ T cells subsets elicited after repeated vaccination with seasonal TIVs. Several studies including a recent one by Richards *et al*. reported that individuals who were repeatedly vaccinated mount less robust antibody responses, due to blunted or diminished $CD4^+$ T cell response [279]. In contrast, we observed significant boost in A(H1N1)pdm09-specific long-lived central memory (Tcm) $CD4^+$ T cells and persistently high memory $CD4^+$ T cells secreting multiple cytokines following priming with the AS03-adjuvanted pandemic vaccine and repeated annual vaccination. Since memory $CD4^+$ T cells can rapidly proliferate and differentiate into effector T cells upon

antigen encounter, they may provide long-lasting protective immunity. However, enhanced T-cell responses (in terms of secreting multiple cytokines) do not prevent influenza virus infection but reduce the severity of influenza illness. Importantly repeated seasonal vaccination rather than single season vaccination was shown to be associated with high vaccine effectiveness against influenza hospitalization [280].

Influenza memory $CD4^+$ T cell can recognize internal viral proteins and more conserved epitopes in the surface HA and NA glycoproteins of multiple influenza subtypes. Additionally, they provide effective immune protection by mediating heterosubtypic immunity, which is generated by previous infection or vaccination and can alter the immune response to subsequent infection with distinct viral strains. Generated heterosubtypic immunity target conserved viral proteins and provide cross-protections between distinct influenza strains. This can be essential in a situation where the circulating influenza strain drifts from the vaccine strain or during a pandemic where there is an absence of neutralizing antibodies [281, 282]. Having demonstrated in **paper I** that a single dose of AS03-adjuvanted pandemic vaccine elicited cross-reactive multifunctional A(H1N1)pdm09-specific $CD4^+$ T cell responses against the homologous and heterologous A/H1N1 strains. We extended our investigation in **paper II** by separately assessing the cross-reactive $CD4^+$ T cell responses to conserved viral external (HA and NA) or internal (M1, NP and PB1) epitopes following repeated vaccination. In murine studies cross-reactive $CD4^+$ T cells were shown to provide broadly protective heterosubtypic influenza immunity [283, 284]. After 3−4 repeated vaccinations, we observed a decline in IL-2 secreting $CD4^+$ T cell responses against the viral internal epitopes, resulting in a trend of increased IFN-γ secreting $CD4^+$ T cell dominance, which is in agreement with previous report [285]. Long-term exposure to influenza through vaccination or infection may direct the T-cell responses towards conserved epitopes that are repeatedly recognized by influenza-specific memory T cells. In **paper II** we observed high frequencies and maintenance of pre-vaccination IFN-γ secreting $CD4^+$ T cells against conserved external epitopes with no further boost after seasonal vaccination like what we have shown with antibodies and Th1 responses and in agreement with a study by Yang *et al.* [286].

Live attenuated influenza vaccine induced immune responses

In Norway apart from the elderly, children make up a large proportion (75%) of influenza-associated hospitalization annually [18]. WHO and several other countries including the US recommend influenza vaccination of infants and children between 6 months and 5 years of age to reduce the risk of severe influenza complications. In Norway annual seasonal influenza vaccination is recommended for high-risk children and not for healthy children, and with the reported associated risk of narcolepsy in children following vaccination with the A(H1N1)pdm09 vaccine [287], many parents are skeptical in having their children vaccination against influenza. There is little data available in Norway on influenza vaccine immunogenicity in children. In 2012 the licensure of the new live attenuated influenza vaccine (LAIV) for use in children 2-17 years old in Europe provided us with the unique opportunity to characterize LAIV elicited immune responses in children and adults.

Natural influenza infection induces durable, broadly cross-reactive antibody and T-cell responses, however the immune responses induced after influenza vaccination is influenced by several factors, such as vaccine type, age and pre-existing immunity of the vaccine recipients. LAIV mimics natural infection and elicit multifaceted immune responses especially in young children [288]. LAIV was shown to elicits broader longer-lasting humoral and cellular immune response, as opposed to IIVs which is poorly immunogenetic in younger children [126, 289]. Additionally, metanalysis of randomized clinical trial estimated 77% LAIV efficacy against antigenically matched influenza strains and can provide protection against drifted strains in children [290]. There is no established correlate of protection for LAIV and the HI assay underestimate LAIV induced immune protection. Pediatric clinical trials conducted in our group, showed that LAIV elicited persistent humoral and cellular immune responses for at least one year in children [124, 126]. Furthermore, we reported that LAIV induced an early B-cell and cross-reactive $CD8^+$ T-cell responses in the tonsils after LAIV vaccination in children [125, 291].

Children had early T_{FH} responses after LAIV vaccination.

The data presented in **paper III** is an extension of our LAIV pediatric studies, where we aimed to investigate in depth the humoral immune responses and the role of follicular T helper (T_{FH}) cells after LAIV vaccination in children and adults. With the unique access to the local draining lymph nodes shortly after LAIV vaccination, we assessed the LAIV induced T_{FH} cell and antibody responses and whether the T_{FH} cell responses correlate with antibody responses in children and adults.

Studies using human tonsils have shed light on the T_{FH} cells responses against influenza [292, 293]. Amodio *et al.* [292] studying tonsils from children who received IIV, showed significant increase in the frequencies of T_{FH} cells, which was associated with antigen-specific B cell responses. Aljurayyan *et al*. [293] using tonsillar mononuclear cells (TMNCs) from unvaccinated individuals, reported T_{FH} cells activation, proliferation, and differentiation, which correlated with antibody production after *in vitro* stimulation with LAIV. In **paper III** we reported the first study investigating the induction or activation of T_{FH} cell response in tonsils after LAIV vaccination in healthy children and adults. Our immunohistochemistry staining of tonsillar sections showed that B cell follicles and GCs were present in the tonsils of children and adults after immunization with LAIV. Kim *et al*. reported that a $CD4^+$ T cell subset residing at the border between T cell zone and B cell follicles, provided help to memory B cells [294]. In **paper III**, we observed that $CD4^+$ T cells were mostly located in the T cell zone and that $CD57^+$ cells were enriched in the GC, justifying the use of $CD57^+$ as a GC marker.

When we grouped $CD4^+$ T cells based on the expression of T_{FH} cell marker CXCR5 and the GC marker $CD57^+$. We identified a subset of $CD4^+$ T cells ($CXCR5^+CD57^+$) with canonical T_{FH} cells features, expressing T_{FH} surface markers like ICOS and the transcriptional factor Bcl-6. Furthermore, we characterized LAIV induced T_{FH} cell response in our study cohort and we observed moderate yet significant increases in ICOS expressing T_{FH} cells in children and a trend of increase in adults one week after vaccination compared to unvaccinated controls. This may indicate early LAIV-induced activation of B cells in children [124]. We further investigated influenza specific T_{FH} cell responses based on the frequencies and the elevation of ICOS expression by

$CXCR5^{+}CD57^{+}$ $CD4^{+}$ T cells after *ex vivo* stimulation of TMNCs with vaccine antigens. We found that LAIV elicited rapid and more potent influenza A/H1N1 and A/H3N2-specific T_{FH} cell responses in children than in adults, but the influenza B-specific T_{FH} cell responses were of equal magnitude in both children and adults.

LAIV induced early antibody responses in naïve children

We assessed LAIV induced antibody responses in children and adults, by measuring HA-specific antibody responses with the HI assay, and quantified systemic influenza-specific IgG responses and local salivary IgA responses with ELISA. Our data showed that LAIV significantly boosted influenza-specific antibody responses two weeks after vaccination, most potently against the influenza B virus ($P<0.05$), followed by influenza A/H3N2 and A/H1N1 viruses in children. Furthermore, increased in influenza-specific local mucosal IgA responses against influenza A/H3N2 and B strains were found only in children. In adults, however no increase in influenza-specific antibody responses were observed after LAIV. The differences in the immunogenicity of the three vaccine strains and between children and adults observed in **paper III** are in line with previous studies [295, 296].

Primed individuals with HI tires ≥40, less frequently had a measurable increase in anti-HA antibodies following vaccination with LAIV. Since LAIV contains live attenuated infectious virus, the presence of pre-existing antibodies in the upper respiratory tract of primed individuals may inhibit the replication of the attenuated viruses in the vaccine [187]. We observed significant boosts in antibody responses after LAIV in naïve children with no pre-existing antibody titer. In contrast, pre-exposed children and all the adults had detectable pre-existing antibody titers against all the three influenza strains. High pre-existing antibodies in the adults may explain why they responded poorly to LAIV. Primed children were shown to maintain their antibody responses for up to one year without inducing measurable boost after LAIV [124].

Low effectiveness of LAIV in 2013/2014 influenza against A(H1N1)pdm09 virus

Vaccine efficacy studies conducted prior to the 2009 pandemic, showed that LAIV was efficacious against the pre-pandemic seasonal A/H1N1 strains, however a systematic

review and meta-analysis of LAIV studies conducted from 2009 to 2015/2016 influenza seasons revealed that the effectiveness of LAIV against A(H1N1)pdm09 strains was significantly lower (42%) than IIV (72%) [297]. During the 2009 pandemic, Norway vaccinated its population with AS03-adjuvanted monovalent A(H1N1)pdm09 vaccine, which induced durable immune response after a single dose [298]. In our current study thirteen children (27%) and sixteen adults (52%) had been vaccinated with the AS03-adjuvanted A/H1N1pdm09 vaccine in 2009. Importantly a study conducted by Medimmune demonstrated that the A(HIN1)pdm09 virus used in the seasonal LAIV formulation from 2009-2014 possessed a lysine to glutamic (E-K) substitution at position 47 in the HA2 subunit, which resulted in reduced the thermal stability and poor replication fitness of the A(H1N1)pdm09 strain [299]. LAIV showed relatively low vaccine effectiveness (VE), which was more profound during the 2013-2014 influenza season with 13% VE for trivalent LAIV (LAIV3) and 17% VE for quadrivalent LAIV (LAIV4) [123, 300]. Perhaps the combination of pre-existing immunity and the thermal instability of A(H1N1)pdm09 vaccine strain could explain why both primed children and adults in our study cohort responded weakly to the A(H1N1)pdm09 strain. Unfortunately, this resulted in the US. CDC Advisory Committee on Immunization Practices (ACIP) making the interim recommendation that trivalent LAIV should not be used in the 2016-2017 season in the US [301]. Moreover, studies in Europe, showed that LAIV still provided good protection, if not better than IIV in the pediatric population. Hence, LAIV is still recommended for use in children and adolescents (2-17-year-old). LAIV recommendation was reinstated in the US during the 2018-2019 influenza season when the A(H1N1)pdm09 strain was substituted for a more thermal stable A/Slovenia/2903/2015/H1N1 virus [302].

Durable immunity elicited by LAIV

An effective influenza vaccine administered during the start of the influenza season, (normally in October) should induce adequate and durable protection against influenza illnesses in vaccinated individuals throughout the influenza season. Two placebo-controlled pediatric efficacy studies evaluating the duration of immune protection demonstrated that LAIV elicits durable protection up to 18 months post-vaccination in

children [303, 304], this is particularly evident in naïve children. A study investigating the durability of the serum antibody responses after LAIV in children, demonstrated that 92% to 100% of the study participants were seropositive one year after the second dose of LAIV against the influenza B and A/H3N2 strains, respectively. Furthermore, high HI titers were maintained in all the study participants who were immunized with LAIV each season during the four-year study period [305]. This observation was confirmed in our study in **paper III**, where we detected significant increases in HI titers after the second dose of LAIV immunization in children. The HI titers were maintained up to one year post vaccination against all three vaccine strains, with the highest titer observed against the influenza B strain. In the adults however, we observed intertrain variability after LAIV. Most of the adults had high pre-vaccination HI titers and no boosting were observed against influenza A/H1N1 and A/H3N2 strains after LAIV. Interestingly we observed significant increases in HI titers against influenza B strain, which were maintained up to one-year post-vaccination. Previous studies have reported that the influenza B strain contained in LAIV induces both antibody and T cell-mediated responses [124, 295]. Our study showed this could either be due to the lack of pre-existing immunity or better replicative ability of the B strains in humans [306].

High pre-existing mucosal IgA may partially inhibit LAIV virus replication

The mucosal surface of the respiratory tract is the main entry site of influenza viruses, thus influenza-specific antibodies at this site are crucial for preventing viral infection. It is well established that IgG antibodies mediate protection against influenza, however there are only few studies available that report the protective immunity mediated by mucosal IgA antibodies (due to difficulties in acquiring mucosal biological specimen e.g. nasal wash) one study reported that nasal IgA contributes to the efficacy of LAIV and provided vaccine induced protection against the establishment of influenza infection [184]. A study conducted in un-primed children showed that higher IgA and IgG levels were present in nasal wash specimens within two weeks after LAIV and persisted for up to one year [307]. In **paper III** we detected high local mucosal IgA antibodies in the saliva prior to vaccination especially in the adults against all three influenza strains and in primed children against influenza A/H1N1 and A/H3N2

strains. No boost in saliva IgA levels were observed after vaccination. This shows that as we age an accumulation of influenza specific IgA levels in the upper respiratory tract may partially inhibits LAIV virus replication. We did, however, detect a significant increase in saliva IgA levels on day 28 post vaccination against influenza B strain in children. The systemic IgA level was lower than that of local IgA level as expected since systemic IgA originates from mucosal secretions and mucosal IgA is produce locally in the upper respiratory tract [308].

The impact of pre-existing antibodies on LAIV induced immunity

Acquired immunity either by natural infection or through vaccination during an individual's lifetime may determine the magnitude of their immune response to subsequent infections or vaccination [183]. Pre-existing antibodies in the upper respiratory tract may affect the immune responses elicited by LAIV in pre-exposed individuals, as we have shown and previously reported [309]. Coelingh *et al*. reported that increased in LAIV induced HI titer is associated by lower baseline HI titer in young children [309]. In **paper III**, we applied a relatively simple but quantitative method to measure tonsillar influenza specific T_{FH} cell and antibody responses. Our results showed that LAIV elicits more potent A(H1N1)pdm09 and A/H3N2-specific T_{FH} cell and antibody responses in children than in adults, but interestingly the influenza B strain-specific T_{FH} cell and antibody responses were of equivalent magnitude in both children and adults. Additionally, our data also showed that vaccine induced T_{FH} cell responses inversely correlate with age-related pre-existing local IgA levels, and positively correlated with the systemic antibody induction after LAIV. This suggests that pre-existing local IgA at the vaccine administration site could have a negative effect on LAIV induction of T_{FH} cell responses and that antibody responses after LAIV can be predicted by tonsillar T_{FH} cell responses. This may explain the higher effectiveness of LAIV in children compared to adults. In contrast a study found that the infectivity, replication, and immunogenicity of influenza A/H1N1 and A/H3N2 viruses in LAIV were not affected by pre-existing antibodies against the A/H1N1 and AH3N2 strains in children [310], suggesting that pre-existing immunity may not limit the replication of LAIV viruses at the vaccination site.

LAIV versus IIV in children

Both IIV and LAIV are safe and effective in inducing protective immunity against influenza, LAIV is reported to elicited potent cellular immunity and provide superior protection in children, while IIV provides superior protective immunity in adults [311, 312]. However, the immunological mechanism responsible for these differences are currently not fully elucidated. Two studies by Hoft *et al*. compared humoral and cellular immune response induced by LAIV and IIV; and showed that both vaccines induced comparable HA-specific antibody and T-cells responses in children and adults respectively. LAIV was shown to elicit significantly higher T-cell and secretory IgA responses in children, while in adults IIV induced significantly higher HI titer compared to LAIV [289, 313]. However, LAIV and IIV effectiveness studies conducted in adults have yield contradictory results, as some studies have showed that LAIV was as effective as IIV, whereas others observed IIV superiority in adults [314-316]. Mohn *et al.* demonstrated that vaccination of children with LAIV induced ASC producing IgM, which is indicative of naïve B cell population. Importantly LAIV boosted peripheral blood IgG and IgA secreting ASC responses which correlated with the frequency of IgG ASC in the tonsil, additionally boosting in IgG memory B cell and cross-reactive T cell responses were observed after LAIV in children [124-126]. Collective these data and the ones presented in **paper III** support the presence of high affinity class switched neutralizing antibodies in the blood and tonsils. LAIV eliciting recall memory B cell response, potent, durable, and cross-reactive T cell, and antibody responses in children. Similar findings were observed in **papers I and II** after vaccination of HCWs with the A(H1N1)pdm09 vaccines and subsequent annual seasonal influenza vaccination.

Limitations

During the pandemic A/H1N1pdm09 vaccine clinical trial we faced some difficulties inherent in a long term follow up study, such as loss of study participants to follow up which reduced the sample size in the **papers I and II**. In **paper I**, 237 HCWs provided blood samples prior to and 21 days after vaccination which were used in the serology assays. Since both the ASC-ELISpot and the ICS assay were performed using fresh PBMCs, the sample size was reduced to a manageable size to the laboratory workload

capacity for sample processing and to allow both assays to be performed simultaneously. In **paper II**, the long follow-up period limited the sample size. Annual influenza vaccination was recommended, although voluntary, for all HCWs after the 2009 pandemic; therefore, we could not control the number of seasonal vaccinations the HCWs received during the study. We therefore only included fourteen HCWs who were repeatedly vaccinated for 5 years to evaluate the long-term impact of repeated vaccinations, without the bias of missing vaccination history. Further studies are required to confirm our findings in larger populations with well documented vaccination history, and to evaluate both antibody and T-cell responses against other vaccine strains (A/H3N2 and influenza B) after repeated annual vaccination.

In the LAIV clinical trial, the blood sampling volume was restricted in younger children, therefore depending upon the child's weight we collected one or two CPTs per participant, further limiting the number of assays we could perform. One of the limitations of the study in **paper III** was the lack of comparation between the local T_{FH} cell and the circulating T_{FH}-like cell responses after LAIV particularly as LAIV boosted systemic influenza specific antibody responses. Furthermore, we focused on the newly licensed LAIV and did not include tonsillectomy patients vaccinated with seasonal IIVs, which was a missed opportunity. A comparation of T_{FH} cell responses after LAIV and IIV, would have provide a better insight into understanding the difference in the immune responses elicited by both vaccines. Furthermore, investigation of the circulating T_{FH}-like cell responses in the HCWs could have aided in understanding the antibody response after repeated vaccination.

6. CONCLUSIONS

In **paper I**, we reported rapid induction of A(H1H1)pmd09-specific and cross-reactive antibody responses against pre-pandemic A/H1N1 strains after pandemic vaccination. The observed cross-reactive responses could be associated with recalled memory B cells, which provide protective immunity and are important for controlling virus infection. B cell responses assessed in **papers I and II** showed that vaccination with a single dose of the AS03-adjuvanted pandemic vaccine elicited vaccine strain specific and cross-reactive antibodies and B cell responses. Furthermore, high frequencies of A(H1N1)pdm09 specific MBCs were maintained after repeated annual vaccination with seasonal TIV. In **paper I** we showed rapid induction of A(H1N1)pdm09 specific and cross-reactive multifunctional $CD4^+$ T cells after the pandemic vaccine. Our follow up study in **paper II** revealed that repeated influenza vaccination induced persistent A(H1N1)pdm09 specific $CD4^+$ T cells, which were associated with high maintenance of neutralizing antibodies over 5 influenza seasons. The SARS-CoV-2 pandemic has highlighted the increased risk of infection in frontline HCW. Healthcare workers working on clinal wards in close contact with infected patients are constantly exposed to influenza and will benefit from annual influenza vaccination. Therefore, our findings support the current recommendations of annual vaccination against respiratory viruses, especially among higher risk individuals and frontline HCWs.

Although no correlate of protection for LAIV has been established, LAIV elicit immune protection against infection which is likely attributed to the multifaceted combination of serum, mucosal as well as T cell responses. In **paper III**, we found subsets of $CD4^+$ T cells expressing canonical features of T_{FH} cells after LAIV. LAIV rapidly induced potent influenza specific T_{FH} cell responses in children, who had lower pre-existing local antibodies. LAIV elicited rapid and long-term antibody responses to all vaccine viruses in children, but only to the influenza B virus in adult. Our work confirmed that LAIV was more immunogenic as a priming vaccine in a naïve population rather than as boosting vaccine in influenza experienced population.

7. FUTURE PERSPECTIVES

In the current work, we have demonstrated that different influenza vaccines are required in different target groups to induce rapid and durable cross-reactive cellular and humoral immune responses. The GC T_{FH} cell response is important for the generation of high affinity antigen specific antibodies, long-lived plasma cells and MBCs induced after influenza infection or vaccination [150]. We used our unique biobank of human tonsils collected after LAIV and elucidated vaccine induced T_{FH} cell responses *ex vivo* using TMNCs. It would be interesting to further study antigen specific T_{FH} cell responses in depth using the TMNCs derived organoid model to characterize the GC reaction after influenza vaccination. Additionally, giving the relatively easy access to PBMCs, we will broaden our knowledge by evaluating circulating T_{FH} cell responses to determine the relationship between systemic and lymphoid organ T_{FH} cells. However, very few studies have investigated the regulation of the GC reaction by follicular regulatory (T_{FR}) cells during influenza vaccination. T_{FR} cells have been recently discovered in B cell follicles and found to restrict T_{FH} cell mediated antibody production, however their involvement in regulating T_{FH} cells after LAIV vaccination remains to be determined. In future studies, it would be interesting to investigate the presence and distribution of T_{FR} cells in tonsils after LAIV.

In our HCWs cohort, we could extend our work by examining the circulating T_{FH} cell responses and long-lived plasma cells in the bone marrow to elucidate the immunological mechanism of durable immune responses. Furthermore, the HCWs received repeated seasonal TIV and we measured A(H1N1)pdm09 specific immune responses in papers **I and II**. So, investigating the immune responses to A/H3N2 and influenza B strains is important. In the context of the current COVID-19 pandemic, the majority of the Norwegian population including HCWs have received 3 doses of the mRNA vaccine. In future studies it would be interesting to investigate immune responses after repeated vaccination with the same mRNA COVID-19 vaccines and compare with our work with the IIV to help future vaccination policy making.

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9. SUPPLEMENTARY FILES

Supplementary Table 1. Split antigens used in ASC, MBC, ELISpot and Flow assays

Influenza virus name	Subtype	HA Content	Assays	Papers
A/California/7/2009	H1N1	169 μg/ml	ASC, MBC, IFN-γ ELISpot, Flow cytometry, ELISA	I, II, III
A/Brisbane/59/2007	H1N1	269 μg/ml	MBC, IFN-γ ELISpot, Flow cytometry	I
A/New Caledonia/20/1999	H1N1	150.5 μg/ml	ASC, MBC, IFN-γ ELISpot, Flow cytometry	I
A/Texas/36/1991	H1N1	405 μg/ml	ASC, MBC, IFN-γ ELISpot	I
A/Taiwan/1/1986	H1N1	179 μg/ml	Memory B Cell ELISpot	I
A/Brazil/11/1978	H1N1	1:800	Memory B Cell ELISpot	I
A/USSR/90/1977	H1N1	1:200	Memory B Cell ELISpot	I
A/Victoria/361/2011	H3N2	308 μg/ml	Flow cytometry, ELISA	III
B/Hubei-Wujiagang/159/2009	B	223 μg/ml	ELISA	III
B/Massachusetts/2/2012	B		Flow cytometry	III

All the influenza virus antigens are kindly provided by GlaxoSmithKline (GSK-Belgium)

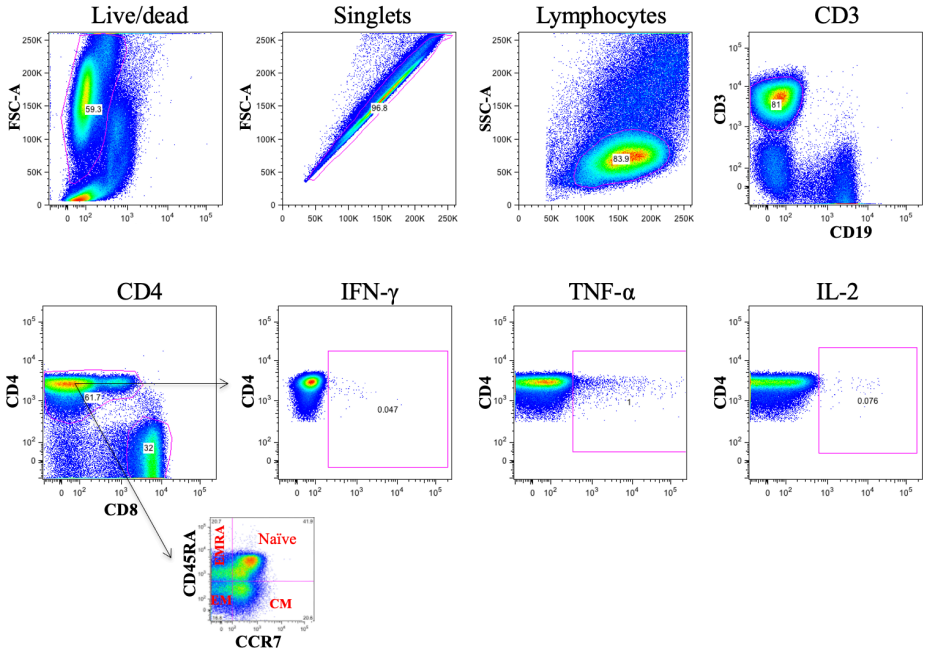
Supplementary Table 2. Coating and detection antibodies used in ELISA

Antibody	Concentration	Supplier
Captured antibody		
Goat anti-human IgG	1.23 mg/ml	Sigma Aldrich/ (1-3382)
Goat anti-human IgA	1.22 mg/ml	Sigma Aldrich (1-0884)
Goat anti-human IgM	1 mg/ml	Sigma Aldrich (1-759)
Immunoglobulin Standards		
IgG	0.477 mg/ml	Sigma Aldrich (1-4506)
IgA	1.3mg/ml	Sigma Aldrich (1-0884)
IgM	1.4mg/ml	Sigma Aldrich (1.8260)
Detection Antibody		
Biotinylated goat anti-human IgG	0,4 mg/ml	Sigma Aldrich (B-1140)
Biotinylated goat anti-human IgA	0,5 mg/ml	Southern Biotech (2050.08)
Biotinylated goat anti-human IgM	0,5 mg/ml	Southern Biotech (2020-08)

Supplementary Table 3. Antibodies used in flow cytometry and IHC

Marker	Fluorochrome	Clone	Isotype	Source/Cat nos.	Papers
Aqua Live/Dead	BV1510			Invitrogen/L34957	I, II, III
Surface marker					
CD3	PE-TR/ECD	UCHT1	m*-IgG1, κ	BD*/562280	I, II
CD4	Per CP Cy 5.5	SK3	m-IgG1, κ	BioLegend/344608	I, II
CD8	APC-Cy 7	SK1	m-IgG1, κ	BioLegend/344713	I, II
CD19	BV-605	HIB19	m-IgG1, κ	BioLegend/302244	I, II
CD56	BV-605	NCAM16.2	m- IgG1, κ	BD/562780	I, II
CD45RA	QDot 655	MEM-56	m-IgG2b, κ	Invitrogen/Q10069	I, II
CD197/CCR7	PE-Cy7	3D12	r*-IgG2a, κ	BD 557648	I, II
Intracellular markers					
IFN-γ	BV 421	4S.B3	m-IgG1, κ	BioLegend/502531	I, II
IL-2	APC	MQ1-17H12	r-IgG2a, κ	BioLegend/500309	I, II
TNF-α	PE	MAb11	m-IgG1, κ	BioLegend/502908	I, II
Markers used in Immunohistochemistry					
Bcl6	-	PG-B6p	m-IgG1, κ	Dako/M7211	III
CD20	-	FL-297	r-IgG, κ*	Santa Cruz/sc15361	III
CD57	-	TB01	m-IgM, κ	Dako/M7271	III
CD68	-	PG-M1	m-IgG3, κ	Dako/M0876	III
CD138	-	MI15	m-IgG1, κ	Dak/ M7228	III
CD278/ICOS	-	SP98	r-IgG1, κ	NP*/NBP2-12499	III
FDC	-	CAN.45	m-IgM, κ	Dako M7157	III
Markers used in tonsillar T_{FH} Cell flow					
CD3	PE-CF564	UCHT1	m-IgG1, κ	BD/ 562280	III
CD4	Per CP Cy 5.5	SK3	m-IgG1, κ	Biolegend/344608	III
CD19	BV-605	HIB19	m-IgG1, κ	Biolengd/302244	III
CD56	BV-605	NCAM16.2	m- IgG1, κ	BD/ 562780	III
CD45RA	QDot 655	MEM-56	m-IgG2b, κ	Invitrogen/Q10069	III
CD185/CXCR5	PE	J252D4	m-IgG1, κ	Biolegend/356904	III
CD278/ICOS	BV421	DX29	m-IgG1, κ	BD/ 562901	III
CD57	FITC	NK-1	m-IgM, κ	BD/555029	III
CD279/PDI	BV711	EH12.1	m-IgG1, κ	BD/ 564017	III
CD154/CD40L	APC Cy7	24-31	m-IgG1, κ	Biolegend/310822	III
Bcl6	PE Cy7	K112-91	m-IgG1, κ	BD/ 563582	III

*m mouse, r rat, BD Becton Dickinson , NP Novus Biological,



Supplementary Figure 1. Flow cytometry gating strategy

Dead cells were excluded from the total cell population using live/dead staining. Single cells and lymphocytes were identified based on the cell size and granularity using forward scatter (FSC) and side scatter (SSC) parameters. $CD19^+$ B cells were excluded and $CD4^+$ and $CD8^+$ T cells were separated $CD3^+$ T cells. To identify the frequencies of cytokine$^+$ $CD4^+$ T cells, total IFN-γ^+, IL-2^+ or TNF-α^+ cells were gated from the $CD4^+$ population. Boolean analysis was used to assess each cytokine combination response, triple producer (IFN-γ^+IL-2^+TNF-α^+), double producers (IFN-γ^+IL-2^+, IFN-γ^+TNF-α^+, IL-2^+TNF-α^+) and single producers (IFN-γ^+, IL-2^+, TNF-α^+). To differentiate the 3 memory subsets; $CD45RA^-CCR7^+$central memory (CM), $CD45RA^-CCR7^-$ effector memory (EM), and $CD45RA^+CCR7^-$late effector memory (EMRA) from $CD45RA^+CCR7^+$ naïve (NA) within $CD4^+$ cells, the gating for CD45RA and CCR7 markers were optimized using the fluorescence-minus-one (FMO) controls containing all the fluorochromes in the T-cell panel except for the one that was measured.

Papers I-III

I

RESEARCH PAPER

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Single dose vaccination of the ASO3-adjuvanted A (H1N1)pdm09 monovalent vaccine in health care workers elicits homologous and cross-reactive cellular and humoral responses to H1N1 strains

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Keywords: AS03, cross-reactivity, healthcare workers, H1N1, immune responses, pandemic influenza, vaccination

Abbreviations: HCW, Healthcare workers

Healthcare workers (HCW) were prioritized for vaccination during the 2009 influenza A(H1N1)pdm09 pandemic. We conducted a clinical trial in October 2009 where 237 HCWs were immunized with a AS03-adjuvanted A(H1N1)pdm09 monovalent vaccine. In the current study, we analyzed the homologous and cross-reactive H1N1 humoral responses using prototype vaccine strains dating back to 1977 by the haemagglutinin inhibition (HI), single radial hemolysis SRH), antibody secreting cell (ASC) and memory B cell (MBC) assays. The cellular responses were assessed by interferon-γ (IFN-γ) ELISPOT and by intracellular staining (ICS) for the Th1 cytokines IFN-γ, interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α). All assays were performed using blood samples obtained prior to (day 0) and 7, 14 and 21 d post-pandemic vaccination, except for ASC (day 7) and ICS (days 0 and 21). Vaccination elicited rapid HI, SRH and ASC responses against A(H1N1)pdm09 which cross reacted with seasonal H1N1 strains. MBC responses were detected against the homologous and seasonal H1N1 strains before vaccination and were boosted 2 weeks post-vaccination. An increase in cellular responses as determined by IFN-γ ELISPOT and ICS were observed 1–3 weeks after vaccination. Collectively, our data show that the AS03-adjuvanted A(H1N1)pdm09 vaccine induced rapid cellular and humoral responses against the vaccine strain and the response cross-reacted against prototype H1N1 strains dating back to 1977.

Introduction

The novel, swine-origin influenza A(H1N1)pdm09 virus was first detected in April 2009 and it caused the first influenza pandemic of the 21st century. The A(H1N1)pdm09 virus was antigenically distinct from the prior seasonal influenza A strains and the majority of the population was immunologically naïve to A (H1N1)pdm09 rendering existing influenza vaccines ineffective against this strain.[1-3] New pandemic vaccines were developed against A(H1N1)pdm09 and they induced sero-protective antibody responses 1-2 weeks after administering a single dose in most healthy adults.[4] Since 2009, the A(H1N1)pdm09 virus has circulated and has been included in the seasonal trivalent influenza vaccines (TIV) as the H1N1 strain.

Antibody responses are a key mediator of sero-protective immunity induced by influenza vaccines.[5] At the start of the pandemic, there were no or little antibody titres against the A (H1N1)pdm09 strain, especially in young adults and children resulting in atypically high rates of severe disease.[2,3] However, people over the age of 60 had higher levels of sero-protective immunity, most likely due to having pre-existing, cross-reactive antibodies from prior exposure to A(H1N1)pdm09-like strains in the distant past.[6] In this regard, infection with A(H1N1) pdm09 has been shown to activate broadly-cross reactive memory B cells that provided protection even in the absence of pre-existing antibody titres.[7] Of interest, recent studies have shown that antibodies specific for the conserved stalk domain of the influenza haemagglutinin were boosted by vaccination and infection with the novel A(H1N1)pdm09 virus and these antibodies have broad cross-reactive neutralizing activity against different group 1 influenza strains.[6,8,9] In addition to antibody responses, T cells play a significant role in anti-influenza immunity. A large percentage of

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T-cell epitopes found in seasonal H1N1 strains in the years preceding the pandemic were conserved in A(H1N1)pdm09, thus were targets of immunological memory.[10] A recent report showed that high frequencies of pre-existing T cells to conserved epitopes on A(H1N1)pdm09 virus were found in people that developed less severe disease, suggesting a key role for cellular immunity in anti-A(H1N1)pdm09 responses.[11]

During the 2009 pandemic, HCWs were prioritized for vaccination in order to maintain the integrity of the healthcare system and to minimize virus transfer to vulnerable patients. In October 2009, we conducted a clinical trial in frontline HCWs to evaluate the safety and immunogenicity of a single dose of a A(H1N1) pdm09 vaccine formulated with the oil-in-water adjuvant AS03. Vaccination commenced 2–3 weeks prior to the peak of pandemic activity. The vaccine was well tolerated and by using the HI assay, we showed that sero-protective responses (titres $\geq$40) were elicited in a majority of subjects (97%) by 2–3 weeks after vaccination.[4] Further studies have shown that influenza vaccines formulated with the oil-in-water adjuvant AS03 to be safe and highly efficacious in children, young adults and the elderly.[4,12-20]

In the current study, we characterized in detail the homologous and cross-reactive humoral and cellular response in HCW after AS03-adjuvanted A(H1N1)pdm09 vaccination. Our results show that vaccination induced serological (HI and SRH) and B cell (ASC and MBC) responses against A(H1N1)pdm09 and protoype seasonal H1N1 vaccine viruses that prevailed in the years preceding the pandemic. Furthermore, by IFN-γ ELISPOT and intracellular cytokine staining assays, we demonstrate that both homologous and cross-reactive Th1 cytokine responses are elicited in HCWs after vaccination with the AS03-adjuvanted A (H1N1)pdm09 vaccine.

Results

In this study we have evaluated the early homologous and cross reactive immune responses to prototype H1N1 vaccine strains dating back to 1977 after a single low dose of pandemic influenza vaccine adjuvanted with the oil-in-water adjuvant AS03 in frontline HCWs. Blood samples were taken at 4 consecutive time points (day 0, 3, 7 and 14 post-vaccination) to evaluate the dynamics of the homologous and cross-reactive immune response to vaccination (**Fig. 1**).

The cross-reactive haemagglutinin inhibition (HI) antibody response after pandemic vaccination

Figure 2 shows the post-vaccination HI response against the homologous A/(H1N1)pdm09-like strain A/California/07/09 (California) and cross-reactivity against 6 seasonal influenza A H1N1 viruses. A sero-protective HI response was defined as an HI titer $\geq$ 40.[21] Prior to vaccination, 13.5% of the subjects had sero-protective HI titres against the homologous California strain with a geometric mean titer (GMT) of 8. Vaccination boosted the California-specific HI response where by day 7, a majority of subjects (78%, GMT = 156) were seroprotected. The HI response continued to increase up to days 14 and 21 post-vaccination with 100% (GMT = 826) and 96% (GMT = 619) of the subjects, respectively having sero-protective HI titres $\geq$40.

The HI assay was used to examine the cross-reactive HI responses against 6 prototype H1N1 vaccine strains; A/USSR/90/77 (USSR), A/Brazil/11/1978 (Brazil), A/Taiwan/1/86 (Taiwan), A/Texas/36/91 (Texas), A/New Caledonia/20/99 (New Caledonia) and A/Brisbane/59/07 (Brisbane). Prior to vaccination, sero-protective HI titres were observed in HCWs against all strains; USSR (26%, GMT = 13), Brazil (26%, GMT = 12), Taiwan (39%, GMT = 24), Texas (56%, GMT = 60), New Caledonia (31%, GMT = 15) and Brisbane (29%, GMT = 14) strains. The post-vaccination HI response peaked on day 14 with 68–94% of subjects having sero-protective titres against USSR (GMT = 116), Brazil (GMT = 145), Taiwan (GMT = 201), Texas (GMT = 796), New Caledonia (GMT = 79), and Brisbane (GMT = 102) strains. Similar HI titres were observed on day 21 post-vaccination with 59–90% subjects having sero-protective titres against USSR (GMT = 84), Brazil (GMT = 110), Taiwan (GMT = 145), Texas (GMT = 653), New Caledonia (GMT = 51) and Brisbane (GMT = 48) strains.

The cross-reactive single radial hemolysis (SRH) response to vaccination

Figure 3 shows the pre- and post-vaccination SRH titres against the homologous California and cross-reactive responses against the New Caledonia and Texas strains. The European Agency for Evaluation of Medicinal Products criterion of protective serological response to influenza vaccines is a SRH titer of $\geq$25 mm^2, which was used as a cut-off for serologic protection. Prior to vaccination, only 4% of the subjects had sero-protective SRH titer of $\geq$25 mm^2 against the Texas strain (GMT = 8), while 24% and 51% of subjects had sero-protective titres against California (GMT = 11) and New Caledonia (GMT = 16) strains, respectively. One week after vaccination, a majority of the subjects (76–84%) had sero-protective SRH titres against New Caledonia (GMT = 35) and California (GMT = 41) strains, while 28% had sero-protective SRH responses against the Texas strain (GMT = 15). The SRH response peaked 2 weeks after vaccination with 92–100% of the vaccinees having sero-protective responses against New Caledonia (GMT = 56) and California (GMT = 73) strains. Lower SRH responses were detected against the Texas strain with only 59% of subjects having sero-protective titres at 2 weeks post vaccination (GMT = 25).

B cellular responses after pandemic vaccination in health care workers

Antibody secreting cell response after vaccination

The humoral response was further characterized by ASC response in peripheral blood mononuclear cells (PBMC) after vaccination. The peak virus-specific ASC response were observed at day 7 post-vaccination (**Fig. 4**), while no ASC responses were observed before vaccination or at days 14 and 21 after vaccination (data not shown). IgG ASCs dominated the anti-California response (mean = 111 ASC per 1 $\times$ 10^5 PBMC) and was

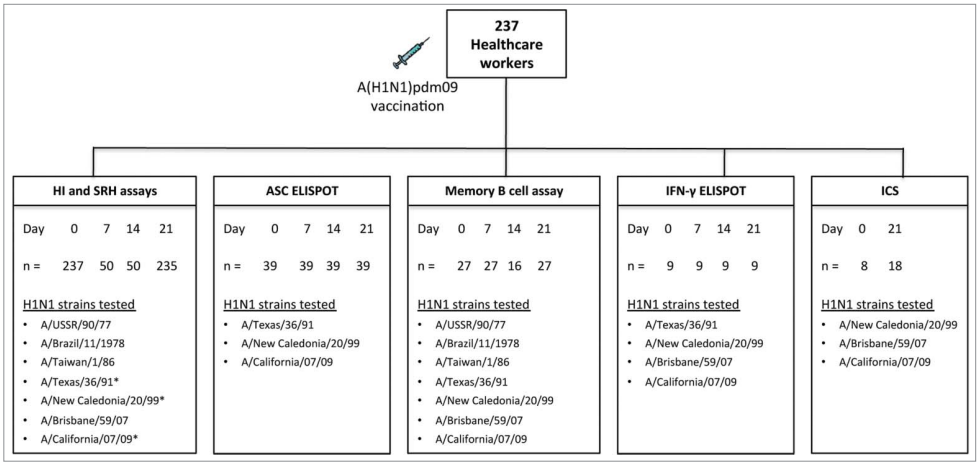

Figure 1. The experimental plan. Two hundred and thirty seven healthcare workers were vaccinated with the 2009 A(H1N1)pdm09 monovalent split virus vaccine (3.75 μg HA) formulated with the oil-in-water adjuvant AS03. The figure shows the number of samples (n) analyzed at each sampling day (Day) for each immunological assay and the H1N1 strains against which the assays were performed. Consecutive blood samples were taken from the same subject at 4 time points (day 0 7, 14 and 21 post-vaccination) for HI, SRH, ASC, memory B cell and IFN-γ ELISPOT assays. For ICS, PBMCs were obtained from 8 subjects before vaccination and a separate cohort of 18 subjects on day 21 post-vaccination. The ASC (n = 39) and ICS (n = 8–18) assays were run on fresh PBMCs, whereas memory B cell (n = 16–27), and IFN-γ ELISPOT (n = 9) assays were run on freeze/thawed PBMCs. * Strains used in the SRH assay.

significantly higher than the IgA ($P < 0.001$) and the IgM ($P < 0.0001$) ASC responses against the same strain (mean = 45 and 27 ASC per 1×10^5 PBMC, respectively). Similarly, significantly higher ($P < 0.0001$) IgG ASCs were detected against Texas and New Caledonia strains (mean = 110 and 86 ASC per 1×10^5 PBMC, respectively) compared with corresponding IgM responses against the same strains (mean = 28 and 23 ASC per 1×10^5 PBMC, respectively). In general, weak IgM ASC responses were detected against all 3 H1N1 strains, which may suggest that relatively low ASC responses were elicited against novel epitopes.

The memory B cell response after vaccination

Memory B cells (MBC) play a key role in anti-influenza immunity. **Figure 5** shows that, prior to vaccination, IgG MBCs were detected against all the influenza A viruses tested with mean frequencies ranging between 114–263 cells per 1×10^6 PBMC and no increase in the response was detected at 7 days after vaccination. The virus-specific IgG MBC frequencies peaked at 14 days after vaccination with the highest responses detected against the Brisbane and California strains (mean 470 and 410 cells per 1×10^6 PBMC, respectively) and the lowest against the Brazil strain (mean 197 cells per 1×10^6 PBMC). A significant increase ($P < 0.05$) in California-specific IgG MBC frequency was observed at day 14 (mean 410 cells per 1×10^6 PBMC) compared to pre-vaccination (mean 200 cells per 1×10^6 PBMC), while the responses against the other strains were not significantly different. Furthermore, we found a significant correlation between pre-vaccination MBC responses against California and the 6 seasonal H1N1 influenza strains with Spearman correlation coefficients (r) ranging between 0.6 and 0.96 (**Table 1**). A significant correlation was also observed between pre-vaccination MBC frequencies and day 7 HI responses against all viruses except the USSR and Brisbane strains (**Table 1**).

Interferon gamma response after vaccination

Figure 6 shows the frequencies of PBMCs secreting IFN-γ in an antigen-specific manner prior to vaccination (day 0) and at 7, 14 and 21 days post-vaccination.

Before vaccination, the highest response was observed against the Texas strain (mean number of IFN-γ^+ cells per 1×10^6 PBMC (mean) = 273) followed by the New Caledonia (mean = 100) and the Brisbane (mean = 51) strains, while the weakest pre-vaccination IFN-γ response was detected against the California strain (mean = 18). At 7 days post-vaccination, an increase in IFN-γ response was detected against the Brisbane (mean = 89), New Caledonia (mean = 170) and Texas (mean = 273) strains, although this was not significantly higher than pre vaccination numbers. No significant increases in the IFN-γ response were observed on days 14 and 21 against any strain. Overall, the weakest IFN- γ response was detected against the California strain, however the response at 21 days post-vaccination (mean = 36) was double that observed before vaccination (mean = 18).

Intracellular Th1 cytokine responses after vaccination

Figure 7 shows frequencies of CD4+ T-cells producing either single (A) or multiple (B) Th1 cytokines against California, New Caledonia and Texas strains before and 21 days after vaccination.

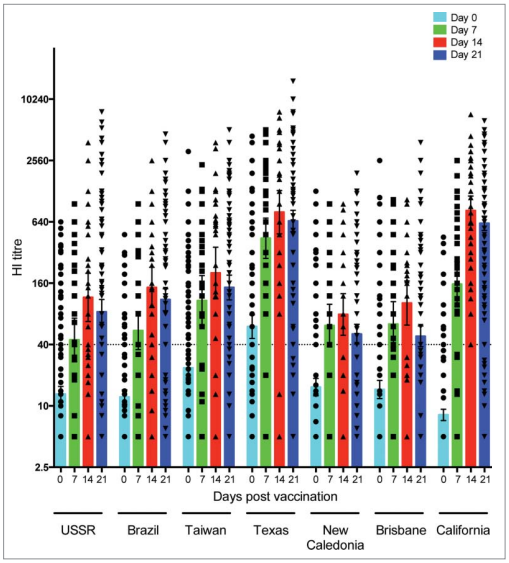

Figure 2. Homologous and cross-reactive haemagglutinin inhibition response after pandemic vaccination. The HI response against the homologous vaccine virus A/California/07/09 and 6 seasonal influenza H1N1 strains A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86, A/Texas/36/91, A/New Caledonia/20/99 and A/Brisbane/59/07 was determined before vaccination (day 0) and at 7, 14 and 21 days after vaccination. The bars show the geometric mean titer with 95% confidence interval and individual responses are presented as symbols. The dotted horizontal line represents a sero-protective HI titer of 40.

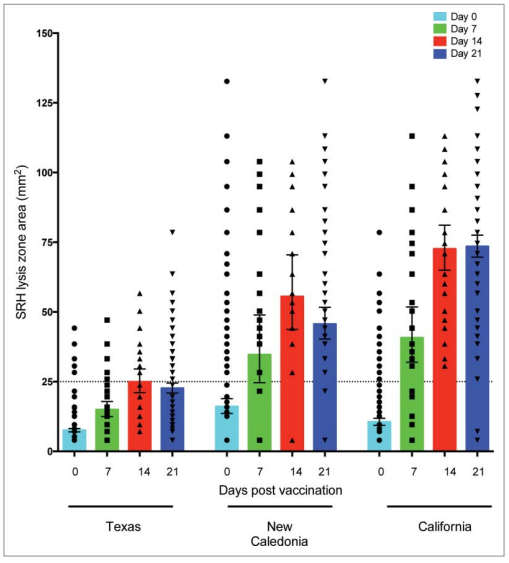

Figure 3. Homologous and cross-reactive single radial hemolysis (SRH) antibody responses after pandemic vaccination. The SRH response against the homologous vaccine strain A/California/7/09 and 2 seasonal influenza strains A/Texas/36/91 and A/New Caledonia/20/99 was determined before vaccination (day 0) and at 7, 14 and 21 days after vaccination. Each symbol represents the SRH titer of one subject, with geometric means and 95% confidence levels indicated. The dotted horizontal line shows the sero-protective SRH titer of 25 mm^2.

Before vaccination (day 0), significantly lower ($P < 0.05$) IFN-γ responses were detected against California compared with the New Caledonia and Brisbane strains. At 21 days post-vaccination, significantly higher ($P < 0.05$) IFN-γ levels were detected against all strains compared with pre-vaccination levels. Before vaccination, significantly lower ($P < 0.05$) frequencies of IL-2 and TNF-α were observed against Brisbane compared with the New Caledonia strain. Vaccination induced a significant increase ($P < 0.05$) in IL-2 response against all 3 strains compared with pre-vaccination levels. Increased TNF-α responses were also detected after vaccination with significantly higher ($P < 0.05$) frequencies detected against New Caledonia and Brisbane strains on day 21 compared with day 0. **Figure 7B** shows the frequency of Th1 cells simultaneously producing one or more cytokine (multi-functional T cells). After vaccination (day 21), significant increases ($P < 0.05$) in both triple and double cytokine producing cells were detected against all 3 strains compared with pre-vaccinaion levels (**Fig. 7B**).

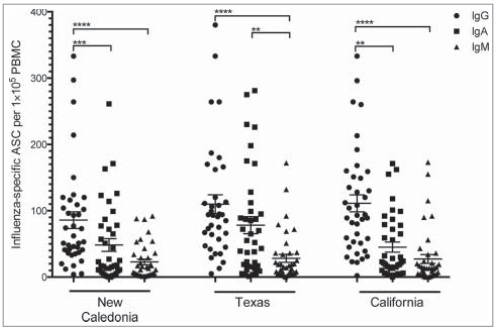

Figure 4. The antibody secreting cell response 7 days after pandemic vaccination. The A/Texas/36/91, A/New Caledonia/20/99 and A/California/07/09-specific IgG, IgA and IgM ASC responses were measured by ELISPOT 7 days after vaccination with AS03-adjuvaned pandemic H1N1 vaccine. The bars represent mean numbers of virus-specific ASCs per 100 000 peripheral blood mononuclear cells (PBMC) ± standard error of the mean. Statistical differences are shown by non-parametric Kruskal-Wallis test. ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

Discussion

The 1918 Influenza H1N1 pandemic killed up to 50 million people and H1N1 strains continued to circulate in the human

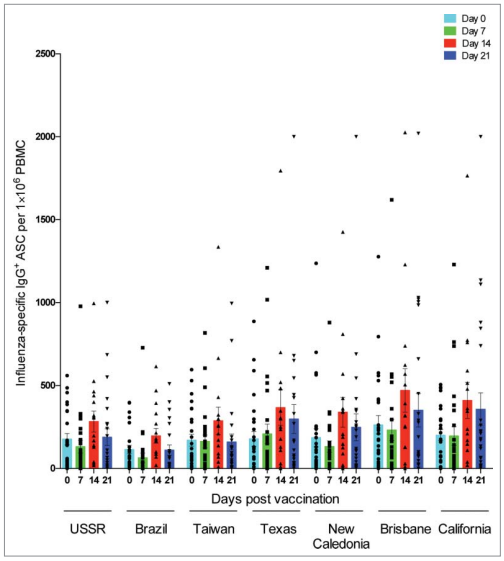

Figure 5. Homologous and cross-reactive memory B cells responses after pandemic vaccination. The pre- and post-vaccination IgG memory B cell responses were determined against the homologous vaccine strain A/California/07/09 and cross-reactive responses against 6 seasonal influenza strains A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86, A/Texas/36/91, A/New Caledonia/20/99 and A/Brisbane/59/07. The vertical bars represent the mean ± standard error of the mean and each symbol represents one subject.

populations until 1957. From 1957 to 1977, H1N1 viruses were not detected in human populations, most likely due to competition from the H2N2 and H3N2 strains. However in 1977, influenza H1N1 re-emerged and circulated as a seasonal virus until the 2009 pandemic.

Phylogenetic analysis of the H1N1 HA gene shows that the A(H1N1)pdm09 strain is highly divergent from the seasonal H1N1 strains, while the seasonal H1N1 strains from 1977 to 2008 are more closely related (**Fig. S1**). Despite the antigenic divergence, infection with the A(H1N1)pdm09 virus induced broad-cross reactive antibody responses against epitopes that are conserved on the HA of seasonal H1N1 and A(H1N1)pdm09 strains.[7,10] Antibody responses directed at common HA epitopes may explain the broad cross-reactivity observed in our cohort following vaccination with the AS03-adjuvanted vaccine. The AS03 adjuvant itself may have contributed to the breath of the cross-reactive response, however the underlying immunological mechanisms for this are not clear. A control group that received a non-adjuvanted A(H1N1)pdm09 vaccine would have shown the benefits of the AS03 adjuvant, however this was not possible as only the AS03 adjuvanted pandemic vaccine was licensed for use in Norway in 2009. We found that 13% of HCW had preexisting HI titres ≥40 to the California strain at baseline, which suggest exposure or subclinical infection with this virus. Almost all HCWs (97%) with preexisting sero-protective HI titres to A(H1N1)pdm09 were under the age of 60, therefore were not exposed to 1918-like H1N1 strains that have been shown to induced cross-reactive antibodies against the A(H1N1)pdm09 virus.[2] However, most of our study cohort (60%) had received the trivalent seasonal influenza vaccines in years preceding the 2009 pandemic[4] and this may have contributed to the preexisting immunity against both the A(H1N1)pdm09 virus and the seasonal H1N1 strains.[2]

When stratified by age, older subjects (persons born before 1957) had similar HI and SRH GMTs against A(H1N1)pdm09 and seasonal H1N1 strains at baseline compared with the younger cohort (**Fig. S2**). This differs from prior reports where higher frequencies of sero-protective antibodies and significantly lower infection rates have been observed in older adults over the age of 60 years.[2] Compared with the general public, the potential for exposure or asymptomatic subclinical infection is higher in HCWs and this may explain relatively high baseline sero-protective rates we observed in younger HCWs.

In this study, we evaluated the serological responses by 2 commonly used assays; HI and SRH with contrasting results. While both assays showed that vaccination induced complete protection

Table 1. Correlation between memory B cell responses to vaccine virus and seasonal H1N1 virus pre- and post-vaccination

	Correlation prior to vaccination*		Correlation post vaccination**	
Strain	**r**	**P**	**r**	**P**
USSR	0.95	0.0004	0.21	ns
Brisbane	0.96	0.0002	0.22	ns
Taiwan	0.82	0.0085	0.63	0.0006
Texas	0.6	0.0963	0.58	0.0023
NC	0.73	0.0304	0.43	0.0325
Brazil	0.95	0.0004	0.45	0.0207
California	NA	NA	0.81	0.0001

*Spearman correlation coefficients (r) between memory B cell responses against the homologous vaccine virus A/California/07/09 and seasonal influenza strains A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86, A/Texas/36/91, A/New Caledonia/20/99 (NC) and A/Brisbane/59/07 prior to vaccination.
**Spearman correlation coefficients between memory B cell response prior to vaccination and haemagglutination inhibition titres at day 7 post-vaccination against A/California/07/09, A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86, A/Texas/36/91, A/New Caledonia/20/99 and A/Brisbane/59/07 strains. ns = not significant. NA = not applicable.

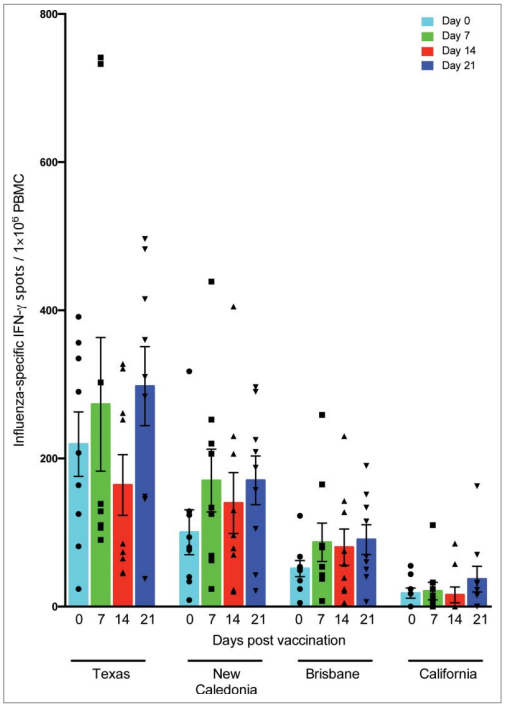

Figure 6. IFN-γ responses against influenza A virus strains after pandemic vaccination. Peripheral blood mononuclear cells (PBMC) obtained from individuals vaccinated with AS03-adjuvanted pandemic H1N1 vaccine were stimulated overnight with split virus antigens from the homologous vaccine virus A/California/7/09 and seasonal H1N1 strains A/Texas/36/91, A/New Caledonia/20/99 and A/Brisbane/59/07 and the IFN-γ response was evaluated by the ELISPOT assay. Each symbol represents one subject and with mean and standard error of the mean indicated.

against A(H1N1)pdm09 by day 14, contrasting results were observed against the Texas strain, with 100% and 59% seroprotection by HI and SRH assays, respectively. Generally there is a good correlation between HI and SRH responses against influenza A viruses[22], therefore the discrepancy observed in our study is intriguing. Perhaps this may reflect different sensitivities of serological assays and measurement of different functionalities in the antibodies against different influenza strains, however further work is required to fully understand the measured differences.

The humoral immune response was further characterized by evaluating the influenza-specific ASC and MBC responses. The day 7 post-vaccination ASC response corresponds to the peak plasmablast $CD19^{+}CD20^{-}CD27^{high}CD38^{high}$ response [23], and in our study, we observed a significant increase in IgG and IgA ASCs against the pandemic and seasonal H1N1 strains. The rapid ASC response one week after vaccination has been shown to be consistent with a recall response originating from activation of cross-reactive MBCs generated by previous influenza infections and/or vaccination.[6,7,24] An important finding in our study is that memory MBCs against A(H1N1)pdm09 strain were detected even before vaccination or widespread circulation of the pandemic virus, at frequencies similar to those observed against recently circulated seasonal H1N1 strains, suggesting cross reaction to conserved epitopes. Furthermore, we observed a significant positive correlation between the pre-vaccination MBC response against A(H1N1)pdm09 and that against the seasonal H1N1 strains (**Table 1**). A significant positive correlation was also observed between the pre-vaccination MBC responses and day 7 HI titres against most of the H1N1 strains tested. Collectively, our results strongly imply and support the suggestion that MBCs targeting A(H1N1)pdm09 exist in the human population and that they arise from prior exposure to seasonal H1N1 strains.[6] These A(H1N1)pdm09-specific MBCs have the capacity to rapidly differentiate into ASCs that secrete IgG antibodies after antigen re-encounter and have broad cross-reactivity.[25-27] The pre-existing MBCs targeting the A(H1N1)pdm09 virus may at least partly explain the fact that rapid sero-protective responses were elicited in a majority of subjects after only a single dose of the pandemic vaccine.[4] Further studies using chimeric virus constructs could evaluate the specificity of the post-vaccination antibody and MBC responses toward the globular head and the relatively well-conserved stalk domains of group 1 HA to confirm that the cross reactivity observed is due to conserved epitopes on the H1 haemagglutinin.[28] In this regard, immunization with chimeric virus constructs derived from novel influenza strains was shown to induce broadly cross-reactive HA stalk-specific antibody responses by ELISA and microneutralization assays.[29,30]

Cellular responses play a significant role in anti-influenza immunity (for a review see ref.[31]). To assess the vaccine induced T-cell activity, we determined the influenza-specific IFN-γ response by ELISPOT and IFN-γ, IL-2 and TNF-α responses by ICS. Both ICS and ELISPOT analysis showed an increase in Th1 cytokine (IFN-γ, IL-2 and TNF-α) responses against both the A(H1N1) pdm09 and seasonal H1N1 strains after vaccination. Both IFN-γ and TNF-α have powerful anti-influenza virus activity and increased levels may help prevent severe influenza illness.[32-34] Furthermore, we observed an increase in homologous and cross-reactive Th1 CD4 T cells simultaneously secreting more than one cytokine (multifunctional T-cells), which are functionally superior to single cytokine producing cells eliciting anti-influenza immunity and conferring protection against lethal influenza infection.[35,36] Furthermore, there was an increase in IFN-γ^{+} IL-2^{+} TNF-α^{+} cells post-vaccination, which have a high proliferative potential and are an important target population for anti-A(H1N1)pdm-09 virus activity.[37] Interestingly, a very low A(H1N1)pdm09-specific IFN-γ response was observed prior to vaccination by both the ELISPOT and ICS assays compared with responses against the seasonal strains. The higher baseline IFN-γ^{+} cell frequencies observed against seasonal H1N1 viruses most likely reflects a recall memory T cell response to prior influenza vaccine and/or infection. In our study, the pandemic split virus antigen used for *in vitro* stimulation consists mainly of A(H1N1)pdm09 HA and NA, which shares only a few T-cell epitopes (12%) with HA and NA of seasonal H1N1 strains [10], hence the pre-vaccination response against A(H1N1)pdm09 would mainly be naïve T cells directed toward novel epitopes. Naïve T cells require sustained

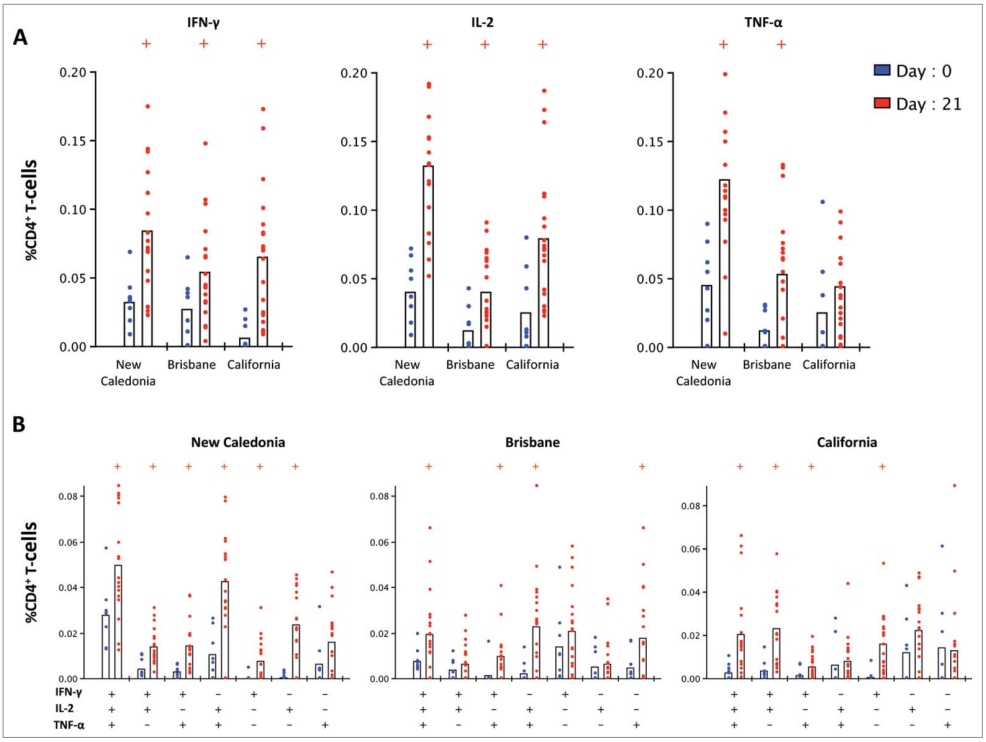

Figure 7. The single (A) and multi-functional (B) $CD4^+$ T-cell cytokine response before and 21 days after pandemic vaccination. Peripheral blood mononuclear cells (PBMC) were obtained from 8 subjects before vaccination (day 0) and from a separate cohort of 18 subjects on day 21 post-A(H1N1)pdm09 vaccination. PBMCs were stimulated overnight with split virus antigens from A/New Caledonia/20/99, A/Brisbane/59/07 and A/California/07/09 viruses and stained for intracellular cytokines (IFN-γ, IL-2 and TNF-α) and the percentage of single cytokine producing (**A**) or muli-functioal (**B**) CD4 T-cells was measured by flow cytometry. +group (day 21) significantly different by Student *t* test from day 0 ($P < 0.05$).

antigen stimulation over days to produce IFN-γ *in vitro* [38], and this may explain the lack of a pre-vaccination IFN-γ response after overnight stimulation in our ELISPOT and ICS assays.

In conclusion, we have shown that the AS03-adjuvanted A (H1N1)pdm09 vaccine induces both humoral and cellular cross-reactive immune responses in HCW and this may have played a key role in eliciting rapid sero-protective immune responses, which contributed significantly to maintaining the integrity of the health-care system during the pandemic.[4] Our results show that immune responses originally primed by exposure to seasonal strains can be recalled after pandemic vaccination and better understanding of mechanisms that result in cross-reactive immune responses may lead to the development of improved influenza vaccines.

Materials and Methods

Participants and study design

In October 2009, 237 frontline healthcare workers at the Haukeland University Hospital, (Bergen, Norway) were vaccinated with a single dose of the AS03 adjuvanted monovalent split virus vaccine (Pandemrix, GlaxoSmithKline, www.clinicaltrials.gov, NCT01003288).[4] All participants provided written informed consent before being included in the study, which was approved by the Regional Ethical committee of Western Norway and the Norwegian Medicines Agency. The inclusion/exclusion criteria for this study are published elsewhere.[4] Consecutive blood samples were taken from the same subject at 4 time points (days 0, 7, 14 and 21 post-vaccination) for the serological (HI, SRH), ASC, memory B cell and IFN-γ ELISPOT assays. For the intracellular cytokine staining (ICS) for Th1 cytokines, PBMCs were obtained from 8 subjects before vaccination and a separate cohort of 18 subjects on day 21 post-vaccination. On days 7 and 14, serological responses were determined in only 50 subjects as a part of a kinetic sub-study (**Fig. 1**). Serum samples were aliquoted and stored at −70°C before use in the serological assays. PBMCs were isolated from a small group of HCWs (n = 39) using Cell preparation Tubes (CPT, BD Biosciences) according to manufacturer's instructions. Fresh lymphocytes were used in the antibody secreting cell ELISPOT assay and for intracellular

cytokine staining of $CD4^+$ T-cells. Remaining lymphocytes were frozen at −70°C and prioritized for use in the memory B cell ELISPOT followed by the IFN-γ ELISPOT assays.

Antibody assays

The haemagglutinin inhibition (HI) antibody response was analyzed against the homologous A/(H1N1)pdm09-like strain (A/California/07/09) and against 6 seasonal influenza A (H1N1) strains (A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86, A/Texas/36/91, A/New Caledonia/20/99 and A/Brisbane/59/07) (**Fig. S1**). Assays were performed in duplicate and the geometric mean titer (GMT) was calculated. The pre- and post-vaccination, influenza-specific HI antibody response was determined by the HI assay using 8 HA units of each virus strain and 0.7% turkey red blood cells, as described earlier.[4] HI titers were defined as the reciprocal of the dilution exceeding 50% haemagglutination. Negative titers were assigned a value of 5 for calculation purposes.

The single radial hemolysis (SRH) responses against A/Texas/36/91, A/New Caledonia/20/99 and A/California/07/09 strains were conducted at the University of Siena, Italy, as previously described.[39-41]

B cell assays

The virus-specific IgG, IgA and IgM antibody secreting cell (ASC) response against A/California/07/09, A/Texas/36/91 and A/New Caledonia/20/99 split virus antigens was determined pre and post-vaccination by ELISPOT assay using fresh PBMCs as described earlier.[23] The numbers of IgG, IgA and IgM ASCs were evaluated at 7 days post-vaccination, as this has previously been shown to be the peak response after inactivated influenza vaccination.[23]

The virus-specific IgG memory B cell (MBC) response against A/California/07/09, A/Brisbane/59/07, A/Texas/36/91 and A/New Caledonia/20/99 split virus antigens and A/USSR/90/77, A/Brazil/11/1978, A/Taiwan/1/86 whole virus was quantified by ELISPOT as described earlier.[42] Results are presented as virus-specific IgG MBC cells per 1×10^6 PBMCs.

Interferon gamma ELISPOT assay

The influenza-specific IFN-γ response pre and post-vaccination was examined by using 96 well plates pre-coated with anti-IFN-γ antibodies according to the manufacturer's instructions (Mabtech AB, Sweden). PBMCs (4×10^5 cells per well) were added in RPMI-1640 medium supplemented with 10% fetal calf serum with negative control (medium alone) and the split virus influenza H1N1 antigens from; A/New Caledonia/20/99, A/Texas/36/91, A/Brisbane/59/07 and A/California/7/09 (X179a). Plates were incubated overnight (37°C, 5% CO_2) and developed the following day. The plates were read using an Immunoscan™ reader and associated software (CTL-Europe). The negative control was subtracted from the influenza-specific response.

Intracellular cytokine staining (ICS) for multi-functional $CD4^+$ T cell responses

PBMCs from vaccinated subjects were stimulated overnight with A/California/7/09 (X179a), A/New Caledonia/20/99 and A/Brisbane/59/07 split virus antigens and the cells were stained for intracellular Th1 cytokines IFN-γ, IL-2 and TNF-α and the percentage of single, or multiple cytokine producing $CD4^+$ T-cells were analyzed by flow cytometry as described earlier.[43]

Statistical assays

Differences in the IFN-γ, ASC and MBC ELISPOT responses were analyzed by non-parametric Kruskal-Wallis test. Correlations between pre-vaccination MBC responses against the A(H1N1)pdm09 virus and seasonal influenza strains were determined by Spearman correlation coefficient analysis. The Kruskal-Wallis and Spearman correlation analysis were performed by GraphPad Prism version 6.00 for Mac (GraphPad software, La Jolla, CA, USA). Differences between intracellular cytokine responses at days 0 and 21 were determined by the student *t* test and a partial permutation test by using SPICE version 5.1 software, as described earlier.[44] $P < 0.05$ was considered as significant for all statistical tests.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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II

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ARTICLE OPEN

Augmented CD4$^+$ T-cell and humoral responses after repeated annual influenza vaccination with the same vaccine component A/H1N1pdm09 over 5 years

Mai-Chi Trieu[1,2], Fan Zhou[1,2], Sarah Larteley Lartey[1,2], Saranya Sridhar[3,6], Siri Mjaaland[2,4] and Rebecca Jane Cox[1,2,5]

Annual seasonal influenza vaccination is recommended for high-risk populations and often occupational groups such as healthcare workers (HCWs). Repeated annual vaccination has been reported to either have no impact or reduce antibody responses or protection. However, whether repeated vaccination influences T-cell responses has not been sufficiently studied, despite the increasing evidence of the protective roles of T-cell immunity. Here, we explored the impact of repeated annual vaccination with the same vaccine strain (H1N1pdm09) over multiple seasons in the post-2009 pandemic era and showed that repeated vaccination increased both T-cell and humoral responses. Using the T-cell FluroSpot and intracellular cytokine-staining, the hemagglutination inhibition (HI), and the memory B-cell (MBC) ELISpot assays, we investigated pre- and postvaccination T cells, antibodies, and MBCs in a cohort of HCWs repeatedly vaccinated with H1N1pdm09 for 5 years (pandemic vaccination in 2009 and subsequently annual seasonal vaccination containing H1N1pdm09 during 2010–2013). We found that the prevaccination H1N1pdm09-specific T cells, antibodies, and MBCs were significantly increased after 3–4 repeated vaccinations and maintained at high levels throughout seasons 2012 and 2013. The cross-reactive IFN-γ-secreting CD4$^+$ cells recognizing conserved viral external or internal epitopes were also maintained throughout 2012 and 2013. Repeated vaccination improved the multifunctional memory CD4$^+$ responses. Particularly, the IFN-γ$^+$TNF-α$^+$CD4$^+$ T cells were boosted following each vaccination. HI antibodies were significantly induced after each vaccination over 5 years. Our findings indicate a broad impact of repeated annual vaccination, even with the same vaccine component, on the influenza-specific T-cell and humoral immunity and support the continuing recommendation of annual influenza vaccination.

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INTRODUCTION

Influenza virus remains a major health challenge due to its continuous ability to evade the hosts' immunity. Annual seasonal influenza vaccination is the main method of prophylaxis for high-risk populations and healthcare workers (HCWs) providing protection against influenza A/H1N1, A/H3N2, and B viruses.[1] In 2009, a novel H1N1 virus (H1N1pdm09) emerged and caused the first pandemic of the twenty-first century. HCWs were prioritized for pandemic vaccination to protect their patients and themselves, and maintain the integrity of the healthcare system.[2] The AS03-adjuvanted monovalent H1N1pdm09 vaccine was used during the pandemic in Norway and provided protection against laboratory-confirmed influenza infection and hospitalization.[3] The H1N1pdm09 virus continued to circulate after 2009 replacing earlier H1N1 strains and was therefore included in the seasonal vaccines as the A/H1N1 component during seasons 2010−2016.

Antibodies directed against the main viral surface glycoprotein, hemagglutinin (HA), can neutralize the influenza virus. The hemagglutination inhibition (HI) assay has been widely used to evaluate the HA-specific antibody responses. An HI titer of 40 is established as a surrogate correlate of protection against influenza at a 50% protective threshold.[4] Inactivated influenza vaccines are standardized by the quantity of HA of each strain and induce HI antibodies after vaccination. Moreover, T cells have recently gained more recognition for their protective roles. Preexisting influenza-specific interferon (IFN)-γ-secreting CD4$^+$ or CD8$^+$ T cells can recognize conserved viral epitopes and provide cross-protection from heterosubtypic influenza A viruses, even in the absence of protective antibodies.[5–8]

Importantly, influenza vaccines have been used for decades; however, the long-term impact of repeated annual vaccination on antibody responses is not fully understood[9–11] and there are limitations of our knowledge of its impact on T-cell responses. The emergence of the H1N1pdm09 virus and its inclusion as the A/H1N1 component in the seasonal vaccines for multiple years provided a unique opportunity to investigate the impact of repeated vaccination. Previously, we investigated the impact of repeated annual vaccination upon preexisting influenza-specific CD8$^+$ and CD4$^+$ T cells prior to two consecutive influenza seasons in HCWs who were either repeatedly vaccinated or only received a pandemic vaccination.[12] In the current study, we further explored the impact of annual vaccination on T cells, particularly

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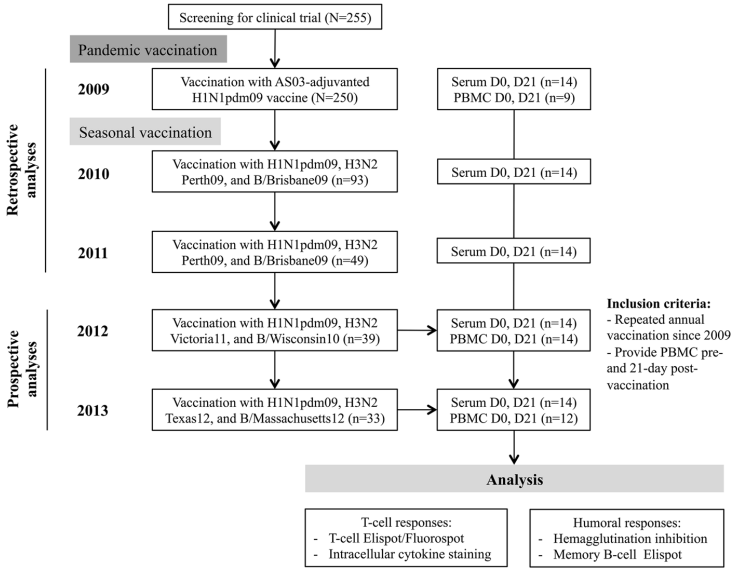

Fig. 1 The study flow chart. Healthcare workers (HCWs) were vaccinated with a single dose of the AS03-adjuvanted monovalent pandemic H1N1pdm09 vaccine (European Clinical Trials Database, EudraCT 2009-016456-43; www.clinicaltrials.gov, NCT01003288). During 2010–2013 seasons, HCWs were vaccinated every year with the trivalent seasonal inactivated vaccines, containing the H1N1pdm09 virus as the A/H1N1 component during the whole study period. The A/H3N2 and B strains included in the seasonal vaccines changed between seasons during 2010–2013. The figure shows the inclusion criteria for this study, which were repeated annual vaccination between 2010 and 2013, and provision of peripheral blood monocular cells (PBMC) prevaccination (D0) and 21 days postvaccination (D21) in 2012 and 2013 seasons

$CD4^+$ T cells, and humoral immunity by assessing paired pre- and postvaccination T cell, antibody, and memory B-cell (MBC) responses in repeatedly vaccinated HCWs over 5 years. We have extended our previous findings to show that repeated annual vaccination with the same strain augmented both humoral and CD4 T-cell responses, maintained the cross-reactive IFN-γ-secreting $CD4^+$ T cells recognizing viral external and internal epitopes, while increasing multifunctional memory $CD4^+$ responses. Our findings have implications for the seasonal influenza vaccination strategy and vaccine development.

RESULTS

Study population

Fourteen HCWs (mean age 41.2 years old, range 30–63 years), who received the AS03-adjuvanted pandemic vaccine in 2009 and subsequently annual seasonal vaccination during seasons 2010–2013, were included in this study (Fig. 1). Most HCWs (12/14) were female, worked on a clinical ward, and had history of previous seasonal vaccination before 2009 (Supplementary-Table 1).

Maintenance of H1N1pdm09-specific IFN-γ-secreting T cells

The magnitude of preexisting and vaccine-induced H1N1pdm09-specific T cells was assessed before and after seasonal vaccination in 2012 and 2013 by measuring the IFN-γ-, interleukin(IL)-2-, or double-cytokine IFN-γIL-2 secretion against the split H1N1pdm09 virus and the H1N1-specific internal protein peptide pools: matrix 1 (M1), nucleoprotein (NP), and polymerase-basic 1 (PB1).

All cytokine-secreting T cells against the split virus were maintained at high levels throughout the two seasons, although no significant boost of these responses was observed after vaccination (Fig. 2a). The IFN-γ-secreting T cells recognizing the three internal proteins were maintained throughout 2012 and 2013 (Fig. 2c, e, g). However, we observed a decline in IL-2-secreting T cells recognizing M1 and NP after 1 year and a low but stable level of double-cytokine-secreting cells, leading to an increased IFN-γ/IL-2 ratio over time (Fig. 2d, f). Whereas IFN-γ dominated the responses against PB1, as there was almost no IL-2 or double-cytokine secretion (Fig. 2g, h). The dominant IFN-γ trend was not observed in the H1N1pdm09-specific T cells against the split virus (containing mainly surface glycoproteins), which had a balanced IFN-γ/IL-2 response (Fig. 2b).

Maintenance of cross-reactive IFN-γ-secreting $CD4^+$ T cells

The influenza-specific cross-reactive $CD4^+$ T-cell responses were investigated using separate CD4 external or internal peptide pools. We found that the cross-reactive IFN-γ- or IL-2-secreting $CD4^+$ T cells against external epitopes were maintained, while there was a trend of increased double-cytokine responses after vaccination ($p = 0.094$) (Fig. 3a). The cross-reactive IFN-γ- or double-cytokine-secreting $CD4^+$ T cells against internal epitopes were maintained throughout the two seasons, whereas the IL-2-secreting cells declined after vaccination in 2012 ($p = 0.088$) and further in 2013 ($p = 0.003$) (Fig. 3e), resulting in the shift toward IFN-γ dominance over time (Fig. 3f). However, the trend of predominant IFN-γ was not observed in the cross-reactive responses to external epitopes (Fig. 3b).

To further examine this trend, T-cell responses were assessed in a control group of HCWs who were only vaccinated with the AS03-adjuvanted H1N1pdm09 vaccine in 2009 and received no further seasonal vaccinations. We have previously reported a decline in all cytokine-secreting cross-reactive $CD4^+$ T cells against internal epitopes and H1N1pdm09-specific T cells after a year in these HCWs (Fig. 3g and Supplementary-Fig. 1a).[12] We assessed the $CD4^+$ T cells against external epitopes and observed a decline in

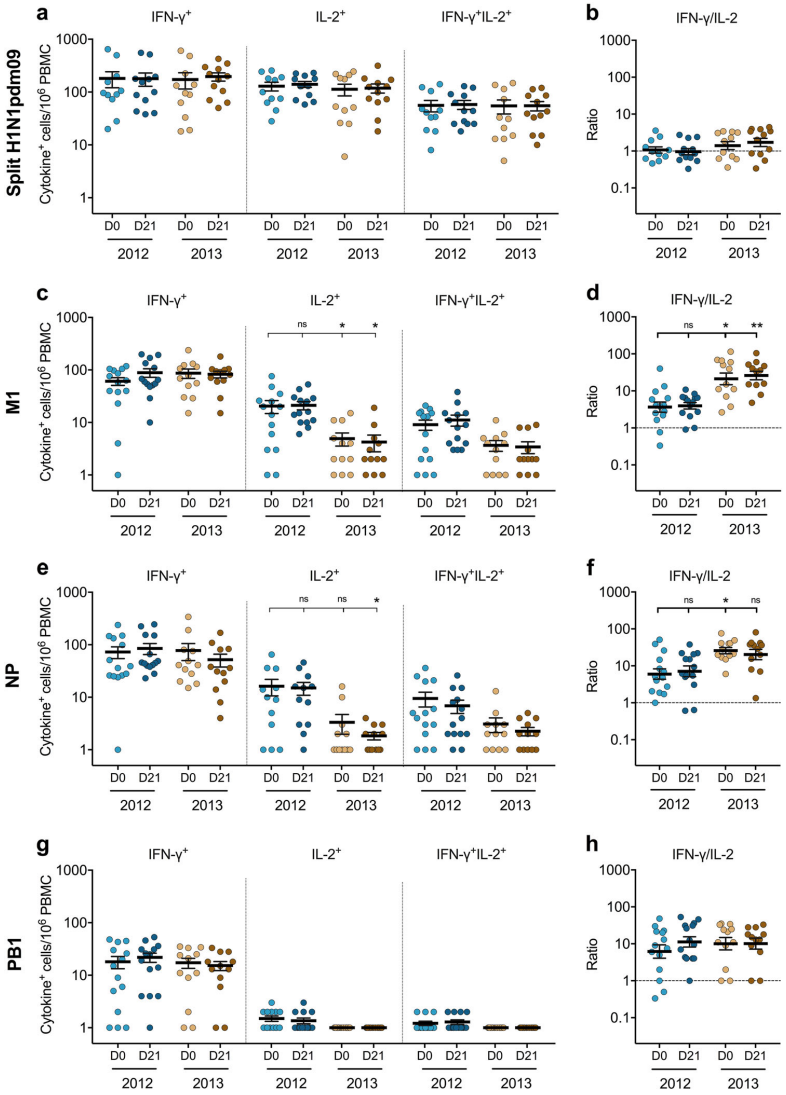

Fig. 2 The magnitude of H1N1pdm09-specific T-cell responses during seasons 2012 and 2013. The single IFN-γ- or IL-2-, or double-cytokine IFN-γIL-2-secreting T cells against **a** the split H1N1pdm09 virus, the H1N1-specific viral internal proteins: **c** M1, **e** NP, or **g** PB1 prevaccination (D0) (light) and at 21 days postvaccination (D21) (dark) were measured in the T-cell FluoroSpot assay. Each symbol represents one individual's response with the horizontal lines representing the mean magnitudes of cytokine-secreting cells per 10^6 PBMC with standard error of the mean (s.e.m.). The IFN-γ to IL-2 ratio was calculated for each participant at each time point and the mean ratios with s.e.m. are shown against **b** the split H1N1pdm09 virus, the viral internal proteins: **d** M1, **f** NP, or **h** PB1. The dotted line indicates the IFN-γ/IL-2 ratio of 1 showing a balanced IFN-γ and IL-2 response. An IFN-γ/IL-2 ratio above or below 1 shows a predominant IFN-γ or IL-2 response, respectively. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$

double-cytokine secretion after a year, although not significant (Fig. 3c). A balanced IFN-γ/IL-2 response against the CD4 external epitopes and split H1N1pdm09 virus was found in this control group (Fig. 3d and Supplementary-Fig. 1b). However, no trend of increasing IFN-γ/IL-2 ratio in the $CD4^+$ T cells specific for viral internal epitopes was observed, supporting the impact of repeated vaccination in favoring IFN-γ responses (Fig. 3h).

Vaccine-induced boost of multifunctional IFN-γ^+ and memory $CD4^+$ T cells

Next, we explored the quality of H1N1pdm09-specific $CD4^+$ T cells before and after vaccination in 2012 and 2013 by assessing the cytokine profile and memory subsets against the split H1Npdm09 antigen in the intracellular cytokine-staining assay ($n = 5$). The cytokine profile included seven cytokine-secreting subsets: triple

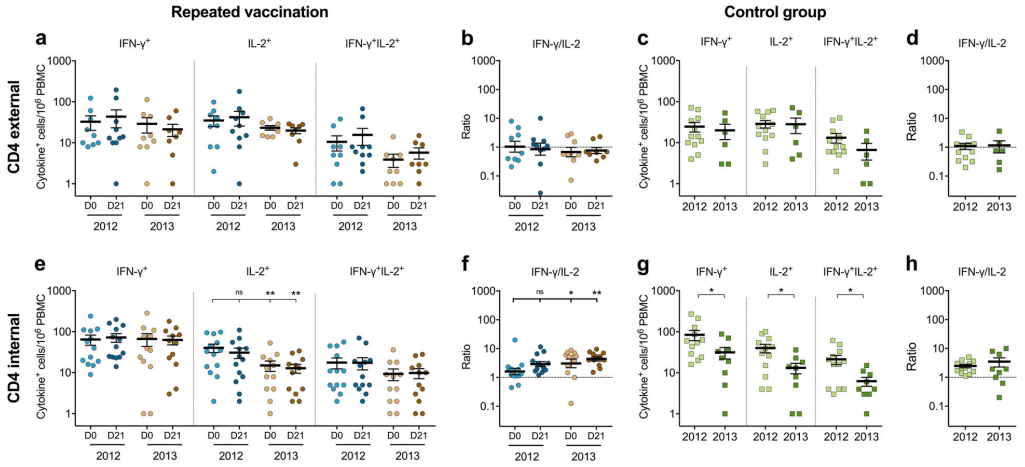

Fig. 3 The magnitude of cross-reactive CD4$^+$ T-cell responses during seasons 2012 and 2013. The single IFN-γ- or IL-2-, or double-cytokine IFN-γIL-2-secreting T cells against the CD4-specific conserved epitopes from viral external/internal proteins were measured in the T-cell FluoroSpot assay **a** and **e** prevaccination (D0) (light) and at 21 days postvaccination (D21) (dark) in HCWs with annual repeated vaccination (round) or **c** and **g** prior to the influenza season in 2012 (light) and 2013 (dark) in a control group of HCWs who were only vaccinated with the AS03-adjuvanted pandemic H1N1pdm09 vaccine in 2009 (square).[12] Each symbol represents one individual's response with the horizontal lines representing the mean magnitudes of cytokine-secreting cells per 10^6 PBMC with standard error of the mean (s.e.m.). The IFN-γ to IL-2 ratio was calculated for each participant at each time point and the mean ratios with s.e.m. are shown against the CD4-specific external/internal epitopes **b** and **f** in HCWs with repeated vaccination or **d** and **h** a control group. The dotted line indicates the IFN-γ to IL-2 ratio of 1 showing a balanced IFN-γ and IL-2 response. An IFN-γ to IL-2 ratio above or below 1 shows a predominant IFN-γ or IL-2 response, respectively. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$

producer (IFN-γ$^+$IL-2$^+$ tumor-necrosis-factor (TNF)-α$^+$), double producers (IFN-γ$^+$IL-2$^+$, IFN-γ$^+$TNF-α$^+$, IL-2$^+$TNF-α$^+$), and single producers (IFN-γ$^+$, IL-2$^+$, TNF-α$^+$). Higher frequencies of double producers were found after vaccination in 2012 ($p = 0.031$) and 2013 ($p = 0.173$) (Fig. 4a). Detailed analysis of double producers showed that the frequencies of IFN-γ$^+$TNF-α$^+$CD4$^+$ T cells were significantly boosted after each seasonal vaccination in 2012 ($p = 0.021$) and 2013 ($p = 0.046$) (Fig. 4b). The frequencies of prevaccination IL-2$^+$TNF-α$^+$CD4$^+$ cells were higher in 2013 than 2012, although not significant ($p = 0.08$).

We assessed the CD45RA$^-$CCR7$^+$central memory (CM) and CD45RA$^-$CCR7$^-$effector memory (EM) CD4$^+$ T-cell subsets secreting IFN-γ or IL-2. We found that the long-lived CD4$^+$ CM T cells secreting IFN-γ ($p = 0.042$) or IL-2 ($p = 0.038$) were boosted after vaccination in 2012, waned throughout the year although persisting at comparatively higher levels than prevaccination (Fig. 4c). Following 2013 vaccination, these responses were boosted, but not significantly. The IFN-γ$^+$ or IL-2$^+$CD4$^+$ EM T cells remained stable throughout the two consecutive seasons (Fig. 4e). Interestingly, higher frequencies of double producers IFN-γ$^+$TNF-α$^+$CD4$^+$ CM ($p = 0.009$) and EM ($p = 0.030$) T cells were observed after vaccination in 2012, albeit not significant in 2013 ($p = 0.223$ and 0.079, respectively) (Fig. 4d, f).

Enhanced quantity and quality of T cells after repeated vaccinations

We further investigated the long-term impact of repeated vaccination on T cells during the 5 study-years by retrospectively analyzing the H1N1pdm09-specific T-cell responses after pandemic vaccination in 2009[13] and comparing to the response after seasonal vaccination in 2012 and 2013. Pre- and post-2009 pandemic vaccination data from nine HCWs who were subsequently annually vaccinated during 2010–2013 were included, of which four HCWs had repeated paired pre- and postvaccination data. The magnitude of prevaccination H1N1pdm09-specific IFN-γ-secreting T cells was significantly higher in 2012 ($p = 0.016$) and 2013 ($p = 0.025$) compared to pre-pandemic vaccination in 2009, although no significant boost was observed at 21 days post-vaccination in these seasons (Fig. 5a, Supplementary-Fig. 2a).

By comparing cytokine profiles across these three seasons, we found a significant increase in multifunctional CD4$^+$ T cells (secreting 2−3 cytokines) after repeated vaccinations (Fig. 5b, Supplementary-Fig. 2b), indicating an enhanced T-cell quality over the 5 years.[14,15] The frequencies of triple producer ($p = 0.008$), and double producers IFN-γ$^+$TNF-α$^+$ ($p = 0.018$) and IL-2$^+$TNF-α$^+$ ($p = 0.034$) CD4$^+$ T cells prevaccination were significantly higher in 2013 than in 2009. Interestingly, the IFN-γ$^+$TNF-α$^+$ and IL-2$^+$TNF-α$^+$CD4$^+$ responses were boosted following 2009 vaccination ($p = 0.031$). In contrast, the frequencies of single IL-2-secreting T cells in the last two seasons were significantly lower than in 2009 ($p = 0.008$). The prevaccination cytokine-profile radar chart (Fig. 5c) shows that repeated vaccinations skewed the CD4$^+$ T-cell responses toward IFN-γ dominance and the IL-2 responses were shifted from single to multifunctional IL-2 (double IL-2$^+$TNF-α$^+$ or triple IFN-γ$^+$IL-2$^+$TNF-α$^+$ co-producers).

Maintenance of high humoral responses after repeated vaccinations

Throughout the 5 years of our study, the H1N1pdm09-specific HI antibodies were significantly boosted after each vaccination (Fig. 6a). After the second vaccination, HI antibodies persisted above the 50% protective threshold (HI titers ≥ 40) for 1-year postvaccination in all HCWs, except one who had chronic respiratory and neurological conditions. The prevaccination HI titers gradually increased from 2009 to 2012, then were maintained between 2012 and 2013. The antibody fold-induction between pre- and postvaccination titers was highest after the adjuvanted pandemic vaccination in 2009 then declined after multiple vaccinations (Fig. 6b). The fold-induction after seasonal vaccination in 2012 and 2013 was significantly lower than in earlier seasons.

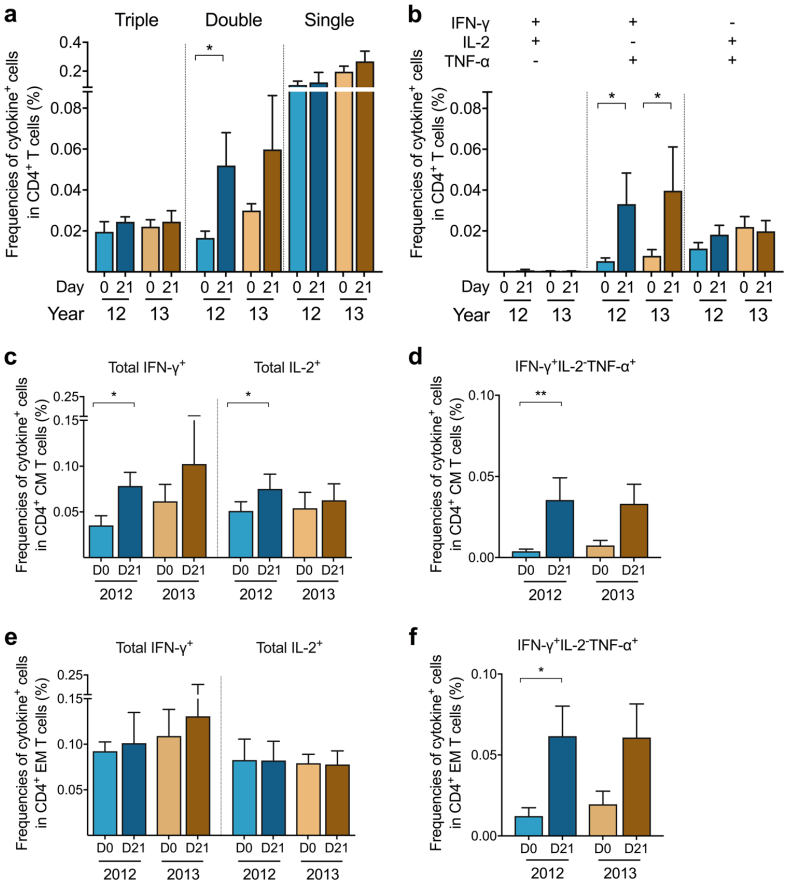

Fig. 4 The quality of $CD4^+$ T-cell responses during seasons 2012 and 2013. The cytokine profile of H1N1pdm09-specific $CD4^+$ T cells was assessed by IFN-γ/IL-2/TNF-α intracellular cytokine-staining (ICS) assay prevaccination (D0) (light) and at 21 days postvaccination (D21) (dark) in the 2012 and 2013 seasons. **a** The mean frequencies (%) of the triple producer (IFN-γ^+IL-2^+TNF-α^+), double producers (IFN-γ^+IL-2^+, IFN-γ^+TNF-α^+, IL-2^+TNF-α^+), and single producers (IFN-γ^+, IL-2^+, TNF-α^+) with standard error of the mean (s.e.m.) are shown as bars. **b** The mean frequencies with s.e.m. are presented for each of the three double-producer subsets. The memory $CD4^+$ T-cell responses were assessed by differentiating the three memory subsets $CD45RA^-CCR7^+$ central memory (CM), $CD45RA^-CCR7^-$effector memory (EM), and $CD45RA^+CCR7^-$ late effector memory (EMRA), from $CD45RA^+CCR7^+$ naïve T cells within the $CD4^+$ population, then IFN-γ^+ or IL-2^+ cells were gated within $CD4^+$ CM or EM population (see gating strategy in Supplementary-Fig. 4). The mean frequencies with s.e.m. of the total IFN-γ- or IL-2-secreting $CD4^+$ **c** CM or **e** EM T cells pre- and postvaccination in seasons 2012 and 2013 are shown. The mean frequencies with s.e.m. are presented for the IFN-γ^+IL-2^-TNF-α^+ subset of the $CD4^+$ **d** CM or **f** EM T cells. *$p < 0.05$, **$p < 0.01$

We further assessed the H1N1pdm09-specific MBC responses in HCWs pre- and postvaccination in 2012 and 2013 ($n = 5$ due to limited PBMC availability), while the 2009 data were retrospectively analyzed from nine HCWs.[13] The prevaccination H1N1pdm09-specific MBCs was significantly higher in 2012 and 2013 than in 2009 (Fig. 6c). Notably, MBCs were significantly boosted following pandemic vaccination in 2009 ($p = 0.031$), but not after seasonal vaccination in 2012 and 2013. The fold-induction after vaccination was higher in 2009 than in 2013 ($p = 0.061$) (Fig. 6d).

We investigated the overall impact of repeated vaccination against H1N1pdm09 on immune responses using radar charts. An increase in both humoral and T-cell immunity specific for H1N1pdm09 prevaccination were observed after 3−4 vaccinations (Fig. 6e). Interestingly, MBCs and double producers $CD4^+$ T cells continue to increase from 2012 to 2013. The fold-induction of the vaccine-induced responses postvaccination was highest after adjuvanted pandemic vaccination in 2009 (Fig. 6f).

DISCUSSION

There is limited knowledge about the long-term impact of repeated annual influenza vaccination on the influenza-specific T-cell immunity despite decades of use of influenza vaccine. Here, we provide unique data on humoral and T-cell responses after repeated annual vaccination with the same H1N1pdm09 strain from its introduction in 2009 and over four subsequent seasons.

Repeated vaccinations resulted in an increase in both the quantity and the quality of H1N1pdm09-specific T-cell responses. One study suggested that >20 IFN-γ-secreting T cells/10^6 PBMC as detectable protective influenza-specific T cells at a population level.[8] In our repeatedly vaccinated HCW cohort, the

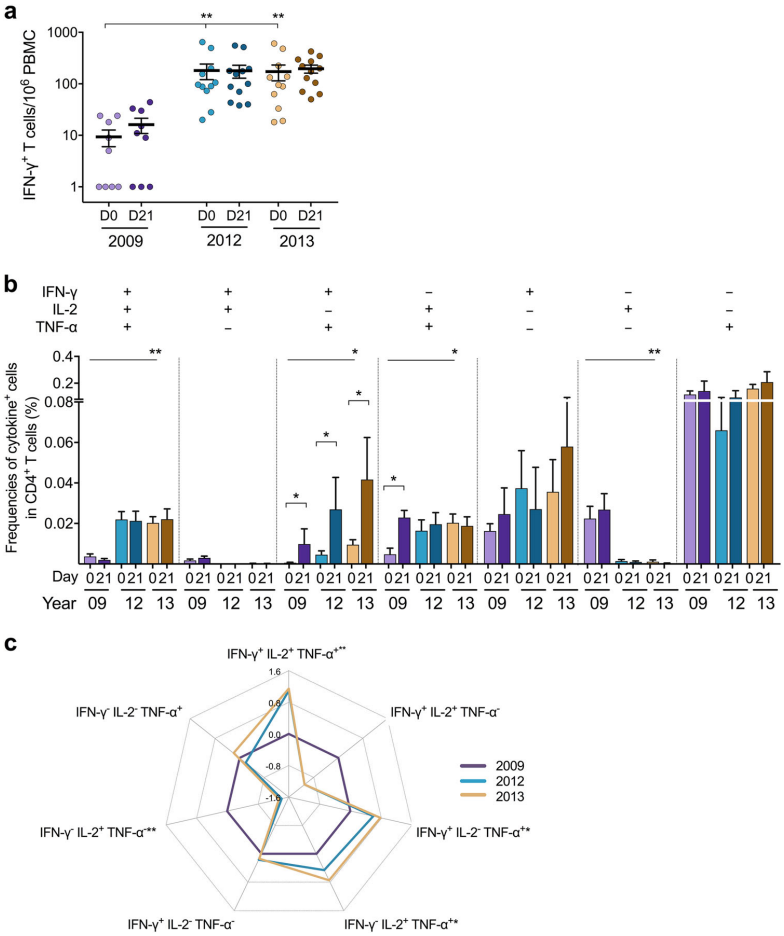

Fig. 5 The impact of repeated annual vaccinations on the magnitude and the quality of H1N1pdm09-specific T cells over 5 years. **a** The magnitude of H1N1pdm09-specific IFN-γ-secreting T cells prevaccination (D0) (light) and at 21 days postvaccination (D21) (dark) were enumerated in the IFN-γ T-cell ELISpot assay in 2009 ($n = 9$), and the IFN-γ/IL-2 FluoroSpot assay in 2012 and 2013. Each symbol represents one individual's response with the horizontal lines representing the mean magnitudes of IFN-γ-secreting cells per 10^6 PBMC with standard error (s.e.m.). **b** The cytokine profile of H1N1pdm09-specific $CD4^+$ T cells was assessed by the IFN-γ/IL-2/TNF-α intracellular cytokine-staining (ICS) assay pre- and postvaccination in 2009, 2012, and 2013. The mean frequencies (%) with s.e.m. of each of the seven cytokine-combination subsets are shown as bars. **c** Changes in the prevaccination cytokine profile of $CD4^+$ T cells after 3–4 years of repeated vaccination. The fold-changes of responses between 2009 (reference) and 2012 or 2013 were calculated for each participant and the log-transformed means of fold-changes are presented in a radar chart. Value above or below 0 represents an increase or decline of response, respectively. *$p < 0.05$, **$p < 0.01$

prevaccination IFN-γ-secreting T cells increased significantly from non-detectable (5 cells/10^6 PBMC) to persistently high numbers (95 cells/10^6 PBMC) after 3−4 vaccinations, although we do not have T-cell data from 2010 and 2011 to confirm whether these cells increased continuously after each year of repeated vaccination until 2012 and 2013, similarly to the antibody responses. Whereas in the absence of repeated vaccination, a decline in H1N1pdm09-specific cytokine-secreting T cells from 2012 to 2013 was observed in single vaccinated individuals (Supplementary-Fig. 1a).[12] Moreover, the quality of $CD4^+$ T cells was improved with higher multi-cytokine-secreting responses, associated with superior function compared to single-cytokine producers.[14,15] The boost of IFN-γ^+TNF-α^+CD4^+ responses after each vaccination in 2009, 2012, and 2013 suggests a continuous increase of these responses. We hypothesize that the magnitude of T cells may reach a plateau after 3−4 repeated vaccinations, like the antibodies, while the quality of T cells continues to improve. Although this study did not assess the quality of antibodies, our earlier report showed that repeated vaccination maintained the antibody avidity.[16] This implies that repeated vaccination may have a broader impact on immune responses, which are not usually assessed in conventional vaccine immunogenicity studies.[17] Future studies should therefore include diverse immunological assessments to better understand vaccine immunogenicity in populations with different influenza exposure backgrounds.

We demonstrated that the H1N1pdm09-specific HI antibodies were boosted after each vaccination during the 5 study-years. The prevaccination antibodies increased each year until 2012 and

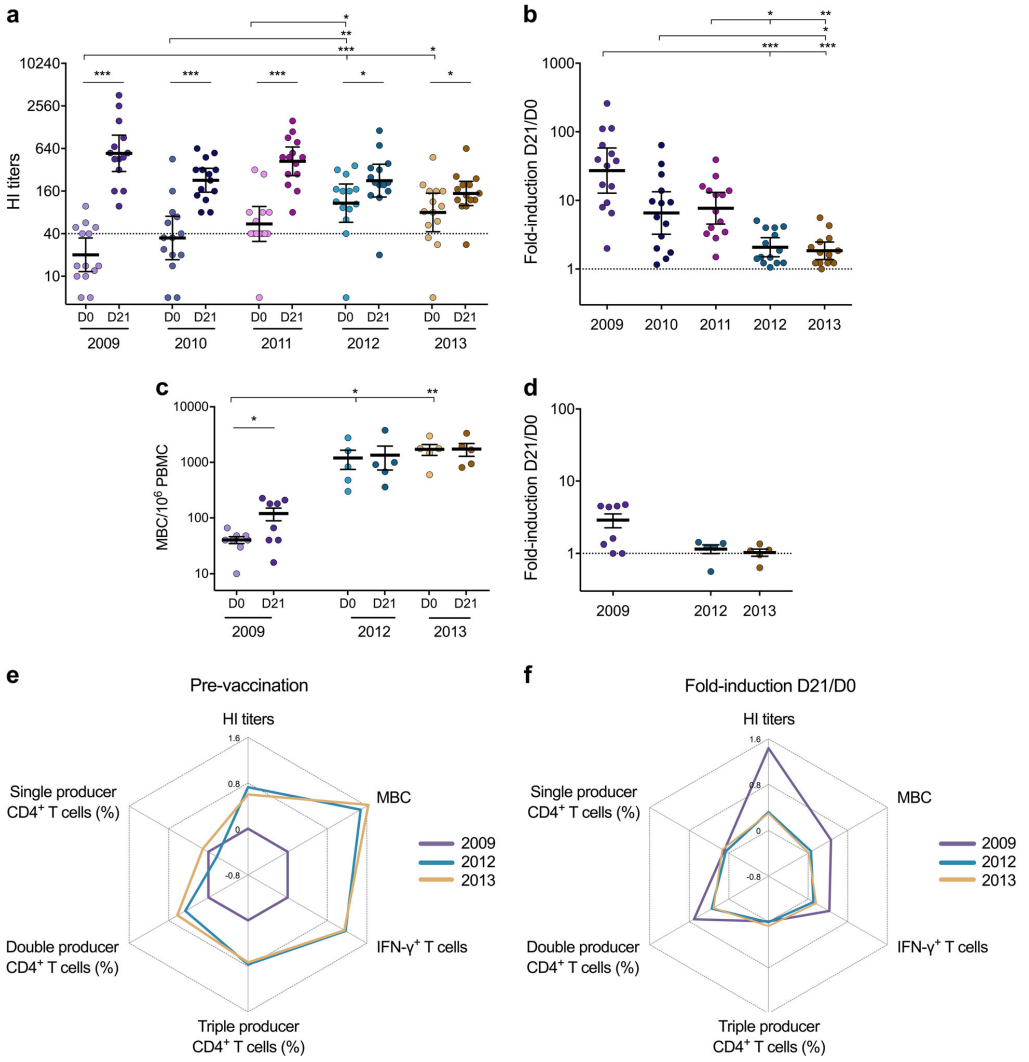

Fig. 6 The overall impact of repeated annual vaccinations on T-cell and humoral responses. **a** The 5-year H1N1pdm09-specific antibody responses following pandemic and repeated seasonal vaccinations were measured in the hemagglutination inhibition (HI) assay. Each symbol represents one individual's response with the horizontal lines representing the geometric mean HI titer with 95% confidence interval (CI) prevaccination (D0) (light) and at 21 days postvaccination (D21) (dark) each year. The dotted line indicates the 50% protective threshold defined as HI titer ≥ 40. **b** The HI antibody fold-induction between pre- and postvaccination (D21/D0) titers was calculated for each participant at each time point. The horizontal lines represent the geometric mean HI antibody fold-induction with 95% CI. **c** The H1N1pdm09-specific memory B-cell (MBC) responses pre- and postvaccination in seasons 2009, 2012, and 2013 were measured in the MBC ELISpot assay. The horizontal lines represent the mean magnitudes of the H1N1pdm09-specific MBCs per 10^6 PBMC with standard error of the mean (s.e.m.). **d** The MBC fold-induction between pre- and postvaccination (D21/D0) was calculated for each participant at each time point. The horizontal lines represent the mean MBC fold-induction with s.e.m. **e** The fold-changes of prevaccination antibodies, MBCs, and T cells between 2009[13] and 2012 or 2013 were calculated for each participant and the log-transformed means of fold-changes are presented in a radar chart. Value above or below 0 represents an increase or decline of response, respectively. **f** The log-transformed mean fold-induction of T-cell and humoral responses between pre- and postvaccination (D21/D0) each year (2009, 2012, and 2013) is presented in a radar chart. The higher the value is above 0, the higher the vaccine-induced response is after vaccination. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

2013 persisting above the 50% protective threshold, whereas the antibody fold-induction postvaccination declined and was lowest in the last two seasons compared to previous seasons. This suggests that repeated vaccinations sustain high protective antibody levels rather than boosting them. The long-term antibody persistence is probably due to activation of MBCs that can undergo continuous proliferation and differentiation to maintain constant levels of plasma cells and antibodies.[18] T cells

also contribute to induction and maintenance of antibodies by providing help to activate naïve and MBCs.[19] This hypothesis was supported by the significantly higher H1N1pdm09-specific MBCs and T cells observed after 3−4 repeated vaccinations. Moreover, the decline in antibody fold-induction observed in later seasons may be due to the presence of high prevaccination HI antibodies, which may block the vaccine epitopes, resulting in a reduction of B- and T-cell activation,[20–22] and therefore limit the boosting ability of subsequent vaccinations.

The controversy of annual influenza vaccination policy involves conflicting reports on the impact of repeated vaccination, which reported either no significant interference or a reduction in antibody responses and vaccine effectiveness (VE).[9–11,23,24] In studies that found that repeated vaccination led to decreased protection, this was mainly related to the H3N2 viruses. The H3N2 subtype has been associated with lower VE and undergoes more frequent antigenic changes compared to the H1N1 and B viruses included in the seasonal vaccines.[25] A recent study investigating the impact of repeated vaccination against H1N1pdm09 on VE suggests that VE was highest after 2−3 vaccinations and reduced in vaccinees immunized with >3 vaccinations.[11] Our study did not find a reduction in H1N1-specific immune responses after 3−4 vaccinations, although protection was not assessed. Our findings show the maintenance of H1N1pdm09-specific antibodies, MBCs and T cells at high levels with improved quality of $CD4^+$ T cells after 3−4 repeated vaccinations. However, enhanced T-cell responses do not prevent infection, the outcome in most studies investigating protection use, but reduce the severity of illness.[5–8] We suggest that broader outcome measures of protection, such as severity scores or time to recover, should be incorporated into future studies together with various immunological assessments to better evaluate the impact of repeated vaccination. Taken together, we support the continuation of the current recommendation of annual influenza vaccination, even with the same vaccine component, to provide protection against all circulating seasonal strains. Our findings point to the importance of inclusion of influenza vaccination history, when evaluating vaccine immunogenicity and effectiveness.

Remarkably, the long-lived H1N1pdm09-specific $CD4^+$ CM T cells were boosted after vaccination. Since CM cells can rapidly proliferate and differentiate into effector cells upon antigen encounter,[26] the persistent $CD4^+$ CM and EM responses following repeated vaccination may provide long-lasting protection. These findings agree with our previous report[12] and provide an explanation for the long-term maintenance of IFN-γ^+ and multifunctional $CD4^+$ T cells in repeatedly vaccinated HCWs, which was not observed in HCWs immunized with only pandemic vaccination. Our findings suggest that repeated vaccination optimizes the quantitative responses while shifting the influenza-specific immunity towards long-term memory and multifunctional responses. However, this hypothesis needs to be verified with the other influenza vaccine strains, H3N2 and B viruses, which change more frequently than H1N1 viruses.

Influenza-specific $CD4^+$ T cells can recognize conserved epitopes from heterosubtypic influenza A viruses and provide cross-protection[5] and are boosted after a single vaccination.[13,27,28] We extended these findings by separately investigating the cross-reactive $CD4^+$ responses to conserved viral external or internal epitopes following repeated vaccination, as these cells may be phenotypically different with distinct potential functions.[29–32] After 3−4 repeated vaccinations, a decline in IL-2 responses resulting in a trend of increased IFN-γ dominance against viral internal epitopes was observed, which agrees with previous reports.[33,34] Long-term exposure to influenza through vaccination or infection may shape the T-cell responses towards conserved epitopes that are repeatedly recognized by influenza-specific memory T cells. We hypothesize that these responses are multifunctional with predominantly IFN-γ and a low level of IL-2. Interestingly, analyses of the quality of H1N1pdm09-specific $CD4^+$ T cells after repeated vaccinations provided a potential explanation supporting this hypothesis. We found the boosting of IFN-γ^+TNF-α^+CD4^+ T-cell responses following each vaccination, the increased multi-cytokine-secreting and decreased single IL-2-secreting cells after 3−4 repeated vaccinations. The ratio of IFN-γ to IL-2-secreting cells, calculated using the cytokine profile data showed that the H1N1pdm09-specific $CD4^+$ T cells remained IL-2 enriched in 2009 but changed toward predominantly IFN-γ secretion in 2012 and 2013 (Supplementary-Fig. 3). However, whether this IFN-γ dominance in cross-reactive $CD4^+$ T cells indicates a greater potential for help and/or other functions will need to elucidate in further studies.

We faced difficulties due to the long follow-up period, such as losing participants and missing samples. The strict inclusion criteria that only HCWs repeatedly vaccinated for 5 years greatly limited the sample size of our study. However, this design allows us to study the long-term impact of repeated vaccinations on immune responses while clarifying the effect of vaccination sequence without the bias of missing vaccination history. As HCWs who were repeatedly vaccinated for 5 years were identified in the last two seasons, the T-cell responses in 2009 were retrospectively evaluated. Although the low number of participants with accessible data and the lack of 2010 and 2011 information on T cells limited our observation, the immune responses after repeated vaccinations were undoubtedly enhanced. As memory T-cell responses are rapidly generated, we cannot dismiss the possibility of T-cell expansion at 7–14 days,[13] time points that were not investigated in this study. Further studies are required to confirm our findings in larger populations with documented vaccination history, and to evaluate both antibody and T-cell responses against other vaccine strains after repeated annual vaccination.

In conclusion, we provide a unique overview of the long-term impact of repeated annual influenza vaccination against the same vaccine strain on humoral and T-cell immunity. Repeated vaccinations with H1N1pdm09 not only maintained the high magnitude of strain-specific HI antibodies and T cells, and cross-reactive IFN-γ^+CD4^+ T cells, but also increased MBC and multifunctional $CD4^+$ T-cell responses. This study highlights a broad immunological impact of repeated vaccination and supports the current recommendation of annual seasonal influenza vaccination. Our findings suggest that routine collection of influenza vaccination history and diverse immunological approaches should be included when evaluating vaccine immunogenicity and effectiveness.

METHODS

Study population and sampling

An open-label 5-year extension of a single-arm clinical trial was conducted in HCWs (Haukeland University Hospital, Norway) vaccinated with the 2009 AS03-adjuvanted pandemic H1N1pdm09 vaccine (www.Clinicaltrials.gov, NCT01003288). The study was approved by the regional ethics committee (REKVest-2012/1772) and the Norwegian Medicines Agency.[35] All participants provided written informed consent before inclusion and new consent for the follow-up blood samples. During 2010–2013, HCWs were annually vaccinated with the non-adjuvanted seasonal trivalent inactivated vaccine (Vaxigrip, Sanofi Pasteur or Influvac, Abbott Laboratories) containing the H1N1pdm09 as the A/H1N1 component and different A/H3N2 and B viruses (Fig. 1). Serum samples were collected pre- and 21 days postvaccination for each season from 2009 to 2013. HCWs who provided additional PBMC samples pre- and 21-day postvaccination in 2012 and 2013 were included in this study.

Sera were separated from clotted blood and stored at −80 °C until analyzed. PBMC were isolated and cryopreserved at −150 °C in 90% fetal bovine serum/10% dimethyl sulfoxide until analyzed.[12]

Hemagglutination inhibition (HI) assay

Receptor destroying enzyme-treated sera were tested in duplicate with 0.7% turkey red blood cells (TRBC) and eight HA units of inactivated A/California/07/2009 (H1N1) antigen, as described previously.[35] The HI titer was the reciprocal of the highest serum dilution causing 50% inhibition of hemagglutination. Titers <10 were assigned a value of 5 for calculation purposes. Sera <40 were screened for nonspecific binding and pre-adsorbed with TRBC before re-analyzing.

T-cell FluoroSpot assay

PBMC were stimulated with the split H1N1pdm09 antigen, the H1N1-specific M1, NP, or PB1 peptide pools (BEI Resources), or the conserved CD4 internal or external peptide pools to measure the IFN-γ- and/or IL-2-secreting T-cell responses, as described earlier.[12,36] The M1, NP, or PB1 peptide pools included overlapping epitopes that covered the complete sequence of the three proteins. The CD4 peptide pools were chemically synthesized and consisted of HLA−class-II-restricted T-cell epitopes from internal or external viral proteins that are conserved among influenza A subtypes with high prevalence and HLA−supertype coverage (Supplementary-Tables 2, 3).[36] Cytokine-secreting cells were counted and the background from non-stimulated cells was subtracted from stimulation responses.

Intracellular cytokine-staining (ICS) assay

PBMC were stimulated with the split H1N1pdm09 antigen to measure the IFN-γ-, IL-2- and/or TNF-α-secreting $CD4^+$ T-cell responses, as described previously.[12] Data were acquired on an LSRFortessa flow cytometer and analyzed in FlowJo version-10 (see Supplementary-Fig. 4 for the gating strategy).

Memory B-cell (MBC) ELISpot assay

The H1N1pdm09-specific IgG^+MBC responses were measured by the ELISpot assay, as described elsewhere.[37]

Statistical analyses

Comparisons of the T-cell responses assessed in the FluoroSpot or ICS assays in 2012 and 2013 and the 5-year log-transformed HI antibody titers were performed using the nonparametric repeated-measure Friedman test, followed by the Dunn−Bonferroni post-hoc test. The retrospective data for IFN-γ-secreting T cells, cytokine profile and MBCs in 2009 were compared to the prospective data in 2012 or 2013 using the nonparametric Kruskal−Wallis or Mann−Whitney test, as appropriate, with Bonferroni correction. Adjusted p values < 0.05 were considered statistically significant. Analyses were performed in SPSS-Statistics version-24 and visualized in Prism version-7.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

R.J.C. and S.M. conceived and designed the study. M.-C.T. and S.L.L. developed the assays and performed the experiments. M.-C.T. analyzed the data. M.-C.T., R.J.C., F.Z., and S.S. interpreted the data. M.-C.T., R.J.C., F.Z., S.S., and S.M. contributed to writing of the manuscript. All authors read and approved the final version of the paper.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper on the *npj Vaccines* website (https://doi.org/10.1038/s41541-018-0069-1).

Competing interests: The authors declare no competing interest.

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Papers I-III

III

The Journal of Infectious Diseases

MAJOR ARTICLE

Live-Attenuated Influenza Vaccine Induces Tonsillar Follicular T Helper Cell Responses That Correlate With Antibody Induction

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Background. Influenza remains a major threat to public health. Live-attenuated influenza vaccines (LAIV) have been shown to be effective, particularly in children. Follicular T helper (TFH) cells provide B-cell help and are crucial for generating long-term humoral immunity. However the role of TFH cells in LAIV-induced immune responses is unknown.

Methods. We collected tonsils, plasma, and saliva samples from children and adults receiving LAIV prior to tonsillectomy. We measured influenza-specific TFH-cell responses after LAIV by flow cytometry and immunohistochemistry. Systemic and local antibody responses were analysed by hemagglutination inhibition assay and enzyme-linked immunosorbent assay.

Results. We report that LAIV induced early (3–7 days post-vaccination) activation of tonsillar follicles and influenza-specific TFH-cell (CXCR5+CD57+CD4+ T cell) responses in children, and to a lesser extent in adults. Serological analyses showed that LAIV elicited rapid (day 14) and long-term (up to 1 year post-vaccination) antibody responses (hemagglutination inhibition, influenza-specific IgG) in children, but not adults. There was an inverse correlation between pre-existing influenza-specific salivary IgA concentrations and tonsillar TFH-cell responses, and a positive correlation between tonsillar TFH-cell and systemic IgG induction after LAIV.

Conclusions. Our data, taken together, demonstrate an important role of tonsillar TFH cells in LAIV-induced immunity in humans.

Keywords. antibody responses; tonsils; influenza; LAIV; T_{FH} cells.

Influenza virus infects all ages and can cause major respiratory illness such as fulminant pneumonia. Influenza viruses are estimated to infect 5%–10% of adults and 20%–30% of children annually, resulting in up to 650 000 deaths globally [1]. Vaccination is the most cost-effective public health strategy to combat annual seasonal influenza [2]. Inactivated influenza vaccine (IIV) is used worldwide, and live-attenuated influenza vaccine (LAIV) is currently licensed for use in United States, Canada, and Europe (Ann Arbor backbone-LAIV), as well as in Russia and India (Leningrad backbone-LAIV). Currently used seasonal IIV induces strain-specific antibodies up to 6 months postvaccination, but it generally does not elicit broadly protective antibodies or long-lived memory B-cells [2, 3]. In contrast, LAIV elicits persistent antibodies, memory B-cell and $CD4^+$ T-cell responses, as well as cross-reactive $CD8^+$ T cells for up to 1 year in young children [4–8]; however, LAIV is less efficacious in adults [9]. The immunological mechanisms for the better effectiveness of LAIV in children than in adults are not fully understood.

The germinal center (GC) response is vital in the generation of high-affinity antibodies, long-lived plasma cells, and memory B cells after vaccination. Follicular T helper (T_{FH}) cells are a subgroup of $CD4^+$ T cells that help antigen-activated B cells through proliferation and affinity maturation inside follicles and GCs [10–17]. Follicular T helper cells express chemokine receptor CXCR5, inducible T-cell costimulator (ICOS), programmed cell death-1 (PD1), and transcriptional factor Bcl6 as canonical features. A subset of T_{FH} cells localized in follicles and GCs expresses CD57 [18, 19]. Recent studies revealed a transient T-cell type, designated as circulating T_{FH}-like cells ($CD4^+CXCR5^+CXCR3^+$ T cells [20–22] or $CD4^+CXCR5^+PD1^+ICOS^+CD38^+$ T cells [23]), in the peripheral blood at 7 days after seasonal IIV. These cells provided help to memory B cells and correlated with the plasmablast and antibody responses after IIV [20–23]. However, it is not known whether LAIV elicits

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T_{FH}-cell responses in humans, and, given their vital role in the induction of long-term humoral immunity, activation of T_{FH} cells is important in the development of new influenza vaccines.

Tonsils are located at the entrance of the upper respiratory tract and are compartmentalized organs where follicles and GCs develop in response to antigens, such as intranasally administered LAIV. We have previously shown that LAIV augments the local salivary immunoglobulin (Ig)A and tonsillar B-cell responses in children [5]. In this study, we conducted a clinical trial in children and adults to answer the following questions: (1) whether LAIV elicits T_{FH}-cell responses in tonsils; (2) are there differences in the kinetics and magnitude of tonsillar T_{FH}-cell responses in children and adults; (3) and whether the LAIV-induced T_{FH}-cell responses correlate with local and systemic antibody responses. Here, we show that LAIV rapidly elicited T_{FH}-cell and antibody responses in children and, to a lesser extent, in adults. Live-attenuated influenza vaccine-induced T_{FH}-cell responses were inversely associated with pre-existing local antibodies, but they positively correlated with antibody induction after vaccination. Our findings will help to improve understanding of the immunogenicity and effectiveness of LAIV in age groups with different pre-existing immunity.

MATERIAL AND METHODS

Study Design

Forty children (3–17 years old) and 37 adults (18–51 years old) were enrolled in the study after recruitment from the Ear-Nose-Throat outpatient clinic at Haukeland University Hospital, Norway. All subjects were patients scheduled for elective tonsillectomy due to chronic tonsillitis, tonsillar hypertrophy, or both but otherwise healthy. The study was approved by the Ethical Committee and the Medicines Agency. All participants or their guardians provided written informed consent before inclusion in the study (NCT01866540, www.clinicaltrials.gov).

Thirty-four children and 31 adults were vaccinated with trivalent LAIV (Fluenz; AstraZeneca) during the influenza season 2013–2014. The children and adults were divided into 3 groups and vaccinated on specific days before their scheduled tonsillectomy. Age- and gender-matched unvaccinated subjects (6 children and 6 adults) were enrolled as controls.

Vaccine and Sampling

Fluenz contained 10^7 fluorescent focus units (FFUs) of live-attenuated A/California/7/2009-like (H1N1), A/Texas/50/2012 (Victoria-like H3N2), and B/Massachusetts/2/2012 strains. The vaccine was administered intranasally as 0.1 mL per nostril. Children under 9 years old (n = 27) received 2 doses LAIV at a 4-week interval as per the manufacturer's recommendation. Children ≥9 years old and adults received a single LAIV dose.

Palatine tonsils were collected during tonsillectomy in phosphate-buffered saline. Tonsillar mononuclear cells (TMNCs) were isolated from 1 tonsil by Ficoll gradient centrifugation after mechanical disruption and cryopreserved at −150°C until use [5]. The other tonsil was cut into blocks, fixed in 4% formaldehyde, paraffin-embedded, and stored at 4°C until use.

Blood samples were collected before vaccination, on the day of tonsillectomy, and up to 1 year after vaccination for vaccinees, or only on the day of tonsillectomy for controls. Plasma samples were separated, aliquoted, and stored at −80°C until use.

Saliva samples were collected from the lower buccal mucosa before vaccination, on the day of tonsillectomy, and up to 1 year after vaccination for vaccinees using the OraSure Oral Specimen Collection Device (OraSure Technologies). The samples were stored at −80°C until use.

Hemagglutination Inhibition Assay

Plasma was treated with receptor-destroying enzyme (RDE; Seiken) before performing preabsorption with packed turkey red blood cells (TRBCs) to remove nonspecific agglutinins. The treated plasma samples were analyzed in duplicate (starting dilution 1:10) with 8 hemagglutinating units of inactivated homologous vaccine strains and 0.7% TRBCs, as previously described [5]. The hemagglutination inhibition (HI) titer was determined as the reciprocal of the highest plasma dilution giving 50% inhibition of hemagglutination. Negative titers (<10) were assigned a value of 5 for calculation purpose.

Enzyme-Linked Immunosorbent Assay

Influenza virus-specific Igs were quantified in plasma and saliva samples using the enzyme-linked immunosorbent assay (ELISA) developed in-house, as previously described [5]. Serially diluted plasma and saliva samples were analyzed in Maxi Sorp 96-well plates coated with 2 μg/mL split antigen from 3 vaccine viruses (kindly provided by GlaxoSmithKline, Wavre, Belgium). Immunoglobulin G, IgA, or IgM concentrations were interpolated from standard human IgG, IgA, or IgM curves, respectively. For calculation purposes, negatives were assigned as 0.05 μg/mL in plasma and 1 ng/mL in saliva.

T-Cell Phenotyping, Stimulation, and Flow Cytometry

For T-cell phenotyping, TMNCs were thawed and rested overnight in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640 medium containing L-glutamine, penicillin, streptomycin, and amphotericin B; Lonza) supplemented with 10% fetal bovine serum (HyClone). For influenza-specific T-cell ex vivo stimulation, rested TMNCs were incubated with 5 μg/mL split antigen from each vaccine virus and anti-CD28 (CD28.2) and anti-CD49d (9F10) antibodies, brefeldin A, and monensin (BD Biosciences).

Rested or stimulated TMNCs were stained with anti-CD3 (UCHT1), anti-CD4 (SK3), anti-CD19 (HIB19; BioLegend), anti-CD56 (NCAM16.2), anti-CD45RA (MEM-56; Thermo Fisher Scientific), anti-CXCR5 (J252D4; BioLegend), anti-CD57 (NK-1), anti-ICOS (DX29), anti-PD1 (EH12.1), and anti-CD40L (24–31; BioLegend) fluorescence-conjugated

antibodies. To detect Bcl6, the surface-stained cells were fixed and permeabilized with FoxP3/Transcription factor staining buffer set (eBioscience), followed by staining with anti-Bcl6 (K112-91) fluorescence-conjugated antibody. All samples were incubated with LIVE/DEAD fixable dead cell stain kit (Thermo Fisher Scientific) and pooled human sera. All antibodies used in flow cytometry were from BD Biosciences, unless otherwise specified. Cells were acquired on LSRFortessa cell analyzer (BD Biosciences). Data were processed using FlowJo software (version 10.4.2 for Mac; TreeStar).

Immunohistochemistry

Paraffin-embedded tonsil sections were single stained with anti-Bcl6 (PG-B6p), anti-CD4 (4B12), anti-CD20 (FL-297; Santa Cruz), anti-CD57 (TB01), or anti-ICOS (SP98; Novus Biologicals), followed by horseradish peroxidase-conjugated secondary antibody. All antibodies were from Dako, unless otherwise specified. Stained sections were counterstained with hematoxylin and analyzed using light microscopy and Cytation 5 (BioTek Instruments).

Statistical Analyses

Biological replicates were used in all experiments, unless otherwise stated. Elevations of median fluorescence intensity from flow cytometry, HI titers, and Ig concentrations from ELISA were Ln transformed before statistical tests. Sidak's multiple comparisons or multiple *t* tests with desired false-discovery rate of 1% were performed in a two-way analysis of variance. Non-parametric Spearman correlations were tested and linear fitting curves were plotted when Spearman $P < .10$. Fisher's exact analysis was performed with 2 × 2 contingency table. All statistical analyses were performed with GraphPad Prism 7.

RESULTS

Study Design

Thirty-four children and 31 adults were divided into 3 groups and vaccinated with LAIV on specific days before their scheduled tonsillectomy: Group 1 (2–5 days, 7 children and 15 adults), Group 2 (6–9 days, 15 children and 8 adults), and Group 3 (10–22 days, 12 children and 8 adults) (Figure 1 and Supplementary Table). Twenty-seven children <9 years old received a second dose of LAIV 4 weeks after the first dose. Nine children and 18 adults had received the 2009 pandemic influenza vaccine, and 4 children and 5 adults had received prior seasonal influenza vaccine(s). Six unvaccinated children and 6 unvaccinated adults were enrolled as controls. None of the vaccinees or controls had received LAIV before this study. Tonsils and plasma samples were collected from all subjects on the day of tonsillectomy. Sequential pre- and postvaccination plasma and saliva samples were collected from all vaccinees (Figure 1).

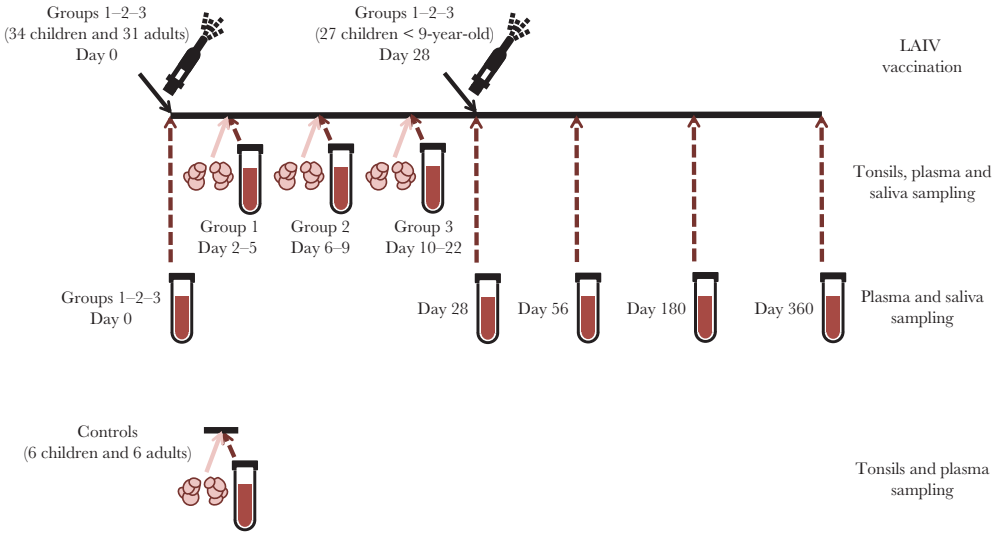

Figure 1. Illustration of the study design. Forty children (3 to 17 years old) and 37 adults (18 to 51 years old) were enrolled in this study. Thirty-four children and 31 adults were vaccinated with trivalent live-attenuated influenza vaccine (LAIV) (Fluenz; AstraZeneca) during the influenza season 2013–2014. The children and adults were randomized into 3 groups and vaccinated on specific days before their scheduled tonsillectomy: Group 1 (2–5 days, 7 children and 15 adults), Group 2 (6–9 days, 15 children and 8 adults), and Group 3 (10–22 days, 12 children and 8 adults). Age- and gender-matched unvaccinated subjects (6 children and 6 adults) were enrolled as controls. Children (n = 27) under 9 years old received the second dose LAIV at day 28. Tonsils, plasma, and saliva samples were collected during tonsillectomy. In addition, plasma and saliva samples prevaccination (day 0) and 28 days, 56 days, 6 months (day 180), and 12 months (day 360) postvaccination were collected for all vaccinated subjects (Groups 1–3).

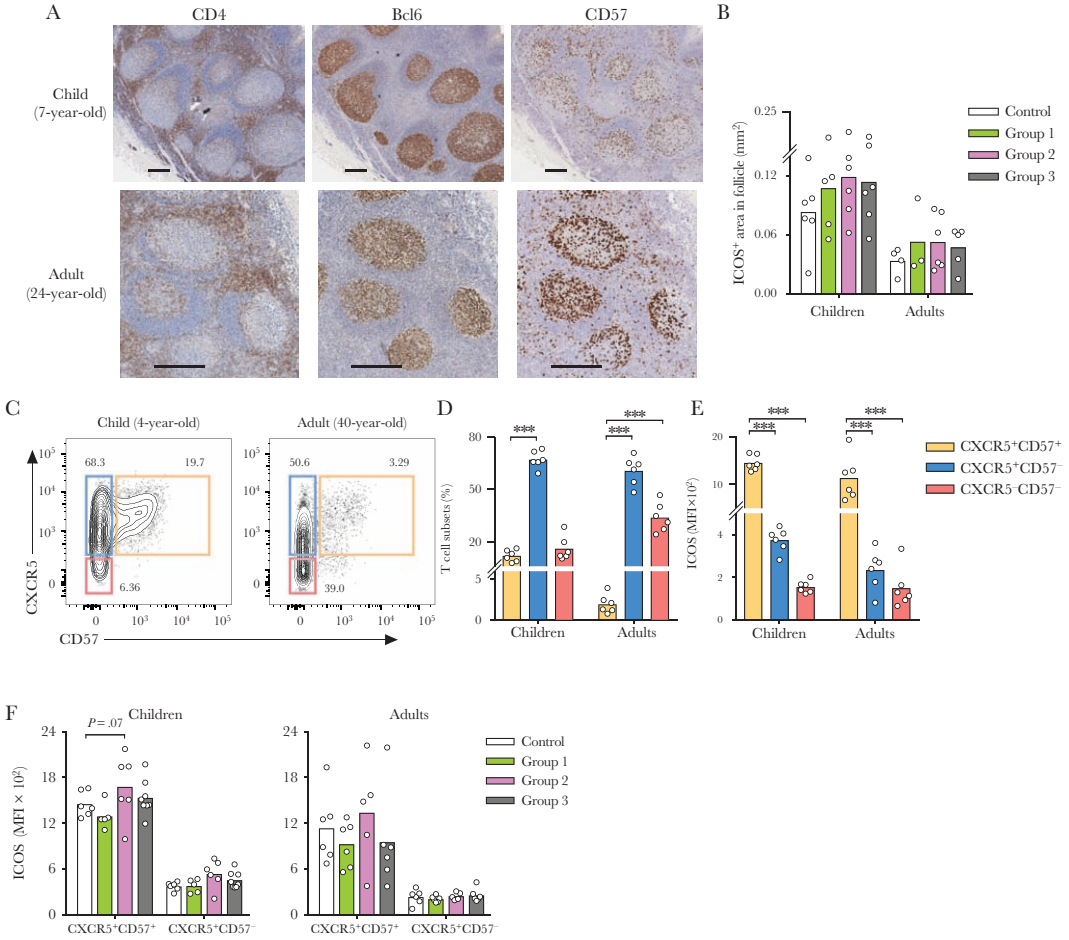

Figure 2. Activation of follicular T helper cells after live-attenuated influenza vaccine. (A) Representative immunohistochemistry images (original magnification ×4) of sections stained with CD4, Bcl6, or CD57 from a child (7 years old, top) and an adult (24 years old, bottom). Stained sections were counterstained with hematoxylin. Scale bar indicates 400 μm. (B) A summary of inducible T-cell costimulator (ICOS)$^+$ area inside follicles from controls and vaccinees in Groups 1–3 in children (n = 22) and adults (n = 18). Follicles were measured based on CD20 and hematoxylin staining. (C) Representative CD4$^+$ T-cell subsets gating based on CXCR5 and CD57 in a child (4 years old, left) and an adult (40 years old, right). Tonsillar mononuclear cells were pregated as CD3$^+$CD19$^-$CD56$^-$CD4$^+$CD45RA$^-$ (pregating in Supplemental Figure S1). Numbers adjacent to outlined areas indicate frequencies of CD4$^+$ T cells in each subset. (D) A summary of the distribution of the CD4$^+$ T-cell subsets in 12 unvaccinated children and adults. (E) The expression of ICOS in median fluorescence intensity (MFI) in CD4$^+$ T-cell subsets: CXCR5$^+$CD57$^+$ (orange), CXCR5$^+$CD57$^-$ (blue), and CXCR5$^-$CD57$^-$ (red) from unvaccinated children and adults. (F) A summary of ICOS expression in MFI from controls and vaccinees in Groups 1–3 in children (n = 25) and adults (n = 23). The mean values are shown as bars, and each symbol represents 1 subject (B and D–F). Sidak's multiple comparisons between CD4$^+$ T-cell subsets were performed in two-way analysis of variance ([ANOVA] D and E). Multiple *t* tests with desired false discovery rate of 1% between vaccinated and unvaccinated subjects were performed in two-way ANOVA (B and F). Data were from 6 independent experiments. ***, $P < .001$.

Live-Attenuated Influenza Vaccine Elicits Rapid Tonsillar Follicular T Helper Cell Responses in Children

Immunohistochemistry (IHC) staining of tonsil sections showed that follicles (CD20$^+$ area) and GCs (Bcl6$^+$ area) were present in both children and adults tonsils after LAIV. CD4$^+$ T cells were mostly found in T-cell zones but occasionally also within GCs. CD57$^+$ cells were enriched inside GCs (Figure 2A). In this study, using IHC, we observed an increase in ICOS$^+$ area inside follicles after LAIV, indicating that LAIV activated follicles in children 7–14 days postvaccination (Figure 2B). However, no change in the number or size of follicles or GCs was found (data not shown). To further study the T_{FH} cells, flow cytometry was used. Tonsillar CD4$^+$ T cells were gated into 3 subsets based on CXCR5 and CD57 expression in flow cytometry. On average, 12.06% and 1.95% of CD4$^+$ T cells

were $CXCR5^+CD57^+$ in children and adults, respectively (Figure 2C and D). The $CXCR5^+CD57^+$ cells expressed the canonical T_{FH}-cell markers ICOS, PD1, and Bcl6 (Figure 2E and Supplementary Figure S2), hence the $CXCR5^+CD57^+$ cells are bona fide T_{FH} cells [17]. Overall, children vaccinated with LAIV had higher ICOS expression on T_{FH} cells compared with controls; however, the cell frequency remained unchanged (Figure 2F and data not shown).

To assess influenza-specific T_{FH}-cell responses after LAIV, we ex vivo-stimulated TMNCs with vaccine antigens and measured ICOS expression as a surrogate of T_{FH}-cell activation [23, 24]. Children had significantly increased ICOS expression in T_{FH} cells as early as 3 days postvaccination against H1N1 and H3N2 antigens, and at day 7 against the B antigen (Figure 3A). The T_{FH} cells in adults had significantly increased ICOS expression 7 days postvaccination, against all 3 antigens (Figure 3B). Furthermore, we calculated the total LAIV-induced T_{FH}-cell responses in each individual (Delta ICOS × $CXCR5^+CD57^+$ %). Live-attenuated influenza vaccine induced significant T_{FH}-cell responses against all 3 viruses in children but only against B virus in adults (Figure 3C). Overall, our data showed that LAIV elicits rapid antigen-specific tonsillar T_{FH}-cell responses in children and, to a lesser extent, in adults.

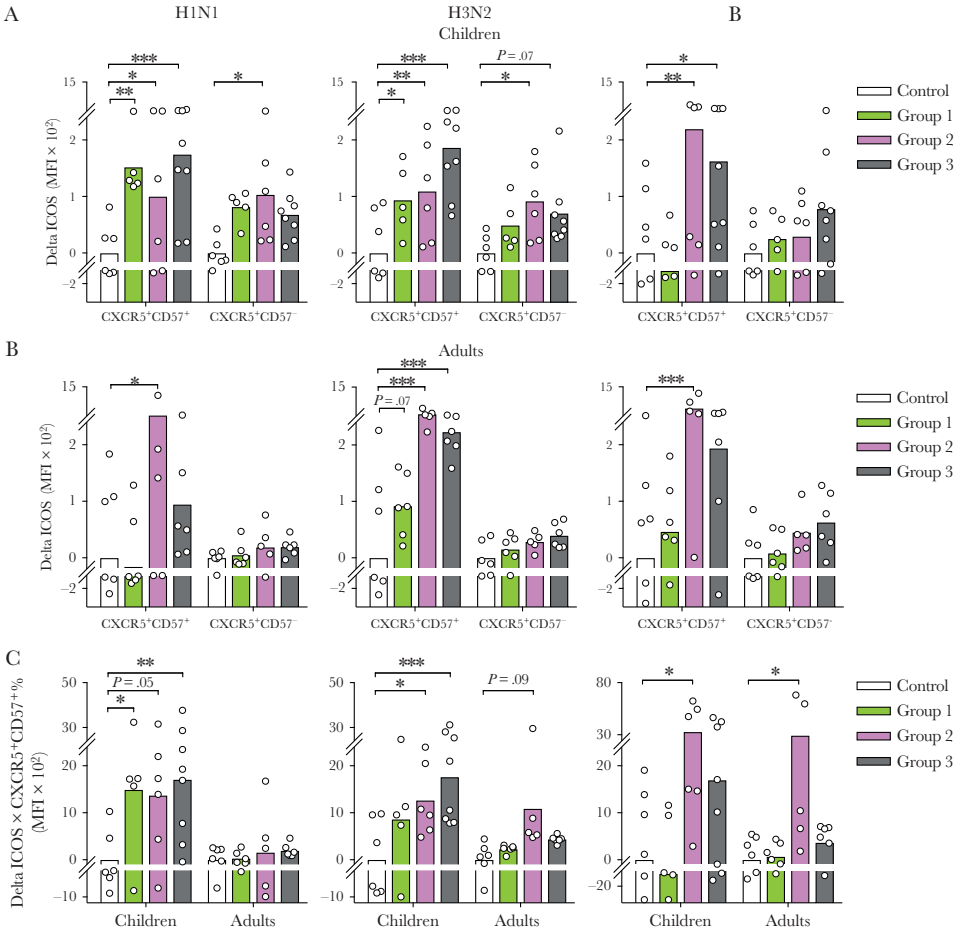

Figure 3. Live-attenuated influenza vaccine elicits influenza-specific follicular T helper (T_{FH})-cell responses. (A and B) Influenza-specific inducible T-cell costimulator (ICOS) expression increases (Delta ICOS in median fluorescence intensity [MFI]) in $CD4^+$ T-cell subsets from controls and vaccinees in Groups 1–3 in children (n = 25; A) and adults (n = 23; B). (C) Vaccine-induced total influenza-specific T_{FH}-cell responses were calculated as the elevation in T_{FH}-cell activity (Delta ICOS × $CXCR5^+CD57^+$ %). Tonsillar mononuclear cells were rested and stimulated with influenza split antigens from A/California/07/2009-like (H1N1) virus (left) or A/Victoria/361/2011-like (H3N2) virus (center) or B/Massachusetts/2/2012 virus (right). Results from vaccinees (Groups 1–3) are presented as arbitrary values relative to unvaccinated subjects (Control). The mean values are shown as bars, and each symbol represents 1 subject. Multiple *t* tests with desired false discovery rate of 1% between vaccinated and unvaccinated subjects were performed in two-way analysis of variance. Data were from 6 independent experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

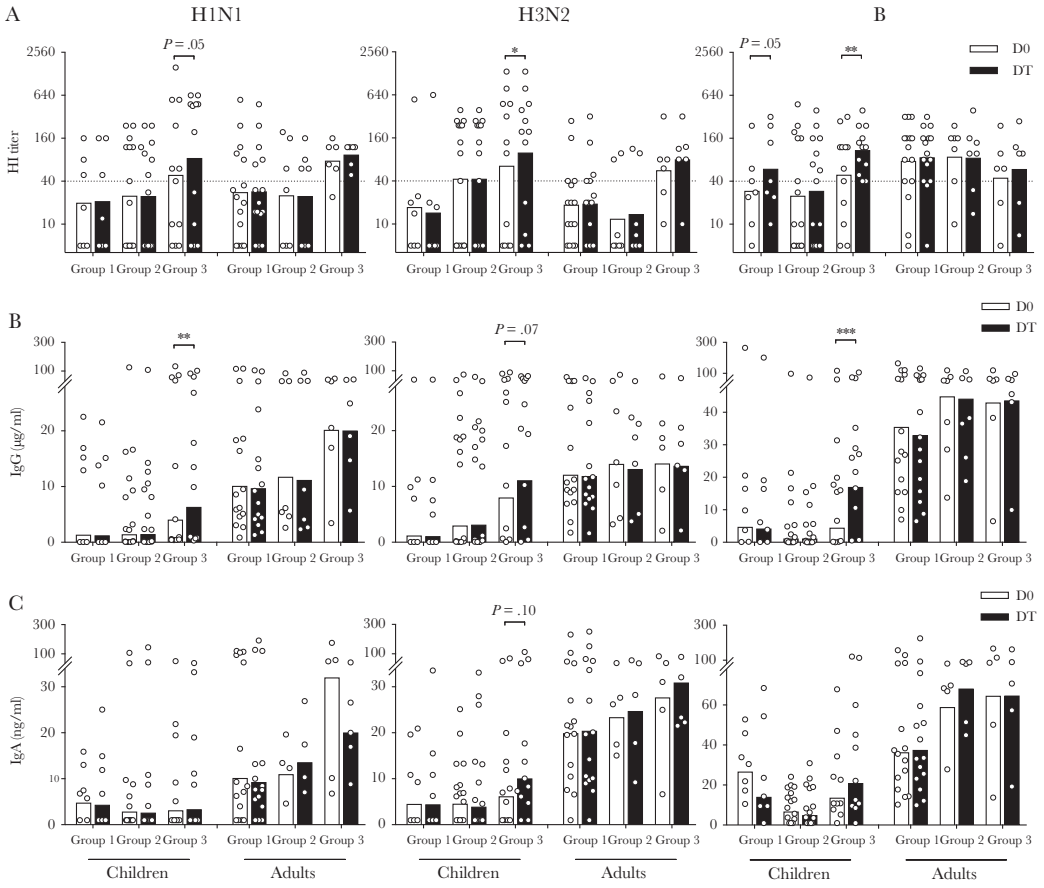

Figure 4. Live-attenuated influenza vaccine elicits influenza-specific antibodies within 14 days in children. Hemagglutinin-specific antibodies (hemagglutination inhibition [HI] titer; A) and total influenza-specific antibodies (immunoglobulin [Ig]G; B) were measured using plasma samples before (D0) and after vaccination (DT) from vaccinees in Groups 1–3. (C) Virus-specific local antibodies (IgA) were measured using saliva samples before (D0) and after vaccination (DT) from vaccinees in Groups 1–3. Antibodies were tested against A/California/07/2009-like (H1N1) virus (left) or A/Victoria/361/2011-like (H3N2) virus (center) or B/Massachusetts/2/2012 virus (right). The geometric mean values are shown as bars, and each symbol represents 1 subject. Antibody titers and concentrations were Ln transformed in statistical analyses. Multiple *t* tests with desired false discovery rate of 1% between pre- and postvaccination titers were performed in two-way analysis of variance. The horizontal dotted lines indicate HI titer of 40 (A). Duplicates were performed in all experiments. *, $P < .05$; **, $P < .01$.

Live-Attenuated Influenza Vaccine Elicits Early Antibody Responses in Children

Having established that LAIV induced rapid T_{FH}-cell responses, we assessed early systemic and local antibody responses after vaccination. Hemagglutinin (HA)-specific systemic antibodies were quantified using the HI assay (Figure 4A). In children, increases in HI titers to H1N1, H3N2, and B viruses were observed as early as day 14 (Group 3) after LAIV. In adults, no increase in HI titers was observed. Neutralizing antibodies measured by microneutralization assay showed similar kinetics (data not shown). Influenza-specific antibodies were quantified using ELISA. In children, we observed increases in systemic IgG against all 3 viruses 14 days after LAIV (Figure 4B), but we only observed marginal increases in local salivary IgA against H3N2 virus (Figure 4C). Adults had higher preexisting (D0) antibodies than children, but no increase after LAIV. Our data, taken togther, show that LAIV elicits early influenza-specific antibody responses in children, but not in adults.

Live-Attenuated Influenza Vaccine Elicits Long-Term Antibody Responses in Children

We assessed the antibody responses up to 1 year after LAIV. After immunization, 6, 10, and 19 of 34 children, and 3, 2, and 5 of 31 adults seroconverted (≥4-fold increase from prevaccination HI titers) to H1N1, H3N2, and B viruses, respectively. The HI titers

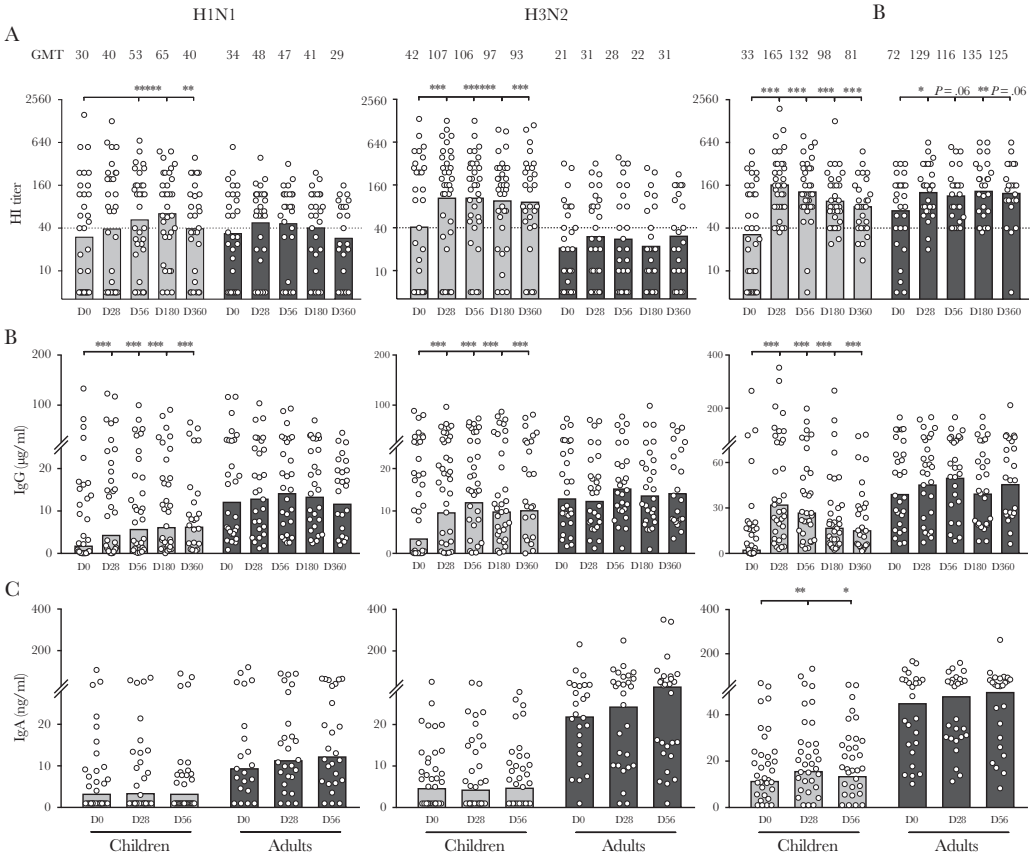

Figure 5. Live-attenuated influenza vaccine elicits long-term influenza-specific systemic and local antibodies in children. Systemic hemagglutinin-specific antibodies (hemagglutination inhibition [HI] titer; A) and total influenza-specific antibodies (immunoglobulin [Ig]G; B) were measured using plasma samples prevaccination (D0) and 28 days (D28), 56 days (D56), 6 months (D180), and 12 months (D360) postvaccination. Local influenza-specific antibodies (IgA; C) were measured using saliva samples before vaccination (D0) and 28 days (D28) and 56 days (D56) after vaccination. Antibodies were tested against A/California/07/2009-like (H1N1) virus (left) or A/Victoria/361/2011-like (H3N2) virus (center) or B/Massachusetts/2/2012 virus (right). The geometric mean titers are shown as bars, and each symbol represents 1 subject. Antibody titers and concentrations were Ln transformed in statistical analyses. Sidak's multiple comparisons between prevaccination (D0) and each time point postvaccination (D28, D56, D180, and D360) were performed in two-way ANOVA. The geometric mean HI titers (GMT) at each time point are noted above the graphs, and the horizontal dotted lines indicate HI titer of 40 (A). Duplicates were performed in all experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

after LAIV were maintained above protective titer (≥40) for 1 year in children against all 3 viruses but only against influenza B in adults (Figure 5A).

We quantified the influenza-specific systemic IgG (Figure 5B), IgA, and IgM (Supplemental Figure S3). In children, LAIV elicited significant increases in IgG against all 3 antigens at 28 days postvaccination, which were maintained throughout the year. In contrast, adults showed no increase in antibodies after LAIV (Figure 5B). Similar trends, although of lower magnitudes, were observed with IgA responses (Supplemental Figure S3A). Live-attenuated influenza vaccines also induced an IgM response in children against influenza B (Supplemental Figure S3B).

Next, we measured local salivary antibodies up to 56 days after vaccination. Live-attenuated influenza vaccine significantly increased the local IgA to influenza B virus in children; however, no change was observed in adults (Figure 5C). In summary, our data showed that in children, LAIV induced long-term antibody responses to all 3 viruses, but local antibodies were only elicited against influenza B virus. In adults, only influenza B-specific antibody responses were observed after LAIV.

Follicular T Helper Cell Responses After Live-Attenuated Influenza Vaccine Correlated With Antibody Responses

Our next aim was to assess whether LAIV-induced T_{FH}-cell responses correlated with the age of the subject or pre-existing

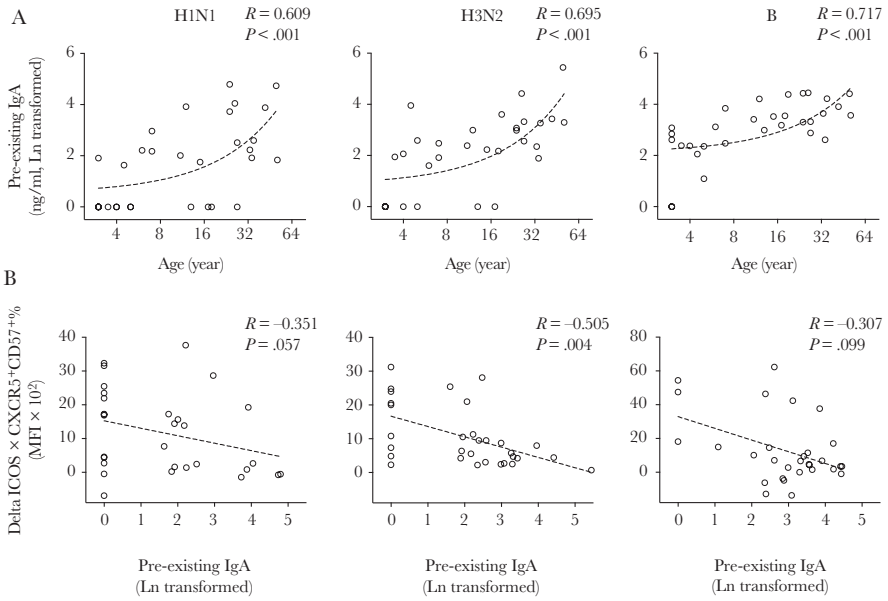

Figure 6. Pre-existing local antibodies correlate with age and follicular T helper (T_{FH})-cell responses after live-attenuated influenza vaccine (LAIV). (A) Pre-existing influenza-specific local antibodies (immunoglobulin [Ig]A) in saliva samples from children and adults correlated strongly with their ages. (B) The LAIV-induced T_{FH}-cell responses (Delta inducible T-cell costimulator [ICOS] × $CXCR5^+CD57^+$ %) inversely correlate with pre-existing influenza-specific local antibodies (IgA). The T_{FH}-cells activity and antibodies were tested against split antigens from A/California/07/2009-like (H1N1) virus (left) or A/Victoria/361/2011-like (H3N2) virus (center) or B/Massachusetts/2/2012 virus (right). Pre-existing IgA concentrations in saliva samples were Ln transformed in statistical analyses. Linear fitting curve was plotted as dotted line when nonparametric Spearman $P < .10$. Spearman r and P values are noted for each correlation. MFI, median fluorescence intensity.

local antibody levels. First, we observed an increase in the pre-existing influenza-specific salivary IgA concentration with an increase in age (Figure 6A). Notably, pre-existing salivary IgA concentrations inversely correlated with LAIV-induced T_{FH}-cell responses (Figure 6B), suggesting that high influenza-specific local IgA concentrations present in adults may limit the tonsillar T_{FH}-cell responses.

Next, we tested whether tonsillar T_{FH}-cell responses elicited after LAIV correlated with systemic antibody induction postvaccination. It is interesting to note that LAIV induced influenza-specific T_{FH}-cell responses correlated with the systemic IgG fold-induction 28 days after vaccination (D28/D0), but not HI titers, against H1N1 and H3N2 antigens (Figure 7A). We further dissected antibody responses by stratifying vaccinees as naive (HI titer <40) or experienced (HI titer ≥40) before vaccination. We were intrigued to find that T_{FH}-cell and antibody responses correlated more closely in naive individuals for influenza A (H1N1 and H3N2) viruses. In contrast for influenza B virus, T_{FH}-cell and antibody responses significantly correlated only in experienced vaccinees (Figure 7B). Similar patterns were also observed with antibody responses 56 days after LAIV (Supplemental Figure S4). The correlations between T_{FH}-cell and systemic IgG responses were confirmed using Fisher's exact test (Supplemental Figure S5). Our data, taken together, demonstrate that LAIV-induced tonsillar T_{FH}-cell responses correlated with systemic antibody responses after vaccination.

DISCUSSION

The mechanisms by which vaccines, particularly live-attenuated viral vaccines, elicit persistent antibody responses remains unclear. Understanding the underlying immunological mechanisms that mediate long-term immunity is critical in designing improved vaccines. Follicular T helper cells play a critical role in GC formation and the development of high affinity antibody and B-cell responses [13], thus vaccines that successfully promote and maintain T_{FH} cells are most likely to induce persistent antibody responses. Our data show, for the first time, that LAIV rapidly activated follicles and tonsillar T_{FH} cells in children and, to a lesser extent, in adults. We also observed rapid and long-term (up to 1 year) antibody responses to all 3 vaccine viruses in children, but only to influenza B virus in adults. The magnitude of tonsillar T_{FH}-cell responses was inversely correlated with pre-existing local IgA antibodies. More important, significant correlations were observed between systemic antibodies and tonsillar T_{FH}-cell responses after LAIV.

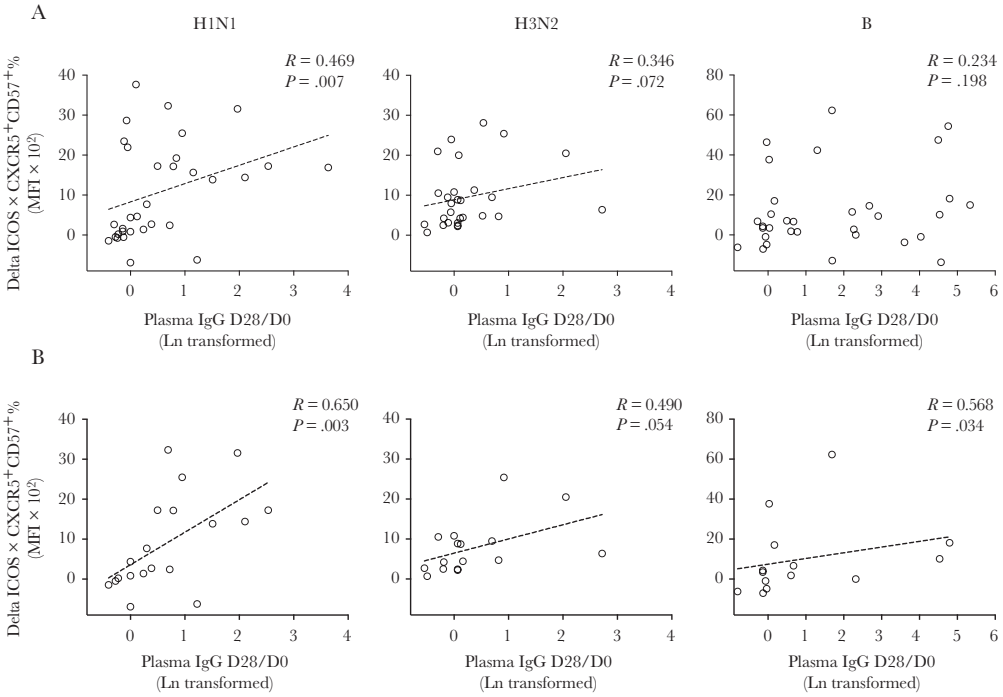

Figure 7. Influenza-specific follicular T helper (T_{FH})-cell responses after live-attenuated influenza vaccine (LAIV) correlate with antibody responses. (A) The correlations between LAIV-induced T_{FH}-cell responses (Delta inducible T-cell costimulator [ICOS] × $CXCR5^+CD57^+$ %) and systemic antibody fold induction (plasma immunoglobulin [Ig]G D28/D0) from all vaccinated children and adults. (B) Influenza A viruses specific T_{FH}-cell and antibody responses correlate in naive individuals (D0 hemagglutination inhibition [HI] <40), whereas influenza B virus-specific T_{FH}-cell and antibody responses correlate in pre-exposed individuals (D0 HI ≥40). The T_{FH}-cell responses and antibody fold induction were tested against split antigens from A/California/07/2009-like (H1N1) virus (left) or A/Victoria/361/2011-like (H3N2) virus (center) or B/Massachusetts/2/2012 virus (right). Systemic antibody fold inductions (plasma IgG D28/D0) were Ln transformed in statistical analyses. Linear fitting curve was plotted as dotted line when nonparametric Spearman $P < .10$. Spearman r and P values are noted in each correlation. MFI, median fluorescence intensity.

Recent studies have shed light on the tonsillar T_{FH}-cell responses against influenza in humans [25, 26]. In children receiving IIV, Amodio et al [25] found a significant increase in the frequencies of T_{FH} cells, which were associated with influenza-specific, antibody-secreting cells. Aljurayyan et al [26] in vitro stimulated TMNCs from unvaccinated individuals with attenuated influenza viruses and reported T_{FH}-cell activation, proliferation, and differentiation, which correlated with antibody production. In our study, using IHC, we showed that a substantial number of $CD4^+$ and $CD57^+$ cells are present inside follicles, in agreement with previous observations [18, 19]. Immunohistochemistry showed that follicles inside tonsils were activated after LAIV, indicated by the elevated ICOS expression. However, no change in follicle number or size was found after vaccination, suggesting the absence of a strong GC reaction. Flow cytometric analyses also showed higher ICOS expression in the T_{FH}-cell ($CXCR5^+CD57^+CD4^+$ T cell) population, suggesting increased T_{FH}-cell activation, although the T_{FH}-cell frequencies remained unchanged after LAIV. The lack of increase in T_{FH}-cell frequencies after LAIV vaccination may be attributed to the replicating nature of the LAIV with a relatively low antigen dosage ($10^{7.0 \pm 0.5}$ FFU) compared with IIV (commonly 15 μg of HA per strain). However, when LAIV-specific T_{FH}-cell responses were ex vivo stimulated, we observed potent T_{FH}-cell responses against all 3 LAIV viruses in children and against the B virus in adults (Figure 3). Future studies on tonsillar T_{FH}-cell and circulating T_{FH}-like-cell responses after LAIV will help gain insights into the relationship between these lymph node and systemic T_{FH} cells and to establish whether circulating T_{FH}-like cells are a possible biomarker for the immunogenicity of LAIV.

To dissect humoral responses after LAIV, we quantified systemic influenza-specific antibodies as HA-specific functional and binding antibodies in plasma samples. In addition, local IgA were measured in saliva samples (Figures 4 and 5). The HI titer of 40 has been widely used as a surrogate correlate of protection [27], although an HI titer of 110 was suggested for protection against H3N2 in children [28]. Recent studies have shed light on the protective roles of nonneutralizing antibodies. For example,

neuraminidase and internal protein-specific antibodies may contribute to elimination of infection through limiting progeny virion release and mechanisms such as antibody-dependent, cell-mediated cytotoxicity [29, 30]. Meanwhile, mucosal IgA provides protection against the initial establishment of infection [4]. We found significant increases in influenza-specific antibodies 14 days after LAIV, which were maintained for up to 1 year in children to all 3 viruses. However, in adults, LAIV only elicited antibodies to the B virus. These differences in the immunogenicity among vaccine viruses observed are consistent with previous findings [31], and they could be due to differences in virus replication efficiency in the upper respiratory tract. In addition, although we only observed low local IgA responses after LAIV using saliva samples (Figures 4 and 5), future studies could measure IgA in nasal washes to gain more direct evidence of mucosal antibody responses after LAIV.

During the 2009 pandemic, Norway vaccinated its population with AS03-adjuvanted pandemic vaccine, which induced durable antibody responses [32]. Among the vaccinees in our cohort, 27% of children and 52% of adults had received this pandemic vaccine. We observed higher pre-existing H1N1 HI titers in these individuals (P = .03 in children and P = .002 in adults) (Supplemental Figure S6), consistent with our previous findings [33]. The higher frequency of adults receiving pandemic vaccine may help explain the higher H1N1-specific pre-existing antibodies and subsequently the lower antibody responses after LAIV in adults compared with children. In future studies, a comparison of T_{FH}-cell responses elicited by IIV and LAIV may provide better insight into understanding differences in the immune responses elicited by parenteral and intranasal influenza vaccines.

In Europe, LAIV is only licensed and recommended for children 2–17 years old, because LAIV elicits more potent and longer-lasting immune responses in children than in adults [2, 33]. Our analyses revealed 3 correlations: (1) the pre-existing local IgA correlates with age; (2) LAIV-induced T_{FH}-cell responses inversely correlate with the pre-existing local IgA; and (3) systemic antibody responses after vaccination correlate with T_{FH}-cell responses. Our data, taken together, may explain why LAIV works better as priming vaccine in children [34]. The low pre-existing local antibody levels at the site of LAIV administration in children may aid virus replication, thus leading to the generation of rapid T_{FH}-cell responses and subsequent long-term humoral responses after vaccination. In contrast, higher pre-existing local antibodies in older individuals, likely due to previous exposure or vaccination, may partially inhibit the initial LAIV virus replication. As a result, LAIV elicits lower T_{FH}-cell activation in adults, which provides insufficient B-cell help, and consequently lower humoral responses after vaccination. We found that LAIV-induced T_{FH}-cell responses inversely correlated more strongly with pre-existing local IgA than age, implying that LAIV functions optimally as a priming vaccine on no or low background immunity. It is interesting to note that T_{FH}-cell and antibody responses correlate significantly in naive vaccinees with H1N1 and H3N2 viruses but with B virus in pre-exposed vaccinees. This could be explained by influenza B virus being better adapted to humans than influenza A viruses, thus lower T_{FH}-cell activation is needed for antibody induction (as confirmed in the Fisher's exact test) (Supplementary Figure S5). Further studies with a larger cohort are needed to validate our findings.

CONCLUSIONS

In this study, we assessed the kinetics and magnitude of LAIV-induced T_{FH}-cell, systemic, and local antibody responses in children and adults. In this study, for the first time, we have shown that LAIV elicits rapid tonsillar T_{FH}-cell responses to all 3 influenza vaccine viruses, which correlated with the long-term systemic antibody increases after vaccine. Our data will contribute to the understanding of mechanisms that govern LAIV-induced immune responses in pediatric and adult populations.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. F. Z., R. D. P., and R. J. C. conceived the project and designed the experiments. R. J. C., K. G.-I. M., and K. A. B. conducted the clinical study and collected samples. F. Z., S. A. S., and S. L. conducted the experiments. F. Z. and S. L. analyzed the data. F. Z., S. L., K. A. B., K. G.-I. M., R. D. P., and R. J. C. prepared and edited the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Errata for

Humoral and cellular immune responses after pandemic and seasonal influenza vaccination in humans

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

07.05.2022 Sarah Jalloh

09.05.22

Errata List

Page 9 Remove s: "(HCWs)" – corrected to "(HCW)"

Page 10 Replacing lower case letter with upper case letter: "alveolar macrophages" and "acetyl salicylic acid" – corrected to "Alveolar macrophages" and "Acetyl salicylic acid"

Page 10 Misspelling: "DICS" – corrected to "DISC"

Page 12 Missing abbreviation: "added LRT- Lower Respiratory Tract"

Page 12 Repeated word: "remove NK cell Natural killer cell from abbreviations"

Page 15 Missing s: "Paper I & II" – corrected to "Papers I & II"

Page 17 Nonbold words: "cycl**e**, pandemic**s**, vaccin**es**"- corrected to "cycle, pandemics, vaccines"

Page 17-25 Missing word: "binding sites influenza H1, H3 and B HAs" – corrected to "binding sites in influenza H1, H3 and B HAs"

Page 17-40 Misspelling: "different of B cells"- corrected to "differentiation of B cells"

Page 22 Wrong number: "11 NAs" corrected to "9 NAs"

Page 23 Removed extra e: "HA and e NA" corrected to "HA and NA"

Page 24 Missing italics: "Wilson et al"- corrected to Wilson *et al.*"

Page 24 Bold letter: "(A)" – corrected to "**(A)**"

Page 24-25 Replacing lower case letter with upper case latter: "Has" – corrected to "HAs"

Page 25 Misspelling: "amino acids residues"- corrected to "amino acid residues"

Page 25 Replacing upper case letter with lower case letter: "The Figure is adapted" – corrected to "The figure is adapted"

Page 27 Wrong figure letter: "€" – corrected to **"(E)"**

Page 28 Missing word: "updating of influenza vaccine"- corrected to "updating of annual influenza vaccine"

Page 28 Missing words and a comma: "human viruses can result a novel influenza virus"- corrected to "human viruses, which can result in a novel influenza virus"

Pages 28 Misspelling: "(which rare occurs)" – corrected to "(which rarely occurs)"

Page 31 Missing words: "that of 1918, when severe disease and 80% mortality were reported" – corrected to "that of the 1918 pandemic, when severe diseases and 80% mortality were reported"

Page 31 Missing word: "a later found" – corrected to "later reports found"

Page 32 Wrong number: "A/H7N6" – corrected to "A/H7N9"

Page 33 Missing word: "which result is pyrolysis of infected cells" – corrected to "which result in pyrolysis of infected cells"

Page 36 Misspelling: "induced after influenza infection and vaccine"- corrected to "induced after influenza infection and vaccination"

Page 36 Misspelling: "which is tack carried out by" – corrected to "which is a task carried out by"

Page 37 Misspelling: "ULT" – corrected to "LRT"

Page 38 Remove s: "(MBCs)" – corrected to "(MBC)"

Page 39 Missing superscript: CD4+ T cells, [142]" – corrected to $CD4^+$ T cells, [142]"

Page 41 Spacing: ".Acute" – corrected to ". Acute"

Page 43 Abbreviated words not defined: "ECDC, CDC and FDA" – corrected to European Centre for Disease Prevention and Control, Centers for Disease Control and Prevention and Food and Drug Administration"

Page 43 Remove s: "(NAIs)" – corrected to "(NAI)"

Page 44 Abbreviated word not defined: "the US"- corrected to the United State"

Page 44 Replacing upper case letter with lower case letter: "Pregnant women" – corrected to "pregnant women"

Page 46 Swapped and missing word: "increase vaccine global supply"- corrected to "increase the global vaccine supply"

Page 46 Replacing upper case letter with lower case letter: "History" – corrected to "history"

Page 49 Wrong Table number: "Table 1.3" – corrected to "Table 1.2"

Page 49 Missing word: "licensed the USA in 2013" – corrected to "licensed in the USA in 2013"

Page 50 Misspelling: "Adjuvants are added to vaccines to enhances the host immune response" – corrected to "Adjuvants are added vaccines to enhance the host immune response"

Page 50 Misspelling: "vaccine dose produced per egg" – corrected to "vaccine doses produced per egg"

Page 50 Misspelling, full stop and comma missing: "rending seasonal influenza vaccine ineffective Within 3 months after the emergence of the novel A(H1N1)pdm09 virus several pandemic vaccines were developed " – corrected to "rendering seasonal influenza vaccine ineffective. Within 3 months after the emergence of the novel A(H1N1)pdm09 virus, several pandemic vaccines were developed"

Page 51 Missing number: "A/California/7/2009" - corrected to "A/California/07/2009"

Page 51 Replacing lower case letter with upper case letter: "strategic Adversary Group of Experts (SAGE)" – corrected to "Strategic Adversary Group of Experts (SAGE)"

Page 52 Replacing upper case letter with lower case letter: "Based" – corrected to "based"

Page 54 Missing word: "T cells have been reported as potential markers COP" – corrected to "T cells have been reported as potential markers of COP"

Page 56 Spacing: "A(H1N1) pdm09" – corrected to "A(H1N1)pdm09"

Page 57 - 59 Misspelling: "the Helsinki Declaration and were conducted under good clinical practice" – corrected to "the Helsinki Declaration and was conducted under good clinical practice"

Page 57 Missing word: "therefore were able manage a study of this magnitude." – corrected to "therefore were able to manage a study of this magnitude."

Page 58 Misspelling: "Health care work" – corrected to "Healthcare workers"

Page 58 Remove word: "licensed for the use in children" – corrected to "licensed for use in children"

Page 61 Spacing: A(H1N1) pdm09 and D 28 – corrected to "A(H1N1)pdm09 and D28"

Page 62 Missing number: "A/California/7/2009" - corrected to "A/California/07/2009"

Page 62 Replacing lower case letter with upper case letter: "the northern hemisphere" – corrected to "the Northern Hemisphere"

Page 62 Missing full stop: October 2013 to February 2014" – corrected to "October 2013 to February 2014."

Page 63 Missing word: "the MBC ELISpot (n = 27), IFN-γ ELISpot (n = 9) assays" – corrected to "the MBC ELISpot (n = 27) and IFN-γ ELISpot (n = 9) assays"

Page 65 Abbreviated words not defined: "RT" – corrected to "room temperature (RT)"

Page 65 Remove s: "the SRH assays – corrected to "the SRH assay"

Page 67 Missing words: "local IgA levels in saliva were measured in children and adult and performed as previously describe" – corrected to "local IgA levels in saliva were measured in children and adult using ELISA and performed as previously describe"

Page 67 Remove word: "The HI assay underestimated the immune antibody responses induced by LAIV" – corrected to "The HI assay underestimated antibody responses induced by LAIV"

Page 70 Missing full stop: "Supplementary Table 1"- corrected to Supplementary Table 1."

Page 74 Missing word: "The sections dehydrated" – corrected to "The sections were dehydrated"

Page 74 Missing s: "The three-brown area" - corrected to "The three brown areas"

Page 76 Spacing: "P <.10" -corrected to "P<10"

Page 77 Spacing: "A(H1N1) pdm09 strain" – corrected to "A(H1N1)pdm09 strain"

Page 77 Misspelling: "when all the HCWs having antibody titers above the protective threshold" -corrected to "when all the HCWs had antibody titers above the protective threshold"

Page 78 Misspelling: "Since the ASC responses was shown to peak on day 7"- corrected to "Since the ASC responses were shown to peak on day 7"

Page 78 Remove s: "ASCs"- corrected to "ASC"

Page 81 Misspelling: "influenza specific T_{FH} cells response" – corrected to "influenza specific T_{FH} cell responses"

Page 82 Spacing: "T_{FH}cell responses" – corrected to "T_{FH} cell responses"

Page 83 Remove abbreviation: "antibody secreting cells (ASCs)/plasma-blast – corrected to "antibody secreting cells/plasma-blast"

Page 83 Remove s: "ASCs"- corrected to "ASC"

Page 83 Missing full stop: "The study reported by Li *et al.* [ref] These ASCs produce functional antibodies" – corrected to "The study reported by Li *et al.* [ref]. These ASCs produce functional antibodies"

Page 84 Remove s: "MBCs"- corrected to "MBC"

Page 84 Missing word: "The frequencies IgG-specific MBCs"- corrected to "The frequencies of IgG-specific MBCs"

Page 84 Missing words: "Maintenance of memory B cell responses"-corrected to "Maintenance of memory B cell responses after repeated vaccination"

Page 84 Missing italics: "Goel et al"- corrected to Goel *et al.*"

Page 85 Missing word and misspelling: "which resulted rapid induce of $CD4^+$ T cells" – corrected to "which resulted in rapid induction of $CD4^+$ T cells"

Page 86 Missing word: "Whereas they had more than 300 subjects and stimulated whole influenza virus" – corrected to "Whereas they had more than 300 subjects and stimulated with whole influenza viruses"

Page 86 Missing comma: "Rosendahl Huber *et al*"- corrected to "Rosendahl Huber *et al.*"

Page 86 Misspelling, Missing comma and brackets: "A/H1N1pmd09 split antigen which was further boosted" – corrected to "A(H1N1)pdm09 split antigen, which was further boosted"

Page 87 Missing word: "further boosted after pandemic vaccination" – corrected to "further boosted after the pandemic vaccination"

Page 87 Spacing: "IFN- γ" – corrected to "IFN-γ"

Page 87 Missing italics: "Richards et al – corrected to "Richards *et al.*"

Page 88 Missing comma: "differentiate into effector T cells upon antigen encounter they may provide long-lasting protective immunity"- corrected to "differentiate into effector T cells upon antigen encounter, they may provide long-lasting protective immunity"

Page 88 Bold word: "paper I"- corrected to "**paper I**"

Page 88 Missing hyphen "cross reactive"- corrected to "cross-reactive"

Page 88 Missing word: "which in agreement with previous study" – corrected to "which is in agreement with previous study"

Page 88 Missing italics: "Yang J et al" -corrected to "Yang *et al.*"

Page 89 Missing words: "influenza vaccination is influenced by several factors, such as vaccine type, age and pre-existing of vaccine recipients"- corrected to "influenza vaccination is influenced by several factors, such as vaccine type, age and pre-existing immunity of the vaccine recipients"

Page 89 Misspelling: "we reported the LAIV induced an early B cell and cross-reactive $CD8^+$ T cell responses" – corrected to "we reported that LAIV induced an early B cell and cross-reactive $CD8^+$ T cell responses"

Page 90 Bold word: "paper III" – corrected to "**paper III**"

Page 90 Misspelling and Missing word: "expression T_{FH} surface marker" – corrected to "expressing T_{FH} cell surface markers"

Page 92 Spacing: "A (H1N1)pdm09" – corrected to "A(H1N1)pdm09

Page 92 Spacing: "A(H1N1)pdm09strain." – corrected to A(H1N1)pdm09 strain."

Page 92 Remove full stop: "the U.S." – corrected to "the US"

Page 95-95-98 Bold words: "papers 1 and II" – corrected to "**paper I** and **II**"

Page 95 Misspelling: "237 HCWs provides" – corrected to "237 HCWs provided"

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