# The immunogenicity of an intranasal adjuvanted pandemic avian influenza vaccine in murine model

by
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# Abstract

Influenza vaccination remains the primary method for the prevention of influenza and the severe complications related to the disease. Development of an intranasal vaccination strategy will not only prevent the disease but also block the viral entry to the host and the horizontal transmission. Thus, intranasal pandemic vaccines may induce of humoral and cellular immune responses at the both systemic and mucosal levels. In this study, we investigated and compared the mucosal, humoral and cytokine immune responses induced in mice after intranasal vaccination with subunit influenza vaccine formulated with or without a novel "c-di-GMP" adjuvant.

Forty BALB/c mice were divided into two equally sized groups. Mice were vaccinated intranasally with two doses at three week interval of 15 μg of A/Vietnam/1194/2004 (H5N1) non-adjuvanted subunit vaccine or c-di-GMP adjuvanted antigen. Nasal wash and serum samples were collected at various time points after vaccination and used in the ELISA, whilst sera was in addition used in the HI assay. Supernatants from *in vitro* activated splenocytes were used to determine the concentration of IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-17 by Bio-plex bead immunoassay.

In this study, we showed that intranasal immunisation of subunit vaccine formulated with c-di-GMP adjuvant induced a more rapid mucosal and humoral immune response than the subunit vaccine administered alone. Additionally, the study showed that the presence of the mucosal adjuvant elicited a Th1 profile, which suggests promotion of cell-mediated immune response. On the other hand, immunization with subunit vaccine alone induced low antibody responses and a Th2-type immune response.
The results obtained in this study indicate that c-di-GMP is a promising adjuvant for the future development of intranasal pandemic influenza vaccines.

# Acknowledgements

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Nam Cong Pham

# Abbreviations

| | |
|---|---|
| ADCC | Antibody-Dependent Cell-mediated Cytotoxicity |
| AEC | 9-amino 3-ethyl carbazole |
| Ag | Antigen |
| APC | Antigen Presenting Cell |
| ASC | Antibody Secreting Cell |
| BSA | Bovine Serum Albumin |
| C-di-GMP | Cyclic dimeric GMP |
| $CD4^+$ | Cluster Determinant 4 positive cell |
| $CD8^+$ | Cluster Determinant 8 positive cell |
| CTL | Cytotoxic T Lymphocyte |
| DC | Dentritic Cell |
| dIgA | Dimeric IgA |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ER | Endoplasmic Reticulum |
| FBS | Foetal Bovine Sera |
| GMT | Geometric Mean Titre |
| HA | Haemagglutinin |
| HI | Haemagglutination Inhibition Assay |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IM | Intramuscular |
| IN | Intranasal |
| LM | Lymphocyte medium |
| M1 | Matrix protein type 1 |
| M2 | Matrix protein type 2 |
| MHC | Major Histocompatibility Complex |
| NA | Neuraminidase |
| NCS | Newborn Calf Serum |
| NICs | National Influenza Centres |
| NP | Nucleoprotein |
| NS1 | Non Structural protein 1 |
| NS2 (NEP) | Non Structural protein 2 (Nuclear Export Protein) |
| NW | Nasal Wash |

| | |
|---|---|
| PA | Polymerase Acid |
| PB1 | Polymerase Base 1 |
| PB2 | Polymerase Base 2 |
| PBS | Phosphate Buffer Saline |
| PMA | Phorbol Myristate Acetate |
| | |
| RDE | Receptor Destroying Enzyme |
| RNP | Ribonucleoprotein |
| | |
| sIgA | Secretory IgA |
| ssRNA | Single strand RNA |
| | |
| T | Tween |
| Tc | cytotoxic T cell |
| TCR | T cell receptor |
| TGF | Transforming growth factor |
| Th | T helper |
| Thp | T helper precursor |
| | |
| WHO | World Health Organization |
| WHO CCs | World Health Organization Collaborating Centres |

# Contents

# 1. Introduction

## 1.1 Introduction to influenza

Influenza is a highly contagious acute respiratory infection and is one of the major causes of disease worldwide. Many years of research have produced extensive knowledge about the influenza virus, vaccines and immunity, but the virus is still capable of surprising us as witnessed by the avian influenza sporadic human transmission and swine origin influenza A (H1N1) ongoing pandemic. In annual influenza epidemics 5-15 % of the world population are infected with upper respiratory tract infection resulting in between 250 000 and 500 000 deaths [1]. Influenza infections in high-risk groups (elderly and chronically ill individuals) cause the highest number of hospitalizations and deaths [1]. At unpredictable time intervals, a novel influenza A virus with the ability to infect humans may emerge and cause a worldwide influenza outbreak (pandemic). This has occurred three times during the 20$^{th}$ century. The Spanish flu pandemic (1918) was caused by influenza A virus H1N1 and had the highest mortality, causing 40-50 million deaths worldwide. The Asian flu pandemic (1957) was caused by the virus subtype H2N2, which resulted in a total number of deaths that probably exceeded 1 million people. The Hong Kong flu pandemic (1968-70), caused by virus type H3N2, had a lower death rate estimated at 500,000 deaths [2]. Now 41 years later, the WHO ha declared a swine origin influenza A(H1N1) pandemic.

In 1997, 18 people were infected with H5N1 from infected poultry and six of these died. The Hong Kong authorities took swift action and destroyed all poultry in the province stopping further human infection. In mid-2003, the outbreak of a highly pathogenic H5N1 influenza virus began in south-east Asia. More than 120 million birds died or were destroyed after the initial spread of H5N1 in poultry [5]. To date 433 people have been infected mainly through direct contact with infected birds and the death rate is approximately 60%. All prerequisites for an influenza pandemic defined by WHO have been met H5N1, except efficient human-to-human transmission. The human infection by H5N1 led to an extensive research into influenza and new pandemic vaccine candidates [3, 4].

## 1.2 The influenza virus

Influenza virus belongs to the family *Orthomyxoviridae* and is divided into three genera, namely influenza A, B and C. Such a division is based on antigenic differences in their internal proteins, nucleoproteins (NP) and matrix (M) [6]. The current thesis will only discuss influenza A virus as this is the focus of the work. Influenza A is further classified into subtypes which is based on its surface glycoproteins, HA (Haemagglutinin) and NA (Neuraminidase). To date, there are sixteen known subtypes of HA (H1-16) and nine subtypes of NA (N1-9), all of which are found in birds. Influenza A viruses are found in a whole range of vertebrates with waterfowl as the main reservoir [7], see fig. 1.1.

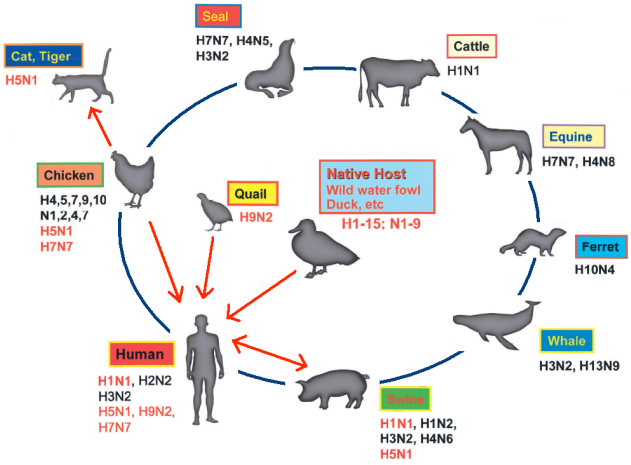


Figure 1.1 **Ecology of influenza A.**
Subtypes shown in red are most likely candidates to start the next pandemic. Adapted from [8].

### 1.2.1 Nomenclature

The nomenclature system for influenza virus is defined by the WHO as follows: type of virus, virus host (if non human), geographical isolation area, isolation number, the year isolation, and the subtypes of HA and NA in parenthesis. For instance, A/duck/Hunan/795/2002 (H5N1) which describes that it is influenza A virus, isolated from chicken in Hong Kong in 1997, with an isolation number 33 and subtype H5N1. The same nomenclature system is used for humans, but the host is omitted, e.g. A/Brisbane/10/2007 (H3N2) [73].

## 1.2.2 Viral structure and genome

Influenza A is an enveloped virus with a dense layer of protein spikes (HA and NA) [6]. Influenza A has eight negative sense ssRNA (single strand RNA) linear segments. Each of these segments expresses essential proteins that participate in the virus life cycle (fig. 1.2 and table 1.1) [6]. The two major surface glycoproteins HA and NA will be described in more detail below.

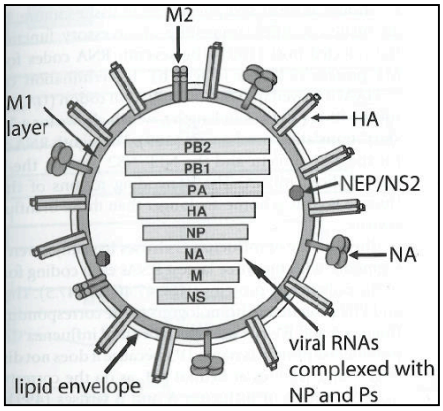


Figure 1.2 **A schematic figure of the influenza A virus that shows the RNA segments and their expressed proteins and where these proteins are localised in the virus.** HA, NA and M2 protein are embedded in the lipid layer. M1 protein is detected beneath the envelope. Nuclear export protein/non structural (NEP/NS) is also associated with the virus. The nucleoproteins/polymerases form a complex with the RNA [6].

### 1.2.2.1 Haemagglutinin (HA)

Haemagglutinin (fig. 1.3) is named after its ability to agglutinate erythrocytes (haemagglutination) [10]. The HA has two major functions in the virus life cycle. Firstly, HA binds to the sialic acid-containing receptor on the host cell surface bringing the virion in close proximity to the host cell membrane such that the infection process can be initiated. Secondly, HA induces the fusion of the endocytosed virion with the endosomal membrane allowing release of the viral nucleocapsid into the cytoplasm [10]. Although, there are differences in the HA subtypes, they all have similar conformation and structure [1]. The HA is the primary target for neutralizing antibodies. Therefore the HA is the main component of vaccines and current vaccines are thus standardised according to the HA concentration [10].

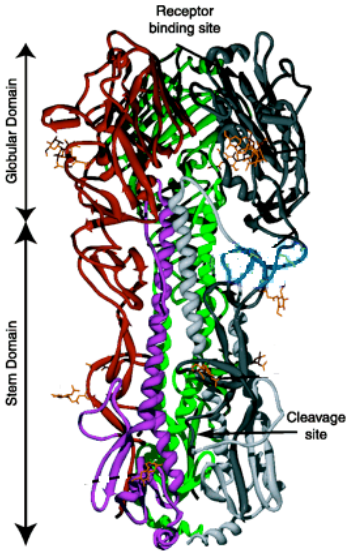


Figure 1.3 **The HA is shown in its trimeric form.** Modified from [51].

### 1.2.2.2 Neuraminidase (NA)

Neuraminidase is a homotetrameric glycoprotein with neuraminidase enzymatic activity. Each enzyme subunit consists of 453 amino acids arranged in a six β-sheet system, where each system is organized in four antiparallel β sheets like blades in a propeller [11]. The NA major functions are helping the virus to penetrate through the mucus layer to the respiratory epithelium [74] and the NA is also responsible for enzymatic removal of sialic acid from the host cell surface which allows the release of the virus from the host cell [75]. NA is the second most important surface antigen and is the target of antiviral agents, Zanamivir and Oseltamivir [12]. The NA is present in influenza vaccines but its concentration is not quantified.

| **Segment number** | **Gene product** | **Major function** |
|---|---|---|
| 1 | Basic polymerase protein 2, PB2 | Part of the polymerase complex, polymerase activity, RNA cap binding |
| 2 | Basic polymerase protein 1, PB1 | Part of the polymerase complex, transcriptase activity. |
| 3 | Acidic polymerase, PA | Part of the polymerase complex, transcriptase activity. |
| 4 | Haemagglutinin, HA | Surface glycoprotein that binds to sialic acid on the host cell surface, fusion with the cellular membrane. Major target for antibodies. |
| 5 | Nucleoprotein, NP | Encapsulates the viral RNA in the RNP. |
| 6 | Neuraminidase, NA | Release of the progeny viral particles from infected cells. |
| 7 | Matrix protein 1, M1 | Viral matrix protein, promotes vRNP nuclear export. |
| | Matrix protein 2, M2 | Membrane protein that has $H^+$ ion channel activity. |
| 8 | Nonstructural protein 1, NS1 | Regulation of cellular and viral protein expression. |
| | Nonstructural protein 2, NS2 (NEP) | RNP nuclear export. |

Table 1.1 **Influenza A gene products and their functions.** Adapted from [6].

## 1.3 Influenza epidemiology

Influenza virus undergoes continuous antigenic variations of the surface glycoproteins, HA and NA, which enable the virus to re-infect its host. These antigenic variations are essential for the influenza evolution and survival, and arise by two mechanisms: antigenic drift and antigenic shift. Antigenic drift causes interpandemic influenza which are localized outbreaks or epidemics; whereas the latter, antigenic shift results in a pandemic influenza [19].

### 1.3.1 Antigenic drift and epidemics

The influenza virus genomes consist of RNA segments which are transcribed by their own RNA polymerase that lacks proofreading ability. This results in a higher potential for accumulation of point mutation in the HA and NA surface glycoproteins that may lead to changes in the antigenicity of these proteins, termed antigenic drift. Some mutations cause changes that permit the virus to evade immune recognition, neutralization by antibodies generated against previous viral strains and leading to repeated interpandemic outbreaks (fig. 1.4). Due to the antigenic drift in the most recent circulating seasonal influenza viruses A (H1N1), A (H3N2) and influenza B viruses, the vaccines' antigen composition has to be annually adjusted [20].

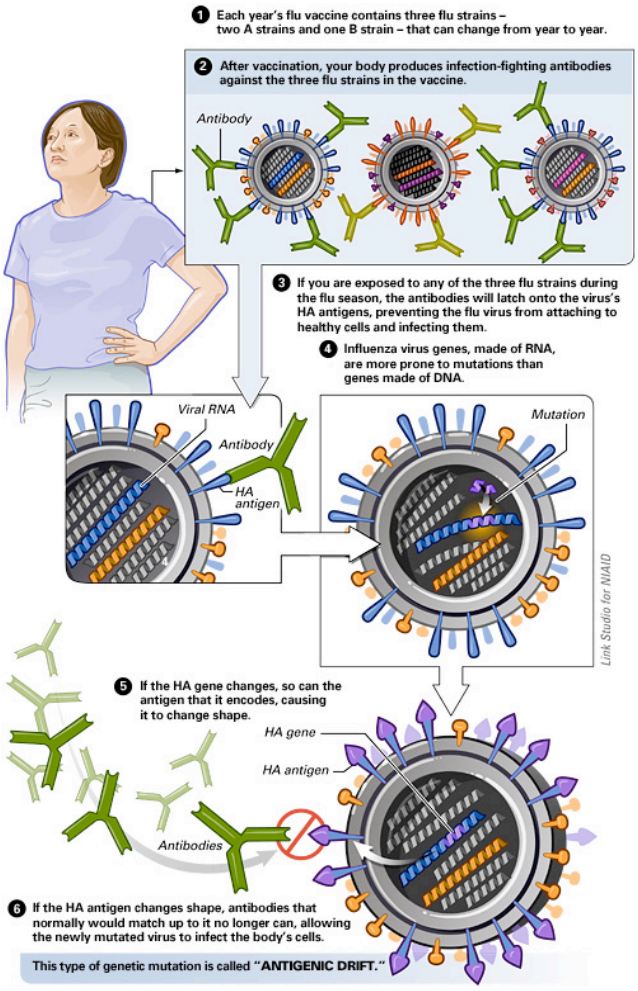


Figure 1.4 **Antigenic drift.** Illustration courtesy of "National Institute of Allergy and Infectious Diseases" [18].

Antigenic drift leads to interpandemic outbreaks and typically new viruses prevail for two to five years before being replaced by a different variant [7]. Influenza is a seasonal respiratory disease. In the northern hemisphere, influenza outbreaks usually occur in the period November to April, and between May to September in the southern parts of the world. During the winter months in the northern hemisphere, it is believed that the low relative indoor humidity prolongs the survival of influenza in aerosols and may contribute to the seasonal pattern [20]. An epidemic is characterized when the number of influenza like illnesses increases above the threshold for the normal number of influenza like illnesses in this period [37].

In Norway, the monitoring of the influenza activity is obtained by reports from the general practitioners [22]. In Norway, a seasonal influenza outbreak causes approximately 1300 deaths annually [29].

### 1.3.2 Antigenic shift and pandemic

Antigenic shift occurs when a novel influenza A virus is introduced into the human population. The new virus results in a high infection rate in the immunologically naïve population as it is antigenically distinct from the previously circulating strains, leading to a pandemic [20, 23]. This has occurred three times in the last century and a pandemic has just started in 2009 as described in the "Introduction to influenza". Wild birds are the natural reservoir of all influenza A subtypes. Antigenic shift is typically a reassortment between human and avian viruses that caused the 1957 and 1968 pandemics (is though to occur in pigs). Another

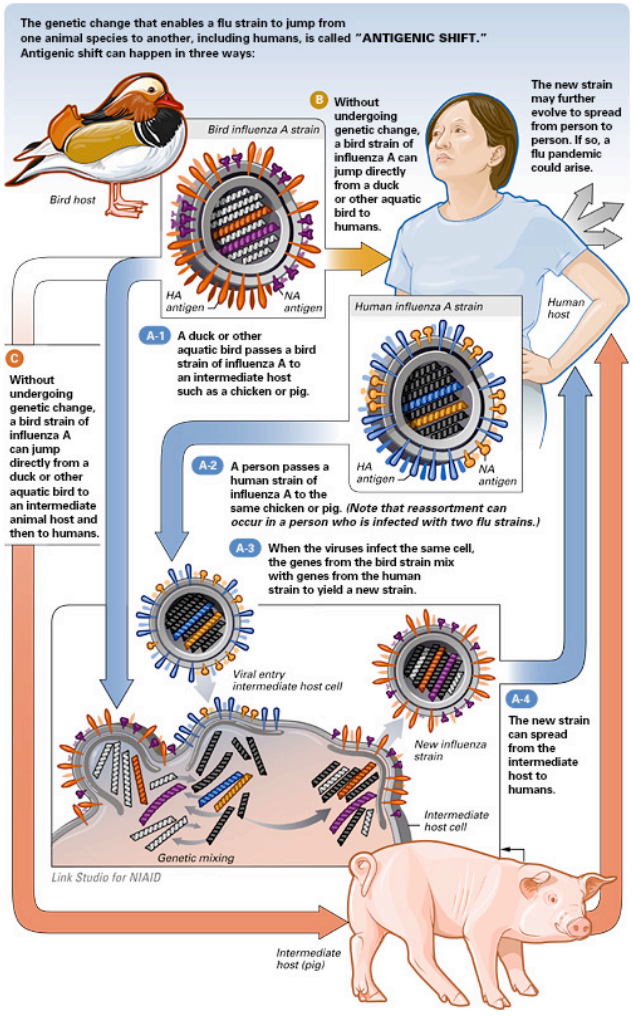


Fig. 1.5 **Antigenic shift.** Illustration courtesy of "National Institute of Allergy and Infectious Diseases" [24].

mechanism for antigenic shift is the direct transmission of swine or avian influenza virus to humans and their establishment in the human population, see figure 1.5. By using phylogenic evidence, the "Spanish influenza" is thought to have been caused by the direct introduction of an avian virus into the human population [7]. The segmented nature of the virus allows for reassortment of two viruses which co-infect one cell.

A pandemic is an epidemic of infectious disease that spreads throughout the world. The World Health Organization (WHO) has defined three prerequisites for an influenza pandemic [26]: 1) a novel influenza virus emerges in the general population to which it is immunologically naïve, 2) this novel virus must be able to replicate in humans and cause disease, and 3) the virus must have effective human-to-human transmission.

The WHO has divided the occurrence of a pandemic into six phases (fig. 1.6). Phase six signifies the declaration of a new influenza pandemic. Since the 1968 influenza pandemic, experts at the WHO and elsewhere believe that the world is now closer to another pandemic. The declaration of a pandemic of swine origin influenza A(H1N1) in June 2009 confirmed this fear. The WHO ascertains that the world is presently in phase 3 for H5N1: This new H5N1 influenza virus is capable of spreading from animals to humans but, fortunately, it does not transmit from humans to humans efficiently [27].

| | | |
|---|---|---|
| Inter-pandemic phase | Low risk of human cases | 1 |
| New virus in animals, no human cases | Higher risk of human cases | 2 |
| Pandemic alert | No or very limited human-to-human transmission | 3 |
| New virus causes human cases | Evidence of increased human-to-human transmission | 4 |
| | Evidence of significant human-to-human transmission | 5 |
| Pandemic | Efficient and sustained human-to-human transmission | 6 |

Figure 1.6 **Phases in the development of an influenza pandemic and the current status for avian H5N1 virus** [27].

## 1.4 WHO surveillance system

WHO utilizes The WHO Global Influenza Surveillance Network to monitor which influenza strains are the most likely potential strains to cause an outbreak during the next influenza season. The WHO Global Influenza Surveillance Network is mainly composed of National Influenza Centres (NICs) and four WHO Collaborating Centres (WHO CCs). NICs, which consist of 125 institutions from 96 countries, takes samples from 175 000 patients who have influenza-like-illness [45]. The isolates are forwarded to WHO CCs for antigenic and genetic analyses [45].

Twice annually, the WHO recommends the strains for inclusion in influenza vaccines for the subsequent influenza season. Annually, approximately 500 million doses of influenza vaccine, based on the WHO's recommendation are produced.
To date, the WHO has registered 433 cases of influenza H5N1 infection with 262 deaths. Most of the incidents have occurred in South-East Asia, Indonesia, Vietnam, Thailand and South China. The only other country with a substantial number of infection and deaths is Egypt [46]. See table 1.2.

| Year | Country/Region | Number of cases | Number of deaths |
|---|---|---|---|
| 2003 | China, Vietnam | 4 | 4 |
| 2004 | Thailand, Vietnam | 46 | 32 |
| 2005 | Cambodia, China, Indonesia, Thailand, Vietnam | 98 | 43 |
| 2006 | Azerbaijan, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Thailand, Turkey | 115 | 79 |
| 2007 | Cambodia, China, Egypt, Indonesia, Lao People's Democratic Republic, Myanmar, Nigeria, Pakistan, Vietnam | 88 | 59 |
| 2008 | Bangladesh, Cambodia, China, Egypt, Indonesia, Vietnam | 44 | 33 |
| 2009 | China, Egypt, Vietnam | 38 | 12 |

Table 1.2 **The number of human cases and deaths from influenza A H5N1 since 2003**. Adapted from [46] (8th of June 2009).

## 1.5 Widespread illness

The main route of human infection with H5N1 is direct contact with infected poultry, or with surfaces and objects contaminated by their faeces. Many households in rural areas keep small poultry flocks. These roam freely inside the homes or share the outdoor area with playing children and are a significant cause of infection. Moreover, many households in Asia depend on poultry for income and food. When signs of illness appear in a flock, birds will be sold or slaughtered and consumed. This is a problem as exposure to virus often occurs during preparation of poultry for cooking, slaughtering, butchering, and defeathering [3].

## 1.6 Clinical manifestation of influenza

### 1.6.1 Seasonal influenza

Influenza infection can be either symptomatic or asymptomatic, and does not cause persistent or latent infection [7]. Transmission of influenza virus from person to person occurs mainly via droplets in the air and small particles excreted when infected individuals cough or sneeze. The influenza virus normally infects epithelial cells of the upper respiratory tract. The incubation period generally is between one to four days [20], and the infected individual is infectious from the day symptoms occur and until three to five days afterwards. The clinical picture varies, but generally it is characterized by sudden onset of high fever, headache and severe malaise, myalgia, sore throat, non-productive cough, and rhinitis. Healthy people recover within one to two weeks without requiring any medical treatment. Influenza poses a serious risk in the very young children (<2 years old), the elderly (>65 years old) and people suffering from medical conditions such as diabetes, cancer, lung disease, kidney and heart problems. Infection may lead to severe complications of underlying diseases, pneumonia and death in people within these groups and these subjects are therefore recommended for annual vaccination. Influenza can cause viral pneumonia or secondary bacterial pneumonia caused by infections such as *Streptococcus pneumoniae, Staphylococcus aureus* and *Hemophilus influenzae* [15].

### 1.6.2 Avian influenza

H5N1 influenza infection in humans is still poorly understood. Current data has indicated that the incubation period for H5N1 may be longer than seasonal influenza, from two to eight days and possibly as long as 17 days. Currently, WHO has recommended an incubation period of seven days. Initial symptoms of H5N1 influenza are similar to influenza-like symptoms, but exceptions have been reported in some patients such as diarrhoea, vomiting, chest pain, abdominal pain, and bleeding from the nose and gums. Another symptom that appears to be more common in H5N1 influenza than in seasonal influenza is watery diarrhoea without blood. The development of manifestations in the lower respiratory tract is observed in many patients early in illness. Other symptoms that are commonly seen in patients are respiratory distress, hoarse voice, and crackling sound when inhaling [3]. The patients infected by H5N1 virus often are subjected to a very aggressive clinical course. They often develop primary viral pneumonia and multi-organ failure. The disease is characterized by rapid deterioration and high fatality [35].

### 1.6.3 Novel influenza A (H1N1) virus

Recently a novel influenza A/(H1N1) virus has infected humans and has caused a serious influenza outbreak. By 29$^{th}$ of June 2009 more than 70893 infected cases (of these 311 deaths) distributed in 115 countries have been officially reported [48]. The influenza A/(H1N1) virus is new, and it has not been found in pigs or humans before. The influenza A/(H1N1) viruses that have been characterized so far, have showed to be sensitive to oseltamivir, but resistant to amantadine and rimantadine [49]. The WHO raised the current level of influenza pandemic alert to phase 6 [50], declaring the start of a new pandemic.

## 1.7 The immune response to influenza

### 1.7.1 Innate immune response

The early line of defence against pathogens is provided by the innate immunity (also known as natural or native immunity). The innate immune system serves two important functions. It initiates the response to microbes that may prevent, control, or eliminate infection of the host. It stimulates as well the adaptive immune response to pathogens (i.e. influenza). The innate immune response does not have the ability to "remember" upon repeated exposure to an antigen and is non-specific [31].

### 1.7.2 Adaptive immune response

In contrast to innate immunity, adaptive immunity is antigen-specific and is capable of recognizing and providing a more rapid and vigorous response to repeated exposures of the same microbe. Adaptive immunity consists of two types, humoral and cell-mediated immune responses. The first is mediated by antibodies produced by B cells, which is required for the neutralization of the influenza virus and therefore for the prevention of clinical influenza infection [86]. The latter is characterized by activation of T cells. Recovery from infection is mediated by cytotoxic T cells that induce lysis of influenza infected cells [36]. There is a connection between these two arms of adaptive immune system, which is essential to mount an effective immune response, see fig. 1.7.

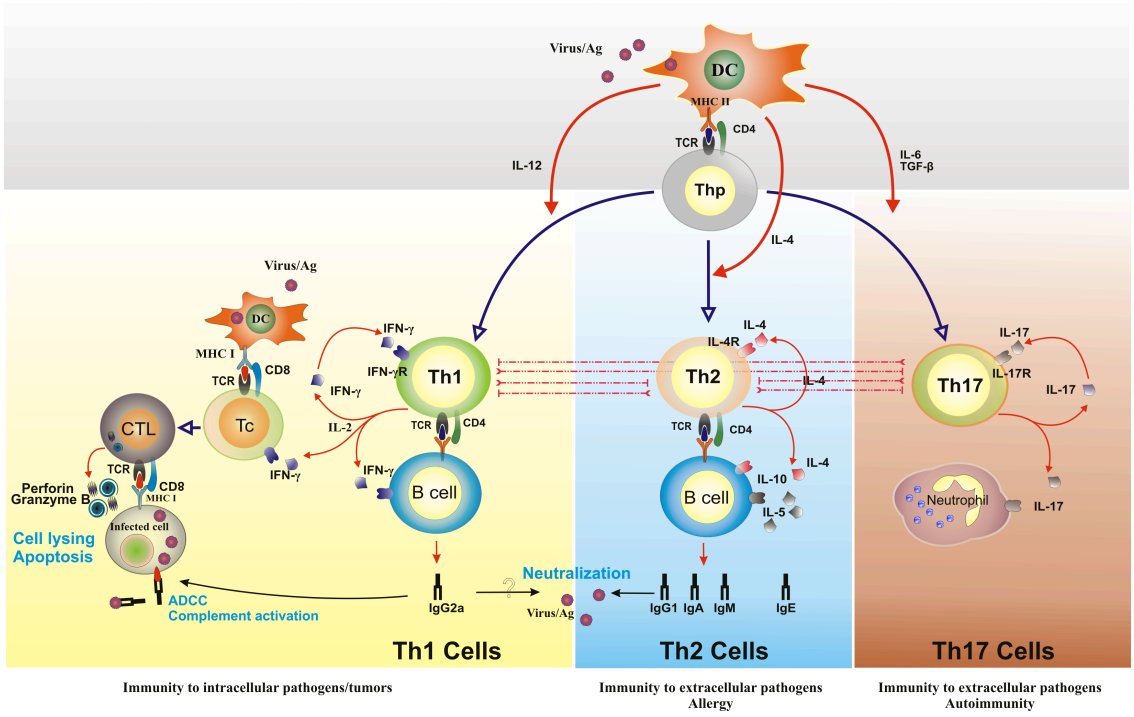


Figure 1.7**A schematic overview of the adaptive immune response to influenza virus.** The scheme describes the interactions between the different cell types that are involved in the immune response. Abbreviations: ADCC: antibody-dependent cell-mediated cytotoxicity, Ag: Antigen, APC: Antigen presenting cell, CD: Cluster of differentiation, CTL: Cytotoxic T lymphocyte, DC: dentritic cell, Ig: Immunoglobulin, IFN: Interferon, IL: Interleukin, MHC: Major histocompatability complex, Tc: cytotoxic T cell, TCR: T cell receptor, TGF-β: Transforming growth factor, Th: T helper cell, Thp: T helper precursor. Kindly provided by Dr. Abdullah Madhun.

### 1.7.2.1 Cell-mediated immune response

Cell-mediated immunity consists mainly of two components, $CD4^+$ and $CD8^+$ T cells. The former, also called T helper (Th) cell, secrete different cytokines which play the key role in the regulation of the humoral and cellular immune response [28]. While $CD8^+$ cells mediate cytotoxic responses.

#### 1.7.2.1.1 $CD4^+$ T cell

$CD4^+$ T cells can differentiate into subsets of effector cells, Th1, Th2, and Th17 cells. The characteristics of these subsets are based on how they are induced, which cytokines they secrete and their effector mechanisms. Induction of Th cells requires antigen presentation via class II major histocompatability complex (MHC) molecule by dentritic cells (DCs) or macrophages. In addition, the type of cytokines that are secreted by DCs determine the Th polarization of the immune response to Th1 or Th2 dominance. Differentiation of the Th precursor to the Th1 subsets is promoted by IL-12 that is secreted by DC, while IL-4 induces the differentiation of Th precursor to Th2 subsets (reviewed in [33]). Cytokines (IFN-γ and IL-2) that are secreted by Th1 cells, are necessary for the development of cell-mediated immunity. Additional, IFN-γ cytokine induces the isotype-switch in B cells to IgG2a antibody and both are therefore used as a marker of a Th1 response [25]. Conversely, differentiation and proliferation of B cells and isotype-switching of antibodies (IgE, IgG1 and IgA) are stimulated by Th2 cytokines (IL-4, IL-5, and IL-10), which are important in the humoral immune response [24, 25]. Th2 cytokines and IgG1 antibody are used as indicators of the Th2 profile. The cytokines produced by Th cell subsets result in the development and expansion of their respective subsets. Therefore, cytokines secreted by one subset promotes further differentiation of that subset and inhibits the proliferation of the other subset [81].

Th17 cell is classified as the third subset of differentiated effector $CD4^+$T cells in mice. These cells secrete the pro-inflamatory cytokine, IL-17, and is involved in the promotion of dentritic cell and neutrophil maturation, which are important in the contribution of the protection against bacteria and fungi [82]. Differentiation of Th17 cell is inhibited in the presence of IFN-γ and IL-4 [34].

#### 1.7.2.1.2 $CD8^+$ T cell

Differentiation of naïve $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) require antigen recognition that is displayed by class I MHC molecules on the surface of the infected cell and stimulation by Th1 cytokines. The CTL´s antigen receptor must recognize peptide-MHC class I on the infected cell before the CTLs kill the infected cell by two main mechanisms. Firstly, CTLs release complexes of perforin and granzymes that enter the infected cell. The granzymes penetrate into the cytoplasm of the infected cell and induce apoptosis [77]. Secondly, FasL receptor that is expressed on CTLs, engages with Fas receptor on the surface of infected cells, and induces apoptosis [34].

The memory cells of both $CD4^+$ and $CD8^+$ cells can reactivate and give a rise to a more rapid immune response if the same antigen is re-encountered. The CTLs provide broad cross-reactivity against drifted influenza strains because they recognize the internal viral proteins, which are highly conserved among influenza viruses [89].

#### 1.7.2.2 The humoral immune response

The initiations of humoral immune response occur by the recognition of antigens by B cells. Membrane immunoglobulin M (IgM) and IgD receptors on naïve B cells bind to antigens. When this occurs together with the essential stimulations by Th cells, the B cells become activated. Activated B cells undergo clonal expansion (proliferation; expansion of the clone of antigen-specific lymphocytes) and differentiate into antibody-secreting plasma cells, memory cells and progeny of clones that produce other Ig isotypes (e.g., IgG or IgA). Some plasma cells migrate to the bone marrow where they live for many years and continue to produce low levels of antibodies for long periods, which provide long-lasting antibody-mediated immunity. Memory cells play an important role in the secondary antibody responses by mount rapid responses after re-encounter with the same antigen [17]. Progenies of clones that produce other Ig isotypes (isotype-switching) are determined by cytokines, which are secreted by helper T cells and this process is essential for the development of the humoral response. IgG antibodies serve three different effector functions [52]: 1) opsonization of antigens that leads to phagocytosis by neutrophils and macrophages, 2) antibody-dependent cell-mediated cytotoxicity which is mediated by natural killer cells, and 3) activation of the classical pathway of complement.

#### 1.7.2.3 Mucosal immunity

The mucosal immune system serves as the first line of defense against influenza virus that enters into humans, via mucosal surfaces. The mucosal arm of immunity is mediated by dimeric IgA (dIgA) antibody. DIgA is a composition of two IgA molecules held together at the end of the Fc region by J-chain. The poly-Ig receptor on the surface of the mucosal epithelial cell recognizes the J-chain and transfers the dIgA to the lumen, which is termed secretory IgA (sIgA) [9]. The protection against influenza virus in the mucosal surfaces of the upper respiratory tract is mediated by sIgA, which neutralizes the virus by forming an antigen-antibody complex that blocks the receptor-binding site on HA without causing tissue damage. Another antibody class that contributes to this

neutralization is IgG, which transudes to the mucus by diffusion. The sIgA antibodies have broader cross-reactivity than IgG and therefore provide protection against drifted viruses. In the infected epithelial cell, dIgA antibodies that are crossing through the cell may bind to the newly synthesized viral proteins and inhibit the viral assembly process. The lung parenchyma lacks sIgA system, therefore the protection against influenza pneumonia is dependent mainly on serum-derived IgG [85].

## 1.8 Vaccination

A vaccine is a biological preparation that induces protective immunity against microbial diseases. To date, influenza vaccination remains the primary method for the prevention of influenza and the severe complications related to the disease. There are a multiple parameters that contribute to the variation of the efficacy of influenza vaccination, e.g. the route of vaccination, the type of vaccine, the immunologic status, the age of the recipient, and the match of vaccine to the circulating strain [21]. The primed individuals that were vaccinated parenterally with killed virus provide a rapid systemic immune response in the blood and a poor immune response at mucosal sites. In order to induce a stronger mucosal immune response, the vaccine can be administered intranasally [13]. Intranasal vaccination strategy does not only prevent the disease but also blocks early infection. The horizontal transmission to susceptible hosts is thus reduced. There are other benefits that are associated to this mucosal vaccination. It is easy to administer. It does not have to be administered by qualified health personal. It is widely accepted by the public. However, intranasal vaccination of antigen alone is poorly immunogenic. Therefore, mucosal adjuvant can be utilized to overcome this problem [44]. To date, the market has no vaccine formulation containing mucosal adjuvant approved for human use, which highlights the urgent need of effective mucosal adjuvants. In the face of most infections, induction of both mucosal and systemic responses after vaccinations is important [53].

### 1.8.1 Inactivated vaccines

Inactivated vaccines consist of three main formulations, e.g. whole virus, split virus and subunit vaccine. Inactivation of whole virus vaccine is performed by using chemical agents (e.g. formalin, β-propiolacetone, or heat) [14]. Inactivated whole virus vaccine is no longer in use due to the increased risk of side reactions. Split virus vaccine is prepared by disruption of the viral membrane by using other chemical agents (e.g. ether) [54]. Previous studies have reported that split virus vaccine is less immunogenic but had less side-effect than the whole virus vaccine [14, 55]. The subunit vaccine used in current study was composed of antigens (HA and NA surface proteins) purified from the influenza viruses (A/Vietnam/1194/2004) and administered with an adjuvant (described below).

### 1.8.2 Adjuvant

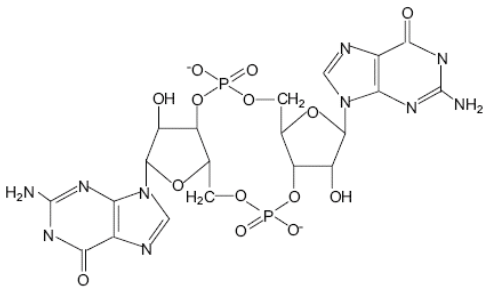

Figure 1.8 **Molecular structure of c-di-GMP** [47].

The function of an adjuvant is to stimulate the immune response without having any specific antigenic effect in itself. Adjuvant is a substance which enhances T cell activation by promoting the accumulation and activation of antigen presenting cells (APCs) at a site of antigen exposure [43]. There are other adjuvants that have different properties, e.g. aluminium containing, cholera toxin (CT), heat-labile toxin (LT of E. coli) and CpG (dinucleotides) adjuvants. Aluminium containing adjuvants induce primarily the humoral immune response [57]. Their mechanism of action is considered to be divided into three parts: 1) involving delayed clearance of the antigen from the injection site [58], 2) stimulating the recruitment and activation of APCs by induction of local inflammatory tissue [59], and 3) facilitating antigen uptake by phagocytosis in APCs by conversion of soluble antigens into a particulate form [60]. CT and LT adjuvant have similar structure to one another and are an ADP-ribosylating enterotoxins that can cause diarrhea. Using CT or LT as a mucosal adjuvant can result in enhancement of antigen-specific secretory and systemic antibody responses, and amplification of cellular immunity [61, 62]. CpG dinucleotides consist of cytosine and guanine nucleotide that are connected by a phosphate and are present in the

prokaryotes's genome [64, 65]. During an infection, the CpG acts as a danger signal to the innate immune system and triggers strong immune response [63].

The selected adjuvant in this research is an autoinducer bis(3',5')-cyclic dimeric GMP (c-di-GMP), which is represented as one of the cell-to-cell signalling systems in bacteria [66]. The molecule structure of c-di-GMP is shown in figure 1.8. Experiments have shown that increased levels of c-di-GMP correlate with increased bacterial aggregation and biofilm formation [67, 68]. Karaolis et al. have shown that c-di-GMP can act as a danger signal on eukaryotes and it stimulates the expression costimulatory molecules and cytokines by dentritic cells in humans [69, 70]. C-di-GMP has shown in mice to have immunomodulator effect that stimulates the immune system in the prevention of lethal bacterial infections (e.g. *S. aureus*) [70]. Increased survival rates and reduction in bacterial counts in lung and blood were observed when the mice are administered c-di-GMP intranasally and subcutaneously before an intratracheal challenge with *Klebsiella pneumonia* [72]. More recently, research conducted by Ebensen et al. have shown that intranasal [44] and subcutaneous [71] vaccinations of mice with β-galactosidase adjuvanted with c-di-GMP induced strong humoral and cellular immune responses with balanced Th1/Th2 profile. Thus, c-di-GMP is a promising adjuvant for development of intranasal pandemic influenza vaccines.

## 1.9 Aim of the study

In recent years, the world has been threatened by a high pathogenic H5N1 virus, which has the potential to cause a pandemic. This influenza virus has a mortality rate approximately 60 % but, fortunately, it has not evolved the ability to transmit from human to human. Thus, it is necessary to have a good vaccine available if this virus evolves further.

Previous reports have shown that paranteral vaccination induces rapid systemic immune response, but poor mucosal immune response. In contrast, an effective intranasal vaccine may induce local immune response which blocks influenza virus at the entry site and reduces further transmission. Intranasal vaccination of influenza antigen alone is poorly immunogenican effective and safe mucosal adjuvants are needed. Studies by Ebensen et al. [44] have shown promising results for the mucosal adjuvant c-di-GMP. Intranasal vaccine that induces a rapid stimulation of both mucosal and systemic immune responses will provide an important measure in combating influenza pandemic.

The aim of this study was to investigate and compare the mucosal, humoral and cytokine immune responses induced in mice after intranasally vaccination with influenza A(H5N1) subunit vaccine adjuvanted with c-di-GMP and non-adjuvanted subunit vaccine.

# 2. Materials

## 2.1 Animals

| Name | Supplier |
|---|---|
| BALB/c mice, female | Taconic M&B A/S, Denmark |

## 2.2 Vaccine

| Name | Supplier |
|---|---|
| Concentrated Subunit NIBRG-14 vaccine (A/Vietnam/1194/2004) | Archimedes Development Ltd, United Kingdom |

## 2.3 Antigen

| Name | Supplier |
|---|---|
| Whole virus, NIBRG-14 | Archimedes Development Ltd, United Kingdom |

## 2.4 Anesthesia

| Name | Cat. no | Supplier |
|---|---|---|
| Rompun vet (20 mg/ml) | 023446 | Bayer, Germany |
| Ketalar (50 mg/ml) | 150086 | Pfizer, USA |

## 2.5 Antibodies

| 2.5.1 Capture antibody | Cat. no | Supplier |
|---|---|---|
| Goat anti-mouse IgA | 1040-01 | Southern Biotechnology, USA |
| Goat anti-mouse IgG | 1030-01 | |
| Goat anti-mouse IgG1 | 1070-01 | |
| Goat anti-mouse IgG2a | 1080-01 | |
| Goat anti-mouse IgM | 1020-01 | |

| 2.5.2 Immunoglobulin standards | Cat. no | Supplier |
|---|---|---|
| Mouse IgA (1 mg/ml) | M1421 | Sigma, USA |
| Mouse IgG (1 mg/ml) | I5381 | |
| Mouse IgG1 (0,5 mg/ml) | 0102-14 | Southern Biotechnology, USA |
| Mouse IgG2a (1mg/ml) | M9144 | Sigma, USA |
| Mouse IgM (1 mg/ml) | M3795 | |

| 2.5.3 Biotin antibody | Cat. no | Supplier |
|---|---|---|
| Biotin-anti-mouse IgA | 1040-08 | Southern Biotechnology, USA |
| Biotin-anti-mouse IgG | 1030-08 | |
| Biotin-anti-mouse IgG1 | 1070-08 | |
| Biotin-anti-mouse IgG2a | 1080-08 | |
| Biotin-anti-mouse IgM | 1020-08 | |

## 2.6 Kits

| Name | Cat. no | Supplier |
|---|---|---|
| Mouse Cytokine Grp I X-Plex Assay (Cytokine 6-plex – IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ) | X6000006RJ | Bio-Rad Laboratories, USA |
| Bio-plex Reagent Kit | 171-304000 | |
| Bio-plex Calibration Kit | 171-203060 | |

## 2.7 Plates

| Name | Cat. no | Supplier |
|---|---|---|
| Greiner Microplatte (ELISA) | 6500, F | Greiner, Germany |
| Multiscreen HTS, BV (Bio-plex) | MSBVN1250 | Millipore, United Kingdom |
| 96 V-well polystyrene microwell plates (HI) | 249570 | Nunc, Denmark |

## 2.8 Reagents

| Name | Cat. no | Supplier |
|---|---|---|
| Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) | 1.00242 | Merck, Germany |
| Con A (Concanavalin) | C5275 | Sigma, USA |
| Di-Sodium hydrogen phosphate anhydrous ($Na_2HPO_4$) | 1.06586.2500 | Merck, Germany |
| DMF (dimethylformamide) | 10322 | BHD AnalaR, England |
| Extravidin Peroxidase Conjugate | E-2886 | Sigma, USA |
| FBS (foetal bovine serum) | 14-710F | BioWhittaker, Belgium |
| Glacial acetic acid ($CH_3CO_2H$) | 1.00063.1000 | Merck, Germany |
| Hydrogen peroxide 30 % ($H_2O_2$) | H-1009 | Sigma, USA |
| Lymphoprep | 1001967 | Nycomed Pharma AS, Norway |
| NCS (Newborn Calf Serum) | ECS0070L | Euroclone |
| OPD (o-Phenylenediamine) (10 mg tablets) | P-8287 | Sigma, USA |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 1.04873 | Merck, Germany |
| PSF (penicillin/streptomycin/fungizone) | 17-745E | BioWhittaker, Belgium |
| RPMI 1640 + L-Glutamine | 21875-034 | Gibco, UK |
| Trypan blue stain (0,4 %) | 17-942E | BioWhittaker, Belgium |
| RDE | | Denka Seiken, Tokyo, Japan |
| Turkey blood | 07801 | The National Institute for Biological Standards and Control, United Kingdom |
| Tween 20 (polyoxyethylene sorbitan monoaurate) | P-1379 | Sigma, USA |

## 2.9 Solutions and buffers

### 2.9.1 ELISA solutions

| Name | Description |
|---|---|
| Citric acid<br>(0,1M $C_6H_8O_7$) | - 11,4 g $C_6H_8O_7$<br>- 500 ml $ddH_2O$ |
| Dibasic sodium phosphate<br>(0,2M $Na_2HPO_4$) | - 14,2 g $Na_2HPO_4$<br>- 500 ml $ddH_2O$ |
| Phosphate citrate buffer<br>(pH 5,0) | - 257 ml 0,2M $NaHPO_4$<br>- 243 ml 0,1M $C_6H_8O_7$<br>- 500 ml $ddH_2O$ |
| OPD solution<br>(Ortho-phenyldiamine dihydrochlorid) | - 10 mg OPD (tablet)<br>- 25 ml Phosphate citrate buffer |
| Sulphuric acid<br>(1M $H_2SO_4$) | - 70 ml 98 % $H_2SO_4$<br>- 430 ml $ddH_2O$ |

### 2.9.2 Buffers and medium

| Name | Description |
|---|---|
| Lymphocyte medium (LM) | - 86 ml RPMI 1640 medium supplemented L-glutamine<br>- 1 ml 100x non essential amino acids<br>- 1 ml 1 M Hepes pH 7.4<br>- 1 ml PSF<br>- 10 ml heat-inactivated FBS |
| PBS (10x)<br>(Phosphate Buffered Saline)<br>(pH 7,2) | - 400 g NaCl<br>- 10 g KCl<br>- 72 g Na2HPO4<br>- 12 g KH2HPO4<br>$ddH_2O$ ad 5 L |
| PBS/FBS<br>(PBS containing 10 % FBS) | - 90 ml PBS<br>- 10 ml FBS |
| PBS/NCS<br>(PBS containing 20 % NCS) | - 80 ml PBS<br>- 20 ml NCS |
| PBS/T<br>(PBS containing 0,05 % Tween) | - 500 ml<br>- 4500 ml dH2O<br>- 2,5 ml Tween 20 |

# 3. Methods

## 3.1 Experimental protocol

### 3.1.1 Mice

In the current study, fifty five BALB/c mice (6-8 week old, female) were used which was approved by "Forsøksdyrutvalg" and conducted according to the Norwegian Animal Welfare Act. All animals used were housed in a specific pathogen free, temperature 21˚C with 12 hour light/dark cycles and food and water ad libitum at the animal housing facility (Vivarium), at the University of Bergen.

### 3.1.2 Immunization

Forty mice were randomly divided into two equal groups, where one group was vaccinated intranasally with 15 µg of A/Vietnam/1194/2004 (H5N1) subunit vaccine adjuvanted with 5 µg c-di-GMP, whilst a second group was immunized with subunit vaccine alone. In addition, fifteen mice received only PBS which served as a control group.

Intranasal vaccination of mice was performed as follows. Mice were primary anaesthetized and laid on their back before the required volume of vaccine was given in droplet into the nostrils. The total vaccine volume was divided into two portions for each nostril cavity. To ensure that the vaccine was only administered intranasally a maximum volume of 5.5 µl per nostril was administered. The adjuvanted vaccine was administered 4.5 µl per nostril 3 times and the non adjuvanted vaccine 5.5 per nostril twice.

### 3.1.3 Sacrifice

The mice were individually placed in a chamber and carbon dioxide ($CO_2$) was used as an asphyxiant for euthanasia.

## 3.2 Sampling and spleen collection

Weekly blood samples could not be collected from the mice because of their size. To obtain a total overview of the kinetics of the immune response, each group was divided into four subgroups, with samples collected as shown in the experimental outline (fig. 3.1).

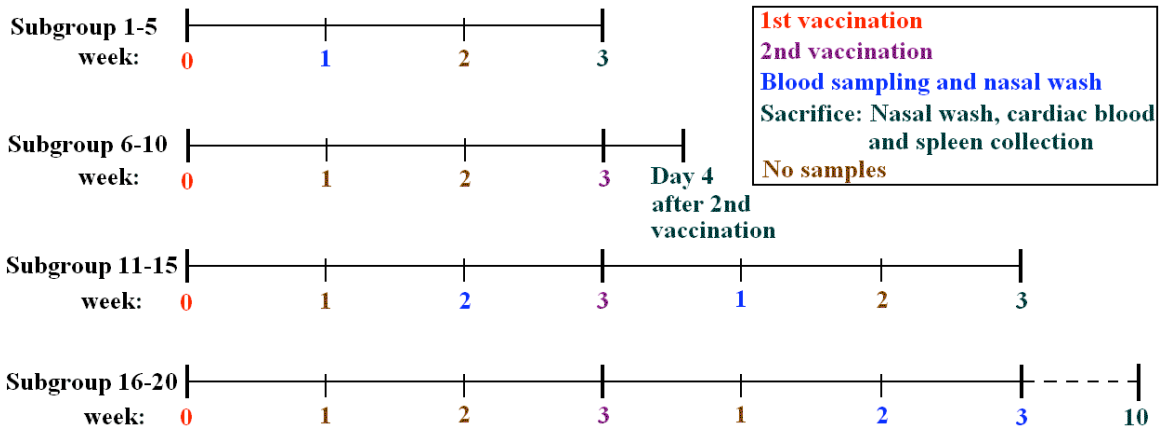


Fig. 3.1 **The experimental outline.** Mice were vaccinated intranasally with two doses at three weeks interval with either the subunit vaccine with adjuvant or alone. Peripheral blood and nasal washes was collected on blood sampling days (blue font). Cardiac blood, nasal washes and the spleen were collected on the day of sacrifice (green font).

### 3.2.1 Collection of nasal wash (NW) samples

The mouse was restrained by holding the scruff of the neck and the tail. Sterile PBS/BSA (0.35 ml) was flushed in large droplets into the mouth and out through the nose and collected in a petri dish (fig. 3.2). Each sample was collected in a separately labelled eppendorf tube and immediately placed on ice. The NWs were then stored at -80 ˚C until processing.

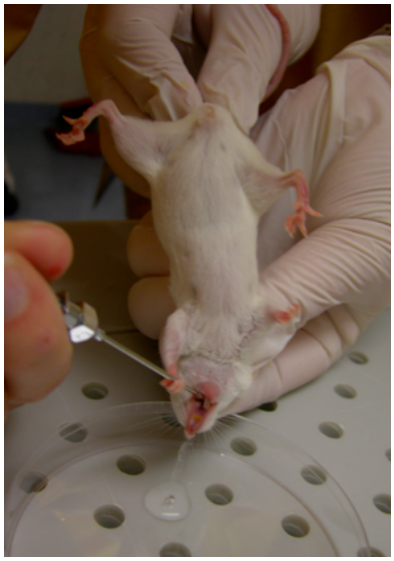

Figure 3. 2 **Collection of NW samples.**

### 3.2.2 Peripheral blood sampling

The mouse was placed in a tube and the hind leg was exposed by holding the skin between the tail and thigh. The fur on the leg was shaved using a scalp blade (fig. 3.3) and a 23 gauge needle was used to puncture the saphenous vein. A labelled microvette was used to collect the venous blood (50-100 µl). After the blood was taken, pressure was applied to the vein to stop the blood flow.

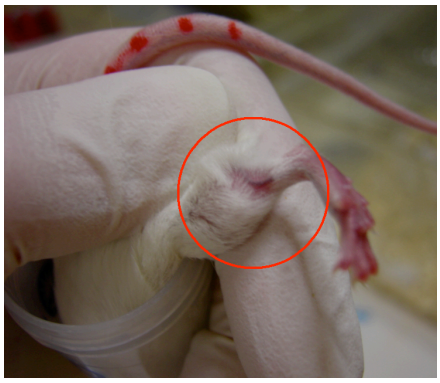

Figure 3.3 **The saphenous vein, where the peripheral blood was taken.**

### 3.2.3 Cardiac blood sampling (sacrifice)

Cardiac blood samplings were only collected on the sacrifice days. After euthanasia, the mouse was pinned to a dissection plate and the chest was opened (from the throat to the abdomen). Blood was collected directly from the heart by cardiac puncture using a 23 g needle with a 2 ml syringe (fig. 3.4). The blood was collected in a labelled eppendorf tube.

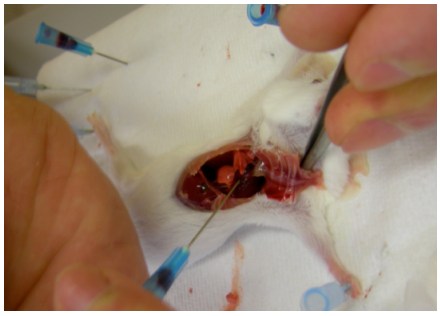

Figure 3.4 **The cardiac blood sampling.**

### 3.2.4 Separation of sera

The blood was allowed to clot at room temperature for approximately five hours. The clotted blood was then centrifuged for 10 minutes at 1000 g before the serum was transferred to a new microtube and stored at -80 °C until tested by ELISA and HI assays.

### 3.2.5 Spleen collection (sacrifice)

The spleen was removed aseptically from dissected mice and transferred to a tube containing 3 ml of sterile PBS. The tissues were kept cold until further processing.

### 3.2.6 Isolation of lymphocytes from spleen

The spleen was placed in a sterile petri dish and punctured with a 23 gauge needle. The spleen cells were washed out of the spleen with 3 ml PBS/FBS into the petri dish. Afterwards, the cells were collected in a 15 ml tube. The wash procedure was repeated with a new 3 ml PBS/FBS medium to achieve a total volume of 6 ml cell suspension. The spleen was flushed until all cells were removed from the spleen when its colour turned greyish. The procedure was performed in a LAF-bench at room temperature.

The 6 ml cell suspension was carefully laid onto 3 ml of Lymphoprep in a 15 ml centrifuge tube. The tube was centrifuged at 800 g for 30 minutes at room temperature with no brake. The distinct band containing the lymphocytes was carefully removed using a pasteur pipette and transferred to a new 15 ml centrifuge tube. Cold PBS/FBS medium was added to make a total volume of 8 ml. The lymphocytes were washed twice with 8 ml of PBS/FBS by centrifuging at 250 g for 10 minutes at 4 $^{\circ}$C. The spleen cells were re-suspended in lymphocyte medium (LM) to a final volume of 2 ml. The isolated lymphocytes were counted by trypan blue exclusion (0.4 %) using flow cytometry. The concentrations of lymphocytes were adjusted in LM to 1.0 x $10^7$ cells/ml and immediately activated *in vitro*.

## 3.3 Immunological Assays

### 3.3.1 *In vitro* activation of lymphocytes

One hundred micro litres of isolated splenic lymphocytes (1.0 x $10^6$ cells) and one hundred micro litres of appropriate activation medium were added into 96-wells flat bottom tissue culture plates and incubated for 72 hours at 37 ˚C in a humidified atmosphere of 5 % $CO_2$. Two different mediums were utilized to activate the lymphocytes; (1) influenza (H5 virosomal antigen) activation medium containing 10 µg HA/ml or (2) a mitogen (PMA) activation medium containing 10 µg PMA/ml. The latter was used as a positive control. Cells with only LM were used as negative control. After incubation, the content of the wells were transferred to a V well bottom plate and centrifuged at 300 g for 10 minutes. The supernatants were transferred to a new plate and stored at -80 ˚C until analysed in Bio-plex bead immunoassay.

### 3.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The 96-well ELISA plates were coated with 100 µl/well of 2 µg/ml whole influenza, A/Vietnam/1194/2004(H5N1), or with 1/1000 dilution of goat anti-mouse capture antibody (IgA, IgG, IgG1 or IgG2a) in PBS overnight at 4 °C. The plates were blocked with 200 µl/well PBS/NCS (20 %) for 1 hour at room temperature. Mouse NW was diluted in two-fold dilutions, ranging from 1:5 and 1:10. Five folds dilution, 1/50 and 1/250 dilutions were prepared for mouse sera. Mouse antibody standard was diluted in two-fold dilutions, starting from 50 ng/ml to 0.781 ng/ml and each dilution in duplicates was added to each plate. The diluted sample and antibody standard were added (100 µl/well) to the appropriate wells in duplicate. After 2 hours incubation for NW samples or 1.5 hour sera at room temperature, the plates were washed 6 times with PBS/T (0.05 %) using Nunc Elisa washer. Goat anti-mouse antibody specific-biotinylated conjugate diluted in PBS/NCS (1/500) were added 100 µl/well and incubated for 1 hour at room temperature. The plates were washed 6 times with PBS/T and incubated for an additional hour 100 µl/well of extravidin peroxidase diluted 1:1000 in PBS/NCS (20 %). The plates were washed 6 times with PBS/T, prior to addition of the substrate 100 µl/well (10 mg OPD, 25 ml 0.05 phosphate citrate buffer pH 5, 20 µl $H_2O_2$) for development. After incubating the plates for 10 minutes in the dark, the reaction was stopped with 100 µl/well of 1M $H_2SO_4$. The Labsystem Multiscan reader was used to read the optical densities of the wells (OD) at 492 nm. The influenza specific antibody concentrations were calculated in µg/ml using standard curves plotted as log-log graphs in the Ascent program (Ascent Software Version 2.6).

### 3.3.3 Haemagglutinin inhibition assay (HI assay)

#### 3.3.3.1 Preparation of turkey red blood cells

The turkey blood was centrifuged in 250 g for 10 minutes at 4 ˚C. The supernatant was removed before 4 ml of sterile PBS was added and centrifuged as described above. This procedure was repeated until supernatant was clear (no haemolysed red blood cells). The turkey blood cells diluted in 0.7 % (v/v) in PBS for use in the virus titration and HI assays.

#### 3.3.3.2 Treatment of sera

To remove non-specific inhibitors in the serum, 240 µl RDE (reconstituted in PBS) was added to 60 µl sera (volume 5:1) and incubated at 37 ˚C overnight. The remaining RDE was heat-inactivated by incubation of the treated serum at 56 ˚C for 30 minutes. The serum was allowed to cool to room temperature.

#### 3.3.3.3 Virus titration

This procedure is used to determine the amount of influenza virus and therefore is necessary for standardising the amount of virus in the HI assay.

Fifty micro litres of PBS were added to each well in a V-bottomed microtiter plate. Then 50 µl of virus suspension was added to the first row and mixed and a 2-fold dilution series was made the rest of the rows. The last 50 µl was discarded. Control wells contained no virus. 50 µl/well of 0.7 % red blood cells was added to each well and incubated at room temperature for 30 minutes. The plates were read by tilting, which allowed the non-agglutinated red blood cells to run downwards. HA titres were calculated as the reciprocal of the dilution of virus giving 50 % haemagglutination. The virus titre was adjusted to be 8 HA units (HAU)/50 µl.

#### 3.3.3.4 HI assay

The HI assay was run in a 96-wells V-bottomed microtiter plate. A two fold dilution of RDE treated sera (from an initial of 1/8 dilution) were made in PBS giving a final volume of 50 µl, as described under virus titration. Fifty micro litres of the standardized virus (8 HAU/50 µl) was added to each well and incubated at room temperature for 1 hour. Then 50 µl of 0.7 % of turkey red blood cells were added to each well and incubated at room temperature for 30 minutes. The haemagglutination inhibition titre was read by tilting the plates, which allowed the nonagglutinated red blood cells to run. The HI titre was calculated as the reciprocal of the highest dilution of serum needed to inhibit 50 % haemagglutination.

### 3.3.4 Bio-plex bead immunoassay

The bio-plex bead immunoassay was performed according to the manufacturer's protocol.

The wells of the supplied plates were pre-wet with 100 µl of assay buffer A before 25 µl of vortexed-bead suspension was added. The beads were washed twice with 100 µl of Working Wash Solution. Standards were prepared according to the manufactures instruction and added to the appropriate wells. 25 µl/well of supernatant from *in vitro* activated cells and 25 µl/well assay diluents (LM) were added to the remaining wells. The plate was sealed and incubated on an orbital shaker (Heidolph titramax 100) at room temperature for 30 seconds at 1,100 rpm, then for 45 minutes at 300 rpm. The liquid was removed by washing three times with 100 µl of Working Wash Solution. Biotinylated detector antibody (biotin: anti IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-17) was added to each well (25 µl/well) and incubated at room temperature on an orbital shaker for 30 seconds at 1,100 rpm, then for 30 at 300 rpm. The plate was washed three times with 100 µl Working Wash Solution before 25 µl of Vortex Streptavidin-PE was added and incubated at room temperature on the orbital shaker (30 seconds at 1,100 rpm subsequently 10 minutes at 300 rpm). Then the plate was washed three times more with 100 µl PBS/T before assay buffer (100 µl/well) was added to each well and the plate was put on the orbital shaker for 30 seconds at 1,100 rpm. The plate was analysed in the Luminex 100$^{TM}$ instrument and the concentration of the cytokines were calculated using standard curves by Bioplex manager 5.0.

## 3.4 Statistical analysis

The following formulas were used to calculate the results (statistic value) of the experiment:

Mean $$\bar{x} = \frac{\sum x}{n}$$

Standard deviation $$SD = \sqrt{\left[\frac{\sum (x - \bar{x})^2}{n - 1}\right]}$$

Standard error of the mean $$SEM = \frac{SD}{\sqrt{n}}$$

Geometric mean $$GM = \text{anti}\log\left[\frac{\sum (\log x)}{n}\right]$$

# 4. Results

In the current study, we have investigated the local and systemic immune responses elicited in mice after intranasal vaccinations with influenza A H5N1 vaccine. The animals were randomly divided into two groups of 20 mice each on the basis of the vaccine formulations: subunit vaccine adjuvanted with c-di-GMP or subunit vaccine alone. Additionally, 15 mice were used as a control group (vaccinated with PBS). The experimental design (immunization and sampling days) is described in Methods (section 3).

There were no detectable influenza-specific immune response in control animals and therefore, the results are not presented.

## 4.1 The antibody response after the intranasal vaccinations

A number of immunological assays were used to analyse the influenza specific response. ELISA assay was used to quantify the influenza specific antibodies in the serum (IgG, IgG1, IgG2a, and IgA) and NW (IgA), before and after influenza vaccination. The haemagglutinin inhibition (HI) assay was conducted to measure the titre of anti-HA antibodies elicited after vaccination.

### 4.1.1 The influenza-specific antibody response quantified by ELISA assays

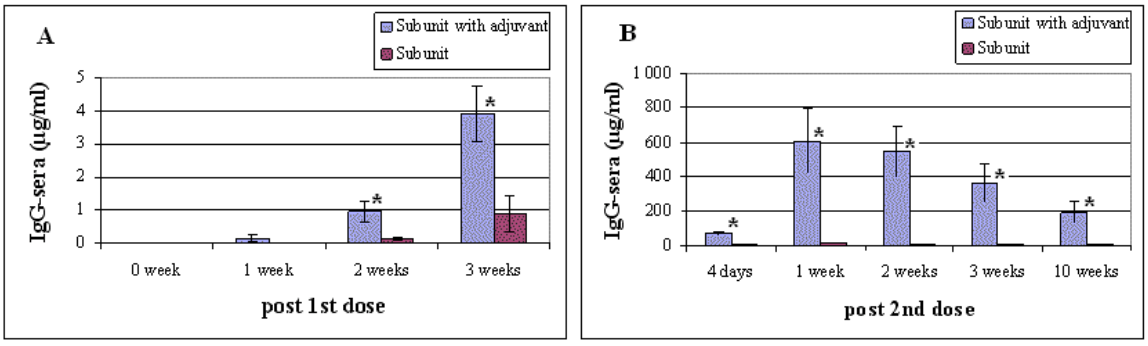


Figure 4.1 **Serum influenza-specific IgG concentrations after the first vaccination (A) and the second vaccination (B)**. The IgG concentrations after vaccinations with 15 µg HA with c-di-GMP adjuvant (blue) or alone (purple) at various sampling intervals. The results are represented as the mean concentration (µg/ml) of 5 mice ± SEM. Differences were statistically significant at a *P* value of <0.05 (*) when compared with the mice vaccinated with subunit alone.

One week after the first immunization (fig. 4.1 A), low concentrations of IgG (0.2 µg/ml) were detected in the group that received subunit antigen adjuvanted with c-di-GMP. The IgG concentration continued to increase, with concentrations of 1 µg/ml and 4 µg/ml observed at the second and the third week after vaccination. On the other hand, no or very low IgG concentrations (0.1 µg/ml after the second week) were detected in the mice immunized with subunit alone, although an increase (0.9 µg/ml) was observed in the third week. However, the IgG level was more than 7-fold (second week) and 4-fold (third week) higher in the group that was immunized with adjuvant vaccine compared to the group that received subunit vaccine alone.

The IgG levels were boosted for both mouse groups after the second vaccination, but the highest concentrations were observed in the mouse group immunized using cdiGMP as a mucosal adjuvant (fig. 4.1 B). The level of IgG significantly increased (approximately 20-fold) four days after the second vaccination (fig. 4.1 B) compared the group immunized with subunit alone (approximately 5-fold). For both groups, the IgG levels peaked one week after the second vaccination, although significantly higher (44-fold higher) levels were observed in the group receiving subunit with c-di-GMP (603 µg/ml), than the group receiving the vaccine without adjuvant (14 µg/ml). The IgG levels declined in the following sampling points in both groups although the group with adjuvant still had significantly higher IgG levels than the group without adjuvant.

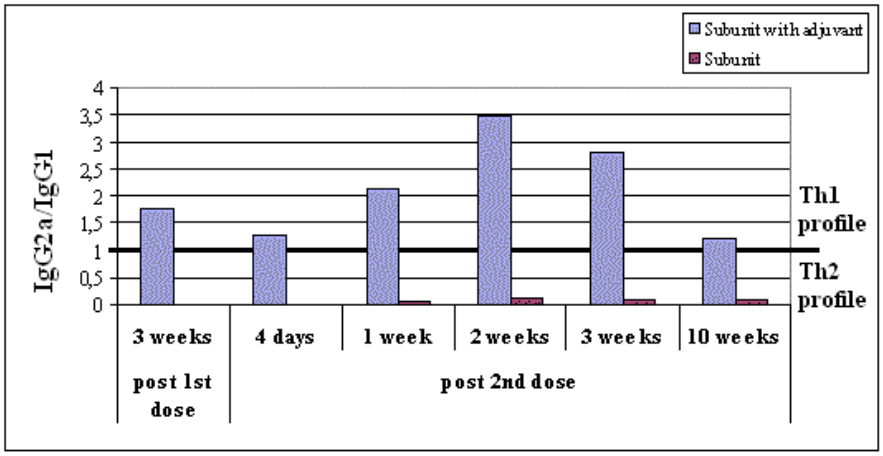


Figure 4.2 **Ratio between IgG2a/IgG1 after vaccination of the groups.** Serum samples were first analyzed at the third week after the first vaccination and at various time intervals after the second vaccination. The IgG2a/IgG1 ratios are calculated by dividing the IgG2a concentration with IgG1. A ratio over 1 is classified as Th1 biased profile, whereas less than 1 is characterized by a Th2 profile. The results of each group are presented as the ratio of 5 mice.

Due to the low IgG concentrations in sera at the first and second week after the first vaccination, the quantifications of IgG subclasses were not performed until the third week after the first dose. In the adjuvant group, IgG2a dominated the response throughout the study as shown by IgG2a/IgG1 ration (Fig. 4.2). On the other hand, the response in the non-adjuvanted vaccine group was not detected until one week after the second immunization and was mainly IgG1.

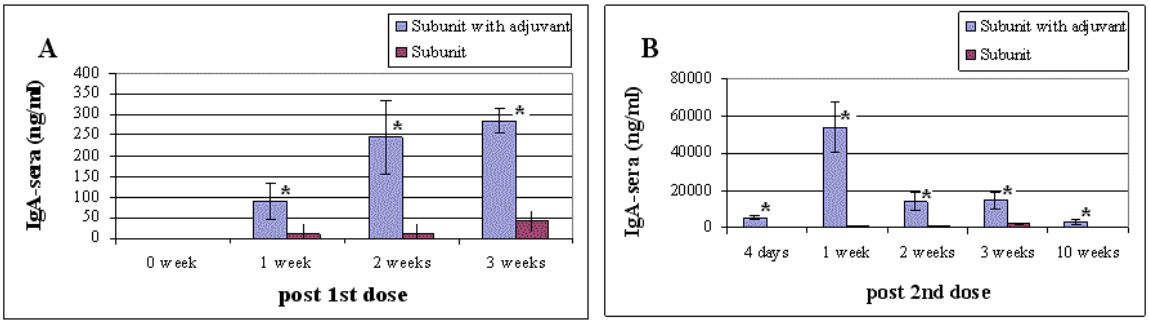


Figure 4.3 **Serum influenza-specific IgA concentrations after the first vaccination (A) and the second vaccination (B).** The IgA concentrations after vaccination at various sampling intervals for the mouse group that received formulated vaccine with adjuvant (blue) and the mouse group that received subunit vaccine alone (purple). The results are represented as the mean concentration (µg//ml) of 5 mice ± SEM. Differences were statistically significant at a *P* value of <0.05 (*) when compared with the mice vaccinated with subunit alone.

One dose of c-di-GMP adjuvanted vaccine elicited significantly higher serum IgA antibody levels than non-adjuvated vaccine (fig. 4.3 A), with the peak response observed three weeks after vaccination in both groups. The IgA antibody concentrations continued to increase up to three weeks post vaccination in the animal group that received antigen adjuvanted with c-di-GMP (245 ng/ml at the second week and 283 ng/ml at the third week). In contrast, the group without adjuvant showed no increase in the IgA concentration until the third week post vaccination (42 ng/ml) (fig. 4.3 A).

Four days after the booster vaccination (fig. 4.3 B), 18-fold increase in the IgA levels was observed in adjuvated vaccine group compared to the third week after the first dose. On the other hand, although the serum IgA concentration also increased in the non adjuvanted group (274.8 ng/ml), the increase was lower than in the adjuvanted group (7-fold increase). In the group immunized with the adjuvant formulation, the IgA level peaked one week after the second vaccination with a concentration of approximately

54000 ng/ml and a significant decline was observed on the following sampling points. On the other hand, the peak IgA response after the second vaccination with the non-adjuvanted vaccine was later at three weeks. C-di-GMP adjuvanted vaccine induced significantly higher IgA levels than non-adjuvanted vaccine through the study period.

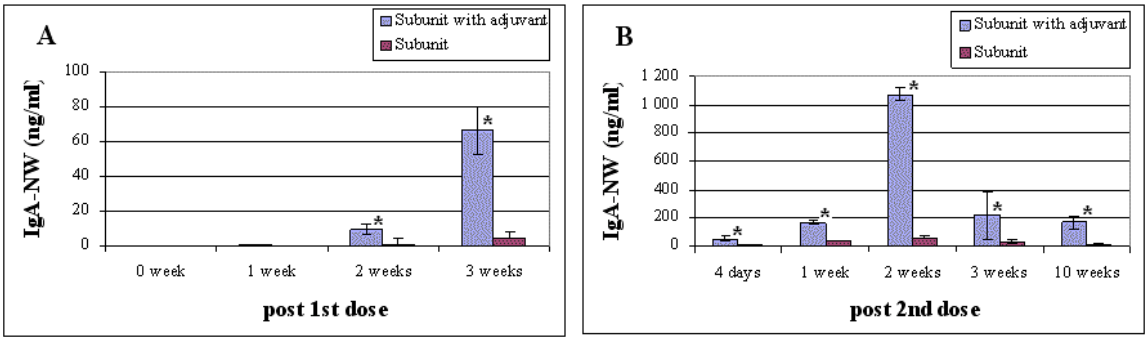


Figure 4.4 **Nasal wash influenza-specific IgA after the first vaccination (A) and the second vaccination (B).** IgA concentrations at various sampling intervals for the mouse group that received 15 µg HA adjuvanted vaccine (blue) or vaccine alone (purple). The results are represented as the mean concentration (ng/ml) of 5 mice ± SEM. Differences were statistically significant at a *P* value of <0.05 (*) when compared with the mice vaccinated with subunit alone.

One dose of intranasal immunization with adjuvant vaccine elicited a detectable IgA response in nasal wash as early as the first week, with the peak response observed in the third week (fig. 4.4 A). Very low concentrations (1 ng/ml) of IgA were detected after one dose of non-adjuvanted vaccine (fig. 4.4 A). After one vaccine dose, the IgA response was significantly higher in the c-di-GMP subunit vaccine group compare to subunit alone group.

After the second vaccination, a small increase in the IgA level was observed for the group without adjuvant. In contrast, there was initially a sight decline in IgA concentration for the group with adjuvant (fig. 4.4 B). However, this group showed a 3-fold increase one week (167 ng/ml) compared to 4 days (54 ng/ml) after the second vaccination. By the second week, both groups reached the peak with the adjuvant vaccine group having significantly higher IgA level (approximately 17 times) than the group without adjuvant. At weeks 3 and 10, IgA concentrations declined in both groups. Immunization with antigen formulated c-di-GMP induced significantly higher levels of NW IgA than antigen alone through the study.

### 4.1.2 Serum antibody response measured by HI assay

The HI is a method for analyzing anti-HA response in serum and is commonly used for assessing the immunity after influenza vaccination. In human, a serum HI titre ≥ 40 provide protection against seasonal influenza virus infection in 50 % of the population.

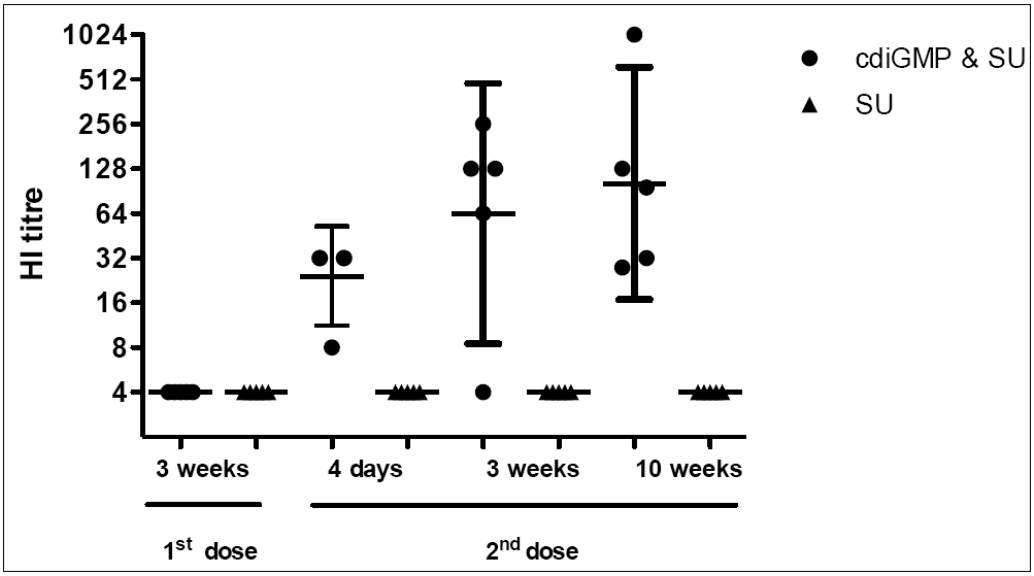


Figure 4.5 **The HI antibody response after vaccinations**. The individual HI titres and the GMT of the group ± 95 % confidence interval at the various sacrificing weeks after one and two doses for the mouse group that received subunit vaccine added adjuvant (ovals) and the mouse group that received subunit vaccine alone (triangles). The results are represented as the geometric mean of 5 mice ± 95 % CI.

After the first vaccination, no HI titers higher than the detection limit of the assay (HI titer =4) were observed in both groups (fig. 4.5). In the adjuvant group, GMT started to increase after the second vaccination, but an HI titers ≥ 40 were not detected until the third week after the second vaccination. A further increase in GMT (GMT >100) was noted 10 weeks after the second dose. None of the mice in the subunit alone group had an HI titer > 4 at any time point after the first and the second vaccination.

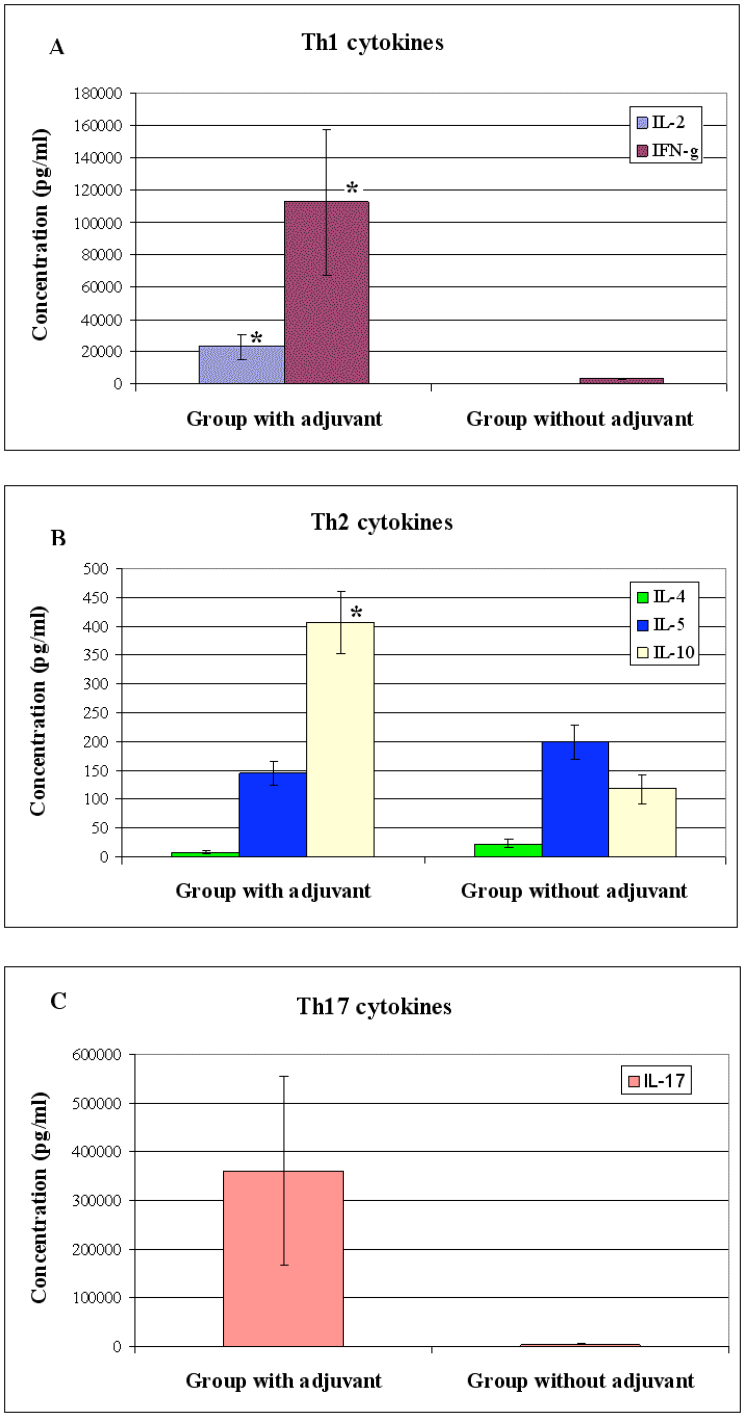


Figure 4.6 **Concentration of cytokines in supernatants from *in vitro* activated splenic lymphocytes.** The cytokines are separated based on their activity in the Th-profile immune response. (A) Th1-cytokines (IL-2 and IFN-γ), (B) Th2-cytokines (IL-4, IL-5 and IL-10), and (C) Th17 (IL-17) are shown for two groups of mice, those vaccinated with and those without adjuvant, three weeks after the second vaccination. It should be noted that there is a variation in the scale of the y-axis. The average concentration (pg/ml) of cytokine is shown from 5 mice per group ± SEM. Differences were statistically significant at a *P* value of <0.05 (*) when compared with the mice vaccinated with subunit alone.

## 4.2 The cytokine immune response

Bio-plex bead immunoassay was conducted to determine the concentration of IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-17 in supernatants from *in vitro* activated lymphocytes. IFN-γ and IL-2 are regarded as indicators of a Th1 profile, whereas IL-4, IL-5, and IL-10 are considered as markers for a Th2 immune response. The Th17 response is characterised by the cytokine IL-17.

The mouse group receiving the vaccine with adjuvant had a significantly higher Th1 (IL-2 and IFN-γ) cytokines response compared to the group that did not received adjuvant (462-fold higher IL-2 and 34-fold higher IFN-γ) (fig. 4.6 A). However, higher concentrations of IL-4 and IL-5 were observed in the non adjuvanted group compared to the adjuvanted group. In contrast, the bio-plex bead immunoassay showed higher levels of IL-10 (approximately 4-fold) and of IL-17 (>100-fold higher) in the group with adjuvant compared to the non-adjuvanted vaccine group (fig. 4.6 B and fig. 4.6 C).

## 4.3 Summary of the results

After the first vaccination, the ELISA IgG and IgA concentrations in sera were low in the two vaccine groups. The IgA level in sera reached the peak at the first week after the second dose, in contrast to the IgA in nasal wash, which peaked two weeks after the second dose. An early booster effect (four days vaccination) was noticed in the group vaccinated with adjuvant and was reflected by high HI titres and high levels of serum antibodies. The IgG subclass distribution did not change after the second vaccination, with predominance of IgG2a in the group that was immunized with adjuvant and IgG1 in the group without adjuvant. However, a significantly high IgG2a/IgG1 ratio in the group with adjuvant was detected at the second and third week after the second dose. The cytokine response was dominated by Th1 (IL-2 and IFN-γ) in the adjuvanted vaccine group, whilst by Th2 (IL-4 and IL-5) in the non-adjuvanted vaccine group. Furthermore, c-di-GMP adjuvanted vaccine induced higher levels of IL-10 and IL-17 than non-adjuvanted vaccine group.

# 5. Discussion

Influenza vaccination remains the primary method of prevention of influenza and the severe complications related to the disease. Influenza virus enters the host via the upper respiratory tract. Thus, an important area of research is the development of new influenza vaccines focusing on the induction of humoral and cellular immune responses at the both systemic and mucosal levels. Intranasal vaccination stimulates mucosal immune responses and prevents disease by blocking the virus at the site of entry to the host and preventing horizontal transmission. Subunit influenza vaccine is less immunogenic, but also less reactogenic than whole virus vaccine. Therefore, the use of adjuvant with subunit vaccine has the advantage of enhancing the immune response and overcoming the low immunogenicity. Ebensen et al. [44] have found intranasal vaccination with antigen formulated with c-di-GMP adjuvant, showed a significantly enhancement of IgG titre in sera, higher sIgA antibodies in the lung and a strong cellular immune response dominated by a Th1 response. Thus in the current murine study, this promising adjuvant was used to formulate an intranasal influenza A (H5N1) subunit vaccine. One mouse group was vaccinated intranasally with 15 μg of A/Vietnam/1194/2004 (H5N1) subunit vaccine with 5 μg mucosal adjuvant, c-di-GMP, while the other mouse group was immunized with the subunit vaccine alone.

## 5.1 Formulation of subunit vaccine with c-di-GMP adjuvant induced a rapid humoral immune response.

Virus neutralizing antibodies play a key role in resistance to infection and in the prevention of illness [86]. Induction of a high influenza-specific antibody level is desirable after vaccination, and for seasonal influenza strains an HI titre ≥40 correlates with protection [83]. In our study, higher serum IgG and IgA levels were observed after the first vaccination for the group immunized with c-di-GMP vaccine, indicating that the adjuvant induced a more rapid and stronger humoral immune response after one dose. Thus the use of adjuvant may reduce the time interval between vaccination and induction of protective immunity, which is of great importance in a pandemic scenario. A significantly higher serum antibody response was also observed after the booster dose, suggesting that this mucosal adjuvant further enhanced humoral immunity. Similarly, others have found higher IgG concentration after the first vaccination and

significantly higher levels after the booster dose in the mice immunized intranasally with β-Gal and c-di-GMP adjuvant compared to animals vaccinated with β-Gal alone [44].
Furthermore, a significantly higher antibody response in mice vaccinated with adjuvant vaccine than in mice immunized with subunit alone was found by the tenth week. This suggests that mucosal adjuvant in the vaccine stimulated a long-lasting humoral immunity.

The HI titre is a surrogate correlate of protection for seasonal influenza strains. We found that none of the vaccine formulations elicited HI titres after the first vaccination. Thus confirming in this murine model the need for two doses of influenza vaccine to mount a satisfactory antibody levels in naïve individuals [84]. However, as early as four days after the booster dose, the presence of the mucosal adjuvant in the vaccine enhanced the HI titer, which correlated with the increase of antibody levels in sera at the same time points (ELISA). An HI titre ≥40 was not detected until three weeks after the second dose and the GMT was over 100 by week 10, suggesting that two doses of subunit vaccine formulated with c-di-GMP adjuvant are required to obtain a sufficiently protective immune response in serum.
Hagenaars et al. have shown that intranasal immunization with 5 μg subunit vaccine in female C57-BL/6 mice did not induce a detectable HI titer, which corresponded to no detectable influenza specific IgG concentration in sera [87]. In our study, a low HI titre in mice immunized with subunit alone also correlated with the low serum antibody levels measured by ELISA. These characteristics together may indicate that non-adjuvanted subunit vaccine administered intranasally is poorly immunogenic.

## 5.2 The subunit vaccine with c-di-GMP adjuvant induced a more rapid and higher mucosal immune response than the subunit administered alone.

Influenza viruses enter the body via the upper respiratory tract, where the mucosal immune system providing the first line of defence against infection. The mucosal arm of immunity is mediated by secretory IgA antibodies, which are the major neutralizing antibody against mucosal pathogens. However, sIgA antibodies do not only block influenza virus infecting mucosal epithelial, but also viral processes inside an epithelial cell [85]. Correlation of protection is less clear for mucosal antibodies [13]. The current study showed a detectable IgA response in the NW from mice immunized with c-di-GMP adjuvanted vaccine as early as one week after the first vaccination. This antibody level increased strongly by the second week and was significantly higher at the third week compared to the non-adjuvanted vaccine group. A rapid mucosal response after just one intranasal vaccination can be a great advantage in a pandemic, because it can contribute to a rapid prevention of further transmission. After the booster dose, the IgA level in NW was significantly higher and peaked by the second week, and a long lasting local response was observed at 10 week post booster dose. This is one week later than in the IgA concentration in sera. A possible explanation can be that IgA antibodies antibody secreting cells circulate systematically before homing to mucosal sites.
Only low IgA concentrations in NW were observed for the mouse group immunized with subunit vaccine alone [87]. Interestingly, low IgA levels were also observed in C57-BL/6 mice after intranasal vaccination with 5 µg subunit vaccine. Lethal challenge of these mice showed that only 1 of 5 mice survived but still had considerable weight lost.

The findings supported by Asanuma et al. who have demonstrated that BALB/c mice intranasally vaccinated with 0.1 µg HA antigen combined with 0.1 µg cholera toxin B subunit (CBT) and 1 month later boosted with an increased dose (1.0 µg HA antigen adjuvanted with 0.1 µg CTB) induced high levels of HA-specific (IgA in NW and IgG in sera). In a protective efficacy study, no virus was detected in the upper (NW) or lower (lungs) respiratory tracts [95]. Interestingly, the mice immunized with mucosal adjuvant elicited also high IgA antibody level in NW and IgG antibody level in sera. This may be

a good reason to conduct a lethal challenge on the mice immunized with mucosal adjuvant to determine the productive efficacy.

## 5.3 The presence of c-di-GMP in the formulation induced a Th1 biased profile.

In mice, the T helper cell polarization after antigen exposure is indicated by the cytokine response and the IgG subclasses. High levels of INF-γ and IL-2 cytokines and IgG2a antibodies indicate a Th1 immune response, whereas high concentrations of IL-4, IL5 and IL-10 cytokines and IgG1 antibodies are characterized as a Th2 immune response. The type of the Th immunity is crucial for the outcome of an influenza infection. Induction of Th1 response promotes cell-mediated immunity [25], which is important in viral clearance and recovery from the infection [38]. Conversely, the Th2 immune response enhances humoral immunity, which contributes to neutralization of influenza virus and therefore prevention of clinical influenza infection [86]. In our study, an IgG2a dominance was observed throughout the experiment in the group immunized with adjuvanted vaccine. We found significantly higher Th1 cytokine (IL-2 and IFN-γ) levels after the second dose in the adjuvanted than non adjuvanted vaccine formulation. Similarly, IL-2 and IFN-γ have been found to dominate the systemic response after influenza infection [78]. Formulation of the vaccine with c-di-GMP gives a stronger cell-mediated immune response. Our group has previously shown that vaccination of mice with one and two doses of whole virus vaccine elicited a predominant IgG2a antibody response in sera [90]. Furthermore, the IgG2a is the most potent isotype in the immune response to viral infection [88]. Th1 responses are desirable after influenza vaccination because they are more cross-protective than Th2 responses. This is due to the recognition of highly conserved internal proteins (NP and M) by CTLs [89].

A low IgG2a/IgG1 ratio and much higher Th2 cytokine (IL-4 and IL-5) levels than Th1 cytokine (INF-γ and IL-2) concentrations were detected in the absence of adjuvant. Similar results have also been observed in C57-BL/6 mice and it has been suggested that the exclusive induction of IgG1 may be explained by the absence of viral genomes [87]. However, others have found that the Th2 response in immunized mice does not confer protection against virus replication in the lungs [92]. Therefore, in the current study it

would be interesting to determine the protective efficacy by using a highly pathogenic H5N1 virus challenge in both groups, immunized with and without c-di-GMP adjuvant.

Our group has earlier shown that mice vaccinated with whole inactivated virus vaccine induced a mixed Th1/Th2 cytokine response after the second dose [93]. Interestingly, the results of the current study shown that the use of c-di-GMP as mucosal adjuvant induced not only high levels of Th1 cytokines, but also proportionally high levels of Th2 cytokines (IL-4 and IL-5) and a high concentration of IgA antibodies in both locally and systematically. This indicates that the presence of the mucosal adjuvant also effectively stimulates the humoral immune response.

In the group vaccinated with adjuvanted vaccine, the concentration of IL-10 cytokine (Th2 profile) was significantly higher than in the group vaccinated without adjuvant. IL-10 cytokines are secreted by Th2 cells and contribute to inhibition of cytokine production by Th1 cells [79]. On the other hand, Th1 cells can also secret IL-10, but this is in order to "self-regulation" [76, 94]. In mice, the function of IL-10 cytokine is thought to prevent pathology induced by inflammatory responses. It also enhances survival, proliferation and antibody production of B cells [79].

The presence of the c-di-GMP adjuvant induced a significant higher IL-17 than the subunit vaccine alone. IL-17 has been shown to be important in the protection against bacteria and fungi [82], but not virus. However, IL-17 has a proinflammatory activity, which is involved in the recruitment of neutrophils. IL-17 is also able to stimulate Th2 cytokine production, which may amplify allergic inflammation [42].

A number of other mucosal adjuvants are currently being tested for intranasal formulation of vaccines. Different mucosal adjuvant elicits various Th type responses. ISCOM and LT stimulate both Th1 and Th2 responses. Cog adjuvant promotes strongly Th1 response whilst CT adjuvant stimulates Th2 response [16]. In our study, the mucosal c-di-GMP showed to induced mainly Th1 response and a weaker Th2 response.

# 6. Conclusions

In this study, we found that intranasal vaccination with subunit vaccine administered with c-di-GMP adjuvant induced a predominant Th1-type response, which may enhance the CTL immune response and thus the clearance of the viral infection. In contrast, non-adjuvanted vaccine elicited a Th2 response, but only low antibody titers. The presence of mucosal adjuvant c-di-GMP in the vaccine elicits a significantly more rapid and significantly stronger mucosal immune response than subunit vaccine alone. Our study confirmed that this formulation induced a stronger humoral and cell-mediated immunity in naïve mice as well. The results obtained in this study indicate that c-di-GMP is a promising adjuvant for the future development of intranasal pandemic influenza vaccines including the current pandemic H1N1 strain.

## 6.1 Future prospective

- We found that this mucosal adjuvant induced immunological memory and long-lasting immunity at 10 weeks post second vaccination. Further work should address the duration of the long-lasting immunity.

- C-di-GMP induced high levels of Th1 cytokines and IgG2a, and this may indicate an efficient induction of cellular immunity. However, the influenza subunit vaccine adjuvanted with c-di-GMP may also induce a cytotoxic response and this should be evaluated in a cytotoxicity assay.

- C-di-GMP used as mucosal adjuvant in an intranasal vaccine has shown to induce a stronger systemic and mucosal immunity. It will be important to determine the antigen to adjuvant ratio which will contribute to mapping the adjuvant's function and toxicity.

- A lethal challenge after intranasal vaccination with c-di-GMP adjuvant should be performed. This will provide a direct evidence of the protective efficacy and allow evaluation of the immunological parameters as correlates of protection.

- C-di-GMP as a mucosal adjuvant gives a good immune response in mice, but it is important to evaluate the adjuvant in other animal species to build a preclinical dossier to allow progression to human clinical trial.

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