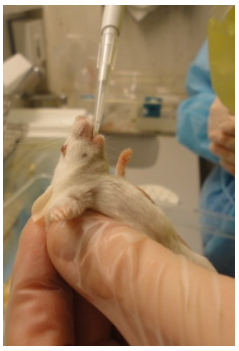
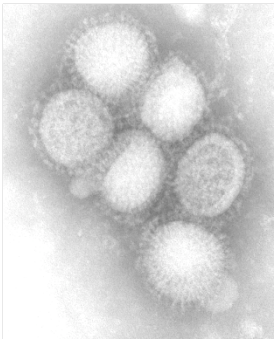
Evaluation of the Sublingual Route for Administration of Influenza Vaccines in a Murine Model

---

Ingrid Hjetland Gjeraker

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Pharmacy

University of Bergen
Centre for Pharmacy
and
Influenza Centre, The Gade Institute
2011

Front page illustration:

Left: Sublingual vaccination of a mouse. Photo: Gabriel Pedersen

Right: The influenza A H1N1 virus. Source:

http://scienceblogs.com/effectmeasure/2009/11/pics_of_the_flu_virus_and_some.php

Acknowledgements

The work in the following thesis was conducted at the Influenza Centre, The Gade Institute from August 2010 to May 2011 with the support from the Faculty of Medicine and Dentistry and the Centre for Pharmacy, University of Bergen.

First, I would like to thank my supervisors, Gabriel Pedersen, Rebecca Cox and Geir Breidholdt, for all your help and encouragement during this thesis. Your knowledge and guidance are highly appreciated. Next, I would like to thank Signe Svindland for helping me with the vaccination and the sampling of the mice and Åsne Jul-Larsen, Rishi Pathirana, Turid Helen Felli Lunde and Mona Nilsen for crucial help in the lab.

Further, my thanks go to Thomas Ebensen and Carlos Guzmán at Helmholtz Centre for Infection Research for teaching us the sublingual vaccination technique.

I would also like to thank my co-students at the Gade Institute for the cooperation and good conversations during the year. Additionally, I also have to thank all my pharmacy co-students and good friends for support and encouragement, and for five wonderful years at University of Bergen.

Finally, a lot of gratitude goes to my friends and family, especially my sister and my boyfriend, for putting up with me during the last stage of my thesis.

I could never have done this without any of you.

Bergen, May 2011

Ingrid Hjetland Gjeraker

Abbreviations

ADCC	Antibody-dependant cell-mediated cytotoxicity
APC	Antigen presenting cell
ASC	Antibody secreting cell
B7	Co-stimulatory molecules
BALB/c	Bagg albino (inbred mouse strain)
BSA	Bovine serum albumin
C	Control mice
CA	Cold adapted
CB	Cardiac blood
CD	Cluster of differentiation
c-di-GMP	Bis (3',5')-cyclic dimeric guanosine monophosphate
CHMP	The Committee for Medicinal Products for Human Use
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
Fas	Death receptor
FasL	Death receptor ligand
FBS	Foetal bovine serum
FM	Flow medium
g	Gravitational force
GISN	Global Influenza surveillance network
GMT	Geometric mean titre
HA	Heamagglutinin
HAU	Haemagglutinin units
HI	Haemagglutination inhibition
HPAIV	Highly pathogenic avian influenza virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IN	Intranasal
ISCOMs	Immune stimulating complexes
LAIV	Live attenuated influenza virus
LN	Lymph node
LT	*Escherichia coli* heat-labile toxin
LWM	Lymphocyte wash medium
M-cell	Microfold cell
M1	Matrix protein
M2	Ion channel protein
MALT	Mucosa associated lymphoid tissue
MEM	Minimal Essential Medium
MHC	Major histocompatibility complex

MLM	Mouse lymphocyte medium
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NAI	Neuraminidase inhibitors
NALT	Nasal associated lymphoid tissue
NIBSC	National Institute for Biological Standards and Control
NCS	Newborn calf serum
NK	Natural killer cells
NP	Nucleoprotein
NS	Non-structural protein
NW	Nasal wash
OD	Optical density
OPD	Ortho-phenylediamine dihydrochloride
PA	Polymerase protein acidic
PAMP	Pathogen associated molecular pattern
PB1/PB2	Polymerase protein basic
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with Tween 20
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol myristate acetate
PR8	A/Puerto Rico/8/34 (H1N1)
PRR	Pattern recognition receptors
PSA	Penicillin/Streptomycin/Amphotherecin
PW	Perm wash
RBC	Red blood cells
RDE	Receptor destroying enzyme
RG-14	A/Vietnam/1194/2004 (H5N1)
RG-6	A/Anhui/1/05 (H5N1)
RNA	Ribonucleic acid
RPMI	Roosewell Park Memorial Institute
SIgA	Secretory immunoglobulin A
SL	Sublingual
SRH	Single radial haemolysis
ssRNA	Single stranded ribonucleic acid
TBM-H	Peroxidase substrate (ELISPOT)
Tc	Cytotoxic T cell
TCR	T cell receptor
TGF	Transforming growth factor
Th	Helper T cell
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
X179a	A/California/07/2007 (H1N1)
WHO	World Health Organisation

Abstract

Influenza is a one of the most important respiratory viruses, and it infects millions of people every year. Due to mutational changes in the virus genome (antigenic drift), influenza cause occasional pandemics. Pandemic influenza strains arise when gene segments from two or more influenza viruses re-assort (antigenic shift), leading to a novel virus. Vaccination is still the most effective way of preventing influenza, and the recent H1N1 pandemic emphasised the need for effective pandemic vaccines that can induce rapid protection in an immunologically naïve population. Mucosally administered vaccines are an attractive approach for delivery of influenza vaccines since they are needle-free and have the ability to induce mucosal immune responses. Intranasal vaccination against influenza has been used for decades, however, an inactivated intranasal influenza vaccine was recently associated with Bell's Palsy (facial nerve paralysis). Sublingual vaccination (application under the tongue) can be a novel alternative for mucosal administration of influenza vaccines.

Avian influenza subtypes have previously shown to be poorly immunogenic in man, thus an effective adjuvant is needed to boost the vaccine effect. In this study we have vaccinated BALB/c mice intramuscularly, intranasally or sublingually with two doses, three weeks apart, of a virosomal H5N1 influenza vaccine (2 μg of haemagglutinin) alone or in combination with 7.5 μg of the novel mucosal adjuvant c-di-GMP. Serum, saliva and nasal wash samples were analysed for influenza specific antibodies using the ELISA and haemagglutination inhibition (HI) antibodies were detected in serum and saliva using the HI assay. Splenocytes were used in the memory B cell ELISPOT, and stimulated *in vitro* before the cytokine profiles were measured by multiplex bead assay. In addition, influenza stimulated splenocytes were fixed and stained intracellularly for cytokines, and the frequency of cytokine producing cells was determined using multiparametric flow cytometry.

The intramuscular, the intranasal and the sublingual routes all induced strong immune responses both in the humoral and the cellular immune assays when the virosomes were combined with c-di-GMP adjuvant. The non-adjuvanted vaccine induced lower immune responses as compared to the adjuvanted vaccine, irrespective of administration route. After the first vaccine dose, intramuscular administration of the

adjuvanted vaccine showed the highest IgG antibody response. In contrast, after the second vaccine dose, the intranasal adjuvanted group showed the highest responses in all assays. A strong local humoral immune response together with systemic IgG and IgA antibodies was elicited in both the sublingual and the intranasal adjuvanted groups. Interestingly, the intranasal adjuvanted group showed a dominant Th1 profile, whereas the sublingual adjuvanted group showed a more balanced Th2/Th1 profile. In addition, high levels of IL-17 (a Th17 cytokine) were produced in both the mucosal administered vaccines groups. The frequency of multifunctional $CD4^+$ T cells was highest in the intranasal adjuvanted group, but also sublingual vaccination of virosomes combined with c-di-GMP induced high frequencies of multifunctional T cells.

This is the first study to report that sublingual vaccination with H5N1 virosomes induces both humoral and cellular immune responses. These results demonstrate that the sublingual route is a promising way of administering influenza vaccines; we therefore suggest further investigation of influenza vaccines administered sublingually.

Contents

ACKNOWLEDGEMENTS 3
ABBREVIATIONS 4
ABSTRACT 6
CONTENTS 8

1 INTRODUCTION 11
1.1 THE INFLUENZA VIRUS 11
1.1.1 Taxonomy, Structure and Nomenclature 11
1.1.2 Replication cycle 13
1.2 EPIDEMIOLOGY 14
1.2.1 Antigenic Drift and Shift 14
1.2.3 Tropism and Ecology 16
1.2.4 Clinical manifestation 17
1.3 THE IMMUNE RESPONSE TO INFLUENZA 18
1.3.1 The Innate Immune System 18
1.3.2 The Adaptive Immune System 19
1.3.2.1 Cellular Immunity 19
1.3.2.2 Humoral Immunity 21
1.3.2.3 Mucosal immunity 24
1.4 PROPHYLAXIS AND TREATMENT 25
1.4.1 Antiviral drugs 25
1.4.2 Influenza Vaccines 26
1.4.2.1 Evaluation of Influenza Vaccines 27
1.4.2.2 Safety 27
1.4.2.3 Propagation of Influenza Virus 28
1.4.2.4 Live Attenuated Influenza Vaccines 28
1.4.2.5 Inactivated Influenza Vaccines 29
1.4.2.6 Virosomal vaccines 30
1.4.2.7 Mucosal vaccines 31
1.4.2.8 Sublingual vaccination 32
1.4.2.9 Vaccine Adjuvants 33
1.5 AIMS OF THE STUDY 36

2 MATERIALS 37
2.1 Mice 37
2.2 Vaccines and viruses 37
2.3 Anaesthetics and other drugs 37

2.4 Blood ... *37*
2.5 Antibodies ... *37*
2.6 Reagents and chemicals ... *38*
2.7 Bio-plex Kits ... *40*
2.8 Plates/equipment ... *40*
2.9 Instruments: ... *40*
2.10 Computer Software ... *41*
2.11 Solutions, buffers, medium ... *41*
2.12 Disposable consumables ... *43*

3 METHODS ... 44
3.1 EXPERIMENTAL PROTOCOL ... 44
3.1.1 Mice ... *44*
3.1.2 Virosomal vaccine ... *45*
3.1.3 Adjuvant ... *45*
3.1.4 Vaccination ... *46*
3.1.5 Collection of Venous Blood ... *47*
3.1.6 Collection of Nasal Wash ... *48*
3.1.7 Collection of Saliva ... *49*
3.1.8 Collection of Cardiac Blood and Tissue ... *49*
3.1.9 Separation of sera ... *50*
3.2 IMMUNOLOGICAL ASSAYS ... 50
3.2.1 Isolation of lymphocytes ... *50*
3.2.2 Antibody ELISA ... *51*
3.2.3 Memory B-cell ELISPOT ... *52*
3.2.4 Haemagglutination Inhibition Assay ... *53*
3.2.5 In vitro activation of lymphocytes ... *54*
3.2.6 Multiplex bead assay ... *55*
3.2.7 Intracellular cytokine staining ... *55*
3.3 STATISTICAL ANALYSIS ... 57

4 RESULTS ... 58
4.1 HUMORAL IMMUNE RESPONSE ... 58
4.1.1 Haemagglutination inhibition (HI) assay ... *58*
4.1.2 ELISA ... *61*
4.1.2.1 The mucosally administered vaccines induce a local antibody response ... 61
4.1.2.2 High concentrations of influenza-specific antibodies were detected in the serum ... 66
4.1.2.3 IgG1 and IgG2a subclass responses ... 69

4.1.3 Mucosal vaccination induces high percentages of influenza-specific IgG memory B cells 70

4.2 CELLULAR IMMUNE RESPONSE 73

4.2.1 Cytokine profiles 73

4.2.1 The induction of multifunctional T cells after vaccination 75

5 DISCUSSION 78

5.1 INDUCTION OF HUMORAL IMMUNITY 79

5.1.1 Haemagglutination inhibition titres induced after vaccination 79

5.1.2 Intranasal and sublingual vaccination induce local IgA responses in the nasal wash and saliva 80

5.1.3 Intranasal vaccination induce systemic IgG and IgA responses 83

5.1.4 Memory B cell ELISPOT 84

5.2 INDUCTION OF CELLULAR IMMUNITY 85

5.2.1 Cytokine profiles and IgG subclasses 85

5.2.2 Multifunctional T cells 87

5.3 LIMITATIONS OF THE STUDY 88

5.4 CONCLUSIONS 90

5.5 FURTHER RESEARCH 90

6 REFERENCES 92

1 Introduction

1.1 The Influenza virus

Influenza virus is a major respiratory virus in humans. The virus causes annual outbreaks, normally during late autumn or early winter in the temperate climate zones of the northern and southern hemispheres. The influenza virus is spreads by aerosols from coughing and sneezing, and every year the World Health Organisation (WHO) estimates that 20 % of children and 5 % of adults worldwide are infected. Each year, influenza causes excess mortality, hospitalisation and absenteeism and therefore has a huge social and economical impact on society. Influenza can also cause pandemics at unpredictable intervals, and previous influenza pandemics have caused millions of deaths worldwide [1].

1.1.1 Taxonomy, Structure and Nomenclature

The influenza virus is a member of the *Orthomyxoviridae* family. It is an enveloped virus with either spherical or filamentous (pleomorphic) morphology with a diameter of 80-120 nm. There are three different types of influenza virus: A, B and C. The three influenza types have antigenic differences in the structural proteins matrix protein (M) and nucleoprotein (NP) (figure 1.1). The genome of influenza A and B have eight negative stranded RNA segments. Type A and B viruses commonly cause human disease, whereas influenza C causes a mild illness. Influenza A can be further subdivided according to structural differences in the surface glycoproteins; haemagglutinin (HA) and neuraminidase (NA) of which 9 subtypes of NA and 16 subtypes of HA have been identified [2, 3]. HA is the most abundant surface glycoprotein on the influenza virus, and has several functions. Firstly, it is responsible for the attachment of the virus to the host cell, via binding to sialic acid on the surface of epithelial cells. Secondly, it promotes fusion between the viral envelope and the host cell and third, as the name suggests, the protein haemagglutinates red blood cells. All these functions make HA one of the most important determinants of viral pathogenicity [4, 5]. HA is activated by cleavage of the protein into two subunits (HA_1 and HA_2) that are held together by a disulphide bond. Most antibodies to HA

neutralize the virus and there are 5 antigenic sites on the globular head [6]. The other surface glycoprotein is NA, which is responsible for cleavage of sialic acid on glycoproteins. This promotes infection by mediating release of newly formed influenza virons from the host cell. NA may also play an important role in the initiation of infection, by removing decoy receptors in the airway epithelium [7].

A standardised nomenclature for influenza viruses includes the following: type (A, B or C), the species it was first isolated from (if non human), place of original isolation, isolation number, and surface antigen (HA and NA). An example is A/Vietnam/1194/2004 (H5N1), which is a strain of influenza type A, H5N1, isolated from man in Vietnam in 2004 [8].

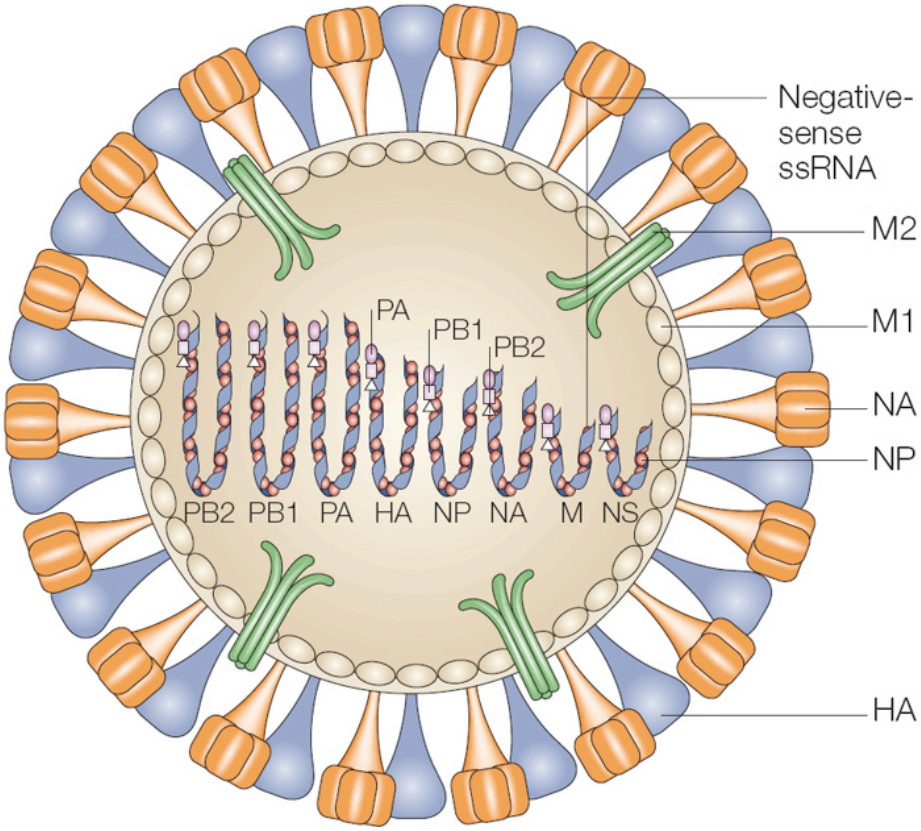


Figure 1.1: Schematic illustration of the influenza virion

The surface antigens haemagglutinin (HA) and neuraminidase (NA) radiate from the viral envelope. M2 is embedded in, whereas M1 lines, the viral envelope. The eight gene-segments code for different viral proteins and are encompassed by nucleoproteins (NP) and have the polymerase complex (PA, PB1 and PB2) attached. From reference [9].

1.1.2 Replication cycle

Briefly, the replication cycle of influenza virus starts with the binding of HA to sialic acid on the glycoproteins of the host epithelial cells (figure 1.2). After binding the virus is endocytosed by the host cell. The acidic environment in the endosome leads to a conformational change in the HA, which exposes the hydrophobic region of HA_2 and promotes fusion of the viral and endosomal membranes. The M_2 proteins form a proton channel, thus facilitating influx of protons into the endosome and further acidification of the viral envelope, resulting in final viral uncoating. The nucleocapsid is released into the cytosol, and transported into the nucleus of the host cell. Here, viral RNA is transcribed into viral mRNA and positive-stranded RNA that serve as templates for new viral negative-stranded RNA segments. Since influenza is a negative stranded RNA virus, it carries its own RNA-dependent RNA polymerase (comprising of PA, PB1, PB2 and NP). But the enzyme lacks the ability to cap and methylate the mRNA, so it steals the 5' cap region from host mRNA, using it as a primer for viral mRNA, allowing it to bind to the ribosome. After transcription viral mRNA enters the cytosol where it is translated into viral proteins. The surface glycoproteins (HA and NA) and M_2-protein are further processed in the endoplasmic reticulum and in the golgi apparatus, and transported to the cell surface after processing. The newly replicated genomic segments associate with the polymerase and the NP protein in the nucleus, forming nucleocapsids. The nucleocapsids are transported to the cytoplasm with help from the NS_2 protein, and assemble with surface proteins and matrix proteins before budding and release of new virus, approximately 8 hours after infection [10].

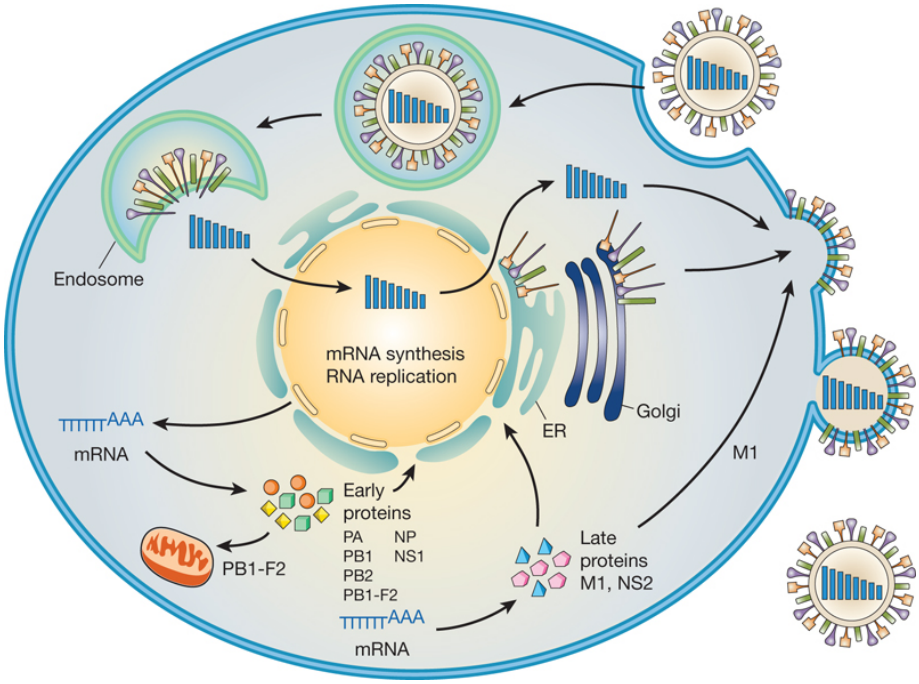


Figure 1.2: Replication cycle of influenza virus

Replication starts with binding of the virus to the host cell. The virion is further uncoated, and the viral RNA is released and transported to the nucleus where synthesis of viral mRNA and replication of RNA is carried out. Viral mRNA is translated into viral proteins and the newly synthesised viral particles assemble and bud from the host cell. See text for more details. From reference [11].

1.2 Epidemiology

1.2.1 Antigenic Drift and Shift

The influenza virus undergoes constant antigenic genetic change, namely antigenic drift and antigenic shift. These are mechanisms to avoid the host's immunity. Antigenic drift is point mutations in the gene segments, due to the high error rate of the RNA-dependent RNA polymerase and the lack of proof reading. The mutations can cause changes throughout the virus genome, but the most important antigenic changes occur in the surface glycoproteins (HA and NA). This result in pre-existing antibodies in a vaccinated or previously infected subject only partially recognising the antigenically changed HA and NA. The antibodies are therefore prevented from eliciting their neutralizing activity and host immunity is wane. Antigenic drift is the cause of for annual influenza epidemics and results in the need for annual vaccination.

Antigenic shift is a reassortment of gene segments, which can cause major changes in the influenza A virus. This can happen when a host (e.g. a pig) is infected with two or more different subtypes of influenza A. Antigenic shifts are not produced by influenza B viruses because they have only one subtype of HA and NA and predominantly infect humans. These antigenic shifts can lead to novel viruses that are able infect humans, and can lead to influenza pandemics, because the human population is immunologically naïve to the new virus (reviewed in [12]).

During the 20th century three influenza pandemics occurred, in 1918, 1957 and 1968. The "Spanish flu" in 1918 caused up to 50 million deaths worldwide. This pandemic was caused by an H1N1 virus [13], which was highly virulent due to extensive replication in the lungs [14] followed by high incidence of viral pneumonia. These two virulent features were mainly caused by the HA (causing specific receptor binding in the lungs) and the polymerase complex (reviewed in [15]). Another characteristic of the virus was that it mainly caused illness and deaths in young adults [16]. In 1957 a novel H2N2 virus caused a pandemic called the Asian influenza and replaced the H1N1 virus. Only eleven years later, in 1968, a new shift in influenza A, H3N2, occurred in Hong Kong and thereby called the Hong Kong influenza pandemic [1]. Together the Asian influenza and the Hong Kong influenza caused approximately 2 million deaths. In 1977, the H1N1 virus reappeared in Russia, however, it was restricted to persons under the age of 25 [1] and thus not defined as a pandemic. The last pandemic occurred in 2009 and was caused by an influenza A H1N1 virus of swine origin, also called "Swine flu". The virus was first discovered in Mexico, but rapidly spread throughout the world and approximately 200 million people were infected worldwide [17]. Luckily, the pandemic strain generally caused a mild and self-limiting disease, and the average case fatality rate was at 0.15 – 0.25 %. Nonetheless the high incidence of deaths in young people compared to other age groups, did pose a reason for alertness [17].

Another influenza subtype has caused great concerns, namely the highly pathogenic avian influenza A H5N1 virus. This influenza subtype primarily affects birds, but sporadic transmission to other species (e.g. man or swine) has occurred. The first case

of influenza A H5N1 in humans was reported in Hong Kong in 1997 [18]. This virus infected 18 people of who 6 died. However, the virus was eradicated by mass culling of all the poultry in Hong Kong. In 2003, the virus reappeared causing zoonosis and has continued to infect man. The average mortality rate since 2003 has been 60%, and the highest mortality rates have been reported in young people [19]. The different H5 viruses that have evolved from A/goose/Guandong/96 (H5N1), can be designated into clades based on a phylogenetic characterisation and sequence homology of the HA gene. The WHO defined 10 clades (0-9) of the H5N1 virus in 2008, and as the virus continues to evolve different sub-clades arise [20]. To date, there has not been efficient transmission of H5N1 between humans [21], but if the H5N1 virus undergoes antigenic shift with a human influenza virus or adapts its receptor affinity to allow upper respiratory tract infections, a future H5N1 pandemic virus can be a fact. Considerable research aims at finding the best H5N1 vaccine to prepare for this potential future pandemic.

1.2.3 Tropism and Ecology

The natural reservoir for influenza A viruses is aquatic birds, where one can find all types of HA and NA [2, 3, 22]. Influenza virus can be transmitted from aquatic birds to other species like humans, pigs, dogs, horses and ferrets, but only a limited numbers of subtypes have established themselves in the different species. The strains circulating in humans have surface molecules from HA (H1, H2 and H3) and NA (N1 and N2), but also avian H5, H7 and H9 viruses have caused zoonosis in man. In pigs, viruses containing HA (H1, H2, H3) and NA (N1 and N2) have been isolated although the H1N1 and H3N2 are the most frequently circulating subtypes (reviewed in [2]).

The different influenza virus subtypes have adapted to different species. HA is the main determinant of tissue tropism, and thereby the infectivity, pathogenicity and virulence [4]. HA binds to sialic acid, and the type of glyco-conjugation of the sialic acid in the tissue can determine what type(s) of viruses that can infect a specific tissue and/or species (tropism). The HA of avian and equine influenza viruses bind to $\alpha(2,3)$-linked sialic acid receptors, whilst the HA in most human influenza viruses

bind to α(2,6)-linked sialic acid receptors [23]. The reason avian influenza less efficiently infects and spreads in humans can be due to the HA binding specificity and may also increase the severity of avian influenza disease in humans. Influenza virus subtypes normally circulating in humans most often cause uncomplicated infection of the upper respiratory tract, where α(2,6)-linked sialic acid receptors are predominant. In contrast, the lower respiratory tract has mainly α(2,3)-linked sialic acid receptors, and avian influenza therefore has a tendency to cause lower respiratory tract infections in man. Pigs have both types of α(2,6) and α(2,3) containing cells in their trachea. Consequently, both types of viruses can infect this species [24], and pigs can thus work as a mixing vessel for new influenza strains.

An important determinant of influenza virulence is the cleavage of HA into HA_1 and HA_2 [25]. The virus is dependent upon host proteases to cleave the HA, allowing fusion of virus with the host cell membrane. In some highly pathogenic avian influenza viruses (HPAIV) the HA has a polybasic cleavage site. HA can therefore be cleaved by a number of proteases in different tissues and the viruses' ability to infect other types of tissues increases, resulting in systemic spread.

1.2.4 Clinical manifestation

The average incubation time for influenza is 2 days, but can vary from 1-4 days. After the infection the virus can cause asymptomatic to severe illness and ultimately death. The elderly, people with chronic heart, metabolic and respiratory diseases and immune deficiencies are more prone to severe illness. The symptoms of influenza are usually fever, fatigue, sore throat, runny nose, cough and myalgia (muscle pain). Cardiac involvement, neurological syndromes and secondary infections such as bacterial pneumonia and myostitis can be complications of influenza, but are rare in healthy adults. Primary viral pneumonia can also occur. An acute influenza infection in young children is often more serious than in adults, because young children have less experience of influenza, and the infection is often accompanied by higher fever, gastrointestinal symptoms, otitis media, myostitis and croup [10].

1.3 The Immune Response to Influenza

The immune system is divided into the *innate* immune system and the *adaptive* (acquired) immune system. In the first line of defence against foreign antigens, we find the innate immunity comprising of immediate recognition of pathogen patterns based on general specificity, whilst the adaptive immune system requires longer time to be activated, but it is more specific and is characterised by immunological memory. Both the innate and the adaptive immune systems are acting closely together in the prevention and eradication of influenza virus from the body.

1.3.1 The Innate Immune System

Innate immunity is acting early in the response to microbial agents like viruses and bacteria. It includes the physical and chemical barriers of the body, like the skin and the mucosal surfaces, phagocytes, natural killer cells (NK-cells) and circulating plasma proteins. In addition, the innate immune system produces inflammatory cytokines that initiates several defence mechanisms including recruitment of neutrophils, macrophages and lymphocytes to the infected site, and cytokine production that helps activate the adaptive immune system.

The innate immune system recognises microbes via pathogen-associated molecular patterns (PAMPs) that bind to pattern recognition receptors (PRRs). The specificity and diversity of these receptors is limited and they include Toll-like receptors (TLR), C-type lectin receptors, scavenger receptors, Nod-like receptors and N-formyl Met-Leu-Phe receptors among others. All these receptors are found on phagocytic cells (macrophages, neutrophils and dendritic cells (DCs)), either on the plasma membrane or in the cytoplasm. In addition, TLRs are also mainly found on endothelial cells. The internal TLRs 3 and 7 are important for recognising viral genomic material from e.g. influenza virus [26]. After a microbe has been recognized by a PRR, the effector cells of the innate immune system can either phagocytose the microbe or kill the infected cell. Neutrophils and macrophages are phagocytes that can engulf and digest microbes by phagocytosis whereas NK-cells, which are derived from the common lymphoid progenitor cells, can recognise stressed and infected cells and initiate killing of these

cells. NK-cells are therefore very important in the initial defence against intracellular microbes, such as viruses.

Circulating plasma proteins can also recognise PAMPs. These proteins include complement, pentraxin and collectin. Pentraxin and collectin work by opsonisation of microbes and activation of the complement system (via the classical pathway and lectin pathway respectively). The complement system works by opsonising microbes, killing microbes by lysis and activating leukocytes by inflammatory mediators. Complement proteins have been shown to contribute in the protection against influenza [27].

Pro-inflammatory cytokines (e.g. TNF-α, IFN-γ and IL-1) and chemokines are an important part of the innate immune system, and helps to activate the adaptive immune system. Type I interferons (IFN-α and IFN-β) are also important cytokines in the innate immune response, and provides a powerful defence against influenza virus by creating a cellular antiviral state, preventing new cells from being infected [28]. In addition, type I interferons work as potent stimulators of the adaptive immune system [29, 30].

1.3.2 The Adaptive Immune System

Five to ten days after primary infection, the adaptive immune system is fully activated [31]. The activation is highly dependant on help from innate immune responses, especially cytokine help and antigen presentation by DCs. The adaptive immune system has two arms: the humoral and the cellular arms, represented by B-cells and antibodies, and T-cells respectively. The two arms are closely connected and dependent on each other.

1.3.2.1 Cellular Immunity

T-lymphocytes are the effector-cells of cellular immunity. T-lymphocytes can be divided into $CD4^+$ and $CD8^+$ T cells by the distinct surface molecules, also called helper T-lymphocytes and cytotoxic T-lymphocytes (CTL) by their main mode of action after activation.

Both $CD4^+$ and $CD8^+$ T cells develop from the same precursor, the common lymphoid progenitor cell. The main the development of T cells occurs in the thymus without exposure to foreign antigens. The mature T cell migrates to the peripheral lymphoid organs, where it scans professional antigen presenting cells (APCs) for a peptide with affinity for its specific T-cell receptor (TCR). The activation that occurs when the immunological synapsis containing major histocompatibility complex (MHC) and TCR complex forms, may stimulate the lymphocyte to proliferate and further differentiate.

$CD4^+$ T cells have CD4 co-receptors that bind to MHC class II molecules. MHC class II are mainly found on professional APCs and bind peptides processed in endosomes, i.e. extracellular proteins endocytosed by the APC. The activation and maturation of APCs (e.g. increased expression of MHC class II and co-stimulatory molecules) are stimulated by innate immune signalling (via TLRs or other innate immune receptors). The most important APCs are DCs, macrophages and B-cells. When a $CD4^+$ T cell meet an APC with an MHC class II peptide complex which it has affinity for, the T cell is activated and can undergo clonal expansion and differentiation into an effector cell and/or memory cell. In order to activate naïve $CD4^+$ T cells, signals are required from co-stimulatory molecules B7-1/CD80 and B7-2/CD86 (mainly found on activated DCs) and cytokines (IL-12 or IL-4) in addition to the signals the TCR complex generate. The $CD4^+$ T cells then start to produce large amounts of IL-2, a cytokine working as a growth factor for T cells in an autocrine and/or paracrine manner.

$CD4^+$ T cells can differentiate into at least four different subsets; Th1, Th2, Th17 and Treg. The different subsets vary in what types of cytokines they secrete, and therefore in effector functions. Tregs are regulatory T cells, which are important in the suppression of other T cells (mainly via IL-10, IL-35 and TGF-β) and thereby induction of immunological tolerance (reviewed in [32]). After an infection with microbes that activate macrophages and NK-cells, production of IL-12 and IFN-γ is induced, which subsequently promote differentiation of $CD4^+$ T cells into Th1 cells. Th1 cells predominantly produce IFN-γ, IL-2 and TNF-α. TNF-α and IFN-γ are pro-inflammatory cytokines inducing macrophage activation and promoting phagocytosis

of microbes. In mice, Th1 cytokines stimulate IgG2a antibody class-switch. The Th2 subset is encouraged by helminthic infections and allergens, which induce production of IL-4 from mast cells or DCs. Th2 cells mainly produce IL-4, IL-5, IL-10 and IL-13 [33], and induce differentiation of B-cells into plasma cells and the subsequent production of antibodies. Th2 especially stimulates the production of IgE, IgM, IgA and IgG1 antibodies. Both Th1 and Th2 boost their own subsets (by autocrine signalling) and regulate the other subset by the production of inhibitory cytokines [34]. The third main subset of $CD4^+$ T cells is Th17. These cells produce IL-17, which can stimulate neutrophils and induce phagocytosis of extracellular pathogens. The Th17 subset has also shown to induce inflammation and autoimmunity [31].

Some $CD4^+$ T cells have the ability to produce more than one cytokine simultaneously, and are hence called multifunctional T cells. T cells producing three cytokines (triple producers) have also been shown to produce more of each cytokine per cell as compared to single cytokine producing T cells [35]. It has been demonstrated that these multifunctional Th cells can be a good correlate for vaccine protection in vaccination against *Leischmania major* [36], and it is speculated if multifunctional Th cells also are important in the protective effect of influenza vaccines [35].

$CD8^+$ T cells recognise MHC class I peptide complexes by its TCR, and differentiate into CTLs after the appropriate stimulation with co-receptors and cytokines (e.g. IL-2). MHC class I is present on the surface of all nucleated cells in the body (with a few exceptions), and present intracellular peptides. After a CTL has become activated, the target cell (e.g. a virus infected cell) is killed by either granzyme/perforin complexes or Fas/FasL interactions, which both induce apoptosis. CTLs are therefore central in the defence against intracellular pathogens such as viruses.

1.3.2.2 Humoral Immunity

B-lymphocytes and antibodies (immunoglobulin, Ig) (figure 1.3) are the main features of humoral immunity. B-lymphocytes recognize extracellular microbes (antigens) and can differentiate into plasma cells, which secrete antibodies (antibody secreting cells

(ASC)). Antibodies can bind to and neutralise microbes as well as activate macrophages and complement.

B-lymphocytes develop from the same precursor as all blood cells, the pluripotent haematopoietic stem cell. B cells develop in the bone marrow, and enter the spleen and other secondary lymphoid tissues via the blood as immature B cells. Here they mature before re-circulating between the blood and the secondary lymphoid organs until the naïve B cells encounter a specific antigen. If T cell help by an activated Th cell to cognate antigen is provided, antigen-specific B cells will differentiate into short-lived IgM-producing plasma cells while others will form germinal centres where they proliferate and undergo affinity maturation and class-switch. Affinity maturation is a process where the Ig V (variable) genes undergo somatic hypermutation, and the B cells with high-affinity Ig survive the selection, before differentiation into a memory B cell or into a plasma cell.

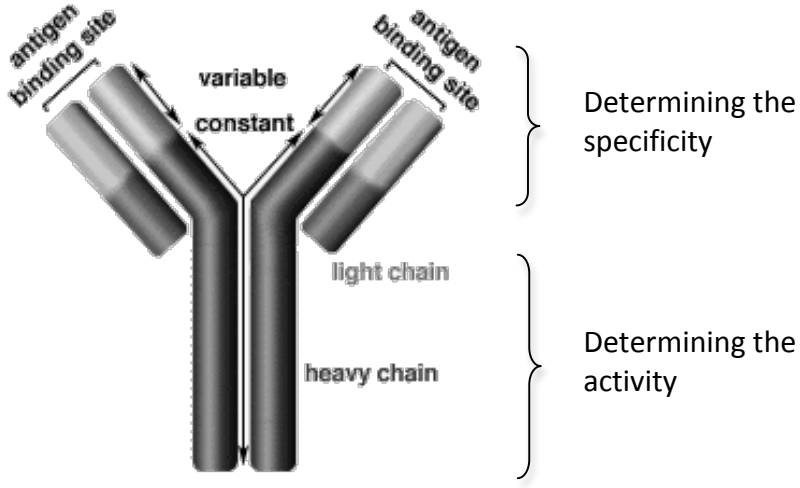


Figure 1.3: Antibody structure

Antibodies consist of two heavy chains and two light chains joined together by disulfide bridges (not shown). Each heavy and light chain has variable and constant regions, and the antigen binds to highly diverse parts of the variable regions. Modified from reference [37].

Naïve B cells express IgM and IgD receptors on their surface. The first time a naïve B cell encounter an antigen (either by infection or vaccination), IgM is the first antibody secreted followed by a weak IgG response (primary response). IgM is secreted as a pentamer and mainly function as a complement activator. The subsequent exposure of

the same antigen, the memory B cells is rapidly activated and the antibody response becomes faster and stronger than the first response (secondary response) (figure 1.4).

The change from one antibody class to another (e.g. IgM to IgG) occurs when B cells undergo class-switch. Class-switch is irreversible because the gene rearrangement is done by deletion. IgG antibodies dominates the secondary response, and is secreted as a monomer. In mice, four different subclasses of IgG can be secreted (IgG1, IgG2a, IgG2b and IgG3). Which IgG subclass produced, is largely dependent on the Th polarisation (either Th1 or the Th2 skewed response, as discussed above). IgG can opsonise bacteria and virus in the extracellular fluid, activate complement and mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Other antibody classes are IgA (an essential part of mucosal immunity, described below) and IgE. IgE is important for the defence against helminths and is also partly responsible for allergic reactions (immediate hypersensitivity).

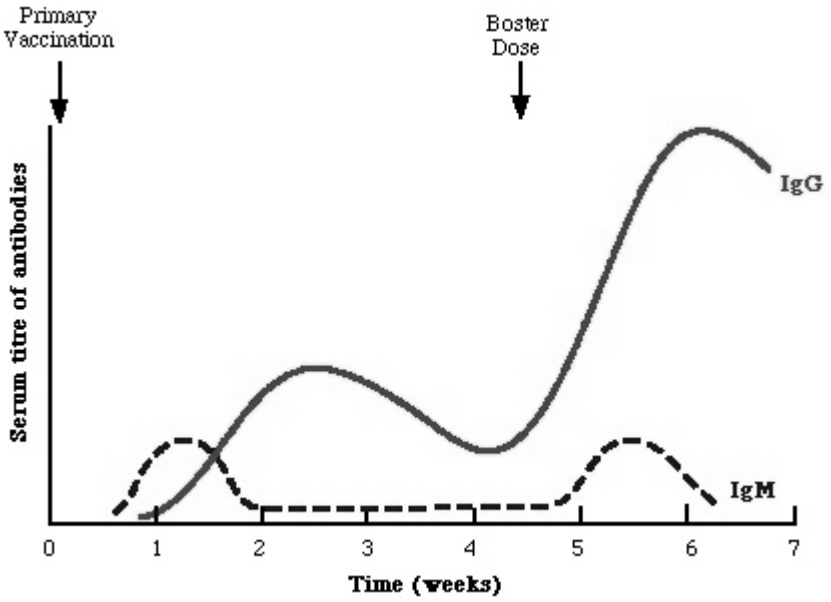


Figure 1.4: Primary and secondary antibody immune response

Primary vaccination/infection is characterised by an initial IgM response, which may during the course of events develop into a weak IgG response. The secondary response (or booster dose) is much more rapid and is dominated by IgG antibodies. Modified from reference [38].

1.3.2.3 Mucosal immunity

The entry site for influenza virus is the mucosal epithelium in the nose and upper respiratory tract. The mucosal sites in the body have associated specialised lymphoid tissue; the mucosal immune system (figure 1.5). This comprises of mucosal associated lymphoid tissues (MALT), which in the nose are called nasal associated lymphoid tissues (NALT).

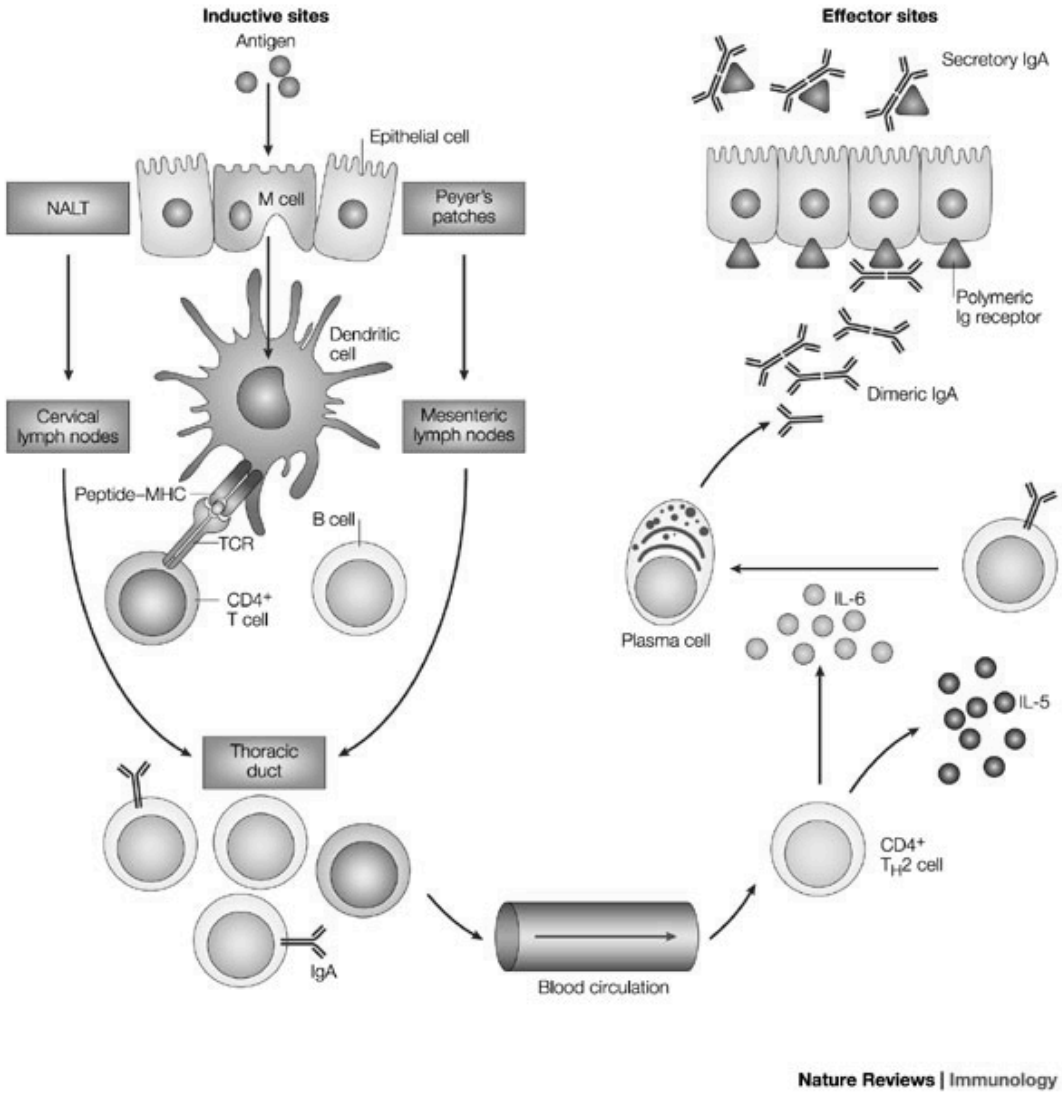


Figure 1.5: Mucosal immunity

Details are described in the text. From reference [39].

Microfold cells (M-cells) can be found in between the mucosal epithelial cells. These cells are in close contact with sub-epithelial lymphocytes and DCs. Antigens that come in contact with the mucosal surface are transcytosed by M-cells and taken up by

DCs at the basolateral side of the epithelium. The DCs are transported to the draining lymph node where they activate $CD4^+$ T cells, which subsequently get the ability to activate B-cells (as previously described). The DCs found in MALT have a special ability to induce $CD4^+$ T cells to produce transforming growth factor β (TGF-β) and IL-10, stimulating IgA class-switch in B-cells [40, 41]. Large amounts of IgA are secreted by plasma cells as dimers and/or polymers, joined together by a J-chain. Before transportation to the lumen, the IgA dimmer/polymer is bound to a polymeric Ig receptor (pIgR). The IgA-pIgR complex is transported across the epithelial cell, and by proteolytic cleavage the SIgA is released. The secretory component (SC) is the secreted part of pIgR, which is covalently bound to SIgA. This binding makes SIgA more stable than other antibodies [41]. On the epithelial surface it can bind to and neutralise pathogens and toxins, preventing them from entering the host [31, 42].

1.4 Prophylaxis and Treatment

Symptomatic treatment with analgesics, anti-pyretics, cough relief and anti-congestive drugs are the most effective way of dealing with the influenza. Antiviral drugs (e.g. Tamiflu and Relenza) can also be used to treat influenza, but are only efficient if used the first 48 hours after the symptoms has presented. Prevention of influenza disease by vaccination is therefore the most important way to minimize the impact of influenza.

1.4.1 Antiviral drugs

Antiviral drugs can be used as prophylaxis and treatment for influenza virus. There are two classes of anti-influenza antiviral drugs, neuraminidase inhibitors (NAI) and M2 ion channel blockers. A recent Cochrane Review showed that NAIs have high effectiveness against influenza when given prophylaxically or post-exposure, but when given as treatment of influenza symptoms the effectiveness was much lower [43]. Resistance towards influenza antiviral drugs due to mutations in the influenza virus is a growing problem, thus widespread use in healthy adults during seasonal influenza is not recommended. In a future pandemic with novel influenza virus, antiviral drugs will be an important part of the treatment [43].

There are two NAIs on the market in Norway, oseltamivir (Tamiflu™) and zanamivir (Relenza™). A novel NAI in, named peramivir, is currently in clinical trials for intravenous administration [44], however an Emergency Use Approval was issued by the U.S. Food and Drug Administration during the H1N1 pandemic in 2009 [45]. The NAIs are active against both Influenza A and B viruses, and they are indicated for use in the first 48 hours of influenza symptoms and prophylactically after exposure to clinical influenza [46]. They work by inhibiting the enzyme function of NA, thus preventing the influenza virus infection and replication [47]. Several resistance mutations have been described against oseltamivir in both seasonal H1N1 [48] and H5N1 viruses [49]. Resistance to zanamivir has also been reported, but is very rare [50].

Another type of antiviral treatment against influenza is the M2 channel blockers, which inhibits acidification of the virion and prevents uncoating of the virus. These drugs are not much in use because of resistance problems [51].

1.4.2 Influenza Vaccines

Seasonal vaccination is most important in the elderly (people older than 65 years), children and individuals with chronic conditions [52]. In 2003, nearly 300 million seasonal vaccines were manufactured, and the number is increasing every year [53]. In recent years, the fear of an influenza H5N1 pandemic placed pandemic influenza vaccines in the spotlight. A considerable effort has been put into development of pandemic vaccines, which allowed an effective pH1N1 to be rapidly manufactured and deployed. Over 350 million pH1N1 vaccine doses were administered globally during the 2009 pandemic [54].

The influenza virus is continuously changing every year due to antigenic drift. The WHO created a global surveillance system, called Global Influenza Surveillance Network (GISN), to collect global antigenic and genetic data for biannual influenza vaccine composition to be decided [55]. GISN consists of National Influenza Centres that collects samples from patients with influenza like symptoms and WHO

Collaborating Centres who analyse the samples to decide whether a previously circulating strain or a new strain should be incorporated into the annual vaccine [56]. A lot of research has also focused on the development of pre-pandemic vaccines and so far five pre-pandemic H5N1 vaccines have been licensed (reviewed in [57]).

1.4.2.1 Evaluation of Influenza Vaccines

The Committee for Medicinal Products for Human Use (CHMP) provided a note on requirements for influenza vaccines, to standardize the criteria for production, and for evaluating tolerance and immunogenicity, of seasonal influenza vaccines [58]. As a standard, evaluation of influenza vaccines sera should be collected prior to vaccination and 3 weeks post vaccination. In the sera, functional antibodies against the vaccine strain must be detected by haemagglutination inhibition assay (HI assay) and/or single radial haemolysis (SRH) (the latter not discussed here). For the HI-assay, seroconversion is described as negative pre-vaccination serum and a post-vaccination serum antibody titre ≥40 or as a fourfold increase in post vaccination antibody titre (considered significant). Seroprotective titres are defined as anti-haemagglutinin antibodies ≥40; which is regarded as a surrogate correlate of protection for seasonal influenza [58]. It is uncertain if this correlate applies to pandemic influenza vaccines [59], however, in the absence of other surrogate correlates of protection, serum HI titres ≥40 are also used to evaluate pandemic influenza vaccines [59]. In addition other immunological methods can be used to evaluate the immunogenicity of pandemic vaccines including measurement of neutralising antibodies, antibody kinetics and cell-mediated immunity [59].

1.4.2.2 Safety

During the last 50 years, hundreds millions of doses of influenza vaccines have been administered worldwide. The manufacturing requirements and the accepted level of adverse drug reactions (ADR) for influenza vaccines in Europe are set by the European Medicines Agency [59]. The most common ARD after intramuscular influenza vaccination are mild local reactions such as pain and redness at the injection site and systemic flu-like symptoms [60]. But also more serious ADR like anaphylactic shock and GuillaIN-Barrés syndrome (a peripheral nerve system

disorder) have been reported at very low frequencies (reviewed in [61]). The incidence of these ARDs must be assessed alongside the immunogenicity in clinical trials prior to licensing and in a phase IV study post licensing (pharmacovigilance).

1.4.2.3 Propagation of Influenza Virus

The majority of influenza vaccines are produced in embryonated hens' eggs. The virus replicates in the allantoic cavity, and the allantonic fluid is harvested. Next, the virus is purified and inactivated by formaldehyde or β-propiolactone [62, 63]. To get maximal virus yield, a "high-growth" influenza A virus strain is used as a donor strain. This strain is called PR8 (A/Puerto Rico/8/34) and has been used since the 1970s to create reassortant influenza A viruses that grow well in eggs [63]. A problem with propagating H5N1 virus in eggs is that the virus is highly virulent and kills the embryo. Reverse-genetics technology has made it possible to overcome this problem by removing the poly-basic cleavage site of HA associated with virulence [64]. There are a number of issues with the use of embryonated hens' eggs, i.e. egg allergy, possible bacterial contamination leading to delay in vaccine supply and the initial problem of growing H5N1 in eggs. Therefore, alternative ways of producing virus for influenza vaccines are currently under investigation. An attractive alternative to eggs is to grow virus in cell cultures, and different types of continuous cell lines have been used to produce influenza virus that are used in licensed vaccines (reviewed in [65] and [66]). Influenza subunit antigens grown in plant-cells are another approach that is under current investigation, which avoids the need to propagate live viruses [67].

1.4.2.4 Live Attenuated Influenza Vaccines

Live attenuated vaccines are developed by growing viruses in cell culture for repeated passages and at low temperatures, in this way making the virus cold adapted (CA), reducing the virus' ability to replicate at the temperature of the lower respiratory tract (37°C). Live attenuated influenza vaccines (LAIV) is registered in the USA and Europe as intranasal vaccines, called FluMist® and Fluenz®, respectively, which both are seasonal trivalent vaccines [68, 69]. The virus replication is restricted to the nasal cavity where the temperature is lower compared to the rest of the body. One dose of a trivalent seasonal LAIV has proved sufficient for protection against clinical

infection [70], and has been found more effective in preventing influenza infections than a trivalent inactivated seasonal influenza vaccine (administered intramuscularly) [71]. There are however, some limitations with the LAIV. It is only is approved for use by non-pregnant persons in the age group 2-59 years [72], should not be used by immunocompromised persons and viral shedding can occur after administration [73]. FluMist® must be kept frozen at -18°C [72], which could lead to difficulties with distributing the vaccine in developing countries. When it comes to pandemic LAIV both successful animal studies and phase I clinical trials have been conducted [72], but H5N1 CA reassortant showed the lowest immunogenicity of all LAIV that have been manufactured [74].

1.4.2.5 Inactivated Influenza Vaccines

There are different types of inactivated influenza vaccines; whole virus, split (chemically disrupted), subunit (isolated surface antigens) and virosomal vaccines (figure 1.6). Virosomes are discussed in section 1.4.1.6. Whole virus vaccines are more immunogenic than the split and subunit vaccines, but they can give undesirable ADRs because of the higher reactogenicity [75]. Whole virus vaccines are prepared by inactivation with formaldehyde or β-propiolactone of purified virus. Split virus vaccines (also called subvirion vaccines) are prepared by adding a detergent (e.g. deoxycholate or Triton X-100) to the whole inactivated virus, resulting in disruption of the lipid membrane, followed by further purification. Split vaccines show lower reactogenicity and fewer side effects as compared to whole virus vaccines. The split virus vaccine is more immunogenic than subunit, but less immunogenic than whole virus vaccine. The subunit formulation contains only the purified viral surface antigens HA and/or NA [63]. This formulation elicits fewer ADRs, but often shows lower immunogenicity than the other formulations and an adjuvant is therefore often required to elicit a sufficient immune response (adjuvants are described below) [76]. In addition to the inactivated vaccines, viral vector vaccines and DNA vaccines are under current investigation as novel ways of delivering influenza vaccines [77].

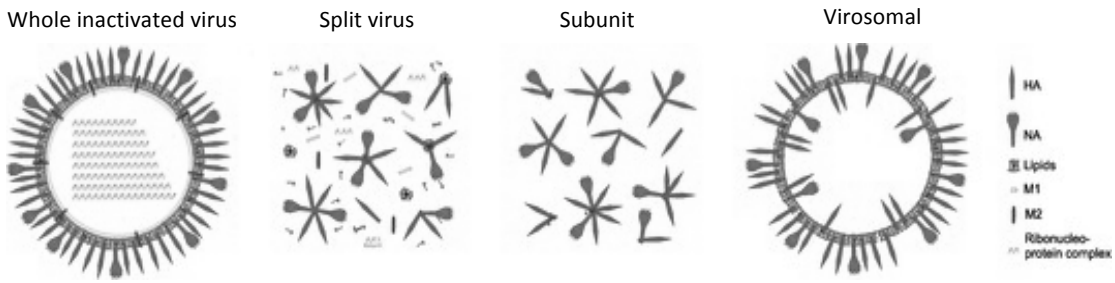


Figure 1.6: Inactivated influenza vaccines

The whole virus vaccine is inactivated by formaldehyde or β-propiolactone and it shows great immunogenicity, eliciting both humoral and cellular immune response. The split virus vaccine contains most of the components of the whole virus, but the lipid membrane is chemically disrupted. Subunit vaccine only contains the influenza surface antigens HA and NA. The virosomal vaccine is a reconstituted viral envelope containing the surface antigens HA and NA, but no genetic material. (HA = Haemagglutinin, NA = Neuraminidase, M1 and M2 = Matrix proteins). Modified from reference [78].

1.4.2.6 Virosomal vaccines

Virosomes are virus-like particles, made from reconstituted influenza viruses. The virosomes lack the genetic material and the viral matrix proteins and NP, and can therefore not replicate like the native influenza virus. Proper reconstituted viral envelopes contain the influenza surface antigens HA and/or NA, and have a retained receptor-binding and membrane fusion activity [79]. Thus, the virosomes interact extensively with B-lymphocytes, and are presented by APC like the native virus [79]. This implies that the antigens are distributed in the cytosol, as well as in endosomal compartments, and can therefore be presented by both classes of MHC molecules, leading to activation of $CD4^+$ and $CD8^+$ T-cells [80, 81]. Activation of $CD8^+$ T-cells is important for the elimination of virus from an infected person. Virosomes have been shown to provide enhanced immune responses in elderly and in persons that have little or no previous exposure to influenza as compared to subunit vaccines [82, 83]. Another advantage of the virosomal vaccine is that adjuvants (both amphiphilic and lipophilic) can be incorporated in the virosomal membrane [79].

Earlier pandemics have shown that children and young adults are at high risk of developing severe illness from pandemic influenza [1, 17]. Virosomal vaccines have been shown to be both well tolerated and highly immunogenic in children (reviewed in [84]), and are therefore favourable for pandemic vaccine formulations. Vaccination of elderly people is important both for pandemic and seasonal influenza. Elderly people have lower immune responses, primarily due to decreased T-cell activity. After influenza vaccination, the immune response shifts toward a Th2 cytokine production, which is related to a reduction in CTL activity [85]. Therefore, to make an efficient vaccine for elderly, the vaccine should induce both humoral and cellular immune responses [86]. A virosomal vaccine could overcome this issue.

1.4.2.7 Mucosal vaccines

Different routes of administering vaccines mucosally are used today and/or currently under investigation. Oral administration of polio and cholera vaccines and intranasal administration of influenza vaccines has been used for decades. Mucosal vaccination has many advantages over intramuscular vaccination, particularly when it comes to a respiratory pathogen such as influenza. When a vaccine is applied at a mucosal site, a local as well as a systemic immune response is induced [87]. The mucosal immune response in the respiratory tract, represented mainly by IgA, can protect against an influenza virus infection at the site of viral entry. It has also been shown that secretory antibodies (SIgA) are more cross-reactive against different strains of influenza than IgG [88, 89]. A highly cross-reactive vaccine could reduce the need for annual vaccination, and render the population immune for a possible future pandemic. The avoidance of needles is also beneficial in a number of ways. More people would choose to be vaccinated with influenza, including those with needle phobia. Self-vaccination would be easier, the need for health care personal minimised reducing the cost of vaccination. Serious injection-site adverse reactions, such as anaphylaxis could be avoided, and the risk of systemic side effects decreased. Today, intranasal (IN) influenza vaccines are licensed in USA and Europe [68, 69]. In Switzerland an intranasal inactivated virosomal influenza vaccine was withdrawn from the market due to an association with a serious side effect called Bell's Palsy (idiopathic facial paralysis) [90]. It has not yet been agreed on whether it was the vaccine antigen or the mucosal adjuvant (*Escherichia coli* heat-labile toxin (LT)) that caused this side

reaction, but since intranasal LAIV has not been associated with the same ADR it is most likely due to the mucosal adjuvant. However, the nasal epithelium is localised close to the CNS, and redirection of viral antigen [91, 92] and mucosal adjuvants (LT and cholera toxin) [93, 94] to the olfactory bulb and olfactory nerves after IN vaccination of mice has been reported. It is therefore important to investigate new ways of administering mucosal vaccines and new mucosal adjuvants that can be used both in intranasal and sublingual formulations.

1.4.2.8 Sublingual vaccination

Sublingual (SL) delivery is the administration of a substance under the tongue (from Latin, *sub lingua* = under the tongue) (figure 1.7). This route of administration have been used for decades [95], and today many drugs are administered sublingually. SL administration can be used for both local and systemic treatment [96]. In recent years, SL tablets have also been used for administration of proteins and peptides, including immunotherapy against allergic rhinitis and it is considered as a safe administration route (reviewed in [97]). After intranasal vaccination was associated with Bell's Palsy [90], SL influenza vaccination represents a novel and attractive approach as an alternative to the intranasal route. In previous studies, SL administration of influenza showed no antigen redirection to the olfactory bulb in the brain, therefore minimising the risks of neurological side effects like Bell's Palsy [91, 92]. In addition, no cases of anaphylactic shock after SL administration have been reported (reviewed in [98]).

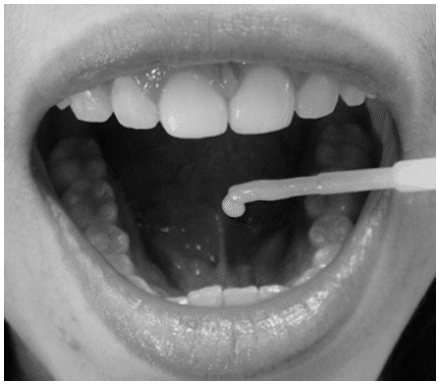


Figure 1.7: Sublingual administration
From reference [99].

1.4.2.9 Vaccine Adjuvants

Adjuvants are used to improve the immunogenicity of a vaccine. The word adjuvant comes from *adjvuo,* which means help in Latin. Formulations of vaccines with adjuvants improves the immune response [100]. Another advantage of adjuvants is that they can allow antigen dose sparing, which may be crucial if only limited quantities of antigen are available for a pandemic vaccine. The H5 influenza virus vaccines have elicited lower immunogenicity in humans than the seasonal vaccines, and a good adjuvant is therefore needed [101]. Adjuvants are often classified into two groups based on their mechanism of action; delivery systems and immune potentiators [102]. The delivery systems often elicit a depot effect, which prolongs vaccine antigen presentation to lymphocytes. This can be obtained by increasing the time APCs and antigen are exposed to each other at the injection site, or by protecting the antigen from breakdown (e.g. liposomes) [100]. The immune potentiators work by activating APCs and induce the secretion of pro inflammatory mediators that improve the immune response. Activation of APCs most commonly happens through binding of PAMPs to PRRs on the APCs and many bacterial and several viral ligands therefore could be potential adjuvants. There are also PRR-independent adjuvants that can stimulate the immune response [103].

Some frequently used adjuvants are aluminium salts, oil-IN-water emulsions and Immune Stimulating Complexes (ISCOMs) and the novel adjuvant used in this project, Bis (3',5')-cyclic dimeric GMP (c-di-GMP), will be described in the next sections.

Aluminium salts

Aluminium salts are the most common adjuvant type used in human vaccines, including influenza vaccines, and until 2009 it was the only adjuvant type licensed in the United States [100, 104]. Different aluminium salts are used, including aluminium hydroxide and aluminium phosphate, but often aluminium containing adjuvants are called only *alum* [105]. Even though aluminium salts have been used for decades, their mechanism of action is not fully clarified. It has been suggested to have a depot effect, so that the antigen is slowly released from the injection site; cause local inflammation, attracting APCs to the injection site and activate them; and adsorption of antigen to the aluminium salt results in particles and not a soluble antigen, so that

uptake into APCs are increased [106]. All these mechanisms have been reviewed in [105]. Regarding the activation of DCs, which are the most important APCs, the activation seems to occur via a monocytic precursor [107]. In addition, the NLRP3 inflammasone have been shown to be an important mediator of the alum adjuvant effect [108]. The aluminium salts elicits a Th2 skewed response [109].

Emulsions (oil in water)

Emulsions occur when two immiscible liquids are mixed together and held stable by an emulsifier. Oil-IN-water emulsions are small droplets of oil dispersed in a watery phase. These are effective adjuvants, widely used together with influenza antigens. The mechanisms of how these adjuvants work are unknown, but different possible explanations could be a depot effect (i.e. the antigen is retained at the injection site), local induction of cytokines leading to APC maturation and/or prolonged presentation of the antigen to APCs [100]. Oil-IN-water adjuvanted vaccine can also elicit cross-protective antibody responses, which can be favourable, both in seasonal and pandemic vaccines [110-113].

There are different preparations of oil-IN-water emulsions. An MF59 adjuvanted vaccine is licensed in Europe [114]. The use and safety issues regarding MF59 is reviewed in [100] and it has shown to be an adjuvant with good immune stimulating effect and good safety profile [115]. Another oil-IN-water emulsion is AS03. This preparation is also showing potent adjuvant activity in combination with influenza virus antigen [100, 116], and was used in the pH1N1 vaccine (Pandemrix®) [117]. However, an increased incidence of narcolepsy in children has been reported in Finland after mass vaccination with Pandemrix® [118], thus questioning the safety of AS03.

ISCOMs and ISCOMATRIX

ISCOMs (Immune Stimulating Complexes) were first described in 1984 as “A novel structure for antigenic presentation of membrane proteins from enveloped viruses” by Morein et al. [119]. ISCOMs are composed of quillaia saponin, cholesterol, phospholipids and an associated antigen (e.g. influenza antigen) [120]. ISCOMATRIX® is ISCOM without the incorporated antigen. It has been shown that both ISCOMs and ISCOMATRIX® can elicit both humoral and cellular immune

responses [121] and the mechanism of action classifies ISCOMs as both immune potentiators and delivery systems [120] (classification by O'Hagan and Valiante (2003)[102]). The ISCOMATRIX® adjuvant has been evaluated in human clinical trials, and it was found to have an acceptable safety profile. In addition, no autoimmune or anaphylactic reactions were reported [120, 122]. A novel generation of ISCOMs, the Matrix-M, has been found to potentiate the immune response following vaccination with influenza H5N1 virosomes in murine models [123, 124].

C-di-GMP

Bis (3',5')-cyclic dimeric GMP (c-di-GMP) is a bacterial intracellular signalling molecule. It has been identified in several different bacterial species, but not in higher eukaryotes. Therefore, c-di-GMP presumably works as a danger signal to the innate immune system. C-di-GMP has been shown to be an effective adjuvant (reviewed in [125]), and it has also shown good potential as a mucosal adjuvant [126]. C-di-GMP has significant immune stimulatory properties, and stimulates both humoral and cellular responses. *In vitro* it can stimulate DC expression of MHC class II, co-stimulatory molecules B7-1/B7-2 and maturation marker CD83 [127]. It also induces production of pro-inflammatory cytokines and chemokines. *In vivo*, c-di-GMP has been shown to recruit monocytes and granulocytes [127]. The exact mechanisms of action still remain unknown, but it has been proposed that c-di-GMP is detected in the cytosol by immunosurveillance pathways, similar to those that sense DNA in the cytosol [128]. The safety profile *in vivo* has not yet been assessed for the c-di-GMP adjuvant. However, *in vitro* studies show no lethal cytotoxicity in rat kidneys cells or in human neuroblastoma [125].

1.5 Aims of the study

Mucosal immunisation provides local immunity, which can prevent influenza infection at the portal of entry. In addition, mucosal vaccines can be administered without the use of needles, and are thus attractive for use in developing countries. Intranasal influenza vaccines have been used for decades, but the only licensed adjuvanted intranasal vaccine was withdrawn from the market due to the association with Bell's Palsy (a facial nerve paralysis). Vaccination under the tongue (sublingual vaccination) may provide a safe alternative to intranasal vaccination and requires further investigation.

Mucosal vaccines have earlier been shown to induce a weak immune response when administered without an adjuvant. Virosomes (virus like particles) are more immunogenic than subunit vaccines [82], and also induce cell-mediated immunity [81].To further boost the immune response we combined a virosomal vaccine with the promising mucosal adjuvant c-di-GMP.

The aim of this study is to evaluate the sublingual route for administration of an H5N1 virosomal influenza vaccine, alone and in combination with the promising mucosal adjuvant c-di-GMP. Therefore we compared the immunogenicity induced by sublingual, intranasal and intramuscular administration of the vaccines in mice. Both the local and systemic humoral response and cellular immunity were studied.

2 Materials

Name	Supplier
2.1 Mice	
BALB/c – 6-8 weeks old, albino, female	Charles River Laboratories, Germany
2.2 Vaccines and viruses	
Influenza H5 virosomal vaccine, 322 µg HA/mL (A/Vietnam/1194/2004 (H5N1)) NIBRG-14	Crucell B.V., Holland
2.3 Anaesthetics and other drugs	
Rompun® Vet (Xylazine) (20 mg/mL)	Bayer, Germany
Ketalar (Ketamine) (50 mg/mL)	Pfizer, USA
Pilocarpine HCL (0.125 mg/mL), P6503-5g	Sigma, USA
Euthanasia:	
CO_2-chamber	Scanbur A/S, Denmark
2.4 Blood	
Turkey red blood cells (10 %) in PBS	National Institute for Biological Standards and Control (NIBSC), United Kingdom

2.5 Antibodies

Name	Catalogue number	Supplier
Capture antibodies		
Goat anti-mouse IgA (1 mg/mL)	1040-01	SouthernBiotech, USA
Goat anti-mouse IgG (1 mg/mL)	1030-01	SouthernBiotech, USA
Goat anti-mouse IgG1 (1 mg/mL)	1070-01	SouthernBiotech, USA
Goat anti-mouse IgG2a (1 mg/mL)	1080-01	SouthernBiotech, USA
Immunoglobulin standards		
Mouse IgA (1 mg/mL)	M1421	Sigma, USA

Mouse IgG (1 mg/mL)	15381	Sigma, USA
Mouse IgG2a (1 mg/mL)	M9144	Sigma, USA
Mouse IgG1 (0.5 mg/mL)	0102-14	SouthernBiotech, USA

Biotinylated antibodies

Goat anti-mouse IgA (0.5mg/mL)	1040-08	SouthernBiothech, USA
Goat anti-mouse IgG (0.5mg/mL)	1030-08	SouthernBiothech, USA
Goat anti-mouse IgG1 (0.5 mg/mL)	1070-08	SouthernBiothech, USA
Goat anti-mouse IgG2a (0.5 mg/mL)	1080-08	SouthernBiothech, USA
Goat anti-mouse pIgR (0.5 µg/mL)	BAF 2800	R & D Systems

Fluorophore-conjugated antibodies

Rat anti-mouse CD8	557654	BD Biosciences, USA
Rat anti-mouse CD4	553047	BD Biosciences, USA
Rat anti-mouse IFN-γ	554413	BD Biosciences, USA
Rat anti-mouse IL-2	554428	BD Biosciences, USA
Rat anti-mouse TNF	557644	BD Biosciences, USA
Hamster anti-mouse CD3e	551163	BD Biosciences, USA

2.6 Reagents and chemicals

Name	**Cat. No**	**Supplier**
Anti-CD28 (Hamster anti-mouse)	553294	BD Biosciences, USA
BD FCblock™ (mouse)	553142	BD Biosciences, USA
BD CompBeads Negative Control (FBS)	51-90-9001291	BD Biosciences, USA
BD CompBeads Anti-rat	51-90-9000949	BD Biosciences, USA
Bovine serum albumin (BSA)	A-6793	Sigma, USA
Casy®Ton	043-90037P	Schärfe System GmbH, Germany
Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$)	1.00244	Merck, Germany
Dimethylformamide (DMF)	10322	BHD AnalaR, England
Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4)	1.06586	Merck, Germany
Extravidin Peroxidase (Extravidin PO)	E-2886	Sigma, USA
Fix/Perm solution (BD Cytofix/Cytoperm)	51-2090KZ	BD Biosciences, USA
Foetal bovine serum (FBS)	14-701F	Bio Whittaker, Belgium

Foetal bovine serum (FBS) Gold	A15-151	PAA, Austria
Glacial acetic acid ($C_2H_4O_2$)	1-06268	Merck, Germany
Golgi plug	51231KZ	BD Biosciences, USA
Hepes buffer (1M)	H0887	Sigma, USA
Hydrogen peroxide, H_2O_2, 30 %	H1009	Sigma USA
Ionomycin	10634	Sigma, USA
Lymphoprep ™	1114545	Axis-Shield PoC A/S, Norway
Minimal Essential Medium (MEM) non essential amino acids (100x)	11140-035	GIBCO, UK
Mercaptoetanol (2-ME)	M-7522	Sigma, USA
Newborn Calf Serum (NCS)	ECS0070L	Euroclone, Italy
Ortho-phenylediamine dihydrochloride (OPD, 10 mg)	P-8287	Sigma, USA
Pansorbin	507858	Calbiochem/Merck, USA
Penicillin/Streptomycin/Amphoterecin (PSA)	17-745E	Bio Whittaker, Belgium
PMA (phorbol myristate acetate)	P8139	Sigma, USA
Pokeweed mitogen	L8777	Sigma, USA
Potassium di-hydrogen phosphate (KH_2PO_4)	1.04847	Merck, Germany
Receptor destroying enzyme (RDE)	340122	Denka Seiken CO, Japan
RPMI (Roosewell Park Memorial Institute) medium	21875-034	GIBCO, UK
Sodium acetate trihydrate (CH_3COONa)	1-06267	Merck, Germany
Sodium Azide	S8032-25G	Sigma, USA
Sodium chloride (NaCl)	1.06404	Merck, Germany
Sodium pyruvate (100 mM)	S8636	Sigma, USA
Sulphuric acid 18.4 M (H_2SO_4)	112080	Merck, Germany
TMB-H Peroxidase substrate	01028101	Moss, inc., USA
Tween 20: Polyoxylene-Sorbitan monolaurate	P-1379	Sigma, USA

2.7 Bio-plex Kits

Mouse Cytokine Grp I X-Plex Assay (Cytokine 7-plex - IL-2, IL-4, IL-5, IL-10, IL17, IFN-γ, TNF-α) Cat. No X6000006RJ		Bio-Rad Laboratories, USA
Bio-plex Reagent Kit	(171-304000)	
Bio-plex Calibration Kit	(171-203060)	

2.8 Plates/equipment

Name

Elisa plates, F-bottom	(655001)	Greiner, Germany
Elispot – Multiscreen®, HA plates	(MAHA N45 50)	Millipore, UK
Cell-culture plates, Nunclon™ surface	(143982)	Nunc Brand Products, DK
Multiplex – Multiscreen®, HTS™, BV	(MSBVN1250)	Millipore, UK
HI – V96 MicroWell™ Plates	(249570)	Nunc Brand Products, DK
Nunc Immuno™ Wash 12 Plate washer		Nunc Brand Products, DK
Microvette® CB 300- System for capillary blood collection	(16.440.100)	Sarstedt, Germany

2.9 Instruments:

Name	**Supplier**
BD FACS Canto ™ Flow Cytometer (No.337175)	BD Biosciences, USA
Bio-Plex ™ 200 System Powered by Luminex XMAP ™ Technology	Bio-Rad Laboratories, USA
CASY® Cell Counter	Schärfe System GmbH, Germany
ELISA plate reader, Multiscan MS	Labsystems, USA
Forma Scientific" bio-freezer Forma 8438 (-80°C)	LabTrader, USA
Heidolph Titramax 100 Vibrating platform shaker	Heidolph Instruments, Germany
Immunoscan ™ Elispot reader	C.T.L Europe GmbH, Germany
Microplate washer, ELx450 HT	BioTek, USA
Knf Lab Laboport vacuum pump	Bio-Rad Laboratories, USA
Centrifuges:	
Heraeus Labofuge 400R – FunctionLine	Thermo Scientific
Eppendorf Centrifuge 5810 R (no. 0036331)	Eppendorf International
Eppendorf Centrifuge 5424, 230V (no. 0006928)	Eppendorf International

2.10 Computer Software

Ascent Software Version 2.6	(ELISA)	Labsystems, USA
Program BioPlex manager 5	(Multiplex)	Bio-Rad, USA
FlowJo	(Intracellular cytokine staining)	TreeStar inc., USA
ImmunoSpot 4.0 Academic and Immunoscan Professional (ELISPOT)		C.T.L, Europe, Germany

2.11 Solutions, buffers, medium

Mouse Lymphocyte medium (MLM), 100 mL

- 86 mL RPMI 1640 medium supplemented L-glutamine
- 1 mL 10 mM nonessential amino acids
- 1 mL 1 M Hepes pH 7.4
- 1 mL 100 mM sodium pyruvate
- 1 mL PSA
- 100 µL $5x^{10-5}$ M 2-ME
- 10 mL heat-inactivated FBS Gold

10x Phosphate Buffered Saline (PBS) 1L

- 85 g NaCl
- 2.50 g KH_2PO_4
- 6.85 g $NaHPO_4$

Add dH_2O to a total volume of 1000 mL. pH should be 7.4 ± 0.2

PBS/NCS (20 %) 250 mL

- 50 mL NCS (newborn calf serum) filtered through a 0.45 µm filter to 200 mL sterile PBS

PBS/FBS (5 %) 250 mL

- 12.5 mL FBS (foetal bovine serum) to 237.5 mL sterile PBS

1x PBS/Tween 0.05% (5 L)

- Dilute 0.5 L sterile 10x PBS in 4.5 L dH_2O
- Add 2.5 mL Tween 20

PBS/BSA (0.05%) 50 mL

- 0.025 g BSA (bovine serum albumin) to 50 mL sterile PBS

Cell count mixture

- 10 mL Casy®Ton mixture
- 20 µl isolated cell suspension

Intracellular cytokine staining

Mitogen medium

- 1 mL negative control medium
- 2 µl PMA (10 ng/mL)
- 5 µl Ionomycin (250 ng/mL)

Influenza medium

- 1 mL (A/Vietnam/1194/2004 (H5N1) NIBRG-14) virosome (2.5µg HA/mL) in MLM
- 2 µl Golgi plug (Brefeldin A)
- 2 µl anti-CD28

Negative control medium

- 1 mL MLM
- 2 µl Golgi plug (Brefeldin A)
- 2 µl anti-CD28

Flow medium (FM)

- 93 mL PBS
- 5 mL FBS
- 2 mL Sodium Azide 5% (w/v) (5 g Sodium Azide in 100 mL milli-Q H_2O)

Perm wash solution (PW)

- 10 mL Perm Wash
- 90 mL ddH_2O

In vitro activation of lymphocytes

Mitogen medium

- 1 mL negative control medium
- 2 µl PMA (10 ng/mL)
- 5 µl Ionomycin (250 ng/mL)

Influenza medium

- 1 mL (A/Vietnam/1194/2004 (H5N1) NIBRG-14) virosome (2.5µg HA/mL) in MLM

Negative control medium

- 1 mL MLM

ELISPOT solutions

2X mitogen mix

- 4.8 mL MLM
- 100 µl Pansorbin, washed and diluted 1:100 in MLM
- 100 µl Pokeweed mitogen, diluted 1:100 in MLM

ELISA solutions

Di-sodium hydrogen phosphate 0.2 M (1L)

- 28.30 g di-sodium hydrogen phosphate (Na_2HPO_2) 0.2 M

  Add ddH_2O to 1000 mL

Citric acid 0.1 M (1L)

- 21.01 g citric acid monohydrate ($C_6H_8O_7H_2O$). Add ddH_2O to 1000 mL

Phosphate citrate buffer (pH 5.0 – 1L)

- 257 mL 0.2 M Na_2HPO_2
- 243 mL 0.1 M $C_6H_8O_7 \cdot H_2O$. Add ddH_2O to 1000 mL

OPD (Ortho-phenylediamine dihydrochloride) solution

- Solve 10 mg OPD in 25 mL phosphate citrate buffer
- Add 20 µl H2O2 immediately before adding to plate. Keep dark

1M H_2SO_4 (1L)

- 54.40 mL 18.4M sulphuric acid
- 949.50 mL ddH_2O

2.12 Disposable consumables

Acetate foil for microtest well plates (82.1586)	Sarstedt, Germany
Centrifuge tube 15 mL	VWR International, USA
Elisa dilution tubes 1.3 mL (102270)	Greiner Bio-One, Germany
Falcon tubes 50 mL	BD Labware, USA
Microtubes 0.5 mL	Sarstedt, Germany
Microtubes 1.5 mL	Axygen Biosciences, USA
Needles, 23 G, 21 G	Braun, Germany
Paper towels for Elisa and Elispot	VWR International, Norway
Petri dishes (90 mm) 391-0875	VWR International, Norway
Pipettes	Thermo Labsystems
Scalpels No 22	VWR International, Norway
Syringe (vaccination) Micro-Fine ™ 0.3 mL (320830)	BD Biosciences, USA
Syringe 1 mL BD Plastipak 300013	BD Medical, Spain
Syringe 2 mL BD Plastipak 300186	BD Medical, Spain
Syringe 50 mL BD Plastipak 300866	BD Medical, UK
Syringe Filter 0.45 µm	Millipore, DK
Syringe Filter 0.20 µm	Whatman, UK
Thermo Labsystems Finnpipette Novus, multichannel	Termo Scientific, USA

3 Methods

3.1 Experimental protocol

3.1.1 Mice

Forty-two BALB/c female mice (6-8 weeks old) were acclimatized in their cages for seven days and were housed at 21°C and with 12-hour light/dark cycles. Mice were divided into groups of six mice according to a numerical system. Each group was vaccinated with the NIBRG-14 virosomal vaccine (2μg HA) either intramuscularly (IM), intranasally (IN) or sublingually (SL) with or without adjuvant (c-di-GMP (7.5 μg)) (table 3.1). One group was given only adjuvant as a control and PBS IN. Immunisation, sampling and euthanasia were carried out at shown in figure 3.1. All handling of the mice was carried out according to The Norwegian Regulation on Animal Experimentation ("Forsøksdyrforskriften"), and approved by the national Animal Research Committee (approval No. 2010-2742).

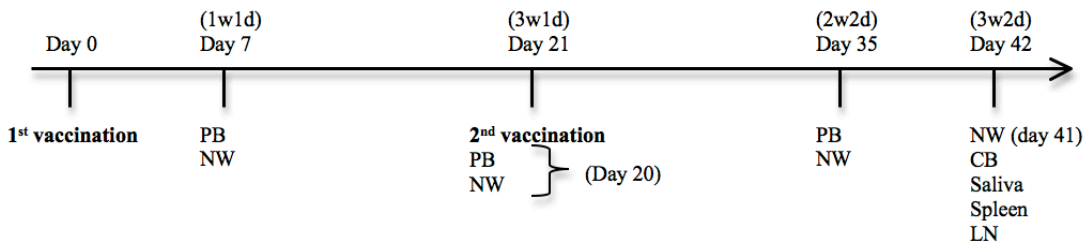


Figure 3.1: Vaccination and sampling timeline

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant three weeks apart. C-di-GMP in PBS was administered IN to controls (C). Peripheral blood (PB) and nasal wash (NW) were collected at days 7, 20 and 35 post first immunisation. NW was also collected at day 41 (the day before the sacrifice). Saliva, cardiac blood (CB) and spleens were collected on the sacrifice day.

Table 3.1: Overview of vaccination groups

A total of 42 BALB/c mice were vaccinated, divided into groups of six. One group received only c-di-GMP as controls. (+) indicates with adjuvant, (-) indicates no adjuvant.

Administration route	c-di-GMP	
Intramuscular (IM)	+ (6 mice)	- (6 mice)
Intranasal (IN)	+ (6 mice)	- (6 mice)
Sublingual (SL)	+ (6 mice)	- (6 mice)
Control (adjuvant only) (C)	+ (6 mice)	

3.1.2 Virosomal vaccine

The vaccine strain used in this study was influenza H5 NIBRG-14, derived from A/Vietnam/1194/2004 (H5N1). The strain was produced using reverse genetics, and the virus was grown in the allantoic cavity of embroynated hen's eggs. The vaccine was formulated as virosomes at Crucell where the virus was inactivated using beta-propiolactone and a detergent (octaethyleneglycol monododecylether) was used to solubilise the HA, NA and phospholipids of the virus. Next the surface antigens NA and HA were purified and mixed with the phospholipid component lecithin. The virosomes were then generated by the stepwise removal of detergent followed by a spontaneous incorporation of HA and NA into the phospholipid bilayer.

3.1.3 Adjuvant

The adjuvant used was bis (3',5')-cyclic dimeric GMP (c-di-GMP) provided by Helmholtz Centre for Infection Research, Germany. This is a chemical compound synthesized according to established protocols [129, 130], and purified by high-performance liquid chromatography as described by Ebensen et al. [126].

3.1.4 Vaccination

Intranasal vaccination

Both SL and IN vaccinations were carried out on anesthetised mice. The anaesthesia used was a 160 µl mixture of xylazine (Rompun Vet®) 1 mg/mL and ketamine (Ketalar®) 10 mg/mL in sterile PBS given subcutaneously. After approximately 10-15 minutes the mice were checked for level of narcosis. For IN administration the mice were placed in supine position, and 3.5 µl vaccine was administered into each nostril using a thin micropipette, giving a total volume of 7 µl vaccine (2 µg HA of virosomal vaccine with or without 7.5 µg of c-di-GMP adjuvant). After vaccine administration, the mice were kept in the same position for at least 5 minutes to make sure the vaccine was absorbed (figure 3.2).

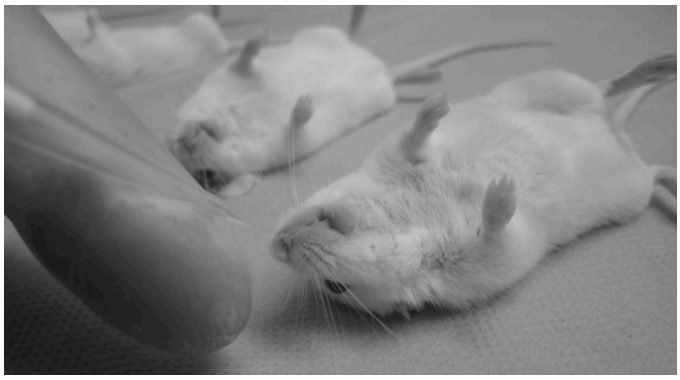


Figure 3.2: Intranasal vaccination

When anesthetised, the mice were placed in supine position and 3,5 µl of vaccine (2 µg HA of virosomal vaccine with or without adjuvant) was applied into each nostril. The mice were kept in supine position for at least 5 minutes after application of the vaccine, to allow vaccine absorbance.

Sublingual vaccination

For SL administration the mice were held in a head-up vertical position as illustrated (figure 3.3.), and a micropipette was used to apply 7 µl vaccine (2 µg HA of virosomal vaccine with or without 7.5 µg of c-di-GMP adjuvant) under the tongue. After application the mice were placed in anteflexion (sitting with their head bend over their lower extremities) for at least 20 minutes after vaccination to prevent the mice from swallowing the vaccine.

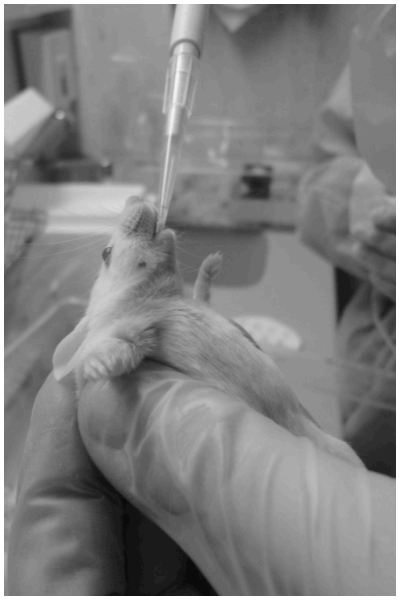


Figure 3.3: Sublingual vaccination

When anesthetised, the mice were held in a head-up vertical position as illustrated, and 7 µl vaccine (2 µg HA of virosomal vaccine with or without 7.5 µg of c-di-GMP adjuvant) were placed under the tongue of the mice.

Intramuscular vaccination

For IM vaccination the mice were restrained in 50 mL plastic tubes, which had been punctured in advance. The hind leg was exposed and 50 µl of vaccine (2 µg HA of virosomal vaccine with or without 7.5 µg of c-di-GMP adjuvant) was injected intramuscularly into the quadriceps muscle.

3.1.5 Collection of Venous Blood

The saphenous vein on the hind leg was used to obtain blood samples (figure 3.4). The mice were placed in a plastic tube and the hind leg was exposed. The fur over the vein was removed using a scalpel before the vein was punctured with a needle. A microvette capillary was used to collect 50-100 µl of blood from each mouse on each blood-sampling day.

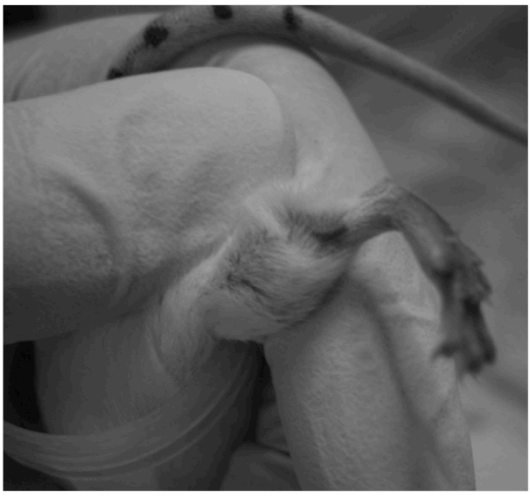


Figure 3.4: The saphenous vein used for blood sampling

The hind leg was first gently shaved to expose the vein. A needle was used to puncture the vein and the blood collected in a microvette capillary. The picture was kindly provided by Monica Trondsen.

3.1.6 Collection of Nasal Wash

Mice were restrained by holding them in the scruff upside-down over a petri dish (figure 3.5). To collect samples from the nasal cavity, 350 µl sterile PBS/BSA 0.05% was flushed from the mouth through the nostrils of the mice using a 1 mL syringe and a feeding tube. Two flushings were performed before the samples were collected in a microtube and put on ice. The samples were frozen at -80°C until used in the ELISA assay (3.2.2).

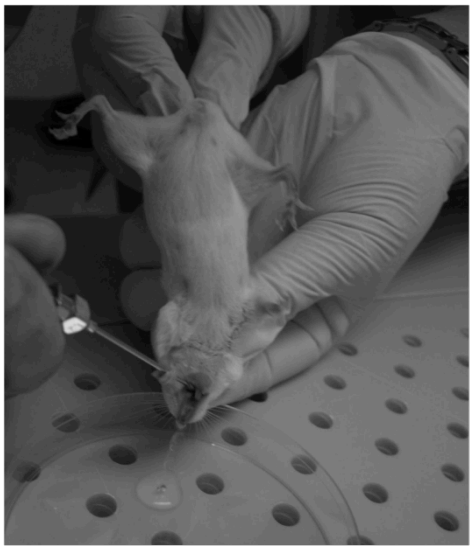


Figure 3.5: Collection of nasal wash

The mice were held steadily upside-down over a petri dish, the nasal cavity flushed with 350 µl PBS/BSA (0.05%) twice. The picture was kindly provided by Monica Trondsen.

3.1.7 Collection of Saliva

On the sacrifice day, mice were anesthetized with 120 µl mixture of xylazine (Rompun Vet®) 1 mg/mL and ketamine (Ketalar®) 10 mg/mL in sterile PBS given subcutaneously. After anaesthesia had occurred, 10 µg pilocarpine in 80 µl PBS was given intraperitoneally and each mouse was placed in a petri dish. After approximately 5 minutes the mice started secreting saliva, and up to 50 µl saliva from each mouse was collected with a micropipette. The samples were frozen at -80°C until used in the HI (3.2.4) and ELISA (3.2.2) assays.

3.1.8 Collection of Cardiac Blood and Tissue

Mice were euthanized using CO_2, and fastened to a dissection plate with pins. Cardiac puncture was performed directly after euthanasia, and the cardiac blood was collected in an Eppendorf tube using a 23 G needle and a 2 mL syringe. Next, the spleen and cervical lymph nodes were aseptically dissected out and transferred to tubes containing sterile PBS/FBS.

3.1.9 Separation of sera

The blood samples were left in the fridge for 2-3 hours to clot. The clotted blood was centrifuged at 845 g for 10 minutes. Then sera were isolated, transferred to microtubes and frozen at -80°C until used in the HI (3.2.4) and ELISA (3.2.2) assays.

3.2 Immunological assays

3.2.1 Isolation of lymphocytes

Lymphocytes were isolated from the spleen. All work with lymphocytes was carried out in a laminar airflow cabinet using aseptic techniques.

Spleen

The spleen was placed in a petri dish and dichotomised using two bent 21 g needles. Splenocytes were washed out of the spleen and flushed with PBS/FBS until a volume of 6 mL cell suspension was obtained.

Lymphoprep

Lymphocytes were separated on three mL of lymphoprep in a 15 mL centrifuge tube, layering 6 mL of cell suspension carefully on top of the lymphoprep. The tube was centrifuged at 800 g for 30 minutes at room temperature with the centrifuge brake off. After centrifugation the distinct band of mononuclear cells was transferred into a new 15 mL centrifuge tube. The lymphocytes were washed (290 g for 10 minutes at 4°C) twice in PBS/FBS (5%). The splenocytes were re-suspended in 2 mL lymphocyte medium, respectively. The cells were counted using a CASY® cell counter. Cell suspensions were adjusted to $1x10^7$ cells/mL by adding lymphocyte medium.

3.2.2 Antibody ELISA

ELISA (Enzyme linked immunosorbent assay) is an immunological method used for detection and quantification of antibodies. The method is based on antigen-antibody interactions and a secondary biotinylated antibody that can bind an enzyme-linked avidin with specificity for biotin. A colorimetric substrate allows measurement of concentration of influenza-specific antibodies, using spectrophotometric equipment.

ELISA-plates were coated with 100 µl/well of 2 µg/mL of influenza H5 NIBRG-14 virosomal antigen or 1/1000 capture antibody (IgG, IgG1, IgG2a or IgA) diluted in sterile PBS. The plates were incubated at 4°C overnight. The next morning, the coating solution was removed and plates were blocked with 200 µl/well with PBS/NCS (20 %) for 1 hour at room temperature. Sera and antibody standards were diluted (5-fold and 2-fold dilutions starting at 50 ng/mL, respectively) in PBS/NCS (20%). Wells were emptied of blocking solution (flicked out in the sink) and 100 µl/well of serum and standard dilutions were added in duplicate. The plates were incubated for 1.5 hours at room temperature. After incubation, the plates were washed 6 times in PBS/Tween (PBS/T) 0.05% using the Microplate washer. Next, 100 µl/well of goat anti-mouse (IgG, IgG1, IgG2a, IgA or pIgR) specific-biotinylated antibody diluted 1/500 (1/50 for pIgR) in PBS/NCS (20%) was added and the plates were incubated for 1 hour at room temperature. After incubation, the plates were washed 6 times as described above. Then, 100 µl/well of Extravidin PO diluted 1/1000 in PBS/NCS (20%) was added and the plates were incubated for 1 hour at room temperature. Again, the plates were washed 6 times as previously described. The OPD substrate was prepared by adding 1 tablet (10 mg) of OPD to 25 mL 0.05M phosphate citrate buffer pH 5, protected from light. Immediately before adding the OPD-substrate to the plate, 20 µl H_2O_2 were added to the solution. One hundred µl/well of OPD-substrate was then added to the plate. After 10 minutes incubation in the dark, the reaction was stopped with 100 µl/well of 1M H_2SO_4. Subsequently, the absorbance (OD) at 492 nm was read using the ELISA plate reader and the Ascent software. The background (OD-values in blank wells) was subtracted from all values and standard curves were prepared as log-log graphs in the Ascent program using linear regression ($R>0.99$). These standard curves were used to calculate the

influenza-specific antibody concentration (in ng/mL and μg/mL) in serum, nasal wash and saliva by interpolation. For pIgR, no standard was available and the results are only given in OD-values (absorbance at 492 nm).

3.2.3 Memory B-cell ELISPOT

Memory ELISPOT is used to detect and quantify antibody-secreting memory B-cells in splenocytes and lymph node cells after polyclonal stimulation with mitogen. The protocol was adapted from [131]. Total IgG specific and influenza antigen-specific memory cells were determined.

Splenocytes were isolated as described in section 3.2.1. The lymphocytes were diluted to 1 x 10^6 cells/mL in mouse lymphocyte medium (MLM) and 0.5 mL cell suspension was added to each well of a 24-well Nunclon™ Surface plate. Each sample was added to four wells (two for mitogen stimulation and two for non-stimulated controls) and the plates were incubated at 37°C with 5% CO_2 whilst preparing the mitogen mixture. A 2x mitogen mix was made up in MLM, 0.5 mL was added to two of the four wells per sample, the remaining two wells were non-stimulated control wells and 0.5 mL MLM was added. The plates were wrapped in plastic to limit evaporation and incubated for 6 days at 37°C with 5% CO_2. On day 5, 96-wells ELISPOT plates were coated with 100 μl/well of 2 μg/mL influenza antigen (RG-14 virosomes) and 1:500 of anti-mouse IgG, diluted in sterile PBS. One hundred μl/well of sterile PBS was added to one row to serve as a negative control. The plates were incubated overnight at 4°C. The next day, the coating-solution was flicked out, and the plates were blocked with 150 μl/well of MLM for at least 2 hours at 37°C with 5% CO_2. Both stimulated and non-stimulated cells were re-suspended and collected in 15 mL tubes and centrifuged for 10 minutes at 300g at 4°C. Each pellet was re-suspended in 10 mL lymphocyte wash medium (LWM) and again centrifuged for 10 minutes at 300g at 4°C. The supernatant was removed and each pellet was re-suspended in 2 mL MLM and centrifuged for 10 minutes at 300g at 4°C. The supernatant was removed and each pellet was re-suspended in 4 mL MLM (final concentration of 25000 cells/100μl). The blocking buffer was flicked out of the ELISPOT plate and 100 μl/well of MLM was added to each well. Subsequently, re-suspended cells were

added (100 µl/well) to the top row of the plate, and 2-fold diluted down the plate. The final row was left blank (no cells) as a negative control. Splenocytes were added to the plates coated with RG-14/anti-mouse IgG. The plates were incubated overnight (16-20 hours) at 37°C and 5% CO_2. The next day, the plates were washed once with PBS (to avoid cell lysis) and six times with PBS/T (0.05%). The first four washes were quick to avoid cross-contamination between wells. For the last two washes the plates were soaked for 2-5 minutes and a vacuum was used to suck PBS/T through the plates. After washing, 100 µl/well of biotIN-conjugated goat anti-mouse IgG diluted 1:1000 in PBS/T and filtered through a 0.2 µm syringe filter were added to the plates. Plates were then incubated for 2 hours at room temperature. Subsequently, 100 µl of filtered extravidIN-PO in PBS/T (1:1000) was added to each well and incubated for 1 hour at room temperature. The plates were washed again as previously described. The reaction was developed with 100 µl/well of TMB-H for approximately 5 minutes, and the plates were washed thoroughly under running tap water to stop the reaction. Plates were left to dry in the dark for 2 days. The membranes were punched out on sealing tape, the wells scanned and spots were counted using the ELISPOT Immunospot reader. Any spots in negative control wells were subtracted from corresponding antigen-stimulated wells.

3.2.4 Haemagglutination Inhibition Assay

Treatment of sera

Receptor-destroying enzyme (RDE) solution was reconstituted in 20 mL sterile according to the manufacturers' instructions. Sera and saliva were diluted 1:5 and 1:1 in RDE and incubated at 37°C overnight to remove nonspecific inhibitors of haemagglutination. The remaining RDE was heat inactivated by further incubation at 56°C for 30 minutes and sera were cooled to room temperature before use.

Preparation of turkey red blood cells (RBC)

Turkey red blood cells were washed repeatedly (167 g for 10 minutes at 4°C) until the supernatant was clear (no haemolysed RBC). A 0.7 % v/v RBC suspension was prepared by adding cold PBS.

Virus titration (Haemagglutination assay)

An initial virus titration was performed to determine the appropriate dilution of virus. For the haemagglutination assay 50 µl PBS was added to each well of a V-well bottom microtitre plate. Then, 50 µl virus suspension was added to the first row, and 2-fold diluted across the plate with the final 50 µl being discarded. Then 50 µl of 0.7 % turkey RBC was added to each well and the plate was incubated for 30 minutes at room temperature. The titre was read as the reciprocal of virus dilution that gave 50 % agglutination. The virus titre was adjusted to be 8 HA units (HAU)/50 µl.

Haemagglutination inhibition test

50 µl PBS was added to each well on a V-well bottom microtitre plate. Then 50 µl of RDE-treated sera/saliva was added to the first row, and 2-fold diluted across the plate, the final 50 µl being discarded. Then 50 µl of standardized virus (8 HAU/50 µl) was added to each well and the plate incubated at room temperature for 1 hour. Thereafter 50 µl 0.7 % turkey RBC was added to each well and the plate was incubated for 30 minutes before readout. The haemagglutination inhibition titre (HIT) was read as the reciprocal of the serum dilution that gave 50 % agglutination.

3.2.5 *In vitro* activation of lymphocytes

The cytokine response from isolated splenocytes can be detected after *in vitro* stimulating cells with influenza antigen. Cytokines accumulate in the supernatant and the concentration can be measured by Illuminex assay.

Influenza A/Vietnam/1194/2004 (H5N1) virosomal antigen diluted in lymphocyte medium to a final concentration of 2.5 µg HA/mL, mitogen medium (PMA and ionomycin) and negative control medium (medium alone) were prepared and 100 µl/well added to a 96-wells flat-bottom tissue culture plate. Next, 100 µl/well of cell suspension containing 1.0×10^6 lymphocytes, was added to the plate. The plate was incubated for 72 hours at 37°C with 5% CO_2. After incubation, 200 µl from each well was transferred to a 96-well V-shaped bottom plate and centrifuged for 10 minutes at 300 g. Supernatants were then transferred to a new plate and frozen at -80°C until tested in the multiplex bead assay (3.2.6).

3.2.6 Multiplex bead assay

The multiplex assay was used to determine the concentration of IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ and TNF-α from supernatants of *in vitro* stimulated splenocytes according to the manufacturers' protocol (Bio-Rad, USA). Cytokine standards were diluted 4-fold and put on ice until used. 575 µl of 10x coupled beads stock was diluted in 5.175 mL Assay Buffer, gently vortexed and 50 µl were added to each well. Next, the plate was washed with wash buffer (100 µl/well) twice by suction. After the wash, 50 µl of each standard and 50 µl of each sample were added to the appropriate wells. Two wells served as blanks containing cell medium alone. The plate was then sealed with a plastic adhesive film and left to incubate in the dark for 30 minutes on a platform shaker (300 rpm). Subsequently, 300 µl detection antibody was diluted in 2700 µl detection antibody diluent. The plate was washed three times by suction and 25 µl diluted detection antibody was added to each well. The plate was incubated as described above for 30 minutes. Again the plate was washed three times by suction. Sixty µl StreptavidIN-PE was diluted in 5 940 µl Assay Buffer A, and 50 µl were added to each well. The plate was incubated as described above for 10 minutes and washed three times by suction. Subsequently, 125 µl of Assay Buffer was added to each well, the plate was sealed and the beads re-suspended by shaking at 1100 rpm for 30 seconds. Thereafter the plate was read on the luminex instrument and a standard curve was created using Bioplex Manager 5.

3.2.7 Intracellular cytokine staining

Lymphocytes produce cytokines upon antigen presentation by an APC. By blocking the Golgi apparatus of the lymphocytes, antigen-specific cytokines accumulate inside the cells and can be measured by intracellular cytokine staining using flow cytometry.

Splenocytes were isolated (3.2.1), and the cell concentration was adjusted to 1.0×10^7/mL. One hundred µl of the appropriate medium (influenza medium (RG-14 virosomal antigen, diluted to a concentration of 2.5 µg HA/mL in MLM), mitogen medium or negative control medium) and 100 µl of cell suspension was added into each well of a 96-wells-flat bottom tissue culture plate and incubated for 17 hours at 37°C with 5% CO_2.

Fixation and staining

The cells were transferred to a 96-wells V-shape well plate and centrifuged at 240 g for 5 minutes at 4°C. After centrifugation, the cells were washed twice with 200 µl Flow Medium (FM) and centrifuged as described above. The supernatants were removed and the cells re-suspended well. Next, 100 µl of 10 µg/mL BD FCblock™ in FM was added and the plate incubated for 15 minutes at 4°C. Then, 100 µl FM was added and the plate centrifuged as previous described. The supernatant was removed and the cells re-suspended well. Subsequently, 100 µl of Fix/Perm solution was added and the plate was incubated for 20 minutes at 4°C. The cells were washed twice by re-suspending the cells in 200 µl Perm Wash (PW) and centrifuged at 240 g for 5 minutes at 4°C. The compensation beads were prepared (described below) in parallel with the centrifugation. The supernatant was removed and the cells thoroughly re-suspended. 50 µl/well fluorophore-conjugated rat-anti mouse antibodies (diluted 1/50 in PW) were added and the plate was incubated for 30 minutes at 4°C. After the incubation, 150 µl PW was added and the plate was centrifuged at 380 g for 5 minutes. Subsequently, the plate was washed twice with PW and centrifuged at 380 g for 5 minutes. The cells were re-suspended in 250 µl FM and analysed on the flow cytometer after compensating.

Compensations

The compensation beads were prepared by adding 3 drops of anti-rat + 3 drops of control beads to 1 mL of PW, then centrifuging at 1500 g for 2 minutes and re-suspended in 350 µl PW. 50 µl beads were added to 6 different tubes (one for each fluorophore). Next, 1 µl of fluorophore-conjugated antibodies was added to each of the different compensation tubes and incubated for 30 minutes at 4°C (simultaneous to the cell incubation). After the incubation, 950 µl PW were added to each compensation tube. Then the compensation tubes were centrifuged at 1500 g for 2 minutes and re-suspended in 250 µl FM before running the compensation tubes in flow cytometer. The samples and compensation tubes were stored protected from light for a maximum of 72 hours before flow cytometric analysis.

3.3 Statistical analysis

To assess if the different groups had statistically significant differences, analysis were performed in Prism 5.0d for Mac OS X (GraphPad Software) using one-way ANOVA with Bonferroni adjustment for multiple-group comparison. P-values ≤ 0.05 were considered significant. For the HI-assay ± 95 % confidence interval was determined using Prism.

4 Results

In this study we have analysed and compared the humoral and cellular immune responses in BALB/c mice to a virosomal influenza A/Vietnam/1194/2004 (H5N1) virus vaccine, administered sublingually (SL), intranasally (IN) and intramuscularly (IM) with (+) or without (-) the mucosal adjuvant c-di-GMP (see table 3.1). Sera, nasal wash (NW), saliva and spleens from the mice were used in different immunological assays to analyse the immune response after vaccination. The results are divided into humoral immune response and cellular immune responses, despite these arms of the immune system being closely related.

4.1 Humoral Immune response

Antibodies are the effectors of humoral immunity. The different antibody classes have different effector functions and can be found at distinct anatomic sites. We have focused on IgG, the class dominating the secondary immune response (see figure 1.3), and on IgA, which is important in mucosal immunity. The enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of IgG, IgG1, IgG2a and IgA in the serum, IgA and IgG in the saliva and IgA in the NW. In addition, the local mucosal antibody response was evaluated by measuring the pIgR in the saliva and the NW. The functional antibody activity was assayed by determining the titre of the haemagglutination inhibition (HI) antibodies in the serum and the saliva. To enumerate the influenza-specific memory B cells in splenocytes the ELISPOT assay was used after *in vitro* polyclonal stimulation. In the following sections, the HI, ELISA and memory B-cell responses are presented.

4.1.1 Haemagglutination inhibition (HI) assay

To compare the different routes of administration and to assess the efficacy of the mucosal adjuvant c-di-GMP, the HI antibody titre in the sera and the saliva were measured at day 42 (euthanasia time-point). Sera were analyzed for antibodies against the homologous vaccine strain (A/Vietnam/1194/2004 (H5N1) NIBRG-14) (RG-14). HI titres ≥ 40 have been considered a surrogate correlate of protection for seasonal

influenza strains in man. Thus HI titres ≥ 40 were considered as protective titres, even though this assay has limitations when assessing pandemic vaccines in immunised mice.

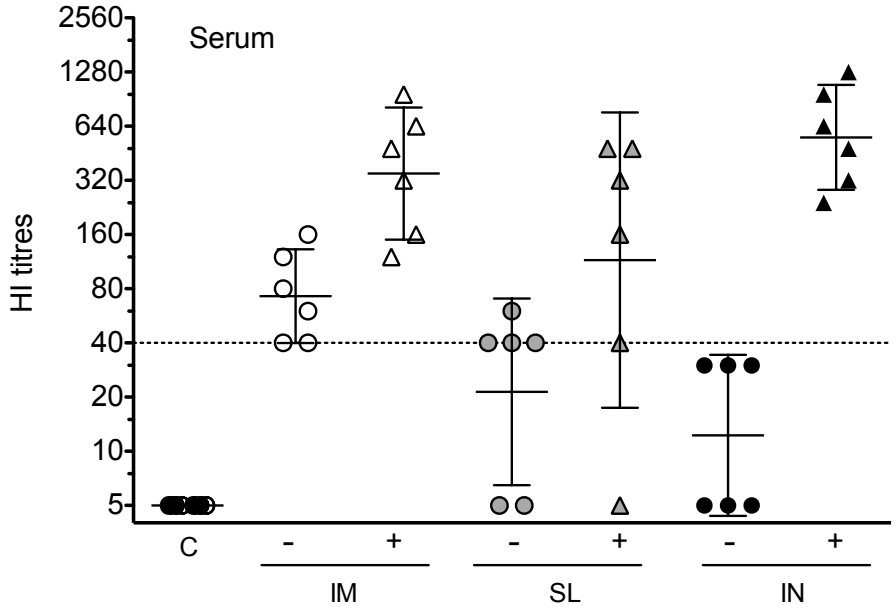


Figure 4.1: Haemagglutination inhibition titres in serum 3 weeks after 2nd dose

BALB/c mice were vaccinated intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Sera were isolated from blood collected three weeks after the second vaccine dose, and assayed for haemagglutination inhibition titres against the vaccine strain (A/Vietnam/1194/2004 (H5N1) NIBRG-14) (RG-14). Error bars indicate geometric mean ± 95 % confidence interval. The dotted line represents the "protective HI titre" of 40.

Sera isolated from cardiac blood were tested for HI-antibodies against the RG-14 strain. The c-di-GMP adjuvanted virosomal H5N1 vaccine induced HI-titres ≥ 40 against the vaccine strain (RG-14) by all administration routes (except from one non-responder in the SL+ group) (figure 4.1). The IN+ group showed the highest geometric mean titre (GMT = 550) and all mice in this group had titres ≥ 320. The groups that were vaccinated with virosomes alone had generally lower GMTs than their respective adjuvanted groups but in the IM- group, all mice had protective HI-titres ≥ 40. Control mice had no detectable HI titres. Comparing the different routes of administration, it was found that the IN+ group had the highest HI titres (GMT = 550), followed by the IM+ (GMT = 350) and the SL+ (GMT = 115) groups.

Saliva samples from all mice in each group were pooled together and tested for HI antibodies against the vaccine strain (RG-14) (figure 4.2). None of the groups had HI GMT over the threshold correlated with protection (HI titres ≥40). The IN+ group had the highest HI GMT (GMT = 13.2) followed by the SL+ group (GMT = 9.2) and the IM+ group (GMT = 4.7). Among the non-adjuvanted groups, only the IM- group had a response (GMT = 3.2). No titres were seen in the non-adjuvanted mucosal groups and the control group.

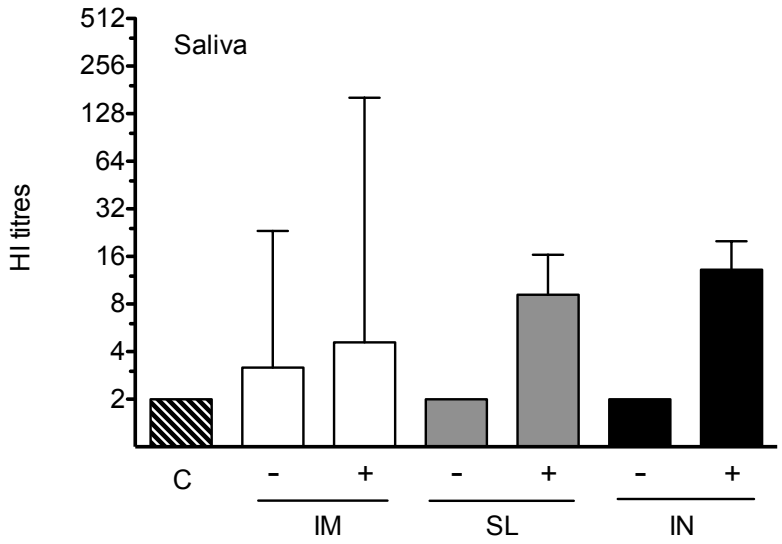


Figure 4.2: Haemagglutination inhibition titres in saliva detected three weeks after the second dose

BALB/c mice were vaccinated intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Saliva samples were collected after i.p. injection with pilocarpine on the day of sacrifice, and the samples from all mice in each group were pooled to provide enough sample volume. The salivary HI assay was conducted three times and bars indicate geometric mean +95 % confidence interval.

4.1.2 ELISA

The enzyme linked immunosorbent assay (ELISA) was used to determine the antibody concentrations in the serum and the nasal washes from all sampling days and the saliva from the day of euthanasia (see figure 3.1 for a detailed overview of sampling days).

4.1.2.1 The mucosally administered vaccines induce a local antibody response

A local immune response in the mucosa of the upper respiratory tract can prevent influenza infection. SIgA is the most important antibody class in the mucosal immunity, and have shown to elicit neutralising activity towards influenza [132]. SIgA has also been shown to be more cross reactive (i.e. cross clade activity) compared to other antibody classes [88]. Therefore, influenza-specific IgA antibodies were quantified in the nasal wash (NW) and the saliva samples.

Saliva was collected from the mice three weeks after the second dose of vaccine (day 42) and the concentrations of influenza (RG-14) specific IgG and IgA antibodies were determined (figure 4.3). IgG antibodies were detected in all administration groups except the control group, and the IN+ group had the highest IgG concentration (mean = 260 ng/mL). However, it was not statistically significantly higher than that found in the other adjuvanted groups. Only the IN+ group showed significantly higher ($p \leq 0.05$) levels of IgG compared to its respective non-adjuvanted group. IgA was found in much higher concentrations than IgG in the SL and IN groups (mean = 8300 ng/mL for the SL+ group and 11700 ng/mL for the IN+ group), whilst in the IM groups IgA was only detected at very low levels (mean = 8 ng/mL for the IM+ group). Both the IN+ and SL+ groups had significantly ($p \leq 0.05$) higher levels of IgA than all the non-adjuvanted groups, but no significant differences in IgA concentration were found between the adjuvanted mucosal groups.

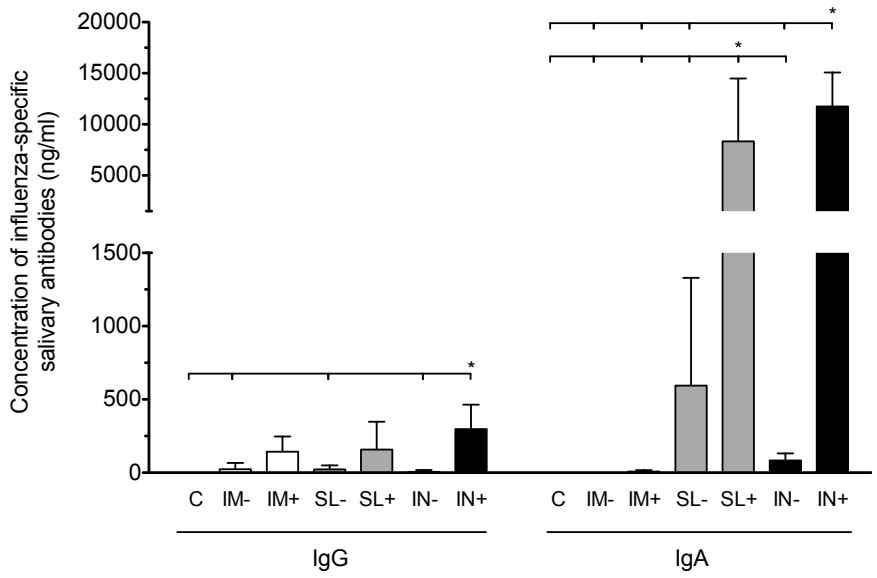


Figure 4.3: Concentration of IgG and IgA in saliva samples three weeks after second vaccine dose

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Saliva was collected from the mice after i.p. injection of pilocarpine, and analysed for IgG and IgA to investigate the local immune response. Each column represents the mean IgA concentration from six mice; error bars show the standard deviation. (*) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, $p \leq 0.01$).

Nasal wash (NW) samples were collected by flushing 350 µl PBS/BSA (0.05%) through the mouth and nose of the mice (figure 3.5) and the samples were analysed for influenza-specific IgA antibodies (figure 4.4). After the first dose, IgA was only detected in the mucosally vaccinated groups and three weeks after the first dose there was no significant difference between SL+ and IN+. After the second dose, a higher immune response was elicited and both SL and IN administration induced high concentrations of IgA in the nasal cavity (up to 1200 ng/mL for the SL+ group and 2800 ng/mL for the IN+ group). No IgA antibodies were detected in NW samples from control mice at any time point. The IN+ group had significantly higher ($p \leq 0.0$) IgA concentrations than all other groups after the second dose. No IgA was detected in mice only receiving c-di-GMP. After the second dose, the intramuscular also group had low concentrations of IgA antibodies in the nasal cavity but surprisingly, higher concentrations were found in the non-adjuvanted than the adjuvanted group.

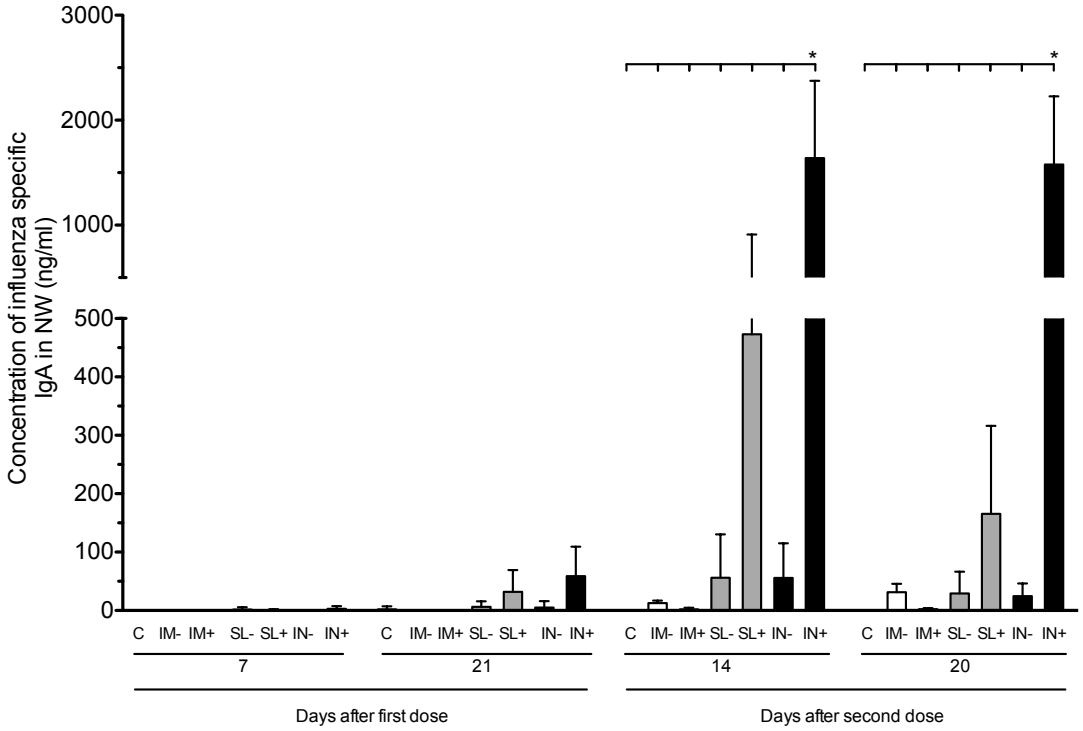


Figure 4.4: Concentration of influenza specific IgA in the nasal wash samples after vaccination

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Nasal washes from all sampling days were analysed for influenza-specific IgA by the ELISA. Each column represents the mean IgA concentration from six mice; error bars show the standard deviation. (*) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.001$).

pIgR is also called the secretory component, and can reflect the amount of locally secreted IgA (SIgA). The pIgR was detected using an ELISA in the saliva and NW samples (figure 4.5 and 4.6). Since the IgA response in NW was higher after the second dose, only samples from two and three weeks after the second dose was tested for pIgR. The OD-values of pIgR were generally lower than the total IgA, but there is a clear association between the OD-values detected for IgA and pIgR both in NW and saliva samples, when we consider each mouse. The saliva samples had the highest amount of both total IgA and pIgR as compared to NW.

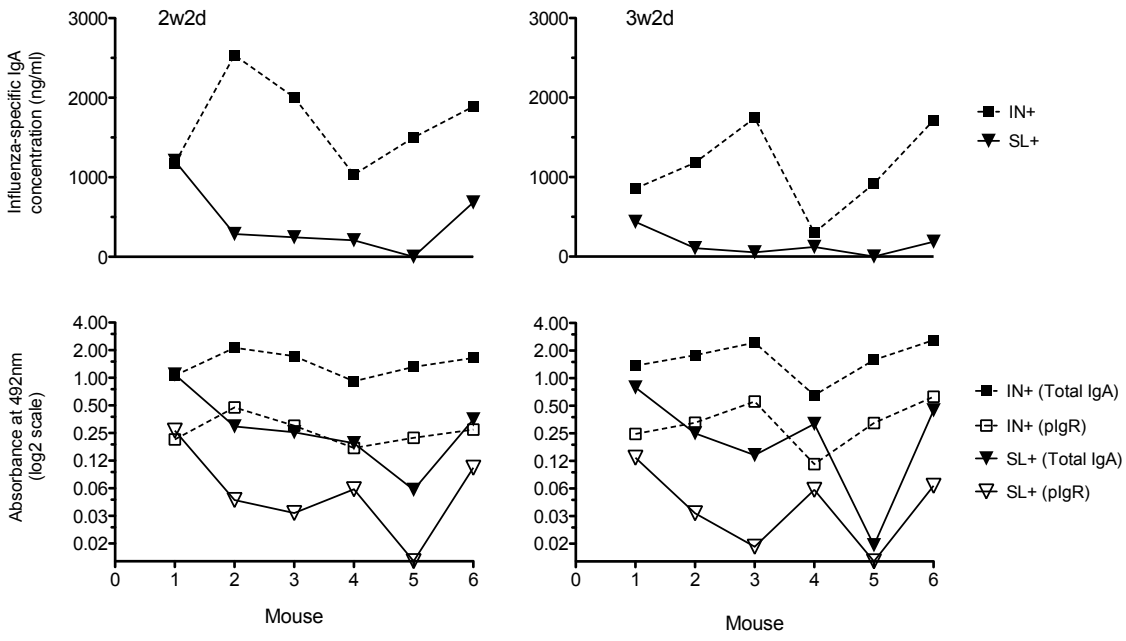


Figure 4.5: Influenza-specific IgA and pIgR in nasal wash samples

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Nasal washes from mice sampled two weeks (2w2d) and three weeks (3w2d) after second dose were analysed for influenza-specific IgA and pIgR using ELISA. Total influenza-specific IgA concentrations and pIgR detected in NW collected from mice in the IN+ and SL+ group, two and three weeks after the second dose. Samples were diluted 1/50, and absorbance read at 492 nm.

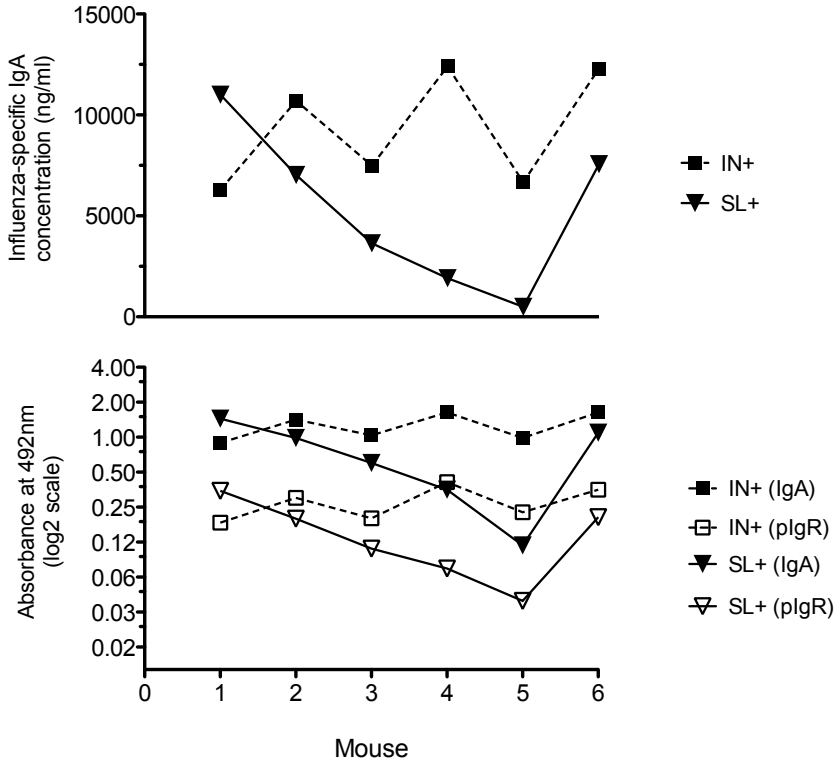


Figure 4.6: Influenza-specific IgA and pIgR in saliva samples

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Total influenza-specific IgA concentrations and pIgR detected in saliva collected from mice in the IN+ and SL+ group, three weeks after the second dose. Samples were diluted 1/500, and absorbance read at 492 nm. Each data point represents each individual mouse in the IN+ and SL+ groups.

Overall, both SL and IN administration elicited high IgA concentrations at the portal of entry for influenza virus, and large amounts of SIgA were secreted in saliva.

4.1.2.2 High concentrations of influenza-specific antibodies were detected in the serum

To measure the systemic humoral immune response, the concentration of IgA and IgG were measured in the serum isolated from peripheral and cardiac blood.

The concentrations of serum IgA were measured in samples taken at all time points (figure 4.7). The highest concentrations of IgA three weeks after the first dose (3w1d) were detected in the SL+ and IN+ groups. After the second dose, the IgA concentrations increased in all groups and the highest concentration was measured two weeks after second dose (2w2d) (mean = 30 μg/mL in the IN+ group). Three weeks after second dose (3w2d) the concentrations had decreased in all the vaccine groups. The IgA concentration in the IN + group were significantly higher ($p \leq 0.05$) than in the IN- group throughout the study. Likewise, a significantly higher ($p \leq 0.05$) IgA concentration was found in the SL+ as compared to the SL- group two weeks after the second dose. When comparing the mucosal administration routes, no significant differences were found, but both the IN+ and SL+ groups had significantly higher IgA concentrations in the serum that the IM+ group at two weeks after second dose ($p \leq 0.001$). Control mice had no detected IgA in the serum at any time point.

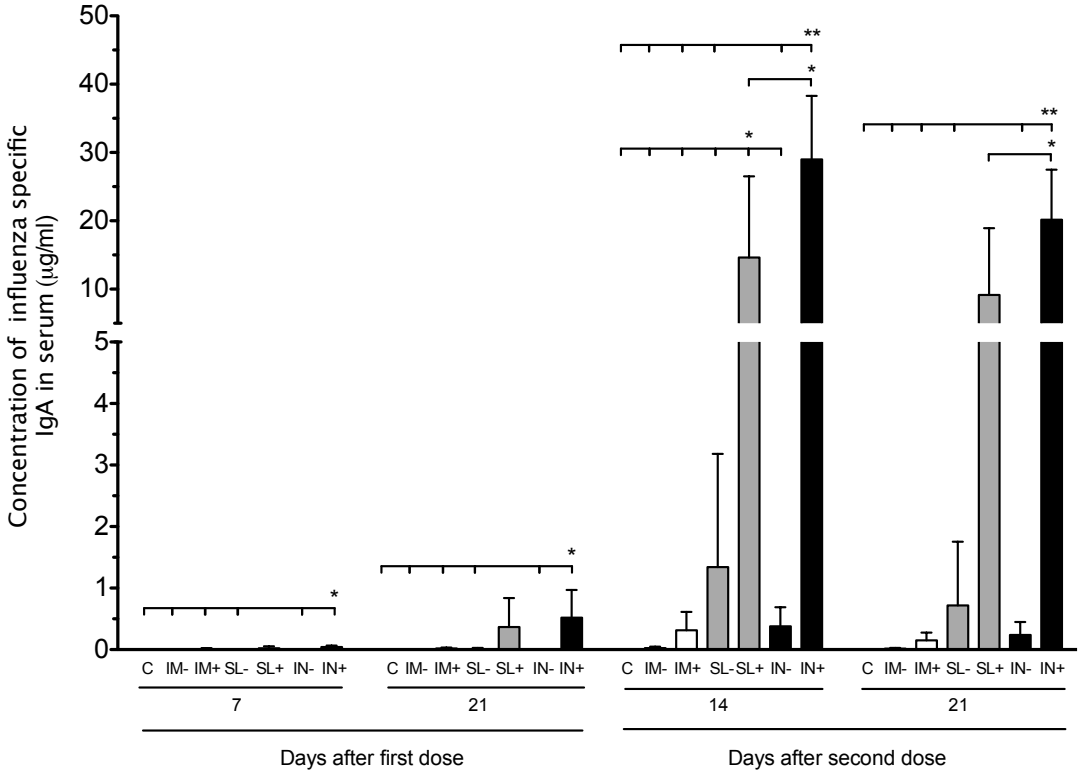


Figure 4.7: Concentration of influenza-specific IgA in the serum samples after 1st and 2nd immunisation

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Sera were separated from blood collected at day 7 and 21 after the first vaccine dose, and day 14 and 21 after the second vaccine dose, and tested for influenza-specific IgA antibodies. Each column represents the mean IgA concentration from six mice; error bars indicate the standard deviation. (*) and (**) indicate statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.05$ **$p \leq 0.001$).

IgG is the most abundant antibody in the serum and most tissues, and serves many important functions such as opsonisation and neutralisation of antigens, activation of complement (the classical pathway) and antibody-dependent cell-mediated cytotoxicity (ADCC). High concentrations of influenza-specific antibodies were found, especially after the second dose (up to 3500 µg/mL in the IN+ group) (figure 4.8). After the first dose, the IM+ group had the highest IgG response with a mean concentration of 40 µg/mL, statistical significantly different from all other groups one week after the first dose, and from all groups except the SL+ and IN+ groups three

weeks after the first dose. However, after the second dose of vaccine the IN+ group showed the highest IgG response, being statistically significantly ($p \leq 0.001$) higher than all other groups. Three weeks after the second dose, only the IN+ group had significantly higher IgG concentrations as compared to the non-adjuvanted group. SL administration did not show significant differences between the adjuvanted and the non-adjuvanted group. When considering the virosomes alone, IM administration induced the highest IgG concentrations, however, not significantly different from the other non-adjuvanted groups. IgG antibodies were not detected in control mice at any time point.

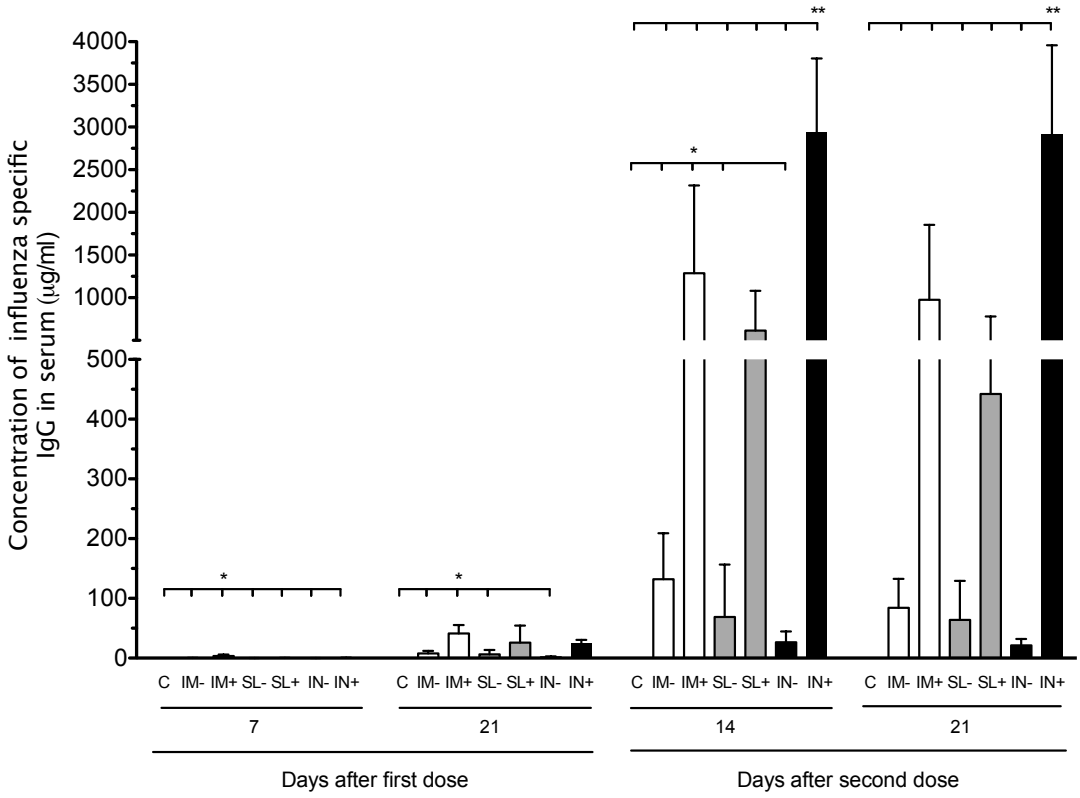


Figure 4.8: Concentration of influenza-specific IgG in serum from all sampling days

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Sera were separated from blood collected at day 7 (1w1d), 21 (3w1d), 35 (2w2d) and 42 (3w2d), and tested for influenza-specific IgG antibodies. Each column represents the mean IgG concentration from six mice; error bars indicate the standard deviation. (*) and (**) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.01$ **$p \leq 0.001$).

4.1.2.3 IgG1 and IgG2a subclass responses

Sera collected two weeks after the second dose (2w2d), at the peak of the IgG response, were analysed for two IgG subclasses; IgG1 and IgG2a (figure 4.9 and table 4.1). The IN+ group had the highest mean concentrations of both IgG1 (mean = 591 µg/mL) and IgG2a (mean = 1651 µg/mL), giving an IgG2a/IgG1 ratio at 2.79. The other mucosal administered group with c-di-GMP, the SL+ group, had much lower concentrations than the IN+ group. However, the IgG2a/IgG1 ratio was still over 1 (IgG2a/IgG1 = 1.63), indicating a balanced but slightly Th1 biased response. IM administration with the adjuvant had almost similar IgG1 concentrations (mean = 580 µg/mL) as compared to the IN+ group, but the concentrations of IgG2a was much lower (mean = 251 µg/mL) giving an IgG2a/IgG1 ratio at 0.72. Administration of the non-adjuvanted virosomes induced an IgG2a/IgG1 ratio below one (IM- = 0.08; SL- = 0.09; IN- = 0.09), indicating a Th2 skewed response. In contrast, when c-di-GMP was included, mucosal administration produced a Th1 polarised response, whilst IM administration showed a more balanced profile with a slightly Th2 polarisation.

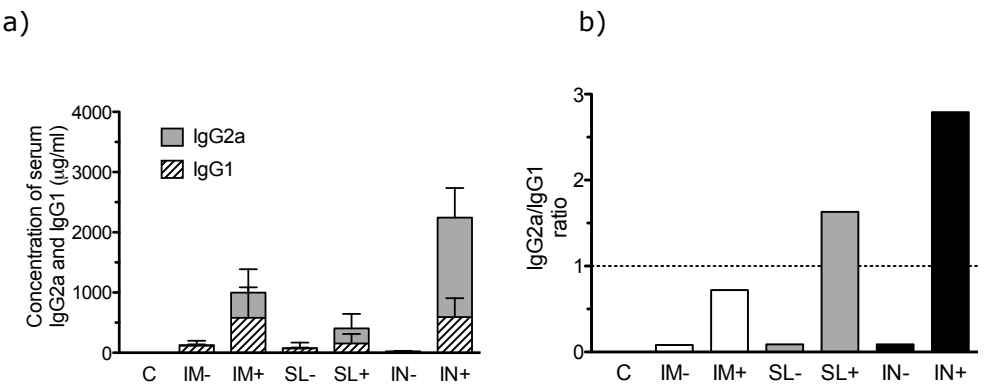


Figure 4.9: The concentrations of IgG1 and IgG2a and the ratio (between them) after the second vaccination

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), intranasally (IN) or sublingually (SL) with (+) or without (-) adjuvant. Controls (C) were only given c-di-GMP in PBS IN. Sera from mice collected at two weeks after the second dose were tested for the IgG subclasses, IgG1 and IgG2a. A) Concentration (µg/mL) of IgG2a and IgG1 in sera. The bars show IgG1 and IgG2a concentration on top of each other, the whole bar indicates the total IgG concentrations. B) IgG2a/IgG1 ratios. Columns show the relationship between IgG2a and IgG1. Values above 1 (dotted line) indicate a Th1 skewed immune response.

Table 4.1: The IgG2a/IgG1 ratio and the T helper cell response

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), intranasally (IN) or sublingually (SL) with (+) or without (-) adjuvant. Controls (C) were only given c-di-GMP in PBS IN. Sera from mice collected at two weeks after the second dose were tested for the IgG subclasses, IgG1 and IgG2a. The ratios between IgG2a and IgG1 were determined for each vaccination group; IgG2a/IgG1 <1 indicate a Th2 polarised response; IgG2a/IgG1 >1 indicate a Th1 polarised response.

Group	IgG2a/IgG1	Th1 or Th2
Mock	0.00	-
IM-	0.08	Th2
IM+	0.72	Th2
SL-	0.09	Th2
SL+	1.63	Th1
IN-	0.09	Th2
IN+	2.79	Th1

4.1.3 Mucosal vaccination induces high percentages of influenza-specific IgG memory B cells

After vaccination with an influenza vaccine, the induction of immunological memory is important for protection against future influenza epidemics and pandemics of the same or similar influenza subtypes. We therefore measured the influenza-specific memory B-cell response in lymphocytes isolated from the spleen after polyclonal differentiation and proliferation by mitogen stimulation (figure 4.10 and 4.11). The IN+ and SL+ groups had the highest number of spots indicating memory B-cells able to secrete IgG antibodies against the vaccine strain (A/Vietnam/1194/2004 (H5N1) NIBRG-14). The IN+ group showed significantly ($p \leq 0.05$) higher percentages of influenza (RG-14) specific cells of total IgG positive cells (up to 6.5 %) compared to all the other groups. The controls and the SL- group had no detectable memory B cells specific for RG-14, and the IM+ group had only barely detectable numbers. As in the IgA NW ELISA, the IM- group had higher percentages of influenza (RG-14) specific cells of total IgG positive cells (up to 1 %) as compared to the IM+ group.

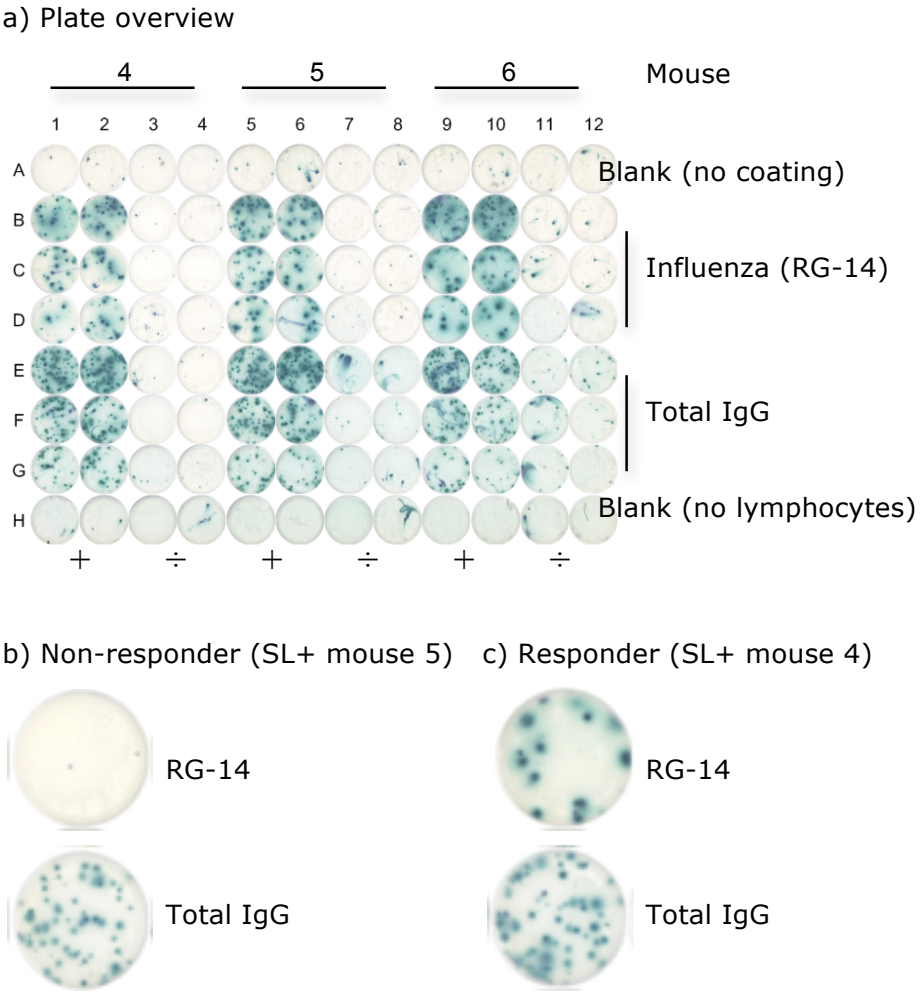


Figure 4.10: ELISPOT plates after development

Lymphocytes from spleens were isolated and stimulated with mitogens. a) To determine the amount of influenza-specific memory B-cells, non-stimulated controls (÷, column 3-4, 7-8, 11-12) were subtracted from the stimulated wells (+, column 1-2, 5-6, 9-10). Row A and H contain only blank wells without coating and lymphocytes respectively. Rows B-D were coated with virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine and row E-G were coated with IgG capture antibody. The plate depicted represents IN+, mouse 4-6. b) Pictures of single wells from a non-responder in the SL+ group. The upper well was coated with RG-14 virosomes; the lower well was coated with IgG capture antibodies to measure the total IgG secreting cells. c) Pictures of single wells from a responder in the SL+ group. The upper well was coated with RG-14 virosomes to measure the influenza-specific IgG secreting cells, the lower well was coated with IgG capture antibodies to measure the total IgG secreting cells.

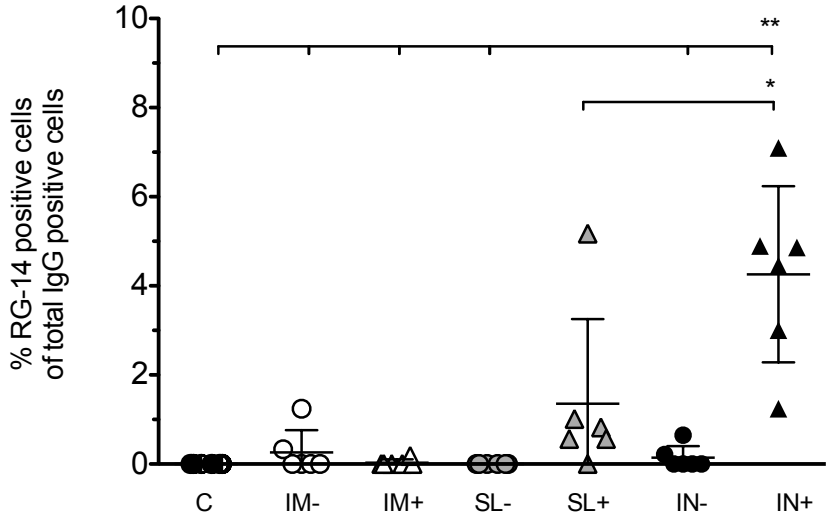


Figure 4.11: Memory B cell ELISPOT (IgG secreting cells)

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), sublingually (SL) or intranasally (IN) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Influenza-specific memory B cells in the spleen were detected by memory ELISPOT after polyclonal differentiation and proliferation by mitogens. ELISPOT results are shown as percent RG-14 influenza-specific memory B-cells secreting IgG of total IgG secreting memory B-cells. (*) and (**) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.01$ **$p \leq 0.0001$).

Overall, the humoral immune response was good in all three administration routes, but the IN+ group had the highest response after two doses with the virosomal vaccine. The SL+ group had also good responses, however, one mouse in the SL+ group (mouse 5) had lower responses than the other mice in the same group (table 4.2). IM vaccination induced high systemic humoral immune responses, but the local IgA production was poor. The inclusion of c-di-GMP gave statistical significantly higher responses than the non-adjuvanted group in all humoral assays when considering the IN administration, and in saliva IgA and serum IgA when considering the SL administration.

Table 4.2: The humoral response in the SL+ group three weeks after the second dose
BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine sublingually (SL) with c-di-GMP. The grey area (mouse number 5) is a non-responder/low-responder through all the different humoral assays.

SL+ mouse	Serum	ELISA					ELISPOT
	HI titre	Serum IgG (µg/mL)	Serum IgA (µg/mL)	NW IgA (ng/mL)	Saliva IgG (ng/mL)	Saliva IgA (ng/mL)	(% RG-14 of Total IgG)
1	80	796	27.2	438	442	17400	0.82
2	480	740	10.7	118	79.7	11100	1.01
3	160	137	2.53	62	8.2	4527	0
4	320	678	4.44	168	355	3820	5.17
5	5	7	0	6.8	24.5	1152	0.56
6	40	296	9.97	201	40.0	12000	0.56

4.2 Cellular Immune response

T cells are central in controlling immune responses. Helper T lymphocytes ($CD4^+$ T lymphocytes) stimulate both inflammation and antibody production, and also help to activate the cytotoxic T lymphocytes (CTL), which are important for destroying influenza infected cells.

4.2.1 Cytokine profiles

Cytokine profiles can reveal the Th polarisation of an immune response after vaccination. IL-2, INF-γ and TNF-α indicate a Th1 skewed response, IL4-, IL-5 and IL-10 indicate a Th2 skewed response and IL-17 indicates a Th17 response. To assess the cytokine profile of the different administration routes with or without c-di-GMP, splenocytes isolated three weeks after the second dose were stimulated *in vitro* with RG-14 virosomal influenza antigen for 72 hours. The concentrations of IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ and TNF-α in the cell supernatants were determined in the

multiplex bead assay (figure 4.12). Non-stimulated cells were incubated with medium alone and used as negative controls.

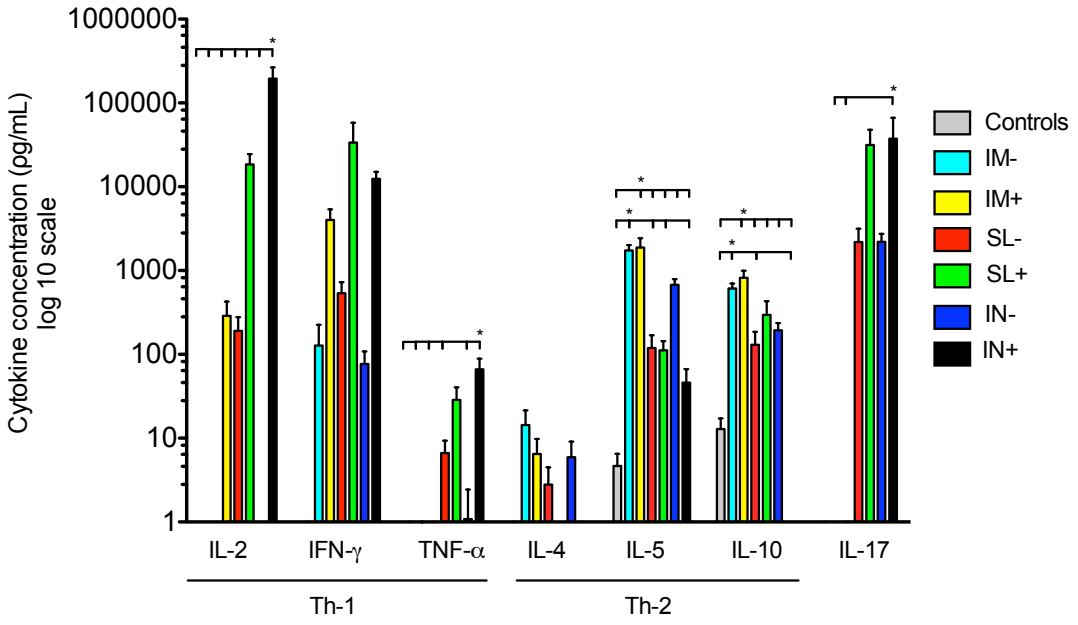


Figure 4.12: Bioplex results depicting cytokine production from *in vitro* activated lymphocytes.

BALB/c mice received two doses of virosomal influenza A/Vietnam/1194/2004 (H5N1) NIBRG-14 vaccine intramuscularly (IM), intranasally (IN) or sublingually (SL) with (+) or without (-) adjuvant. C-di-GMP in PBS was administered IN to controls (C). Splenocytes were isolated three weeks after second dose and incubated for 72 hours with 2.5 µg/mL H5N1 virosomal RG-14 before harvest of supernatant for analysis. The concentrations of cytokines in the negative controls were subtracted from the cytokine concentration of stimulated cells. Each column represents mean values from six mice (Except for the control group which is only for three mice) and error-bars indicate standard error of the mean. (*) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.05$).

The IN+ group had the highest concentration of the Th1 cytokines IL-2 (mean = 195 426 pg/mL) and TNF-α (mean = 66,3 pg/mL), both being significantly higher ($p \leq 0.05$) as compared all to the other vaccination groups. No IL-2 was detected in the control group and the non-adjuvanted IM and IN groups. The highest concentration of IFN-γ was found in the SL+ group (mean = 33 772 pg/mL), however no statistically

significant differences were seen between the different routes of administration. The Th2 cytokines were found in lower concentrations than the Th1 cytokines in the mucosal vaccinated groups. The IM+ and IM- groups showed significantly higher ($p \leq 0.05$) concentrations of both IL-5 and IL-10 than observed in most of the other groups. However, IL-5 and IL-10 cytokines were also measured in the control group that only received c-di-GMP intranasally, whereas no other cytokines were detected the control mice. Together this indicates that mucosal vaccination with RG-14 virosomes and c-di-GMP induces a Th1 skewed immune response (which also is consistent with the IgG2a and IgG1 concentrations found in the ELISA).

The Th17 response was evaluated by measuring the IL-17 concentration (figure 4.12). Only the IN and SL vaccinated groups had a detectable IL-17 production, and the c-di-GMP adjuvanted groups had the highest concentrations (the IN+ group mean = 37412 pg/mL; the SL+ group mean = 31511 pg/mL). However, only the IN+ group had significantly higher concentrations as compared to the non-responding IM+ and IM- groups.

4.2.1 The induction of multifunctional T cells after vaccination

The ability of $CD4^+$ T lymphocytes to produce two or more cytokines simultaneously (multifunctional T-cells) may play a role in the protection against influenza virus. We have assessed the ability of mouse splenocytes to produce IL-2, IFN-γ and TNF-α alone or in combination after stimulation with the virosomal H5N1 vaccine (RG-14). Lymphocytes were fixed and subsequently stained for the intracellular cytokines and measured using multiparametric flow cytometry (figure 4.13). All c-di-GMP adjuvanted groups produced all three cytokines, whereas no cytokine producing cells were detected in non-adjuvanted groups and the control group (results not shown). Overall, the IN+ group had the highest frequency of influenza-specific cytokine producing $CD4^+$ T cells (figure 4.13b). The fraction of triple producers, double producers and single producers are described in pie charts (figure 4.13a). The IM+ group had the largest fraction of triple producers; approximately 30 % of the $CD4^+$ T cells produce all three cytokines. The SL+ group has the largest fraction of double producers and IN+ has the largest fraction of single producers of the three groups. The IN+ group still had a higher fraction of double producers as compared to single

producers. This however, does not reflect the frequency of cytokine producing T cells but the distribution of the single, double and triple producers from the total cytokine producing cells. When focusing on the frequency of influenza-specific $CD4^+$ T cells, the IN+ group had the highest amount of triple producers (mean = 0.4 %) compared to IM+ and SL+, where the mean frequency was 0.2 % and 0.1 %, respectively. The IN+ group also had a high a frequency of cells producing two cytokines; average 3 % of $CD4^+$ T cells produced both IL-2 and TNF-α. These two cytokines were also produced simultaneously by average 1.5 % of $CD4^+$ T cells in the SL+ vaccination group with c-di-GMP. Further, the SL+ group had few or no cells producing IFN-γ and TNF-α or IFN-γ and IL-2 simultaneously. The single-cytokine producing cells produced mainly TNF-α and the highest frequencies of these cells were observed in the IN+ and SL+ groups.

a)

b)

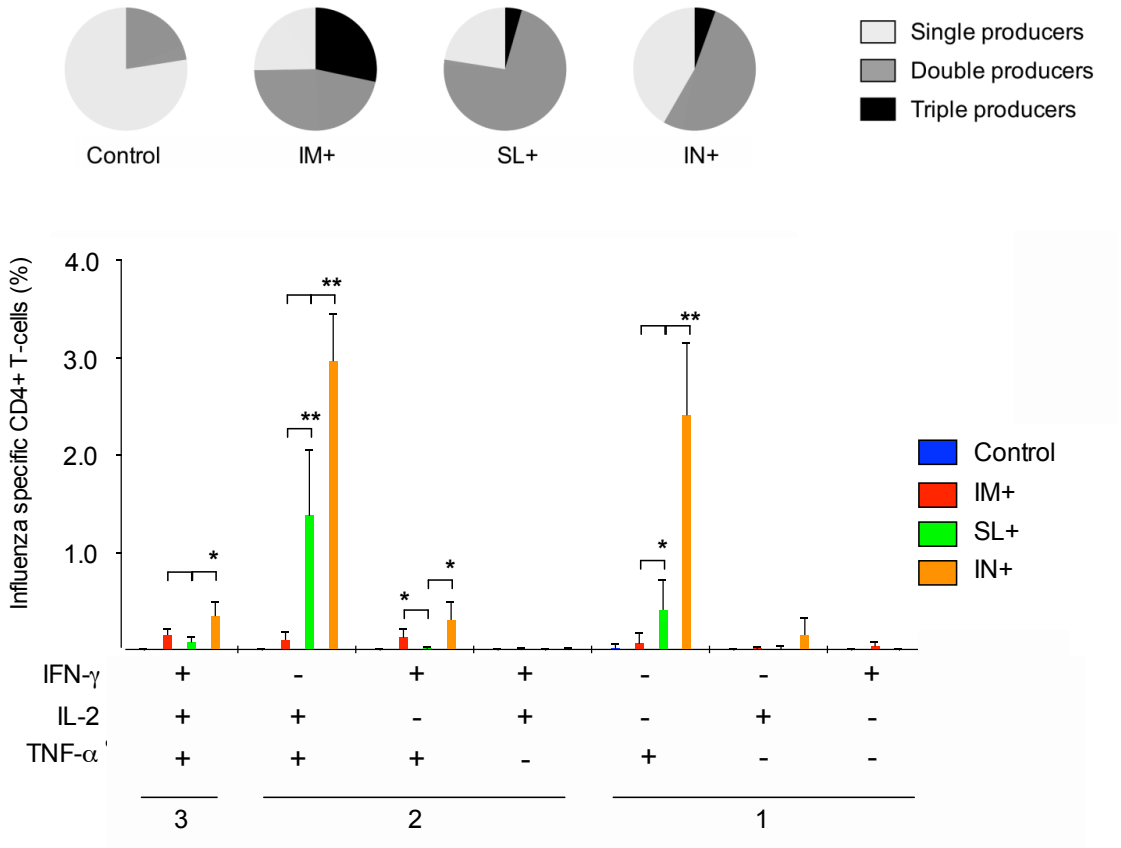


Figure 4.13: Multiparametric flow cytometry detection of intracellular cytokines

Splenocytes isolated three weeks after second dose were stimulated with the virosomal vaccine (A/Vietnam/1194/2004 (H5N1) NIBRG-14) for 17 hours. After fixation and

intracellular staining for IFN-γ, TNF-γ and IL-2, the cytokines were detected by flow cytometry. a) Pie charts showing fractions of single producers, double producers and triple producers in the control group and the adjuvanted groups. b) The percentage of $CD4^+$ T cells that have produced cytokines after stimulation with virosomal influenza vaccine. The non-adjuvanted groups had no or little cytokine production (not shown). Error bars indicate standard deviation. (*) and (**) indicates statistical significant differences between the groups (one-way ANOVA with Bonferroni correction, *$p \leq 0.05$ **$p \leq 0.01$). (IM+ = intramuscularly with adjuvant, IN+ = intranasally with adjuvant, SL+ sublingually with adjuvant.)

All together, a cell-mediated immune response was induced after all three types of immunisation, however, the mucosal routes induced higher concentrations of Th1 and Th17 cytokines and higher frequencies of $CD4^+$ T cells than the intramuscular route. As seen in the humoral assays, the IN+ group generally showed the highest response, followed by the SL+ group.

5 Discussion

The first transmission of H5N1 (avian influenza) from birds to man was registered in Hong Kong in 1997 [133]. Subsequently, sporadic cases of H5N1 infection in man have occurred in Asia and other countries with a large poultry industry, and the mortality rate has been high (≥ 60 %) [19]. No effective transmission of H5N1 from man to man has yet been reported, but could occur if H5N1 re-assorts with another viral subtype [134], and H5N1 thus poses a substantial pandemic threat.

The ideal pandemic influenza vaccine should be easy to administer, cheap and provide long-term and cross-reactive protection, both systemically and mucosally, after one vaccine dose. Mucosal vaccination fulfils many of these requirements, and is therefore an attractive approach. Since no needles are involved in mucosal vaccination, the public acceptance and compliance rate would increase, and vaccine induced herd immunity would be high. Needle-free vaccination is also beneficial for use in the developing countries where blood borne infections are prevalent. Intranasal administration of influenza vaccines has been investigated for some time, but the close localisation of the nose to the brain and the clear association between an intranasal inactivated influenza vaccine and Bell's Palsy [90], requires investigation of new mucosal routes of administration. Sublingual administration is used for many drugs (e.g. nitro-glycerine and desensitisation therapy for different allergies [135]), but only one study has looked into the sublingual route for administration of influenza vaccines [91].

A problem with avian influenza vaccines in man is that they have low immunogenicity [136-138]. To overcome this issue, high antigen doses (up to 90 μg HA) or an effective adjuvant have been used. Two vaccine doses have also been necessary to induce antibody levels associated with protection. Virosomes are virus like particles lacking genetic material, and have been shown to be very immunogenic and elicit high levels of protective antibodies and induce activation of $CD4^+$ and $CD8^+$ T cells [81]. In addition, virosomes can be less reactogenic as compared to whole virus vaccines (reviewed in [86]). Virosomes are also promising for mucosal vaccination [139].

In this study we have found that IN administration of the virosomal vaccine combined with c-di-GMP generally induced the highest humoral and cellular immune responses. However, SL administration of the c-di-GMP adjuvanted vaccine elicited a mucosal immune response and can thus provide an alternative to the intranasal route. The only previous study on SL administration of influenza vaccines also depicted a great potential of the SL route [91].

5.1 Induction of humoral immunity

5.1.1 Haemagglutination inhibition titres induced after vaccination

Antibodies can neutralize influenza virus and thus prevent infection [140]. Induction of antibodies in the sera and locally in the respiratory tract is one of the main goals after vaccination against influenza. However, in the requirements set by the European Medicines Agency for licensing of seasonal influenza vaccines in man, the only surrogate correlate of protection are related to serum antibody responses (e.g. HI titres ≥40) [58]. Even though this threshold may not be applicable for pandemic strains like avian influenza, it is recommended that vaccines against these strains should be able to meet the same criteria as seasonal vaccines. HI titres ≥ 40 are therefore used as indicative of sero-protection in this study (in both the serum assay and the saliva assay).

We found that all c-di-GMP adjuvanted groups had serum HI GMT ≥40 after two vaccine doses. IN vaccination with adjuvant gave the highest GMT as compared to both SL and IM vaccination with adjuvant (figure 4.1). This elevated immune response as compared to IM vaccination is consistent with previous work conducted by our group using c-di-GMP in combination with a subunit H5N1 or a plant produced H5 vaccine [141] and (unpublished data). In another study, where a different adjuvant (Matrix-M) but the same influenza virosomal vaccine was used, IM vaccination induced higher HI-titres than IN vaccination [123] illustrating the different functions of the two adjuvants. In our study, IN administration with adjuvant was the only adjuvanted group showing substantially higher serum HI GMT as

compared to the non-adjuvanted group. Among the adjuvanted groups, SL administration showed the lowest serum HI GMT, although this may have been influenced by one mouse in the SL+ group, which had no response in the assay. This mouse also responded poorly in many of the other assays and this was most likely due to difficulties in the SL vaccination in this mouse (see table 4.2). When considering the virosomes alone, only IM immunisation induced serum HI titres ≥ 40. This emphasises the need for an adjuvant when the vaccine is administered mucosally.

When we tested the pooled saliva samples from each group for HI antibodies (figure 4.2), the IN+ group showed the highest response, followed by the SL+ and IM+ groups. No groups had HI titres ≥40, which is the titre surrogate correlate of protection for HI-antibodies in human sera [59]. But no surrogate correlate of protection is based on HI titres in saliva; hence the salivary HI titres could be sufficient for protection. SIgA antibodies have shown the ability to neutralise virus [132], thus the HI antibodies in the saliva are likely to have local origin. However, the fact that both IM vaccination groups induced HI-antibodies in saliva, indicates an association with salivary IgG antibodies, which have been shown to have the ability to transudate from the serum to the upper respiratory tract [142]. The fact that we pooled the samples made the assay sensitive to outliers. From table 4.2, we can se a great variability in the SL+ group, which could have affected our results. However, the saliva HI GMTs correspond to the serological assays we conducted, hence the overall result is not much affected by the pooling.

5.1.2 Intranasal and sublingual vaccination induce local IgA responses in the nasal wash and saliva

IgA antibodies are the dominant antibody of the mucosal immune system, and are locally secreted as dimers and polymers (pIgA) joined together by a J-chain and secreted together with the soluble part of the polymeric Ig receptor (pIgR) [143]. Secretory IgA (SIgA) can neutralise influenza virus at the portal of entry, before the virus is able to establish an infection [132]. In addition, when the pIgA is trancytosed across the epithelium, also intracellular viruses can be neutralised [143]. SIgA has

also shown to be more cross-reactive than IgG, and can thus have neutralising activity against drifted influenza strains [88, 143].

In this study, one vaccine dose elicited only detectable IgA antibodies in the nasal wash samples from the mucosally immunised groups (figure 4.4), in agreement with previous findings using an IN adjuvanted vaccine [123, 141]. The secondary response after IN vaccination was much stronger than after SL vaccination when c-di-GMP was included, and the IN+ group showed significantly higher concentrations of IgA in the nasal wash as compared to the SL+ group. In contrast, the non-adjuvanted mucosal groups had similar IgA concentrations. Surprisingly, for the IM administered vaccines, the non-adjuvanted group showed a low response after the second dose, whilst the group that received virosomal vaccine with c-di-GMP IM had only barely detectable levels. However, the levels of local IgA in the upper respiratory tract after IM vaccination were much lower than in the to SL and IN groups, and would probably not have protected the mice from infection [144].

In a murine study of intranasal vaccination with seasonal H1N1 and subsequent challenge with the same virus, an influenza-specific IgA concentration of 15 ng/mL found in the nasal wash correlated with no or reduced viral replication in the nasal cavity [144]. If we apply this concentration to our results, most of the mice in the mucosally administered groups would be protected from severe illness. But due to possible differences in assay protocols and since we use a different antigen and adjuvant, it is difficult to compare these studies. Therefore the next step would be to perform a highly pathogenic avian H5N1 challenge study in mice to assess the correlation between SIgA and protection.

The concentrations of influenza-specific IgG and IgA were also measured in saliva (figure 4.3). IgA was only found in the groups receiving mucosally administered vaccine. A study in monkeys, which investigated the protective efficacy of an intranasal whole virus vaccine (NIBRG-14) combined with Ampligen® adjuvant, showed that high levels of IgA in the saliva correlated with low viral titres in the nose and throat and protection from pneumonia after highly pathogenic virus challenge [145]. Differences in the ELISA make it difficult to compare Ichinohe et al. with our

study; however, it shows the importance of IgA in saliva. We also measured the salivary IgG concentrations, and not surprisingly, much lower IgG concentrations were found in saliva as compared to serum IgG at the same time-point. No significant differences were found between the adjuvanted groups, whilst only the IN vaccination route showed a statistically significant difference between the adjuvanted and the non-adjuvanted group. The concentration of salivary IgG (figure 4.3) correlates with the salivary HI GMT (figure 4.2); the mucosal adjuvanted groups had the highest response in both assays, whilst the IM- group had the highest response of the non-adjuvanted groups.

There were no statistically significant differences in salivary IgA concentrations between the SL+ and IN+ group, whereas significantly higher NW IgA was found in the IN+ group than in the SL+ animals. The reason for this could be an increased IgA response in the nose after IN administration of the vaccine, which we do not see after SL administration.

The amount of influenza-specific pIgR was measured in nasal wash and saliva samples from all mice to identify the amount of locally secreted IgA (SIgA). Only the SL+ and IN+ group had detectable levels at the dilution used in the assay (figure 4.5 and 4.6). The relationship between IgA and pIgR reflected the levels of SIgA as has been reported previously [146]. Compared to total IgA, SIgA was detected at lower OD-values in all samples, indicating that not all IgA antibodies found in the upper respiratory tract are actively transported into the lumen by pIgR. The remaining IgA may have been serum derived monomeric IgA or polymeric IgA only connected with the J chain, which had entered the upper respiratory tract mucosa [147]. The gap between IgA and pIgR could also be due to different sensitivity in the assays. Nonetheless, the results clearly show that the c-di-GMP adjuvanted vaccine induced influenza-specific SIgA antibodies both when administered intranasally and sublingually.

5.1.3 Intranasal vaccination induce systemic IgG and IgA responses

The induction of systemic antibodies are important to eliminate influenza virus that have breached the barriers of the innate immune system and escaped the SIgA antibodies in the mucosa of the respiratory tract.

IgG antibodies have a superior specificity and serve many important functions in protection against influenza. High levels of serum IgG are associated with a reduction of viral shedding [148] and can cross the alveolar walls of the lungs, and thereby protect against viral pneumonia [149]. In this study, the IM+ group had significantly higher IgG concentrations than all the other groups after the first vaccine dose. However, after the second dose, we found significantly higher concentrations of IgG in the IN+ group as compared to the IM+ and SL+ groups (figure 4.8). However, no significant difference was found between SL and IM vaccination when adjuvanted with c-di-GMP. In contrast, previous studies have shown that IM administration of a virosomal influenza vaccine with Matrix-M adjuvant, and IM administration of a whole influenza virus vaccine, induced higher IgG concentrations in sera than IN administration [123] and (unpublished data). When we considered the virosomes alone, the IM- group showed the highest IgG concentration, consistent with what we found in the serum HI assay. Again, the need for a good mucosal adjuvant is highlighted. A previous study in mice showed that an IgG concentration of 38 μg/mL in the serum was associated with undetectable levels of virus in mouse lung washes after viral challenge with seasonal H1N1 [144]. Interestingly, in our study the mean IgG concentration in serum was higher in all groups after two vaccine doses (except the IN- group), than the concentration associated with undetectable levels of virus in the lungs. But as previously discussed, comparison of ELISA results between different labs are difficult because of the different protocols used.

The IN+ group had significantly higher serum IgA concentrations after one vaccine dose as compared to all other groups, except the SL+ group (figure 4.7). Two weeks after the second dose the SL+ group also had IgA concentrations significantly higher than the concentrations found in all other groups, except the IN+ group. The peak IgA response was, in all groups, found two weeks after the second dose, and a small decrease in IgA concentration was observed from two weeks to three weeks after the

second dose. The decreasing concentration is possibly caused by declining numbers of IgA producing short-lived plasma cells or the short half-life of IgA antibodies in serum. Due to the high levels of serum IgA in the mucosally vaccinated groups compared to the intramuscular vaccinated groups, it is possible that some of these antibodies have a mucosal origin.

5.1.4 Memory B cell ELISPOT

A population of B cells activated by antigen are able to survive for a longer period of time and are called memory B cells. Memory B cells can induce rapid antibody responses after subsequent encounter with the same antigen, and are therefore an important part of the immune response after vaccination. In addition, memory B cells can be an important source of cross-reactive antibodies [150, 151]. The enumeration of memory B cells may also give an indication of the longevity and magnitude of the vaccine response, since they are formed in germinal centres where long-loved plasma cells also are formed. After differentiation into memory B-cells, some of these cells stay in the germinal centre of the lymph nodes, whilst others re-circulate between the spleen and lymph nodes [152].

The SL+ and IN+ vaccines induced differentiation into memory B cells (figure 4.11), again illustrating the efficiency of c-di-GMP as a mucosal adjuvant. However, only IN vaccination showed significantly higher numbers of influenza-specific IgG secreting memory B cells as compared to the other vaccination groups. The IM- and the IN- group also had a memory B cell response, albeit low, whilst no response was observed in the IM+ group. Interestingly, we saw a higher response in the non-adjuvanted IM group than in the adjuvanted IM group, consistent with the results seen in the NW IgA ELISA (figure 4.4). It was also surprising that IM vaccination induced such low numbers of RG-14 specific IgG secreting memory B cells, since the IgG serum concentrations in the IM+ group was similar to that of the SL+ group. However, it is unclear if the numbers of memory B cells correlate with antibody concentrations in sera [131], and other findings suggest that memory B cells and ASC plasma cells are independent of each other [153]. To assess the amount of ASC after vaccination, we should have used the direct ELISPOT assay.

5.2 Induction of cellular immunity

T cells are essential for the prevention and recovery from influenza virus infection [140], however, there are no international standards for measurement of cell-mediated immune (CMI) responses after influenza vaccination and no correlate of protection based on CMI has been established.

5.2.1 Cytokine profiles and IgG subclasses

Vaccines should preferably activate both the Th1 (IgG2a, IL-2, IFN-γ and TNF-α) and Th2 subsets (IgG1, IL-4, IL-5, and IL-10) of T helper cells, since both are important for elimination of influenza virus from the host [154, 155]. We found serum IgG2a domination in both the SL+ and IN+ groups, which indicates a Th1 polarised response (figure 4.9). This is supported by the high concentrations of the Th1 cytokines IL-2 and IFN-γ in the supernatants of influenza stimulated splenocytes (figure 4.12). However, the IN+ group had a stronger polarisation towards Th1 as compared to the SL+ group. This is consistent with other studies carried out using intranasal administration with adjuvants where a Th1 skewed response is elicited [156], whilst sublingual immunisation gives a more balanced Th1/Th2 response [92]. In contrast, the IM+ group produced similar amounts of IgG1 and IgG2a, and cytokines from both the Th1 and Th2 subsets were found upon *in vitro* stimulation of splenocytes, depicting a balanced Th1/Th2 response. Another study conducted in our group, where a different adjuvant was used, found a balanced Th response in mice both when vaccinated intranasally and intramuscularly [123]. All the non-adjuvanted groups produced an excess of IgG1 as compared to IgG2a (figure 4.9). In addition the non-adjuvanted groups produced lower Th1 responses, but similar Th2 cytokine levels as compared to the SL+ and IN+ groups. Thus, the virosomes alone induced a Th2 skewed response whilst inclusion of c-di-GMP induced a more balanced (IM) or Th1 biased response (SL and IN). Both Th1 cytokines and IgG2a antibodies are important mediators in promoting antibody-dependant cell-mediated cytotoxicity (ADCC) [157, 158], suggesting that SL and IN vaccination combined with c-di-GMP would elicit greater protection and be able to eradicate virus-infected cells more effectively than IM vaccination and all the non-adjuvanted vaccine groups.

IL-10 is considered a Th2 cytokine, and inhibits Th1 differentiated cells. IL-10 is also associated with enhanced IgA class-switch, thus increasing the production of IgA (reviewed in [41]). In spite of this, we found similar concentrations of IL-10 in all groups, except for the IN+ group where no IL-10 was detected. This can perhaps be explained by the substantial Th1 polarisation of the IN+ group. However, also the control group (which received only c-di-GMP alone) had detectable levels of IL-10, indicating no association between IL-10 and influenza-specific IgA production in this study. Another Th2 cytokine associated with IgA class-switch is IL-4 (reviewed in [41]). Similar to IL-10, no IL-4 was detected in the SL+ and IN+ groups despite the high concentrations of IgA found both locally and systemically in these groups. In addition, TGF-β is a cytokine that induces IgA class-switch [159, 160], and it would have been interesting to measure this cytokine in the supernatant of *in vitro* stimulated lymphocytes. This could have explained the high levels of IgA in the mucosally immunised mice. Also the concentration of the respective cytokines can be different between local compartment lymphoid tissues and the spleen, therefore sampling of cervical lymph nodes would have given us a better picture of the local CMI. Other explanations for the weak association between high IgA concentration and IgA inducing cytokines could be due to incorrect stimulation, lack of sensitivity of the assay and/or sampling time point.

Th17 is a newly discovered subset of Th cells and has been associated with inflammation and protection against extracellular pathogens and helminths [161]. Recent findings have shown that a Th17 cytokine (IL-17) is also important in the immune response towards pathogens at mucosal sites. IL-17 has been detected in murine lungs after infection with influenza [162]. In our study, high levels of IL-17 were found in the mucosally vaccinated mice, whereas no IL-17 was detected in mice receiving the vaccine intramuscularly (figure 4.12). Similarly, a previous study has found high levels of IL-17 after intranasal vaccination [163]. The importance of Th17 cells in influenza infections is debatable, but a challenge study using influenza PR8 (H1N1) found an improved survival rate in mice with increased Th17 cytokine concentration in the lungs [164]. If we extrapolate this to our results, the IN and SL vaccinated mice would be protected against influenza associated illness and death. In contrast, in a previously study of Matrix-M adjuvanted virosomal vaccine conducted

in our group, the IL-17 production from influenza stimulated splenocytes did not correlate with the protection against lethal challenge with influenza [124]. Nonetheless, these results are the first to show that SL administration of influenza vaccines can induce a Th17 response.

5.2.2 Multifunctional T cells

Induction of $CD4^+$ T cells producing more than one cytokine simultaneously has been shown to correlate with the protection against infections other than influenza [36, 165, 166]. In addition, multifunctional T cells have been shown to produce higher levels of cytokines as compared to single cytokine producing T cells [35].

In this study, we have shown that mucosal vaccination in combination with c-di-GMP induces high frequencies of influenza-specific multifunctional T cells (figure 4.13). The IN+ group had the highest frequency of influenza-specific $CD4^+$ T cells producing three cytokines (TNF-α, IFN-γ and IL-2) and any combination of two cytokines. Among the double-producers, the TNF-α^+IL-2^+ cells showed the highest frequencies in both the adjuvanted mucosal groups (IN+ and SL+) with adjuvant. The same double-producers have been found in high frequencies after influenza vaccination studies in our group, using a different adjuvant and the same virosomal antigen [123], and using the same c-di-GMP adjuvant but a different H5 antigen [141]. In contrast, other studies investigating vaccines against *Leischmania Major* and *Mycobacterium Tuberculosis*, found a double cytokine producer response dominated by TNF-α^+IFN-γ^+ cells [36, 165, 166]. This can indicate that influenza vaccination mainly induces TNF-α^+IL-2^+ cells; however, also TNF-α^+IFN-γ^+ cells were found in the IN+ and the IM+ groups (figure 4.12b). Among the single cytokine producing cells, the TNF-α^+ cells were most abundant in all groups. IL-2^+ cells had lower frequencies, and IFN-γ^+ cells were only barely detectable. Thus, IFN-γ is mainly produced together with other cytokines. IFN-γ, TNF-α and IL-4 have been shown to increase the pIgR gene expression [167-169], which could explain the higher concentrations of IgA and pIgR in the nasal washes and the saliva in the IN+ and SL+ groups (figure 4.5). The frequencies of antigen-specific cytokine producing (triple, double and single producers) cells in our study are higher than previously reported [36, 165, 166], indicating that c-di-GMP is an effective mucosal adjuvant.

We found that the SL+ and IN+ groups had higher frequencies of $CD4^+$ T cells producing cytokines than the IM+ group, indicating that mucosal vaccination with c-di-GMP is highly effective at inducing $CD4^+$ T cell responses. Nonetheless, as illustrated in the pie charts (figure 4.12a) despite the lower frequencies, a higher proportion of cells in the IM+ group than the mucosal groups produced all three measured cytokines simultaneously, hence higher levels of cytokines could have been produced from these cells [35].

5.3 Limitations of the study

In this study we have assessed the immune response after vaccination with an H5N1 virosomal vaccine. There is, however, no established surrogate correlate of protection after vaccination against avian H5N1 [59]. The next step in evaluating the vaccine is to assess the protective efficacy, and evaluate and correlate the immune response to protection from illness and death. Anyhow, we have previously reports that two IM doses of 1 μg HA of the virosomal vaccine alone can protect mice against HPAI [124]. In the present study, all groups receiving the c-di-GMP adjuvanted vaccine had higher immune responses than the IM- group, suggesting that these groups would also be protected from HPAI challenge.

When vaccinating the mice intranasally and sublingually, it is important that the whole vaccine volume is absorbed in the nasal cavity and under the tongue, respectively. If some of the vaccine is swallowed, the immune response may be drastically decreased due to acidic and enzymatic degradation of the antigen and/or adjuvant in the stomach. Should any antigen reach the mucosa in the gastrointestinal tract intact, an immune response may be induced, but at the wrong immunological site. However, we used a small volume (7 μl), which previously has been reported to be retained in the sublingual mucosa [91], and by placing the mice in positions that should prevent them from swallowing the vaccine, we reduced this risk to a minimum. Similar problems apply to IN vaccination, however, protocols for IN vaccination have been optimised in our lab.

Overall, the IN+ group elicited the highest immune response in all immunological assays. The SL+ group showed a similar, but generally lower response. This may be due to difficulties in performing the SL vaccination. In addition, the antigen uptake under the tongue can be different from the antigen uptake in the nose, and the NALT has been shown a superior mucosal site to the other MALTs (reviewed in [39]). Also salivary enzymes, which not are present in the nasal cavity, could play a role in the reduced immunogenicity in SL as compared to IN administration of the c-di-GMP adjuvanted virosomal vaccine.

The vaccination technique can also be one of the reasons for the variability between mice within one vaccine group, especially the SL groups (see table 4.2). The sampling (especially NW sampling) technique, e.g. spills or incorrect technique, could also explain some of the variability between the mice. Nutritional status and stress levels can have a huge impact on the immune status [170-172], hence the immune response after vaccination could be altered differently for an individual mouse, depending upon the stress level after transport and handling of the mice. Variability within the SL+ group can be one of the reasons for the lack of statistically significant differences between the SL+ and the SL- groups. Furthermore, this is the first sublingual study in our group; hence some of the protocols were not fully optimised. The present study will therefore form a pilot study for future development of assays and vaccination protocols.

5.4 Conclusions

In this study, we were the first to evaluate the sublingual route for administration of an H5N1 virosomal influenza vaccine, and found further evidence that this route of administering influenza vaccines can be an attractive alternative to intranasal vaccination. In addition, we evaluated the c-di-GMP adjuvant, and found that, for all administration routes, the combination of c-di-GMP and the virosomal vaccine induced higher immune responses than the virosomes alone, thus illustrating the need for a good mucosal adjuvant.

Both the mucosal c-di-GMP adjuvanted vaccination groups induced strong humoral (both systemic and local) and cellular immune responses, with a Th1 polarisation, and high frequencies of influenza specific multifunctional $CD4^+$ T cells. The intramuscular route showed similar systemic antibody responses, but lower local antibody concentrations, as compared to the mucosal routes. In addition, the intramuscular route induced a Th2 polarisation irrespective of the inclusion of c-di-GMP adjuvant. Interestingly, lower frequencies of influenza-specific multifunctional $CD4^+$ T cells were found after intramuscular vaccination, than for the mucosal vaccination groups. All three administration routes induced serum HI GMT $\geq$40 when the virosomes were combined with c-di-GMP, thereby fulfilling the CHMP criteria for seasonal vaccines in man [58].

5.5 Further research

In this study, we have evaluated the humoral and cellular immune response after SL, IN and IM vaccination of an H5N1 virosomal vaccine with or without c-di-GMP in a murine model. To get a more detailed evaluation of the SL route, further studies characterising the immune response in more detail (e.g. microneutralisation assay, $CD8^+$ T cell and NK cell assays and long-term immunity), and survival after lethal viral challenge, are needed. In addition, it would have been interesting to further investigate the local immune response, by isolating cells from the appropriate draining lymph nodes for each administration route and assess the cell-mediated immunity (e.g. *in vitro* activation, multifunctional T cells).

Cross-clade immunity is important for pandemic vaccines as the H5N1 are antigenically distinct in different geographical locations, thus further investigation of the cross-reactivity of the virosomal vaccine combined with c-di-GMP is necessary. We therefore suggest evaluation of cross-protection, both by assessing cross-reactive immune responses and by lethal viral challenge with heterologous influenza strains.

In a pandemic situation, one of the possible challenges would be limited amount of antigen for vaccine production, and the antigen dose in each vaccine dose should be as low as possible. We used a low dose of antigen (2 μg HA) and previous studies in our group have shown that c-di-GMP had a dose sparing effect (unpublished data). We would suggest a dose-efficacy study using the virosomal antigen and c-di-GMP to further assess the dose sparing potential of this adjuvant.

The eventual goal for all vaccines is to obtain marketing authorisation for human use. Today, no mucosal adjuvants are licensed, however, most mucosal vaccines require an effective adjuvant. C-di-GMP has in this study shown great potential as a mucosal adjuvant, and we therefore suggest further toxicity studies and further immunogenicity studies in larger animal models (ferrets and monkeys) to build a non-clinical dossier.

Further investigation of the SL route should also address formulation issues. Since the vaccine is to be administered in the mouth, organoleptic properties (e.g. taste, odour and colour) are important for compliance and should be thoroughly investigated. The viscosity and solubility of the formulated vaccine are also important, as the vaccine should stay under the tongue for as long as possible to increase absorption of antigen and adjuvant. Also, the formulation should (if formulated as a tablet) rapidly disintegrate when placed under the tongue.

6 References

1. Kilbourne, E.D., *Influenza pandemics of the 20th century.* Emerg Infect Dis, 2006. **12**(1): p. 9-14.
2. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.
3. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.
4. Bosch, F.X., et al., *The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses.* Virology, 1979. **95**(1): p. 197-207.
5. Belser, J.A., et al., *Influenza A virus transmission: contributing factors and clinical implications.* Expert Rev Mol Med, 2010. **12**: p. e39.
6. Wilson, I.A., J.J. Skehel, and D.C. Wiley, *Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution.* Nature, 1981. **289**(5796): p. 366-73.
7. Matrosovich, M.N., et al., *Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium.* J Virol, 2004. **78**(22): p. 12665-7.
8. WHO, *A revision of the system of nomenclature for influenza viruses: a WHO memorandum.* Bull World Health Organ, 1980. **58**(4): p. 585-91.
9. Horimoto, T. and Y. Kawaoka, *Influenza: lessons from past pandemics, warnings from current incidents.* Nat Rev Microbiol, 2005. **3**(8): p. 591-600.
10. Murray, P.R.P., K.S. Rosenthal, and M.A. Pfaller, *Orthomyxoviruses*, in *Medical microbiology*. 2009, Mosby Elsevier: Philadelphia. p. 583-591.
11. Neumann, G., T. Noda, and Y. Kawaoka, *Emergence and pandemic potential of swine-origin H1N1 influenza virus.* Nature, 2009. **459**(7249): p. 931-9.
12. Zambon, M.C., *The pathogenesis of influenza in humans.* Rev Med Virol, 2001. **11**(4): p. 227-41.
13. Taubenberger, J.K., et al., *Initial genetic characterization of the 1918 "Spanish" influenza virus.* Science, 1997. **275**(5307): p. 1793-6.
14. Tumpey, T.M., et al., *Characterization of the reconstructed 1918 Spanish influenza pandemic virus.* Science, 2005. **310**(5745): p. 77-80.
15. Watanabe, T. and Y. Kawaoka, *Pathogenesis of the 1918 pandemic influenza virus.* PLoS Pathog, 2011. **7**(1): p. e1001218.
16. Glezen, W.P., *Emerging infections: pandemic influenza.* Epidemiol Rev, 1996. **18**(1): p. 64-76.
17. Girard, M.P., et al., *The 2009 A (H1N1) influenza virus pandemic: A review.* Vaccine, 2010. **28**(31): p. 4895-902.
18. Claas, E.C., et al., *Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus.* Lancet, 1998. **351**(9101): p. 472-7.
19. WHO. *Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO.* 2011; Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_02_09/en/index.html.
20. WHO. *Continuing progress towards a unified nomenclature system for the highly pathogenic H5N1 avian influenza viruses.* 2009; Available from: http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/.

21. Ungchusak, K., et al., *Probable person-to-person transmission of avian influenza A (H5N1).* N Engl J Med, 2005. **352**(4): p. 333-40.
22. Hinshaw, V.S., R.G. Webster, and B. Turner, *The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl.* Can J Microbiol, 1980. **26**(5): p. 622-9.
23. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin.* Virology, 1983. **127**(2): p. 361-73.
24. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.
25. Steinhauer, D.A., *Role of hemagglutinin cleavage for the pathogenicity of influenza virus.* Virology, 1999. **258**(1): p. 1-20.
26. Koyama, S., et al., *Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination.* J Immunol, 2007. **179**(7): p. 4711-20.
27. O'Brien, K.B., et al., *A Protective Role for Complement C3 Protein during Pandemic 2009 H1N1 and H5N1 Influenza A Virus Infection.* PLoS One, 2011. **6**(3): p. e17377.
28. Ehrhardt, C., et al., *Interplay between influenza A virus and the innate immune signaling.* Microbes Infect, 2010. **12**(1): p. 81-7.
29. Kohlmeier, J.E., et al., *Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge.* Immunity, 2010. **33**(1): p. 96-105.
30. Stark, G.R., *How cells respond to interferons revisited: from early history to current complexity.* Cytokine Growth Factor Rev, 2007. **18**(5-6): p. 419-23.
31. Abbas, A.K., S. Pillai, and A.H. Lichtman, *Cellular and molecular immunology*. 2010, Philadelphia: Saunders Elsevier. VIII, 566 s.
32. Belkaid, Y. and W. Chen, *Regulatory ripples.* Nat Immunol, 2010. **11**(12): p. 1077-8.
33. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
34. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones.* J Exp Med, 1989. **170**(6): p. 2081-95.
35. Kannanganat, S., et al., *Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells.* J Virol, 2007. **81**(16): p. 8468-76.
36. Darrah, P.A., et al., *Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major.* Nat Med, 2007. **13**(7): p. 843-50.
37. University, A. *The Biology Project, Antibody structure*. 2000; Available from: http://www.biology.arizona.edu/immunology/tutorials/antibody/structure.html.
38. MicrobiologyBytes. *Humoral Immunity*. 2004 [cited 2011; Available from: http://www.microbiologybytes.com/iandi/3b.html.
39. Kiyono, H. and S. Fukuyama, *NALT- versus Peyer's-patch-mediated mucosal immunity.* Nat Rev Immunol, 2004. **4**(9): p. 699-710.
40. Massacand, J.C., et al., *Intestinal bacteria condition dendritic cells to promote IgA production.* PLoS One, 2008. **3**(7): p. e2588.

41. Brandtzaeg, P., *Role of secretory antibodies in the defence against infections.* Int J Med Microbiol, 2003. **293**(1): p. 3-15.
42. Brandtzaeg, P., *Mucosal immunity: induction, dissemination, and effector functions.* Scand J Immunol, 2009. **70**(6): p. 505-15.
43. Jefferson, T., et al., *Neuraminidase inhibitors for preventing and treating influenza in healthy adults: a Cochrane review.* Health Technol Assess, 2010. **14**(46): p. 355-458.
44. Castillo, R., L.E. Holland, and D.A. Boltz, *Peramivir and its use in H1N1 influenza.* Drugs Today (Barc), 2010. **46**(6): p. 399-408.
45. FDA. *FDA Authorizes Emergency Use of Intravenous Antiviral Peramivir for 2009 H1N1 Influenza for Certain Patients*. 2009 [cited 13.05.11; Available from: http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm187813.htm.
46. Legemiddelhåndboka. *Norsk legemiddelhåndbok: L1.4.4.9 Zanamivir*. 2010; Available from: http://www.legemiddelhandboka.no/xml/.
47. Rang, H.P. and M.M. Dale, *Rang and Dale's pharmacology*. 6th ed. 2007, [Edinburgh]: Churchill Livingstone. xiii, 829 p.
48. WHO, *Update on oseltamivir-resistant pandemic A (H1N1) 2009 influenza virus: January 2010.* Wkly Epidemiol Rec, 2009. **85**(6): p. 37-40.
49. Smith, J.R., *Oseltamivir in human avian influenza infection.* J Antimicrob Chemother, 2010. **65 Suppl 2**: p. ii25-ii33.
50. van der Vries, E., F.F. Stelma, and C.A. Boucher, *Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus.* N Engl J Med, 2010. **363**(14): p. 1381-2.
51. Hatakeyama, S., *[Drug-resistant influenza viruses: an overview]*. Nippon Rinsho, 2010. **68**(9): p. 1671-8.
52. WHO. *Fact sheet: Influenza (sesonal)*. 2010; Available from: http://www.who.int/mediacentre/factsheets/fs211/en/index.html.
53. IFPMA. *Global Usage of Influenza Vaccines*. 2009; Available from: http://www.ifpma.org/Influenza/index.php?id=4181.
54. WHO. *Safety of pandemic A (H1N1) influenza vaccines*. 2010; Available from: http://www.who.int/vaccine_safety/topics/influenza/pandemic/h1n1_safety_assessing/Jun_2010/en/.
55. IFPMA. *The WHO Global Influenza Surveillance Network*. 2008; Available from: http://www.ifpma.org/Influenza/index.php?id=4220.
56. WHO. *WHO Global Influenza Surveillance Network*. 2010; Available from: http://www.who.int/csr/disease/influenza/surveillance/en/.
57. Prieto-Lara, E. and A. Llanos-Mendez, *Safety and immunogenicity of prepandemic H5N1 influenza vaccines: a systematic review of the literature.* Vaccine, 2010. **28**(26): p. 4328-34.
58. EMA. *NOTE FOR GUIDANCE IN HARMONISATION OF REQUIREMENTS FOR INFLUENZA VACCINES*. 1996; Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003945.pdf.
59. EMA. *GUIDELINE ON INFLUENZA VACCINES PREPARED FROM VIRUSES WITH THE POTENTIAL TO CAUSE A PANDEMIC AND INTENDED FOR USE OUTSIDE OF THE CORE DOSSIER CONTEXT*. 2007; Available from:

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003872.pdf.
60. Felleskatalogen. *Influvac, Abbot*. 2011; Available from: http://www.felleskatalogen.no/.
61. Lehmann, H.C., et al., *GuillaIN-Barre syndrome after exposure to influenza virus.* Lancet Infect Dis, 2010. **10**(9): p. 643-51.
62. Cox, R.J., K.A. Brokstad, and P. Ogra, *Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines.* Scand J Immunol, 2004. **59**(1): p. 1-15.
63. Bridges, C.B., et al., *Inactivated influenza vaccines*, in *Vaccines*, S.A.S. Plotkin, Editor. 2008. p. 265-290.
64. Webby, R.J., et al., *Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines.* Lancet, 2004. **363**(9415): p. 1099-103.
65. Bardiya, N. and J.H. Bae, *Influenza vaccines: recent advances in production technologies.* Appl Microbiol Biotechnol, 2005. **67**(3): p. 299-305.
66. Genzel, Y. and U. Reichl, *Continuous cell lines as a production system for influenza vaccines.* Expert Rev Vaccines, 2009. **8**(12): p. 1681-92.
67. Chichester, J.A., L.R. Haaheim, and V. Yusibov, *Using plant cells as influenza vaccine substrates.* Expert Rev Vaccines, 2009. **8**(4): p. 493-8.
68. EMA. *Fluenz(TM) Live attenuated influenza vaccine, nasal*. 2010; Available from: http://www.ema.europa.eu/docs/no_NO/document_library/EPAR_-_Product_Information/human/001101/WC500103709.pdf.
69. FDA. *Approved products > FluMist*. 2010; Available from: http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm094047.htm.
70. Piedra, P.A., et al., *Trivalent live attenuated intranasal influenza vaccine administered during the 2003-2004 influenza type A (H3N2) outbreak provided immediate, direct, and indirect protection in children.* Pediatrics, 2007. **120**(3): p. e553-64.
71. Belshe, R.B., et al., *Live attenuated versus inactivated influenza vaccine in infants and young children.* N Engl J Med, 2007. **356**(7): p. 685-96.
72. WHO. *Acute Respiratory Infections (Update September 2009)*. 2009; Available from: http://www.who.int/vaccine_research/diseases/ari/en/index1.html#.
73. Block, S.L., et al., *Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5-49 years of age.* Vaccine, 2008. **26**(38): p. 4940-6.
74. Girard, M., L. Palkonyay, and M.P. Kieny, *Report of the 4th meeting on the "Evaluation of pandemic influenza prototype vaccines in clinical trials." World Health Organization, Geneva, Switzerland, 14-15 February 2008.* Vaccine, 2008. **26**(39): p. 4975-7.
75. Barry, D.W., et al., *Comparative trial of influenza vaccines. I. Immunogenicity of whole virus and split product vaccines in man.* Am J Epidemiol, 1976. **104**(1): p. 34-46.
76. Dooley, M. and K.L. Goa, *Adjuvanted influenza vaccine.* BioDrugs, 2000. **14**(1): p. 61-9.
77. Kopecky-Bromberg, S.A. and P. Palese, *Recombinant vectors as influenza vaccines.* Curr Top Microbiol Immunol, 2009. **333**: p. 243-67.

78. Amorij, J.P., et al., *Development of stable influenza vaccine powder formulations: challenges and possibilities.* Pharm Res, 2008. **25**(6): p. 1256-73.
79. Huckriede, A., et al., *The virosome concept for influenza vaccines.* Vaccine, 2005. **23 Suppl 1**: p. S26-38.
80. Bungener, L., et al., *Virosome-mediated delivery of protein antigens to dendritic cells.* Vaccine, 2002. **20**(17-18): p. 2287-95.
81. Bungener, L., et al., *Virosomes in vaccine development: induction of cytotoxic T lymphocyte activity with virosome-encapsulated protein antigens.* J Liposome Res, 2002. **12**(1-2): p. 155-63.
82. Conne, P., et al., *Immunogenicity of trivalent subunit versus virosome-formulated influenza vaccines in geriatric patients.* Vaccine, 1997. **15**(15): p. 1675-9.
83. Gluck, R., et al., *Immunogenicity of new virosome influenza vaccine in elderly people.* Lancet, 1994. **344**(8916): p. 160-3.
84. Gasparini, R. and P. Lai, *Utility of virosomal adjuvated influenza vaccines: a review of the literature.* J Prev Med Hyg, 2010. **51**(1): p. 1-6.
85. McElhaney, J.E., *The unmet need in the elderly: designing new influenza vaccines for older adults.* Vaccine, 2005. **23 Suppl 1**: p. S10-25.
86. Calcagnile, S. and G.V. Zuccotti, *The virosomal adjuvanted influenza vaccine.* Expert Opin Biol Ther, 2010. **10**(2): p. 191-200.
87. Kozlowski, P.A., et al., *Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women.* Infect Immun, 1997. **65**(4): p. 1387-94.
88. Waldman, R.H. and F.M.S.P.A. Wigley, Jr., *Specificity of respiratory secretion antibody against influenza virus.* J Immunol, 1970. **105**(6): p. 1477-83.
89. Asahi-Ozaki, Y., et al., *Secretory IgA antibodies provide cross-protection against infection with different strains of influenza B virus.* J Med Virol, 2004. **74**(2): p. 328-35.
90. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
91. Song, J.H., et al., *Sublingual vaccination with influenza virus protects mice against lethal viral infection.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1644-9.
92. Cuburu, N., et al., *Sublingual immunization induces broad-based systemic and mucosal immune responses in mice.* Vaccine, 2007. **25**(51): p. 8598-610.
93. van Ginkel, F.W., et al., *Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues.* J Immunol, 2000. **165**(9): p. 4778-82.
94. van Ginkel, F.W., et al., *EnterotoxIN-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract.* Infect Immun, 2005. **73**(10): p. 6892-902.
95. Mantle, J.A., et al., *Isosorbide dinitrate for the relief of severe heart failure after myocardial infarction.* Am J Cardiol, 1976. **37**(2): p. 263-8.
96. Smith, I., J.A. Rees, and A.J. Winfield, *Pharmaceutical practice*. 4th ed. 2009, Edinburgh: Churchill Livingstone Elsevier. XIX, 643 s.
97. Radulovic, S., et al., *Sublingual immunotherapy for allergic rhinitis.* Cochrane Database Syst Rev, 2010(12): p. CD002893.

98. Kweon, M.N., *Sublingual mucosa: A new vaccination route for systemic and mucosal immunity.* Cytokine, 2011. **54**(1): p. 1-5.
99. PNAS. *In this issue*. 2008; Available from: http://www.pnas.org/content/105/5/1387/F2.small.gif&imgrefurl=.
100. Atmar, R.L. and W.A. Keitel, *Adjuvants for pandemic influenza vaccines.* Curr Top Microbiol Immunol, 2009. **333**: p. 323-44.
101. Manzoli, L., et al., *Immunogenicity and adverse events of avian influenza A H5N1 vaccine in healthy adults: multiple-treatments meta-analysis.* Lancet Infect Dis, 2009. **9**(8): p. 482-92.
102. O'Hagan, D.T. and N.M. Valiante, *Recent advances in the discovery and delivery of vaccine adjuvants.* Nat Rev Drug Discov, 2003. **2**(9): p. 727-35.
103. Aguilar, J.C. and E.G. Rodriguez, *Vaccine adjuvants revisited.* Vaccine, 2007. **25**(19): p. 3752-62.
104. Dubensky, T.W., Jr. and S.G. Reed, *Adjuvants for cancer vaccines.* Semin Immunol, 2010. **22**(3): p. 155-61.
105. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants.* Curr Opin Immunol, 2009. **21**(1): p. 23-9.
106. Vogel, F.R. and S.L. Hem, *Immunological adjuvants*, in *Vaccines*, S.A.S. Plotkin, Editor. 2008, Saunders: Philadelphia. p. 59-71.
107. Kool, M., et al., *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells.* J Exp Med, 2008. **205**(4): p. 869-82.
108. Li, H., et al., *Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3.* J Immunol, 2008. **181**(1): p. 17-21.
109. Serre, K., et al., *Molecular differences between the divergent responses of ovalbumIN-specific CD4 T cells to alum-precipitated ovalbumin compared to ovalbumin expressed by Salmonella.* Mol Immunol, 2008. **45**(13): p. 3558-66.
110. Leroux-Roels, I., et al., *Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial.* Lancet, 2007. **370**(9587): p. 580-9.
111. Leroux-Roels, I., et al., *Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine.* PLoS One, 2008. **3**(2): p. e1665.
112. Yang, P., et al., *Cross-clade protection against HPAI H5N1 influenza virus challenge in BALB/c mice intranasally administered adjuvant-combined influenza vaccine.* Vet Microbiol, 2010. **146**(1-2): p. 17-23.
113. Moris, P., et al., *H5N1 Influenza Vaccine Formulated with AS03(A) Induces Strong Cross-Reactive and Polyfunctional CD4 T-Cell Responses.* J Clin Immunol, 2010.
114. O'Hagan, D.T., *MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection.* Expert Rev Vaccines, 2007. **6**(5): p. 699-710.
115. O'Hagan, D.T., et al., *MF59 adjuvant: the best insurance against influenza strain diversity.* Expert Rev Vaccines, 2011. **10**(4): p. 447-62.
116. Madhun, A.S., et al., *An adjuvanted pandemic influenza H1N1 vaccine provides early and long term protection in health care workers.* Vaccine, 2010. **29**(2): p. 266-73.
117. Legemiddelverket. *Pandemrix, Summary of Product characteristics*. 2011 13.05.11]; Available from:

http://www.legemiddelverket.no/custom/Preparatsok/prepSearch____80333.aspx?SearchID=80983466-c4da-4182-994e-edb8cb463d60.

118. THL. *National Narcolepsy Task Force Interim Report 31 January 2011*. 2011; Available from: http://www.thl.fi/thl-client/pdfs/dce182fb-651e-48a1-b018-3f774d6d1875.
119. Morein, B., et al., *Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses.* Nature, 1984. **308**(5958): p. 457-60.
120. Drane, D. and M. Pearse, *The ISCOMATRIX (TM) adjuvant*, in *Immunopotentiators in modern vaccines*, V.E.J.C. Schijns and D.T. O'Hagan, Editors. 2006, Elsevier Academic Press: Amsterdam. p. xi, 371 s.
121. Sjolander, A. and J.C. Cox, *Uptake and adjuvant activity of orally delivered saponin and ISCOM vaccines.* Adv Drug Deliv Rev, 1998. **34**(2-3): p. 321-338.
122. McKenzie, A., M. Watt, and C. Gittleson, *ISCOMATRIX() vaccines: Safety in human clinical studies.* Hum Vaccin, 2010. **6**(3).
123. Madhun, A.S., et al., *Intramuscular Matrix-M-adjuvanted virosomal H5N1 vaccine induces high frequencies of multifunctional Th1 CD4+ cells and strong antibody responses in mice.* Vaccine, 2009. **27**(52): p. 7367-76.
124. Pedersen, G., et al., *Matrix-M adjuvanted virosomal H5N1 vaccine confers protection against lethal viral challenge in a murine model.* Influenza and Other Respiratory Viruses, 2011: p. no-no.
125. Chen, W., R. Kuolee, and H. Yan, *The potential of 3',5'-cyclic diguanylic acid (c-di-GMP) as an effective vaccine adjuvant.* Vaccine, 2010. **28**(18): p. 3080-5.
126. Ebensen, T., et al., *The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant.* Clin Vaccine Immunol, 2007. **14**(8): p. 952-8.
127. Karaolis, D.K., et al., *Bacterial c-di-GMP is an immunostimulatory molecule.* J Immunol, 2007. **178**(4): p. 2171-81.
128. McWhirter, S.M., et al., *A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP.* J Exp Med, 2009. **206**(9): p. 1899-911.
129. Ross, P., et al., *The cyclic diguanylic acid regulatory system of cellulose synthesis in Acetobacter xylinum. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives.* J Biol Chem, 1990. **265**(31): p. 18933-43.
130. Fan, Z. and R.A. Jones, *Synthesis of cyclic dinucleotides by an H-phosphonate method in solution.* Nucleosides & Nucleotides, 1996. **15**(11-12): p. 1679-1686.
131. Crotty, S., et al., *Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system.* J Immunol Methods, 2004. **286**(1-2): p. 111-22.
132. Taylor, H.P. and N.J. Dimmock, *Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG.* J Exp Med, 1985. **161**(1): p. 198-209.
133. Claas, E.C., et al., *Human influenza virus A/HongKong/156/97 (H5N1) infection.* Vaccine, 1998. **16**(9-10): p. 977-8.

134. Boni, M.F., et al., *Modelling the progression of pandemic influenza A (H1N1) in Vietnam and the opportunities for reassortment with other influenza viruses.* BMC Med, 2009. **7**: p. 43.
135. Moingeon, P., et al., *Immune mechanisms of allergen-specific sublingual immunotherapy.* Allergy, 2006. **61**(2): p. 151-65.
136. Bresson, J.L., et al., *Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial.* Lancet, 2006. **367**(9523): p. 1657-64.
137. Goji, N.A., et al., *Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant.* J Infect Dis, 2008. **198**(5): p. 635-41.
138. Lin, J., et al., *Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial.* Lancet, 2006. **368**(9540): p. 991-7.
139. Yao, Q., et al., *Intranasal immunization with SIV virus-like particles (VLPs) elicits systemic and mucosal immunity.* Vaccine, 2002. **20**(19-20): p. 2537-45.
140. Kohlmeier, J.E. and D.L. Woodland, *Immunity to respiratory viruses.* Annu Rev Immunol, 2009. **27**: p. 61-82.
141. Madhun, A., *Intranasal cdi-GMP-adjuvanted plant-derived H5 vaccine induces high frequencies of multifunctional Th1 CD4+ cells and strong mucosal and systemic antibody responses in mice.* Vaccine (in press), 2011.
142. Wagner, D.K., et al., *Analysis of immunoglobulin G antibody responses after administration of live and inactivated influenza A vaccine indicates that nasal wash immunoglobulin G is a transudate from serum.* J Clin Microbiol, 1987. **25**(3): p. 559-62.
143. Brandtzaeg, P., *Induction of secretory immunity and memory at mucosal surfaces.* Vaccine, 2007. **25**(30): p. 5467-84.
144. Asanuma, H., et al., *Long- and short-time immunological memory in different strains of mice given nasally an adjuvant-combined nasal influenza vaccine.* Vaccine, 2007. **25**(39-40): p. 6975-80.
145. Ichinohe, T., et al., *Intranasal administration of adjuvant-combined vaccine protects monkeys from challenge with the highly pathogenic influenza A H5N1 virus.* J Med Virol, 2010. **82**(10): p. 1754-61.
146. Brokstad, K.A., et al., *IgA, IgA subclasses, and secretory component levels in oral fluid collected from subjects after parental influenza vaccination.* J Infect Dis, 1995. **171**(4): p. 1072-4.
147. Mazanec, M.B., et al., *Transport of serum IgA into murine respiratory secretions and its implications for immunization strategies.* J Immunol, 1989. **142**(12): p. 4275-81.
148. Hovden, A.O., et al., *Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice.* Scand J Immunol, 2005. **62**(4): p. 342-52.
149. Ramphal, R., et al., *Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis.* Infect Immun, 1979. **25**(3): p. 992-7.
150. Wrammert, J., et al., *Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection.* J Exp Med, 2011. **208**(1): p. 181-93.

151. Corti, D., et al., *Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine.* J Clin Invest, 2010. **120**(5): p. 1663-73.
152. Abbas, A.K., S. Pillai, and A.H. Lichtman, *B cell activation and antibody production*, in *Cellular and molecular immunology*. 2010. p. 215-241.
153. Amanna, I.J., N.E. Carlson, and M.K. Slifka, *Duration of humoral immunity to common viral and vaccine antigens.* N Engl J Med, 2007. **357**(19): p. 1903-15.
154. Kistner, O., et al., *A whole virus pandemic influenza H1N1 vaccine is highly immunogenic and protective in active immunization and passive protection mouse models.* PLoS One, 2010. **5**(2): p. e9349.
155. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
156. Saluja, V., et al., *Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant.* AAPS J, 2010. **12**(2): p. 109-16.
157. Huber, V.C., et al., *Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza.* Clin Vaccine Immunol, 2006. **13**(9): p. 981-90.
158. von Bubnoff, A., *Antibodies: beyond neutralization.* IAVI Rep, 2010. **14**(1): p. 8-12.
159. Coffman, R.L., D.A. Lebman, and B. Shrader, *Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes.* J Exp Med, 1989. **170**(3): p. 1039-44.
160. Sonoda, E., et al., *Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production.* J Exp Med, 1989. **170**(4): p. 1415-20.
161. Weaver, C.T., *Th17: The ascent of a new effector T-cell subset. Preface.* Eur J Immunol, 2009. **39**(3): p. 634-6.
162. Hamada, H., et al., *Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge.* J Immunol, 2009. **182**(6): p. 3469-81.
163. Zygmunt, B.M., et al., *Intranasal immunization promotes th17 immune responses.* J Immunol, 2009. **183**(11): p. 6933-8.
164. McKinstry, K.K., et al., *IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge.* J Immunol, 2009. **182**(12): p. 7353-63.
165. Forbes, E.K., et al., *Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice.* J Immunol, 2008. **181**(7): p. 4955-64.
166. Tchilian, E.Z., et al., *Immunogenicity and protective efficacy of prime-boost regimens with recombinant (delta)ureC hly+ Mycobacterium bovis BCG and modified vaccinia virus ankara expressing M. tuberculosis antigen 85A against murine tuberculosis.* Infect Immun, 2009. **77**(2): p. 622-31.
167. Sollid, L.M., et al., *Interferon-gamma enhances expression of secretory component, the epithelial receptor for polymeric immunoglobulins.* J Immunol, 1987. **138**(12): p. 4303-6.
168. Loman, S., et al., *InterleukIN-4 and interferon-gamma synergistically increase secretory component gene expression, but are additive in stimulating secretory immunoglobulin A release by Calu-3 airway epithelial cells.* Immunology, 1999. **96**(4): p. 537-43.
169. Schjerven, H., P. Brandtzaeg, and F.E. Johansen, *A novel NF-kappa B/Rel site in intron 1 cooperates with proximal promoter elements to mediate TNF-*

alpha-induced transcription of the human polymeric Ig receptor. J Immunol, 2001. **167**(11): p. 6412-20.

170. Brown, R.E., *Interaction of nutrition and infection in clinical practice.* Pediatr Clin North Am, 1977. **24**(1): p. 241-52.
171. Avitsur, R., J.W. Mays, and J.F. Sheridan, *Sex differences in the response to influenza virus infection: modulation by stress.* Horm Behav, 2011. **59**(2): p. 257-64.
172. Ha, C.L. and B. Woodward, *Depression in the quantity of intestinal secretory IgA and in the expression of the polymeric immunoglobulin receptor in caloric deficiency of the weanling mouse.* Lab Invest, 1998. **78**(10): p. 1255-66.