Live Attenuated Influenza Vaccine (LAIV) Immunization in Children and Adults: Lesson for Development of Universal Influenza Vaccine

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Thesis for the degree of Philosophiae Doctor (PhD)
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I am dedicating my thesis to my mother, my first teacher who taught me the very first word, the very first alphabet and the numbers that I count now.

You belongs to my every breathe…. I love you!!

To Shahnaj Binu, Sihar and Suhair (my lovely family)

Scientific Environment

The PhD project is funded at the Faculty of Medicine and Dentistry, University of Bergen. By collaboration with the KG Jebsen Center for Influenza vaccine research, I was solely involved at the Influenza Centre in Bergen. The whole scientific expertise and the knowledge I have learnt during my Ph.D. at the Influenza Centre were guided and directly supervised by the Professor Rebecca Jane Cox Brokstad, University of Bergen (UiB). The project managed by Professor Cox and she was my main supervisor, while Professor Bjarne Bogen from University of Oslo acted as my co-supervisor. The PhD project was conducted for four years (2014-2018) and was part of a larger clinical trial studying the immune responses after live attenuated influenza vaccine conducted in 2012-2014 as collaborative work between the Influenza Centre, the Paediatric clinical trial unit and the Ear Nose and Throat (ENT) departments at the Haukeland University Hospital (HUH). All the necessary experiments and the present work in the dissertation were carried out at the laboratory of the Influenza Centre, Department of the Clinical Science (K2). The mandatory courses were attended mainly from the Faculty of Medicine, UiB.

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Shahinul Islam

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Abstract

According to the WHO (World Health Organization), one billion peoples are infected annually of whom three to five millions become severely ill and 250-500 000 deaths worldwide (He, Wang et al. 2013) although the latest research reported 291-645 000 deaths each year (4.0-8.8 per one hundred thousand individuals). Occasional pandemics cause even higher rates of mortality. Controlling influenza infection is a frontline problem for human health. Vaccination is considered the best strategy for reducing influenza infection. However, antigenic drift of influenza requires updating of the vaccine each year to match the circulating virus strains to provide optimal vaccine efficacy. Currently, two different types of vaccines are in clinical use; trivalent inactivated vaccines (TIV) and trivalent live attenuated influenza vaccines (LAIV). Quadrivalent LAIV is also approved and used in some countries. The majority of currently approved seasonal influenza vaccines are TIV, delivered intramuscularly or deep subcutaneously, which can further be subdivided as split-virion vaccines and subunit vaccine based on their formulations (Toback, Levin et al. 2012). On the other hand, only one LAIV is licensed in the USA and Europe for specific at risk populations. The vaccine compromises live attenuated influenza viruses produced by reverse genetics that can replicate efficiently (Coelingh, Luke et al. 2014). Moreover, live attenuated, cold-adaptive, trivalent influenza virus vaccine is administered intranasally representing a convenient, safe and effective approach for the prevention of influenza in children (Belshe, Mendelman et al. 1998). However, strain-matched vaccines often lag behind the antigenic changes in the virus and in the event of a pandemic, there is a time lag of at least six months before the vaccine is available (Epstein and Price 2010). Thus, the concept of 'universal influenza vaccine' is under discussion to reduce all influenza A virus infections by providing broad cross-reactive (heterosubtypic) immunity. Several studies have shown that LAIV can boost virus-specific cytotoxic T lymphocytes, as well as mucosal and serum antibodies and induce broad cross-protection against heterologous human influenza A viruses (He, Wang et al. 2013). Although the integrated approach provides evidence of cross-protective immunity, the underlying mechanism is poorly understood.

This research project therefore mainly addressed some of the fundamental research questions of how LAIV provides protective immunity. We have been shown a significant elevated neutralizing antibody response after LAIV in children measured by haemagglutinin inhibition (HI) assay. Further we dissected haemagglutinin (HA) to head and stalk antibody responses, we observed in children that LAIV significantly elicited H3 head specific antibodies. H1 stalk specific antibodies were also increased but not significantly. In contrast in adults, LAIV did not boost antibody responses (*Paper I*).

We found that H1N1pdm09 virus specific humoral immunity was not boosted in general, although NAI responses were elevated in children. CD4 T-cell responses in blood were also induced against H1N1 vaccine strain. Influenza specific IFN-γ responses increased in children as well *(Paper II)*. The influenza B strain specific IFN-γ responses increased both locally (TMNC) and systemically (PBMC). LAIV resulted in a significant increase in CD8+ T-cell responses post-vaccination in the tonsils suggesting LAIV is able to induce cross-reactive local CD8+ T-cells in the upper respiratory tract *(Paper III)*. In contrast to the observation in USA, our overall results illustrated in this thesis correspond to the response found in other European countries, like UK and Finland. Our finding suggest that the H1N1 vaccine strain in LAIV may have protected children through NAI and T cellular responses, suggesting support for continued use of live attenuated influenza vaccine for children. The multifaceted immune response following LAIV immunization in children suggests LAIV could be used as future universal vaccine for children.

Introduction (motivational)

Research at the Influenza Centre, Bergen has for many years focused on better understanding of influenza virus and vaccines that help to address the control of future influenza infection. The head of the centre, Professor Rebecca Jane Cox Brokstad has a long-standing interest in influenza research. Professor Cox and her collaborators have conducted clinical trials registry and built biobanks from vaccinated children and adults, which provides a unique possibility to investigate live attenuated influenza vaccine (LAIV) specific immunity in both groups. By combining this unique biobank with the expertise on clinical vaccinology, immunology and gold-standard serological methodology residing within the group of Professor Cox, I tried to address the mechanisms of immunity induced by LAIV. The project integrates advanced functional vaccine studies with analysis of Norwegian children and adult samples, and involves local, national, and international collaboration. As such, I believe this project meets many of the research objectives of the KG Jebsen Centre for Influenza research, with translational and clinical research on immunology as well as virology and universal vaccine development strategy as priority areas.

List of publications

I. Islam S, Mohn KG, Krammer F, Sanne M, Bredholt G, Jul-Larsen A, et al. Influenza A haemagglutinin specific IgG responses in children and adults after seasonal trivalent live attenuated influenza vaccination. ***Vaccine. 2017;35:5666-73***.

II. Islam S*, Fan Zhou, Sarah Larteley Lartey, Mohn KG, Krammer F, Cox RJ, Brokstad KA. Functional Immune Response to Influenza H1N1 in Children and Adults after Live Attenuated Influenza Virus Vaccination. ***Revision resubmitted to Scandinavian Journal of Immunology.***

III. Mohn KG, Brokstad KA, Islam S, Offing F, Tønde IC, Årstad MJ, Cox RJ et al. Early induction of cross-reactive CD8+ T-cell responses in tonsils after LAIV vaccination in children. ***Manuscript ready to submit to The Journal of Infectious Disease.***

List of Abbreviations

ADCC	Antibody depended cellular cytotoxicity
ASA	Acetyl salicylic acid
ASC	Antibody secreting cells
CDC	Centers for Disease Control and Prevention
CF	Complement fixation
CPT	Cell preparation tube
cRNA	Complimentary ribonucleic acid
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
ENT	Ear Nose and Throat
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FFU	Fluorescent focus units
GC	Germinal centre
GMT	Geometric mean titre
HA	Haemagglutinin
HI	Haemagglutination inhibition
HRP	Horseradish peroxidase
HLA	Human leukocyte antigen
HUH	Haukeland University Hospital
IAV	Influenza A virus
IFN	Interferon gamma
IIV	Inactivated influenza vaccine
IL	Interleukin
LAIV	Live attenuated influenza vaccine
LAIV3	Trivalent Live attenuated influenza vaccine
LAIV4	Tetravalent Live attenuated influenza vaccine
MBC	Memory B cells
MDV	Master donor virus
MDCK	Madin-darby canine kidney cells
MHC	Major histocompatibility complex
MN	Micro-neutralization assay
MOI	Multiplicity of Infection
MVS	Master virus strain
NA	Neuraminidase
NAA	Nucleic acid amplification
NAI	NA inhibitors
NEP	Nuclear export protein

NIAID	National institute of allergy and infectious diseases
NIPH	Norwegian institute of public health
NK	Natural killer
NLSs	Nuclear localization signals
NP	Nucleoprotein
NPIs	Non-pharmaceutical Interventions
NS	Non-Structural
OD	Optical density
OPD	O-phenylenediamine dihydochloride
OAS	Original antigenic sin
PA	Polymerase acid
PAMPs	Pathogen associated molecular patterns
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBMCs	Peripheral blood mononuclear cells
PNA	Peanut agglutinin
RDE	Receptor destroying enzyme
RIG-I	Retinoic acid-inducible gene-I
RIV	Recombinant influenza vaccine
RMT	Resident memory T-cells
SA	Sialic acid
SFUs	Spot-forming units
SRID	Single radial immunodiffusion
TBA	Thiobarbituric acid
TCID	Tissue culture infectious dose
TCR	Toll-like receptor
Tfh	T follicular helper cells
Th	T helper
TLR	Toll-like receptors
TMNC	Tonsillar mononuclear cell
TNF	Tumor necrosis factor
VLPs	Virus-like particles
vRNA	Viral ribonucleic acid
WHO	World Health Organisation

Contents

1 Introduction

1.1 Influenza A Virus

Influenza virus is a member of the *Orthomyxoviridae* virus family and is a single stranded negative sense RNA virus with eight gene segments. Four types of influenza virus have been identified (A, B C and D). Types A and B are commonly circulating in humans and causes disease, whereas, type C is associated with mild discomfort mainly in children. Influenza type D virus has recently been isolated from pigs and cattle [1]. Influenza A viruses infects several mammals and bird species and animals, many of them are domesticated like pigs, horses, ducks, chicken and turkey. The natural reservoir for influenza A is believed to be waterfowl, aquatic birds and occasionally the virus is also identified and isolated in wild aquatic mammals like seals and whales. Influenza B and C viruses are mainly human pathogens [2, 3]. The influenza A genome contains eight RNA segments coding for at least 17 proteins including 2 surface glycoproteins, HA (Haemagglutinin) and NA (Neuraminidase). Classification of influenza A viruses into subtypes is based on these two surface glycoproteins. Currently, there are 18 known subtypes of HA (H1-H18) and 11 subtypes of NA (N1-N11) and allowing different subtypes (H/N combinations) e.g. H1N1, H5N1 or H3N2 [1, 4].

1.1.1 Viral Structure

Influenza virus is mainly pleomorphic but also found spherical in structure, but elongated filamentous forms can also be observed. New progeny virus measures 80-120 nm in diameter. The virion is enveloped with a lipid bilayer containing surface glycoproteins HA and NA and the ion channel (M2). Influenza A virus (IAV) has eight segments of negative sense RNA genome which range between 890-2341 base pairs in length [5]. Each RNA segment encodes at least one essential protein. The RNA encodes segments as for the following genes Polymerase basic 2 (PB2), Polymerase basic 1 (PB1), Polymerase acid (PA), HA, Nucleoprotein (NP), NA,

Matrix (M) and Non-Structural (NS) [6]. A schematic diagram of influenza A virus is illustrated below in Figure 1.

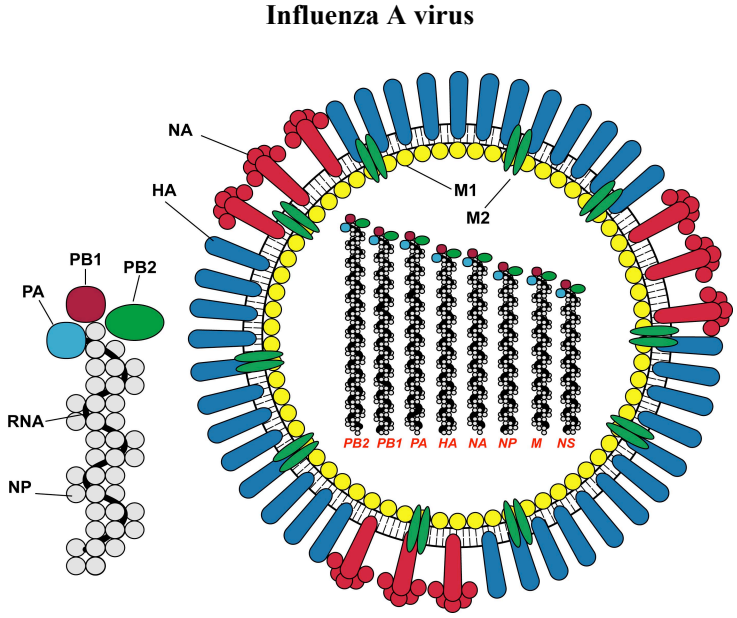


Figure 1. Illustration of Influenza A virus structure showing the important surface glycoproteins, the location of the others virus proteins and the 8 negative sense RNA segments.

1.1.2 Haemagglutinin

Haemagglutinin (HA) is the major surface glycoprotein of influenza A virus. The HA is a trimer composed of three monomeric HA proteins The HA is produced as an immature polypeptide chain or precursor (HA0), which is cleaved by the host's proteases to produce two distinct subunits; the head (HA1) and the stalk (HA2) The globular head (HA1) contains the receptor binding site and the main antigenic sites and is prone to mutation enabling the virus to escape the hosts immune response. This process of accumulated mutations introduces changes in the main epitopes is called 'antigenic drift'. The HA2 together with N-and C-terminal HA1 residues form the

most conserved transmembrane stalk domain. The stalk is functionally responsible for fusion of viral and endosomal membranes. The function of the HA is to facilitate binding of the virus to the host cell's sialic acids (SA). Sialic acids are widely distributed on many different cells in the respiratory tract, such as the epithelial cells, dendritic cells and alveolar macrophages. The binding affinity of the virus depends upon the type of sialic acids and their associated oligosaccharides, with human influenza viruses preferentially binding α2,6 linked SA [6].

1.1.3 Neuraminidase

The second surface glycoprotein of influenza virus A is neuraminidase; a homotetrameric structure with a mushroom shape and highly conserved active sites. The NA protein is coded by the 6th segment of RNA. It comprises 470 amino acid residues and several domains; the globular head domain that is connected to stalk domain rooted in the viral membrane by a hydrophobic transmembrane region. The NA stalk can vary in length depending on adaption to the host, but the enzymatic active site and the calcium binding sites lies on the NA head and potentially stabilizes the NA structure at low pH in the presence of calcium ion [7-9]. The function of NA is well characterized and involved in different phases of the virus infection life cycle. Neuraminidase is mainly involved in releasing progeny virions by cleaving sialic acids from the cell surface and facilitating the virus release from the infected cells [10]. Importantly, NA is also involved in virus entry and actively plays a role in binding the virus to the host cell membrane [11]. NA cleaves sialic acid from the respiratory mucine, releasing the virus and therefore helping the virus to reach the target cells [10]. Interestingly, in mucus NA facilitate the cleavage of the α-ketosidic linkage between the terminal sialic acid and neighbouring sugar residue to facilitate virus in reaching the target cells, whereas HA binds to the sialic acid residues to promote the virus entry. The receptor destroying function of NA, and its complementary activity to HA of binding to the sialic acid receptor shows the importance of their co-operation and/or competition for infection [8].

1.1.4 Internal Virus Proteins

Nucleoprotein (NP) and the polymerase complex proteins (PA, PB1 and PB2) are the internal proteins that play important roles in viral transcription and replication. The polymerase complex is a heterotrimer formed by PB1 with the PA on one side and by the N-terminal domain of PB2 on the other side [12]. The nucleoprotein encapsidates each viral RNA (vRNA) segment and support viral RNA synthesis. Viral mRNA transcription from vRNA is initiated by the primers and also generated through PA dependent cap snatching of host mRNAs [13]. The PB2 generally binds to the capped RNAs [14] and PB1 performs the actual RNA synthesis [15-17]. The polymerase complex lacks proof reading capability, which consequently results in a relative high gene mutation rate, contributing to antigenic drift and is a major determinant of viral virulence and host adaptation. However, the molecular structure of the polymerase complex has been elucidated and future interpretation of their function by inhibition and/ or blocking could improve antiviral strategies [18].

1.1.5 Matrix And Non-structural Proteins

Matrix proteins, M1 and M2, are encoded from a single gene segment 7 (M). M1 is the structural component with lipid binding properties and interacts with the lipid membrane of influenza virus. The main function of M1 is export of the vRNPs to the cytosol from the nucleus which occurs at the later stages of influenza infection [19]. Moreover, the post-translational cellular phosphorylation is necessary to prevent re-uptake of the vRNPs to the nucleus by M1 [20]. M2 is a transmembrane protein, which act as an ion channel that makes a pore in the viral envelope. M2 plays a crucial role in the early stages of viral replication by promoting an acidic environment to facilitate viral uncoating [21]. The M2 also functions at the later stage of infection, M2 protein is important in viral budding and allows release of new virions for further infection [22].

The two non-structural internal influenza proteins are NS1 and NS2, which are alternative splicing products encoded from the smallest gene (segment 8) of the viral

genome. In brief, NS1 is a pleiotropic virulence factor that interacts with multiple cellular components. It plays an important role in interfering with the type I interferons produced by the host system to repress innate antiviral mechanisms and thereby inhibit host IFN responses [23, 24]. NS1 can interfere with the host cell mRNA processing unit to repress nuclear export of cellular mRNA [25] and interfere with mRNA translation to favour viral protein translation [26]. NS2 was re-named as nuclear export protein or NEP recently. NEP is involved in helping M1 to export vRNP from the nucleus to the cytoplasm [27]. Both NEP and more specifically NS1 are considered as potential target for the development of antiviral drugs.

1.1.6 Influenza A Virus Molecular Structure And Replication Cycle

The spherical influenza A virus with its genome segments inside is covered by an outer host cell derived lipid bilayer membrane containing the glycoproteins HA and NA; at a ratio of one NA to four HAs, and also the transmembrane ion channel M2. The HA head, with its receptor binding domain, attaches to N-acetylneuraminic acid of the host respiratory epithelium cells through α2,6 linkage in humans. Many bird viruses has high affinity to the α2,3 linkages, while pig adapted virus may binds both α2,3 and α2,6 sialic acids. NA also helps the virus to reach the target cell by enhancing cleavage of terminal sialic acid from its neighbouring sugar residue [28]. These molecular properties of surface glycoproteins facilitate virus attachment to the host cells and initiate the virus entry through clathrin mediated endocytosis process. The transmembrane M2 ion channel plays an important role in virus entry thereafter. The M2 ion channel, found only in influenza viruses, is responsible for triggering the change to the acidic environment in endosomal vesicles by pumping the hydrogen ions inside the virus particle from the endosome and lowering the pH. The acidic change facilitates a conformational change in the HA structure catalysed by the host protease (serine) to expose the fusion peptide. The fusion peptide in the HA stalk is cleaved and allows the triple α-helix bundle to extended leading to fusion of the viral and endosomal membranes. This fusion enables the virus uncoating allowing release of the viral genome segments into the host cell cytoplasm. Once the viral genome is

released into the cytoplasm, transport signal or nuclear localization signals (NLSs) translocate and direct the vRNP to the nucleus (reviewed in [29, 30]).

Most negative sense RNA viruse replication occurs in the cytoplasm, but influenza virus is different (Figure 2). Influenza virus performs its replication cycle in the nucleus of the host cell by interacting with host cell nuclear machinery [31]. Influenza viral genome transcription is initiated by the viral RNA dependent RNA polymerase complex and through viral mRNA synthesis. Newly transcribed mRNAs are then transported to the cytoplasm for translation. The replication is also carried out by producing a positive strand complimentary RNA (cRNA) from viral genome to use as an intermediate template, which is then transcribed to vRNA. Newly replicated vRNA coated in nucleoprotein and the RNA polymerase, are then directed to the infected cell membrane for assembly. The newly formed virions are then released by budding process with help from NA.

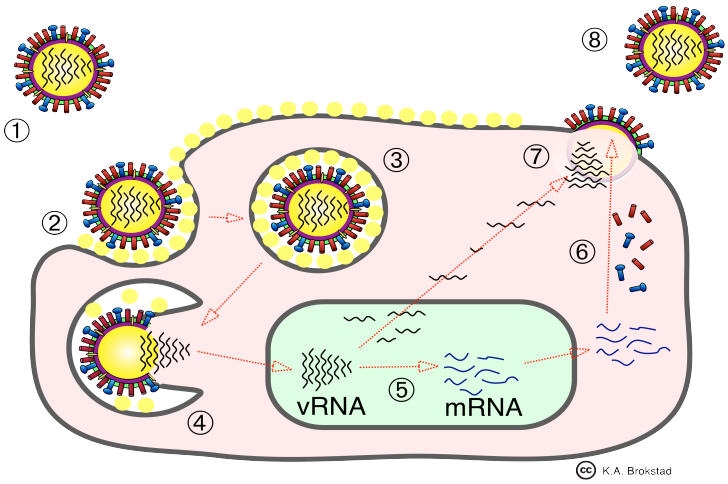


Figure 2. Illustration of influenza A virus life cycle, 1) free virion, 2-3) attachment and endosomal uptake, 4) genome release, 5) viral replication to vRNA and mRNA, 6) synthesis of viral proteins and glycoproteins, 7) assemble and 8) release.

1.2 Natural Infection by Influenza Virus And Burden of Disease

1.2.1 Influenza Virus History: Epidemics And Pandemics

Influenza associated illness have been observed and reported for many centuries, although the causative virus was not identified until the 1930s [32], first in pigs and later in human [33]. The established reservoir of most subtypes of influenza A viruses are aquatic birds, except H17 and H18 which are found in bats, although a wide range of species are also susceptible such as pigs, horses and humans. Phylogenetic analysis suggests that the known mammalian influenza A viruses are mostly derived from the avian reservoir [34]. In man, the virus is able to infect all year round globally, but the large wave of infection is often seen in the winter months in the Northern and Southern hemispheres. Worldwide evolutionary studies illustrate distinct patterns of influenza infection with different circulating strains. Therefore the southern and northern hemispheres have significant differences in dominant prevailing influenza strains [35]. The frequent migration of aquatic bird is often responsible for transmission of influenza both intra- and inter-species including zoonotic transmission to man. The high density of human population, open yard poultry production (hens, ducks etc.), live-poultry or animal market provides the ideal condition for influenza virus natural reassortment. The transmission of influenza between avian species has increased particularly in Asia, which also increases possible zoonosis. Poultry farms can be a source of highly pathogenic influenza virus, which can infect poultry workers and potentially spread to others [36]. Pigs are also considered as the ‘mixing vessel’ containing both receptors for avian (sialic acids with α-2,3-galactose linkage) and human (α-2,6-galactose linkage) viruses.

The potential of influenza viruses is due to the antigenic variation in the two major surface glycoprotein of the virus; the HA and NA. The variation occurs due to the point mutations (including insertion, substitution and deletions) in HA and NA genes, in a process known as ‘antigenic drift’. As influenza virus lacks proof reading machinery this allows the genetic variation to be transcribed during the viral replication cycle. If an antigenic change in the surface glycoproteins occurs that

allows the virus strain to escape neutralization by existing immunity against the previously infected strains.

Epidemic; Epidemics are the major wave of seasonal infection, which occur due to **'antigenic drift'** of the circulated strain. Due to the genetic alterations in the viral genome, the novel virus is often closely related to previously circulating strains, resulting in sporadic localized outbreaks or epidemics. Amino acid changes in the antigenic sites in HA and NA allow the virus to escape existing immune defences. Current epidemics are mostly caused by influenza A H3N2 strain, Influenza A H1N1 or the influenza B virus.

Pandemic; A novel virus can arise as a result of reassortment of circulating and animal viruses or multiple influenza infections in a host at the same time. This novel virus can then spread globally if the population is immunologically naïve and this is referred to as **'antigenic shift'.** This causes a pandemic, which is a large-scale global outbreak with a novel virus that overcomes a weak community resistance. The result is associated with global transmission with high morbidity and high mortality. Several pandemic outbreaks have occurred with millions of deaths. The 1918 pandemic was caused by the H1N1 subtype, resulting in over 20 million deaths and is also known as Spanish flu. Thereafter, pandemics occurred in 1957 (Asian pandemic, H2N2 subtype), and 1968 (Hong Kong pandemic, H3N2 subtype), in 1977 the re-emergence of the H1N1 subtype (Russian pandemic) and very recently 2009 pandemic (Swine flu).

1.2.2 Global Flu Burden

The Spanish flu in 1918 was the deadliest influenza pandemic known to man, and is thought to have infected approximately one-third of the world's population, at that time 500 million people and with at least 20-50 million death estimated [37]. As a consequence, the World Health Organisations (WHO) influenza surveillance system was adopted in 1947 and has for more than 80 years continuously measured the burden of influenza around the world. The annual global burden depends upon the circulating virus types and subtypes for influenza A, status of the immunity in the

population and vaccination coverage, alongside the vaccine match to circulating strains. Annually influenza infection related illness, absenteeism, hospitalization and deaths have great socio-economic impact. Seasonal influenza infection is mostly associated with affecting elderly and people in high-risk groups, whereas, pandemic influenza is often associated with high mortality rates across the population particularly in the young and elderly. Although the elderly did not suffer high mortalities rates during the swine 2009 pandemic, probably due to cross-reactive antibody from previous older H1N1 strains [38]. Recent studies demonstrated the actual number of influenza death was underestimated in the 2009 pandemic. The WHO reported approximately 250-500 thousands of deaths annually from seasonal influenza infection, which is 3.8-7.7 individuals per one hundred thousand people. The latest research reported that seasonal influenza causes 291-645 thousands of deaths, which is 4.0-8.8 per one hundred thousand individuals [39]. In the USA alone, the Centers for Disease Control and Prevention (CDC) estimates 9.2 -35.6 million illnesses with influenza symptoms and 140-710 thousands of hospitalization, with the mortality reported as between 12-56 thousands each year since 2010 [40]. In children, the influenza A subtypes (H1N1 and H3N2) and Influenza B cause higher prevalence rates of cough, wheezing, vomiting and convulsions compare to the children with other respiratory infections [41]. The influenza related illness causes a heavy global burden especially due to high rates of illness in the high-risk groups (Table 1), like young children, elderly people and pregnant women. Importantly, influenza-like illness has great socio-economic impact on children and their families due to need for healthcare and absence from day care or the work place. Thus, the direct effect on children and indirectly on families causes a heavy financial burden [42-44].

Table 1: High risk groups recommended for annual vaccination against seasonal influenza

Norwegian recommendation (NIPH)	International recommendation (WHO)
Pregnant women • After 12 weeks of pregnancy (2^{nd} 3^{rd} trimester) and during 1^{st} trimester in some cases	**Pregnant women** • Highest priority for increased risk of severe disease and fatal outcome due to influenza, which causes stillbirth, neonatal death, pre-term delivery and low birth weight
Children and adults with • Diabetes mellitus, type 1 and 2 • Chronic respiratory disease • Chronic cardiovascular disease • Chronic liver failure • Chronic renal failure • Chronic neurological disease or injury • Immunodeficiency disorders • Severe obesity (BMI over 40) • Other severe or chronic illness evaluated on an individual basis by a doctor	**Children aged < 6 months** • Cannot be vaccinated but can be protected specifically through vaccination of their mothers during pregnancy **Children < 2 years old** • Should be target group **Children 2-5 years old** • The group have high burden of disease. When available, LAIV immunization showed higher protection and broader protection **Person with specific chronic disease**
Elderly > 65 years	**Elderly individuals ≥ 65 years of age** • Highest risk of influenza associated mortality
Subjects in nursing homes and sheltered accommodation	
Others • Health professional with patient contact • Household contacts of immunosuppressed patients • Pig farmers who have regular contact with live pigs	**Others** • Heath-care workers • International travellers

* NIPH stands for Norwegian Institute of Public Health and the information provided [45]
* The information based on the WHO's (World Health Organization) position paper published in November 2012 [46]

1.3 Immunity Against Influenza A Virus (IAV) Infection

Infection with influenza virus occurs generally in the upper respiratory tract through the oral or nasal cavity with brief encounter of the mucous layer, which covers the epithelial cells. The mucosal immune system is therefore the first line of defence against influenza infection. Mucosal immunity has specialized defence mechanism providing protection against invasion of potential mucosal pathogens, like influenza virus [47]. Some highly pathogenic influenza virus successfully can invade the mucous layer and attach to the epithelial cells, where the front line defence is initiated [48].

1.3.1 Innate And Adaptive Immunity

The innate immune system provides [49] immunity by recognition of the pathogen associated molecular patterns (PAMPs) of IAVs by pathogen recognition receptors (PRRs). Retinoic acid-inducible gene-I (RIG-I) and toll-like receptor (TLR) are PRRs which cause activation of hundreds of genes that also are known as ISGs or IFN-stimulating genes, responsible for induction of the innate immune defence (Figure 3). PRRs can distinguish self from non-self-molecules within the infected cells and also cause the secretion of type I IFN, pro-inflammatory cytokines, eicosanoids and chemokines. By playing distinct roles, inflammatory cytokines and eicosanoids induce the early symptoms of infection in host but also inform the adaptive immune system about the influenza infection. At the same time, chemokines are produced and secreted at the site of infection, which leads to recruitment of others immune cells including natural killer (NK) cells to facilitate phagocytic viral clearance. Together with IFNs-I producing macrophages, DCs and pDCs, phagocytic cells of the innate immune system provide protection by clearing virus-infected cells through complex mechanisms. Based on the location, macrophages are the one of the earliest cells that responds to the influenza virus. The importance of macrophages against influenza infection is very critical but essential for protection. They internalize the virus and enhance lysosomal degradation, remove the degraded debris of apoptotic cells. And

most importantly, macrophages help to induce adaptive immune response by antigen presentation to the naïve T-cells.

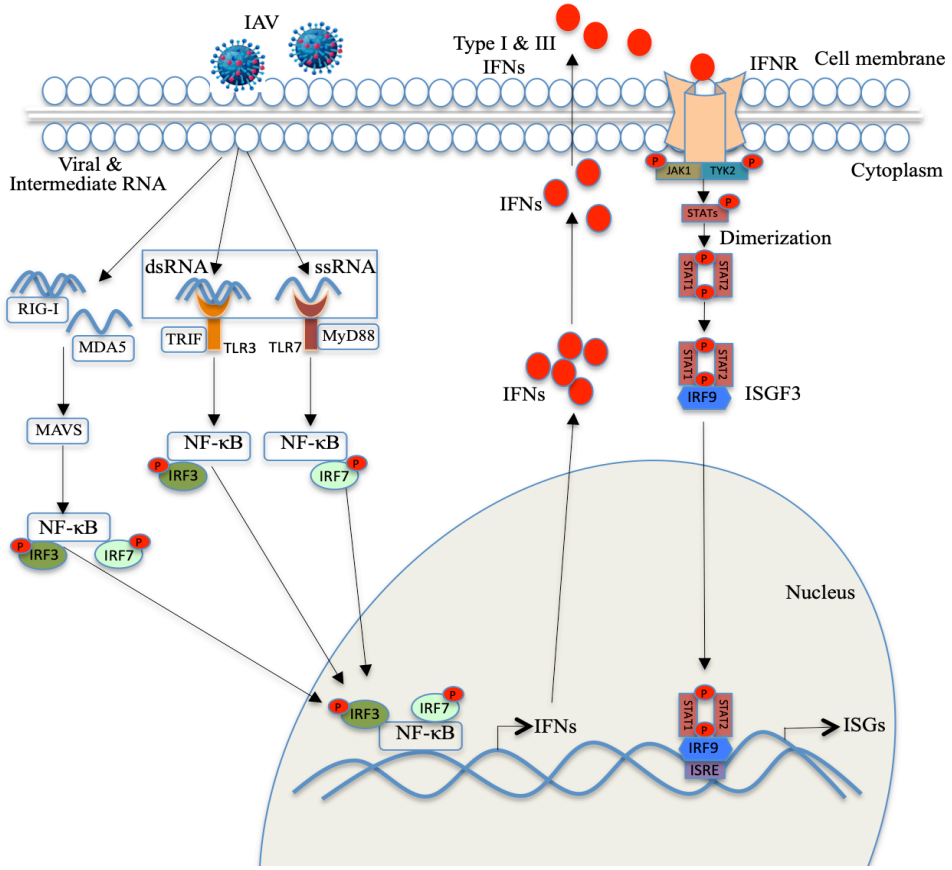


Figure 3. Activation of innate immune system against influenza A virus (IAV) infection. Influenza virus recognized by PRRs PAMP/DAMP and initiates signal transduction and a cellular pathway, reaching transcriptional factors in the machinery regulating antiviral genes.

If a virus penetrates the innate immune defence barrier, infection can successfully be established. Thus, the ultimate clearance of the virus or virus-infected cell is dependent on the adaptive immunity. Adaptive immunity comprises both B- and T-cells that play important roles against IAV infection. B-cells produce antibodies to combat infection and reduce viral replication. Antibodies directed to the HA head region can neutralize the virus preventing infection of the host cell. Antibodies can

also mediate other effector functions to kill influenza virus infected cells, known as antibody dependent cellular cytotoxicity (ADCC) [50-52].

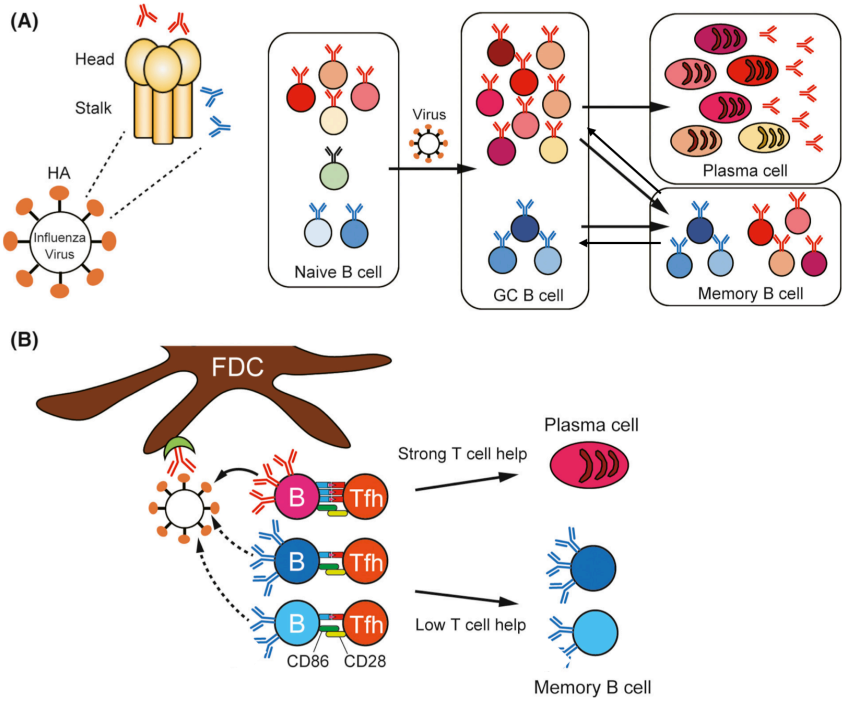
CD8+ T-cells are activated from T-cell zone enriched with migrated DCs and differentiated into cytotoxic T-lymphocytes (CTLs). Through the infection signal, CTLs initiates signals to produce cytokines and immunomodulatory molecules to restrict influenza virus replication. Cytotoxic T-lymphocytes are therefore involved in reducing viral shedding by killing the infected cells [53]. CD4+ T-cells are another major type of immune cells that play key roles in the adaptive immune system. CD4+ T-cells are also known as T-helper (Th) cells and can differentiate into Th1, Th2, Th17 and regulatory T-cells among others [54]. In response to influenza virus infection, CD4+ T-cells become activated, differentiate and predominantly produce co-stimulatory molecules, cytokines to regulates T-cell response [55]. They are importantly involved in regulation of T-cell mediated B-cell response [56] and sometimes promotes killer cells to combat influenza virus [57]. Tfh cells are another specialized subsets of CD4+ T-cells exclusively found in lymphoid tissue and necessary for germinal centre formation in secondary lymphoid organs (SLOs). Germinal centre B-cells proliferate rapidly [58] and produce high affinity antibodies with great diversification and thus provide immune response often induced by immunization against influenza [59]. Tfh and FDC (follicular dendritic cell) cells also regulate the process to produce antibody secreting plasma cells and memory B-cells [60].

1.3.2 Humoral Immunity

Humoral Immunity is the immunological responses provided by B-cell and by secreted antibodies. In influenza studies, the haemagglutination inhibitory (HI) antibody titre $\geq$ 40 has been used as a correlate of protection after infection or vaccination, and has been widely used for vaccine licensing for decades. B-cells are originating from the bone marrow [61]. The naïve, but mature B-cells expresses surface IgD antibodies along with IgM when circulates in the blood and lymphoid tissue. After antigen recognition, the B-cells migrate to T-cell rich area of lymphoid

tissue. Here the B-cells become activated by T-helper and Follicular T-helper cells, and they can proliferate and differentiate into antibody secreting plasma cells (ASC) and memory B-cells [62]. Upon re-exposure to antigen, secondary immune responses are initiated found by mainly memory B cells. While the primary immune response often are dominated by IgM, the secondary immune response shows signs of isotype switching, producing additional IgG, IgA and IgE antibodies. IgM is secreted as a pentameric antibody, but often have low affinity and avidity in contrasts with the higher affinity antibodies expressed by memory B-cells [63].

Antibody-secreting plasma cells can be either short-lived or long-lived. They produce antibodies, which are strain specific and can be detected in the blood within 5-7 days of infection or vaccination depending on previous priming [64, 65]. IgA and IgG antibodies play important roles in influenza immunity. IgA antibodies provide the initial immune defence at the portal of viral entry; the respiratory mucosa, whereas IgG provides systemic protection against influenza virus infection [66, 67]. MBC in comparison, activate and differentiate into plasma cells when activated by an antigen to mount the specific antibody response (Figure 4) [68, 69].



modified from https://doi.org/10.1111/imr.12640

Figure 4. Schematic illustration of how influenza virus infections lead to HA head/stalk specific B-cells activation, differentiation, proliferation and memory B-cell generation. *A,* Antibodies to immunodominant viral epitopes are marked in red (anti-haemagglutinin (HA) head domain), and antibodies towards conserved viral epitopes are marked in blue (anti-HA stalk domain). *B,* Illustration shows the recruitment of broadly reactive GC B-cells into the memory compartment mediated by T-cells help. Low affinity broadly reactive GC B-cells are more prone to be recruited into memory B-cell compartment.

This strategy is exploited in priming and booster strategies for influenza vaccines [70]. Interestingly, MBC circulates from bone marrow to the periphery and the lymphoid organs for many years and may be for life-time.

1.3.3 Cellular Immunity

Cellular immunity is the effector function of both CD4+ and CD8+ T-cells. Antigen presenting cells (APCs) such as dendritic cells (DC) present antigenic peptide fragments bound to the major histocompability complex (MHC). T-cells require the antigen to be processed and presented by either MHC-I on circulating T-cells or

MHC-II on APCs before antigen recognition. In addition to antigen recognition, naïve T-cells also need stimulus from DC to become activated. The progenitor T-cells produced in the bone marrow are transported to the thymus to be matured along with the complex process of T-cell receptor (TCR) genes and diversified membrane marker expression. Proliferation and differentiation occurs to generate functionally distinct subpopulations mainly CD4+ and CD8+ T-cells. CD4+ T-cells are principle component of immune regulation [71] that become activated by recognising the antigenic peptide bound and expressed with co-stimulatory molecules with the MHC class II. Importantly, APCs (DC) can also present peptides to the CD8+ T-cells via MHC class I producing cytotoxic CD8+ T-cells, which are important in viral clearance. Activated T-cells are thereafter regulated by the production of different cytokines that also co-ordinate the T-cells clonal expansion and differentiation into both effector and memory cells.

CD4+ T-cells plays key role in influenza prevention through the effector functions, which are involved in viral clearance either by directly killing infected cells or helping other cell types including B-cell stimulation and cytotoxic CD8+ T-cell activation. Activated B-cells produce antibodies, whereas macrophages destroy the ingested microbes upon activation. The important feature of the naive CD4+ T-cells is that they differentiate into different subsets upon interaction with APCs and regulate cellular immunity with distinct functions of the subtypes based on the infection environment, types of APCs and co-stimulatory molecules through specific cytokine production [72]. Naive CD4+ TCR coupled with CD3 initiate activation through antigen-MHC II complex that induces the downstream signalling pathway.

Consequently, naive cells proliferate and differentiate into specific effector cells. Thus major subsets are the Th1 and Th2 cells. Both Th1 and Th2 cells are differentiated from naive CD4+ T-cells upon IL-12 and IFN-γ secretion [73], whereas, IL-4 and IL-2 are critical for Th2 differentiation [74]. The signalling pathway for Th1 differentiation starts with large amounts of IL-12 production from APCs, which in turn induces NK-cells to produce IFN-γ [75]. Th1 cells mainly secrets IFN-γ and IL-2, but also TNF- □, and are essentially involved in elimination

of intracellular pathogen. IFN-γ in particular is involved in activation of macrophages and microglial cells; the mononuclear phagocytes that enhanced phagocytic activity [76]. TNF□□ potentially accelerates apoptosis and thereby acts as an immunosuppressive agent. One study reported increased TNF-□ activation after influenza infection by NA which is thought to be involved in host response to mediate apoptosis [77]. The most potent cytokine produced by Th1 cells is IL-2 that promotes effector CD8+ T-cells for cytotoxic action [78]. IL-2 is an important growth factor that plays a role in T-cell growth but also increases the production of CD8+ memory T-cells. Thus by ensuring sufficient production of memory cells that circulate in the blood, lymphoid organs or periphery, IL-2 facilitates more rapid and strong secondary immune responses after antigen priming [79]. Interestingly, thymus derived regulatory T-cells need IL-2 to survive and thus plays a major role in immune suppression upon Treg activation [80]. Th2 cells are characteristically distinguished by their cytokine production of IL-4, IL-5, IL-10 and IL-13. Among them, the important function of IL-4 is to promote B-cells to IgE switching and secretion in the mucous against infection inflammation [81]. IL-10 causes inhibition of innate immune cells, as well as Th1 cells leading to maintenance of homeostasis after clearance of infection [82].

Recent studies have also identified others subtypes of differentiated CD4+ T-cells besides the classical Th1 and Th2, including Th17, Tfh, iTreg, Tr1, Th3, Th22 and with very recently Th9. The orchestration of naive CD4+ T-cells differentiation is illustrated below in Figure 5, involving direct and indirect cellular immunity after interaction with antigen presenting dendritic cells.

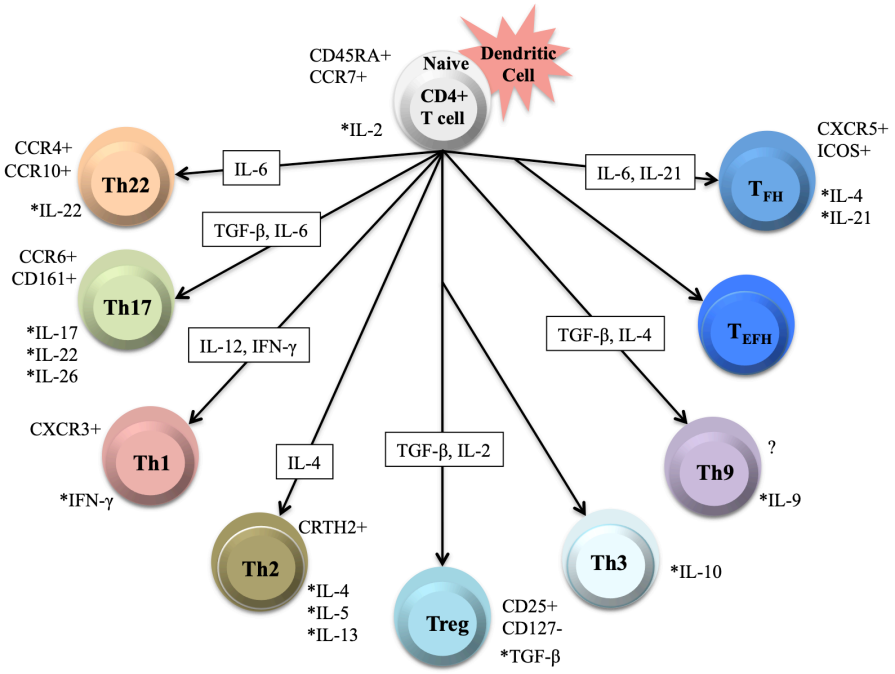


Figure 5. The naïve CD4+ T-cells differentiation into subtypes, which are characterized by the cytokines secreted.

Th17 CD4+ T-cells have been found to be increased after intranasal influenza vaccination in mice and contributed to increased morbidity rather than viral clearance [83]. Interestingly, in hospitalized patients infected with severe pandemic H1N1 high level of Th17 mediators were found [84]. Follicular T-helper cell subsets of CD4+ T-cells have been studied in recent years in influenza immunity as they have a role in promoting B-cell responses upon infection. CXCR5+CD4+T (Tfh) differentiate from CSCR5-CCR7+CD4+ naïve cells and play significant role in producing long-lived memory B-cells in the germinal centre. Based on the cytokine environment, Tfh-cells are also grouped into Tfh1, Tfh2 and Tfh10 with distinct functions. IFN-γ secretion by Tfh1 promotes IgG2, whereas Tfh2 produce IgG1 and IgE with the help of IL-4. Most importantly, Tfh10 facilitates mucosal defence against infection by IgA production upon IL-10 secretion [85].

CD8+ T-cells or CTLs (cytotoxic T-lymphocytes) mediate their function in clearing influenza-infected cells by two distinct pathways; cell lysis and apoptosis. Activated CD8+ T-cells primarily detect infected influenza cells by their TCR receptor and cause direct cytotoxic lysis by the Fas-dependent pathway or by perforins [86]. Secondly, clearance of virus-infected cells occurs through secretion of different cytokines [87, 88].

1.3.4 Target Immune response by Live Attenuated Influenza Vaccine (LAIV)

Live attenuated influenza virus is strain with very low pathogenicity and replication ability and can be used as vaccine, administered as nasal spray at the upper respiratory tract. Similar to the natural infection, LAIV induce multifaceted immune responses including plasma cells to produce both neutralizing and non-neutralizing antibodies. Importantly, LAIV can elicit cellular immunity (CD4+ and CD8+ T-cells) in children [89, 90]. LAIV mimics natural influenza infection, also induces broad cellular response and shown protection in animals against heterosubtypic influenza strains [91-93].

Cell mediated immune protection against influenza by boosting CD8+ T-cells and CD4+ T-cells after LAIV immunization has been shown. And thus proved the concept of LAIV that the vaccine has potential impact on universal influenza vaccine development [94, 95]. Priming, pre-existing immunity and age plays important roles in the immunogenicity of LAIV. LAIV boosts T-cell responses in children that correlate with protection. The evidence clearly suggests that effector CD8+ T-cells cannot prevent infection but play crucial roles in reducing disease severity by killing and elimination of the virus infected cells to clear the infection. Effector CD8+ T-cells also facilitates viral clearance by recruiting both innate and others adaptive immune cells through secretion of anti-viral cytokines and chemokines. Recovery from highly pathogenic influenza A H7N9 virus is associated with memory CD8+ T-cell response that provided protection [96]. Influenza specific memory CD8+ T-cells were found as a correlate of protection against the pandemic influenza in the absence of cross-reactive neutralizing antibodies [95].

Memory T-cells after LAIV immunization also showed robust induction of cellular immunity that causes reduction of the viral shedding although the underline mechanism has not been fully explained yet [97]. A key feature of memory T-cells is of cross-protection. Immediately after the viral clearance, the majority of effector plasma cells undergo apoptosis but a few remain in circulation as long-lived memory T-cells [98]. Upon recognition of a similar infection, memory cells initiate a rapid but massive clonal expansion and differentiate into the secondary effector T-cells to robustly clear the infection. Based on their functionality, memory T-cells are also grouped into effector memory T-cells and central memory T-cells. The central memory T-cells in the secondary lymphoid organ do function after meeting presented antigenic peptides and act more rapidly. They are activated T-cells that sit dormant in its position with low metabolic activity and wait to be reactivated. Upon reactivation, the memory T-cells become quickly active and can go directly into action. The effector memory cells are functionally like newly stimulated effector T-cells in the blood and non-lymphoid tissue [99]. The non-circulating tissue-resident memory T-cells subset (RM) has also been recently characterized and provides the greatest protection against influenza infection [100]. RMT-cells enter into the tissue during their effector function phase and last until they recognize the antigenic peptides after selective expression of CD69 and CD103 [101, 102]. It is believed that resident-memory T-cells protect against influenza at the mucosal site but both CD4+ and CD8+ RMT-cells are increased in the lungs of humans after recovery and may be a correlate of protection against influenza virus infection [100-104].

LAIV boosts memory B-cells responses in children as well and robustly induced antibodies to closely related influenza strains. Thus, B-cell mediated humoral immunity plays a crucial role to develop protective immunity against influenza infection through the activation of the cellular compartment by the help of different cytokines and chemokines production. Therefore, increased knowledge on LAIV studies will have great implications in future vaccine development.

The conserved HA stalk specific immunity after LAIV vaccination is thought to be more heterosubtypic, which can provide cross-protection [105-107]. Several studies

demonstrated that stalk antibodies also recruit non-neutralizing antibodies to mediate cytotoxic destruction of infected cells, such as recruitment of NK-cells facilitates ADCC [51]. Therefore, the HA stalk is also considered as a potential target for future vaccine development [108-110]. Therefore, elucidation of the specificity of the stalk epitopes B-cells and T-cells and investigation of their function as well in downstream signalling will have the great importance. Furthermore, HLA or human leukocyte antigen complex encoding the MHC class-I or MHC class-II is crucial in antigenic peptide recognition. The HLA typing that customizes the epitopes (antigenic fragment) specificity often found to increase CD8+ and CD4+ T-cell responses and provide cross-protection [111, 112]. The approach can cover the large human population and could be useful for development of future vaccine development [113].

1.4 Availble Diagnostic tools, Preventive measures and Treatment of Influenza

Diagnosis of influenza virus infection is often critical from a clinical perspective [114, 115]. The symptoms of influenza are associated with fever, muscle aches, headache, dry cough, sore throat, nasal congestion and runny nose that often provide discomfort to the infected individual and share clinical symptoms with others respiratory diseases including adenovirus, pneumoniae and respiratory syncytial viruses. General influenza infection associated discomfort resolves within a week and most people do not require medical assistance but hospitalization may be required based on the disease severity. Therefore, accurate and timely diagnosis of hospitalized influenza patients is important for initiation of antiviral treatment [116, 117]. To date, several tests have been developed and are widely used for diagnosis of influenza virus infection, requiring respiratory specimen collection and antigen detection or nucleic acid amplification (NAA).

After collection of respiratory specimens, influenza virus can be detected by the rapid tests, like PCR/Q-PCR, PCR ligation. *Nucleic acid amplification test (NAA)* by reverse transcription polymerase chain reaction (RT-PCR) is considered as the gold

standard for use in hospital or clinical environment due to the higher sensitivity. Moreover, influenza virus RNA can be stable for detection for several days by RT-PCR regardless of sample collection, transport and processing time [114]. Virus activation assay is also used as non-rapid test. If the above mentioned diagnosis results are negative or unavailable, *serology* can be used to confirm influenza infection [118]. The common serology assays are, haemagglutination inhibition assay (HI), micro-neutralization assay (MN), complement fixation, enzyme-linked immunosorbant assay (ELISA) and enzyme-linked lectin assay (ELLA), which can be used for measuring both neutralizing and non-neutralizing antibodies [2].

Prevention is the primary measure to control influenza and always considered as more effective than treatment. Prophylaxis is recommended for preventing influenza infection and its severe complications, with annual vaccination recommended for high-risk groups. Although, vaccination rates have increased worldwide the vaccine coverage still needs to be improved to reach the 75% vaccination rate recommended by the WHO [119]. Particularly underdeveloped and developing countries have poor access and consequently poor influenza vaccine coverage rates [120]. These countries are particularly hard hit when a new pandemic emerges. The animal reservoir represents a considerable threat for the emergence of a new pandemic virus. Since, the emergence of the influenza A H7N9 virus in March 2013, China has experienced sixth zoonotic waves with a total of 1567 laboratory-confirmed human cases reported including 615 deaths so far [121, 122]. Vaccination is the most effective means to limit the influenza infection, and the severity of influenza associated morbidity and mortality. Although, antigenic mismatch between a vaccine virus strain and the circulatory strain in a year could potentially cause the lower vaccine efficacy nevertheless influenza vaccine is still cost effective. As we experienced during the 2009 pandemic influenza virus infection, a period of at least six months is required before vaccine is available. For this reason preventive Mitigation strategies like, NPIs (Non-pharmaceutical Interventions) are now recommended when vaccines are not yet available [123, 124]. These include frequent handwashing, good cough hygiene, isolation of infected patients and use of face masks to reduce influenza viral transmission [125, 126]. Influenza virus transmits in humans mainly through air

droplets released by coughing, sneezing or talking with a sick individual. Transmission can be direct or indirect and the virus can be transmitted to healthy individuals up to six feet away [127]. The kindergarten and school children are considered as the main transmitters of influenza virus causing infection in their siblings and family. Healthcare workers in the front-line health care facilities have an increased risk of infection from flu patients. The overall economic burden from influenza and its associated complications is frequently under estimated due to the lack of public awareness or due to ignorance. NPIs limit the spread of highly contagious influenza virus and reduced the flu burden in a society [128].

1.4.1 Influenza Treatment by Vaccination

Through antigenic drift and shift, the influenza virus is capable of escaping the immune system and widely infects humans resulting increased morbidity and mortality [129]. Having elevated anti-influenza antibodies do have an effect on protection and infection severity. Certain group in the "risk groups" population has been identical as more vulnerable to influenza infection, e.g. people aged ≥ 65 years. Influenza vaccination has been recommended for many years for the risk groups in order to reduce mortality and morbidity [130]. Reduction of virus spread through vaccination of school age children can reduces the spread in the community. Influenza vaccination is therefore the only global prophylaxis measure available. Although, some antiviral drugs are commercially available to treat influenza infected individuals.

The first isolation of influenza virus in 1933 by Wilson Smith and his colleagues opened up the door to the production of influenza vaccines [131]. The first inactivated monovalent influenza vaccine was crudely purified from inactivated whole virus in 1942. In 1945, bivalent influenza vaccine containing one influenza A and one B strain was first licensed for use for civilians [132]. Later in 1970s, trivalent influenza vaccine containing two influenza A strains and one B strain were recommended for seasonal vaccination by World Health Organisation (WHO) [133]. In 2012 the U.S. Food and Drug Administration approved the quadrivalent live

attenuated influenza vaccine formulated as nasal spray containing two influenza A and two influenza B strains [134]. The European medicine agency also approved quadrivalent LAIV vaccine in 2013 to use in Europe [135]. Subsequently, a number of manufactures have had their quadrivalent IIVs licensed.

Chemical inactivation of the whole virus was the first method of development of inactivated influenza vaccine (IIV). Live attenuated influenza vaccine is generated through genetic reassortment from the master donor virus (MDV). The stable but attenuated and cold-adapted MDV is used in the reverse genetics system with the surface glycoproteins from the wild-type strain to produce the master virus strain (MVS). Most commercial vaccines are produced by propagating the viruses in fertilized hen's egg. Further preparation can vary, giving whole inactivated virus, split virus or subunit virus vaccine. Other varieties of vaccine can be produced in insect cells or recombinantly. In recent years, the recombinant flu vaccine has been licensed expressing the HA protein of the circulating strains by cloning into baculovirus vectors [136]. Some viruses need strains are genetically enhanced to provide certain immunological property, or give better growth yield or lower morbidity.

The inactivated vaccines are all standardized by the amount of HA, and predominantly HA specific antibodies are induced. On the other hand, LAIV vaccine contains live attenuated viruses that may replicate and induce a multifaceted immune response. IIV is more effective in adults, whereas the LAIV provided better protection in children from 2-9 years of age [137-139].

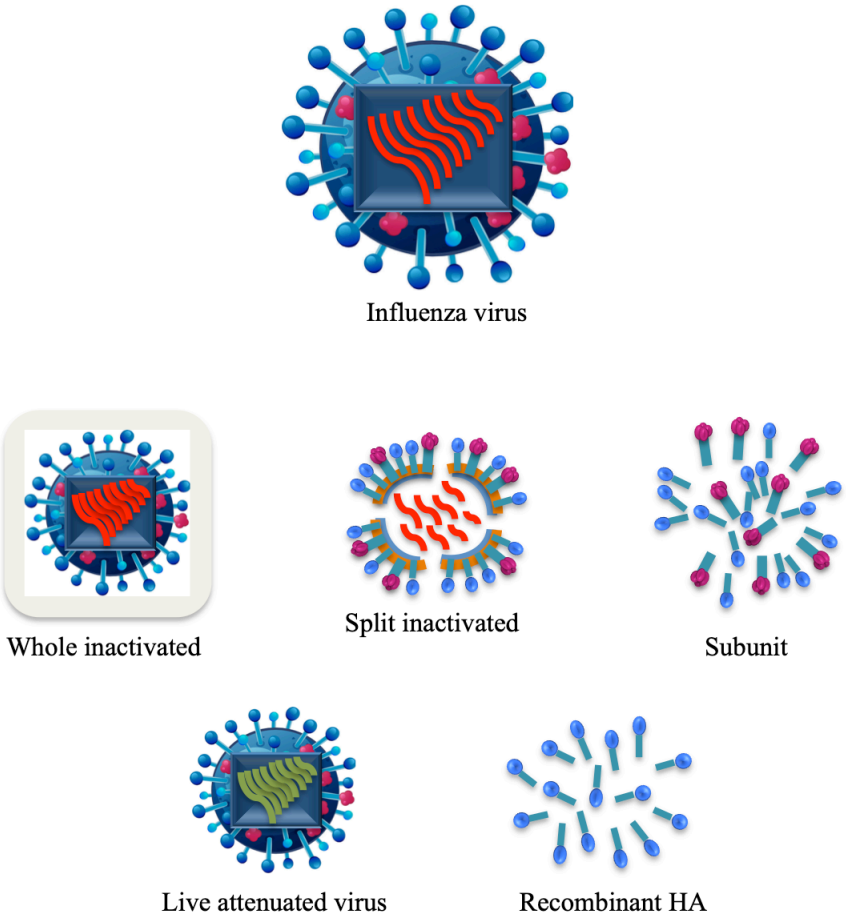


Figure 6. Commonly used vaccine types.

1.4.2 Antiviral Drugs

Two major viral antigens are targets of current antiviral drugs for influenza treatment. The transmembrane protein, M2-ion channel inhibitors were the first antiviral introduced and used in treatment of influenza patients up to 1999 [140]. Amantadine and rimantadine are the approved M2-ion channel inhibitor that inhibit influenza A viruses [141]. The adamantanes, M2 inhibitors function by interfering with viral endocytosis and release of viral RNA into the host cytoplasm that consequently inhibits the viral replication [142, 143]. The first M2-ion channel blocker amantadine

was initially approved to treat influenza A/H2N2 virus, and later for all strains. Rimantadine, the structural analogue of the amantadine, was approved for use against a variety of influenza A strains. In contrast to the amantadine, rimantadine has more antiviral specificity and is extensively metabolized. However, both of these antiviral drugs have been shown to shorten the duration and the severity of influenza A infection when taken within one to two days of developing symptoms. Although, they cannot be used against influenza B strain due to the lack of M2 protein. The major disadvantage of the ion channel inhibitors is they rapidly acquire resistance, particularly in the influenza A H3N2 virus [142] and cannot be used due to high levels of resistance in currently circulating viruses. Moreover, the adamantanes can have an adverse effect on the central nervous system although the risk is lower with rimantadine [144].

NA inhibitors (NAI) were first licensed for influenza treatment in 1999 and were the first structural-based drug designed. Over sixty years of scientific studies resulted in the achievement of the development of NA inhibitor; oseltamivir. Since then, two more NA inhibitors known as peramivir and zanamivir have also been approved by FDA for treatment of circulating influenza viruses and also recommended by CDC as antiviral drugs for hospitalized severely ill patients [145]. NAIs block the release of progeny influenza virus from the infected host cell and thus restrict the spread of new virus infection to neighbouring host cells. Neuraminidase functions at the phase of releasing of progeny virus in the replication cycle, thereby NAIs are most effective at the peak of the influenza virus replication, generally between 24-72 hours of illness. These NAIs are capable of inhibiting influenza B and all Influenza A subtypes, therefore can be used against all influenza strains including both epidemic and pandemic viruses [146]. Some adverse effects have been observed after NAIs. Zanamivir is inhaled and may cause bronchospasms and decreased pulmonary functions, but is otherwise well tolerated [147]. Interestingly, some side effects of oseltamivir have also been reported upon administration such as nausea, vomiting and abdominal pain [148, 149]. A major negative impact has been reported after oseltamivir treatment in adults with renal impairment [150]. However, neuraminidase inhibitors rarely acquire result in development of resistance against any of the

influenza strains. To overcome the seasonal flu burden and the occasional pandemic catastrophe, several antiviral drugs are being developed. Internal proteins inhibitors are also targeted for the new drug development, like the newly approved baloxavir carboxyl, the potent PA inhibitor [151].

1.4.3 Others Prophylaxis

With the moderate effectiveness of current influenza vaccines there is a need for next generation influenza vaccines to induce broad and long lasting immunity. Antibodies found against the conserved epitopes of the HA provide heterosubtypic and cross-reactive immunity. Broadly cross-reactive monoclonal antibodies against the conserved HA stalk domain could be a new treatment or prophylactic measure when a new influenza virus strain emerged. Furthermore, generation of stalk based vaccines could be a novel approach.

1.5 Influenza vaccines

Licensed vaccines are available against seasonal or pandemic influenza to protect against epidemic strains or a new pandemic strain, respectively. Seasonal influenza vaccines combat the annual challenge in every season; and are updated twice a year. The composition of seasonal vaccines is recommended based on geographically dominant strains for the northern and southern hemispheres. Despite only providing moderate protection, vaccination is still the most important and cost-effective means to protect against influenza infection. The target groups for vaccination are people with an increased risk of severe influenza infection, such as those with chronic disease in all age groups, the elderly, pregnant women, health care workers and farmers working closely with swine and avian species. In the USA, CDC recommends annual influenza vaccination for the whole population from the age of 6 months old [152]. The WHO has set pregnant women as the highest priority for vaccination and high priority for children between 6 to 59 months old, elderly people over 50 years of age, individuals with specific chronic medical conditions and health-care workers in every season [153]. Influenza vaccination also have some

contraindications including people with severe allergy to egg proteins, but they can also be vaccinated with recombinant protein or cell culture produced vaccines therefore embryonated hens egg are not the substrate for the vaccine production. Influenza vaccines are also not recommended to children under the age of 6 months due to their immature immune system.

1.5.1 Available Vaccines

Since the historical development of first inactivated vaccine in 1945, the vaccine composition has evolved based on the circulating strains being isolated. Originally the vaccine was monovalent to protect against the circulating influenza A virus, then bivalent vaccines were developed for both influenza A and B strains. Later in 1970s, trivalent influenza vaccines were introduced, when completely new influenza A H1N1 strain with different HA and NA emerged [154]. In 2013, the quadrivalent seasonal vaccine consisting of two influenza B strains were recommended by WHO due to co-circulation of the two lineages B/Victoria and B/Yamagata. Today, the vaccine is mainly produced by propagation in embryonated hens eggs, even though, some inactivated vaccine production using MDCK cell culture.

There are currently two types of approved influenza vaccines, inactivated influenza vaccine or IIV and live attenuated influenza vaccine or LAIV. The first recombinant influenza vaccine (RIV) is trivalent and approved by the FDA in January 2013 that used recombinant DNA technology to produce recombinant HA. Flublok RIV (Sanofi Pasteur) is now available as quadrivalent RIV vaccine in the 2018-2019 seasons in USA.

1.5.2 Inactivated Influenza Vaccine (IIV) Vs. Live Attenuated Influenza Vaccine (LAIV)

Several trivalent and quadrivalent IIVs are available on the market for 2018-19 seasons. Quadrivalent IIVs includes Fluzone (Sanofi Pasteur), Flucelvax and Afluria (Seqires), Fluarix (GlaxoSmithKline) and FluLaval (ID Biomedical Corp. of Quebec). The available trivalent IIVs are Afluria and Fluad from Seqirus and Fluzone

from Sanofi Pasteur. The inactivated vaccine viruses is propagated in the allantoic fluid of embryonated hen's eggs, virus is concentrated and purified before inactivation by beta-priolactone or formaldehyde or by detergent disruption for spilt or subunit vaccines. IIV can be formulated as whole virion or as split virus or subunit (contains HA and NA). The route of administration is either by deep subcutaneous (SC) or intramuscular (IM). Whole inactivated influenza vaccine elicit strong humoral immune responses and the serum antibody response is dominated by the IgG [155], along with mixed cellular responses of Th1 and Th2 cells. Both split and subunit IIVs can potentially induce Th2 cell responses beside strong systemic antibody responses. The principle mechanism of IIVs is to induce neutralizing antibodies to the major surface glycoprotein HA and to a lesser extent to NA. IIV therefore induces higher serum HAI responses compare to LAIV. A previous study has also demonstrated increased serum HAI response in adults by IIV compare to LAIV [156]. Although, IIVs do not mount mucosal immunity, and also have lower induction of plasmablast and plasmablast induced polyclonal antibodies specific to HA compared to LAIV [157].

LAIV is often attenuated by propagation at lower temperature (cold adaptive) leading to adaptive gene mutations. The virus strains can additionally be modified by replacing the genes from low-pathogenic strains. Thus, LAIV share the internal attenuated genes segments which are mutated at least in one or several segments by using serial passage at lower temperatures to produce the master donor virus (MDV) backbone of cold-adaptive (*ca*), temperature-sensitive (*ts*) and attenuated (*att*) phenotype. The MDV backbone then incorporates the HA and NA of annually circulating virus strains for the annual LAIV vaccine [158, 159]. Quadrivalent FluMist (North America) or Fluenz (Europe) LAIV is available for vaccination against influenza infection. LAIV is a licensed as nasal spray and has been used in Russia since 1970s, and licensed in USA from 2003 and later in 2012 in Europe. The vaccine is well tolerated and generally only minor local side effects occur, such as runny and congested nose. The three characteristic benefits of the attenuated virus allow replication at the local infection site of respiratory tract and generate immune response similar to natural infection. In contrast to IIV, LAIV induce stronger

mucosal responses dominated by IgA. T-cellular responses are elicited by LAIV beside strong humoral and mucosal immune responses [160]. LAIV provides superior protection in young children than the adults due to the T-cell responses [90]. Administration of intranasal LAIV elicits long-lasting humoral and cellular responses particularly in the upper respiratory tract (Figure 7).

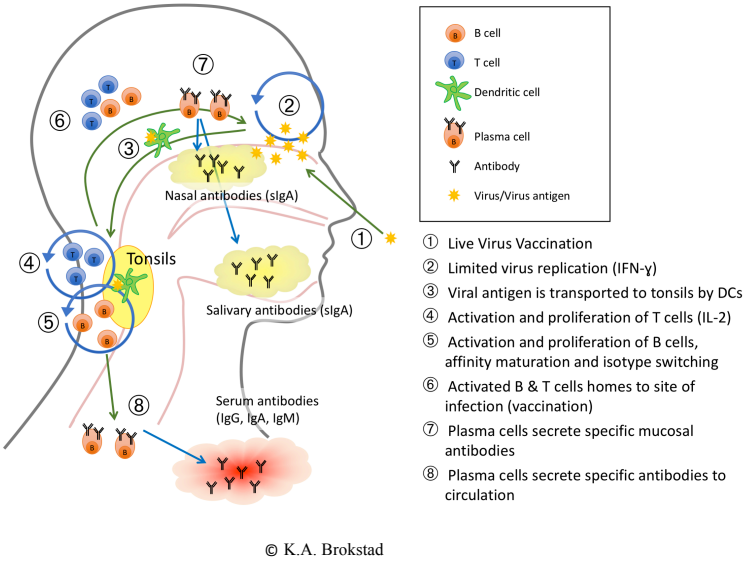


Figure 7. Model of induction of immune responses after live attenuated influenza vaccination (LAIV). (1) Intranasal LAIV immunization; (2) Viral antigen is transported to the tonsils/adenoids by the Dendritic Cells (DCs); (3) Activation and proliferation of T and B-cells in tonsils/adenoids with help from $CD4^+$ T-cells. Affinity maturation of B-cells; (4,5) Activated T and B-cells home to the site of infection or enter the circulation. Plasma cells secrete antibody into the blood and at the mucosal surfaces.

Our previous study reported that LAIV induce cross-reactive and durable T-cells responses in children [161]. LAIV provides protection by promoting HA- specific neutralizing antibodies [162] and can also provide heterosubtypic protection [89]. LAIV is now included in the childhood vaccination program in UK due to the demonstrated effectiveness in children and reduction of community spread of influenza and its associated economic burden [163].

1.5.3 Targeting Influenza Vaccines by Age or Gender

Previous exposures to influenza strains are believed to have a great impact on the immune response to future strains. Seasonal vaccines are annually updated to match the predicted circulating strains and induce strain specific immunity. Current influenza vaccines are standardized by the amount of the most common surface glycoprotein, HA, and the antibody response is mainly directed to the mutable and highly variable globular head domain of HA. These vaccines mainly do not induce an immune response to the immune-subdominant conserved stalk of HA. The frequent antigenic drift of the virus due to the high plasticity of the HA can limit vaccine effectiveness when the circulating strain has drifted. The number of strains and their diversity experienced by an individual in a lifetime including by natural infection or vaccination can impact upon the magnitude of immunity that will develop in the future. When a novel HA is substantially antigenically changed, infection or vaccination an individual can generates response of broadly neutralizing plasmablast toward HA stalk. Moreover, infection with closely related influenza strains induce neutralizing B-cell response to the globular head [164]. One of the key questions that become important to know about the age and exposure history to seasonal and pandemic influenza infection. However, it is not still clear how different prior exposure confers immunity to circulating influenza virus and/or vaccination. Interestingly, a systems analysis suggested that the immune system of an individual is defined by specific exposure frequency, that an individual encounters in their lifetime [165]. From the experience during the last pandemic in 2009, older people who were born in 1940's had less severe infection due to the presence of pre-existing cross-reactive anti pdm2009 antibody [166, 167]. Several studies show that the stalk specific broadly neutralizing antibodies increase with age due to previous experience with divergent influenza viruses [168, 169].

Gender differences also play a key role in response to influenza vaccination, e.g. testosterone can be immunosuppressive [170]. One of the largest risk group recommended for influenza vaccines is the elderly is however immunosenescence; the aging of the immune system and progressive to decline of immune functions with

age. The hallmark of the aging immune system is chronic low-grade pro-inflammatory state and occurs to a much greater extent in females than in male. The performance of the innate immune cells including dendritic cells, macrophages or neutrophils is vastly dysregulated with age under inflammation or pathogenesis [171]. Further animal studies suggest that aging promotes dysregulation of T-cell function; limiting clonal diversity of naive CD+ T-cells and CD8+ T-cells [172, 173], reduce effector memory CD4+ T-cells but with increased central memory T-cells and increased effector memory and effector CD8+ T-cells [173, 174]. There is increasing evidence that the both innate and adaptive immune responses are different among sexes due to exposure to earlier immunological stimuli but are not suitably considered during vaccine development.

The availability of two different influenza vaccines either IIV or LAIV allows targeting of the vaccines to the different age groups in which they are documented to be most effective. IIV shows higher protection against influenza infection in adults compared to LAIV. On the other hand, LAIV vaccination induces better protective immune response in children compared to IIV one of the reasons that the European Medicines Agency did not licence the LAIV for use in adults in Europe. As LAIV is administered intranasally and acts like natural infection, pre-existing immunity in older adults may limit the replication of the LAIV strains. The recommendation of which groups should be prioritized for seasonal vaccines differs between different countries partly based on economic priorities and due to their differing effectiveness in different age groups. The quality of the immune response to vaccination and natural infection should be addressed in future studies in all age group. As the new era for development of both seasonal and pandemic influenza immunization continues, research may explain how and why influenza exposure history, priming and imprinting, the age difference as well as the gender difference influence vaccine uptake, response and outcome. Recent studies demonstrated that potent protection were achieved via antigenic seniority and childhood HA imprinting against next pandemic potential A H7N9 and A H5N1 strains [106, 107, 175] explaining the age distribution of both zoonotic strains and may be useful in predicting the future pandemic potential of a virus [176].

1.6 Next Generation Vaccine Strategy And Immunity Involvement

In principle, next generation vaccines are focused on improvements to current influenza vaccines with the ultimate goal of development of the universal vaccine. A universal vaccine refers to a vaccine concept that should elicit broadly protective immune responses and provide long-lasting protection against seasonal as well as potentially deadly pandemic influenza virus strains. The reason universal vaccines have been nearly impossible to develop lies in the nature of the influenza virus surface glycoproteins, especially the haemagglutinin (HA). Current influenza vaccines are dependent on the induction of the neutralizing antibodies towards the globular head domain, which directly neutralizes the virus preventing attachment. The HA is highly variable among different virus strains and vaccines are most effective if the vaccine virus strain is well matched with the circulating strain. Typically, the available licensed vaccine confers protection against seasonal vaccine ranging from 10%-60% depending on the antigenic match between circulating and vaccine strains, and require annual updating of vaccine strains. Seasonal vaccines elicit strain specific antibodies and provide little to no protection against newly emerged pandemic influenza viruses and so immediate production of a pandemic vaccine is required. The world has already experienced four devastating pandemic in 20^{th} and 21^{st} century and the first recognised pandemic in 1918 was the worst on record. The very recent 2009 pandemic was relatively mild but showed the lack of the entire global capability to produce sufficient number of vaccine doses for the whole world [177, 178]. Thus, the obvious solution discussed during the last decade is for universal vaccine development, which may become achievable in the near future. A consensus among experts on the criteria can be set up as a universal vaccine is needed. Thus scientists from national institute of allergy and infectious diseases (NIAID) at the NIH and collaborators suggest a universal "vaccine with $\geq$ 75% protection against symptomatic influenza disease infected by groups I and II influenza A viruses lasting over a year in all populations" [108]. The major challenges for universal vaccine development are, I. understanding the differentiation of the immune response upon natural infection or vaccination in all ages. II. To map

the evolutionary capabilities of influenza viruses that make the virus to escape the host defence. Several concepts are being studied further to overcome the lack of knowledge, like original antigenic sin (OAS), antigenic seniority and /or antigenic imprinting to achieve the immunological knowledge for the next generation vaccines. Since the discovery of the OAS concept in 1960s by Thomas Francis Jr and colleagues, the theory has been challenged but gained critical information about how the previous exposure to influenza virus infection shapes antibody responses for further infection or vaccination [179, 180]. The 'original antigenic sin' concept explains the influence of the first influenza virus experience on lifelong immunity [181]. Antigenic seniority or antigenic imprinting might be more appropriate as these describe the phenomenon of OAS and how childhood influenza experience takes a superior position over the immune response [182, 183]. The memory responses remain higher than to subsequent infection with a similar strain type [184].

The rapid evolutionary capabilities of influenza virus strains makes it more complicated to design universal vaccines. More conserved epitopes lies in the HA stalk compared to the high mutable globular head domain. Studies have shown that both seasonal and pandemic vaccine can induce broadly cross-reactive antibody responses specific to the HA stalk. The majority of broadly neutralizing antibodies identified target the epitopes on the stalk domain and epitopes are conserved across the group 1 or group 2 [185, 186]. To overcome the limitations of HA head based conventional vaccines, HA stalk based influenza virus vaccines are now considered as potential candidate universal vaccine and have entered phase I human clinical trial. In principle, the next generation HA stalk based vaccine will induce immunity against both group I and group II stalk epitopes and will confer protection against any drifted or shifted influenza virus strain [187-189]. Two different approaches have been described in studies, where, the entire head domain is removed to construct the headless stalk as first strategy. A recent study has shown protection against both group I and group II influenza viruses using mini stalks from group I HA [190, 191]. The second approach is to construct chimeric HAs containing the stalk domain from H1, H3 or influenza B virus with the head domain of exotic virus strains not known to infect humans [192, 193]. Studies in mice and ferrets describe the protective

immune response by sequential vaccination with chimeric HAs. Importantly, immunization by different routes and using adjuvants effectively boosted long-lasting anti-stalk antibodies [194-197].

Besides the HA stalk based approach, other vaccination strategies have been introduced in the last decades that target broad immunity against influenza strain variants. For example, a computational method was used to produce consensus sequences of all strains. Computationally-optimized broadly reactive antigen (COBRA) were generated, where, consensus antigen sequence consisted of conserved epitopes across all isolates combining the most common amino acids undergoing frequent changes which remain in their respective positions. The computationally customised antigen sequences induce cross-reactive antibodies [198, 199]. This strategy has resulted in promising outcome and elicited protective immune responses against pre-pandemic H5N1 and also seasonal H1 and H3 viruses [200, 201]. Immunization with virus-like particles (VLPs) containing HA provides broad protection and protects mice against multiple subtypes of influenza A virus [202]. Moreover, conserved peptide pools from HA were also tested with adjuvants and conjugated bacterial protein to assess the potentiality as next generation vaccine [203, 204]. More recently, live attenuated vaccine is used as the newest strategy that confers broad protection and currently (2017) is undergoing the clinical trial. The approach to use both inactivated vaccine (IIV) priming following live-attenuated vaccine (LAIV) boosting is considered as a novel strategies for the development of the next generation vaccine. Live attenuated vaccination approach had given promising results in pre-clinical setup and hopefully licensed in coming years [205]

2 Aim of the Study

The study aimed primarily to elucidate the humoral and cellular immune responses after live attenuated influenza vaccine (LAIV) in children and adults in blood and tonsils collected during a clinical trial conducted at Haukeland University Hospital (HUH).

The major ***secondary objectives*** are

- To evaluate influenza A HA head and stalk specific antibody responses after LAIV immunization.
- To investigate the HA and NA specific humoral and cellular H1N1pdm09 responses following LAIV vaccination.
- To elucidate the local and systemic cellular responses after intranasal LAIV immunization.

3 Study Design

3.1 Clinical Vaccine Trial

Our LAIV vaccine clinical trial was conducted in children in 2012-13 and in 2013-14 in adults. The clinical trial is approved by the regional ethical committee (REC West 2012/1088) and the Norwegian Medicines Control Agency. The trial was registered in the open access databases; EUDRACT2012-002848-24 and www.clinicaltrials.gov; NCT01866540. The clinical trial involved collaborative work between the Influenza Centre, the Paediatric clinical trial unit and the Ear Nose and Throat (ENT) departments at the Haukeland University Hospital (HUH). Written informed consent from adults and parents (and children over the age of 12) were collected before enrolment in the study. Children and adults were recruited from the elective tonsillectomy lists during their initial outpatients visit.

Fifty-five children were included in the trial and 39 children were vaccinated and 16 unvaccinated children were included as age and sex matched controls. Twenty-four healthy adults were recruited and 20 were vaccinated during 2013-14 season (Figure 8). Children under the age of 9 years old received two vaccine doses at 28 days interval, whereas older children and adults received a single dose. The vaccine was administered intranasally according to the manufacturer's instructions. LAIV was administered as a divided dose of 0.1ml per nostril. After vaccination, all subjects remained under observation by the study staff for at least 30 minutes.

The inclusion criteria were designed for healthy children or adults with no fever or symptoms of influenza-like illness during the 7 days prior to vaccination, subjects with mild or moderate asthma (with daily use of inhaler) were included and females of child bearing age had to have a negative pregnancy test before vaccination. Children or adults were ***excluded*** if they suffered from chronic or serious medical conditions like, unstable asthma, recent influenza or high fever, pregnancy, use of acetyl salicylic acid (ASA) or were taking immunosuppressive therapy. Also subjects who were allergic to the vaccine or its components or children under governmental

custody were excluded. During the time of trial LAIV vaccine was not licenced in Norway, but imported from Finland and UK solely for this study. For all subjects, demographic information was collected such as age, sex, weight and height for children, underlying disease and previous seasonal vaccination or pandemic vaccination.

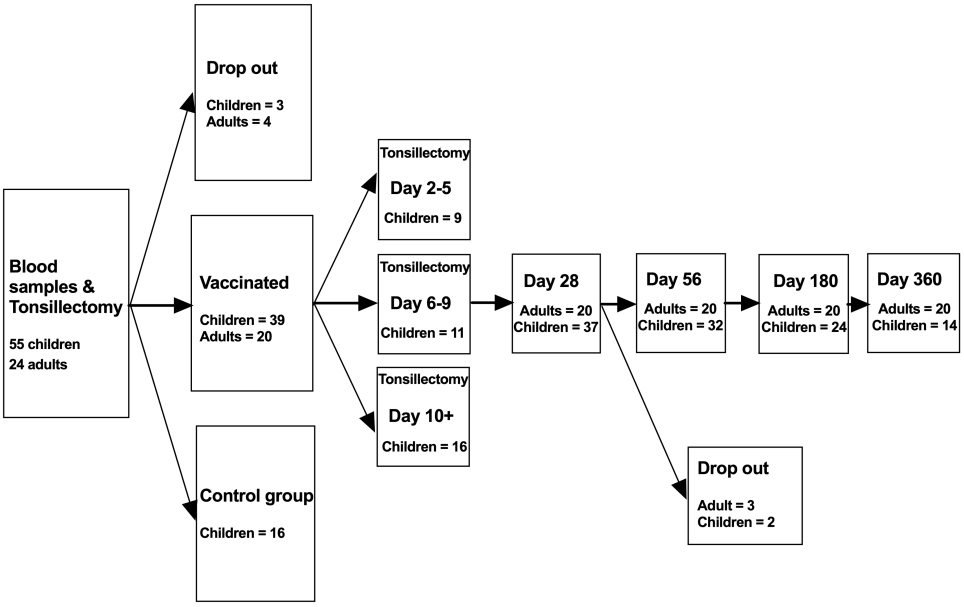
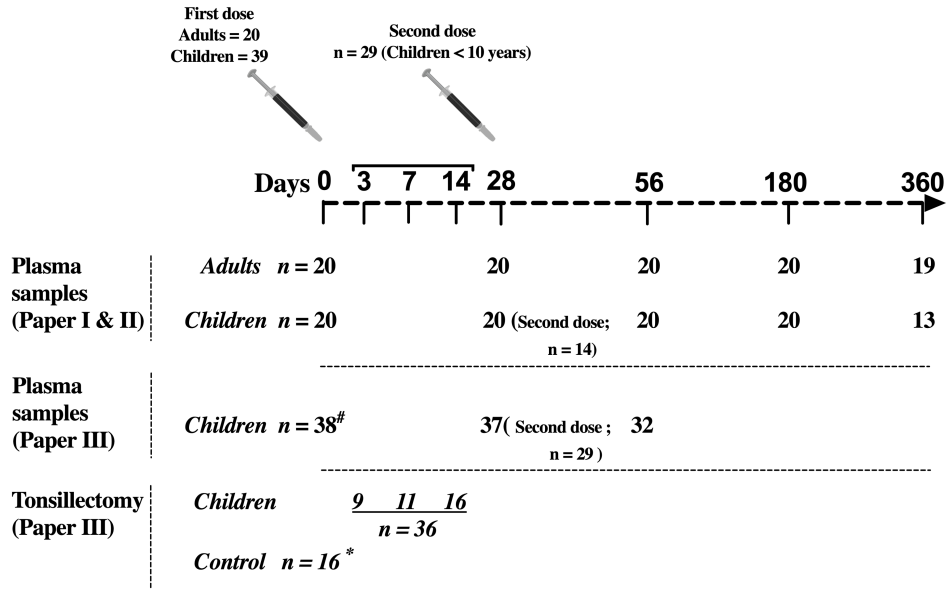


Figure 8. Overview of the live attenuated influenza vaccine clinical trial. The adults and children were recruited from the operation list scheduled for elective tonsillectomy from ear, nose and throat (ENT) department. Subjects were vaccinated with LAIV at 2-5, 6-9 and >10 day prior to tonsillectomy. Blood samples were collected prior to tonsillectomy (day 0), at the time of tonsillectomy and up to one year (day 28, 56, 180 and day 360) after vaccination. A control group of children was included for paper III to provide a pre-vaccination comparison to post vaccination tonsillar T-cell responses.

3.2 Immunization And Sampling in Following Study

Children received one (≥9 years old, n=6) or two doses (<9 years old, n=14) of LAIV (Fluenz, Astra Zeneca, Liverpool, UK) in 2012 at a four-week interval, whereas adults received a single dose in 2013-14 seasons [161]. Blood samples were collected pre-, at elective tonsillectomy, and at regular intervals after vaccination (28, 56, 180

and 360 days). Plasma was aliquoted and frozen for use in the serological assays, as previously described and illustrated in Figure 9 [206]. Cell preparation tubes (CPT, BD) were used to separate peripheral blood mononuclear cells (PBMCs) for the ELISpot assay. Fresh PBMC were immediately separated by centrifugation (density gradient) and used directly in cellular assays in the paediatric population otherwise stored in liquid Nitrogen. During the operation, tonsils were operated as scheduled at 2-21 days after first LAIV immunization and collected (in 0.9% NaCl) by the assigned doctors from ENT department, working as the part of the project. Lymphoprep (Stemcell tech. UK) was used to isolate tonsillar mononuclear cells (TMCs), which were analysed in cellular assays. Briefly, manual disruption of the tonsils was carried out using forceps and scalpel and filtered before density gradient centrifugation separation of lymphocytes.



* Non-vaccinated controls.
\# One vaccinated child provided samples on day of tonsillectomy, but no sample at day 0.

Figure 9. Study design. Adults were vaccinated with one dose of LAIV, whilst children received either 1 (n = 39) or 2 (n = 29 children, age < 10 years old). Plasma samples were collected prior to tonsillectomy and vaccination as pre (day 0) and after tonsillectomy or at 28, 56, 180 and 360 days post vaccination. Tonsil samples were collected at 3, 7 or 14 days post vaccination in children. The figure shows the number of adults and children at each sampling point and the papers (I-III) these samples were used in.

3.3 Participants in the Study

Subjects were intranasally immunized with 0.1 mL per nostril of the seasonal LAIV (Fluenz, Astra Zeneca, Liverpool, UK) in the clinical trial conducted in 2012-14. The exclusion criteria and study details for this clinical trial have been published earlier and are described above [161].

3.4 Vaccine

Trivalent LAIV (Fluenz) contained 10^7 fluorescent focus units (FFU) of A/California/7/2009(H1N1)pdm09-like and A/Victoria/361/2011(H3N2)-like strains in both seasons, with either B/Wisconsin/1/2010-like or B/Massachusetts/2/2012-like in the 2012-13 or 2013-14 seasons, respectively.

3.5 Recombinant Haemagglutinin Proteins

The influenza A haemagglutinin proteins were prepared for use in the ELISA by using the baculovirus expression system (Paper I, Table 1) [207, 208]. The chimeric stalk HAs were prepared by using an irrelevant head domain, from a virus which does not cause human infection. The cH6/1 contained the globular head domain from A/mallard/Sweden/81/02 (H6N1) and the stalk domain from A/PuertoRico/1/34 (H1N1). The cH4/3 contained the H4 globular head domain from A/duck/Czech/1956 (H4N6) in combination with the H3 stalk domain from A/Perth/09 (H3N2).

3.6 Antigens and Peptides

A/California/7/09 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010 split virus antigen from the vaccine strains were kindly provided by GSK (Glaxo Smithkline, Belgium). By mapping the sequence diversity, HLA supertype, prevalence and the influenza isolates spanning from 1934-2009, a panel of T-cell epitopes were selected. The panel contains 33 peptides covering cross-reactive CD4

epitopes and 31 peptides from CD8 [209]. The peptides with the highest sequence stability were further selected from the panel to know the cross-reactive immune response against viral strains. The peptide library was kindly provided by the Norwegian Institute of Public Health (NIPH) consisting of peptides for H1N1pdm09 (see paper II supplementary materials) and cross reactive CD4 and CD8 peptides (see paper III supplementary table). Fmoc chemistry (Mimotopes, Clayton, Australia) were chemically synthesized the peptides supplied in 100% DMSO with a concentration of 20mg/ml. Stock concentration (8µg/ml) were made by diluting in RPMI media and working concentration of 2µg/ml were prepared before running the IFN-γ ELISpot assay.

3.7 Viruses Used

Viruses were propagated in the allantoic cavity of 10 days old embryonated hen's eggs. The allantoic fluid was harvested, clarified and frozen at -80^{o}C until used in the assays as described below. The reassortant A/California/7/2009(H1N1) virus (x179a) was used for the micro-neutralization (MN) assay, the chimeric H1N1 virus (cH9/1 containing the HA stalk from A/California/7/2009(H1N1) and head from A/guinea fowl/Hong Kong/WF10/99 for the virus neutralization (VN) assay, and the reverse genetics A H7N1 virus (NIBRG-127 containing the NA from A/California/7/2009(H1N1) and HA from the equine A/Prague/56 (H7N7) strain) for enzyme-linked lectin assay (ELLA). The wild type A/California/7/2009(H1N1) virus was used for the antibody dependent cellular cytotoxicity (ADCC) assay.

3.8 Statistics

Statistical analyses were performed in ***Paper I*** by linear mixed model using STATA/IC 14.1 for Mac (StataCorp, College Station, TX 77845, USA) where, geometric mean titres with 95% confidence interval were used to investigate statistical differences between different time points after vaccination. GraphPad

Prism, version 6f for Mac (GraphPad Software, San Diego, California) was used for Wilcoxon test for head/stalk distribution between time points after vaccination.

Statistical differences (***Paper II***) from pre-vaccination to 56 days post vaccination in children and adults were analysed using the Wilcoxon-matched pairs signed rank tests. The nonparametric Mann-Whitney test was also used for comparing children and adult antibody responses in multiple assay analysis (GraphPad Prism; v.6f for Mac, GraphPad Software, USA), where, $P < 0.05$ was considered significant. In ***Paper III***, differences between pre- and post-vaccination immune responses were analysed by non-parametric Kruskal-Wallis multiple comparisons test or the Mann-Whitney test using GraphPad Prism version 6 for Mac OS X. The correlation analysis was performed by non-parametric Spearman correlation. $P < 0.05$ was considered significant.

4 Methods & Consideration

4.1 Haemagglutinin Inhibition (HI) Assay

Plasma samples were pre-treated with four volumes of receptor destroying enzymes (RDE) (Seiken, Japan) overnight and heat inactivated at 56^{o}C for 30 minutes. The HI assay was performed in duplicate using serial 2-fold dilutions (starting dilution 1:10) of RDE treated plasma and eight hemagglutinating units of either influenza A/California/04/09 (H1N1), A/Victoria/361/11 (H3N2) or B/Wisconsin/1/2010 and 0.7% (v/v) turkey erythrocytes. The individual HI titres were read as the reciprocal of the highest dilution at which 50% haemagglutination was inhibited. Titres <10 were assigned a value of 5 for calculation purposes. The geometric mean titre (GMT) was calculated for each subject and for each group at each time point.

Methodological Consideration

Quantification of influenza-specific antibodies against different influenza strains is important to know the level of pre-existing immunity and the response to LAIV. Based upon the properties of influenza virus to agglutinate the erythrocyte, the HI assay can be used with human sera to measure the inhibitory antibody concentration [210] . There are several methods to quantify viral strain specific antibodies but the HA inhibition assay is commonly used and immensely reliable to detect antibodies to the circulating viruses in sera. The HI method is globally used for influenza surveillance for determining antigenic properties of emerging viruses. One of the major drawbacks of the HI assay is that the non-specific inhibition of haemagglutination can interfere with the assay. Therefore, sera must be pre-treated with RDE and in some cases sera need to be pre-treated with packed red blood cells to removed non-specific inhibitors which can interfere with the HI assay [211]. All the LAIV plasma samples included in this study were run in the HI assay. In adults an HI titre of 40 is considered to provide protection in 50% of adults. The assay is shown in the illustration below in Figure 10.

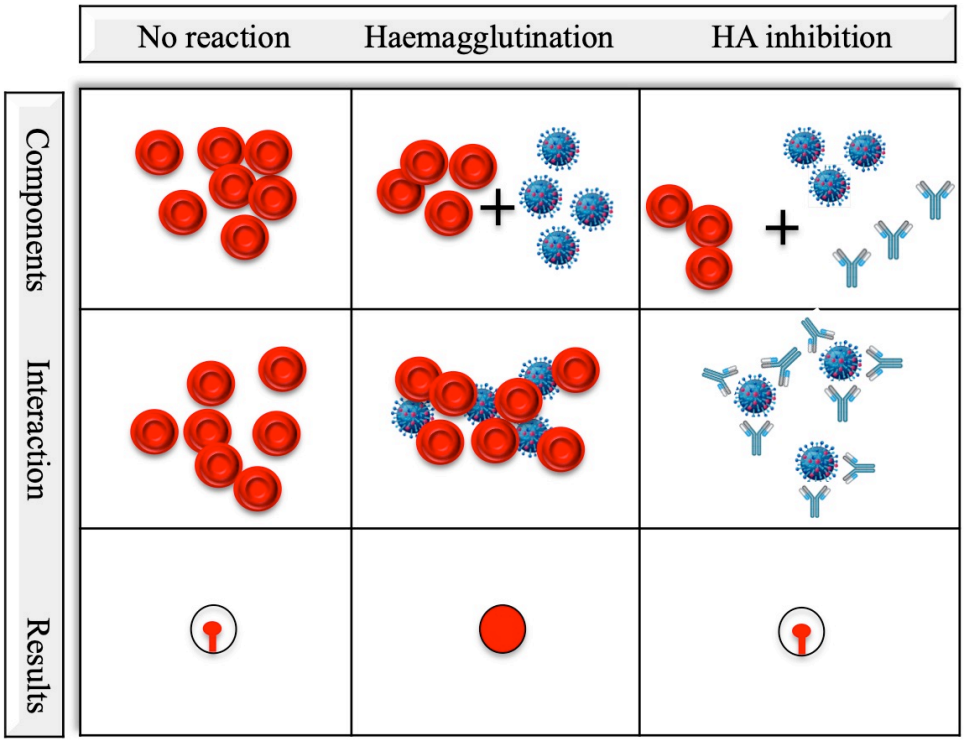


Figure 10. Principle of the haemagglutination inhibition (HI) assay.

4.2 Enzyme-Linked Immunosorbant Assay (ELISA)

Nunc Maxisorp 96-well immunoplates (eBioscience, Inc., USA) were coated with the appropriate HA protein (0.1 µg/well) in phosphate buffered saline (PBS) (Medicago AB, Uppsala, Sweden) overnight at 4°C (paper I, table I). After blocking with PBS containing 5 % skimmed milk powder (Sigma-Aldrich Co., USA), 1 % Bovine Serum Albumin (BSA) (Sigma-Aldrich Co., USA) and 0.1 % Tween-20 (Sigma-Aldrich Co., USA), plasma samples were diluted in 5-fold dilution series from 1:50 to 1:312500 in blocking buffer and incubated for 1 hour at 37 °C. After washing, specific serum antibodies were detected by mouse monoclonal anti-human IgG conjugated with horseradish peroxidase (HRP) (BD; USA) (1:4000 dilution for 1 hour at 37 °C) and developed with 3,3', 5,5' tetramethylbenzidine (BD; USA) for 10 minutes. The reaction was stopped using 100 µl per well of 0.5 M HCl and the plates

were read immediately at 450 nm using BioTek synergy H1 hybrid reader. The end point titres were calculated using the mean of the blank plus three standard deviations as a cut off [105].

Methodological Consideration

The indirect ELISA has been widely used for detection of influenza strain specific antibodies. The method is suitable for detecting antibodies to the coated antigen, e.g. haemagglutinin (HA). Recently, the newer methods are using continuous readout mPLEX-Flu assay to measure the concentration of anti-HA IgG antibodies against HAs from multiple influenza virus strains. The assay is Luminex-based that can be used against up to 50 influenza strain or particularly HA proteins using reduced amount of serum samples [212]. Indirect ELISA is more sensitive than any other conventional assays used previously [213]. As an example, single radial haemolysis (SRH) assay, first used by Weiler, Melletz and Breuninger Peck in 1965 and later used to widely detect antibodies against influenza HA [214, 215]. On the other hand, ELISA was developed at the same time to detect antibody against a variety of viruses [216] and the method has good sensitivity [217] and is more convenient to perform with collected blood samples. In the conventional serological assays, antibodies to influenza A H5 and H7 avian strains require higher biological containment if working with live virus. These problems can be overcome by expressing the HA proteins in the baculovirus system and detecting antibodies in ELISA using influenza strain specific surface antigens [218-220]. We preferentially chose the indirect ELISA to measure influenza specific IgG antibody in the plasma. The protocol has been developed in our lab to measure the antibody responses to different influenza haemagglutinin constructs and measures the optical density (OD) by using colorimetric substrate under spectrometry.

4.3 Micro-Neutralization (MN) Assay

The Microneutralization assay was conducted as previously described [206]. Briefly, plasma samples and control sheep sera were serially diluted from 1:10 using double

dilutions in flat-bottom 96-well cell culture plates (Nunclone Delta surface, USA) before incubation with 100 $TCID_{50}$/50 µl/well of A/California/04/09 (H1N1) (x179a) for 1 h at room temperature. Then, $1.5x10^5$ Mardin Darby canine kidney (MDCK) cells/ml were added in DMEM media contained 1% FBS (filtered) and 1% PSA. 15000 cell/well were plated and incubated for 16-18 hours at 37°C. The propagation of influenza virus was detected using antibody (dilution; 1: 5000) to the nucleoprotein and TMB (3,3′, 5,5′-Tetramethylbenzidine; Thermo Fisher Scientific, USA) before reading at 450nm and 620nm to obtain the final optical density (OD). The Microneutralization titres (IC_{50}) were calculated using the Reed and Muench method.

Methodological Consideration

The microneutralization assay (MN) is the most reliable functional assay to measure the level of neutralizing antibody. Both HAI assay and MN assay primarily detect antibody directed to the HA, although the HAI assay measures antibody which inhibits haemagglutination and can be used with inactivated virus. The MN assay is highly sensitive and specific for detecting functional neutralizing antibodies to a given influenza virus strain [221]. The main advantage of the MN method is that the assay can be rapidly carried out if a novel virus emerges and the infectious virus is available. The drawback of the assay is that the conventional neutralization assay is based on measuring inhibition of cytopathogenic effect in MDCK cells and requires long incubation times. The ELISA based MN assay using microtiter plates with virus-infected cells can provide serum neutralization titres within two days. Initially the 50% tissue culture dose or $TCID_{50}$ is calculated [222] to allow standardization of the virus in the assay. Although, the MN titres is usually correlated with HAI titres, the MN assay is more sensitive and mechanistically relevant in estimation of antibody mediated protection; for an example, HAI titre of > 40 is considered as the protective titre corresponds to the MN titre of >80 (or in some cases > 160) [223, 224].

4.4 Virus-Neutralization (VN) Assay

The virus neutralization assay was conducted with the cH9/1 virus using a 3-day incubation period [225, 226]. Briefly, cell culture plates (Flat-bottom 96-well Nunclone Delta surface, USA) were seeded with 1.5×10^5 MDCK cells and incubated at 37°C overnight. Next, heat-inactivated plasma samples were diluted to 1:10 and 2-fold serially diluted before incubation with cH9/1 (100 $TCID_{50}$/50 µl) for 1 h at room temperature. MDCK cells were washed with PBS, and plasma/virus dilutions were added and incubated for 1-h incubation at 37°C. The mixture was removed, cells were washed with PBS, and 50 µl of serially diluted plasma plus 50 µl infection medium (DMEM medium containing 2.5mg/ml TPCK-treated trypsin (Worthington Biomedical, USA), PSA (100 IU/ml penicillin, 100mg/ml streptomycin and 0.25 µg fungizone; Lonza, Switzerland) and 0.14% bovine serum albumin (Sigma-Aldrich, USA) were added to each well before incubation at 37°C for 72 h. The virus neutralization titres were measured by haemagglutination assay using the supernatant (50 µl) and 50 µl of 0.7% human red blood cells and read after 30 min of incubation at room temperature. The highest dilution of plasma resulting in 100% haemagglutination was read as the neutralizing antibody titre. A value of 5 was assigned to samples below the limit of detection for statistical analysis.

Methodological Consideration

The neutralization assay has uniquely been recently established to target the HA stalk specific neutralizing antibody. The conventional neutralization assay generally measures HA head specific functional antibodies when using human influenza viruses, which interfere with detection of stalk specific antibodies. Therefore, the chimeric HA based influenza virus strain was designed with the stalk combined with an “exotic” head domain derived from influenza virus subtype which does not infect man and an irrelevant N3 neuraminidase [225]. The cH9/1 virus was generously provided by Florian Krammer to use *in vitro* to infect MDCK cells followed by 72 hour incubation to detect antibody to the conserved stalk epitopes. This virus allows detection of functional antibodies specifically to the HA stalk domain rather than the

head specific antibodies due to the exotic head which has not circulated in humans [193].

4.5 Enzyme-Linked Lectin Assay (ELLA)

The ELLA was used to measure antibodies inhibiting the ability of neuraminidase to cleave sialic acid. The reverse genetics influenza A H7N1 virus containing the HA from an equine influenza A H7 strain and NA from A/California/07/09 (H1N1) was used as previously described [227-229]. Briefly, 96-well flat bottom Maxisorb plates (VWR, USA) were coated with 100 µl fetuin (Sigma-Aldrich, USA) in 25 µg/ml coating solution (KPL; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 4°C overnight. Plates were washed three times with T-PBS (PBS with 0.05% Tween-20) before adding serially diluted serum (heat-inactivated at 56°C for 45 minutes) and 24 µl H7N1 virus (1:10 dilution) and incubating at 37°C for 16-18 hours. Plates were washed six times with T-PBS and Horseradish peroxidase (HRP)- conjugated peanut agglutinin (PNA) (Sigma-Aldrich, USA) was added (100 µl, 1 µg/ml) and incubated at room temperate for 2 hours. The plates were then washed three times with T-PBS, and o-phenylenediamine dihydochloride (OPD) (Sigma-Aldrich, USA) substrate (0.5mg/ml) in citrate buffer was added to all wells. After 10 min incubation at room temperature in the dark, the reaction was stopped with 100 µl 1 M sulphuric acid. The plates were read with a microplate reader by spectrophotometry at OD 490 nm. The anti-NA antibody titres (EC_{50}) in the plasma samples were calculated as the reciprocal dilutions of plasma with gave OD values equal to 50% of (OD virus control + OD blank) in four-parameter non-linear regression analysis using GraphPad Prism.

Methodological Consideration

Neuraminidase is the second major surface glycoprotein of influenza virus and plays important roles in both virus attachment and release from infected cells, although, not as abundant as HA. Inhibition of NA activity reduces virus plaque size *in vitro* and NA inhibiting (NAI) antibodies also correlates with protection against H1N1 in

healthy human challenge model [230]. Therefore, NAI antibodies could lead to decreases in viral shedding after infection and also reduce replication of LAIV strains after vaccination. Although, the NA function is crucial and important to prevent influenza, the traditionally established NI assay has not been included in past influenza immunogenicity epidemiology studies. The traditional NI assay was first described by Webster and Laver in 1967 and follows the assay procedure of Warren and Aminoff in 1959 described quantification of free sialic acid [231]. Another NA assay is the thiobarbituric acid (TBA) method but is impractical for routine serology due to the use of hazardous chemicals that convert sialic acid into a chromophore. The miniaturized assay was then developed in 1990 by Lambre *et al.* known as enzyme-linked lectin assay (ELLA) that quantifies the enzyme activity using peanut agglutinin (PNA) [232]. The PNA binds to the galactose moieties that are cleaved and exposed after the NA enzymatic reaction. The NA inhibiting antibodies are quantified after overnight incubation using a colorimetric readout and antibody titres calculated. The ELLA can also be used to compare the antigenic properties between the NAs of the circulating influenza strain.

The ELLA assay is now widely accepted and used as alternative of neuraminidase inhibition (NI) assay, but there are some major considerations that need to be taken into account. First of all, the test sample containing NA specific antibody will probably also bind HA. The presence of HA specific antibodies therefore interfere with NA binding in a competitive way [233] and are often underestimated due to the presence of high quantity of HA on the surface of the virus. To avoid this non-specific inhibition by HA-specific antibodies, we used the reverse genetics generated wild type N1 virus containing antigenically-mismatch HA. Secondly, some NA subtypes enzyme activity is greatly reduced at pH >7.0, therefore the recommended pH 6.5 of the buffer can be adjusted during the virus titration. Overall to minimize the other non-specific inhibition, the serum samples were heat-treated at 56 °C for 45 min. In contrast to the conventional NI assay, ELLA is easier to perform with a large number of samples and easier to read without using harmful chemicals. Most importantly, the other studies demonstrated the assay result is reproducible and plate–to-plate variability is minimum [234, 235].

4.6 Antibody Dependent Cellular Cytotoxicity (ADCC)

The ADCC Reporter Bioassay (Core Kit G7010/G7018, Promega, USA) was used to quantify pre- and post-vaccination ADCC antibodies [51]. MDCK cells as 'target cells' (at $1.5x10^4$ cells/well) were seeded in 96 F-well white tissue culture plates (VWR, USA). After overnight incubation (18-24 hours), cells were infected with wild type A/California/7/2009(H1N1) virus at a multiplicity of infection (MOI) of 3. On the day of the assay, the medium was replaced with assay buffer (RPMI 1640 with 4% (vol/vol) low IgG FBS; Lonza, Switzerland), followed by the addition of 5-fold serial dilutions of plasma (starting at 1:10). The infected cells were incubated together with antibodies at 37 °C for 30 min. The effector cells (Jurkat) at $7.5x10^4$ cells/well were added to the assay plates. After 6 hours incubation at 37 °C, the Bio-Glo™ luminescence assay (Promega, USA) system was used to quantify using a plate reader with glow-type luminescence.

Methodological Consideration

ADCC is a non-phagocytic killing process of an antibody coated infected cell (target cell) conducted by cytotoxic effector cells. ADCC was first described in natural killer cells (NK) by Moller in 1967 [236], but can also involve monocytes, macrophages, dendritic cells, neutrophils and eosinophils. The conserved HA stalk specific antibodies have been shown to increase ADCC activity [225]. After interaction with target cell bound antibodies (typically, IgG, IgE or IgA) through FcγRIIIa (CD16) signals initiate the expression of cell death molecules or release of cytotoxic granules triggering ADCC. NK-cells have a majority of FcγRIII that interact with the influenza specific antibody. Therefore, further understanding of the mechanism of ADCC is crucial to understand the immune response to current vaccines and develop future influenza vaccines. The conventional ADCC assay activates the CD16 receptor in primary NK cells and uses flow cytometry for detection of degranulation and IFN-γ, it is labour intensive and expensive. On the other hand, the ADCC reporter bioassay is commercially available and standardised in a 96-well plate format. The assay has good precision, is biologically relevant and provides good reproducibility

[50]. Importantly, to quantify the pre- and post-vaccination ADCC antibodies, cell culture is not required in the reporter bioassay and the assay is rapid to perform.

4.7 ELISpot Assay

Antigen-specific interferon (IFN) γ^+ cytokine-secreting T-cells were quantified at the single-cell level by the ELISpot assay (Mabtech AB, Sweden) [237]. Optimized libraries of peptides representing unique T-cell epitopes from four of the initial H1N1pdm09 circulating strains were used for measuring H1N1pdm09 specific $CD4^+$ (originating from HA, NA, M1, NP, PB2) and $CD8^+$ (M1, NA, PA and NS2) responses (Paper II). Optimized libraries of broadly reactive $CD4^+$ and $CD8^+$ - conserved peptide pools were used in Paper III. Briefly, 400000 PBMCs or TMNCs per well were stimulated with appropriate $CD4^+$ and $CD8^+$ peptide pools (2 μg/mL), anti-CD3 T-cell activator (positive control), or lymphocyte medium alone (negative control) [209]. Plates were incubated overnight at 37°C, 5% CO_2. Plates were then developed the following day by following the manufacturer's instruction and read using an automated reader (Advanced Imaging Devices), where spot-forming units (SFUs) were counted. The background values were subtracted from the influenza virus-specific (H1N1, H3N2, B or peptide panel) responses.

Methodological Consideration

ELISpot assay was used to quantify the influenza specific T-cell responses pre- and post-LAIV immunization. The ELISpot assay was developed measures IFN- γ release at a single cell level [238]. The advantages of the assay is its high sensitivity, results are consistent in different laboratories and reproducible [239]. The assay has been increasingly used in infectious disease studies for quantitative assessment of antigenic specific T-cell responses from peripheral blood mononuclear cells (PBMC) to understand the role of T-cell immunity after infection or vaccination. Several studies demonstrated that pre-existing CD4+ and CD8+ T-cell responses are highly cross-reactive and provide protection from influenza infection [209, 240]. Therefore, the T-cell mediated responses to highly conserved proteins may guide development of

universal influenza vaccines. On the other hand, the cytokine ELISA assay or cytokine bead array can also measure the cytokine release by the activated T-cells although they are often less sensitive and reproducible.

4.8 Multiplex Cytokine Assay

We used the multiplex assay to analyse the Th1, Th2 and Th17 responses after LAIV in the systemic (blood, PBMC) and local (tonsils, TMC) compartments. Due to the limited volume of blood samples a mixture of three influenza split antigens (2.5 μg/ml) from A/H1N1, A/H3N2 and B were incubated in lymphocyte medium with $1x10^6$ cells/well (TMCs and PBMCs) for 72 hours as previously described [241]. Medium alone was used as a negative control and positive controls contained T lymphocyte mitogen (Phorbol myristate acetate (10ng/ml) and ionomycin (250ng/ml) (Sigma-Aldrich, USA). The supernatant was frozen at -80°C and later thawed for assessing GM-CSF, IL-2, IFN-□, TNF-□, IL-4, IL-5, IL-10, IL-12, IL-13 and IL-17 by the Bio-Plex system. The concentrations of cytokines in the supernatants was analysed by following manufacturer's instructions and interpolation of cytokine concentrations from standard curves. The negative control unstimulated samples were subtracted from the influenza specific responses for data analysis.

Methodological Consideration

Cytokines act as effectors or modulators in the innate or adaptive immune system after influenza infection or vaccination and play an important role in development and control of the immune response. Measuring a broad range of cytokines following influenza infection or vaccination therefore provides an insight into the influenza pathogenicity or immunogenicity [242]. The multiplex cytokine assay is now widely used to allow simultaneous detection of a number of cytokines from the same sample, which is particularly important in studies of young children where only small volumes of blood can be collected. The method is also appropriate for longitudinal studies of influenza responses where changes in cytokines are important [243]. The assay has a higher level of sensitivity and is able to detect circulating multiple

cytokines in a single sample in contrast to the ELISA, which can measure only one protein [244, 245].

5 Summary of Results

The influenza centre conducted a clinical trial on live attenuated influenza vaccine (LAIV) in children and adults scheduled for elective tonsillectomy in the influenza seasons 2012-13 and 2013-14. Plasma samples, and peripheral blood mononuclear cells (PBMCs) were collected before, and up to 1-year after vaccination. Tonsillar mononuclear cells (TMNCs) were collected during tonsillectomy from individuals receiving LAIV, as well as a group of age matched control individuals without vaccination. With these clinical samples, we investigated the kinetics and longitudes of humoral and cellular immune responses after LAIV in children and adults.

Paper I (Vaccine 2017)

Pre and post LAIV vaccination (days 0, 28 56, 6 months and 1 year) plasma samples were used to study the humoral immune responses in children and adults with HI and ELISA assays.

The neutralizing antibody responses against HAs from homologous strains were measured in HI assays. LAIV elicited significant increase in neutralizing antibodies against H3HA up to a year, but not H1HA, in children. LAIV didn't induce antibody responses against H1HA or H3HA in adults. Interestingly, children had higher level of H1HA specific pre-existing antibodies compared to adults, while similar levels of H3HA specific pre-existing antibodies were found in both children and adults.

The cross reactivity of the LAIV elicited antibodies was assessed in ELISA with H1 and H5 for group 1 HA, and H3 and H7 for group 2 HA. Similar to HI results, we observed significant increase in the amount of H3HA specific antibodies, but not H1HA specific antibodies, up to a year after LAIV in children. Of note, the amount of H7HA specific antibodies also significantly increased after LAIV, but no change was found in antibodies against H5HA in children. LAIV didn't induce any noticeable antibody increase in adults. Intriguingly, children had higher and similar level of H1 and H3 specific antibodies, but lower amounts of H5HA and H7HA specific antibodies before LAIV vaccination.

To dissect HA head or stalk domain specific antibody responses, plasma samples were tested in ELISA coated with H1 head, H1 stalk, H3 head or H3 stalk. In children, LAIV elicited significant increase in H3 head specific antibodies, and slight increase in H1 stalk specific antibodies, although not significant. In adults, the amount of antibodies didn't change after LAIV. Notably, children had higher and similar level of H1 and H3 head specific antibodies, but lower level of H1 and H3 stalk specific antibodies before LAIV vaccination.

Paper II (Scand. J. Immunol, revision resubmitted)

LAIV elicits multifaceted immune responses, and we observed increase in H1 stalk specific antibodies in children after LAIV. In paper II, we move on to investigate the LAIV elicited H1N1 specific antibody and T-cell responses more comprehensively, using plasma samples before, 28 and 56 days after LAIV with MN, VN, ELLA, ADCC, and ELISpot assays.

To confirm the H1 stalk specific antibody responses after LAIV in children, we performed MN assay with the vaccine autologous A/H1N1Cal09 virus, and VN assay with a reassortant virus cH9/1N3, which carries a chimeric HA of irrelevant H9 head and H1 stalk, together with an irrelevant N3NA. In agreement with the HI and ELISA results in paper I, no increase in H1N1 specific neutralizing antibodies was found in children or adults. However, VN assay with the cH9/1N3 virus revealed a slightly elevated level of H1 stalk specific antibodies in children after LAIV, confirming the ELISA results from paper I. As expected, LAIV elicited no antibody responses in adults. Interestingly, when children and adults were further stratified, children and adults who had received earlier pandemic or seasonal vaccine(s) had higher pre-existing neutralizing antibody titres in MN assay than individuals who had not received pandemic or seasonal vaccine(s) prior to LAIV vaccination.

Next, we measured the amount of antibodies, which inhibits NA enzymatic activity, before and after LAIV in children and adults with ELLA, in which a reasserted A H7N1 virus carrying irrelevant H7HA and N1NA from the vaccine autologous A/H1N1Cal09 virus. Of note, LAIV elicited significant increases in NA inhibiting

antibodies in children, and to a lesser extend in adults. Interestingly, children had higher level of NA inhibiting antibodies before LAIV vaccination compared to adults.

ADCC plays important roles in eliminating infected cells and reduce disease severity. We continued to quantify the vaccine autologous A/H1N1Cal09 virus specific antibodies, which is capable of inducing ADCC. LAIV induced H1N1 specific ADCC antibody increase in children, but not in adults. Unlike NA inhibiting antibodies, children had lower amount of ADCC antibodies before LAIV vaccination compared to adults.

Last but not least, we used PBMCs from children receiving LAIV to assess the vaccine autologous A/H1N1Cal09 specific T-cell responses. IFN-γ ELISpot was performed using PBMCs with stimuli as Cal09 peptide pools consisting of CD4 epitopes from HA, NA, M1, NP and PB2 proteins, and CD8 epitopes from M1, NA, PA and NS2 proteins. LAIV significantly induced A/H1N1Cal09 specific CD4 T-cell responses revealed by the elevated IFN-γ+ spot forming unit per 10^6 PBMCs in children, but no increase in CD8 T-cell responses was observed.

Paper III (Ready to submit to The Journal of Infectious Disease)

We demonstrated that LAIV elicited antibody responses to H3HA head and H1HA stalk (paper I) in children. In addition, LAIV also induced NA specific and ADCC antibody, as well as CD4 T-cell responses in children up to 1 year after LAIV vaccination. In paper III, we aimed to study the early kinetics of local and systemic, humoral and cellular immune responses after LAIV in children, using plasma, TMNCs and PBMCs from vaccinees during tonsillectomy and samples from unvaccinated age-matched control individuals for comparison, with HI, IFN-γ ELISpot and Multiplex cytokine quantification assays.

Early kinetics of systemic antibody response were measured using plasma samples from the day of tonsillectomy, i.e. 3, 7 and 14 days, together with 28 and 56 days after LAIV with HI assay. In agreement with HI results in paper I, LAIV elicited significant HA specific antibody responses against H3, but not H1 at day 28. In fact, most of individuals had increased H3 specific antibody titre at day 14. Notably,

significant increases in B virus specific antibody titres were found as early as day 14, as well as day 28 and 56.

Next, we measured the local and systemic T-cell responses towards homologous vaccine strains using TMNCs and PBMCs with H1, H3 and B split antigens as stimuli in IFN-γ ELISpot assay. Locally, LAIV elicited T-cell responses against H3 and B viruses at day 14, but not against H1 virus. Systemically, LAIV induced T-cell responses against H1 and B viruses at day 14, but not against H3 virus. In addition, TMNCs and PBMCs were stimulated with H1, H3 and B split antigens pooled together, and cytokines (Th1, Th2 and Th17, GM-CSF) were quantified in the supernatants by Multiplex cytokine quantification assay. Significantly elevated amounts of cytokines, including IL-2, IFN-γ, IL-13 and GM-CSF were secreted from TMNCs 14 days after LAIV to the mixture of H1, H3 and B split antigens. No increase of cytokines from PBMCs was found, probably due to the limited amount of cells available.

Lastly, the breadth of LAIV induced local and systemic T-cell responses were assessed using TMNCs and PBMCs with conserved peptide pools covering CD4 and CD8 epitopes as stimuli in IFN-γ ELISpot assay. In agreement with the results from IFN-γ ELISpot assay using A/H1N1Cal09 split antigen as stimulus, no increases of IFN-γ+ spot forming unit per 10^6 cells was observed against both CD4 and CD8 specific A/H1N1Cal09 epitopes in TMNCs (local). Interestingly, increases of IFN-γ+ spot forming unit was found against the conserved CD4 and CD8 epitopes in TMNCs (local), although due to the limited amount of PBMCs available, no systemic T-cell responses against conserved epitopes were measured.

6 Discussion

Influenza virus causes the respiratory illness, “the flu”, with severe illness, hospitalization and death occurring in all age groups [246, 247]. The World Health Organization (WHO) identifies the elderly, young children, pregnant women and people with chronic medical conditions as high-risk groups (See the table 1). Annually, 20-30% of young children are infected with influenza viruses. Due to the long duration of virus shedding, young children, once infected, become the main transmitters in families and communities [248-252]. During A/H1N1 pandemic in 2009, the attack rates were found consistently higher among children as compared to adults [253]. Studies have proven that mass vaccination of pre-school and school children provides herd immunity in communities, which greatly reduces virus transmission and indirect protection for the elderly [254-258].

Vaccines are a cost-effective prophylactic measure to combat influenza virus and influenza virus related illness in the general public, especially high-risk groups [259, 260]. Current licensed influenza seasonal vaccines are available as inactivated, live attenuated, and recombinant-HA vaccines. The recombinant-HA vaccines were recently licensed for use in adults aged 18 to 49 years old in the US [261, 262], to provide an alternative for individuals allergic to eggs. For children and the majority of adults, the inactivated influenza vaccine (IIV) and the live attenuated influenza vaccine (LAIV) are the two main options available on the market.

LAIV recommendation and application in children

The cold-adapted, temperature-sensitive LAIV viruses replicate in the upper respiratory tract to mimic natural infection. Hence, LAIV induces broader and multifaceted immune responses including antibodies in both the blood and the upper airway mucosa [263] and T-cells, especially in children [264, 265] compared to IIV which mainly induces vaccine strain specific systemic antibody responses. In a meta-analysis comparing systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy between LAIV and IIV, Beyer *et al.* studied 18 randomized comparative clinical trials and demonstrated that LAIV induced significantly lower

levels of serum HI antibodies and significantly higher levels of local IgA antibodies than IIV. However, no significant difference was found between LAIV and IIV when assessing clinical efficacy [266]. Ambrose *et al.* summarized studies directly comparing the efficacy of LAIV and IIV in children and adults: in children 6 months to 18 years old, LAIV was more protective; in individuals 17-49 years old, IIV is similarly or more efficacious compared to LAIV, however LAIV provides greater against mismatched A/H3N2 strain. In older adults above 60 years old, limited data suggest LAIV and IIV are similarly effective [137]. In addition, *Nichol et al.* reported lower frequencies of influenza infection by a mismatched influenza virus strain in adults vaccinated with LAIV in a randomized controlled trial [267]. In children, LAIV demonstrated better cross-protection against mismatched strains [89, 139, 268, 269].

LAIV vaccine has been recommended for healthy children from 2 to 17 years old in the UK since 2012, under the terms of a universal childhood influenza program following the recommendation of the Joint Committee on Vaccination and Immunization [270]. The direct and indirect impact of vaccination has been studied, where reduced influenza incidence rates were reported in adults [271] but direct effect in children of 4-11 years old group. In areas where children were vaccinated, influenza activity was consistently low and the indirect cost effectiveness of the program was observed [272, 273]. Interestingly, limited transmission and virus spreading were strongly associated with vaccination [274]. During the season 2014/15, moderate LAIV effectiveness was reported against the circulating A/H3N2 and drifted B strain [275]. The following season was dominated by influenza A/H1N1pdm09 but reduced influenza infection was observed among the vaccinated school-age children [276]. The A/H1N1pdm09 was from the new genetic subgroup (6B.1 and 6B.2) during this season and thus LAIV vaccine effectiveness was reported against a mismatched strain [277]. Since then, the UK continuously monitors the uptake and impact of the LAIV program in school children [278]. Finland introduced the LAIV in their national vaccination program from the influenza season 2015/16 for children 24 to 35 months old and they found LAIV effectively combated laboratory-confirmed influenza. Thereafter, Finland also recommended LAIV as an alternative

prophylaxis to inactivated vaccine in children [279]. Interestingly, the USA suspended LAIV vaccine based on the report of the US Centers for Disease Control and Prevention (CDC), where no significance evidence of vaccine effectiveness was found against A/H1N1pdm09 at the end of 2015/16 season [280]. Potentially due to a mutation in HA that led the thermal instability of the A/H1N1pdm09 LAIV vaccine strain used in 2009-2014, reported by *Caspard H et al*. [281]. The US Advisory Committee on Immunization Practice therefore temporarily withdrew the recommendation of LAIV for the 2016/17 and 2017/18 seasons. In Norway, LAIV is also recommended for high-risk children between 2-17 years old with underlying medical conditions, but the vaccine is not regularly used [282].

To better understand the early kinetics as well as the longevity of LAIV induced humoral and cellular immune responses, we conducted a clinical trial of LAIV in children in the season 2012-13 and season 2013-14 in both children and adults. Individuals were intranasally vaccinated 3, 7 or 14 days before scheduled elective tonsillectomy with seasonal trivalent LAIV. Plasma and peripheral blood mononuclear cells (PBMCs), and saliva were collected before and up to 1 year after vaccination, as well as on the day of tonsillectomy. Tonsils were collected and TMNCs were isolated on the day of tonsillectomy from children and adults. Antibody responses were measured using plasma samples with a panel of standard and newly established assays. Systemic and local T-cell responses were characterized using PBMCs and TMNCs, respectively, with IFN-γ ELISpot assay and multiplex cytokine measurement.

Systemic humoral immune responses after LAIV

Neutralizing antibodies prevent influenza virus entry into host cells, and the initial establishment of infection. Therefore, the ability of a vaccine to elicit protective neutralizing antibodies has been the gold standard measurement of a successful vaccine. The HI assay measures HA receptor binding site specific antibodies, and an HI titre of 40 has been widely used as a surrogate correlate of protection in influenza vaccine studies, based on its correlation with a 50% reduction in the risk of contracting influenza in healthy adults [283]. The MN assay directly measures

antibody inhibiting virus entry and MN titres consistently correlated with HI titres. Therefore, we assessed whether LAIV elicited neutralizing antibody responses against homologous strains using HI (papers I and III) and MN (paper II) assays. LAIV elicited significant increase in neutralizing antibodies against H3 and B, but not the H1 virus in children.

Sasaki et al. reported that LAIV induced cross-reactive plasmablast response towards HAs of heterovarient H1N1 strains [157]. We measured the cross-reactive antibody responses against H1, H3, H5 and H7 HAs in ELISA. We detected significant increase in H3 and H7, but not H1 or H5, specific antibodies [206]. All 18 serotypes of HAs found in influenza A viruses can be divided into two groups, largely attribute to the relative conserved stalk region, and H1 and H5 are both in group I, while H3 and H7 are in group II. Therefore, we further dissected antibodies into HA head or stalk specific, and found LAIV elicited significant increase in H3 head specific antibodies and a slight increasing trend in H1 stalk specific antibodies in children (paper I). In addition, VN assay with a re-assortant virus cH9/1N3 also revealed a slightly elevated level of H1 stalk antibodies after LAIV (paper III).

Recently, functional non-neutralizing antibodies' contribution to shortening viral shedding and reduced disease severity has been more and more appreciated. With ELLA and ADCC reporter assays, we demonstrated that LAIV elicited significant increases in N1NA inhibiting antibodies and H1N1 specific ADCC antibody increase in children.

HA stalk specific neutralizing antibodies can provide cross-protection by inhibiting a variety of viruses heterologous to vaccine strain from entering host cells. For example, avian influenza H5N1 and H7N1/H7N9 viruses have caused zoonotic outbreaks and sporadic infection in humans, which give rise to potential pandemic threats. The zoonotic H5 and H1 from LAIV A/H1N1pdm09 strain have divergent head domains but share similar stalk, as do the avian H7 and H3 from LAIV H3N2 strain. Therefore, LAIV elicits cross-reactive antibodies, which may provide (partial) protection in case of H5 or H7 pandemics. Non-neutralizing HA stalk specific antibodies can be involved in NK cells activation and complement dependent

cytotoxicity of infected cells. Our observations imply that LAIV may be used to induce stalk based antibodies, although further studies will be required to know if these functional antibodies play a role in protection against H1N1. NA enables the cleavage of HA-sialic acid binding on infected host cells during progeny virion release and virus penetration through mucosal lumen. As a result, NA inhibiting antibodies play important roles in preventing initial establishment of infection and shortening virus shedding. Monto *et al.* reported NA inhibiting antibody titres with no correlation to neutralizing antibody titres measured in HI or MN assays, and therefore were an independent correlate of protection in their clinical trial [284]. Currently there's no standard concentration of NA content in IIV although it should be present, but since LAIV has live viruses, its NA protein content is in a more or less consistent ratio to HA protein, which elicits NA inhibiting antibody responses after LAIV, often better than that after IIV. Although the quadrivalent LAIV monitoring study in UK shows no boost in NAI antibodies, but that could be due to the incompetent replication ability of the H1N1 vaccine strain, especially in competition with two B viruses [285, 286]. However, due to the tight conformational structure among HAs and NAs on the virus membrane, HA specific antibodies and NA specific antibodies may compete with each other for conformational close epitope binding [287-289]. He *et al.* demonstrated that antibody specificity profoundly influences the induction of ADCC, and the interaction among antibodies binding to discrete epitopes on the same antigen can influence the induction of Fc-dependent effector functions [51].

Local humoral immune responses after LAIV

In the work included in this thesis, no local humoral immune response after LAIV was assessed. However, an early study from colleagues analysing the samples from our LAIV clinical trial showed LAIV induced significant local antibody (salivary IgA) responses against H3N2 and B viruses as early as 14 days after vaccination. Influenza specific salivary IgA levels correlated with systemic neutralizing antibody levels measured in HI assay. Notably, LAIV augmented influenza specific antibody secreting cells and local memory B-cell responses in tonsils. Local and systemic memory B-cell responses detected in TMNCs and PBMCs, respectively, correlated with each other [264].

Due to its route of administration, LAIV elicits more predominantly local IgA responses, instead of systemic IgG responses [290, 291]. An early study showed that the immunocompetent cells in the nasal mucosa can spread widely to the distant mucosal site, like salivary glands and could also potentially mediate the effective immunity in tonsils [292]. Belshe *et al.* and Ambrose et al. also reported LAIV elicited local IgA responses in nasal mucosa, which correlated with protection [162, 263]. Therefore, although HI titre of 40 is used as a surrogate correlate of protection in influenza IIV studies [293], a different parameter, or more likely a set of parameters, is needed as correlates of protection to better understand the immunogenicity and efficacy of LAIV [294].

Cellular immune responses after LAIV

CD4 and CD8 T-cell responses have been demonstrated in association with shortened viral shedding and reduced disease severity, in the absence of neutralizing antibodies. In human studies, sero-negative adults showed reduced viral shedding in response to the increased CD8 T-cells [295, 296]. Increased CD8 T-cells mediate the cytotoxic destruction of infected cells. Sridhar *et al.* reported that in the absence of the A/H1N1pdm09 specific antibody, pre-existing late-effector and cytotoxic CD8 IFN-γ T-cells were potentially associated with milder symptoms and less severe illness [95]. *Wang et al.* demonstrated that CD8 T-cell responses were associated with recovery from severe H7N9 disease [96]. However, Wilkinson *et al.* in a human challenge study showed that pre-existing CD4 T-cells correlate with lower virus shedding and less severe influenza symptoms in sero-negative individuals [94].

T-cell receptors recognize antigen epitopes presented on MHC molecules. As a result, T-cell epitopes are in linear structure of peptides 9-11 aa (CD8 T-cells) and 13-25 aa (CD4 T-cells), often much simpler than the conformational structure of B-cell epitopes. In addition, CD4 and CD8 T-cell epitopes can be found not only on the heavily divergent surface glycoprotein HA and NA, but also, perhaps more predominantly, on relatively conserved internal proteins, such as PB1, NP and M1. Consequently, CD4 and CD8 T-cell responses are more cross-reactive against a variety of heterologous influenza strains compared to antibody responses, which is

often influenza strain specific with little cross-reactivity. CD8 T-cells differentiate into cytotoxic T-lymphocytes (CTL), and kill virus infected host cells through cytokines and other effector molecules. On the other hand, different lineages of CD4 helper T-cells can be characterized based on their distinctive cytokine secretion upon antigen stimulation. Th1 cells, secreting IFN-γ, IL-2 and TNFα, are involved in assisting anti-viral CTL responses; Th2 cells, secreting IL-4, IL-5 and IL-13, can promote B-cell responses; Th17 and regulatory T-cells are involved in regulating cellular immunity; follicular T-helper cells, secreting IL-21, mainly provide vital help in B-cell survival, programming and maturation within germinal centres inside secondary lymph nodes [49].

Earlier studies from colleagues using samples from the same LAIV clinical trial provided key knowledge in understanding cellular immune responses in children after LAIV. Firstly, the breadth of T-cell responses after LAIV was tested with two heterologous influenza viruses, A/Solomon Islands/2006 (H1N1) as a historical strain circulated before most of the children in LAIV clinical trial were born, and A/Switzerland/2013 (H3N2) as a future strain circulated after the LAIV clinical trial was performed. Interestingly, increases in Sol/06 specific IFN-γ+ and IFN-γ+IL2+ T-cells, and increases in Swi/13 specific IFN γ+, IL 2+ and IFN-γ+IL2+ T-cells were found after LAIV. As most of the children had little chance of experiencing either of Sol/06 or Swi/13 virus before the LAIV clinical trial, we showed that LAIV elicited broadly reactive T-cell responses against both H1N1 and H3N2 viruses. Next, CD8 T-cell responses were detected using synthetic influenza specific major histocompatibility complex class 1 (MHC-I) restricted peptide pools covering influenza internal proteins PB1, NP and M1. CD8 T-cell responses were highest to NP, and lower to PB1 and M1. NP-specific CD8 cells increased after LAIV and maintained above the pre-vaccination level up to 1 year. When the NP response were further dissected by age, children ≥10 years old showed significantly higher levels of anti-NP CD8 T-cells than those <10 years old [265].

In this thesis, we analysed influenza specific local and systemic cellular immune responses in TMNCs and PBMCs, respectively, using IFN-γ ELISpot assay with LAIV split antigens or peptides as stimuli (paper II and III). Additionally, we

measured the cytokine secretion from TMNCs and PBMCs upon LAIV split antigen stimulation to further dissect Th1, Th2 and Th17 responses after LAIV in children (paper III). We showed that LAIV elicited significant local T-cell responses against H3 and B viruses at day 14, but not against H1 virus. Systemically, LAIV induced T-cell responses against H1 and B viruses at day 14, but not against H3 virus. Furthermore, significantly elevated amounts of Th1 and Th2 cytokines were secreted from TMNCs 14 days after LAIV. No noticeable increase of cytokines from PBMCs was found, probably due to the limited amount of cells available. Lastly, we assessed the breadth of LAIV induced local and systemic T-cell responses using TMNCs and PBMCs with conserved peptide pools covering CD4 and CD8 epitopes. Importantly, LAIV elicited increases of IFN-γ+ cells was against the conserved CD4 and CD8 epitopes in TMNCs (local).

Pre-existing immunity impacts LAIV immunogenicity

Natural infection with influenza viruses occurs in all age groups and triggers multifaceted immune responses. The WHO recommends annual vaccination to combat seasonal influenza, especially in high-risk groups. Due to the proof-reading lacking replication mechanism and vast virus reservoir in migrating aqua and seashore birds, influenza viruses evolve constantly. As a result, immunity gained from last season (from vaccine or natural infection) would not necessarily be protective in the next season, especially in case of influenza antigenic drift and shift. How the pre-existing immunity from last season impacts the immune responses in the next season vaccination and/or infection has been the focus of more and more studies, but underlying mechanisms are still largely unknown.

LAIV mimics natural infection and elicits humoral and cellular immune responses, in which the attenuated virus replication in upper respiratory tract is essential. Therefore, the influence from pre-existing immunity, especially in nasal mucosa, is more profound on LAIV compared to the influence on IIV [156]. Studies have proven that LAIV induces better immune responses and is more efficacious in children, who had lower pre-existing immunity, compared to adults [297]. In addition, LAIV works better as priming vaccine in children with no previous virus/vaccine exposure, while

IIV works ideally as boost vaccine in children with LAIV and/or natural infection history [110].

To better understand how pre-existing immunity impacts on LAIV immunogenicity, we conduced LAIV clinical trial on both children and adults, despite that LAIV is only recommended for children 2-17 years old in Europe. Firstly, children had higher titre of neutralizing antibodies (paper I), as well as NA inhibiting antibodies against H1N1 virus than adults (paper II). More interestingly, children and adults who had received earlier pandemic or seasonal vaccine(s) had higher pre-existing H1N1 specific neutralizing antibody titres than those had not (paper II). On the contrary, adults had higher amount of antibodies cross-reactive to H5 and H7 full length HA, H1 and H3 stalk specific antibodies (paper I), as well as ADCC antibodies (paper II). Here, we speculate that this intriguingly opposite pattern between children and adults among different types of antibodies attribute to the different mechanisms involved in inducing those antibodies. For example, stalk specific and ADCC antibodies are more cross-reactive and can be accumulated with age and multiple exposures (vaccine or infection), whereas neutralizing antibodies and NA inhibiting antibodies are more strain specific and dependent on one successful immune system stimulation, either as the priming strain or potent adjuvant vaccine response. The illustrated speculation is described for children in Figure 11 after LAIV immunization. However, more studies are needed to test our speculation.

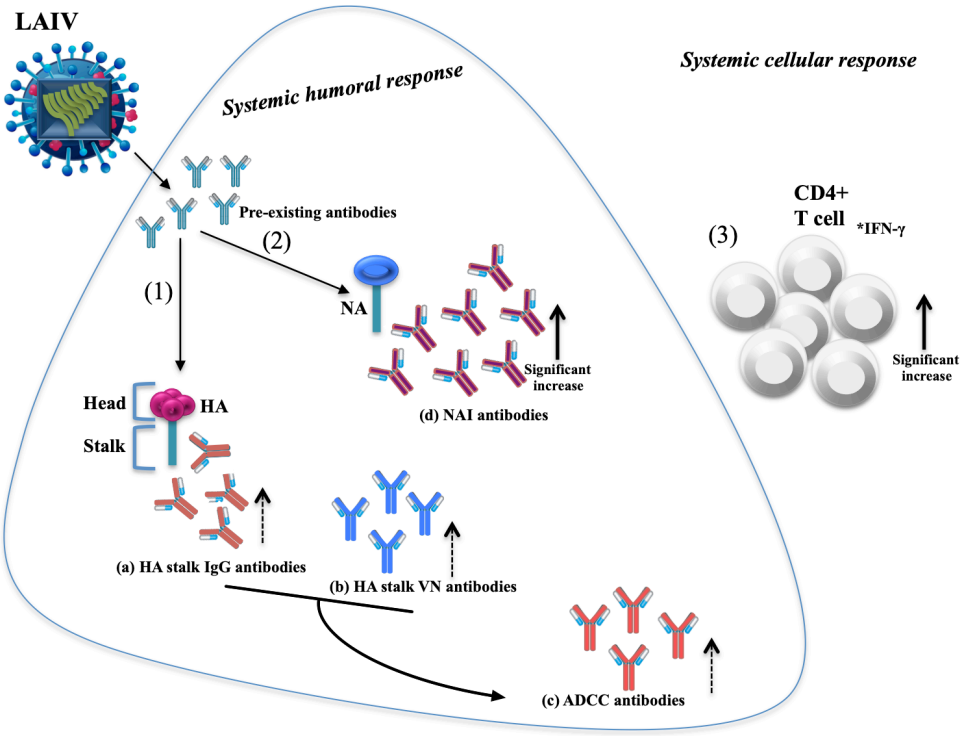


Figure 11. Speculation of how live attenuated influenza vaccine is involved in A/H1N1pdm09 specific immune response in children. Pre-existing antibodies derived from memory B-cells may direct the post-immunization response toward different pathways; (1) HA stalk antibodies (IgG and VN antibodies) increased and thus ADCC antibodies may be elevated (2) LAIV significantly increased NAI antibodies and (3) IFN-γ secreting CD4+ T-cells were boosted after LAIV.

In summary, LAIV requires replication of the vaccine viruses at the site of administration but is influenced by the pre-existing antibodies in children and adults. We explained the H1N1 vaccine strain in LAIV might have been protected children through NAI and T cellular response. Local protection is mediated by both humoral and cellular responses following LAIV immunization, where tonsils play a critical role. The systemic antibody response is broad comprising of both neutralizing and non-neutralizing antibodies following LAIV. A/H1N1pdm09 virus specific humoral immunity was not boosted in general although NAI antibody responses were elevated in children. Thus, NAI assay appears to be a sensitive tool to detect antibody responses after LAIV, where pre-existing HA specific response may not be boosted. Also, CD4 T-cells responses in blood were induced against A/H1N1pdm09 vaccine

strain. In contrast to the observation in USA, our overall study results showed LAIV boosted immune responses and confirm the European effectiveness data from UK and Finland [277, 279]. There is a multifaceted immune responses following LAIV immunization, which supports the continued use of live attenuated influenza vaccine in children.

Limitations of the studies

The studies included in this thesis are part of the clinical trial on trivalent LAIV in children and adults. As a result, our studies inherit limitations of human clinical trial, especially in paediatric population. The number of subjects included and samples collected in the studies are limited by the time and capacity of the facilities involved in the clinical trial. In Norway, participants including parents of children enrolled are not compensated. Blood sampling time points and volumes are restricted especially in children. As a result, immunological assays must be prioritized, if samples were not enough for every assay desired. Serology assays are performed with cyro-preserved plasma samples. Cellular assays, on the contrary, had to be performed with freshly isolated cells to ensure the optimal sensitivity. Therefore, we chose IFN-γ ELISpot assay and multiplex cytokine quantification, which are relatively short and straightforward. However, more in-depth analyses of cellular immune responses were not possible in the work here, but should be considered in future studies.

7 Conclusions

This PhD project aimed to understand the systemic and local immune responses in children and adults recruited into the LAIV clinical trial. We have gained new knowledge of the immunological responses after LAIV, particularly the antibodies including functional antibody responses to HA and NA, non-neutralizing ADCC responses as well as the T cellular responses, which will guide future studies of LAIV. In conclusion, the study has answered our primary aim of investigating the magnitude, quality, breadth and duration of the humoral immune response. The secondary objectives were met as followed as summarized below-

Paper I provided the following knowledge-

- LAIV elicited H3N2 specific HI antibodies in children, but in adults antibodies were not boosted to H3N2 or H1N1 strains and remained below the protective titre both pre- and post-vaccination.

- Intranasal LAIV boosted the H3 specific IgG responses against the full-length and head of HA in children, but not adults. H1 specific IgG antibody responses were also more HA head oriented in children. Although the H3 head specific IgG was dominant in adults, the H1 antibodies were stalk dominant, but LAIV immunization did not boost head or stalk responses.

- Importantly, we observed a trend of boosting of H1 stalk specific IgG antibodies in children after LAIV. Adults had higher levels of pre-existing stalk antibodies (towards both H3 and H1), but these were not boosted.

- Heterologous IgG antibody responses to H5 and H7 were not boosted in either children or adults after LAIV immunization, although children had no H7 cross-reactive antibodies.

In paper II, we further investigated the H1N1 specific neutralizing and non-neutralizing functional antibody responses and found-

- No significant changes were observed for micro-neutralizing (MN) antibodies in either children or adults after LAIV3.
- H1 stalk specific neutralizing antibody titres were high in adults and increased post-LAIV vaccination in children.
- We observed that LAIV boosted neuraminidase inhibition antibodies (NAI) in children, although both children and adults demonstrated NAI titres below the recently published protective level of NAI titre <40 [230].
- Higher ADCC antibody titres were seen in adults compared to children pre- and post-vaccination against A/H1N1pdm09. LAIV immunization did not induce ADCC activity in either children or adults.
- Only H1N1 specific CD4 T-cells are boosted after immunization, with almost undetectable CD8 T-cell responses found in children.

We answered in **Paper III** the following-

- Children had increases in IFN-γ responses to the influenza A virus as well as the conserved CD4+ and CD8+ peptides covering conserved epitopes of isolated influenza A viruses circulating between 1934-2009 after LAIV.
- H1N1 specific IFN-γ response increased significantly at 56 days post-vaccination in PBMC compared to pre-vaccination and non-vaccinated controls but no increase was observed in the TMNC.
- Influenza B strain specific IFN-γ responses increased both locally (TMNC) and systemically (PBMC).
- LAIV vaccination increased local and systemic IFN-γ responses for H3N2, although only blood had a significant increase from days 14 to 56 post-vaccination.

- Interestingly, we showed an increasing trend of broadly cross reactive CD4+ T-cells, although not significant. LAIV resulted in a significant increase in CD8+ T-cell responses post-vaccination in the tonsils.

- We showed the increased cytokine responses in TMNC within 14 days of LAIV post-vaccination demonstrated by Th1, Th2 and GM-CSF subpopulation and compared to control tonsils. Whereas no increase in cytokine responses was observed in PBMC after LAIV and confirming the early the local tonsillar $CD4^+$ T-cell responses.

8 Future Perspective

This thesis utilised the LAIV vaccine trial in children aged between 3 to 17 years old and adults to dissect and compare the humoral immune responses. Functional antibodies were measured in both terms of neutralizing and non-neutralizing antibodies responses. The work was extended to investigate the local or systemic cellular immune response following LAIV administration.

Due to time limitation, we have not studied functional or non-neutralizing antibodies against the H3N2 and B influenza strains in children and adults. We have shown that LAIV boosted IgG responses in children in blood against H3N2. LAIV boosts T-cell response both locally and systemically against influenza B in children. Therefore, in future studies we will investigate the functionality as well as the non-neutralizing antibodies against H3N2 and B strain in children and adults. Influenza NA antibodies may provide protection and could also be an important parameter for future studies of LAIV extending to the H3N2 and B influenza strains. Importantly, influenza NA functional antibodies can be analysed by the plaque reduction assay, which is more sensitive than MN assay, allowing the roles of neutralizing antibody to HA and NA to be dissected.

LAIV is administered intranasally and the tonsils are the local draining lymph nodes. They are compartmentalized organs where germinal centres (GC) are formed for generation of high affinity antibodies and long-lived plasma cells and memory B-cells. Follicular T-helper (T_{FH}) cells are a subgroup of $CD4^+$ T-cells that help B-cells through proliferation and affinity maturation inside GC. For future studies it will be important to measure mucosal antibodies and the T follicular cellular response in the tonsils both using flow cytometry and immunohistochemistry.

LAIV may play a role in reducing the influenza related illness through induction of herd immunity in the population when vaccination rates are high enough [256]. Finally, as LAIV rapidly induces T-cell responses in the tonsil, we may address the issues how LAIV could be a potential candidate as a universal vaccine candidate.

9 Reference

[1] Su S, Fu X, Li G, Kerlin F, Veit M. Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics. Virulence. 2017;8:1580-91.
[2] Vemula SV, Zhao J, Liu J, Wang X, Biswas S, Hewlett I. Current Approaches for Diagnosis of Influenza Virus Infections in Humans. Viruses. 2016;8:96.
[3] Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312:384-8.
[4] Allen JD, Ross TM. H3N2 Influenza Viruses in Humans: Viral Mechanisms, Evolution, and Evaluation. Hum Vaccin Immunother. 2018:0.
[5] Ferhadian D, Contrant M, Printz-Schweigert A, Smyth RP, Paillart JC, Marquet R. Structural and Functional Motifs in Influenza Virus RNAs. Front Microbiol. 2018;9:559.
[6] Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, Wyatt RT. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat Rev Microbiol. 2008;6:143-55.
[7] da Silva DV, Nordholm J, Madjo U, Pfeiffer A, Daniels R. Assembly of Subtype 1 Influenza Neuraminidase Is Driven by Both the Transmembrane and Head Domains. J Biol Chem. 2013;288:644-53.
[8] Shtyrya YA, Mochalova LV, Bovin NV. Influenza virus neuraminidase: structure and function. Acta Naturae. 2009;1:26-32.
[9] Yang J, Liu S, Du L, Jiang S. A new role of neuraminidase (NA) in the influenza virus life cycle: implication for developing NA inhibitors with novel mechanism of action. Rev Med Virol. 2016;26:242-50.
[10] Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol. 2004;78:12665-7.
[11] Wagner R, Wolff T, Herwig A, Pleschka S, Klenk HD. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J Virol. 2000;74:6316-23.
[12] Braam J, Ulmanen I, Krug RM. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell. 1983;34:609-18.
[13] Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, et al. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature. 2009;458:914-8.
[14] Blaas D, Patzelt E, Kuechler E. Identification of the cap binding protein of influenza virus. Nucleic Acids Res. 1982;10:4803-12.
[15] Stevaert A, Naesens L. The Influenza Virus Polymerase Complex: An Update on Its Structure, Functions, and Significance for Antiviral Drug Design. Med Res Rev. 2016;36:1127-73.
[16] Boivin S, Cusack S, Ruigrok RW, Hart DJ. Influenza A virus polymerase: structural insights into replication and host adaptation mechanisms. J Biol Chem. 2010;285:28411-7.

[17] Kobayashi M, Toyoda T, Ishihama A. Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. Arch Virol. 1996;141:525-39.
[18] Gabriel G, Fodor E. Molecular determinants of pathogenicity in the polymerase complex. Curr Top Microbiol Immunol. 2014;385:35-60.
[19] Gomez-Puertas P, Albo C, Perez-Pastrana E, Vivo A, Portela A. Influenza virus matrix protein is the major driving force in virus budding. J Virol. 2000;74:11538-47.
[20] Bui M, Wills EG, Helenius A, Whittaker GR. Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins. J Virol. 2000;74:1781-6.
[21] Pinto LH, Lamb RA. The M2 proton channels of influenza A and B viruses. J Biol Chem. 2006;281:8997-9000.
[22] Rossman JS, Jing X, Leser GP, Lamb RA. Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell. 2010;142:902-13.
[23] Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, et al. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe. 2009;5:439-49.
[24] de Chassey B, Aublin-Gex A, Ruggieri A, Meyniel-Schicklin L, Pradezynski F, Davoust N, et al. The interactomes of influenza virus NS1 and NS2 proteins identify new host factors and provide insights for ADAR1 playing a supportive role in virus replication. PLoS Pathog. 2013;9:e1003440.
[25] Chen Z, Li Y, Krug RM. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. EMBO J. 1999;18:2273-83.
[26] Falcon AM, Fortes P, Marion RM, Beloso A, Ortin J. Interaction of influenza virus NS1 protein and the human homologue of Staufen in vivo and in vitro. Nucleic Acids Res. 1999;27:2241-7.
[27] O'Neill RE, Talon J, Palese P. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO J. 1998;17:288-96.
[28] Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature. 1988;333:426-31.
[29] Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008;26 Suppl 4:D49-53.
[30] Cros JF, Palese P. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. Virus Research. 2003;95:3-12.
[31] Dou D, Revol R, Ostbye H, Wang H, Daniels R. Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. Front Immunol. 2018;9:1581.
[32] Thompson T. Annals of influenza or epidemic catarrhal fever in Great Britain from 1510 to 1837. London,: Printed for the Sydenham society; 1852.
[33] Shope RE. Swine Influenza : I. Experimental Transmission and Pathology. J Exp Med. 1931;54:349-59.
[34] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152-79.
[35] World Health Orgnization. International travel and health. 2018.
[36] Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, et al. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet. 2008;371:1427-34.

[37] Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med. 2002;76:105-15.
[38] Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N Engl J Med. 2009;361:1945-52.
[39] Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet. 2018;391:1285-300.
[40] Centers for Disease Control and Prevention. Disease Burden of Influenza. 2018, May 22.
[41] Yu J, Zhang T, Wang Y, Gao JM, Hua J, Tian JM, et al. [Clinical characteristics and economic burden of influenza among children under 5 years old, in Suzhou, 2011-2017]. Zhonghua Liu Xing Bing Xue Za Zhi. 2018;39:847-51.
[42] Wang X, Cai J, Yao W, Zhu Q, Zeng M. [Socio-economic impact of influenza in children:a single-centered hospital study in Shanghai]. Zhonghua Liu Xing Bing Xue Za Zhi. 2015;36:27-30.
[43] Zhang T, Zhang J, Hua J, Wang D, Chen LL, Ding YF, et al. Influenza-associated outpatient visits among children less than 5 years of age in eastern China, 2011-2014. Bmc Infectious Diseases. 2016;16.
[44] Wang D, Zhang T, Wu J, Jiang YW, Ding YF, Hua J, et al. Socio-Economic Burden of Influenza among Children Younger than 5 Years in the Outpatient Setting in Suzhou, China. Plos One. 2013;8.
[45] (NIPH) NIoPH. Influenza vaccine. 2016.
[46] Organization WH. Weekly epidemiological record 2012.
[47] P.James S. Mucosal Immunity. Encyclopedia of Immunology (Second Edition). 1998.
[48] Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. Nat Rev Immunol. 2014;14:315-28.
[49] Chen X, Liu S, Goraya MU, Maarouf M, Huang S, Chen JL. Host Immune Response to Influenza A Virus Infection. Front Immunol. 2018;9:320.
[50] Parekh BS, Berger E, Sibley S, Cahya S, Xiao L, LaCerte MA, et al. Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay. MAbs. 2012;4:310-8.
[51] He W, Tan GS, Mullarkey CE, Lee AJ, Lam MM, Krammer F, et al. Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus. Proc Natl Acad Sci U S A. 2016;113:11931-6.
[52] Dunand CJH, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, et al. Both Neutralizing and Non-Neutralizing Human H7N9 Influenza Vaccine-Induced Monoclonal Antibodies Confer Protection. Cell Host & Microbe. 2016;19:800-13.
[53] Ho AW, Prabhu N, Betts RJ, Ge MQ, Dai X, Hutchinson PE, et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. J Immunol. 2011;187:6011-21.
[54] La Gruta NL, Turner SJ. T cell mediated immunity to influenza: mechanisms of viral control. Trends Immunol. 2014;35:396-402.

[55] Pape KA, Khoruts A, Mondino A, Jenkins MK. Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells. J Immunol. 1997;159:591-8.
[56] Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4(+) T cells in immunity to viruses. Nat Rev Immunol. 2012;12:136-48.
[57] Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol. 2010;28:445-89.
[58] Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41:529-42.
[59] Aljurayyan A, Puksuriwong S, Ahmed M, Sharma R, Krishnan M, Sood S, et al. Activation and Induction of Antigen-Specific T Follicular Helper Cells Play a Critical Role in Live-Attenuated Influenza Vaccine-Induced Human Mucosal Anti-influenza Antibody Response. J Virol. 2018;92.
[60] Qi H. T follicular helper cells in space-time. Nat Rev Immunol. 2016;16:612-25.
[61] Hayakawa K, Li YS, Wasserman R, Sauder S, Shinton S, Hardy RR. B lymphocyte developmental lineages. Ann N Y Acad Sci. 1997;815:15-29.
[62] Huang KY, Li CK, Clutterbuck E, Chui C, Wilkinson T, Gilbert A, et al. Virus-specific antibody secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model. J Infect Dis. 2014;209:1354-61.
[63] Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. 7th ed. Philadelphia: Elsevier/Saunders; 2012.
[64] Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature. 2008;453:667-71.
[65] Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A. Clonal dissection of the human memory B-cell repertoire following infection and vaccination. Eur J Immunol. 2009;39:1260-70.
[66] Clements ML, Murphy BR. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. J Clin Microbiol. 1986;23:66-72.
[67] Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Jpn J Infect Dis. 2004;57:236-47.
[68] Frolich D, Giesecke C, Mei HE, Reiter K, Daridon C, Lipsky PE, et al. Secondary immunization generates clonally related antigen-specific plasma cells and memory B cells. J Immunol. 2010;185:3103-10.
[69] Inoue T, Moran I, Shinnakasu R, Phan TG, Kurosaki T. Generation of memory B cells and their reactivation. Immunol Rev. 2018;283:138-49.
[70] Lanzavecchia A, Bernasconi N, Traggiai E, Ruprecht CR, Corti D, Sallusto F. Understanding and making use of human memory B cells. Immunol Rev. 2006;211:303-9.
[71] Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133:775-87.
[72] Tao X, Constant S, Jorritsma P, Bottomly K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. J Immunol. 1997;159:5956-63.

[73] Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity. 2003;19:641-4.
[74] Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev. 2000;14:1693-711.
[75] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5:987-95.
[76] Murray HW, Rubin BY, Carriero SM, Harris AM, Jaffee EA. Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular Toxoplasma gondii. J Immunol. 1985;134:1982-8.
[77] Schultz-Cherry S, Hinshaw VS. Influenza virus neuraminidase activates latent transforming growth factor beta. J Virol. 1996;70:8624-9.
[78] Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev. 2006;17:349-66.
[79] Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 2006;441:890-3.
[80] Wuest TY, Willette-Brown J, Durum SK, Hurwitz AA. The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. J Leukoc Biol. 2008;84:973-80.
[81] Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J Immunol. 2001;166:7276-81.
[82] Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol. 2008;180:5771-7.
[83] Maroof A, Yorgensen YM, Li Y, Evans JT. Intranasal vaccination promotes detrimental Th17-mediated immunity against influenza infection. PLoS Pathog. 2014;10:e1003875.
[84] Bermejo-Martin JF, Ortiz de Lejarazu R, Pumarola T, Rello J, Almansa R, Ramirez P, et al. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. Crit Care. 2009;13:R201.
[85] Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. Immunity. 2009;30:324-35.
[86] Topham DJ, Tripp RA, Doherty PC. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol. 1997;159:5197-200.
[87] Sun J, Braciale TJ. Role of T cell immunity in recovery from influenza virus infection. Curr Opin Virol. 2013;3:425-9.
[88] Kagi D, Hengartner H. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr Opin Immunol. 1996;8:472-7.
[89] Hoft DF, Babusis E, Worku S, Spencer CT, Lottenbach K, Truscott SM, et al. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. J Infect Dis. 2011;204:845-53.

[90] He XS, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, et al. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. J Virol. 2006;80:11756-66.
[91] Cheng X, Zengel JR, Suguitan AL, Jr., Xu Q, Wang W, Lin J, et al. Evaluation of the humoral and cellular immune responses elicited by the live attenuated and inactivated influenza vaccines and their roles in heterologous protection in ferrets. J Infect Dis. 2013;208:594-602.
[92] Rekstin A, Isakova-Sivak I, Petukhova G, Korenkov D, Losev I, Smolonogina T, et al. Immunogenicity and Cross Protection in Mice Afforded by Pandemic H1N1 Live Attenuated Influenza Vaccine Containing Wild-Type Nucleoprotein. Biomed Res Int. 2017;2017:9359276.
[93] Jang YH, Seong BL. Cross-protective immune responses elicited by live attenuated influenza vaccines. Yonsei Med J. 2013;54:271-82.
[94] Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med. 2012;18:274-80.
[95] Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med. 2013;19:1305-12.
[96] Wang Z, Wan Y, Qiu C, Quinones-Parra S, Zhu Z, Loh L, et al. Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells. Nat Commun. 2015;6:6833.
[97] Wang Z, Kedzierski L, Nuessing S, Chua BY, Quinones-Parra SM, Huber VC, et al. Establishment of memory CD8+ T cells with live attenuated influenza virus across different vaccination doses. J Gen Virol. 2016;97:3205-14.
[98] Razvi ES, Jiang Z, Woda BA, Welsh RM. Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. Am J Pathol. 1995;147:79-91.
[99] Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708-12.
[100] Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL. Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. J Immunol. 2011;187:5510-4.
[101] Laidlaw BJ, Zhang N, Marshall HD, Staron MM, Guan T, Hu Y, et al. CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. Immunity. 2014;41:633-45.
[102] Piet B, de Bree GJ, Smids-Dierdorp BS, van der Loos CM, Remmerswaal EB, von der Thusen JH, et al. CD8(+) T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. J Clin Invest. 2011;121:2254-63.
[103] Hogan RJ, Usherwood EJ, Zhong W, Roberts AA, Dutton RW, Harmsen AG, et al. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. J Immunol. 2001;166:1813-22.

[104] Turner DL, Bickham KL, Thome JJ, Kim CY, D'Ovidio F, Wherry EJ, et al. Lung niches for the generation and maintenance of tissue-resident memory T cells. Mucosal Immunol. 2014;7:501-10.
[105] Ellebedy AH, Krammer F, Li GM, Miller MS, Chiu C, Wrammert J, et al. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. Proc Natl Acad Sci U S A. 2014;111:13133-8.
[106] Henry Dunand CJ, Leon PE, Kaur K, Tan GS, Zheng NY, Andrews S, et al. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. J Clin Invest. 2015;125:1255-68.
[107] Nachbagauer R, Wohlbold TJ, Hirsh A, Hai R, Sjursen H, Palese P, et al. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. J Virol. 2014;88:13260-8.
[108] Paules CI, Marston HD, Eisinger RW, Baltimore D, Fauci AS. The Pathway to a Universal Influenza Vaccine. Immunity. 2017;47:599-603.
[109] Liu L, Nachbagauer R, Zhu L, Huang Y, Xie X, Jin S, et al. Induction of Broadly Cross-Reactive Stalk-Specific Antibody Responses to Influenza Group 1 and Group 2 Hemagglutinins by Natural H7N9 Virus Infection in Humans. J Infect Dis. 2017;215:518-28.
[110] Krammer F, Palese P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr Opin Virol. 2013;3:521-30.
[111] Hertz T, Oshansky CM, Roddam PL, DeVincenzo JP, Caniza MA, Jojic N, et al. HLA targeting efficiency correlates with human T-cell response magnitude and with mortality from influenza A infection. Proc Natl Acad Sci U S A. 2013;110:13492-7.
[112] Clemens EB, Grant EJ, Wang Z, Gras S, Tipping P, Rossjohn J, et al. Towards identification of immune and genetic correlates of severe influenza disease in Indigenous Australians. Immunol Cell Biol. 2017;95:648.
[113] Falfan-Valencia R, Narayanankutty A, Resendiz-Hernandez JM, Perez-Rubio G, Ramirez-Venegas A, Nava-Quiroz KJ, et al. An Increased Frequency in HLA Class I Alleles and Haplotypes Suggests Genetic Susceptibility to Influenza A (H1N1) 2009 Pandemic: A Case-Control Study. J Immunol Res. 2018;2018:3174868.
[114] van Elden LJ, van Essen GA, Boucher CA, van Loon AM, Nijhuis M, Schipper P, et al. Clinical diagnosis of influenza virus infection: evaluation of diagnostic tools in general practice. Br J Gen Pract. 2001;51:630-4.
[115] Ruest A, Michaud S, Deslandes S, Frost EH. Comparison of the Directigen flu A+B test, the QuickVue influenza test, and clinical case definition to viral culture and reverse transcription-PCR for rapid diagnosis of influenza virus infection. J Clin Microbiol. 2003;41:3487-93.
[116] Prevention CfDCa. Influenza Signs and Symptoms and the Role of Laboratory Diagnostics. 2016.
[117] Boivin G, Hardy I, Tellier G, Maziade J. Predicting influenza infections during epidemics with use of a clinical case definition. Clin Infect Dis. 2000;31:1166-9.
[118] Playford EG, Dwyer DE. Laboratory diagnosis of influenza virus infection. Pathology. 2002;34:115-25.

[119] Bridges CB, Fukuda K, Uyeki TM, Cox NJ, Singleton JA, Centers for Disease C, et al. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2002;51:1-31.
[120] Palache A, Oriol-Mathieu V, Fino M, Xydia-Charmanta M, Influenza Vaccine Supply task f. Seasonal influenza vaccine dose distribution in 195 countries (2004-2013): Little progress in estimated global vaccination coverage. Vaccine. 2015;33:5598-605.
[121] Lu J, Raghwani J, Pryce R, Bowden TA, Theze J, Huang S, et al. Molecular Evolution, Diversity, and Adaptation of Influenza A(H7N9) Viruses in China. Emerg Infect Dis. 2018;24:1795-805.
[122] WHO. Human infection with avian influenza A(H7N9) virus – China: Update. 2018.
[123] CDC. Nonpharmaceutical Interventions (NPIs). 2017.
[124] Aledort JE, Lurie N, Wasserman J, Bozzette SA. Non-pharmaceutical public health interventions for pandemic influenza: an evaluation of the evidence base. BMC Public Health. 2007;7:208.
[125] Organization WH. WHO recommendations on pandemic (H1N1) 2009 vaccines. 2009.
[126] CDC. Key Facts About Influenza (Flu). 2018.
[127] CDC. How Flu Spreads. 2018.
[128] WHO. Reducing transmission of pandemic (H1N1) 2009 in school settings. 2009.
[129] Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. J Infect Dis. 2006;194 Suppl 2:S111-8.
[130] Armstrong BG, Mangtani P, Fletcher A, Kovats S, McMichael A, Pattenden S, et al. Effect of influenza vaccination on excess deaths occurring during periods of high circulation of influenza: cohort study in elderly people. BMJ. 2004;329:660.
[131] <A VIRUS OBTAINED FROM INFLUENZA PATIENTS.pdf>.
[132] Francis T, Jr. Vaccination against influenza. Bull World Health Organ. 1953;8:725-41.
[133] Hannoun C. The evolving history of influenza viruses and influenza vaccines. Expert Rev Vaccines. 2013;12:1085-94.
[134] ADMINISTRATION USFD. February 13, 2015 Approval Letter - FluMist Quadrivalent. 2015.
[135] agency EM. EPAR summary for the public. 2016.
[136] Gomez Lorenzo MM, Fenton MJ. Immunobiology of influenza vaccines. Chest. 2013;143:502-10.
[137] Ambrose CS, Levin MJ, Belshe RB. The relative efficacy of trivalent live attenuated and inactivated influenza vaccines in children and adults. Influenza Other Respir Viruses. 2011;5:67-75.
[138] Ambrose CS, Wu X, Belshe RB. The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination. Pediatr Infect Dis J. 2010;29:806-11.
[139] Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, et al. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N Engl J Med. 1998;338:1405-12.

[140] Hayden FG. Antivirals for influenza: historical perspectives and lessons learned. Antiviral Res. 2006;71:372-8.
[141] Hall M, Brown MD. Evidence-based emergency medicine/systematic review abstract. Are amantadine and rimantadine effective in healthy adults with acute influenza? Ann Emerg Med. 2005;46:292-3.
[142] Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, et al. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J Infect Dis. 2007;196:249-57.
[143] Thomaston JL, Polizzi NF, Konstantinidi A, Wang J, Kolocouris A, DeGrado WF. Inhibitors of the M2 Proton Channel Engage and Disrupt Transmembrane Networks of Hydrogen-Bonded Waters. J Am Chem Soc. 2018.
[144] Hayden FG, Gwaltney JM, Jr., Van de Castle RL, Adams KF, Giordani B. Comparative toxicity of amantadine hydrochloride and rimantadine hydrochloride in healthy adults. Antimicrob Agents Chemother. 1981;19:226-33.
[145] DRUG USF. Influenza (Flu) Antiviral Drugs and Related Information. 2018.
[146] Moscona A. Neuraminidase inhibitors for influenza. N Engl J Med. 2005;353:1363-73.
[147] Freund B, Gravenstein S, Elliott M, Miller I. Zanamivir: a review of clinical safety. Drug Saf. 1999;21:267-81.
[148] Hayden FG, Belshe R, Villanueva C, Lanno R, Hughes C, Small I, et al. Management of influenza in households: a prospective, randomized comparison of oseltamivir treatment with or without postexposure prophylaxis. J Infect Dis. 2004;189:440-9.
[149] Nicholson KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, Mercier CH, et al. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. Lancet. 2000;355:1845-50.
[150] Dobson J, Whitley RJ, Pocock S, Monto AS. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. Lancet. 2015;385:1729-37.
[151] Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, et al. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. N Engl J Med. 2018;379:913-23.
[152] CDC. Influenza Vaccination: A Summary for Clinicians. 2018.
[153] WHO. Vaccine use. 2018.
[154] Treanor JJ. Prospects for Broadly Protective Influenza Vaccines. Am J Prev Med. 2015;49:S355-63.
[155] Cox RJ, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. An early humoral immune response in peripheral blood following parenteral inactivated influenza vaccination. Vaccine. 1994;12:993-9.
[156] Hoft DF, Lottenbach KR, Blazevic A, Turan A, Blevins TP, Pacatte TP, et al. Comparisons of the Humoral and Cellular Immune Responses Induced by Live Attenuated Influenza Vaccine and Inactivated Influenza Vaccine in Adults. Clin Vaccine Immunol. 2017;24.

[157] Sasaki S, Holmes TH, Albrecht RA, Garcia-Sastre A, Dekker CL, He XS, et al. Distinct cross-reactive B-cell responses to live attenuated and inactivated influenza vaccines. J Infect Dis. 2014;210:865-74.
[158] Ambrose CS, Luke C, Coelingh K. Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza. Influenza Other Respir Viruses. 2008;2:193-202.
[159] Haaheim LR, Madhun AS, Cox R. Pandemic influenza vaccines - the challenges. Viruses. 2009;1:1089-109.
[160] Mohn KG, Smith I, Sjursen H, Cox RJ. Immune responses after live attenuated influenza vaccination. Hum Vaccin Immunother. 2018;14:571-8.
[161] Mohn KG, Bredholt G, Brokstad KA, Pathirana RD, Aarstad HJ, Tondel C, et al. Longevity of B-cell and T-cell responses after live attenuated influenza vaccination in children. J Infect Dis. 2015;211:1541-9.
[162] Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis. 2000;181:1133-7.
[163] Pebody R, Sile B, Warburton F, Sinnathamby M, Tsang C, Zhao H, et al. Live attenuated influenza vaccine effectiveness against hospitalisation due to laboratory-confirmed influenza in children two to six years of age in England in the 2015/16 season. Euro Surveill. 2017;22.
[164] Andrews SF, Huang Y, Kaur K, Popova LI, Ho IY, Pauli NT, et al. Immune history profoundly affects broadly protective B cell responses to influenza. Sci Transl Med. 2015;7:316ra192.
[165] Brodin P, Davis MM. Human immune system variation. Nat Rev Immunol. 2017;17:21-9.
[166] Skountzou I, Koutsonanos DG, Kim JH, Powers R, Satyabhama L, Masseoud F, et al. Immunity to pre-1950 H1N1 influenza viruses confers cross-protection against the pandemic swine-origin 2009 A (H1N1) influenza virus. J Immunol. 2010;185:1642-9.
[167] Lv J, Ren ZY, Zhang YY, Liu YE, Gao J, Yao K, et al. Study on age-dependent pre-existing 2009 pandemic influenza virus T and B cell responses from Chinese population. BMC Infect Dis. 2017;17:136.
[168] Nachbagauer R, Choi A, Izikson R, Cox MM, Palese P, Krammer F. Age Dependence and Isotype Specificity of Influenza Virus Hemagglutinin Stalk-Reactive Antibodies in Humans. MBio. 2016;7:e01996-15.
[169] Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, et al. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol. 2013;87:4728-37.
[170] Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. Proc Natl Acad Sci U S A. 2014;111:869-74.
[171] Agrawal A, Tay J, Ton S, Agrawal S, Gupta S. Increased reactivity of dendritic cells from aged subjects to self-antigen, the human DNA. J Immunol. 2009;182:1138-45.

[172] Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, et al. The influence of age on T cell generation and TCR diversity. J Immunol. 2005;174:7446-52.
[173] Hong MS, Dan JM, Choi JY, Kang I. Age-associated changes in the frequency of naive, memory and effector CD8+ T cells. Mech Ageing Dev. 2004;125:615-8.
[174] Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, et al. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. J Immunol. 2004;173:673-81.
[175] Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, et al. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci U S A. 2008;105:5986-91.
[176] Gostic KM, Ambrose M, Worobey M, Lloyd-Smith JO. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. Science. 2016;354:722-6.
[177] CDC. The 2009 H1N1 Pandemic: Summary Highlights, April 2009-April 2010. 2010.
[178] WHO. Pandemic (H1N1) 2009 - update 67. 2009.
[179] Monto AS, Malosh RE, Petrie JG, Martin ET. The Doctrine of Original Antigenic Sin: Separating Good From Evil. J Infect Dis. 2017;215:1782-8.
[180] Davenport FM, Hennessy AV, Francis T, Jr. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J Exp Med. 1953;98:641-56.
[181] Henry C, Palm AE, Krammer F, Wilson PC. From Original Antigenic Sin to the Universal Influenza Virus Vaccine. Trends Immunol. 2018;39:70-9.
[182] Lessler J, Riley S, Read JM, Wang S, Zhu H, Smith GJ, et al. Evidence for antigenic seniority in influenza A (H3N2) antibody responses in southern China. PLoS Pathog. 2012;8:e1002802.
[183] Miller MS, Gardner TJ, Krammer F, Aguado LC, Tortorella D, Basler CF, et al. Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. Sci Transl Med. 2013;5:198ra07.
[184] Fonville JM, Wilks SH, James SL, Fox A, Ventresca M, Aban M, et al. Antibody landscapes after influenza virus infection or vaccination. Science. 2014;346:996-1000.
[185] Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, et al. Antibody recognition of a highly conserved influenza virus epitope. Science. 2009;324:246-51.
[186] Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol. 2009;16:265-73.
[187] Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, et al. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog. 2010;6:e1000796.
[188] Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science. 2011;333:843-50.

[189] Tan GS, Krammer F, Eggink D, Kongchanagul A, Moran TM, Palese P. A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. J Virol. 2012;86:6179-88.
[190] Krammer F. The Quest for a Universal Flu Vaccine: Headless HA 2.0. Cell Host Microbe. 2015;18:395-7.
[191] Valkenburg SA, Mallajosyula VV, Li OT, Chin AW, Carnell G, Temperton N, et al. Stalking influenza by vaccination with pre-fusion headless HA mini-stem. Sci Rep. 2016;6:22666.
[192] Krammer F. Novel universal influenza virus vaccine approaches. Curr Opin Virol. 2016;17:95-103.
[193] Krammer F, Pica N, Hai R, Margine I, Palese P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J Virol. 2013;87:6542-50.
[194] Krammer F, Margine I, Hai R, Flood A, Hirsh A, Tsvetnitsky V, et al. H3 stalk-based chimeric hemagglutinin influenza virus constructs protect mice from H7N9 challenge. J Virol. 2014;88:2340-3.
[195] Margine I, Krammer F, Hai R, Heaton NS, Tan GS, Andrews SA, et al. Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. J Virol. 2013;87:10435-46.
[196] Nachbagauer R, Miller MS, Hai R, Ryder AB, Rose JK, Palese P, et al. Hemagglutinin Stalk Immunity Reduces Influenza Virus Replication and Transmission in Ferrets. J Virol. 2015;90:3268-73.
[197] Goff PH, Eggink D, Seibert CW, Hai R, Martinez-Gil L, Krammer F, et al. Adjuvants and immunization strategies to induce influenza virus hemagglutinin stalk antibodies. Plos One. 2013;8:e79194.
[198] Crevar CJ, Carter DM, Lee KY, Ross TM. Cocktail of H5N1 COBRA HA vaccines elicit protective antibodies against H5N1 viruses from multiple clades. Hum Vaccin Immunother. 2015;11:572-83.
[199] Giles BM, Bissel SJ, Dealmeida DR, Wiley CA, Ross TM. Antibody breadth and protective efficacy are increased by vaccination with computationally optimized hemagglutinin but not with polyvalent hemagglutinin-based H5N1 virus-like particle vaccines. Clin Vaccine Immunol. 2012;19:128-39.
[200] Carter DM, Darby CA, Lefoley BC, Crevar CJ, Alefantis T, Oomen R, et al. Design and Characterization of a Computationally Optimized Broadly Reactive Hemagglutinin Vaccine for H1N1 Influenza Viruses. J Virol. 2016;90:4720-34.
[201] Wong TM, Allen JD, Bebin-Blackwell AG, Carter DM, Alefantis T, DiNapoli J, et al. Computationally Optimized Broadly Reactive Hemagglutinin Elicits Hemagglutination Inhibition Antibodies against a Panel of H3N2 Influenza Virus Cocirculating Variants. J Virol. 2017;91.
[202] Schwartzman LM, Cathcart AL, Pujanauski LM, Qi L, Kash JC, Taubenberger JK. An Intranasal Virus-Like Particle Vaccine Broadly Protects Mice from Multiple Subtypes of Influenza A Virus. MBio. 2015;6:e01044.
[203] Muller GM, Shapira M, Arnon R. Anti-influenza response achieved by immunization with a synthetic conjugate. Proc Natl Acad Sci U S A. 1982;79:569-73.
[204] Ben-Yedidia T, Arnon R. Epitope-based vaccine against influenza. Expert Rev Vaccines. 2007;6:939-48.

[205] ClinicalTrials.gov USNLoM. Safety and Immunogenicity of a Live-attenuated Universal Flu Vaccine Followed by an Inactivated Universal Flu Vaccine. 2017.
[206] Islam S, Mohn KG, Krammer F, Sanne M, Bredholt G, Jul-Larsen A, et al. Influenza A haemagglutinin specific IgG responses in children and adults after seasonal trivalent live attenuated influenza vaccination. Vaccine. 2017;35:5666-73.
[207] Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, et al. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. Proc Natl Acad Sci U S A. 2012;109:2573-8.
[208] Hai R, Krammer F, Tan GS, Pica N, Eggink D, Maamary J, et al. Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. J Virol. 2012;86:5774-81.
[209] Savic M, Dembinski JL, Kim Y, Tunheim G, Cox RJ, Oftung F, et al. Epitope specific T-cell responses against influenza A in a healthy population. Immunology. 2016;147:165-77.
[210] Kaufmann L, Syedbasha M, Vogt D, Hollenstein Y, Hartmann J, Linnik JE, et al. An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers. J Vis Exp. 2017.
[211] WHO. WHO manual on animal influenza diagnosis and surveillance. 2002.
[212] Li D, Wang J, Treanor JJ, Zand MS. Improved Specificity and False Discovery Rates for Multiplex Analysis of Changes in Strain-Specific Anti-Influenza IgG. Comput Math Methods Med. 2019;2019:3053869.
[213] Masihi KN, Lange W. Enzyme-Linked Immunosorbent-Assay for the Detection of Influenza Type-Specific Antibodies. Journal of Immunological Methods. 1980;36:173-9.
[214] Russell SM, McCahon D, Beare AS. A single radial haemolysis technique for the measurement of influenza antibody. J Gen Virol. 1975;27:1-10.
[215] Wang B, Russell ML, Brewer A, Newton J, Singh P, Ward BJ, et al. Single radial haemolysis compared to haemagglutinin inhibition and microneutralization as a correlate of protection against influenza A H3N2 in children and adolescents. Influenza Other Respir Viruses. 2017;11:283-8.
[216] Bishai FR, Galli R. Enzyme-linked immunosorbent assay for detection of antibodies to influenza A and B and parainfluenza type 1 in sera of patients. J Clin Microbiol. 1978;8:648-56.
[217] Masihi KN, Lange W. Enzyme-linked immunosorbent assay for the detection of influenza type-specific antibodies. J Immunol Methods. 1980;36:173-9.
[218] Snyder DB, Marquardt WW, Yancey FS, Savage PK. An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. Avian Dis. 1985;29:136-44.
[219] Abraham A, Sivanandan V, Halvorson DA, Newman JA. Standardization of enzyme-linked immunosorbent assay for avian influenza virus antibodies in turkeys. Am J Vet Res. 1986;47:561-6.
[220] Meulemans G, Carlier MC, Gonze M, Petit P. Comparison of hemagglutination-inhibition, agar gel precipitin, and enzyme-linked immunosorbent assay for measuring antibodies against influenza viruses in chickens. Avian Dis. 1987;31:560-3.

[221] Verschoor CP, Singh P, Russell ML, Bowdish DME, Brewer A, Cyr L, et al. Microneutralization Assay Titres Correlate with Protection against Seasonal Influenza H1N1 and H3N2 in Children (vol 10, e0131531, 2016). Plos One. 2016;11.
[222] Klimov A, Balish A, Veguilla V, Sun H, Schiffer J, Lu X, et al. Influenza virus titration, antigenic characterization, and serological methods for antibody detection. Methods Mol Biol. 2012;865:25-51.
[223] Grund S, Adams O, Wahlisch S, Schweiger B. Comparison of hemagglutination inhibition assay, an ELISA-based micro-neutralization assay and colorimetric microneutralization assay to detect antibody responses to vaccination against influenza A H1N1 2009 virus. J Virol Methods. 2011;171:369-73.
[224] Hsu JP, Zhao X, Chen MI, Cook AR, Lee V, Lim WY, et al. Rate of decline of antibody titers to pandemic influenza A (H1N1-2009) by hemagglutination inhibition and virus microneutralization assays in a cohort of seroconverting adults in Singapore. BMC Infect Dis. 2014;14:414.
[225] Tete SM, Krammer F, Lartey S, Bredholt G, Wood J, Skrede S, et al. Dissecting the hemagglutinin head and stalk-specific IgG antibody response in healthcare workers following pandemic H1N1 vaccination. NPJ Vaccines. 2016;1.
[226] Gauger PC, Vincent AL. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine. Methods Mol Biol. 2014;1161:313-24.
[227] Gauger PC, Loving CL, Vincent AL. Enzyme-linked immunosorbent assay for detection of serum or mucosal isotype-specific IgG and IgA whole-virus antibody to influenza A virus in swine. Methods Mol Biol. 2014;1161:303-12.
[228] Rajendran M, Nachbagauer R, Ermler ME, Bunduc P, Amanat F, Izikson R, et al. Analysis of Anti-Influenza Virus Neuraminidase Antibodies in Children, Adults, and the Elderly by ELISA and Enzyme Inhibition: Evidence for Original Antigenic Sin. MBio. 2017;8.
[229] Gao J, Couzens L, Eichelberger MC. Measuring Influenza Neuraminidase Inhibition Antibody Titers by Enzyme-linked Lectin Assay. J Vis Exp. 2016.
[230] Memoli MJ, Shaw PA, Han A, Czajkowski L, Reed S, Athota R, et al. Evaluation of Antihemagglutinin and Antineuraminidase Antibodies as Correlates of Protection in an Influenza A/H1N1 Virus Healthy Human Challenge Model. MBio. 2016;7:e00417-16.
[231] Warren L. The thiobarbituric acid assay of sialic acids. J Biol Chem. 1959;234:1971-5.
[232] Lambre CR, Terzidis H, Greffard A, Webster RG. Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtitre plates coated with natural substrates. J Immunol Methods. 1990;135:49-57.
[233] Gao J, Couzens L, Eichelberger MC. Measuring Influenza Neuraminidase Inhibition Antibody Titers by Enzymelinked Lectin Assay. Jove-J Vis Exp. 2016.
[234] Couzens L, Gao J, Westgeest K, Sandbulte M, Lugovtsev V, Fouchier R, et al. An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera. J Virol Methods. 2014;210:7-14.
[235] Eichelberger MC, Couzens L, Gao Y, Levine M, Katz J, Wagner R, et al. Comparability of neuraminidase inhibition antibody titers measured by enzyme-

linked lectin assay (ELLA) for the analysis of influenza vaccine immunogenicity. Vaccine. 2016;34:458-65.
[236] Moller E. Cytotoxicity by nonimmune allogeneic lymphoid cells. Specific suppression by antibody treatment of the lymphoid cells. J Exp Med. 1967;126:395-405.
[237] Trieu MC, Zhou F, Lartey S, Jul-Larsen A, Mjaaland S, Sridhar S, et al. Long-term Maintenance of the Influenza-Specific Cross-Reactive Memory CD4+ T-Cell Responses Following Repeated Annual Influenza Vaccination. J Infect Dis. 2017;215:740-9.
[238] Czerkinsky C, Andersson G, Ekre HP, Nilsson LA, Klareskog L, Ouchterlony O. Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J Immunol Methods. 1988;110:29-36.
[239] Schmittel A, Keilholz U, Bauer S, Kuhne U, Stevanovic S, Thiel E, et al. Application of the IFN-gamma ELISPOT assay to quantify T cell responses against proteins. J Immunol Methods. 2001;247:17-24.
[240] Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, et al. Quantification of Antigen-Specific Cd8(+) T-Cells Using an Elispot Assay. Journal of Immunological Methods. 1995;181:45-54.
[241] Eriksson JC, Cox RJ, Szyszko E, Davidsson A, Brokstad KA. Local and systemic cytokine and chemokine responses after parenteral influenza vaccination. Influenza Other Respir Viruses. 2007;1:139-46.
[242] Talaat KR, Halsey NA, Cox AB, Coles CL, Durbin AP, Ramakrishnan A, et al. Rapid changes in serum cytokines and chemokines in response to inactivated influenza vaccination. Influenza Other Respir Viruses. 2018;12:202-10.
[243] Breen EC, Reynolds SM, Cox C, Jacobson LP, Magpantay L, Mulder CB, et al. Multisite comparison of high-sensitivity multiplex cytokine assays. Clin Vaccine Immunol. 2011;18:1229-42.
[244] Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J Gerontol A Biol Sci Med Sci. 2008;63:879-84.
[245] Coughlan L, Lambe T. Measuring Cellular Immunity to Influenza: Methods of Detection, Applications and Challenges. Vaccines (Basel). 2015;3:293-319.
[246] Glezen WP. Emerging infections: pandemic influenza. Epidemiol Rev. 1996;18:64-76.
[247] Linder JA. Influenza-associated deaths among children. N Engl J Med. 2006;354:1317-8; author reply -8.
[248] Nair H, Brooks WA, Katz M, Roca A, Berkley JA, Madhi SA, et al. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. Lancet. 2011;378:1917-30.
[249] Fraaij PL, Heikkinen T. Seasonal influenza: the burden of disease in children. Vaccine. 2011;29:7524-8.
[250] Izurieta HS, Thompson WW, Kramarz P, Shay DK, Davis RL, DeStefano F, et al. Influenza and the rates of hospitalization for respiratory disease among infants and young children. N Engl J Med. 2000;342:232-9.

[251] Heikkinen T, Booy R, Campins M, Finn A, Olcen P, Peltola H, et al. Should healthy children be vaccinated against influenza? A consensus report of the Summits of Independent European Vaccination Experts. Eur J Pediatr. 2006;165:223-8.
[252] Hurwitz ES, Haber M, Chang A, Shope T, Teo ST, Giesick JS, et al. Studies of the 1996-1997 inactivated influenza vaccine among children attending day care: immunologic response, protection against infection, and clinical effectiveness. J Infect Dis. 2000;182:1218-21.
[253] Glatman-Freedman A, Portelli I, Jacobs SK, Mathew JI, Slutzman JE, Goldfrank LR, et al. Attack rates assessment of the 2009 pandemic H1N1 influenza A in children and their contacts: a systematic review and meta-analysis. Plos One. 2012;7:e50228.
[254] Reichert TA, Sugaya N, Fedson DS, Glezen WP, Simonsen L, Tashiro M. The Japanese experience with vaccinating schoolchildren against influenza. N Engl J Med. 2001;344:889-96.
[255] Hodgson D, Baguelin M, van Leeuwen E, Panovska-Griffiths J, Ramsay M, Pebody R, et al. Effect of mass paediatric influenza vaccination on existing influenza vaccination programmes in England and Wales: a modelling and cost-effectiveness analysis. Lancet Public Health. 2017;2:e74-e81.
[256] Fisman DN, Bogoch, II. Have you herd? Indirect flu vaccine effects are critically important. Lancet Public Health. 2017;2:e57-e8.
[257] Plans-Rubio P. The vaccination coverage required to establish herd immunity against influenza viruses. Prev Med. 2012;55:72-7.
[258] Block SL. Role of influenza vaccine for healthy children in the US. Paediatr Drugs. 2004;6:199-209.
[259] Esposito S, Marchisio P, Bosis S, Lambertini L, Claut L, Faelli N, et al. Clinical and economic impact of influenza vaccination on healthy children aged 2-5 years. Vaccine. 2006;24:629-35.
[260] Nichol KL, Mallon KP, Mendelman PM. Cost benefit of influenza vaccination in healthy, working adults: an economic analysis based on the results of a clinical trial of trivalent live attenuated influenza virus vaccine. Vaccine. 2003;21:2207-17.
[261] Cox MM, Izikson R, Post P, Dunkle L. Safety, efficacy, and immunogenicity of Flublok in the prevention of seasonal influenza in adults. Ther Adv Vaccines. 2015;3:97-108.
[262] Cox MM, Patriarca PA, Treanor J. FluBlok, a recombinant hemagglutinin influenza vaccine. Influenza Other Respir Viruses. 2008;2:211-9.
[263] Ambrose CS, Wu X, Jones T, Mallory RM. The role of nasal IgA in children vaccinated with live attenuated influenza vaccine. Vaccine. 2012;30:6794-801.
[264] Mohn KG, Brokstad KA, Pathirana RD, Bredholt G, Jul-Larsen A, Trieu MC, et al. Live Attenuated Influenza Vaccine in Children Induces B-Cell Responses in Tonsils. J Infect Dis. 2016;214:722-31.
[265] Mohn KGI, Zhou F, Brokstad KA, Sridhar S, Cox RJ. Boosting of Cross-Reactive and Protection-Associated T Cells in Children After Live Attenuated Influenza Vaccination. J Infect Dis. 2017;215:1527-35.
[266] Beyer WE, Palache AM, de Jong JC, Osterhaus AD. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and

systemic antibody response, and vaccine efficacy. A meta-analysis. Vaccine. 2002;20:1340-53.
[267] Nichol KL, Mendelman PM, Mallon KP, Jackson LA, Gorse GJ, Belshe RB, et al. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. JAMA. 1999;282:137-44.
[268] Belshe RB, Gruber WC. Prevention of otitis media in children with live attenuated influenza vaccine given intranasally. Pediatr Infect Dis J. 2000;19:S66-71.
[269] Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med. 2007;356:685-96.
[270] meeting Jmot. JOINT COMMITTEE ON VACCINATION AND IMMUNISATION. 2011.
[271] Powers DC, Fries LF, Murphy BR, Thumar B, Clements ML. In elderly persons live attenuated influenza A virus vaccines do not offer an advantage over inactivated virus vaccine in inducing serum or secretory antibodies or local immunologic memory. J Clin Microbiol. 1991;29:498-505.
[272] Baguelin M, Flasche S, Camacho A, Demiris N, Miller E, Edmunds WJ. Assessing optimal target populations for influenza vaccination programmes: an evidence synthesis and modelling study. PLoS Med. 2013;10:e1001527.
[273] Tran CH, Sugimoto JD, Pulliam JR, Ryan KA, Myers PD, Castleman JB, et al. School-located influenza vaccination reduces community risk for influenza and influenza-like illness emergency care visits. Plos One. 2014;9:e114479.
[274] Pebody RG, Green HK, Andrews N, Zhao H, Boddington N, Bawa Z, et al. Uptake and impact of a new live attenuated influenza vaccine programme in England: early results of a pilot in primary school-age children, 2013/14 influenza season. Euro Surveill. 2014;19.
[275] Pebody RG, Green HK, Andrews N, Boddington NL, Zhao H, Yonova I, et al. Uptake and impact of vaccinating school age children against influenza during a season with circulation of drifted influenza A and B strains, England, 2014/15. Euro Surveill. 2015;20.
[276] Pebody RG, Sinnathamby MA, Warburton F, Andrews N, Boddington NL, Zhao H, et al. Uptake and impact of vaccinating primary school-age children against influenza: experiences of a live attenuated influenza vaccine programme, England, 2015/16. Euro Surveill. 2018;23.
[277] Pebody R, Warburton F, Ellis J, Andrews N, Potts A, Cottrell S, et al. Effectiveness of seasonal influenza vaccine for adults and children in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2015/16 end-of-season results. Euro Surveill. 2016;21.
[278] meeting Jmot. JOINT COMMITTEE ON VACCINATION AND IMMUNISATION. 2016.
[279] Nohynek H, Baum U, Syrjanen R, Ikonen N, Sundman J, Jokinen J. Effectiveness of the live attenuated and the inactivated influenza vaccine in two-year-olds - a nationwide cohort study Finland, influenza season 2015/16. Euro Surveill. 2016;21.

[280] Pebody R, McMenamin J, Nohynek H. Live attenuated influenza vaccine (LAIV): recent effectiveness results from the USA and implications for LAIV programmes elsewhere. Arch Dis Child. 2018;103:101-5.
[281] Caspard H, Coelingh KL, Mallory RM, Ambrose CS. Association of vaccine handling conditions with effectiveness of live attenuated influenza vaccine against H1N1pdm09 viruses in the United States. Vaccine. 2016;34:5066-72.
[282] Folkehelseinstituttet. Anbefaling om valg av type influensavaksine til barn i risikogrupper. 2016.
[283] Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond). 1972;70:767-77.
[284] Monto AS, Petrie JG, Cross RT, Johnson E, Liu M, Zhong W, et al. Antibody to Influenza Virus Neuraminidase: An Independent Correlate of Protection. J Infect Dis. 2015;212:1191-9.
[285] Hoschler K, Southern J, Thompson C, Warburton F, Andrews NJ, Miller E, et al. Responses to live attenuated influenza vaccine in children vaccinated previously with Pandemrix (ASO3B adjuvanted pandemic A/H1N1pdm09). Vaccine. 2018;36:3034-40.
[286] Hoschler K, Andrews NJ, Faust SN, Finn A, Pollard AJ, Snape MD, et al. Administration of AS03B-adjuvanted A(H1N1)pdm09 vaccine in children aged <3 years enhances antibody response to H3 and B viruses following a single dose of trivalent vaccine one year later. Clin Infect Dis. 2014;58:181-7.
[287] Johansson BE, Moran TM, Bona CA, Popple SW, Kilbourne ED. Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. II. Sequential infection of mice simulates human experience. J Immunol. 1987;139:2010-4.
[288] Johansson BE, Moran TM, Bona CA, Kilbourne ED. Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. III. Reduced generation of neuraminidase-specific helper T cells in hemagglutinin-primed mice. J Immunol. 1987;139:2015-9.
[289] Wohlbold TJ, Krammer F. In the shadow of hemagglutinin: a growing interest in influenza viral neuraminidase and its role as a vaccine antigen. Viruses. 2014;6:2465-94.
[290] Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol. 1986;24:157-60.
[291] Barria MI, Garrido JL, Stein C, Scher E, Ge Y, Engel SM, et al. Localized mucosal response to intranasal live attenuated influenza vaccine in adults. J Infect Dis. 2013;207:115-24.
[292] Moldoveanu Z, Clements ML, Prince SJ, Murphy BR, Mestecky J. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine. 1995;13:1006-12.
[293] Black S, Nicolay U, Vesikari T, Knuf M, Del Giudice G, Della Cioppa G, et al. Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. Pediatr Infect Dis J. 2011;30:1081-5.

[294] Zhou F, Trieu MC, Davies R, Cox RJ. Improving influenza vaccines: challenges to effective implementation. Curr Opin Immunol. 2018;53:88-95.
[295] Epstein SL, Price GE. Cross-protective immunity to influenza A viruses. Expert Rev Vaccines. 2010;9:1325-41.
[296] McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. N Engl J Med. 1983;309:13-7.
[297] Jegaskanda S, Luke C, Hickman HD, Sangster MY, Wieland-Alter WF, McBride JM, et al. Generation and Protective Ability of Influenza Virus-Specific Antibody-Dependent Cellular Cytotoxicity in Humans Elicited by Vaccination, Natural Infection, and Experimental Challenge. J Infect Dis. 2016;214:945-52.

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Influenza A haemagglutinin specific IgG responses in children and adults after seasonal trivalent live attenuated influenza vaccination

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ABSTRACT

Influenza is a major respiratory pathogen and vaccination is the main method of prophylaxis. In 2012, the trivalent live attenuated influenza vaccine (LAIV3) was licensed in Europe for use in children. Vaccine-induced antibodies directed against the main viral surface glycoprotein, haemagglutinin (HA), play an important role in virus neutralization through different mechanism. The objective of this study was to dissect the HA specific antibody responses induced after LAIV3 immunization to the influenza A viruses in children and adults.

Plasma was collected from 20 children and 20 adults pre- and post-LAIV3 vaccination (up to a year) and analysed by the haemagglutination inhibition (HI) and ELISA assays. We found that LAIV3 boosted the HA specific IgG response against the head and the full-length of H3N2 in children, but not adults. Adults had higher levels of pre-existing stalk antibodies (towards H3N2 and H1N1), but these were not boosted by LAIV3. Importantly, we observed a trend in boosting of H1N1 HA stalk specific antibodies in children after LAIV3. Whereas, heterosubtypic H5 and H7 full-length HA specific antibodies were not boosted in either children or adults. In conclusion, LAIV3 elicited H3-head and low levels of H1 stalk specific antibody responses in children, supporting the prophylactic use of LAIV in children.

1. Introduction

Influenza viruses cause annual seasonal outbreaks or epidemics, with occasional pandemics occurring at unpredictable intervals. Influenza infects all age groups, although the burden of hospitalization is highest in very young children and the elderly [1–3]. Vaccination is the main method of influenza prophylaxis with either inactivated influenza vaccine (IIV) or live attenuated influenza vaccine (LAIV). Although used in Russia for decades, the LAIV was first licensed in the USA in 2003 for children and adults (2–49 years old), and in 2012 in Europe for children (2–17 years old). LAIV is administered as a nasal spray and replicates in the upper respiratory tract, mimicking natural infection and inducing both humoral and cellular immune responses [4]. Trivalent LAIV (LAIV3) has been reported to have a higher efficacy in young children than intramuscular IIV and thus provide greater protection against influenza-associated severe complications [5–8]. Importantly, the immune response after seasonal IIV is strain-specific, whereas LAIV3 provides better protection against mismatched strains [8,9].

The haemagglutinin is the major viral surface antigen, consisting of two domains; the globular head and the stalk domains, with a disulphide bride between C52 and C277 (H3 numbering) being the demarcation line between the two domains [10]. The immuno-dominant globular head contains the receptor-binding site and the antigenic sites, which undergo continuous antigenic drift. The membrane proximal HA stalk is highly conserved and contains a conformation-dependent fusion-peptide [11]. Antibodies directed against the stalk are broadly neutralizing, recognizing divergent and heterosubtypic strains from either group 1 (including H1 and H5) or group 2 (including H3 and H7) viruses [12–14] and provide *in vivo* protection from viral challenge in animal models [14,15]. Antibodies can be boosted to the more conserved but less immunogenic stalk, when a virus has a highly divergent HA

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head from a previously circulating strain, such as the 2009 pandemic or avian H5N1 virus [13,14,16]. Therefore, the conserved HA stalk is a promising target for development of a future universal influenza vaccine.

Conventional IIVs predominantly induce strain-specific antibody to the HA head, but the type of HA response LAIV3 elicits is yet to be fully defined. In this study, we dissected the HA head and stalk-specific antibody responses to the homologous vaccine (H1N1 and H3N2) and heterologous avian (H5N1 and H7N9) viruses after intranasal seasonal LAIV3 vaccination in children and adults.

2. Material and methods

2.1. Participants and study design

Twenty children (3–17 years old) and 20 adults (21–59 years old) were intranasally vaccinated (0.1 mL per nostril) with seasonal LAIV3 (Fluenz, Astra Zeneca, Liverpool, UK). Children received one (≥9 years old, n=6) or two doses (<9 years old, n=14) of LAIV3 in 2012 at a four-week interval [4]. Adults received one dose of LAIV3 in 2013. The study had ethical and regulatory approval (EUDRACT2012-002848-24, www.clinicaltrials.gov; NCT01866540) and the exclusion criteria are published [4]. Plasma was collected at 0 (pre-vaccination), 28, 56, 180 and 360 days post-vaccination, aliquoted and stored at −80 °C.

2.2. Vaccine

LAIV3 (Fluenz) contained 10^7 fluorescent focus units (FFU) of A/California/7/2009(H1N1)pdm09-like and A/Victoria/361/2011 (H3N2)-like strains in both seasons, with either B/Wisconsin/1/2010-like or B/Massachusetts/2/2012-like in the 2012 or 2013 seasons, respectively.

2.3. Recombinant haemagglutinin proteins

The influenza A full-length and chimeric haemagglutinin proteins were prepared by using the baculovirus expression system (Table 1) [16]. The cH6/1 contained the globular head domain from A/mallard/Sweden/81/02 (H6N1) and the stalk domain from A/PuertoRico/8/34 (H1N1). The cH4/3 contained the H4 globular head domain from A/duck/Czech/1956 (H4N6) in combination with the H3 stalk domain from A/Perth/16/09 (H3N2). The HA1 proteins were purchased from eEnzyme, USA and were used as proxy for the head domain.

Table 1
Source of virus antigens.

	Strain	Haemagglutinin (HA)
Group 1	A/California/04/09 (H1N1)	Full-length H1
		H1 HA1 (head proxy)
	Chimeric protein: stalk of A/PuertoRico/8/34 (H1N1), globular head from A/mallard/Sweden/81/02 (H6N1)	Stalk (cH6/1)
	A/Vietnam/1203/04 (H5N1)	Full-length H5
Group 2	A/Victoria/361/11 (H3N2)	Full-length H3
	A/Texas/50/2012 (H3N2)	H3 HA1 (head proxy)
	Chimeric protein: stalk of A/Perth/16/09 (H3N2), globular head from A/duck/Czech/1956 (H4N6)	Stalk (cH4/3)
	A/Shanghai/1/13 (H7N9)	Full-length H7

2.4. Haemagglutination inhibition (HI) assay

The HI assay was conducted using serial 2-fold dilutions of receptor destroying enzyme (RDE, Seiken, Japan) treated plasma (starting dilution 1:10) and 0.7% turkey blood cells, as previously described [4].

2.5. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was conducted using the full-length, head or chimeric HAs as previously described [17]. The end-point titres were calculated using the mean of the blank plus three standard deviations as a cut off [13].

3. Statistics

Statistical differences after vaccination were analysed using the linear mixed model in STATA/IC 14.1 for Mac (StataCorp, USA). The Wilcoxon test was used for the head/stalk distribution after vaccination and the nonparametric Mann-Whitney test for comparing children and adult responses in HI (GraphPad Prism® V6f for Mac, GraphPad Software, USA). The children and adult sampling points were also compared using paired student's *t*-test and *t*-test with Welch correction. $P < 0.05$ was considered significant.

4. Results

Twenty children (median 4.5 years old) and twenty adults (median 33.5 years old) were immunized with seasonal LAIV3 and sequential blood samples were collected up to one year post-vaccination (Fig. 1). Fourteen children (70%) (median age 3.8 years old) received two doses of vaccine, whilst the remaining 6 children (30%) (median age 15.5 years old) and adults received only one dose (Table 2). Ten adults (50%) and nine children (45%) were vaccinated in 2009 with the AS03-adjuvant pandemic H1N1 vaccine (Table 2). One child's (5%) mother was also vaccinated while pregnant in 2009, and only one child (5%) had previously been vaccinated with trivalent IIV.

4.1. LAIV3 boosts H3N2 haemagglutination inhibition antibody responses in children

We examined the plasma HI antibody response against the influenza A H1N1 and H3N2 vaccine strains after LAIV3. An HI titre of ≥40 is considered to protect 50% of individuals from disease. Pre-vaccination, fifteen children (75%) had protective antibody titres against H1N1 (Fig.2A), which were not boosted after vaccination. Five children (25%) remained below the protective HI threshold after the first vaccination, of whom one responded after the second dose (HI titre 69) and the remaining 4 children (20%) did not respond (HI < 10) vaccination. Children had significantly higher HI titres than adults pre- to 180 days post-vaccination. Pre-vaccination, ten adults had HI titres below the protective threshold of whom nine were seronegative (HI < 10). Vaccination did not generally boost antibody titres in adults, except two adults who responded with protective HI titres. No change in fold change in HI titres was observed to H1N1 after LAIV3 (Fig. 2C).

For the H3N2 strain, 10 (50%) of the 20 children had a pre-vaccination HI titre of <40, of whom 8 (40%) were seronegative (HI titre < 10) (Fig. 2B). After the first LAIV3 dose, there was a significant increase ($P < 0.0001$) in HI titres, which remained significantly elevated above pre-vaccination levels after the second dose (day 56) ($P < 0.0001$) and until day 360 ($P < 0.01$). Thirteen (65%) adults had pre-vaccination antibodies below the protective titre (HI<40) to H3N2. LAIV3 elicited protective HI titres in two

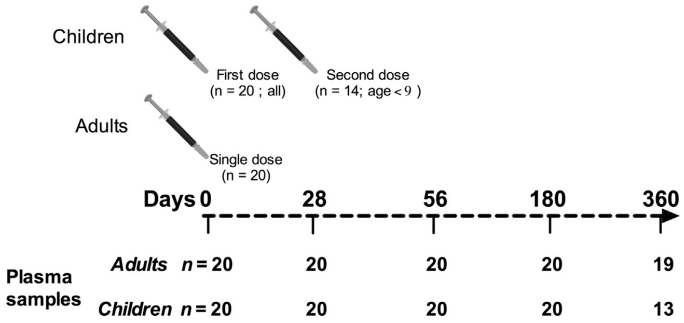

Fig. 1. The study design. Children were vaccinated with 1 (n = 20) or 2 (n = 14 children, age < 9 years old) doses of LAIV, whilst adults received one dose of LAIV. Plasma samples were collected pre (0) and at 28, 56, 180 and 360 days post vaccination. The number (n) of children and adults providing samples at each time point is shown.

Table 2
Baseline demographics of the patient cohort.

	Children	Adults
Number of participants (n)	20	20
Median age by year (Range)	4.5 (3–17)	33.5 (21–59)
Male/Female (% Female)	9/11 (55%)	6/14 (70%)
Single dose (%)	6 (30%)	19 (95%)
Two doses (%)	14 (70%)	1 (5%)
Pandemic vaccination in 2009 (n (%))	9 (45%)	10 (50%)

adults (10%) post-vaccination, only one of which was maintained above the protective titre up to 1 year. Children had significantly higher fold changes after LAIV3 than adults, and these were maintained in children up to day 360 whereas low increases were observed up to day 56 in adults (Fig. 2D). In summary, LAIV3 significantly boosted the HI response to H3N2 in children. Generally, no H1N1-specific HI response was boosted in either children or adults.

4.2. IgG responses to full-length H3N2 were boosted in children after LAIV3

The IgG response was measured by ELISA to the homologous (H1 and H3) and heterologous (H5 and H7) full-length influenza A HAs (Table 1). High pre-vaccination IgG titres to the full-length H1 HA were detected in both the children and adults, and LAIV3 vaccination did not boost these antibodies (Fig.3A). No correlation was observed between pre-vaccination IgG and fold-increase 28 days post-vaccination in children against H1N1 (data not shown).

H3-specific antibodies were detectable pre-vaccination in all adults and in the majority of children to the full-length HA, although 5 children had low levels of antibodies (Fig. 3B). In the children, antibody titres increased significantly ($p < 0.0005$) at day 28 after LAIV3 immunization and were generally maintained up to day 360. Children's IgG titres were significantly higher than adults after one dose and up to one year post-vaccination. No changes in H3-specific antibody responses were observed in the adults after vaccination. Pre-existing IgG antibodies specific for H3 full-length HA significantly but inversely correlated with fold induction in children both at day 28 ($r = -0.8412$, $p < 0.0001$) and day 56 ($r = -0.8618$, $p < 0.0001$).

We further evaluated the heterosubtypic antibody response. H1 and H5 are both group 1 HAs and the H3 and H7 are group 2 HAs, with a similar conserved stalk domain. We observed a trend of higher pre- and up to 180 days post-vaccination antibody titres against the full-length H5 HA (Fig. 3C) in adults compared to children. The H5 antibody titres at day 360 were significantly different ($P < 0.05$) from earlier time points in both adults and children. No change in H3 stalk antibodies was observed after LAIV3 (Fig. 3D). However, adults had significantly higher H7 HA-specific antibody titres pre- and up to 180 days post-vaccination compared to children.

4.3. LAIV3 boosts H3 HA head-specific responses in children

We measured the IgG antibodies to the homologous influenza A vaccine HA heads and stalks using chimeric HAs. The H1 head-specific antibody titres were significantly higher in children than the adults (Fig. 4A) pre-vaccination, although the LAIV3 did not boost these antibodies. Adults only had a slight increase in head antibody but this decreased by day 360. Pre-vaccination, H3 head specific antibodies were comparable between children and adults (Fig. 4B). In children, H3 head specific antibodies increased significantly ($p < 0.05$) after one dose (day 28) and were maintained up to one year post-vaccination (Fig. 4B). However, no boost in H3 head antibodies was observed after vaccination in adults. Children had significantly higher head H3 specific antibodies after LAIV3 immunization than adults.

The stalk-specific antibody response was assessed using chimeric group 1 (cH6/1) and group 2 (cH4/3)) HAs containing exotic head domains derived from avian viruses, which do not cause human infection. For the H1 stalk, adults had significantly higher stalk antibody titres compared to children pre-vaccination and up to day 180 ($p < 0.05$). There was a trend of an increase in the H1 stalk antibody response in children at days 28 and 56 post-vaccination, but not in adults (Fig. 4C). Adults had significantly higher H3 stalk-specific antibodies pre-vaccination than the children. LAIV3 immunization did not boost H3 stalk-specific IgG in children or adults. H3 stalk-specific antibody titres decreased significantly ($P < 0.05$) in adults at days 180 and 360 compared to pre-vaccination titres (Fig. 4D). In summary, circulating H3N2 head-specific IgG responses were boosted and low levels of H1N1 stalk-specific antibody responses were induced in children, but not adults.

4.4. Comparative distribution of head- and stalk-specific IgG after LAIV3

The HA specific IgG distribution was compared by calculating the ratio of the antibodies to the head domain and the stalk domain in children and adults. Children, who had previously been infected or vaccinated with the H1N109pdm virus, but with limited exposure history to other influenza H1s, had more H1 head-specific

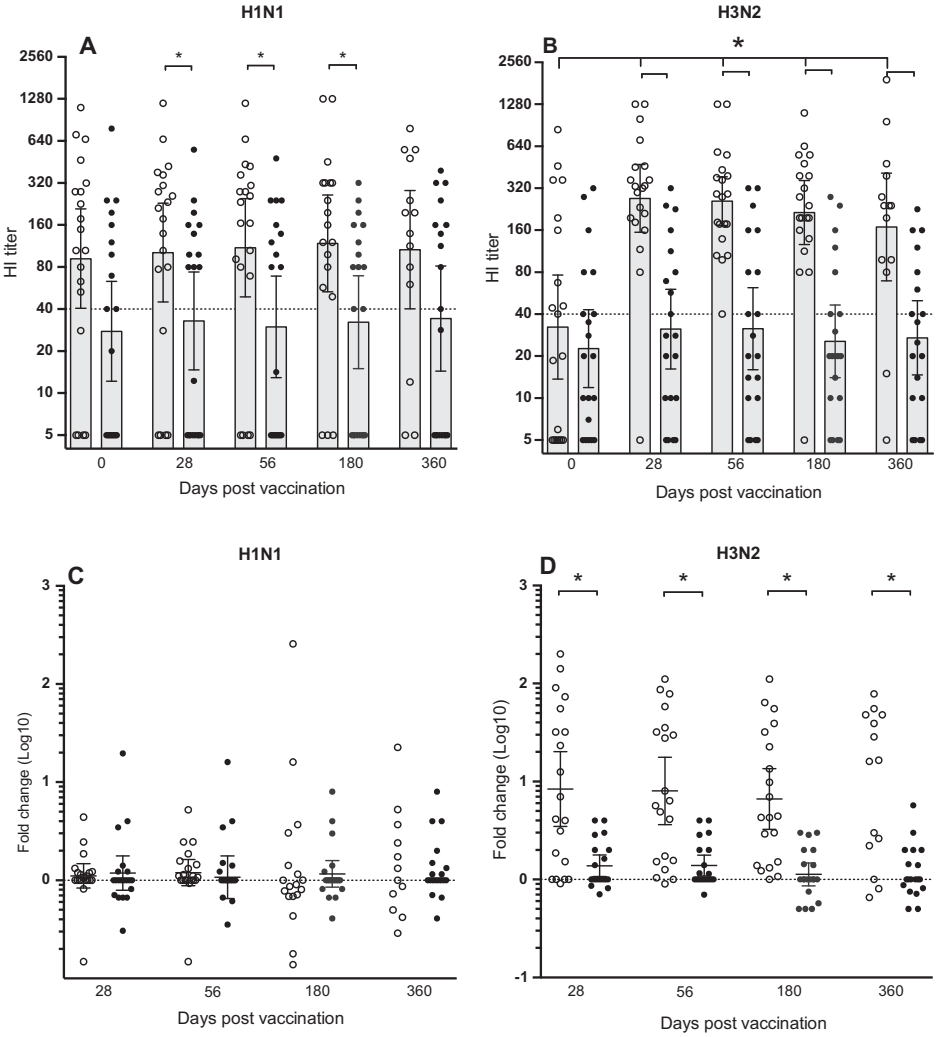

Fig. 2. The haemagglutination inhibition (HI) antibody responses in children and adults after live attenuated influenza vaccination. HI titres were measured towards the homologous influenza A/California/7/09 (H1N1) (A) and A/Victoria//361/11 (H3N2) (B) vaccine strains in both children and adults after live attenuated influenza vaccination. The dotted line shows an HI titre of 40, considered the protective level. The fold increase in children from pre-vaccination antibody titres to H1N1 (C), and H3N2 (D) homologous viruses. Ratios above or below 1 indicate higher or lower post-vaccination HI titres compared to pre-vaccination titres, respectively. Blood was collected at 0 (pre), 28 (after 1st dose), 56 (after 2nd dose in children < 10 years old), 180 and 360 days post vaccination. Each circle represents the HI response of one individual with the bar showing the group geometric mean HI titres ± 95% confidence interval. Open circles are children while filled circles are adults.. The nonparametric Mann-Whitney test was used to investigate statistical significance between children and adults. The linear mixed model was also used to investigate the change from pre-vaccination antibody titres up to one year for both children and adults; $^{*}p < 0.05$.

antibodies pre-vaccination, which decreased post-vaccination. However, in adults who had previously experienced several natural H1N1 infections and/or vaccination, HA stalk-specific antibody dominance was observed (Fig. 4E). The children had significantly higher ratio throughout the study than adults. Both children and adults had a H3 head dominant response (Fig. 4F). An increase in H3 head specific-antibodies after LAIV3 immunization was observed in children, leading to a significantly higher ratio in children than adults post-vaccination.

5. Discussion

The licensure of LAIV in Europe in 2012 expanded available prophylaxis for influenza, offering an attractive easily administered nasal spray vaccine for children. In Norway, annual seasonal influenza vaccination is only recommended for high-risk populations and thus most of our volunteers had not previously received seasonal influenza vaccination. In this study, we dissected the influenza A HA-specific antibody response after the newly licensed

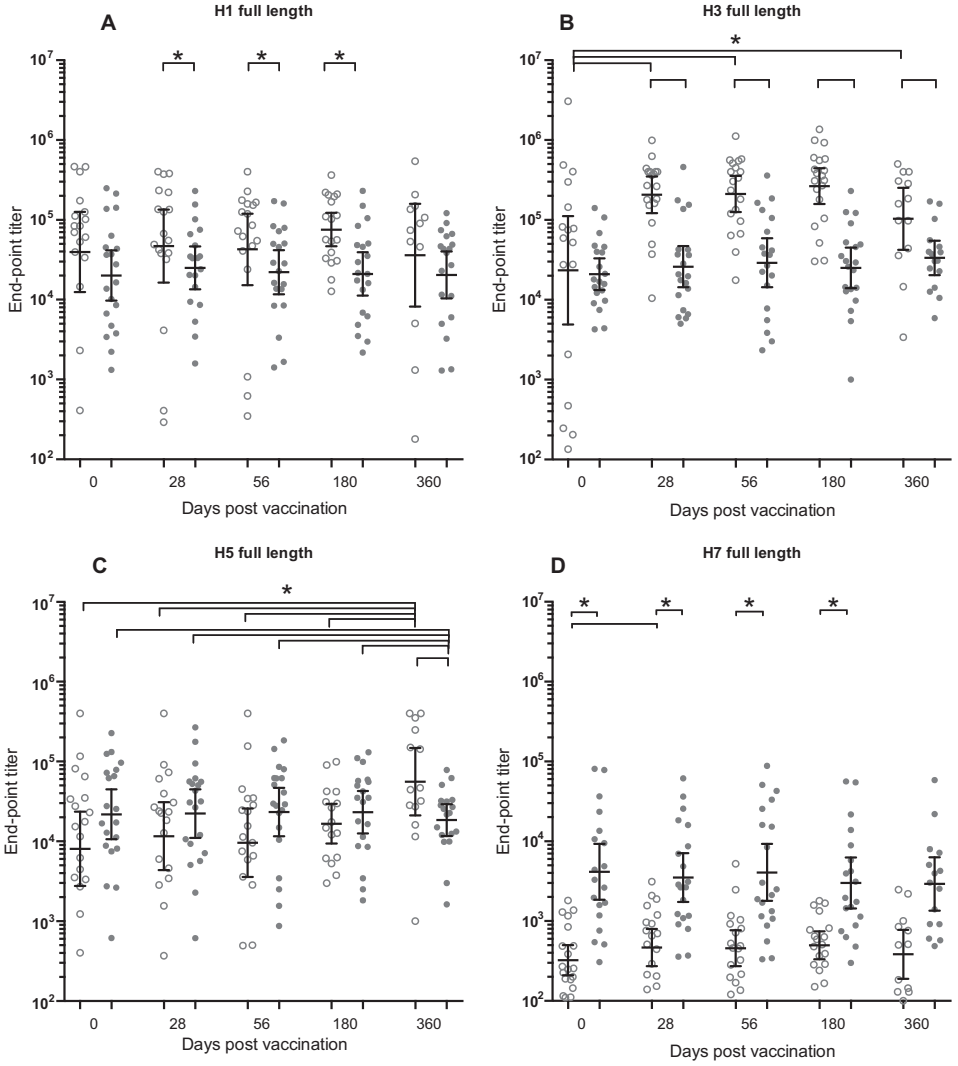

Fig. 3. The haemagglutinin specific antibody response to seasonal and heterosubtypic influenza A viruses. The IgG response to the haemagglutinin of the homologous vaccine strains (A/California/4/2009 (H1N1) (A) and A/Victoria/361/11 (H3N2) (B)) and the avian (A/Vietnam/1203/04 (H5N1) (C) and A/Shanghai/1/13 (H7N9) (D)) viruses was measured by ELISA. Each circle (open = children, filled = adults) represents the endpoint titre of one individual, and the bars show the geometric mean titre ± 95% confidence interval. The sampling points were at day 0 (pre-vaccination) and days 28, 56, 180 and 360-post vaccination. The children and adult sampling points were compared using paired student's *t*-test and *t*-test with Welch correction. $^{*}p < 0.05$.

intranasal LAIV3 vaccination in children and adults. Our results show that LAIV3 boosted the H3-specific antibody responses in children, but not adults, and the antibody response was dominated by antibodies to the HA head domain. A trend of increase in H1 stalk specific antibodies was found in children after LAIV3. Adults who have previously experienced repeated influenza infection had higher pre-existing H1 stalk-specific antibodies, but these were not boosted after LAIV3 immunization.

The golden standard haemagglutination inhibition (HI) assay mainly measures neutralising HA head-specific antibodies with a serum HI titre of 40 considered protective in adults [18]. Although in children HI titres of 110 have been proposed as providing 50% protection to H3N2 after IIV vaccination [19]. We found that LAIV3 only boosted H3N2 HI antibodies (>110) in the children, and these titres were maintained up to a year. Generally, no boost in the HI antibody was observed to the H1N1pdm09 virus in either adults or children, in agreement with our previous findings in children [4]. Pre-existing influenza-specific antibodies, particularly to H1N1pdm09 from previous infection or vaccination, may limit replication of the H1N1pdm09 LAIV strain and therefore restrict stimulation of the immune response [20], but should also provide protection against H1N1pdm09 infection. Interestingly, studies in the USA have shown reduced vaccine effectiveness post-pandemic against H1N1pdm09 after LAIV [21,22] although

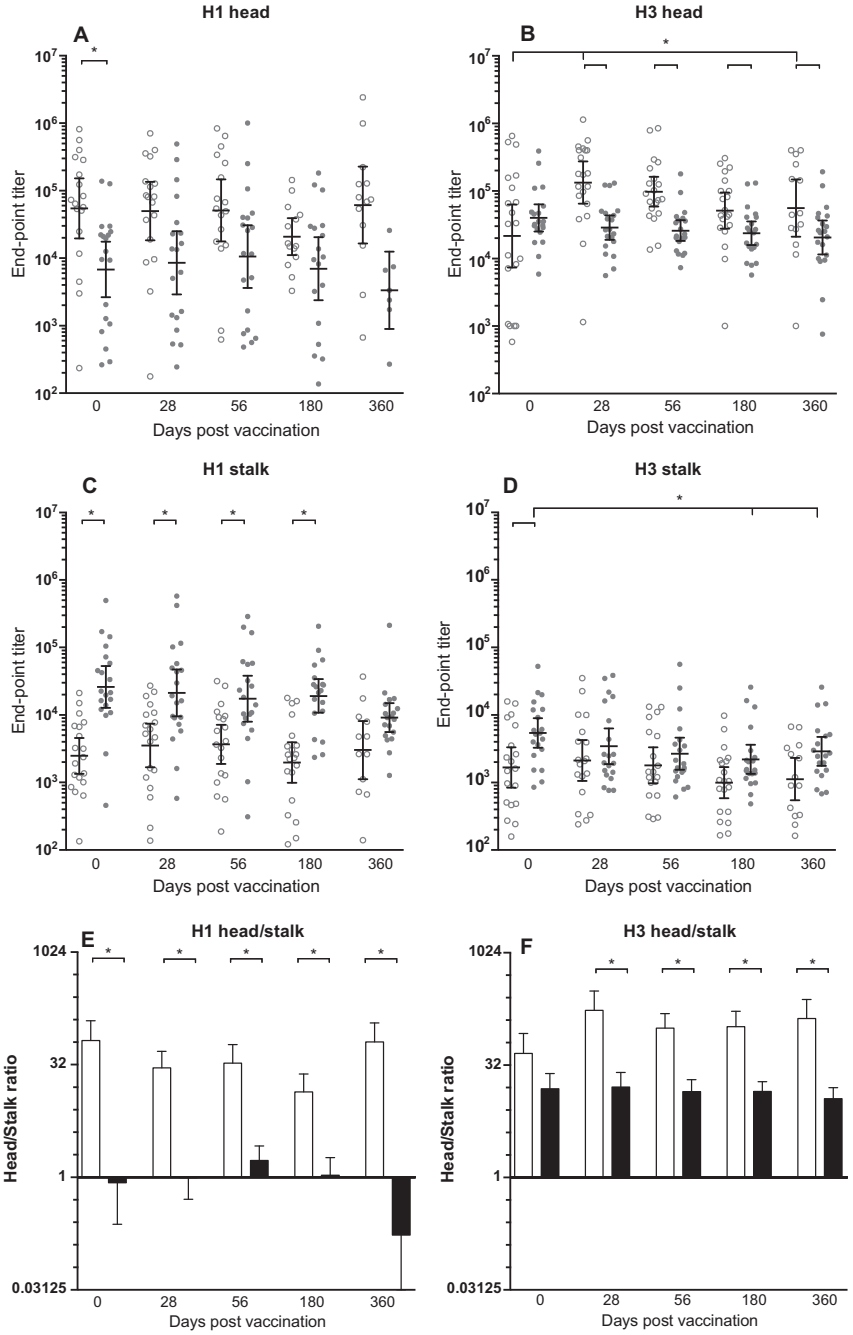

Fig. 4. The IgG HA specific response to the haemagglutinin head and stalk of influenza A H1N1 and H3N2 viruses. The head and stalk IgG responses to the HA of A/California/4/2009 (H1N1) (A, C) and A/Victoria/361/11 (H3N2) (B, D) were measured by ELISA. The time points for sampling were day 0 (pre-vaccination) and days 28, 56, 180 and 360 post vaccination, as indicated on the x-axis. Each circle (open = children, filled = adults) represents the endpoint titre of one individual and the bars show the geometric mean titre ± 95% confidence interval. E (A/California/4/2009 (H1N1)) and F (A/Victoria/361/11 (H3N2)) show the ratio between head and stalk antibody titres on a $\log_2$ scale, where a ratio of 1 shows an equivalent distribution of stalk and head antibody. The bar charts indicate the mean titre ± 95% confidence interval. The children and adult sampling points were compared using paired student's *t*-test and *t*-test with Welch correction. * $p < 0.05$.

in Europe the LAIV has been shown to be effective against laboratory confirmed influenza [23]. The H1Npdm09 LAIV strain used in 2009–2014 had a mutation in the HA (E47 amino acid residue) that potentially led to reduced thermal stability of this strain [21]. As a consequence of lower effectiveness against H1N1, the LAIV is not recommended in the USA by the Advisory Committee on Immunization Practices (ACIP) for the 2016–17 season although in Europe LAIV is still recommended. These findings of no or low increases in H1N1-specific antibodies observed in our study may help explain the lower effectiveness of the H1N1 strain.

We further used the HA proteins from the homologous vaccine strains to dissect the IgG response to the immunodominant head and the subdominant stalk domains. Our data demonstrate increased IgG antibodies after the first dose of LAIV3 in children to the H3 full-length protein and the H3 head, which persisted up to one year. These head-specific antibodies, that can also be detected by the HI assay, have higher neutralizing capacity than stalk-specific antibodies *in vitro* [24]. Furthermore, our recent study reported that the IgG1 subclass is boosted against the H3 head after LAIV3 [25]. Interestingly the full-length H3N2 HA specific IgG corresponds with the head-specific IgG titres in both groups. The titres of stalk-specific antibodies were determined by age and/or previous exposure history. Adults had higher pre-existing H7 specific antibodies with similar titres to the chimeric H3 stalk. In contrast, the children had low or undetectable levels of pre-existing H7 stalk specific antibodies illustrating the lack of group 2 influenza virus exposure [12]. Lower stalk specific antibodies to the group 2 stalk (H3 and H7) than to group 1 stalk were detected in children, possibly due to exposure to mostly one group 2 virus (H3N2) during their life span.

Both children and adults had pre-existing IgG antibodies to the head of H1, which generally did not boost upon vaccination. Higher head specific titres were observed in children compared to adults, reflecting recent influenza infection with an antigenically similar virus. Interestingly, higher pre-existing stalk H1 antibodies were observed in adults than children probably due to sequential exposure to antigenically distinct HAs from group 1 (seasonal and pandemic H1 and H2 viruses) causing selective boosting of antibodies to the highly-conserved stalk, similarly reviewed by Krammer et al. [10]. The HAs of H5N1 and H1N1pdm09 viruses belong to group 1 with similar stalk, but divergent head domains. Higher levels of H5 antibody were found in adults compared to children pre- and post-vaccination, reflecting adults more extensive previous group 1 infection history resulting in higher levels of cross reactive (heter-subtypic) stalk antibodies. As hypothesized, the presence of a novel globular head in the H1N1pdm09 virus skewed the antibody response to the heterologous stalk in adults [10,26,27]. We have earlier reported that pandemic vaccination with H5N1 vaccine containing a highly divergent HA head and a group 1 stalk boosted the neutralizing stalk-specific responses after the first vaccination in adults [14].

In contrast, children have had limited influenza exposures to group 1 HAs. In agreement, Nachbagauer et al. found that HA stalk-reactive antibodies increased with age after repeated exposure to divergent influenza viruses with conserved stalks [28]. Stalk-reactive antibodies are not extensively induced after seasonal IIV [13,29–32], but we found that children with limited previous influenza infection or earlier pandemic vaccination had pre-existing H1 stalk specific IgG that was boosted by seasonal LAIV3 vaccine, although not significantly. This may allow LAIV3 to be used as a priming strategy for a future universal influenza stalk based vaccine.

The continuous antigenic drift and occasional pandemics with the associated time delay in production of pandemic vaccine highlights the need for development of universal influenza vaccines, which can provide broader and longer lasting immunity. This study shows that LAIV can boost stalk-specific antibodies in children in the absence of a boost in head specific responses a finding, which would need to be confirmed in a larger group of children. Nachbagauer et al. also showed that a high dose recombinant HA seasonal vaccine boosted stalk-specific responses in adults 19–49 years old. Recently, Impagliazzo et al. engineered stable trimeric stems, which were effective at inducing broad protection in pre-clinical animal models [33]. Stalk-specific antibodies provide broader cross reactivity through virus neutralisation and by activation of NK cells through FcγR resulting in lysis of target cells. Further studies could evaluate the functionality of the stalk specific antibodies in children and adults by antibody dependent cellular cytotoxicity (ADCC) and neutralization assays, although a recent study found that LAIV did not induce ADCC in children [34].

In conclusions, adults had higher pre-existing H1 HA stalk antibodies, whereas children had H1 head dominant antibodies probably reflecting recent infection with the same H1 strain. LAIV3 mimics natural infection in children eliciting H3N2 HA head and low levels of stalk H1N1 antibodies, confirming LAIV as a priming of young children.

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Conflict of interest

None.

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References

[1] Longini IM, Koopman JS, Monto AS, Fox JP. Estimating Household and Community Transmission Parameters for Influenza. Am J Epidemiol. 1982;115:736–51.
[2] Viboud C, Boelle PY, Cauchemez S, Lavenu A, Valleron AJ, Flahault A, et al. Risk factors of influenza transmission in households. Brit J Gen Pract 2004;54:684–9.
[3] Fleming DM, Cross KW. Respiratory syncytial virus or influenza? Lancet 1993;342:1507–10.
[4] Mohn KG, Bredholt G, Brokstad KA, Pathirana RD, Aarstad HJ, Tondel C, et al. Longevity of B-cell and T-cell responses after live attenuated influenza vaccination in children. J Infect Dis 2015;211:1541–9.
[5] Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 2012;12:36–44.
[6] Block SL, Heikkinen T, Toback SL, Zheng W, Ambrose CS. The efficacy of live attenuated influenza vaccine against influenza-associated acute otitis media in children. Pediatr Infect Dis J 2011;30:203–7.
[7] Ambrose CS, Wu X, Belshe RB. The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination. Pediatr Infect Dis J 2010;29:806–11.
[8] Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007;356:685–96.
[9] Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, et al. Correlates of immune protection induced by live, attenuated, cold-

adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis 2000;181:1133–7.
[10] Krammer F, Palese P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr Opin Virol 2013;3:521–30.
[11] Bullough PA, Hughson FM, Skehel JJ, Wiley DC. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 1994;371:37–43.
[12] Henry Dunand CJ, Leon PE, Kaur K, Tan GS, Zheng NY, Andrews S, et al. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. J Clin Invest 2015;125:1255–68.
[13] Ellebedy AH, Krammer F, Li GM, Miller MS, Chiu C, Wrammert J, et al. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. Proc Natl Acad Sci USA 2014;111:13133–8.
[14] Nachbagauer R, Wohlbold TJ, Hirsh A, Hai R, Sjursen H, Palese P, et al. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. J Virol 2014;88:13260–8.
[15] Krammer F, Pica N, Hai R, Margine I, Palese P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J Virol 2013;87:6542–50.
[16] Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, et al. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. Proc Natl Acad Sci USA 2012;109:2573–8.
[17] S. Tete, F. Krammer, S. Lartey et al. Dissecting the hemagglutinin head and stalk-specific IgG antibody response in healthcare workers following pandemic H1N1 vaccination. http://dx.doi.org/10.1038/npjvaccines.2016.1.
[18] Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) 1972;70:767–77.
[19] Black S, Nicolay U, Vesikari T, Knuf M, Del Giudice G, Della Cioppa G, et al. Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. Pediatr Infect Dis J 2011;30:1081–5.
[20] Ilyushina NA, Haynes BC, Hoen AG, Khalenkov AM, Housman ML, Brown EP, et al. Live attenuated and inactivated influenza vaccines in children. J Infect Dis 2015;211:352–60.
[21] Caspard H, Coelingh KL, Mallory RM, Ambrose CS. Association of vaccine handling conditions with effectiveness of live attenuated influenza vaccine against H1N1pdm09 viruses in the United States. Vaccine 2016;34:5066–72.
[22] https://www.cdc.gov/flu/news/nasal-spray-effectiveness.htm.
[23] Pebody R, Sile B, Warburton F, Sinnathamby M, Tsang C, Zhao H, et al. Live attenuated influenza vaccine effectiveness against hospitalisation due to laboratory-confirmed influenza in children two to six years of age in England in the 2015/16 season. Eurosurveillance 2017;22:18–22.
[24] He W, Mullarkey CE, Duty JA, Moran TM, Palese P, Miller MS. Broadly neutralizing anti-influenza virus antibodies: enhancement of neutralizing potency in polyclonal mixtures and IgA backbones. J Virol 2015;89:3610–8.
[25] Manenti A, Tete SM, Mohn KG, Jul-Larsen A, Gianchecchi E, Montomoli E, et al. Comparative analysis of influenza A(H3N2) virus hemagglutinin specific IgG subclass and IgA responses in children and adults after influenza vaccination. Vaccine 2017;35:191–8.
[26] Sangster MY, Baer J, Santiago FW, Fitzgerald T, Ilyushina NA, Sundararajan A, et al. B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza virus vaccination. Clin Vaccine Immunol 2013;20:867–76.
[27] Thomson CA, Wang Y, Jackson LM, Olson M, Wang W, Liavonchanka A, et al. Pandemic H1N1 influenza infection and vaccination in humans induces cross-protective antibodies that target the hemagglutinin stem. Front Immunol 2012;3.
[28] Nachbagauer R, Choi A, Izikson R, Cox MM, Palese P, Krammer F. Age dependence and isotype specificity of influenza virus hemagglutinin stalk-reactive antibodies in humans. MBio 2016;7:e01996–e2015.
[29] Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature 2008;453:667–71.
[30] Suzuki Y. Selecting vaccine strains for H3N2 human influenza A virus. Meta Gene. 2015;4:64–72.
[31] Andrews SF, Huang Y, Kaur K, Popova LI, Ho IY, Pauli NT, et al. Immune history profoundly affects broadly protective B cell responses to influenza. Sci Transl Med. 2015;7:316ra192.
[32] Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, et al. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol 2013;87:4728–37.
[33] Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, et al. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. Science 2015;349:1301–6.
[34] Jegaskanda S, Luke C, Hickman HD, Sangster MY, Wieland-Alter WF, McBride JM, et al. Generation and protective ability of influenza virus-specific antibody-dependent cellular cytotoxicity in humans elicited by vaccination, natural infection, and experimental challenge. J Infect Dis 2016;214:945–52.

Errata for
Live Attenuated Influenza Vaccine (LAIV) Immunization in Children and Adults: Lesson for Development of Universal Influenza Vaccine

Shahinul Islam

Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

07.10.2019

(date and sign. of candidate) (date and sign. of faculty)

03 October 2019

Errata

Page 2, Figure 1.

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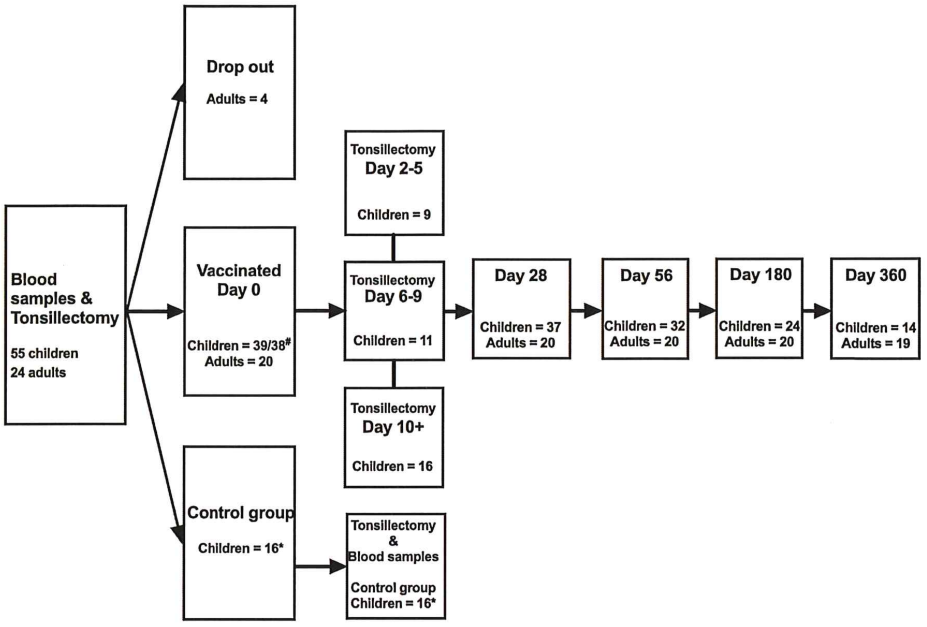

* Non-vaccinated controls
# One vaccinated child provided samples on day of tonsillectomy, but no sample at day 0

Page 97, Reference

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