Immunity to Influenza and Future Vaccination Strategies

Bescherming tegen influenza en toekomstige vaccinatiestrategieën

Rogier Bodewes

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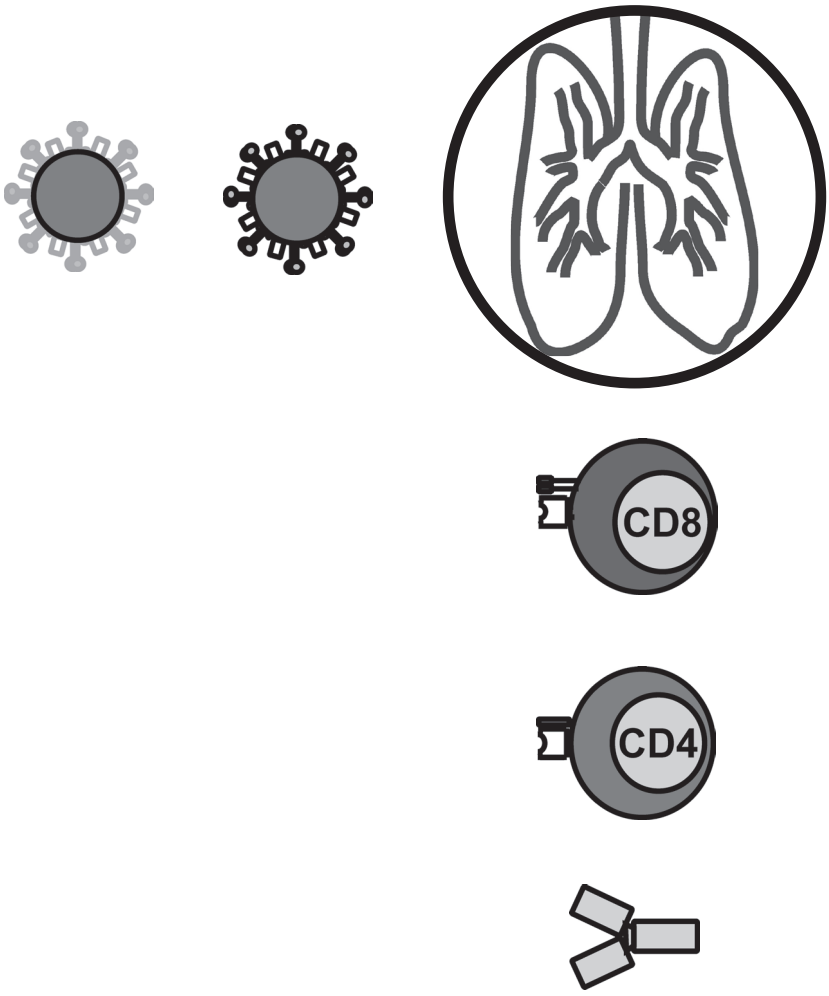
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1 General Introduction and Outline

Partially based on:

Animal models for the preclinical evaluation of candidate influenza vaccines
R. Bodewes, G.F. Rimmelzaan, A.D.M.E. Osterhaus
Expert Reviews Vaccines 9(1), 59-72 (2010)

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Targets for the induction of protective immunity against influenza A viruses
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Viruses 2010, 2, 166-188

Classification of influenza viruses

Influenza viruses are very small pathogens (typically 80-120 nm) belonging to the family of the Orthomyxoviridae together with four other genera: Thogoto virus, Isavirus and influenza B and C viruses. The influenza viruses are distinguished based on the membrane channel protein, genome size and surface glycoproteins. The influenza A viruses are further subdivided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Figure 1). At present, 16 subtypes of the HA and 9 subtypes of the NA are known (1).

Structure of influenza A viruses and their proteins

Influenza A viruses are enveloped single stranded negative sense RNA viruses with a genome consisting of eight gene segments encoding eleven different proteins. These eight RNA segments are independently encapsidated by the nucleoprotein (NP) and associated with the polymerase proteins PB1, PB2 and PA, which together form the ribonucleoprotein (RNP) complex (2). The polymerase proteins are responsible for replication and transcription of vRNA and mRNA respectively (3). The matrix protein M1 functions as a spacer between the RNP complexes and the viral envelope and interacts with both. The viral envelope is derived from the host cell membrane. Two major surface glycoproteins (HA and NA) are inserted and protrude from the viral envelope. The HA is the receptor binding protein, facilitating attachment of the virus particle to the host cell. The HA is synthesized as a precursor polypeptide HA0 which requires proteolytic cleavage into HA1 and HA2 subunits before it becomes functional and virus particles can infect cells. The HA1 subunit contains the receptor-binding pocket and the relatively conserved HA2 unit constitutes the stem region containing the fusion peptide. This fusion peptide plays an important role in pH-dependent fusion of the viral envelope with the endosomal vesicle.

By acting as a receptor-destroying enzyme, the NA plays an important role in the virus replication cycle after budding of new viruses from the infected cell. NA cleaves sialic acid residues, which promotes release of newly produced virus particles from the infected cell. The minor envelope protein, M2, is the result of alternative splicing of mRNA encoding M1. It functions as an ion channel and facilitates the influx of H+ ions into the virus particle. M2 is the target for the antiviral drug amantadine. Two non-structural (NS) proteins are also expressed in the infected cell, NS1 and NS2. NS1 is a multifunctional protein and is known for antagonizing the host cell IFN production and its activity (3). NS2 is involved in nuclear transport of RNP complexes. Recently, the eleventh viral protein was identified which is transcribed from an alternative reading frame of PB1 (PB1-F2) (4). Most likely, this protein plays a role in promoting apoptosis of the infected cell.

As for other virus infections, influenza viral proteins are degraded in the cytosol of the infected cell by the proteasome into peptides. These peptides are transported to the endoplasmatic reticulum where they can bind to MHC class I molecules. The MHC class I peptide complexes are subsequently transported to the surface of the infected cells where they can be recognized by virus specific CD8+ T cells (see below).

Influenza A virus replication cycle

To start virus replication, the virus needs to attach to host cells first. Therefore, attachment of the

virus to the host cell is crucial and is thought to be the major determinant for the host range of influenza A viruses (5). After binding of the HA of the influenza virus particle to the sialic acid residues on the host cell surface, the virus enters the cell through receptor-mediated endocytosis. The low pH in the endocytic compartments activates fusion of the viral membrane with the cell membrane which releases the RNPs into the cytoplasm. After transport of the RNP to the nucleus, the viral RNA is transcribed in the nucleus into messenger RNA and copy RNA. Messenger RNA is used for the translation of viral proteins and copy RNA serves as a template for viral RNA synthesis. Viral RNA and newly formed proteins together constitute new RNPs, which are packaged into new virions. Subsequently, the NA cleaves sialic acid residues from the cell surface to facilitate efficient release of the viral particles.

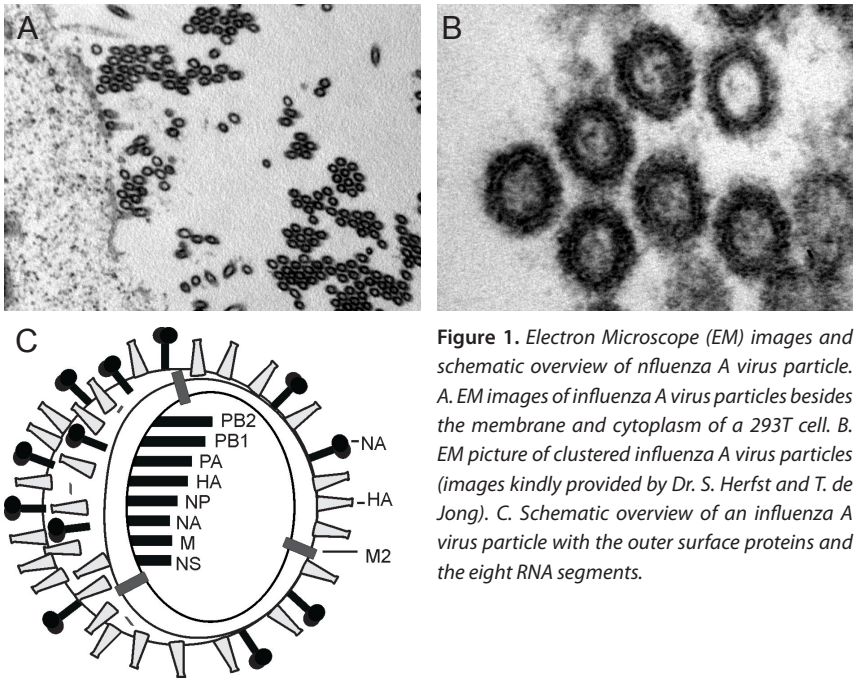


Figure 1. *Electron Microscope (EM) images and schematic overview of nfluenza A virus particle. A. EM images of influenza A virus particles besides the membrane and cytoplasm of a 293T cell. B. EM picture of clustered influenza A virus particles (images kindly provided by Dr. S. Herfst and T. de Jong). C. Schematic overview of an influenza A virus particle with the outer surface proteins and the eight RNA segments.*

Natural hosts of influenza viruses

Wildfowl and shorebirds are the natural reservoir of all subtypes of influenza A viruses (6). Low pathogenic influenza viruses have been isolated from at least 105 wild bird species (7). Also influenza viruses have been isolated from a number of mammalian species, including horses, swine, marine mammals, mink and ferrets. The fact that influenza A viruses cross the species barrier easily is not only demonstrated by the recent influenza A/H5N1 and influenza A/H1N1(2009) infections of humans, but also by the recent isolation of equine influenza A/H3N8 from dogs with severe pneumonia (8). In addition to infection of humans with avian influenza A/H5N1 viruses, a number of animal species have been infected with these viruses. This includes tigers, leopards, cats, dogs, other free-living wild carnivores and a number of bird species (9-11).

Influenza A virus in humans

Influenza-like illness in humans was already recorded more than 2000 years ago by Hypocrates, while the first human influenza A virus was isolated in during the 1932/1933 epidemic by Smith, Andrewes and Laidlaw. At present, influenza A and B viruses continue to circulate among humans, mainly during the winter season of both hemispheres. Clinical signs after infection of humans with epidemic influenza A virus and after infection with influenza A/H1N1(2009) viruses strains include nasal obstruction, cough, fever, anorexia and headache but complications with severe pneumonia do occur. Mainly the young, elderly and immunocompromised people are at risk for developing severe disease after infection with seasonal influenza virus either due to viral pneumonia or secondary bacterial pneumonia causing approximately 250,000 to 500,000 deaths annually (12). In addition to influenza epidemics caused by seasonal influenza viruses, pandemics occur occasionally. Influenza pandemics are caused by influenza A viruses of novel subtypes that emerge after genetic reassortment (antigenic shift) or adaptation to replication in humans of non-human influenza viruses. In the absence of virus-neutralizing antibodies to these novel subtypes of influenza A virus in the human population, these viruses can affect a substantial proportion of the human population when transmitted efficiently from human-to-human. In the last century, three pandemics occurred, which were caused by influenza A viruses of the H1N1, H2N2 and H3N2 subtypes. Recently, influenza A viruses of swine origin have caused the first pandemic of the 21st century (13, 14). These newly emerged pandemic viruses are the result of the exchange of gene segments originating from human, classical swine and avian-like swine influenza viruses and have spread worldwide within a few months (15, 16). In contrast to the efficient human-to-human transmission and the rapid spread of the new influenza A/H1N1 viruses, the highly pathogenic avian influenza A viruses of the H5N1 subtype, first detected in humans in 1997 (17, 18) are transmitted from human-to-human inefficiently so far, although clusters of human-to-human transmission have been reported (19, 20). However, over 500 human cases that have been reported since 2003 of which 60% had a fatal outcome and therefore it is feared that these viruses may adapt and become pandemic in the future (21). In addition to avian A/H5N1 viruses and the new A/H1N1 viruses of swine origin also influenza A viruses of other subtypes have crossed the species barrier and have infected humans recently. In 2000, an avian influenza A/H9N2 virus infected two children in Hong Kong causing only mild disease (22) whereas during an outbreak of highly pathogenic avian influenza A/H7N7 in poultry in the Netherlands, 89 humans were infected of which one died (23).

Clinical symptoms after infection with the highly pathogenic influenza A/H5N1 virus develop in most cases 2-4 days after the last exposure to affected poultry. Clinical signs include fever, cough and shortness of breath. On chest radiographs, evidence of pneumonia is present. The pneumonia seems in most cases of primary viral origin without evidence of bacterial supra infection. Other symptoms are diarrhea, vomiting and abdominal pain. In more severe cases a progressive bilateral pneumonia develops rapidly, with complications that include acute respiratory distress syndrome, renal dysfunction and multiorgan failure. Indications of involvement of the central nervous system were only present in one case. Progressive respiratory failure is in most cases the cause of death, but there is evidence that influenza A/H5N1 viruses can disseminate to other organs (24).

Immunity to influenza viruses

The immune system of mammals has developed several lines of defence to prevent the invasion of pathogens or to limit their replication. Before attachment and infection of the host cells is possible, there are several hurdles to be taken. The innate immune response creates the first line of defence, is broadly-reactive and plays a crucial role in the induction and direction of the adaptive immune response. In addition, the innate immune system is essential for the host during the first 4-7 days after infection until the adaptive response can have an effect. In contrast to the innate immune response, the adaptive immune response is very specific and is able to provide an increased level of protection against reinfection with a certain pathogen.

Innate immune response

The innate immune system recognizes pathogen-associated molecular patterns (PAMP) and endogenous danger signals (25). The major PAMP of the influenza virus is thought to be cytoplasmatic viral RNA species that contain triphosphate groups at their 5' ends (26). Host cells detect the presence of an infecting virus by pattern recognition receptors (PRR) that recognize the PAMP. PRRs are divided into several families, including nucleotide-binding oligomerization domain (NOD)-like receptors, Toll-like receptors (TLRs), and retinoic acid-inducible gene-I (RIG-I)-like helicases. Recognition of PAMP leads to initiation of antiviral signaling cascades that induces an antiviral response, the infected tissue is in an 'alerted state'. This alerted state results in the secretion of cytokines (e.g. type I and III interferons) and secretion of chemokines to attract and activate inflammatory cells (e.g. natural killer cells) and antigen presenting cells (dendritic cells). Especially dendritic cells play a crucial role in the induction of the adaptive immune response as they migrate to the afferent lymph node to present their antigen to T cells.

Table 1. Viral targets for the induction of protective antibody responses

Viral antigen	Mode of action	Comments
HA	Prevents virus attachment to host cells	• Antibodies must have proper specificity • Strain specific
NA	Inhibits enzymatic activity of NA and spread of virus	• Antibodies must have proper specificity
M2	Induction of antibody-dependent cell-mediated cytotoxicity (ADCC) and elimination of infected cells	• M2 is highly conserved • Hyperimmunization induces cross-protective immunity
NP	Largely unknown, complex formation?	• Non-neutralizing • Mode of action and effectiveness unknown

Adaptive immune response

In contrast to the innate immune response, the adaptive immune response is highly specific and is characterized by the development of memory for a specific pathogen. This indicates that upon re-encounter of the host with a certain pathogen the immune response is stronger and more rapidly. The adaptive immune response consists of the humoral antibody-mediated response and the cellular T-lymphocyte-mediated response. Upon recognition of their antigen and specific

stimulation signals, B-lymphocytes start to proliferate and differentiate into plasma cells that are able to secrete antibodies. Multiple isotypes of antibodies exist. For the protection against influenza viruses, mainly antibodies of isotypes IgA, IgG and IgM are important. IgA antibodies can play an important role in the protection against influenza viruses as they are the main class of antibodies in the secretions of the mucous epithelium of the respiratory tract, while IgG antibodies are the prinicipal isotype of blood and extracellular fluid and thought to be the most effective antibodies against infection with influenza A viruses.

T cells can be further subdivided into the CD4+ and CD8+ T cells. CD4+ T cells recognize antigen presented by the MHC-class II molecules and play a crucial role in the regulation of the immune response. CD4+ T cells are divided into various subsets, of which especially T helper 1 and T helper 2 cells are thought to play an essential role in the control of virus infections. T helper 1 cell stimulate the CD8+ T cells by secretion of certain cytokines (27-30), while T helper 2 cells are important for the virus-specific antibody response since they play a role in the optimal activation and early expansion of B cells, the initiation and maintenance of germinal center reaction and the generation of long-lived plasma and memory B-cells (31-34). Furthermore, it has been suggested that CD4+ T cells also can attack virus-infected host cells directly (35).

The latter function is especially executed by virus-specific cytotoxic T lymphocytes (CTL). After initial stimulation and differentiation into CTL with an effector phenotype, CD8+ T cell are able to recognize and kill virus-infected cells that present certain epitopes on the surface of the cell by the MHC class I molecules. CTL can eliminate virus-infected cells through the release of perforin and granzyme or Fas/FasL interaction. In addition, activated CD8+ T cells produce cytokines like IFN-γ and TNF-α which can modulate the immune response

Table 2. Viral targets for the induction of protective T cell responses.

Viral antigens	Type of response	Comments
All viral proteins	CD4+ T helper cell response	• Polarization (Th1/Th2) dependent on antigen delivery • Essential for B-cell and CD8+ CTL responses • Direct action against infected cells • HLA restricted
PB1/PB2/PA/NP/ M1/M2/ NS1	CD8+ CTL response	• Key role in elimination of infected cells • Cytokine production • HLA restriction dictates magnitude of response • Only marginal response to HA

Viral targets for the induction of immunity against influenza A viruses

The envelope proteins are the most important targets for the induction of virus-specific antibodies (Table 1 and Figure 2). The induction of sufficiently high titers of HA-specific antibodies affords sterilizing immunity against infection provided that these antibodies have the proper specificity for the strains causing the infection. The induction of NA specific antibodies also contributes to protective immunity, but since these antibodies interfere with a late step in the virus replication cycle they cannot prevent infection. Furthermore, NA-specific antibodies need to have specificity for the strain causing the infection, like HA-specific antibodies. In contrast, M2-specific antibodies

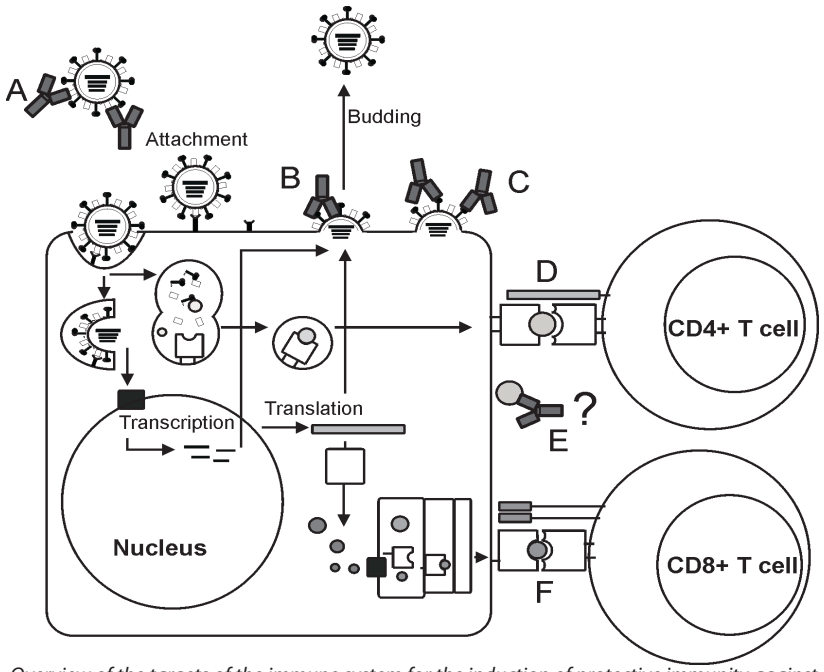


Figure 2. *Overview of the targets of the immune system for the induction of protective immunity against influenza. (A) HA-specific antibodies can bind to the HA on viruses and prevent infection of cells. (B) M2e specific antibodies can bind to M2e on virus-infected cells and induce antibody-dependent cell-mediated cytotoxicity (ADCC). (C) NA specific antibodies inhibit enzymatic activity of NA and thus further spread of newly produced virus particles. (D) Pathogens and proteins are broken down into peptides within acidified endosomes and these peptides bind to MHC Class II, MHC Class II peptide complexes are subsequently transported to the surface of the cell for recognition by CD4+ T cells. (E) The mode of action of NP-specific antibodies is largely unknown. (F) Influenza viral proteins are degraded in the cytosol of the infected cell by the proteasome into peptides that are transported to the endoplasmatic reticulum where they can bind to MHC class I molecules. The MHC class I peptide complexes are transported to the surface of the infected cells for recognition by CD8+ T cells, which subsequently eliminate the infected cell.*

induced after hyperimmunization or passively administered, afford protection against multiple influenza virus strains and even against multiple subtypes of influenza A virus, since this protein is highly conserved. Also M2-specific antibodies do not afford sterilizing immunity since their most important mode of action is through ADCC after binding to infected cells expressing M2 on their surface. The mode of action and the effectiveness of non-neutralizing NP-specific antibodies are not fully understood although it has been demonstrated after hyperimmunization that they afford some protection (36).

In addition to the systemic and/or mucosal antibody responses, also virus-specific T cells contribute to protective immunity against infection (Table 2). T helper cells are directed to virtually all viral structural proteins and polymerases, while virus-specific CTL preferentially recognize internal structural proteins like NP and M1. Since these proteins are highly conserved between subtypes, CTL responses are cross-reactive and are tought to contribute to heterosubtypic immunity.

Heterosubtypic immunity

Infection with influenza virus does not induce lifelong protective immunity against influenza infection in humans, even not against infection with the same subtype. The main reason for this is that influenza A viruses continue to circulate as antigenic drift variants, that have accumulated mutations in antigenic sites of the HA molecule that are recognized by virus neutralizing antibodies. However, the induction of antibodies of the proper specificity will afford strain-specific protection and this strain-specific immunity can be very long lasting (37, 38).

Since subtypes of the influenza viruses are defined by the absence of mutual cross-reactivity of subtype specific antibodies (39), antibodies to one subtype will not afford protection against infection with an influenza virus of another subtype. However, it has been demonstrated that infection with an influenza A virus can induce a certain degree of protective immunity against infection with an influenza A virus of another subtype, although infection cannot be prevented (40). This so-called heterosubtypic immunity was first described more than four decades ago (41). Heterosubtypic immunity induced by infection has shown to be long-lasting (18 months) in the ferret model, which is the gold standard model for human influenza A virus infections (42). The immunologic basis underlying heterosubtypic immunity has been the topic of numerous studies (40). Experiments in multiple knock-out and transgenic mouse models have shown that virus-specific CD4+ T cells (T helper cells), CD8+ Cytotoxic T cells (CTL), mucosal antibodies (IgA) and B cells can contribute to heterosubtypic immunity (36, 43-47). Especially cell-mediated immune responses directed to conserved proteins of influenza A viruses are believed to play an important role (48-51). Indeed, human cytotoxic T lymphocytes (CTLs) responses directed to human influenza A viruses display considerable cross-reactivity with HPAI viruses of the A/H5N1 subtype.(51-53). Consequently, also in humans the presence of cross-reactive CTLs inversely correlated with the amount of viral shedding in the absence of antibodies specific for the virus used for experimental infection.(50) Thus, cross-reactive T cell responses induced after previous infection may afford some degree of protection against influenza A viruses of novel subtypes. There is also evidence that indeed infection with influenza A virus can induce heterosubtypic immunity in humans. Individuals that had experienced an infection with an influenza A/H1N1 virus before 1957 less likely developed flu during the H2N2 pandemic of 1957 (54). In this respect, the disproportional age distribution of severe human H5N1 cases and the relatively high mortality rates new H1N1 influenza virus infections among younger individuals is of interest and at present a matter of debate (55-57). Especially younger individuals are at risk and although other confounding factors cannot be excluded, it is tempting to speculate that young subjects have been infected with seasonal influenza viruses less frequently and therefore have not developed protective heterosubtypic immune responses against infection with the new influenza A/H1N1 virus and the highly pathogenic avian A/H5N1 viruses.

Influenza A virus vaccines

Since seasonal influenza A viruses of the H3N2 and H1N1 subtypes and influenza B viruses cause epidemics annually associated with excess morbidity and mortality mainly among the elderly, immuno-compromised and other high-risk groups, influenza vaccination is recommended for these high-risk groups. Furthermore, due to the higher risk of complications and hospitalizations

secondary to influenza in children (58, 59), annual vaccination of all healthy children 6 to 59 months of age was recommended in various countries including the United States since 2007 (60). Also in Europe, vaccination of children is currently considered and a number of countries already recommend vaccination of healthy children (61). Annual vaccination against epidemic flu has been shown to be (cost-) effective in children since it decreases the burden of disease and the number of hospitalizations due to infection with influenza A viruses (62-66). Most influenza virus vaccines that are currently used against seasonal influenza viruses and against the influenza A/H1N1(2009) virus are prepared by infecting embryonated chicken eggs with influenza virus vaccine strains. Subsequently allantoic fluids of infected eggs are harvested and the egg-derived virus is purified. Depending on the vaccine manufacturer, influenza viruses are inactivated with formaldehyde or ß-propiolactone to prepare a whole inactivated influenza virus vaccine or treated with a detergent to prepare a split or subunit influenza vaccine. Subunit vaccines are, after treatment with a detergent, further purified to remove all viral proteins and lipids except the HA and NA. Live attenuated viruses are typically attenuated by adapting viruses to replicate at lower temperatures, while cold-adapted vaccine strains are subsequently prepared by reassortment with selected epidemic strains to ensure that the vaccine strains contain the proper HA and NA.

To increase the immunogenicity of influenza A virus vaccines, adjuvants can be used. At present, many of these non-specific stimulators of the immune system have been developed for use in influenza A virus vaccines. The value of adjuvants has been especially demonstrated for weak immunogens, like the influenza A/H5N1 virus. Furthermore, it has been demonstrated that the use of adjuvants allows dose sparing of the antigen and broadens the specificity of the antibody responses that are induced (67, 68). In addition to the use of adjuvants, alternative formulations and methods to prepare influenza virus vaccines are in various stages of development or already licensed. Examples are the production of vaccine strains by reverse genetics, the use of virosomes and virus-like particles and the expression of viral genes in recombinant baculoviruses or modified vaccinia viruses (69-74).

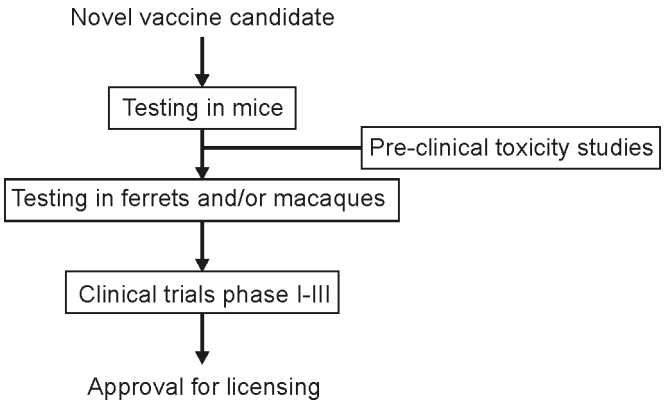


Figure 3. *Stages in the development of novel influenza A virus vaccines*

Use of animal models for influenza virus vaccine research

Ideally, the effectiveness of novel seasonal influenza vaccines is assessed in clinical trials. However, in most cases large numbers of study subjects are required considering the attack rate (5-10%) of influenza viruses, which makes these studies very costly. Therefore, there should be a good justification for studies like this and preclinical evaluation of candidate vaccines is essential for the selection of promising candidates to be tested in clinical trials. For pandemic influenza vaccines it is not possible to assess their effectiveness in humans for obvious reasons: the pandemic viruses are not yet circulating and experimental infections are potentially very dangerous. In those cases only the immunogenicity of candidate vaccines can be determined, but not the protective efficacy of vaccine-induced immune responses. Therefore, to assess their protective efficacy, the preclinical testing of candidate pandemic influenza vaccines in appropriate animal models is essential. In table 3, advantages and disadvantages of various animal species that are commonly used for influenza vaccine research are listed (Table 3) and in Figure 3 stages in the development of novel influenza A virus vaccines are shown.

Table 3. Advantages and disadvantages of animal models most commonly used for the evaluation of candidate vaccines.

Species	Advantages	Disadvantages
Mouse	• Cheap • Short reproduction time • Good availability of immunological reagents • Allows mechanistic studies (inbred species and genetically modified mice) • Genomics approach possible • Evaluation of vaccines in elderly mice possible	• Predictive value not always clear • Most seasonal strains need to be adapted • Pathogenesis does not resembles that of humans • Most strains used are inbred strains • Not suitable for the evaluation of live attenuated influenza A virus vaccines
Guinea pig	Suitable for transmission studies	• Pathogenesis does not resemble that of humans • No clinical signs after infection
Ferret	• Pathogenesis resembles that of humans • Suitable for transmission studies • Pattern of virus attachment resembles that of humans	• Poor availability of immunological reagents • Perhaps more susceptible to influenza A viruses than humans (especially newborn ferrets), sometimes difficult to obtain specified pathogen free animals
Macaque	• Good predictive value of vaccine immunogenicity and protective efficacy • Immune system resembles that of humans • Genomics approach possible	• Expensive • Ethical issues • Need of experienced personnel

Outline of this thesis

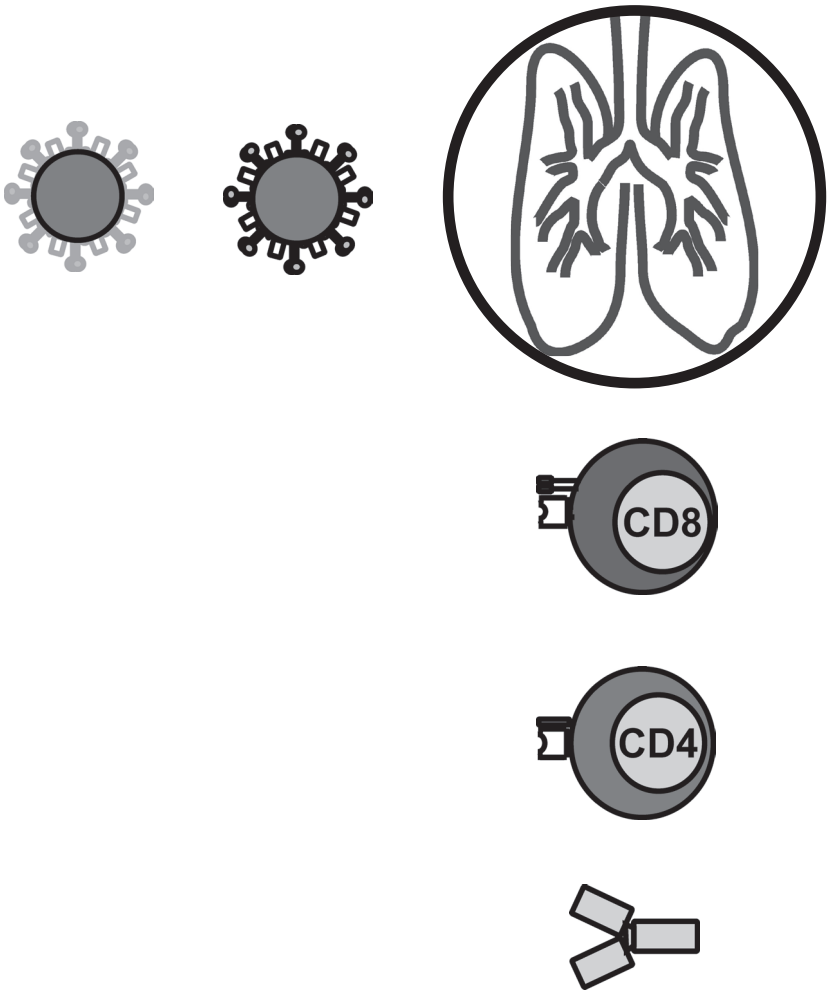
In this thesis, the impact of the use of seasonal and pandemic influenza A vaccines was evaluated in the light of the pandemic threat of the highly pathogenic avian influenza A/H5N1 viruses. In chapter 2, the presence of heterosubtypic immunity against influenza A/H5N1 virus after infection with influenza A/H3N2 virus was evaluated in the mouse model and immunological assays were performed to assess the correlation between survival and the CD8+ T cell response.

Based on these findings and the recent recommendation of public health authorities of a number of countries, including the USA and some European countries, to vaccinate all healthy children 6-59 months of age against seasonal influenza it was questioned whether annual vaccination of young children against seasonal influenza may have a potential downside. Since annual vaccination against seasonal influenza is effectively preventing infection, it also may prevent the induction of immune responses that otherwise could have been induced by infection. To test this hypothesis, mice were vaccinated with either a influenza A/H3N2 subunit vaccine (chapter 3) or a whole inactivated influenza A/H3N2 virus (chapter 4) vaccine before mice were inoculated with influenza A/H3N2 virus. Four weeks after inoculation with the influenza A/H3N2 virus, mice were challenged with an influenza A/H5N1 virus and clinical symptoms, viral load in the lungs and CD8+ T cell immunity were assessed after challenge. In addition to studies in the mouse model, the ferret model was used to demonstrate the presence of heterosubtypic immunity induced by infection with an influenza A/H3N2 virus and to evaluate the effect of vaccination against seasonal influenza viruses on the development of heterosubtypic immunity in this species (chapter 5). Since it can be of great importance for the implementation of adequate vaccination strategies to know at which age children will have antibodies against multipe influenza viruses, the presence of antibodies against seasonal influenza viruses in children in the Netherlands was evaluated to assess the proportion of children that had experienced an influenza virus infection between 0 and 7 years of age. To this end, sera were used from children 0-7 years of age collected during a nation-wide sero-epidomological study (chapter 6). In addition, virus-specific antibody and CD4+ and CD8+ T cell responses of children with cystic fibrosis that were vaccinated against seasonal influenza annually were compared with unvaccinated healthy control children in chapter 7 to evaluate the difference in the virus-specific immune responses between these two groups.

Besides studies involving the impact of the use of seasonal influenza virus vaccines on the development of heterosubtypic immunity, the immunogenicity and protective efficacy of the novel adjuvant CoVaccine HT™ in combination with a whole inactivated influenza A/H5N1 virus vaccine was assessed in chapters 8 and 9 . To this end, *in vitro* experiments with dendritic cells and *in vivo* vaccination experiments were performed in mice and ferrets.

In addition, in chapter 10 the pathogenicity of influenza A/H5N1 virus after intranasal and intratracheal inoculation of ferrets are described.

Collectively, studies presented in this thesis demonstrate the role of infection with seasonal influenza viruses and the use of seasonal inactivated influenza virus vaccines on the development of heterosubtypic immunity and show that the novel adjuvant CoVaccine HT™ in combination with a low dose of antigen can establish protection against influenza A/H5N1 virus after a single vaccination. The potential implications of these findings are discussed in chapter 11.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Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus

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ABSTRACT

The transmission of highly pathogenic avian influenza (HPAI) A viruses of the H5N1 subtype from poultry to man and the high case fatality rate fuels the fear for a pandemic outbreak caused by these viruses. However, prior infections with seasonal influenza A/H1N1 and A/H3N2 viruses induce heterosubtypic immunity that could afford a certain degree of protection against infection with the HPAI A/H5N1 viruses, which are distantly related to the human influenza A viruses. To assess the protective efficacy of such heterosubtypic immunity mice were infected with human influenza virus A/Hong Kong/2/68 (H3N2) four weeks prior to a lethal infection with HPAI virus A/Indonesia/5/05 (H5N1).

Prior infection with influenza virus A/Hong Kong/2/68 reduced clinical signs, body weight loss, mortality and virus replication in the lungs as compared to naïve mice infected with HPAI virus A/Indonesia/5/05. Priming by infection with respiratory syncytial virus, a non-related virus did not have a beneficial effect on the outcome of A/H5N1 infections, indicating that adaptive immune responses were responsible for the protective effect. In mice primed by infection with influenza A/H3N2 virus cytotoxic T lymphocytes (CTL) specific for $NP_{366-374}$ epitope ASNENMDAM and $PA_{224-233}$ SCLENFRAYV were observed. A small proportion of these CTL was cross-reactive with the peptide variant derived from the influenza A/H5N1 virus (ASNENMEVM and SSLENFRAYV respectively) and upon challenge infection with the influenza A/H5N1 virus cross-reactive CTL were selectively expanded. These CTL, in addition to those directed to conserved epitopes, shared by the influenza A/H3N2 and A/H5N1 viruses, most likely contributed to accelerated clearance of the influenza A/H5N1 virus infection. Although also other arms of the adaptive immune response may contribute to heterosubtypic immunity, the induction of virus-specific CTL may be an attractive target for development of broad protective vaccines. Furthermore the existence of pre-existing heterosubtypic immunity may dampen the impact a future influenza pandemic may have.

INTRODUCTION

Highly pathogenic avian influenza A viruses of the H5N1 subtype continue to cause outbreaks in domestic birds and are transmitted regularly from infected poultry to humans. Since 2003, 409 human cases have been reported of which >60% had a fatal outcome (75). It is feared that these viruses adapt to their new host and become transmissible from human to human. Since neutralizing antibodies against these viruses are absent in the human population at large, this may spark a pandemic outbreak.

However, previous infections with influenza A virus of the H1N1 and H3N2 subtypes responsible for seasonal influenza activity, can induce heterosubtypic immunity, which may afford a certain degree of protection against viruses of a novel subtype e.g. H5N1. The induction of heterosubtypic immunity by primary influenza virus infection was already recognized more than four decades ago (41) and has been demonstrated in various animal models including mice (76, 77), pigs (78, 79), ferrets (42), chickens (80) and cotton rats (81), using various combinations of influenza A virus subtypes for priming and challenge infection. There is also direct and indirect evidence for the existence of heterosubtypic immunity in humans. It was demonstrated that individuals that experienced an infection with influenza A(H1N1) virus in preceding years were partially protected from infection with the pandemic H2N2 virus in 1957 (54).

Several lines of evidence indicate that cell-mediated immunity and in particular CD8+ cytotoxic T lymphocytes contribute to heterosubtypic immunity (82, 83). The majority of CTL is directed to conserved epitopes located within the relatively conserved proteins of the virus (84) like the nucleoprotein (NP) and the matrix (M1) protein. The cross-reactive nature of CTL not only has been demonstrated in various animal models but also in man. Human CTL directed to human influenza A virus of the H1N1 or H3N2 subtype can recognize and eliminate cells infected with highly pathogenic avian influenza viruses of the H5N1 subtype (51-53).

A protective effect of virus-specific CTL was demonstrated after adoptive transfer of these cells to naive mice or in mice from which CTL were depleted (42, 47, 83). Also the use of vaccine preparations that induce (cross-reactive) CTL responses supported a protective role of virus specific CTL in heterosubtypic immunity. In humans evidence is sparse, but it was demonstrated that in the absence of virus-specific antibodies, the presence of cross-reactive CTL correlated with reduced viral shedding after experimental infection (50).

Recently we confirmed in a mouse model that a prior infection with influenza A virus X-31 (H3N2) protected against a lethal challenge infection with influenza virus A/Puerto Rico/8/34 (A/PR/8/34) (H1N1), which correlated with anamnestic CTL responses (77). However, influenza virus X-31 and A/PR/8/34 share the gene segments that encode the internal viral proteins which, of course, favors the induction of cross-reactive CTL responses. Therefore, we wished to investigate the protective efficacy of heterosubtypic immunity, induced by infection with a human influenza A virus, against infection with a highly pathogenic avian influenza virus of the H5N1 subtype to mimic the natural situation and the order of infections more closely. For this purpose, influenza viruses A/Hong Kong/2/68 (H3N2) and A/Indonesia/5/05 (H5N1) were used. Again, prior infection with a heterosubtypic strain (H3N2) had a beneficial effect on the clinical outcome of the H5N1 challenge infection

and control of virus replication. The CTL response against the H-2Db restricted CTL epitopes $NP_{366-374}$ and $PA_{224-233}$ primed for an anamnestic CTL response to the H5N1-derived peptide variants that correlated with the observed protection.

MATERIAL AND METHODS

Influenza viruses

Influenza viruses A/Hong kong/2/68 (A/HK/2/68) (H3N2) and A/Indonesia/5/05 (A/IND/5/05) (H5N1) were propagated in Madin-Darby Canine Kidney (MDCK) cells. Infectious titers of the virus stocks were determined in MDCK cells as described previously (85).

Mice

Female specified pathogen free 6-8 weeks old C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany) and age-matched at the time point of challenge infection. Mice were infected intranasally with $5x10^2$ $TCID_{50}$ of influenza virus A/HK/2/68 in a volume of 50µl PBS. Control mice were mock-infected with PBS or were infected with $5x10^6$ $TCID_{50}$ of respiratory syncytial virus (RSV) in a volume of 50ul. Productive infection with influenza virus A/HK/2/68 and RSV was confirmed by virus isolation from the lungs of infected animals on day 4 post infection (p.i.). Four, seven and twenty-eight days after infection, mice were euthanized by exsanguination and their lungs and spleen were resected. After twenty-eight days, remaining mice were subsequently challenged with $2x10^2$ $TCID_{50}$ of influenza virus A/IND/5/05. This is the minimal dose that resulted in a lethal infection in >90% mice reproducibly. Mice were monitored daily for weight loss and morbidity after infection. Four, seven and fourteen days after challenge infection mice were euthanized and their lungs and spleen were resected. Intranasal infections, blood sampling and euthanasia were carried out under anesthesia with isoflurane (3%/O_2). The animals were housed in filter-top cages and had access to food and water ad libitum. During the infection with the influenza A/H5N1 virus, animals were housed in bio-safety level 3 containment facilities. The experimental protocol was approved by an independent animal ethics committee.

Serology

Serum samples were obtained before primary infection, four weeks later and four, seven and fourteen days after challenge infection. After treatment with cholera filtrate and heat-inactivation at 56°C, the sera were tested for the presence of anti-HA antibodies. For this purpose a hemagglutination inhibition assay (HI) was used, following a standard protocol using 1% turkey erythrocytes and four HA-units of either influenza virus A/HK/2/68 or A/IND/5/05 (86). For this purpose a reverse genetics influenza A/IND/5/05 virus was produced from which the basic cleavage site in the HA molecule was deleted. The antibody titers obtained with this virus were comparable with those obtained with the wild type strain (data not shown). Sera were also tested for the presence of virus-neutralizing antibodies specific for the two influenza viruses using a micro virus neutralization (VN) assay with 100 $TCID_{50}$ of the respective viruses (87). Influenza virus A/HK/2/68 specific serum was obtained by injecting a rabbit with sucrose gradient purified virus (88). Hyper-immune

serum obtained from a swan immunized twice with inactivated influenza H5N2 virus A/Duck/Potsdam/1402/86 (Intervet, Boxmeer, the Netherlands) was used as a positive control against the influenza A/H5N1 virus (89).

Lung virus titers

Lungs were snap frozen on dry ice with ethanol and stored at -70°C. Subsequently they were homogenized with a FastPrep-24® (MP Biomedicals, Eindhoven, the Netherlands) in transport medium (Hanks medium (MEM) containing: 10% Glycerol, 100U/ml penicillin, 100µg/ml streptomycin, polymyxin B, Nystatin, Gentamicin, 7,5% NaHCO3, 1M Hepes) and centrifuged briefly. Quintuplicate ten-fold serial dilution of these samples were used to determine the virus titers on confluent layers of MDCK cells as described previously (85).

Virus-specific T cells

Tetramer staining

Single-cell splenocyte suspensions were obtained using 100µm cell strainers (BD, Alphen a/d Rijn, the Netherlands). Red blood cells were removed using erythrocyte lysis buffer (Roche, Almere, the Netherlands). The cells were washed with 2% FCS in PBS and stained for flow cytometry with antibodies: CD3e-PerCP, CD8b.2-FITC (BD Pharmingen, Alphen a/d Rijn, the Netherlands) and APC labeled H-2Db tetramer with the $NP_{366-374}$ epitope ASNENMEVM or an PE labelled H-2Db tetramer with the $NP_{366-374}$ epitope ASNENMDAM (Sanquin Research, Amsterdam, the Netherlands). Cells were analysed using a FACSCalibur with a high throughput sampler in combination with Platemanager and Cellquest Pro software (BD Pharmingen).

Intracellular cytokine staining of splenocytes after peptide stimulation

Single-cell splenocyte suspensions were obtained as described above. CTL epitopes $NP_{366-374}$ (ASNENMDAM and ASNENMEVM derived from influenza virus A/HK/2/68 and A/IND/5/05 respectively) and $PA_{224-233}$ (SCLENFRAYV and SSLENFRAYV, respectively) were purchased as synthetic peptides (immunograde, >70% purity) (from Sanquin Research, Amsterdam, the Netherlands and Eurogentec, Seraing, Belgium, respectively) (90, 91). Four hundred thousand splenocytes were cultured for 6 h at 37°C in the presence of 5µM of peptide in IMDM (Lonza, Breda, the Netherlands) with 5% FCS and Golgistop (BD Pharmingen). The cells were then incubated overnight at 4°C and subsequently intracellular IFN-γ staining was performed. In brief, cells were washed with PBS containing 2% FCS and Golgistop, stained for flow cytometry with monoclonal antibodies: CD3e-PerCP or CD8b.2-FITC (BD Pharmingen), fixed and permeabilized with cytofix and cytoperm (BD Pharmingen) and stained with a monoclonal antibody specific for IFN-γ (BD Pharmingen). Cells were analysed on a FACSCalibur with HTS module in combination with Platemanager and Cellquest Pro software (BD Pharmingen).

Histopathology and immunohistochemistry

After euthanasia, the lungs of the mice were inflated with 10% neutral buffered formalin. After fixa-

tion the lungs were embedded in paraffin, sectioned at 4µm and stained with hematoxylin and eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method with a monoclonal antibody (Clone HB65 IgG2a (American Type Culture Collection)) directed against the nucleoprotein of influenza A virus. a Goat-anti-mouse IgG2a HRP (Southern Biotech, Birmingham, Alabama, USA) was used as secondary antibody. The peroxidase was revealed using diamino-benzidine as a substrate, resulting in a deep red precipitate in the nucleus of influenza A virus infected cells and a less intense red staining in the cytoplasm. The sections were counterstained with hematoxylin.

Statistical analysis

Data for weight loss, viral titers, antibody titers and virus-specific T cell populations were analyzed using the two-sided Student's t test and differences were considered significant at $P<0.05$. Cumulative survival was calculated with the Kaplan Meyer log rank test.

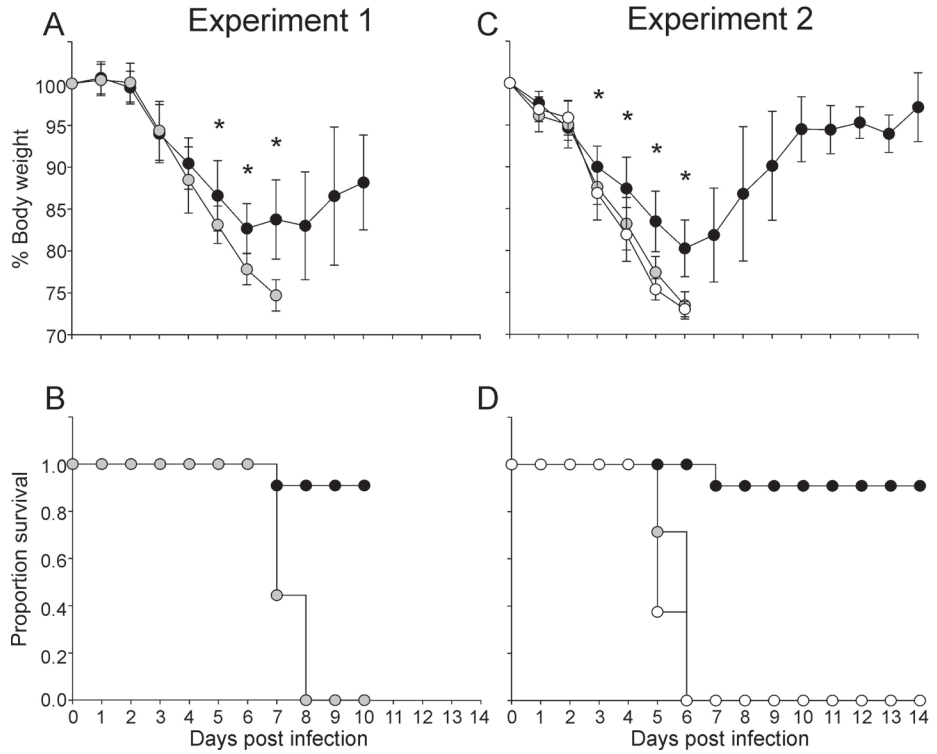

Figure 1. *Bodyweight loss after challenge infection with influenza virus A/IND/5/05 in H3N2-primed mice (black dots) and mock-infected control mice (grey dots) or RSV-primed mice (white dots) (A,C). Survival of these animals after infection with influenza virus A/ IND/5/05 (B,D), which was significantly higher after day 6 for the H3N2-primed mice.*(indicates statistical significant difference (p<0.05)*

RESULTS

Clinical outcome of influenza virus A/IND/5/05 infection

To determine whether primary infection with a human influenza A virus can protect against a subsequent infection with a highly pathogenic avian influenza virus two experiments were performed. In the first experiment the mice were primed with $5x10^2$ $TCID_{50}$ of influenza H3N2 virus A/HK/2/68 or mock-infected with PBS, and four weeks later they were challenged with $2x10^2$ $TCID_{50}$ of influenza H5N1 virus A/IND/5/05. After challenge infection all animals lost weight until day 6. Subsequently, all but one H3N2-primed animals gained weight and these animals had only mild symptoms compared to immunologically naïve control mice (Figure 1A). The proportion survival in the H3N2-primed group was significantly higher than that in the mock-infected group ($p<0.05$)(Figure 1B).

The experiment was repeated with larger groups and an extra control group, primed by infection with respiratory syncytial virus (RSV) to exclude non-specific innate immune responses as basis for the observed protection. The first two days post challenge infection (p.i.) all groups of mice displayed similar weight loss. From day 3 p.i. onwards, the mean body weights between the three groups differed. Four days p.i. H3N2-primed mice had lost 12.6% (SD=3.7) of their body weight, whereas PBS and RSV inoculated mice lost 16.8% (SD=3.1) and 18.1% (SD=3.2) of their bodyweight, respectively (Figure 1C). The weight loss in the H3N2-primed group was significantly lower than in the two other groups ($p<0.05$). On day 6 the mean loss of body weight was 26.5% (SD=1.6) and 27% (SD=0.9) for the PBS and RSV inoculated mice and 19.8% (SD=3.4) for the H3N2-primed mice, which was significantly lower ($p<0.05$) (Figure 1D). From day 6 onwards the latter animals gained weight and fully recovered within two weeks after challenge infection with influenza virus A/IND/5/05 (H5N1).

Between day 4 and 6 p.i. one out of eleven H3N2-primed animal and all animals from the PBS and RSV control groups became less active, showed reduced muscle strength and started to develop respiratory distress, observed as heavy breathing in combination with hunched posture. In combination with a weight loss of more than 20%, animals had to be euthanized for ethical reasons. The survival rate on day 5 p.i. was 100% for the H3N2-primed group and 71.4% and 37.5% for the animals previously inoculated with PBS and RSV respectively (Figure 1D). Eventually, all animals of the PBS and RSV group had to be euthanized (0% survival), whereas only one of the H3N2-primed mice had to be taken out of the experiment (91% survival). This difference in survival rate was statistically significant ($p<0.05$).

Serology

Twenty-eight days after primary infection with influenza virus A/HK/2/68, mice developed GMT HI antibody titers of 905.1 (SD=1.49) and VN antibody titers of 226.3 (SD=1.49) against the homologous strain. The sera did not react with influenza virus A/IND/5/05 in either of the assays.

Lung virus titers

Four and seven days p.i. with influenza virus A/IND/5/05 lung virus titers were assessed. In the first experiment lung virus titers of the H3N2-primed mice were significantly lower at these time points

p.i. than those of unprimed mice ($p<0.05$)(Table 1). In the second experiment the lung virus titers on day 4 p.i were significantly lower for the H3N2-primed animals: $10^{8.1}$ $TCID_{50}$ ($SD=10^{0.4}$) than for the naïve mice of the PBS group: $10^{9.0}$ $TCID_{50}$ ($SD=10^{0.4}$) ($p<0.05$) and RSV group: $10^{8.8}$ $TCID_{50}$ ($SD=10^{0.3}$) ($p<0.05$) (Table 1). Only the H3N2-primed animals survived the infection post day 7 and the mean virus titer in the lungs on that day was $10^{4.6}$ $TCID_{50}$ ($SD=10^{0.7}$). Fourteen days p.i. infectious virus was no longer detectable in the lungs of these animals.

Table 1: Lung virus titers after infection with influenza virus A/IND/5/05 (H5N1)[1]

	Days post infection	4	7	14
Experiment 1	HK/2/68 infection	7.7 ± 0.1[2]	4.8 ± 0.5[2]	n.d.[3]
	Mock infection	8.8 ± 0.5	7.0 ± 0.6	n.d.
Experiment 2	HK/2/68 infection	8.1 ± 0.4[4]	4.6 ± 0.7	<1.5[5]
	Mock infection	9.0 ± 0.4	†	†
	RSV infection	8.8 ± 0.3	†	†

1 titers are expressed as $TCID_{50}$ per gram tissue (Log10)
2 significantly lower than the mock-infected mice ($p<0.05$)
3 n.d. = not done
4 significantly lower than the mock-infected and RSV-infected mice ($p<0.05$)
5 average virus titer below the cut-off value, all animals tested negative by virus isolation
† animals did not survive until these time points.

Detection of virus-specific CTL

Tetramers were used to detect CTL specific for the $NP_{366-374}$ epitope: ASNENMDAM (influenza virus A/HK/2/68) and ASNENMEVM (influenza virus A/IND/5/05). Four and seven days after infection with influenza virus A/HK/2/68 no tetramer-positive T lymphocytes were detected in the spleen (data not shown). However, twelve days p.i. an $NP_{366-374}$ (ASNENMDAM) specific response was observed (Figure 2A). A small fraction of these cells stained positive with both the ASNENMDAM tetramer and the tetramer prepared with the $NP_{366-374}$ epitope (ASNENMEVM) derived from influenza virus A/IND/5/05 (H5N1). Upon challenge infection with this virus, this double positive fraction was selectively expanded (Figure 2B) in the majority of mice.

After challenge infection with the influenza A/H5N1 virus, A/IND/5/05-derived $NP_{366-374}$ (ASNENMEVM)-specific cells were detected as early as day seven p.i. in both experiments. Of all CD8+ T lymphocytes the mean frequency of Tm+ CD8+ cells was 5.3% (SD= 5.7) and 6.2% (SD=4.6) respectively (Table 2). In mock-primed mice this frequency was significantly lower. Two weeks p.i. the frequency of $NP_{366-374}$ specific CTL had declined to 3.6% (SD=2.8).

In addition to tetramer-staining also intracellular IFNγ-staining was used to assess the presence of virus-specific CTL in the spleen. In mice that were primed by infection with influenza virus A/HK/2/68, the stimulation of splenocytes with the $NP_{366-374}$ peptide resulted in the detection of IFNγ in CD8+ T cells in both experiments with mean frequencies of 2.0% (SD=1.1) and 1.8% (SD=0.8), respectively (Table 2). The frequency of IFNγ positive cells was significantly higher than in mock-infected animals ($p<0.05$). Fourteen days p.i. the frequency had declined to 0.4% (SD=0.5). Also the

frequency of IFNγ positive CD8+ T cells specific for the $PA_{224-233}$ epitope (SSLENFRAYV) was significantly higher in H3N2-primed mice than in mock-infected animals (Table 2).

Table 2: $NP_{366-374}$ specific CD8+ T lymphocytes 7 dpi with influenza virus A/IND/5/05 (H5N1)

		Experiment 1		Experiment 2	
epitope	group	IFN-γ+	Tm+	IFN-γ+	Tm+
$NP_{366-374}$ ASNENMEVM	HK/2/68 infection	2.0 ± 1.1[1]	5.3 ± 5.7[1]	1.8 ± 0.8	6.2 ± 4.6
	Mock infection	-0.5 ± 0.2	0.9 ± 0.7	†	†
	RSV infection	n.d.2	n.d.	†	†
$PA_{224-233}$ SSLENFRAYV	HK/2/68 infection	3.0 ± 1.4[1]	n.d.	n.d.	n.d.
	Mock infection	0.2 ± 0.3	n.d.	†	n.d.
	RSV infection	n.d.	n.d.	†	n.d.

1 significantly higher than the mock-infected mice ($p<0.05$)
2 n.d.= not done

Histopathology

Twenty-eight days after infection with influenza virus A/HK/2/68, multifocal hyperplasia and hypertrophy of the bronchiolar epithelium and peribronchiolar lymphocytic infiltrates were visible, but not after PBS or RSV inoculation.

Four days after infection with influenza virus A/IND/5/05, multifocal moderate necrotizing broncho-interstitial pneumonia covering almost complete lobes was observed in the lungs of both the mock- and RSV-infected mice. There was loss of bronchiolar epithelium in combination with necropurulent material in the bronchiolar lumen. Infiltration of inflammatory cells, mainly neutrophils and lymphocytes, was present in the PBS- and RSV-inoculated mice (Figure 3B, C). In contrast, multifocal mild to moderate broncho-interstitial pneumonia with marked inflammatory infiltrates consisting of predominantly lymphocytes and neutrophils (Figure 3A) was observed in the lungs of H3N2-primed mice. The bronchiolar wall was rather conserved although some cellular debris was present in the lumen. In the alveoli, hypertrophy of the type II pneumocytes was seen.

Seven days p.i. the lungs of the H3N2-primed animals still displayed mild to moderate broncho-interstitial pneumonia. The bronchiolar epithelium displayed hyperplasia and hypertrophy (indication of regeneration) and the peribronchiolar and perivascular infiltration of lymphocytes was stronger, also in iBALT formations, compared to day 4. Regeneration of alveoli was visible and a mild lymphocytic infiltrate was found in the alveolar lumen. No data were available for the mock- and RSV-primed mice on day seven and fourteen p.i. with influenza virus A/IND/5/05, since none of these animals survived past day 6.

Detection of virus-infected cells in the lungs by immunohistochemistry

The presence of influenza A/IND/5/05 virus-infected cells was assessed by immunohistochemistry using a monoclonal antibody directed to the viral NP. Four days p.i. with influenza A/IND/5/05 virus, virus-infected cells were abundantly present in the lungs of mock and RSV-primed mice (Figure 4B, C). The infected cells were located in and around lesions. Almost 50% of the bronchioles was

infected with more than 75% of the epithelial cells staining positive for viral antigen. The infected cells in the alveoli were predominantly type II like pneumocytes.

In contrast, only a small number of bronchiolar epithelial cells stained positive in mice that were primed by infection with influenza virus A/HK/2/68, although especially in the alveoli virus-infected cells, mainly type II like pneumocytes, were readily detectable (Figure 4A). Seven days p.i. infected cells only were detected sporadically in the lungs of these mice, which resolved the infection completely by day 14 p.i. (data not shown).

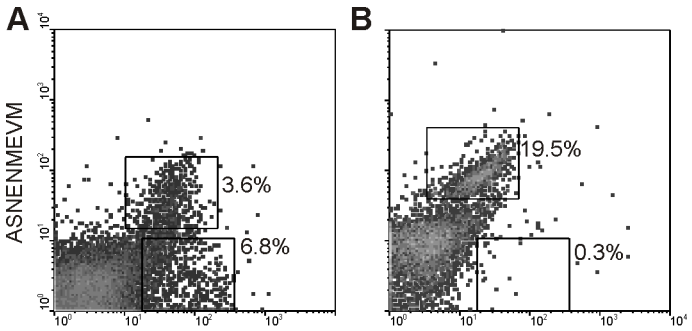

Figure 2. *Expansion of a cross-reactive CD8+ T cell population, induced by primary infection, after subsequent infection with influenza virus A/IND/5/05 (H5N1). On day 12 after infection with influenza virus A/HK/2/68 (H3N2) a CD8+ T cell population was detected that recognized both the A/HK/2/68 derived NP366-374 epitope (ASNENMDAM) and the A/IND/5/05 derived analog (ASNENMEVM) (A). This cross-reactive population was expanded constituting the majority of NP366-374 specific CTL on day 7 after infection with influenza virus A/IND/5/05 (H5N1) (B).*

DISCUSSION

In the present study, the protective efficacy was assessed of heterosubtypic immunity induced after infection with a human influenza A/H3N2 virus against a lethal challenge infection with a highly pathogenic avian influenza A/H5N1 virus. A prior exposure to the human A/H3N2 strain protected mice from severe clinical signs and mortality which correlated with control of virus replication in the lungs and the induction of anamnestic cross-reactive CTL responses upon influenza A/H5N1 virus infection.

Inoculation of naive C57BL/6J mice with influenza virus A/IND/5/05 (H5N1) caused a productive infection of these animals with infectious virus titers four days p.i. of up to 10^9 $TCID_{50}$ per gram of lung tissue. These animals developed a severe necrotizing interstitial pneumonia and eventually succumb to the infection. Although the kinetics of the development of disease differed somewhat between experiment 1 and 2, in both experiments a prior infection with influenza virus A/HK/2/68 (H3N2) had a significant effect on virus replication rates, loss of body weight and mortality rates. In experiment 2 a prior infection with RSV did not prevent influenza A/H5N1 virus-induced disease, indicating that the observed protection was dependent on adaptive immune responses induced by infection with influenza virus A/HK/2/68 (H3N2) rather than a non-specific innate immune response.

In contrast, the protection of H3N2-primed mice against influenza A/H5N1 virus infection corre-

lated with the induction of anamnestic CTL responses specific for the $NP_{366-374}$ (ASNENMEVM) and $PA_{224-233}$ (SSLENFRAYV) epitopes derived from influenza virus A/IND/5/05 (H5N1). These epitopes differ from their counterparts (ASNENMDAM and SCLENFRAYV, respectively) in influenza virus A/HK/2/68 that was used for priming of the animals. Apparently, infection with influenza virus A/HK/2/68 primed for cross-reactive CTL against these two epitopes, in addition to epitopes that are conserved and shared between influenza viruses A/HK/2/68 and A/IND/5/05. A similar result was obtained previously after subsequent infection with influenza viruses A/NT/60/68 (H3N2) ($NP_{366-374}$ ASNENMDAM) and A/PR/8/34 (H1N1) (ASNENMETM) (92).

Consecutive infections with these two variant viruses lead to the selective expansion of cross-reactive CTL responses specific for the $NP_{366-374}$ epitope. After infection with only one of these viruses the cross-reactive CTL only form a minor proportion that is expanded after the second infection with the variant strain. Thus, also with the combination of viruses we used, cross-reactive CTL were selectively expanded after infection with influenza virus A/IND/5/05. A similar result was obtained in vitro with human CTL specific for the HLA-B*3501 restricted $NP_{418-426}$ epitope that differed between human influenza A/H1N1 and A/H3N2 viruses (93).

Although it has been described that the $NP_{366-374}$ epitope is more immunodominant than the $PA_{224-233}$ epitope in a secondary CTL response (94), a stronger response was observed against the $PA_{224-233}$ epitope after infection with influenza virus A/IND/5/05. Possibly, the cross-reactivity of CTL directed to the $NP_{366-374}$ epitope is lower than that of CTL specific for $PA_{224-233}$. Thus, the induction of memory CTL responses induced by infection with influenza virus A/HK/2/68 (H3N2) correlated with protective immunity against infection with influenza A/IND/5/05 virus (H5N1). However, it cannot be excluded that other arms of the adaptive immune response contributed to the observed protection. It is possible that prior infection with an influenza A virus also primed for a secondary T helper cell response.

In addition, it has been demonstrated that antibodies directed to NP (36) or the M2 protein can have a protective effect against challenge infection (95-97). However, the protective role of NP-specific antibodies only could be demonstrated after hyper-immunization of mice with a high dose of recombinant NP in combination with LPS. With post-vaccination rNP-immune serum the protective effect could be transferred to B-cell deficient μMT mice but not to intact recipient C57BL/6J mice.

For M2-specific antibodies the protective effect was demonstrated after hyper-immunization or the transfer of high doses of M2-specific monoclonal antibodies (96, 98). In addition, transfer of post-infection serum to naïve recipient mice failed to protect the animals from infection with a heterosubtypic strain (44) including influenza virus A/IND/5/05 (H5N1) (data not shown). Therefore, it is unlikely that NP or M2 specific antibodies contributed to a great extent to the infection-induced heterosubtypic immunity against influenza virus A/IND/5/05 (H5N1) observed in the present study.

Protection of mice against lethal influenza A/H5N1 virus infection induced by primary infection with a heterosubtypic influenza virus has been demonstrated before (76). However the internal genes of the influenza A/H9N2 and A/H5N1 viruses used for priming and challenge infection respectively were closely related and displayed 98% sequence homology (99). Therefore, this scenario resembled that with the use of influenza X-31 (H3N2) and A/PR/8/34 (H1N1) virus for priming and challenge infection respectively since these two viruses share identical internal genes (77). Here we

demonstrate for the first time that infection with a human influenza virus induces protective immunity to a highly pathogenic avian influenza virus of the H5N1 subtype. The findings reported may have a number of important implications. Although this type of immunity did not protect against infection per se, it contributed to control of virus replication and as a result dampened the clinical impact of the H5N1 infection. The observed protection against the development of severe disease correlated with the induction of cross-reactive CTL responses. Since human CTL raised against seasonal influenza virus strains of the H1N1 and H3N2 subtypes are highly cross-reactive with highly pathogenic avian influenza A/H5N1 viruses it is anticipated that they will contribute to protective immunity against these viruses, when they might become pandemic (51, 52). Similarly, cross-reactive CTL may have contributed to heterosubtypic immunity and a reduction of lethal influenza A/H2N2 cases observed during the pandemic in 1957 (54). It even may be speculated that the history of infections with seasonal influenza viruses and the cross-reactive CTL responses associated with these infections is at the basis of the disproportional age distribution of severe H5N1 cases (100). Last but not least, the induction of cross-reactive CTL may be an attractive target for the development of vaccines that could induce broad-protective immune response, even against influenza A viruses of a novel subtype.

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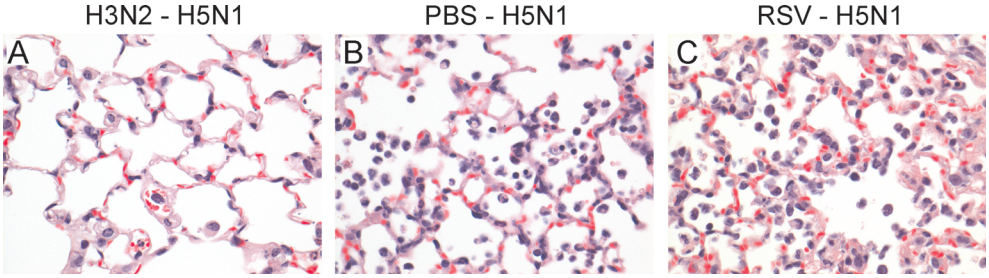


Figure 3. *Histopathology was examined in the lungs of mice after infection with influenza virus A/IND/5/05. Four days after infection the lungs of H3N2-primed animals showed a multifocal mild broncho-interstitial pneumonia with mild inflammatory infiltrates consisting of predominantly lymphocytes and neutrophils (A).*
The mock-infected control mice (B) and RSV-primed mice (C) displayed a multifocal moderate necrotizing broncho-interstitial pneumonia with marked infiltration, consisting of inflammatory cells, mainly neutrophils and lymphocytes, in the alveoli.

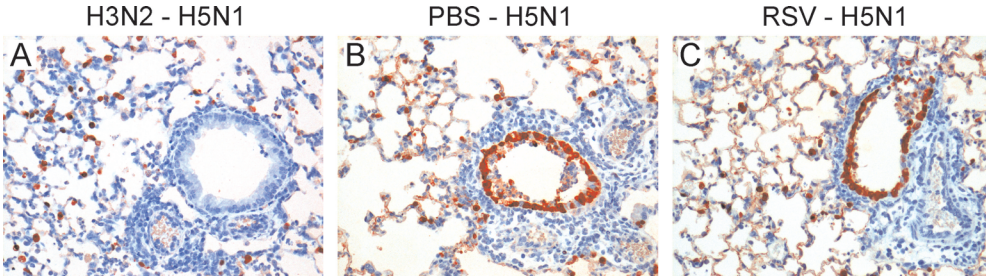
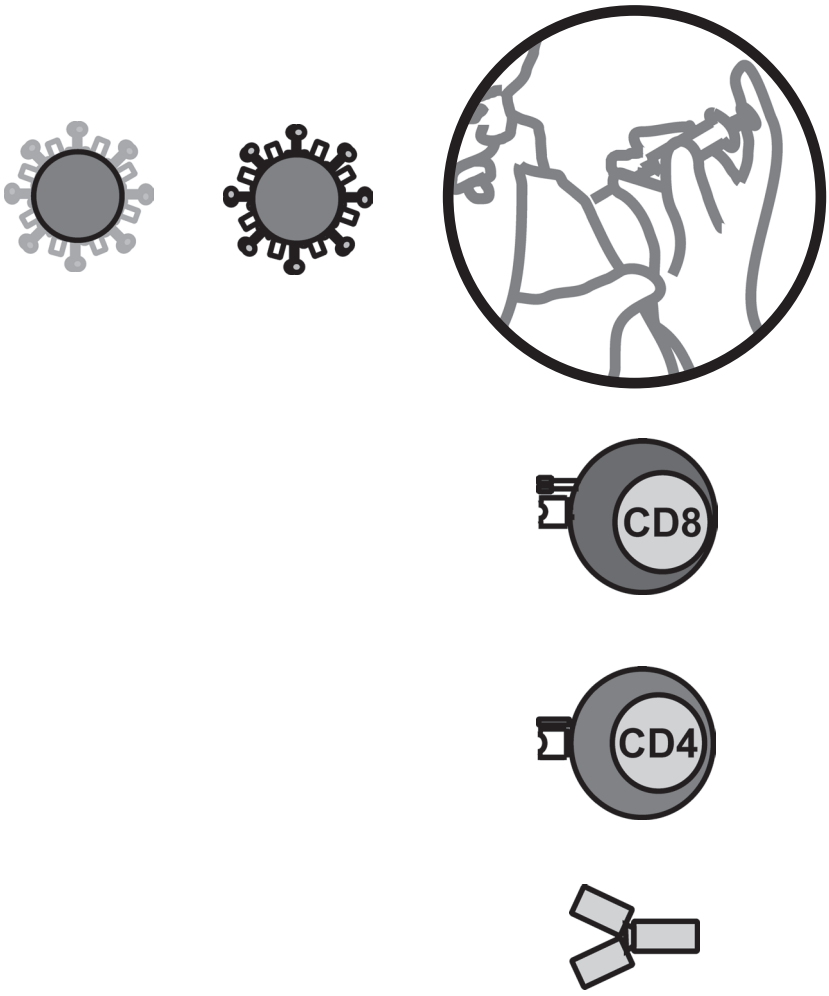


Figure 4. *Detection of virus-infected cells by immunohistochemistry in the lungs of influenza virus A/IND/5/05 (H5N1)-infected mice on day 4 p.i. in mice primed by infection with influenza virus A/HK/2/68 (H3N2) (A), mock-infected mice (B), or RSV-primed mice (C).*


CD8
CD4

3 Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus

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ABSTRACT

Annual vaccination against seasonal influenza viruses is recommended for certain individuals that have a high risk for complications resulting from infection with these viruses. Recently it was recommended in a number of countries including the USA to vaccinate all healthy children between 6 and 59 months of age as well. However, vaccination of immunologically naïve subjects against seasonal influenza may prevent the induction of heterosubtypic immunity against potentially pandemic strains of an alternative subtype, otherwise induced by infection with the seasonal strains.
Here we show in a mouse model that the induction of protective heterosubtypic immunity by infection with a human A/H3N2 influenza virus is prevented by effective vaccination against the A/H3N2 strain. Consequently, vaccinated mice were no longer protected against a lethal infection with an avian A/H5N1 influenza virus. As a result H3N2-vaccinated mice continued to loose body weight after A/H5N1 infection, had 100-fold higher lung virus titers on day 7 post infection and more severe histopathological changes than mice that were not protected by vaccination against A/H3N2 influenza.
The lack of protection correlated with reduced virus-specific CD8+ T cell responses after A/H5N1 virus challenge infection. These findings may have implications for the general recommendation to vaccinate all healthy children against seasonal influenza in the light of the current pandemic threat caused by highly pathogenic avian A/H5N1 influenza viruses.

INTRODUCTION

Since 2003, more than 380 human cases of infection with highly pathogenic avian influenza A virus (IAV) of the H5N1 subtype have been reported to the World Health Organization (WHO) of which more than 60% were fatal (101). Because of the continuous spread of these viruses among domestic birds, the frequent introduction into wild birds and the increasing number of human cases, a pandemic outbreak caused by influenza A/H5N1 viruses is feared (17, 102, 103).

It has been demonstrated in animal models that prior exposure to an IAV can induce heterosubtypic immunity to infection with an IAV of an unrelated subtype (for review see(40)). Also in humans there is evidence that infection with IAV can induce heterosubtypic immunity (54). Individuals that had experienced an infection with an H1N1 IAV before 1957 less likely developed influenza during the H2N2 pandemic of 1957 (54). In particular, the induction of cell-mediated immune responses after infection contributes to protective immunity against infection with heterosubtypic IAVs. The presence of cross-reactive cytotoxic T lymphocytes (CTL) in humans inversely correlated with the amount of viral shedding in the absence of antibodies directed against the virus used for experimental infection (50). It is well documented that seasonal human IAVs and avian IAVs share CTL epitopes located in the internal viral proteins like the nucleoprotein (51-53). Thus, cell-mediated immunity induced by natural infection with seasonal IAVs may confer protection against heterosubtypic pandemic influenza viruses. In this respect, the disproportional age distribution of severe human H5N1 cases is of interest (57). Especially younger individuals are at risk and although other confounding factors cannot be excluded, it is tempting to speculate that young subjects have been infected with seasonal influenza viruses less frequently and therefore have not developed protective heterosubtypic immune responses against infection with the highly pathogenic avian A/H5N1 viruses.

Since seasonal IAVs of the H3N2 and H1N1 subtypes cause epidemic outbreaks annually associated with excess morbidity and mortality mainly among infants, the elderly, immuno-compromised and other high-risk patients, influenza vaccination is recommended for these high-risk groups. In general, the influenza vaccines most frequently used are inactivated vaccines, including subunit preparations that consist of the viral hemagglutinin (HA) and neuraminidase (NA). Due to the higher risk of complications and hospitalizations secondary to influenza in children (58, 59), annual vaccination of all healthy children 6 to 59 months of age was recommended in various countries including the United States since 2007 (60).

However, annual vaccination may prevent the induction of heterosubtypic immunity by infection with seasonal influenza virus strains. In addition, it is unlikely that seasonal inactivated influenza vaccines, unlike live attenuated vaccines, induce heterosubtypic immunity since they induce cross-reactive CTL responses inefficiently (104, 105).

Thus, we hypothesized that vaccination against seasonal flu prevents the induction of cross-protective cell-mediated immunity, which consequently may lead to more severe clinical outcome of infection with a future pandemic virus. Here we show in a mouse model that protective immunity against lethal infection with H5N1 IAV Indonesia/5/05 (IND/05) was induced by infection with H3N2 IAV HongKong/2/68 (HK/68), which was prevented by effective vaccination against the A/H3N2 vi-

rus. The lack of protection against IAV IND/05 correlated with reduced virus-specific CTL responses.

MATERIALS AND METHODS

Viruses

Virus stocks of influenza viruses A/Hong Kong/2/68 (IAV HK/68) and A/Indonesia/5/05 (H5N1) (IAV IND/05) were prepared by infecting confluent Madin-Darby-Canine-Kidney (MDCK) cells. After cytopathologic changes were complete, culture supernatants were cleared by low speed centrifugation and stored at –70°C. Infectious virus titers were determined in MDCK cells as described previously (85).

Vaccine preparation

Influenza subunit antigen derived from IAV X-31 (H3N2) was essentially prepared as described previously (106). X-31 is a reassortant vaccine strain of A/Aichi/2/68 and A/PR/8/34, of which the HA and NA resemble that of IAV HK/68 closely. The purity of the subunit preparations was tested by SDS-polyacrylamide gel electrophoresis and the absence of the nucleoprotein and matrix protein of the subunit preparations was tested by western blotting using monoclonal antibodies against the influenza A nucleoprotein and the influenza A matrix protein. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, USA).

Immunization and infection of mice

Female specified pathogens free 6-8 weeks old C57BL/6J (H-2b) mice were purchased from Charles River (Sulzfeld, Germany). Mice were immunized twice with an interval of four weeks intramuscularly (i.m.) in both hind legs in a total volume of 100µl. Mice (n=19-40 per group) received PBS (phosphate buffered saline) (Groups 1,3 and 4), 15µg subunit vaccine with (Groups 2 and 5) or without (Group 6) 1mg Aluminium hydroxide gel (Alum) (Sigma-Aldrich, Zwijndrecht, the Netherlands) or Alum only (Group 7). Eight days after the second vaccination, four mice of each group were bled and spleens were resected. Four weeks after the second vaccination, mice of groups 2, 3, 6 and 7 were infected intranasally with $5x10^2$ $TCID_{50}$ IAV HK/68 in a volume of 50µl. Four and twelve days post infection (p.i.), 5-7 mice were bled and lungs and spleens were resected. Four weeks after infection with IAV HK/68, all mice except mice of group 1 were challenged with $2x\ 10^2$ $TCID_{50}$ IAV IND/05. A dose of $2x\ 10^2$ $TCID_{50}$ was used because this was the minimal dose resulting in a lethal infection in >90% mice reproducibly. The day before challenge with IAV IND/05, mice of each group (n=2-4) were euthanized and lungs and spleens were resected as well as on day four (n=4-6), seven (n=2-9) and fourteen (n=3-8) days after challenge. Vaccinations, intranasal infections, orbital punctures and euthanasia were performed under anesthesia with isoflurane in O_2. After infection with IAV HK/68 and IAV IND/05, mice were monitored for the presence of clinical signs, including weight loss. All experiments with IAV IND/05 were performed under Biosafety Level 3 conditions. An independent animal ethics committee (DEC consult) approved the experimental protocol before the start of the experiments.

Table 1. Experimental groups and design of the study

Experimental group	Vaccination		Infection	
	Subunit	Adjuvant	HK/68	IND/05
1	-	-	-	-
2	+	+	+	+
3	-	-	+	+
4	-	-	-	+
5	+	+	-	+
6	+	-	+	+
7	-	+	+	+

Serology

Serum samples of mice were collected at various time points during the experiment and tested for the presence of HA-specific antibodies against IAV HK/68 and IAV IND/05 using the hemagglutination inhibition (HI) assay (107) and virus neutralising (VN) antibodies using the VN assay (87). To determine the titer of antibodies against IAV IND/05 before infection with IAV IND/05, a reverse genetics virus was produced from which the basic cleavage site was removed. Antibody titers obtained with this reverse genetics virus was comparable with that against the wild-type strains (data not shown). Positive control serum specific for IAV HK/68 was obtained by injecting a rabbit with sucrose gradient purified virus (88). Hyper-immune serum obtained from a swan immunized twice with inactivated H5N2 influenza virus A/Duck/Potsdam/1402/86 (Intervet, Boxmeer, the Netherlands) was used as a positive control against IAV IND/05(89).

Lung virus titers

Lungs of mice were snap frozen on dry ice with ethanol and stored at –70°C. Lungs were homogenized with a FastPrep-24 (MP Biomedicals, Eindhoven, the Netherlands) in medium consisting of Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, the Netherlands) and centrifuged briefly. Quintuplicate 10-fold serial dilutions of these samples were used to infect MDCK cells as described previously(85). HA activity of the culture supernatants collected 5 days post inoculation was used as indicator of infection. The titers were calculated according Spearman-Karber (108).

Flow cytometry of virus-specific CD8+ T cells

Peptides and intracellular IFN-γ staining

Single cell suspensions of spleens were prepared as described previously (77). CD8+ T cell responses after infection were measured by incubation with peptides representing two immunodominant epitopes of IAVs in C57BL/6J mice (H2-b), $PA_{224-233}$ and $NP_{366-374}$ (91, 109). The peptides of the $PA_{224-233}$ epitope of influenza A virus were manufactured at Eurogentec (Seraing, Belgium),

while peptides of the $NP_{366-374}$ epitope were manufactured at Sanquin Research (Amsterdam, the Netherlands). Four hundred thousand splenocytes were cultured for 6 h at 37°C in the presence of 5μM of either the $NP_{366-374}$ ASNENMDAM (NP_{HK}), $PA_{224-233}$ SCLENFRAYV (PA_{HK}) peptides derived from IAV HK/68 or the $NP_{366-374}$ ASNENMEVM (NP_{IND}) or SSLENFRAYV (PA_{IND}) peptides (derived from IAV IND/05) in IMDM (Lonza, Breda, the Netherlands) with 5% FCS and Golgistop (BD). After incubation, cells were o/n stored at 4°C, stained with monoclonal antibody directed to CD3e-PerCP and CD8b.2-FITC, fixate and permeabilized with Cytofix and Cytoperm and stained with monoclonal antibody specific for IFN-γ-PE (all from BD Pharmingen, Alphen a/d Rijn, the Netherlands). Data were acquired using a FACSCalibur and analysed with Cellquest Pro Software (BD).

Tetramerstaining

Splenocytes were washed and stained with mAbs CD3e-PerCP, CD8b.2-FITC (BD Pharmingen, Alphen a/d Rijn, the Netherlands) and either the Phycoerythrin (PE)-labeled H-2Db tetramer with the immunodominant $NP_{366-374}$ epitope derived from IAV X-31 ASNENMETM (Tm_{X-31}) or IAV HK/68 ASNENMDAM (Tm_{HK}) or the APC labeled tetramer derived from IAV IND/05 $NP_{366-374}$ ASNENMEVM (Tm_{IND}). All tetramers were purchased from Sanquin Research, Amsterdam, the Netherlands. Following incubation with tetramers and mAbs for 20 minutes, cells were washed twice and analysed by flow cytometry using a FACSCanto in combination with FACS Diva software (BD).

Histopathology and immunohistochemistry

After euthanasia, lungs of mice were inflated with 10% neutral buffered formalin. After fixation and embedding in paraffin, lungs were sectioned at 4μm and tissue sections were examined by staining for hematoxylin and eosin (HE). Using an immunoperoxidase method, sequential slides were also stained with a monoclonal antibody directed against the nucleoprotein of IAV (110).

Statistical analysis

Data for weight loss after infection, viral load in the lungs, tetramerstaining, and peptide pulsing were analysed statistically using the two-sided student's T test. Survival was analysed using the Log-rank test. Differences were considered significant at $P<0.05$.

RESULTS

Antibody responses against IAV HK/68 (H3N2) after vaccination

Mice were vaccinated with subunit vaccine with or without Alum or were 'mock' vaccinated (table 1). HI antibody titers were detected 28 days after the first vaccination with subunit and Alum (groups 2 and 5) and in 3 out of 26 mice vaccinated with unadjuvanted subunit vaccine (group 6). Four weeks after the second vaccination, geometric mean titers (GMTs) increased to 244 and 218 in mice from group 2 and group 5, respectively. Four mice of group 6 developed detectable HI-antibody responses with a GMT of 48, the other mice of this group did not seroconvert (figure 1A). Sera of mice were also analysed for the presence of virus neutralizing (VN) antibodies. Four weeks after the second vaccination, mice vaccinated with adjuvanted subunit vaccine developed VN anti-

bodies with a GMT of 38 and 29 in group 2 and group 5 respectively, while only two mice of group 6 developed detectable VN antibody titers (Figure 1B).

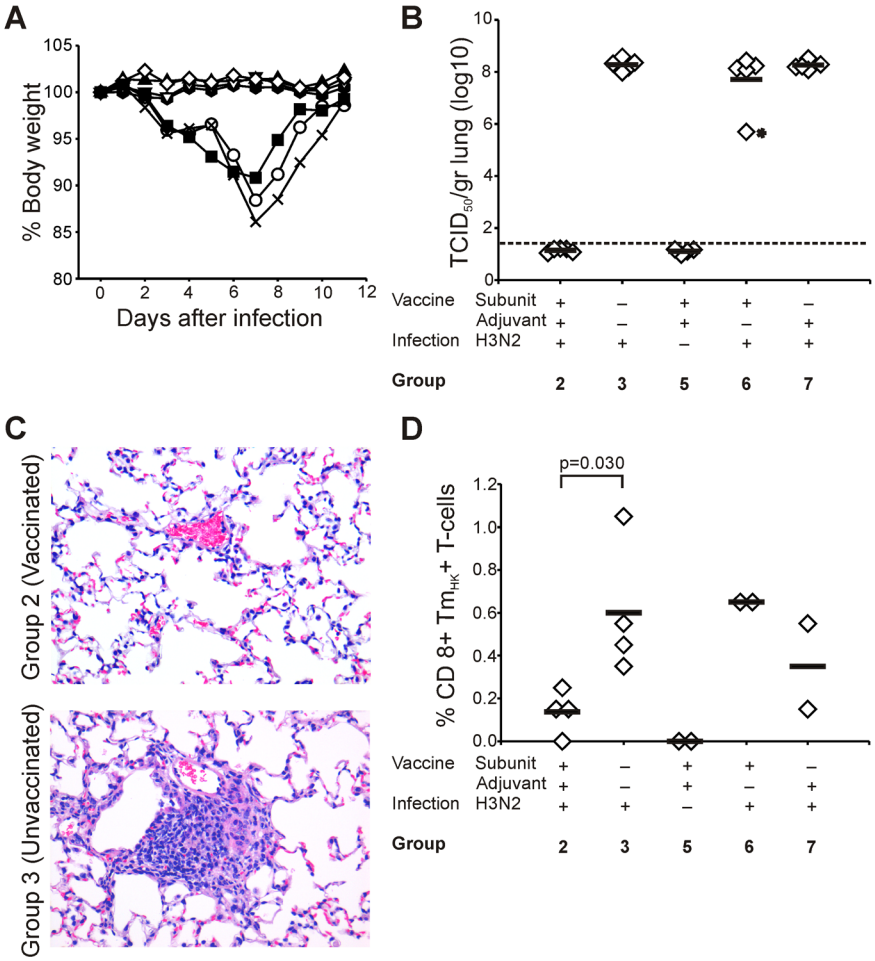


Figure 2. *Outcome of infection with IAV HK/68 (H3N2). Mice were inoculated with IAV HK/68 (groups 2 (▲), 3 (○), 6 (■) and 7 (✕)) or PBS (groups 1 (⬢), 4 (▽) and 5 (◇)). (A) Body weight after infection was determined daily and expressed as the percentage of the original body weight before infection. (B) Lung virus titers measured on day 4 p.i. in mice from the indicated experimental groups. Horizontal bars represent the average titers of five mice. The dotted line represents the cut-off value for obtaining a positive result. *This mouse from group 6 had before infection an HI antibody titer of 40. (C) Vaccination prevented the induction of iBALT after infection. Twenty-eight days post infection with IAV HK/68 iBALT was detected in mice from group 3, but not in mice from group 2. Lung tissue sections were stained with HE. (D) Virus-specific CD8+ T cell responses detected 28 days post infection. Splenocytes of mice from the indicated experimental groups were tested for the presence of CD8+ T cells that bound the H2-Db NP_{HK} Tetramer. Horizontal bars represent the average of 2-4 mice. The difference in %CD8+ Tm+ T cells between groups 2 and 3 was statistically significant (P=0.030).*

Outcome of infection with IAV HK/68 (H3N2)

Mice that developed HI-antibodies against IAV HK/68 (all mice of group 2 and four of group 6) were

protected from weight loss after infection with IAV HK/68, while mice of other groups lost weight until day seven post infection (p.i.) and showed mild clinical symptoms for 2-3 days (Figure 2A). Clinical signs and weight loss after infection correlated well with virus titers in the lungs of infected mice 4 days p.i.. No virus was detected in lungs of mice vaccinated with adjuvanted subunit vaccine, while the average lung virus titer of mock-vaccinated mice was $10^{8.1}$ $TCID_{50}$/gram lung. Similar titers were observed for the mice in groups 6 and 7 with the exception of one mouse in group 6 with a HI antibody titer of 40 induced by vaccination with unadjuvanted subunits that had a lung virus titer of $10^{5.7}$ $TCID_{50}$/gram lung (Figure 2B). The virus titers detected on day 4 p.i. correlated with the absence or presence of virus infected cells in the lungs detected by immunohistochemistry (data not shown).

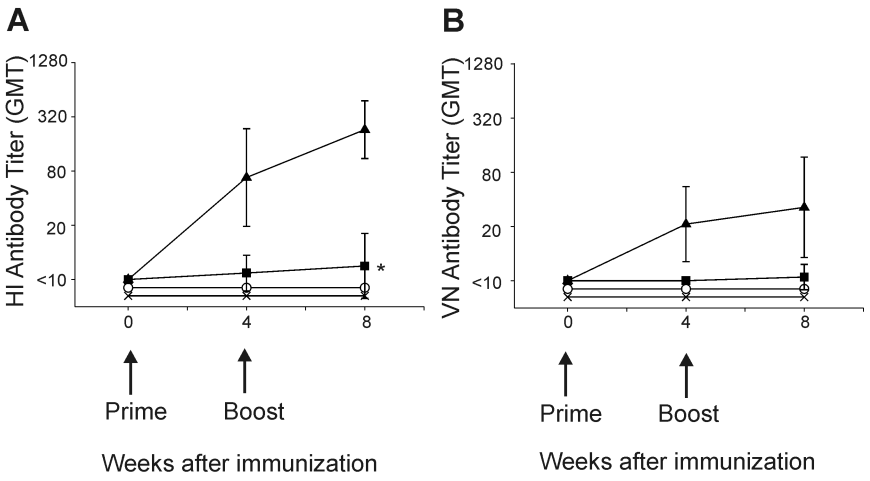


Figure 1. *Induction of serum antibodies against IAV HK/68 (H3N2) by vaccination. Serum antibody levels were determined before and at the indicated time points after vaccination of mice with PBS (groups 1, 3 and 4; ○), subunit vaccine with alum (groups 2 and 5; ▲), subunit vaccine only (group 6; ■) and alum only (group 7; ×) by HI assay (A) and VN assay (B).*

Virus-specific CTL and antibody responses after infection with IAV HK/68 (H3N2)

Four days p.i. with IAV HK/68 the frequency of splenic CD8+ T lymphocytes specific for the $NP_{366-374}$ epitope of IAV HK/68 (CD8+ Tm_{HK}+ T-cells) as determined by tetramer staining remained at background levels in all groups (data not shown).

In all infected mice a raise in the frequency of CD8+ Tm_{HK}+ T-cells was detected twelve days p.i.. No statistically significant differences were observed between the experimental groups. Essentially the same results were observed using intracellular IFN-γ staining after re-stimulation with peptides representing the $NP_{366-374}$ and $PA_{224-233}$ epitopes of IAV HK/68 (NP_{HK} and PA_{HK}). The NP_{HK} and PA_{HK} specific CTL induced by infection with IAV HK/68 cross-reacted to various extents with their counterparts derived from IAV IND/05 (NP_{IND} and PA_{IND}). The cross-reactive nature of a proportion of the $NP_{366-374}$ specific CTL was confirmed by double staining with Tm_{HK} and Tm_{IND} (data not shown). By day 28 p.i. with IAV HK/68, just before challenge infection with IAV IND/05, the frequency of virus-specific CTL in the spleen had declined and virus-specific CTL were not detectable by intracellular

IFN-γ staining. However, Tm_{HK} and Tm_{IND} positive cells were detected in mice that were mock vaccinated prior to infection (group 3). Strikingly, the frequency of Tm_{HK} positive CD8+ T lymphocytes was significantly lower in mice of group 2 that were effectively vaccinated against infection with IAV HK/68 (p=0.030) (Figure 2D).

Vaccination prevents induction of iBALT after IAV HK/68 infection

Following infection with IAV HK/68, no significant lesions were found in lungs of mice vaccinated with adjuvanted subunit vaccine (group 2), whereas mice that were mock-vaccinated or vaccinated with Alum or subunit preparation only (mice of groups 3, 6 and 7) developed a multifocal mild subacute necrotizing bronchopneumonia four days after infection, which on day 12 p.i. progressed into a multifocal moderate chronic necrotizing bronchopneumonia. On day 28 p.i., these mice had developed perivascular moderate proliferation of inducible Bronchus Associated Lymphoid Tissue (iBALT), consisting mainly of mononuclear cells, which was absent in mice effectively vaccinated against infection with IAV HK/68 (Figure 2C).

Effective vaccination prevents heterosubtypic immunity against IAV IND/05 (H5N1)

After infection with IAV IND/05, all mice developed clinical signs (weight loss, ruffled fur, lethargy) from day two p.i. onwards. Mice that developed clinical signs p.i. with IAV HK/68 (groups 3, 6 and 7) lost weight until day 6-7 after infection with IAV IND/05 and then started to gain weight and fully recovered, while mice of other groups, not previously infected with IAV HK/68 (groups 4 and 5) and more strikingly, those effectively vaccinated against infection with IAV HK/68 (group 2) lost significantly more weight (group 2 versus group 3: p=0.0001) on day 7 p.i. with IAV IND/05 and showed more severe clinical signs (lethargy, ruffled fur, hunched posture) than mice of the other groups (Figure 3A). Moribund animals were euthanised when they reached pre-fixed criteria regarding weight loss (>20%) and clinical signs, which was used to determine mortality rates. One mouse out of 10 (10%) of group 2 survived lethal challenge, while all mice but one (91%) of group 3 survived lethal challenge (n=11). This difference in survival rate was statistically significant (p= 0.0003) as was calculated with the Logrank test (Figure 3B). All other mice not previously exposed to IAV HK/68 became moribund, whereas all mice not adequately vaccinated against IAV HK/68 (groups 6 and 7) survived.

Replication of IAV IND/05 (H5N1) in the lungs

The lung virus titers at days four and seven p.i. were compared between groups of IAV IND/05 infected mice. Four days p.i. no significant differences were found between mice of different groups. The average virus titer in mice of group 3 was $10^{7.7}$ $TCID_{50}$/gram lung, which was similar to that observed in mice from group 2 that were effectively vaccinated against IAV HK/68 ($10^{7.6}$ $TCID_{50}$/ gram lung). In contrast, there were significant differences in lung viral titers between mice of the different groups seven days p.i. (Figure 3C). Group 3 mice, not vaccinated against infection with IAV HK/68, had virus titers of $10^{4.8}$ $TCID_{50}$/gram lung while mice of group 2, vaccinated with adjuvanted subunits, had significantly higher virus titers with an average of $10^{6.5}$ (p=0.025), which was similar to that observed in naïve mice infected with IAV IND/05 virus (group 4) or those that were vaccinated

against, but not infected with IAV HK/68 virus (group 5). Mice unsuccessfully vaccinated against IAV HK/68 infection with adjuvant or subunits only also displayed lower lung viral titers (groups 6 and 7).

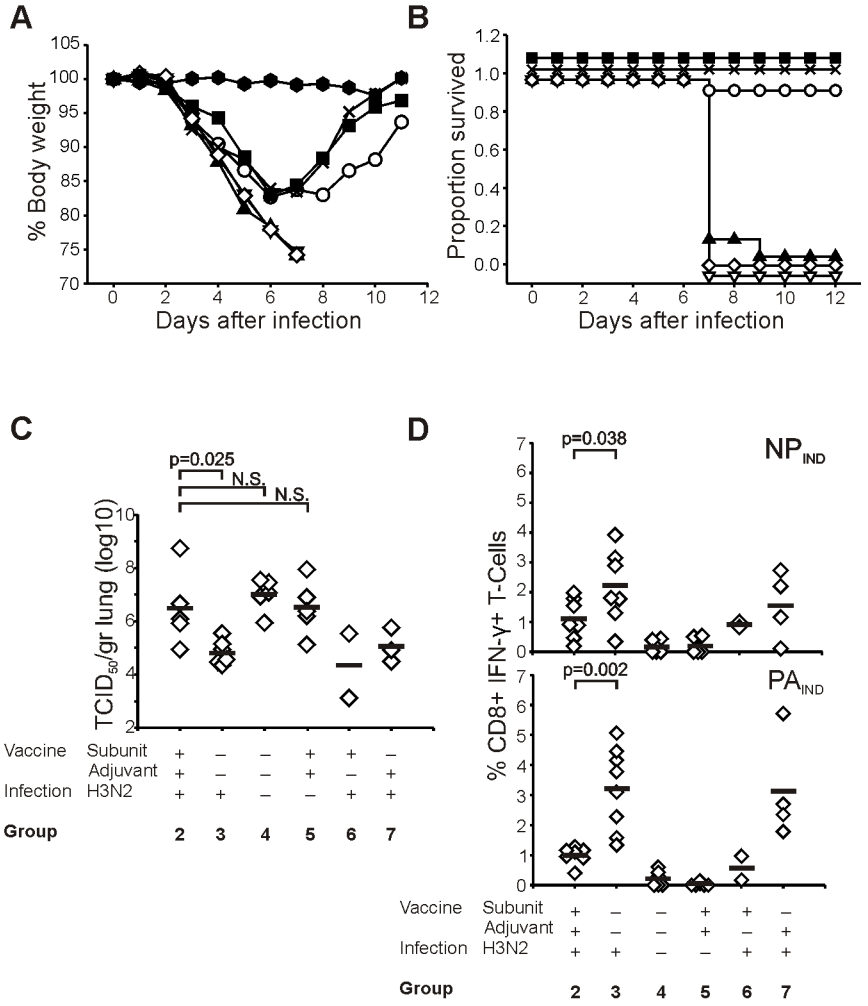


Figure 3. *Outcome of infection with IAV IND/05 (H5N1). Mice were inoculated with IAV IND/05 (groups 2 (▲), 3 (○), 4 (▽), 5 (◇), 6 (■) and 7 (×)) or PBS (group 1 (●)). (A) Body weight after infection was determined daily and expressed as the percentage of the original body weight before infection. (B) Survival rates after infection with IAV IND/05. The proportion of mice from the indicated groups that survived infection is shown in a Kaplan-Meier plot. Moribund animals were euthanized when they reached pre-fixed criteria regarding weight loss (>20%) and disease severity score, which was used to determine mortality rates. (C) Lung virus titers measured on 7 days p.i. in mice from the indicated groups. Horizontal bars represent the average of 2-6 mice. The difference in virus titers between mice of group 2 and group 3 was statistically significant (p=0.025). N.S.: not significant. (D) Virus-specific CD8+ T cell responses on day 7 p.i.. The frequency of CD3+ CD8+ splenocytes specific for peptide $NP_{366-374}$ and $PA_{224-233}$ derived from IAV IND/05 was determined by intracellular IFN-γ staining. The horizontal bars represent the average frequency of IFN-γ+ cells in the CD8+ T cell population of 2-7 mice in the indicated groups. Differences between group 2 and group 3 were statistically significant for both peptides.*

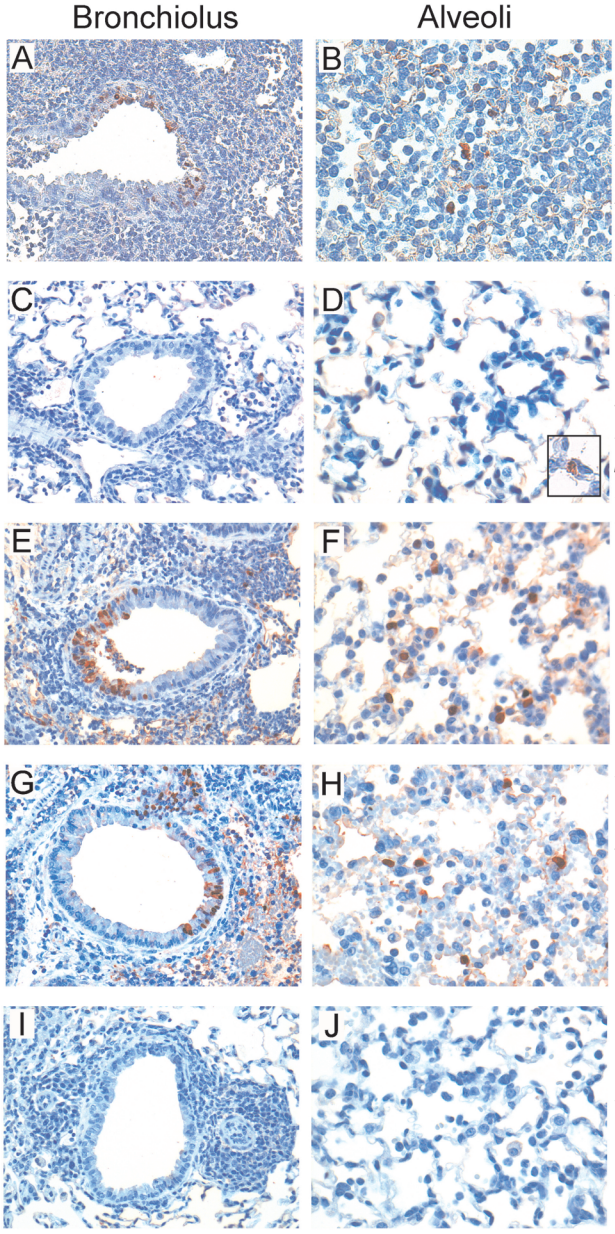


Figure 4. *Histopathological analysis and immunohistochemistry of the lungs of mice infected with IAV IND/05. Mouse lung sections were stained for influenza A virus nucleoprotein. Cytoplasm of infected cells stain red, the nuclei of infected cells stain deep red. In the groups without a history of productive A/H3N2 infection, including group 2 (A,B), infection with IAV IND/05 led to severe histopathological changes and to viral antigen expression in cells of the bronchiolar walls and in the alveoli (group 4: E,F and group 5: G,H). In mice of groups 3 (C,D) and 7 (I,J) that had experienced a productive infection with IAV HK/68 only moderate histopathological changes were observed and virus infected cells were detected sporadically (see insert in panel D).*

Induction of CD8+ T cell responses p.i. with IAV IND/05 (H5N1)

Four and seven days p.i. infection with IAV IND/05, splenocytes were stained for intracellular IFN-γ after incubation with peptides NP_{IND} and PA_{IND}. Four days p.i., no virus-specific CD8+ T cell responses were detected in any of the IAV IND/05 infected mice . However, seven days p.i., anamnestic NP_{IND} and PA_{IND} specific IFN-+CD8+ T-cell responses were observed in mice from group 3, which were significantly lower in mice effectively vaccinated against IAV HK/68 (group 2) (p=0.038 and p=0.002 respectively) (Figure 3D).

Histopathology and detection of infected cells after infection with IAV IND/05 (H5N1)

On day four p.i. with IAV IND/05, mice developed a multifocal severe subacute necrotizing bronchopneumonia, of which the severity was similar for all experimental groups. However, seven days p.i. there were marked differences between the groups. The mock-vaccinated mice or those vaccinated with adjuvant only prior to infection with IAV HK/68 had a multifocal moderate chronic necrotizing

bronchopneumonia characterized by a perivascular core of lymphocytes and plasma cells, proliferation of bronchiolar epithelium and hyperplasia of pneumocytes with a type II appearance. In contrast, mice of groups 4, 5 and especially group 2 had more severe lung pathology characterized by a multifocal to coalescing severe subacute necrotizing bronchopneumonia.
In general, the extent of lung histopathology and the lung virus titers after infection with IAV IND/05 correlated with the presence of virus-infected cells in the lungs as determined by immunohistochemistry. Four days p.i., virus-infected cells were detected in all IAV IND/05 infected mice. In contrast, seven days p.i., antigen positive cells were found sporadically in lungs of mice of groups 3 (Figures 4C-D) and 7 (Figures 4I-J), whereas in the lungs of mice from group 2 (Figures 4A-B), 4 (Figures 4E-F) and 5 (Figures 4G-H) virus-infected cells were still abundantly present.

DISCUSSION

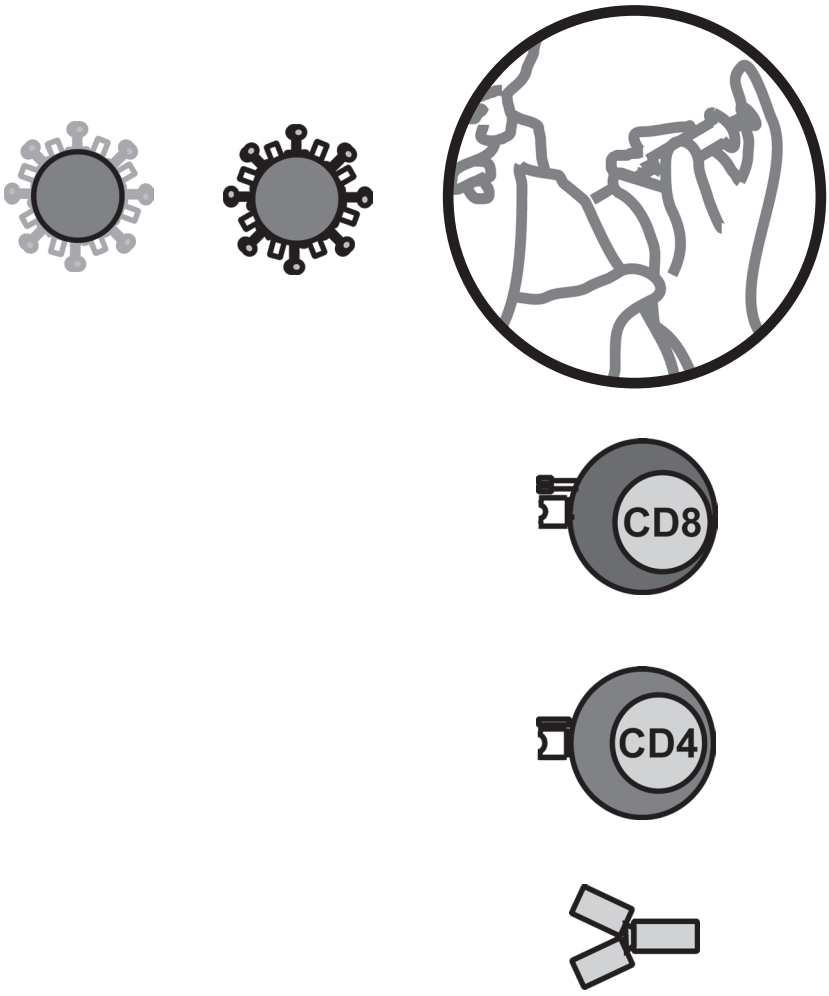
Here we demonstrate that successful vaccination of mice against human IAV HK/68 (H3N2) prevented the induction of heterosubtypic immunity against a lethal challenge with IAV IND/05 (H5N1). As a result, H3N2 vaccinated mice had a fatal clinical outcome of infection with IAV IND/05, associated with higher virus titers and more severe histopathological lesions in the lungs seven days p.i. and reduced virus-specific CD8+ T cell responses compared to mice that experienced a productive, self-limiting infection with IAV HK/68. It has been well established that infection with IAV can induce a certain degree of protective immunity against infection with an heterosubtypic strain of IAV (41). This so-called heterosubtypic immunity was not only demonstrated in animal models (41, 76, 80, 111) but there is also direct and indirect evidence that it exists in humans (50, 54) and that cell-mediated immune responses contribute to this type of immunity (for review see (83)). To test the hypothesis that successful immunization against seasonal influenza could interfere with the induction of heterosubtypic immunity, mice were vaccinated with an Alum-adjuvanted subunit vaccine. The use of an adjuvant was necessary since vaccination with subunit alone induced detectable antibody responses in a small proportion of mice only and would not provide a useful model for successful vaccination against seasonal influenza. Indeed, all mice vaccinated with Alum alone and most mice vaccinated with subunits alone were not protected against infection with A/H3N2 virus. In contrast, all mice vaccinated with adjuvanted subunits, were fully protected against infection with IAV HK/68. This prevented the induction of heterosubtypic immunity against infection with IAV IND/05 normally seen in mice that had experienced a productive IAV HK/68 infection. The severity of the clinical signs and histopathological lesions, the extent of weight loss, lung virus titers and mortality rates of these mice was comparable of those that were immunologically naïve prior to infection with IAV IND/05 (group 4) or that were vaccinated against IAV HK/68 virus, but not subsequently infected with IAV HK/68 virus (group 5).
Four weeks after infection with IAV HK/68 virus, the number of virus-specific CD8+ T cells in the spleen was significantly lower in mice vaccinated against IAV HK/68 than in unvaccinated mice. The differences were not observed at earlier time points p.i.. Further evaluation of the CD8+ Tm_{HK}+ T cells indicated that the numbers of $CD62L^{high}$ and $CD127^{high}$ cells were higher in unvaccinated mice than in vaccinated mice on day 28 p.i. (data not shown). This may indicate that the control of IAV

HK/68 replication in the lungs had prevented the efficient induction of virus-specific central and effector memory CD8+ T cell responses. These results resemble those found in a mouse model for Listeria monocytogenes infection, in which shortening of the duration of the infectious period did not impact the size of the primary CD8+ T cell response, but diminished the memory population of CD8+ T cells (112). The analysis of the CD8+ T cells responses seven days after challenge infection with IAV IND/05 further indicated that indeed prior vaccination against HK/68 (H3N2) prevented the efficient induction of memory CTL responses. Both the secondary response to the NP_{IND} and the PA_{IND} epitope were reduced compared to the responses observed in un-vaccinated mice. Although it has been described that the $NP_{366-374}$ is more immunodominant than the $PA_{224-233}$ epitope in secondary CTL responses (91), a stronger response was observed against the $PA_{224-233}$ epitope after infection with IAV IND/05. This could be explained by the lower cross-reactivity of CTL directed to the $NP_{366-374}$ epitope derived from IAV HK/68 (ASNENMDAM) with that derived from IAV IND/05 virus (ASNENMEVM) compared to the cross-reactivity of CTL specific for the $PA_{224-233}$ epitope as was observed after the analysis of the CTL measured by tetramerstaining p.i. with IAV HK/68 and IND/05 (data not shown). Apart from systemic CTL responses measured in the spleen also local CTL responses may contribute to protective immune responses, such as in the draining lymph nodes and in the lung tissue itself (113, 114, 117). Since the frequency of virus-specific CD8+ T cells in the spleen reflected that in the lymph nodes (45, 115) , we analysed CTL responses in the spleen only. It was of interest to note that infection with IAV HK/68 resulted in the formation of iBALT structures. Prior vaccination against IAV HK/68 infection prevented the formation of iBALT completely. iBALT consists mainly of B cells, T cells and dendritic cells and it has been shown that mice with iBALT but without peripheral lymphoid organs can clear virus infection (116). Although no IAV IND/05 cross-reactive antibodies were detected by VN or HI assay on the day of challenge infection, it is possible that infection with IAV HK/68 induced M2 specific antibodies that potentially cross-reacted with the M2 protein of IAV IND/05. However it is unlikely that these antibodies accounted for the heterosubtypic immunity induced by primary infection with IAV HK/68 (44, 118).

Thus, prior infection with seasonal influenza viruses, which generally results in a self-limiting upper respiratory tract infection, may afford at least partial protection against potentially pandemic heterosubtypic influenza virus strains. Altough vaccination is (cost-) effective in this age group (62-66), it may interfere with the induction of heterosubtypic immunity against potentially pandemic strains of a novel subtype, e.g. H5N1, by creating an immunological "blind spot". Furthermore, the use of adjuvants is considered to increase vaccine efficacy in young children(119). Ideally, seasonal influenza vaccines are used that also induce heterosubtypic immunity (105, 120). More research is required in this field to define vaccine preparations that not only induce protective immunity against seasonal influenza, but also induce heterosubtypic immunity. With the current pandemic threat caused by A/H5N1 viruses this would be highly desirable (121).

Acknowledgements

The authors wish to thank Theo Bestebroer, Peter van Run and Pascal Lexmond for excellent technical assistance.


CD8
CD4

4 Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza A/H5N1 and immunodominance of virus-specific CD8+ T cell responses in mice

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ABSTRACT

Recently we have demonstrated that the use of an experimental subunit vaccine protected mice against infection with a human A/H3N2 influenza virus, but consequently affected the induction of heterosubtypic immunity to a highly pathogenic A/H5N1 influenza virus, otherwise induced by the A/H3N2 infection. Since inactivated whole virus (WIV) vaccines are widely used to protect against seasonal influenza and also contain inner viral proteins like the nucleoprotein, we tested the potential of a WIV vaccine to induce protective immunity against infection with a homologous A/H3N2 (A/Hong Kong/2/68) and a heterosubtypic A/H5N1 influenza virus (A/Indonesia/5/05). As expected, the vaccine afforded protection against infection with the A/H3N2 virus only. In addition, we demonstrated that the use of WIV vaccine for the protection against A/H3N2 infection affected the induction of heterosubtypic immunity otherwise afforded by A/H3N2 influenza virus infection. The reduction of protective immunity correlated with changes in the immunodominance patterns of the CD8+ T cell responses directed to the epitopes located in the acid polymerase ($PA_{224-233}$) and the nucleoprotein ($NP_{366-374}$). In unvaccinated mice that experienced an infection with the A/H3N2 influenza virus, the magnitude of the CD8+ T cell response to both peptides was similar upon a secondary infection with an A/H5N1 influenza virus. In contrast, prior vaccination with WIV affected the immunodominance pattern and skewed the response after infection with influenza virus A/Indonesia/5/05 towards a dominant $NP_{366-374}$ specific response. These findings may have implications for vaccination strategies aiming at the induction of protective immunity to seasonal and/or pandemic influenza.

INTRODUCTION

Influenza viruses are a major cause of respiratory tract infections and responsible for annual excess morbidity and mortality mainly in the elderly, infants and patients with underlying disease (12). In addition to annual epidemics, influenza A viruses occasionally cause pandemics. New influenza A/H1N1 viruses of swine origin have caused the recent pandemic that started in Mexico in the spring of 2009 (13, 14). Because influenza A viruses of various subtypes can be transmitted to humans from animal reservoirs and potentially could cause novel pandemic outbreaks, there is an interest in immunity that could protect against multiple subtypes of influenza A viruses, so called heterosubtypic immunity, as a basis for the development of universal vaccines (120, 122).

This type of immunity induced by infection with influenza A virus was already recognized in the 1960's and has been demonstrated in various animal models (41, 42, 44, 76, 123). Heterosubtypic immunity induced by infection was shown to be long-lasting (42). It does not prevent infection since virus neutralizing antibodies against the viral glycoproteins, the hemagglutinin and neuraminidase, per definition will not cross-react with those of other subtypes. However, heterosubtypic immunity does afford a certain degree of protection and reduces morbidity and mortality otherwise caused by infection with an influenza virus of an alternative subtype (for review see (40)). Evidence for the existence of heterosubtypic immunity in humans is circumstantial but probably contributes to protection against infection with (pandemic) influenza viruses of novel subtypes (50, 54, 124). Heterosubtypic immunity may be relevant especially for protection against infection with highly pathogenic avian influenza viruses like those of the H5N1 subtype that have infected more than people since 1997 with a case fatality rate of 60% (125).

The elucidation of the mechanism of heterosubtypic immunity has been the subject of numerous studies. It has been demonstrated in mice, that multiple arms of the immune system contribute to heterosubtypic immunity, including virus specific CD4+ and CD8+ T cells, local virus specific antibodies and B cells (40, 47, 96, 126-129). Especially CD8+ cytotoxic T lymphocytes (CTL) are thought to contribute to heterosubtypic immunity, because they recognize predominantly cross-reactive epitopes located in conserved proteins like the nucleoprotein and the matrix protein. The cross-reactive nature of influenza virus-specific CTL has been confirmed with T cell populations obtained from mice (130-132) and humans (51-53, 133, 134). Furthermore the presence of CTL immunity correlated with protection against infection and inversely correlated with the extent and duration of viral replication in mice and humans (45, 50, 77).

Since infection with seasonal influenza A viruses induces strong virus-specific CTL responses it could be hypothesized that the use of vaccines that induce protective antibody responses to seasonal influenza viruses, subsequently prevents the induction of virus-specific CTL responses. This could affect the induction of heterosubtypic immunity against infection with viruses of novel subtypes. We recently tested this hypothesis in a mouse model using an alum-adjuvanted subunit preparation to protect against infection with an A/H3N2 influenza virus. Consequently, H3N2-vaccinated mice did not develop heterosubtypic immunity and were no longer protected against a lethal challenge with an A/H5N1 influenza virus (135).

In the present study, we used a whole inactivated influenza A virus vaccine (WIV) based on an A/

H3N2 virus comparable to vaccine preparations used for the vaccination of human subjects to mimic the situation in humans more closely. This vaccine preparation contained also the internal viral proteins like NP and M1 protein in addition to HA and NA. Upon vaccination, this WIV preparation induced protective immunity to a human seasonal influenza A/H3N2 virus and virus-specific CD8+ T cell responses against the conserved proteins, but failed to induce protection against infection with influenza A/H5N1 virus. Furthermore, vaccination with the WIV preparation affected the induction of heterosubtypic immunity induced by experimental infection with a human influenza A/H3N2 virus, similar to the use of adjuvanted subunit vaccine. In addition, the use of WIV affected the immunodominance pattern of the CD8+ T cell response to the epitopes $NP_{366-374}$ and $PA_{224-233}$.

MATERIALS AND METHODS

Viruses

Virus stocks of influenza viruses A/Hong Kong/2/68 (H3N2) (A/HK/2/68) and A/Indonesia/5/05 (H5N1) (A/IND/5/05) were prepared by infecting confluent Madin-Darby canine kidney (MDCK) cells and infectious virus titers were determined in MDCK cells as described previously (135).

Vaccine preparation

Egg-grown concentrated and purified influenza A X-31 virus (a reassortant vaccine strain of A/Aichi/2/68 and A/PR/8/34 of which the hemagglutinin and neuraminidase resemble that of influenza A/HK/2/68 closely) was inactivated by treatment with 0.05% formaldehyde for seven days under continuous stirring at 4°C. After inactivation, antigen was dialyzed against phosphate buffered saline (PBS). The purity of the vaccine preparation was tested by SDS-polyacrylamide gel electrophoresis and the inactivation was confirmed by failure to passage on MDCK cells. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, USA).

Immunization and infection of mice

Six-to-eight weeks old, female, specified pathogens free C57BL/6J (H-2b) mice were purchased from Charles River (Sulzfeld, Germany). Mice were divided in five groups and animals of groups 1 (n=42), 3 (n=35) and 4 (n=17) were mock-immunized intramuscularly twice with an interval of four weeks with 100 µl PBS in two hind legs, while mice of groups 2 (n=47) and 5 (n=23) were immunized twice with 15µg whole inactivated vaccine (WIV) in PBS (total volume 100µl) (Table 1). Eight and 28 days after the first and 28 and 56 days after the second vaccination, four mice of group 1 and 2 were bled and spleens were resected. As a positive control group for the induction of virus-specific CD8+ T cell responses after vaccination, eight mice were also infected with $2x10^4$ $TCID_{50}$ influenza A X-31 intranasally in a total volume of 50µl, which were euthanized eight and 28 dpi.

Four weeks after the second vaccination, mice of groups 2 and 3 were infected intranasally with $5x10^2$ $TCID_{50}$ influenza virus A/HK/2/68 (H3N2) in a volume of 50µl. Mice of groups 1, 4 and 5 were mock-infected with PBS. Following infection, mice were weighed daily to monitor their weight loss as clinical indicator of infection. Four, twelve and 28 dpi six mice of groups 1, 2 and 3 were bled and lungs and spleens were resected. Four weeks after infection with influenza virus A/HK/2/68

(H3N2), all mice except those of group 1 were infected with $2x10^2$ $TCID_{50}$ influenza A/IND/5/05 (H5N1) intranasally. This dose was chosen at it was the minimal dose resulting in a lethal infection in >90% mice reproducibly (135, 136). Mice of group 1 were mock-infected with PBS. The day before infection with influenza virus A/IND/5/05 (H5N1), six mice of each group except group 4 were euthanized and lungs and spleens were resected. After infection with influenza virus A/IND/5/05 (H5N1), mice were weighed daily and monitored for clinical signs. Mice were euthanized four (n=5 per group), seven (n=8 or more per group) and fourteen (n=4 or less per group) days after challenge and spleens and lungs were resected. Vaccinations, intranasal infections, blood withdrawal and euthanasia were performed under anesthesia with isoflurane in O_2. All experiments with influenza A/H5N1 virus were performed under Biosafety Level 3 (BSL III) conditions. An independent animal ethics committee approved the experimental protocol before the start of the experiments.

Table 1. Overview of experimental groups of mice used in this experiment

Experimental group	Treatment of mice		
	X-31 WIV vaccination	Infection with A/HK/2/68 (H3N2)	Infection with A/IND/5/05 (H5N1)
1	-	-	-
2	+	+	+
3	-	+	+
4	-	-	+
5	+	-	+

Serology

Before vaccination, four weeks after the first vaccination, four weeks after the second vaccination and four weeks after infection with influenza A/H3N2 virus, serum samples of mice were collected and tested for the presence of HA-specific antibodies against influenza viruses A/H3N2 and A/H5N1 using the hemagglutination inhibition (HI) assay and virus neutralizing (VN) antibodies using the VN assay as described previously (87, 107, 135).

Lung virus titers

Lungs of mice collected four and twelve dpi with influenza A/H3N2 and four, seven and fourteen days after challenge with influenza A/H5N1 virus were snap frozen and stored at –70°C until further processing. Lungs were homogenized and quintuplicate 10-fold serial dilutions of these samples were used to inoculate MDCK cells as described previously (135). HA-activity of the culture supernatants collected 5 days post inoculation was used as indicator of infection. The titers were calculated according to the Spearman-Karber method (108).

Flow cytometry of virus-specific CD8+ T cells

Preparation of single cell suspensions of lung cells and splenocytes

Twelve and 28 days after influenza A/H3N2 virus infection, mice were bled and subsequently lungs of three to four mice per group were collected in gentleMACS tubes (Miltenyi Biotec, Bergish Gladbach, Germany) with IMDM medium supplemented with 5% FCS, 100µg/mL streptomycin and 100 IU/mL penicillin and a single cell suspension was made following the manufacturers protocol. The lung suspension was then filtered with a 100µM cell strainer (BD Falcon, Erembodegem, Belgium) and subsequently treated with red blood cell lysis buffer (Roche, Mannheim, Germany). Single splenocyte suspensions were prepared as described previously (77).

Tetramerstaining

After vaccination or infection with influenza A/H3N2, single cell suspensions of splenocytes and lung cells were washed and subsequently stained with fluorescently labeled monoclonal antibodies (mAbs) CD3e-PeCy7, CD8b.2-FITC (both BD, Alphen a/d Rijn, the Netherlands), CD62L-APC-Cy7 (BioLegend, San Diego, USA), CD127-Pacific Blue (eBioscience, San Diego, USA) and either the PE-labeled H-2Db tetramer with the immunodominant $NP_{366-374}$ epitope derived from influenza A virus X-31 (ASNENMETM; Tm_{X-31}) or influenza A/HK/2/68 (ASNENMDAM; Tm_{H3N2}) and the APC-labeled tetramer derived from influenza A/IND/5/05 $NP_{366-374}$ (ASNENMEVM; Tm_{H5N1}) (137). Tetramers were purchased from Sanquin Research, Amsterdam, The Netherlands. To exclude dead cells in the analysis, cells of vaccinated or H3N2-infected mice were also stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, the Netherlands). Following incubation with tetramers and mAbs for 20 minutes, cells were washed twice and analyzed with a FACSCantoII and FACS Diva software (all BD).

After H5N1-infection (BSL-III conditions), single cell suspensions of spleens were washed and subsequently stained with mAbs against CD3e, CD8b.2 and with either the PE-labeled H-2Db tetramer Tm_{X-31} or Tm_{H3N2} and the APC-labeled tetramer Tm_{H5N1}. After incubation, cells were washed, fixed with Cytofix (BD) and subsequently acquired on a FACSCalibur and analyzed with Cellquest Pro software (both BD).

Peptides and intracellular interferon gamma (IFN-γ) staining

CD8+ T cell responses were measured by incubation of splenocytes or lung cells with peptides representing two immunodominant epitopes of influenza A viruses in C57BL/6J mice (H2-b), $PA_{224-233}$ and $NP_{366-374}$ (91, 109). Peptides were manufactured at Eurogentec (Seraing, Belgium). Splenocytes or lung cells were cultured for 6 h at 37°C in the presence of 5µM of either the $NP_{366-374}$ ASNENMDAM (NP_{H3N2}), $PA_{224-233}$ SCLENFRAYV (PA_{H3N2}) peptides derived from influenza A/HK/2/68 (H3N2) virus or the $NP_{366-374}$ ASNENMEVM (NP_{H5N1}) or SSLENFRAYV (PA_{H5N1}) peptides derived from influenza A/IND/5/05 virus in IMDM (Lonza, Breda, the Netherlands) with 5% FCS and Golgistop (BD). The PA_{H5N1} peptide is also a known epitope in the influenza A X-31 vaccine strain that was used. After incubation, cells were stored o/n at 4°C and the presence of intracellular IFN-γ in CD8+ T cells was subsequently analyzed as described previously (135). Peptide-specific responses were calculated by subtracting the percentage of CD8+ IFN-γ+ T cells after incubation with medium and Golgistop only from the percentage of CD8+ IFN-γ+ T cells after peptide-stimulation.

Statistical analysis

Data for weight loss after infection, lung-virus loads, tetramerstaining, and peptide-pulsing were analysed statistically using the Mann Whitney Test. Survival was analysed using the Logrank test. Differences were considered significant at $p<0.05$.

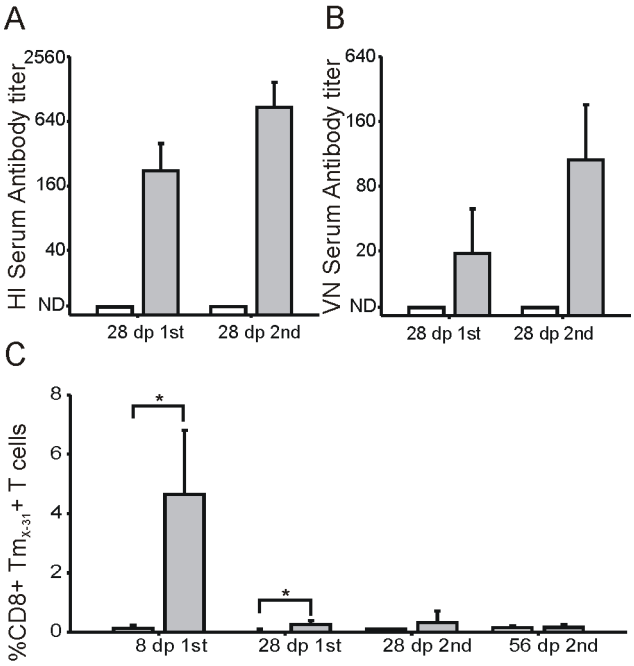

Figure 1. *Antibody and CD8+ T cell responses after vaccination of mice with WIV X-31 (H3N2). Virus-specific serum antibody levels were measured by HI-assay (A) and VN (B) assay. Splenic CD8+ T cell responses directed against the NP366-374 epitope of influenza A X-31 (ASNENMETM) (C). White bars (with s.d.) represent mock-vaccinated mice (groups 1,3 and 4) and grey bars (with s.d.) represent WIV X-31 vaccinated mice (groups 2 and 5). *: p<0.05 compared to mice of group 1.*

RESULTS

Antibody and CD8+ T cell responses induced by vaccination with WIV

Twenty-eight days post administration of the WIV X-31 vaccine, mice of groups 2 and 5 developed HA–specific antibodies against influenza virus A/HK/2/68 (H3N2) as detected in the HI assay (geometric mean titer (GMT) of 234) and VN assay (GMT of 19) (Figure 1A). Upon a second vaccination twenty-eight days later the GMTs increased to 915 and 144 respectively (Figure 1B). Influenza virus-specific antibody responses were detected in none of the mock-vaccinated mice. The induction of virus-specific CTL responses by WIV vaccination was assessed by determining the frequency of CD8+ T lymphocytes in the spleens specific for the $NP_{366-374}$ epitope (ASNENMETM) by tetramer staining using Tm_{X-31}. Eight days after the first vaccination, the mean percentage of CD8+Tm_{X-31}+ T lymphocytes was 4.7 (s.d. 2.2), which was significantly higher than in mock-vaccinated mice ($p=0.02$) (Figure 1C). Subsequently, the frequency of CD8+Tm_{X-31}+ T lymphocytes declined but remained higher than in mock-vaccinated mice (0.25 and 0.05%, respectively $p=0.04$). The booster vaccination did not increase the frequency of $NP_{366-374}$ specific CD8+ T cells. Twenty-eight and 56 days after the second vaccination the percentages of CD8+Tm_{X-31}+ were no longer different from those determined in mock-vaccinated mice ($p=0.13$ and $p=1.00$, respectively). The frequencies of

CD8+Tm_{X-31}+ T lymphocytes in the spleens of control mice infected with influenza virus X-31, were 8.9 (±3.0) and 1.0 (± 0.7) eight and twenty-eight days post infection, respectively. The proportion of cells that expressed CD62L or CD127 did not differ significantly between vaccinated and infected mice (data not shown).

Outcome of infection with influenza virus A/HK/2/68 (H3N2)

Upon infection with influenza virus A/HK/2/68, mock-vaccinated mice developed mild to moderate clinical signs and lost body weight from day 3 to day 7 post infection (Figure 2A). From day 7 onwards, these mice recovered from infection. In contrast, mice vaccinated with the WIV X-31 vaccine (groups 2) did not develop any clinical signs upon infection and did not display loss of body weight, comparable to mock-infected mice (groups 1,4 and 5). The development of clinical signs correlated with virus titers in the lungs four days post infection (dpi). In the lungs of mock-vaccinated mice, the virus replicated to mean titers of $10^{8.6}$ $TCID_{50}$ per gram of tissue (Figure 2B). In contrast, infectious virus could not be detected in the lungs of mice that were vaccinated with the WIV X-31 vaccine preparation and in lungs of mock-infected control mice.

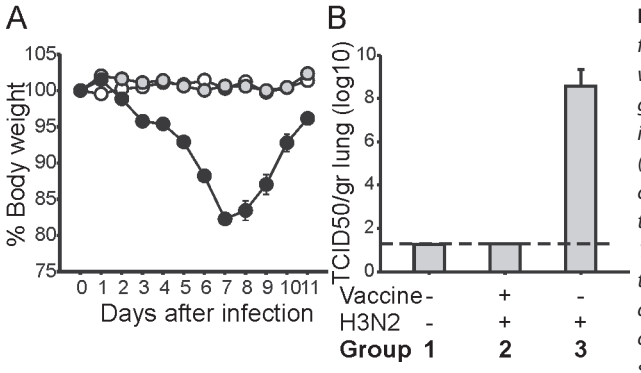

Figure 2. *Outcome of infection with influenza virus A/HK/2/68 (H3N2). Mean weight loss of mock-infected (mice of groups 1, 4 and 5; white circles) or mice infected with influenza A/H3N2 virus (group 2; grey circles and group 3; black circles) ± s.e.m (A). Four dpi lung virus titers were determined of mice of groups 1,2 and 3. Bars represent mean virus titers of four mice per group with standard deviations. The dotted line indicates the cut-off value for obtaining a positive result (B).*

CD8+ T cell responses after infection with influenza A/H3N2

Tetramerstaining

Twelve and 28 dpi with influenza virus A/HK/2/68 (H3N2), the frequency of CD8+ T cells specific for the $NP_{366-374}$ epitope present in influenza virus A/HK/2/68 (H3N2) (ASNENMDAM) was assessed in the spleen and lungs by tetramerstaining. Twelve dpi, the mean percentage of CD8+ Tm_{H3N2}+ T cells in spleens and lungs of mock-vaccinated H3N2-infected mice of group 3 were 0.83 (s.d. 0.36) and 4.00 (s.d. 1.00), respectively. In contrast, the mean frequency of CD8+ Tm_{H3N2}+ T lymphocytes was significantly lower in the WIV-vaccinated mice (0.22 ± 0.10% and 0.93 ± 0.54% in spleens (p=0.004) and lungs (p=0.02) respectively) and comparable to the frequencies found in naïve control mice (Figure 3A and B).

Twenty-eight dpi, the percentage of CD8+ Tm_{H3N2}+ T cells in the spleens and lungs had declined somewhat (Figure 3C and 3D). This percentage in the spleens and lungs was still lower in vaccinated mice H3N2-infected mice compared to unvaccinated/H3N2 infected mice.This difference was

statistically signficant for the spleens (p=0.01) and approached statistical significance for the lungs (p=0.05) at this time point post infection.

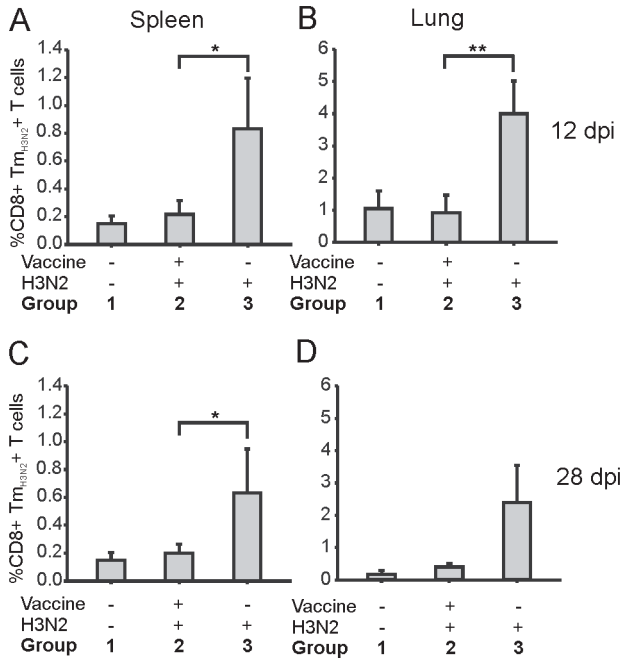

Figure 3. *Virusspecific CD8+ T cell responses after influenza A/HK/2/68 (H3N2) infection. Percentages of CD8+ TmH3N2+ T cells were determined in spleens (A, C) and lungs (B,D) obtained 12 (A,B) and 28 (C,D) days after infection with influenza A/HK/2/68 (H3N2). Bars represent mean percentages of CD8+ TmH3N2+ T cells ± s.d.. *: p<0.05 compared to mice of group 2 and **: p<0.01 compared to mice of group 2.*

4

Cross-reactivity and differentiation of CD8+ TmH3N2+ T cells

The cross-reactivity of CD8+ TmH3N2+ T cells with the epitope variant of the influenza A/H5N1 virus (ASNENMEVM) was analysed using Tm_{H5N1}. Only few CD8+ TmH3N2+ T cells induced after infection with influenza virus A/HK/2/68 (H3N2) cross-reacted with the H5N1-derived epitope. Furthermore, percentages of CD8+ Tm_{H5N1} T cells did not differ between the respective groups after influenza A/H3N2 infection (data not shown).

The CD8+ Tm_{H3N2}+ T cells were also phenotyped according to their expression of CD62L and CD127. $CD62L^{high}/CD127^{high}$ cells were regarded as central memory (CM),$CD62L^{low}/CD127^{high}$ cells as effector memory (EM) and $CD62L^{low/}CD127^{low}$ as effector (E) cells (138). In spleens and lungs of mock-vaccinated, H3N2-infected mice (group 3) 12 and 28 dpi, most CD8+ Tm_{H3N2}+ T cells were of the EM phenotype (Figure 4). The percentage EM CD8+ Tm_{H3N2}+ T cells of these mice was significantly higher than that of X-31 vaccinated mice of group 2 ($p<0.05$) in both spleens (12 and 28 dpi) and lungs (only 12 dpi). Only twelve dpi, the percentage of E CD8+ Tm_{H3N2}+ T cells was higher in mice of group 3 compared to mice of group 2 in both lungs and spleens. On day 28 dpi this population was virtually absent in mice of all groups. Twelve dpi, an increase in the percentages of CM CD8+ TmH3N2+ T cells was observed in the spleens, but not in the lungs, of H3N2-infected mice compared to mock-infected animals or those that were vaccinated and infected (p=0.002). At day 28 dpi these differences between groups were no longer detectable.

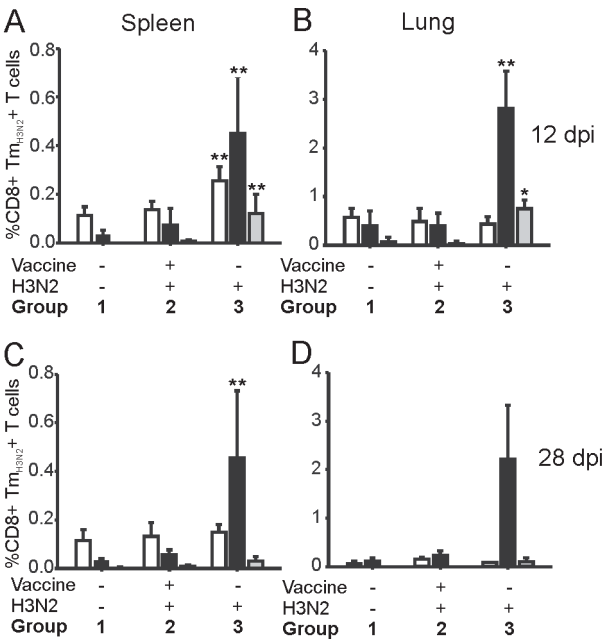

Figure 4. *Differentiation of CD8+ TmH3N2+ T cells. CD8+ TmH3N2+ T cells in spleens (A, C) and lungs (B,D) obtained 12 (A,B) and 28 (C,D) dpi with influenza A/HK/2/68 (H3N2) were divided based on their expression of CD62L and CD127 by flow cytometry. White bars represent central memory cells (CD62L^{high} CD127^{high}), black bars effector memory cells (CD62L^{low} CD127^{high}) and grey bars effector cells (CD62L^{low} and CD127^{low}).*
**: p<0.05 compared to mice of group 2 and **: p<0.01 compared to mice of group 2.*

Specificity of CD8+ T cell response (Intracellular IFN-γ staining)

To assess the cross-reactivity of the CD8+ T cell response with epitope variants present in the influenza virus strain A/Indonesia/5/05 (H5N1), splenocytes and lung cells obtained from mice twelve dpi with influenza virus A/HK/2/68 (H3N2) were stimulated with peptides $NP_{366-374}$ and $PA_{224-233}$ derived from both viruses. Upon infection with the H3N2 strain peptide-specific CD8+ T cells responses were observed in spleens and lungs that also cross-reacted with the peptide from the heterosubtypic strain (Figure 5). The response to NP_{H5N1} peptide (H5N1) was significantly higher in mice that were X-31-vaccinated prior to infection with A/HK/2/68 (H3N2) than in mock-vaccinated mice. Otherwise vaccination reduced the magnitude of the CD8+ T cell response following infection with the H3N2 strain.

Also in the lungs virus-specific CD8+ T cells were observed after infection of mock-vaccinated mice to the homologous $NP_{366-374}$ peptide and the H3N2 and H5N1 $PA_{224-233}$ peptide variants. These responses were significantly higher than those of mice that were vaccinated against the H3N2 strain. Twenty-eight dpi, peptide-specific CD8+ T cells were no longer detectable by intracellular IFN-γ staining in both lungs and spleens of any of the mice (data not shown).

Vaccination prevents heterosubtypic immunity against influenza A/H5N1

Twenty-eight dpi with influenza A/H3N2, mice of groups 2, 3, 4 and 5 were infected with a 90% lethal dose of influenza A/Indonesia/5/05 (H5N1). All H5N1-infected mice developed clinical signs including weight loss from two days post infection onward. Eleven out of 12 unvaccinated, H3N2-infected mice (group 3) lost weight until day six or seven and then started to recover and gained weight. In contrast, all mice of groups 4 and 5 lost weight until they reached humane endpoints for

euthanasia according to animal welfare regulations (weight loss of more than 20% and severe clinical signs). Of the X-31-vaccinated and H3N2 infected mice, eight out of twelve (67%) mice had to be euthanized. Mice of groups 2 and 3 that survived challenge and that were not taken out of the experiment to assess lung virus titers or CD8+ T cell responses continued to gain weight and reached their original body weight 14 dpi. The difference in weight loss and survival between mock-vaccinated and WIV X-31-vaccinated H3N2-infected mice was statistically significant (both $p<0.01$) (Figure 6A and B). No significant differences were observed between virus titers in the lungs of mice of groups 2 and 3 four (data not shown) and seven dpi, but the average virus titer at 7 dpi was the lowest in mice of group 3, which survived the lethal challenge ($p=0.32$) (Figure 6C). No virus was detected in lungs of mice that survived the lethal challenge until14 dpi.

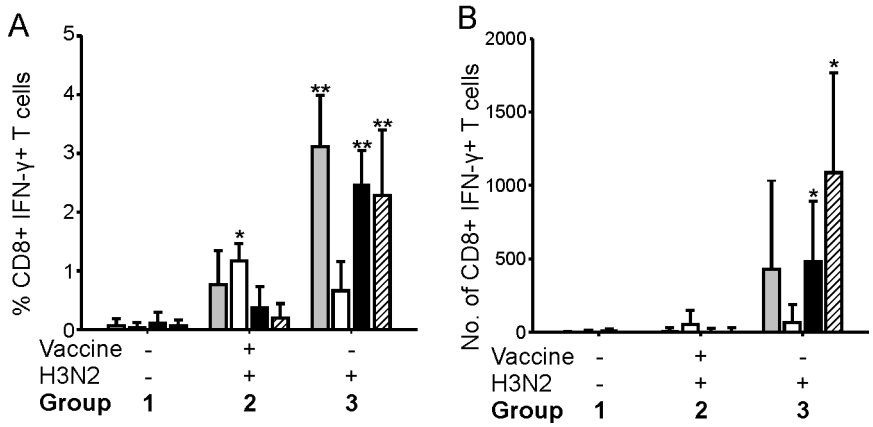

Figure 5. *CD8+ T cell responses to $NP_{366-374}$ and $PA_{224-233}$ as measured by intracellular IFN-γ staining. Responses of CD8+ T cells directed against peptides NP_{H3N2} (grey bars), NP_{H5N1} (white bars), PA_{H3N2} (black bars) and PA_{H5N1} (hatched bars) were detected by intracellular IFN-γ staining 12 days after influenza A/HK/2/68 (H3N2) infection in spleens (A) and lungs (B). Bars represent mean values ± s.d.. *: $p<0.05$ compared to mice of group 2 and **: $p<0.01$ compared to mice of group 2.*

Virus-specific CD8+ T cell responses after H5N1-infection

Seven dpi with influenza virus A/Indonesia/5/05 (H5N1) infection, the presence of CD8+ T cells specific for the NP_{H5N1}, NP_{H3N2} NP_{X-31} and PA_{H5N1} epitopes in the spleen was assessed by tetramerstaining and/or intracellular IFN-γ staining after stimulation with peptides.

In naïve mice, virus-specific CD8+ T cell responses were virtually undetectable seven dpi with influenza virus A/IND/5/05 (H5N1) (Figure 6D). In contrast, a prior infection with influenza virus A/HK/2/68 (H3N2) (group 3 mice) primed for a CD8+ T cell response for both the $NP_{366-374}$ and $PA_{224-233}$ epitope derived from the H5N1 strain (PA_{H5N1}). The magnitude of the response to both epitopes was comparable indicating that the responses were co-dominant. In contrast, the response in group 5 that were vaccinated with WIV X-31 before infection with the A/H5N1 virus was dominated by the $NP_{366-374}$ –specific response. A similar result was observed in mice of group 2 that were WIV-X-31 vaccinated and subsequently infected with influenza virus A/HK/2/68 before infection with influenza virus A/IND/5/05 (H5N1). In this group the response to $PA_{224-233}$ was virtually absent and significantly lower than that observed in mice of group 3 ($p<0.001$).

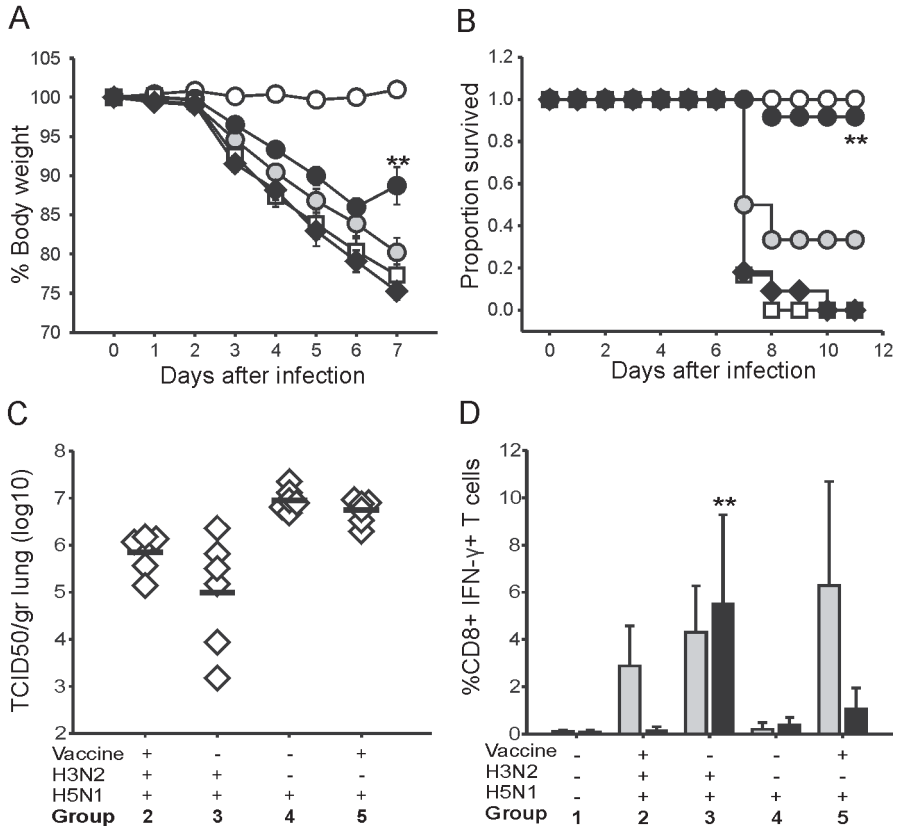

Figure 6. *Outcome of influenza A/IND/5/05 (H5N1) virus infection. Mice were mock-infected (group1; white circles) or infected with influenza A/IND/5/05 (H5N1) (group 2; grey circles, group 3; black circles, group 4; black diamond squares and group 5; white squares), were weighed daily and the mean weight loss ± s.e.m. was calculated (A). Moribund animals were euthanized according to pre-fixed criteria and were recorded as dead to calculate the cumulative survival after H5N1 challenge as shown in the Kaplan Meier curve (B). Lung virus titers were determined 7 dpi with influenza A/IND/5/05 (H5N1). Horizontal bars represent the average titers of each group of mice (C). CD8+ T cells to peptides NPH5N1 (grey bars) and PAH5N1 (black bars) in spleens of mice were measured 7 dpi by intracellular IFN-γ staining (D). The data represent the mean percentage of CD8+ IFN-γ+ T-cells ± s.d..*: p<0.05 compared to mice of group 2 and **: p<0.01 compared to mice of group 2.*

The results obtained with intracellular IFN-γ staining were in agreement with those obtained with $NP_{366-374}$ based tetramer staining (table 2), illustrating that the response to the $NP_{366-374}$ epitope was cross-reactive in mice of group 2, 3 and 5. In addition, this method allowed the assessment of the cross-reactivity of the CD8+ T cells that were induced against this epitope. Prior infection with influenza virus A/HK/2/68 (H3N2) (containing $NP_{366-374}$ epitope ASNENMDAM) did not predispose for the induction of CD8 + T cells cross-reactive with NP_{H5N1} (ASNENMEVM). In contrast, vaccination with WIV-X-31 (containing ASNENMETM), primed for the induction CD8+ T cell responses not only cross-reactive with the H5N1 variant of the epitope ASNENMEVM but also with the H3N2 variant ASNENMDAM (table 2).

Table 2. Specificity and cross-reactivity of $NP_{366-374}$ specific CD8 T cells in spleens of mice 7 days post infection with A/IND/5/05 (H5N1) as detected by tetramer-staining.

Group	CD8+ Single Tm+ T cells			CD8+ Double Tm+ T cells	
	NP_{X-31+}	NP_{H3N2+}	NP_{H5N1+}	NP_{X-31+} NP_{H5N1+}	NP_{H3N2+} NP_{H5N1+}
2	1.1 (1.0)*	0.7 (0.9)	0.8 (0.7)	1.2 (1.8)	0.8 (1.2)
3	n.d.	1.0 (0.9)	2.0 (2.7)	n.d.	0.1 (0.1)
4	n.d.	0.1 (0.1)	0.0 (0.1)	n.d.	0.0 (0.0)
5	2.9 (3.3)	0.6 (0.5)	0.9 (0.9)	3.6 (6.9)	4.1 (6.6)

* data are represented as percentage Tm+ cells within the CD8+ T cell population (standard deviation)
n.d. : no data collected

DISCUSSION

In the present study, it was demonstrated that vaccination of mice with an inactivated influenza A/H3N2 vaccine induced protective immunity to infection with a corresponding A/H3N2 virus. By preventing productive infection with influenza virus A/HK/2/68 (H3N2) the protective potential of heterosubtypic immunity afforded by infection with the A/H3N2 virus was severely reduced. As a result, WIV X-31/H3N2 infected vaccinated mice suffered more from clinical signs as weight loss and displayed higher mortality rates after infection with the highly pathogenic influenza virus A/IND/5/05 (H5N1) than unvaccinated/H3N2 infected mice. In addition, WIV X-31 vaccination altered immunodominance patterns of CD8 T cell responses induced after infection with the A/H5N1 virus and the extent of viral shedding.

In a previous study we have shown that the use of an experimental subunit vaccine exclusively containing the hemagglutinin and neuraminidase of influenza virus X-31 prevented the induction of heterosubtypic immunity against an influenza A/H5N1 virus infection, otherwise induced by infection with influenza virus A/HK/2/68 (H3N2) (135). Since the viral inner proteins were absent, this vaccine preparation failed to induce CD8+ T cell responses to e.g. the NP.

In this study, we evaluated the use of whole inactivated virus vaccines for several reasons: first, WIV vaccines are more immunogenic than subunit vaccines and are widely used in humans as unadjuvanted influenza vaccines. Secondly, since WIV vaccines contain inner viral proteins it was anticipated that they also induce CD8+ T cell responses to these conserved proteins (104), albeit inefficiently, and therefore may afford some level of heterosubtypic immunity. Indeed, vaccination with WIV X-31 induced detectable virus-specific CD8+ T cell responses, probably through cross-priming (139), in addition to virus-specific antibody responses and protected mice from infection with the corresponding H3N2 influenza virus A/HK/2/68. However, the T cell responses to the $NP_{366-374}$ epitope were transient and only detected shortly after the first vaccination. In addition, they were modest compared to the responses observed after infection with influenza virus X-31. Furthermore, vaccination severely impaired the CD8+ specific T cell response to $NP_{H3N2,}$ PA_{H3N2} and PA_{H5N1}, but not the response to NP_{H5N1} after infection with influenza virus A/HK/2/68 (H3N2). Apparently there is cross-reactivity between the X-31 (ASNENMETM), the H3N2 (ASNENMDAM) and the H5N1 (ASNEN-

MEVM) variant of the $NP_{366-374}$ epitope, which was also reported by others (92).
Vaccination with WIV largely prevented the formation of central and especially effector memory cells specific for $NP_{H3N2,}$ otherwise induced after infection with influenza virus A/HK/2/68 (H3N2).
However, vaccination did prime for a secondary CD8+ T cell response specific for the heterologous $NP_{366-374}$ epitope derived from influenza virus A/IND/5/05 (H5N1) (ASNENMETM vs ASNENMEVM). Apparently these two variants are antigenically similar and are highly cross-reactive and CD8+ T cell responses were induced of which the relative responses to the $NP_{366-374}$ and $PA_{224-233}$ epitopes resembled that of a typical secondary influenza virus specific CTL response in C57BL/6 mice (91). Nevertheless, WIV X-31 vaccination did not afford protection against the development of clinical signs and mortality caused by A/IND/5/05 infection and did not reduce virus replication in the lung.
In contrast, a primary infection with influenza virus A/HK/2/68 (H3N2) afforded partial protection against infection with influenza virus A/IND/5/05 (H5N1). These mice started to regain body weight seven dpi onward and fully recovered. This protection correlated with the induction of co-dominant CD8+ T cell responses against the NP_{H5N1} and PA_{H5N1} epitopes. Although the difference with naïve mice of group 4 and WIV-vaccinated mice of group 5 was striking, it is uncertain if the response to especially the $PA_{224-233}$ epitope was responsible for the protective effect of a prior A/H3N2 infection. Firstly, it has been demonstrated that the $PA_{224-233}$ epitope was presented by professional antigen presenting cells like dendritic cells, but not by virus-infected epithelial cells (140), indicating that $PA_{224-233}$ specific CD8+ T cells are unable to eliminate virus-infected cells from the lung efficiently. In addition, it was shown that vaccination with DCs loaded with the $PA_{224-233}$ peptide induced an epitope-specific CD8+ T cell response but failed to control a subsequent infection with influenza virus or even delayed virus clearance (140). Secondly, only a minority of $NP_{366-374}$ specific CD8+ T cells induced after infection with influenza virus A/HK/2/68 (H3N2) was cross-reactive with the peptide variant derived from the H5N1 virus, which could explain the co-dominant $PA_{224-233}$ specific response. Although the infection with influenza virus A/IND/5/05 (H5N1) resulted in the selective expansion of cross-reactive $NP_{366-374}$ specific cells, this response is much lower than observed after a secondary infection with an infuenza virus carrying the identical epitope sequence (141). In the absence of a dominant response to $NP_{366-374}$, the response to $PA_{224-233}$ can become co-dominant (141).
Vaccination with WIV-X-31 (group 2) completely abrogated the response to the $PA_{224-233}$ epitope observed after consecutive infections with influenza viruses A/HK/2/68 and A/IND/ 5/05, but not the response to the $NP_{366-374}$ epitope. With regard to the response to the latter epitope, two contradictory mechanisms could be at work simultaneously: first, vaccination could reduce the induction of CD8+ T cells specific for NP_{H3N2} (ASNENMDAM) cross reactive with NP_{H5N1} (ASNENMEVM) as was also observed after vaccination with a subunit vaccine (135). Second, WIV vaccination could prime for a secondary response to the $NP_{366-374}$ as was seen for the mice of group 5.
In addition, vaccination may prevent the induction of other arms of the adaptive immune response otherwise induced by infection like virus-specific CD4+ T cells, antibodies to the extracellular domain of the matrix protein (M2e) (43, 46, 47, 96, 123, 126, 127, 129, 142-144) and the induction of tertiary lymphoid structures (116). Although it has been suggested recently that also antibodies to the nucleoprotein contribute to heterosubtypic immunity (36), it is unlikely that they played a role

in the present study since all vaccinated mice developed NP-specific antibodies which did not correlate with protection against H5N1 infection (data not shown).
Collectively, this study demonstrates that vaccination with WIV X-31 induces virus-specific CD8+ T cell responses, induces protective immunity against homologous infection with influenza A/H3N2 and primes for secondary CD8+ T cell responses after infection with A/H5N1 influenza virus.
However, despite its capacity to induce memory CD8+ T cell immunity, vaccination with WIV, like the use of subunit vaccines, did not afford protection against influenza A/H5N1 infection.
In addition, the use of WIV vaccines affected the induction of heterosubtypic immunity by infection, like the use of subunit vaccine (135). Thus, regardless of the nature of the inactivated vaccine that is used for the protection against seasonal influenza, the induction of heterosubtypic immunity is affected. This may be an unwanted effect of seasonal influenza vaccination using inactivated vaccines and is at present a matter of debate as it was recently recommended in a number of countries including the USA to to vaccinate all healthy children 6-59 months of age against seasonal influenza (60, 61, 145, 146).
The interference with the induction of heterosubtypic immunity correlated with changes in the immunodominance patterns of the virus-specific CD8+ T cell response. However it is unlikely that these changes were responsible for the lack of protection per se and most likely reflect differences in imprinting of CD8+ T cell responses by the absence or presence of priming of $NP_{366-374}$ specific T cell memory responses. Detailed analysis of the outcome of immune responses upon consecutive infections with influenza viruses of different subtypes may provide better insight in the pros and cons of the use of inactivated vaccines in immunologically naïve subjects.

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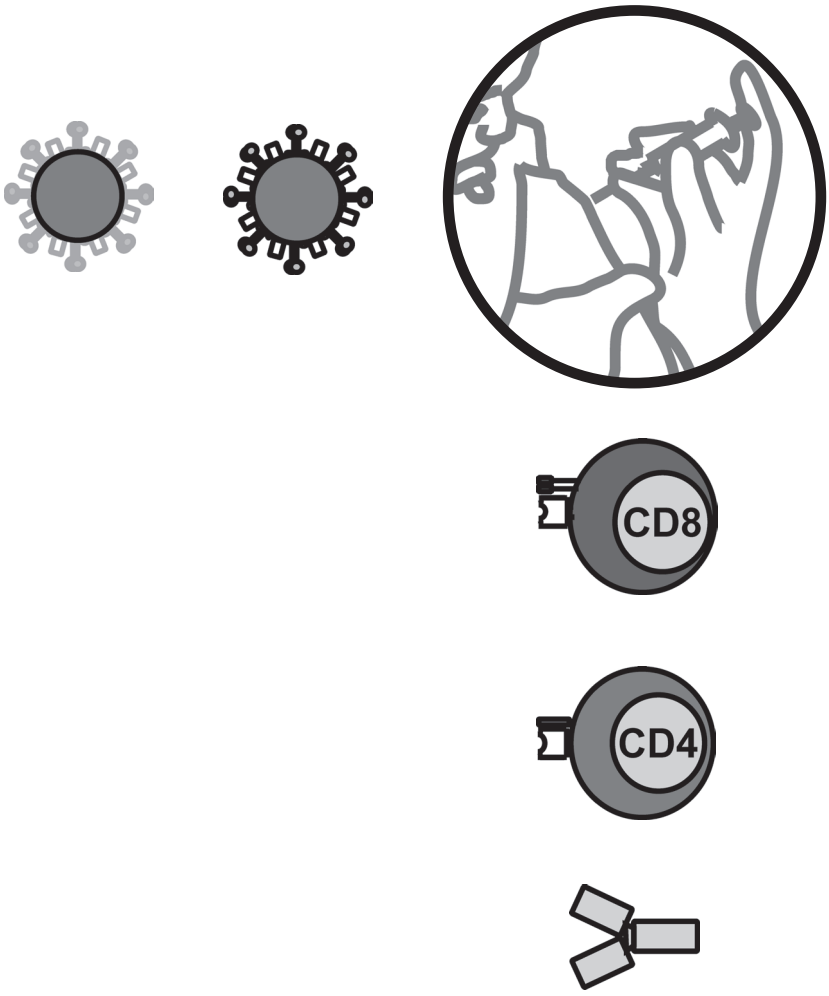
CD8
CD4

5

Vaccination against seasonal influenza A/H3N2 reduces the induction of heterosubtypic immunity against influenza A/H5N1 in ferrets

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ABSTRACT

Infection with seasonal influenza viruses induces a certain extent of protective immunity against potentially pandemic viruses of novel subtypes, also known as heterosubtypic immunity. Here we demonstrate that infection with a recent influenza A/H3N2 strain induces robust protection in ferrets against infection with a highly pathogenic avian influenza virus of the H5N1 subtype. Prior H3N2 virus infection reduced H5N1 virus replication in the upper respiratory tract, clinical signs, mortality, and histopathological changes associated with virus replication in the brains. This protective immunity correlated with the induction of T cells that cross-reacted with H5N1 viral antigen. We also demonstrated that prior vaccination against influenza A/H3N2 reduces the induction of heterosubtypic immunity otherwise induced by infection with the influenza A/H3N2 virus. The implications of these findings are discussed in the context of adopting vaccination strategies and vaccine development aiming at the induction of immunity to pandemic influenza.

INTRODUCTION

Since highly pathogenic influenza A viruses of the H5N1 subtype continue to circulate among domestic bird populations and cause fatal infections of humans occasionally, a pandemic outbreak with these viruses is still feared (21). To become pandemic, these viruses need to adapt to their new mammalian host and become transmissible from human to human. Apparently the influenza A/H1N1(2009) viruses that originated in swine, possessed these properties and were responsible for the first influenza pandemic in the 21st century.

Probably the most effective measure to reduce the impact of influenza pandemics is the development and use of vaccines. Several candidate vaccines have been developed against influenza A/H5N1 viruses, which in some cases contain adjuvants to make these vaccines more efficacious and to achieve dose-sparing (147-150). Also against the influenza A/H1N1(2009) virus vaccines were developed. Although these vaccines were efficacious (151, 152), they arrived late and after the peak of the pandemic (153), which precluded profiting optimally from the protection these vaccines would have afforded. Therefore, there is interest in the development of more universal vaccines (121, 154), that could induce protective immunity to influenza A viruses of various subtypes, including influenza A/H5N1. Full understanding of the heterosubtypic immunity that is induced by infection with influenza A viruses may help developing such vaccines. Indeed, prior infection with an influenza A virus can reduce morbidity and mortality caused by infection with an influenza A virus of another subtype, including influenza A/H5N1 as was demonstrated in various animal models (40, 78, 80, 136, 155). Thus, individuals previously infected with seasonal influenza viruses may be less susceptible to developing severe disease after infection with (pandemic) viruses of a novel subtype than immunogenically naïve subjects (54). This might, at least in part, also explain the disproportionate age distribution of severe cases of infection with influenza A/H5N1 (57) and A/H1N1(2009) viruses (55) with higher incidences in subjects of young age which had not yet developed robust heterosubtypic immunity after one or more infections with seasonal influenza viruses. Elucidating the basis of heterosubtypic immunity has been the topic of numerous studies (for review see (40)).

It is generally accepted that arms of the immune system other than serum-antibodies to hemagglutinin and neuraminidase contribute to heterosubtypic immunity, like CD4+ and CD8+ T cells specific for conserved viral proteins, mucosal antibodies and B cells (40, 43, 47, 123, 136).

So far, infection-induced heterosubtypic immunity to highly pathogenic influenza A/H5N1 viruses has been studied predominantly in mice (76, 80, 136). Since the pathogenesis of influenza virus infections of mice differs from that of humans and results obtained in mice are considered to be of limited predictive value for humans (156), we wished to study the induction of heterosubtypic immunity to H5N1 influenza viruses with seasonal influenza virus in ferrets, which resemble the human situation more closely. In addition, we wished to investigate if vaccination of ferrets against seasonal influenza A/H3N2 virus would interfere with the induction of heterosubtypic immunity to influenza A/H5N1 viruses otherwise induced by infection with influenza A/H3N2 virus as was demonstrated recently in mice (135, 157).

To this end, ferrets were vaccinated with a subunit vaccine based on a recent A/H3N2 influenza virus vaccine strain or mock-vaccinated and subsequently inoculated with a matching influenza

A/H3N2 virus strain or not. Four weeks after inoculation with the influenza A/H3N2 strain, all ferrets were infected with the highly pathogenic H5N1 strain A/Indonesia/5/2005 to assess the presence of heterosubtypic immunity and the effect H3N2 vaccination had on the development of this immunity. It was shown that vaccination against A/H3N2 virus infection affected the induction of heterosubtypic immunity to H5N1 influenza virus. This may have implications for the universal recommendation to vaccinate all healthy children in some countries and highlights the need for the development of broadly protective vaccines.

MATERIALS AND METHODS

Influenza A viruses

Influenza virus A/Indonesia/5/2005 (H5N1, clade 2.1, A/Ind/5/05) was propagated in confluent MDCK cells. After cytopathic changes were complete, culture supernatants were harvested and cleared by low speed centrifugation and stored at −80°C. Influenza virus A/Brisbane/010/2007 (H3N2) was propagated in the allantoic cavity of 11-days old embryonated chicken eggs. Allantoic fluid was harvested two days post inoculation, cleared by low speed centrifugation and stored at -80°C. Virus titers were determined in MDCK cells as described previously (85). All experiments with the influenza A/H5N1 virus were performed under Bio Safety Level (BSL)-3 conditions.

Vaccine preparation

Preparation and purity testing of egg-grown, influenza A virus subunit antigen (SU) derived from influenza A/Uruquay/716/2007 (NYMC X-175-C, H3N2) was performed essentially as described previously (106, 135). Influenza NYMC X-175-C is the H3N2-vaccine strain for influenza seasons 2008-2009 and 2009-2010 and is closely related to the vaccine reference strain of these seasons, influenza A/Brisbane/010/07 (H3N2). The viral nucleoprotein and matrix protein could not be detected in the vaccine preparation by SDS-PAGE and coomassie brilliant blue staining, while small traces were detected using Western blot analysis and on a silver stained SDS-PAGE gel.

Ferrets

Healthy young adult outbred female ferrets (Mustela putorius furo; between 6 and 12 months of age) were purchased from a commercial breeder. Ferrets were tested for the presence of serum antibodies against recent influenza A/H1N1 and A/H3N2 viruses and for the presence of antibodies against influenza virus A/Indonesia/5/2005 (H5N1) by hemagglutination inhibition assay. Ferrets were also tested for the presence of antibodies against Aleutian disease virus and when the absence of these antibodies was confirmed, ferrets were randomly assigned to one of four experimental groups (see below). During the experiment, ferrets were housed in groups and received food and water ad-libitum. An independent animal ethics committee (DEC consult) approved the experimental protocol before the start of the experiments.

Immunizations and infection of ferrets

Fourty ferrets were divided over four groups of 10 animals each. Ferrets of groups 2 and 3 were im-

munized twice with 15 µg influenza subunit vaccine in combination with Titermax Gold adjuvant (Sigma-Aldrich, Saint Louis, USA) with an interval of four weeks, while ferrets of groups 1 and 4 were mock-vaccinated with phosphate buffered saline (PBS). The experimental groups are listed in table 1. All animals were tested simultaneously in a single experiment. Vaccinations were performed in the quadriceps muscles of the left hind leg in a total volume of 0.25 ml under anaesthesia with ketamine. Four weeks after the second immunization, ferrets of groups one and two were inoculated intranasally with $1x10^6$ $TCID_{50}$ influenza virus A/Brisbane/010/2007 (H3N2) in a total volume of 0.5 ml PBS, while ferrets of groups 3 and 4 were inoculated with PBS. Four weeks after inoculation with the influenza A/H3N2 virus, two ferrets of each group were euthanized and lungs, nasal cavity and trachea were taken out for histopathological evaluation, while the eight remaining ferrets of each group were inoculated intranasally with $5x10^6$ $TCID_{50}$ influenza virus A/Ind/5/05. All inoculations were performed under anaesthesia with ketamine/medetomidine (reversed with atipamezole). During infection experiments, ferrets were checked daily for the presence of clinical signs. Before and two, four and seven days post inoculation (dpi) with the influenza A/H3N2 virus and before and two, four, six and seven dpi with the H5N1 influenza virus, ferrets were weighed and nasal and pharyngeal swabs were collected while anesthesized with ketamine. Seven days after inoculation with influenza A/H5N1 virus or earlier when ferrets became moribund, animals were weighed and subsequently killed by exsanguination while under anesthesia with ketamine and medetomidine. Necropsies were performed according to standard procedures and samples of the olfactory bulb, the cerebrum, lungs (all lobes of the right lung and the accessory lobe), spleen and duodenum were collected for determination of virus titers and evaluation of histopathological changes.

Table 1. Experimental groups

Group	Vaccination	Primary infection	Challenge infection
1	mock	A/H3N2 virus[2]	A/H5N1 virus[3]
2	H3N2 vaccine[1]	A/H3N2 virus	A/H5N1 virus
3	H3N2 vaccine	mock	A/H5N1 virus
4	mock	mock	A/H5N1 virus

1 subunit vaccine derived from influenza A/Uruquay/716/2007 (NYMC X-175-C, H3N2) adjuvanted with Titermax Gold adjuvant

2 influenza virus A/Brisbane/010/07 (H3N2)

3 influenza virus A/Indonesia/5/05 (H5N1

Serology

Serum samples were collected before, twenty-eight days after the first and second vaccination and twenty-eight days after infection with influenza virus A/Brisbane/010/07 (H3N2). Sera were stored at –20°C until use. Sera were tested for the presence of antibodies against influenza virus X-175-C (H3N2), A/Brisbane/010/07 (H3N2) and A/Indonesia/5/05 (H5N1) using the hemagglutination inhibition assay (HI-assay) performed with four hemagglutinating units of virus and 1% turkey erythrocytes and a micro virus neutralization assay (VN-assay) performed with 100 $TCID_{50}$ of the respective virus as described previously (107, 158).

Preparation of viral antigens for T cell proliferation assay

Influenza A viruses A/Brisbane/010/07 (H3N2) and A/Ind/5/2005 (H5N1) were propagated in MDCK cells and purified and concentrated by isopycnic density centrifugation. Subsequently virus was inactivated by dialysis against PBS containing 0.1% formaldehyde for 4 days under continuous stirring at room temperature. After inactivation, antigen was dialysed against PBS. The purity of the antigens was tested by SDS-PAGE and inactivation was confirmed by failure to passage on MDCK cells. The protein concentration was determined using a BCA Protein Assay kit (Pierce).

T cell proliferation assay

Blood samples were collected from the jugular vein of ferrets 28 days after the second vaccination and 28 days after infection with influenza A/H3N2 virus and isolation of peripheral blood mononuclear cells (PBMC) and the T cell proliferation assay was performed as described previously (159). In brief, cryopreserved PBMC were thawed, washed and labelled with 0.3µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5 minutes in PBS (Invitrogen, Breda, the Netherlands). After washing the cells were resuspended in RPMI 1640 (Cambrex, East Rutherford, USA) containing 10% (v/v) fetal calf serum, penicillin (100 µg/ml), streptomycin (100 U/ml) and L-glutamine (2mM) and seeded (5x 105 cells per well) in a 96-well round-bottom plate in the presence or absence of 50 ng of inactivated influenza A/Brisbane/010/07 or A/Indonesia/5/2005 antigen and incubated at 37°C/5% CO_2. After two days supernatant of Concanavalin A-stimulated ferret lymph node cells was added (160). For each condition duplicate samples were tested. After four more days, cells were incubated with a monoclonal antibody directed to human CD8 (OKT-8)-eFluor 450 (eBioscience, San Diego, USA) and LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, the Netherlands). Cells were subsequently fixed and permeabilized with Cytofix and Cytoperm (BD Pharmingen, Alphen a/d Rijn, the Netherlands) and incubated with a Alexa Fluor 647-labelled monoclonal antibody specific for human CD3 (PC3/188A) (Santa Cruz Biotechnology, Santa Cruz, USA). Fluorescence of cells was assessed by flow cytometry using a FACSCanto-II and analysed with FACS Diva software (BD). The specific proliferation of CD3+CD8- cells for each antigen was calculated by subtracting the mean number of CD3+CD8-CFSElow cells of the medium controls from the mean number of CD3+CD8-CFSElow cells stimulated with antigen derived from A/Brisbane/010/07 (H3N2) or A/Ind/5/05 (H5N1). The same calculation was performed for CD3+CD8+ cells.

Virus titers in tissue and nasal and pharyngeal swabs

Tissue samples of ferrets were collected and snap frozen on dry ice with ethanol and stored at –70°C until further processing. Tissue samples were weighed and subsequently homogenized with a FastPrep-24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, The Netherlands) and centrifuged briefly.

After collection of nasal and pharyngeal swabs, swabs were stored at -70°C in the same medium as used to homogenize lung samples. Quadruplicate 10-fold serial dilutions of swab and tissue samples were used to infect MDCK cells as described previously (85). HA activity of the culture super-

natants collected 5 days post infection was used as indicator of infection. The titers were calculated according to the Spearman-Karber method and expressed as $^{10}\log TCID_{50}$ per gram for tissue or per milliliter (ml) for swabs (108).

Histopathology and immunohistochemistry

Tissues from ferrets euthanized 28 days after inoculation with influenza A/Brisbane/010/07 (H3N2) and 7 days after inoculation with influenza A/Ind/5/05 (H5N1) were examined macroscopically for the presence of lesions and lungs (after inflation with 10% neutral buffered formalin), liver, brain, spleen and duodenum were fixed with 10% neutral buffered formalin. After fixation and embedding in paraffin, tissues were sectioned at 4 µm and tissue sections were examined by staining with hematoxylin and eosin (HE). Using an immunoperoxidase method, serial lung tissue sections were also stained with a monoclonal antibody directed against the nucleoprotein of the influenza A virus for the detection of virus-infected cells in the respective tissues (110).

Statistical analysis

The presence of statistical significant differences in virus titers in nasal and pharyngeal swabs between groups after inoculation with influenza A/Brisbane/010/07 (H3N2) was assessed using the Mann-Whitney U test, while differences in the number of CD3+CD8-CFSElow cells and differences in virus titers in nasal and pharyngeal swabs and weight loss after challenge with influenza A/H5N1 virus were calculated using the Games-Howell test. Differences were considered significant when P values were less than an α of 0.05.

RESULTS

Antibody responses after vaccination

Twenty-eight days after the first vaccination with Titermax-adjuvanted X-175-C subunit preparation, 11 out of 20 ferrets of groups 2 and 3 had developed HA-specific antibodies against influenza A/Brisbane/010/07 (H3N2) as measured with the hemagglutination inhibition (HI) assay (geometric mean titer, GMT, 28), while no antibodies were detected in mock-vaccinated ferrets of groups 1 and 4 (Figure 1A). Twenty-eight days after the second vaccination, virus-specific HI antibodies were detected in all ferrets of groups 2 and 3 (GMT 192). In the virus-neutralization assay, antibodies against influenza A/Brisbane/010/07 (H3N2) were detected in 12 out of 20 ferrets of groups 2 and 3 (GMT 15) 28 days after the first vaccination and in 16 out of 20 ferrets of groups 2 and 3 28 days after the second vaccination (GMT 52) (data not shown). Of all ferrets tested, only a small number of ferrets showed a discrepancy in the presence of antibodies as measured in the VN and HI assay (VN -, HI +), which most likely is caused by the low antibody levels in these ferrets. Four weeks after inoculation with influenza A/Brisbane/010/07, antibodies against influenza A/Brisbane/010/07 (H3N2) virus were only detected in all vaccinated and/or infected animals. In addition, no antibodies against influenza A/Ind/5/05 were detected in any of the sera of the animals with the HI and VN assay (data not shown).

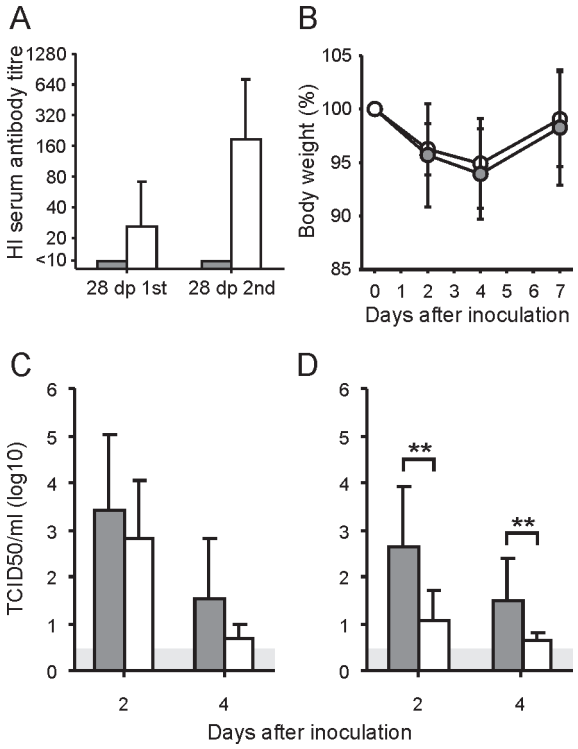


Figure 1. *Antibody responses after vaccination and outcome of infection with influenza A/H3N2 virus. Mean virus-specific antibody responses ± S.D. as measured with the hemagglutination inhibition assay 28 days after the first and 28 days after the second vaccination for unvaccinated animals (shaded bars) and vaccinated animals (open bars) (A). Mean weight loss of vaccinated (open circles) and unvaccinated animals (shaded circles) after inoculation with influenza A/H3N2 virus (B). Virus titers of nasal (C) and pharyngeal swabs (D) collected 2 and 4 dpi with influenza A/H3N2 virus. Shaded bars represent mean results ± S.D. for unvaccinated animals and open bars represent mean results ± S.D. for vaccinated animals. **, P<0.01 compared with unvaccinated animals. The grey area indicates the detection limit of the assay.*

5

Outcome of inoculation with influenza A/H3N2 virus

Following inoculation with influenza virus A/Brisbane/010/07 (H3N2), unvaccinated ferrets of groups 1 developed mild to moderate clinical signs including sneezing, decreased appetite and weight loss (mean percentage of weight loss 6% at day 4). The ferrets of group 2 displayed similar weight loss (mean percentage of weight loss 5% at day 4, Figure 1B), although their clinical signs were less severe than those observed in group 1. The observed differences in clinical signs correlated with differences in virus titers in pharyngeal swabs collected 2 and 4 days after inoculation of ferrets of both groups. Mean virus titers in pharyngeal swabs collected 2 and 4 dpi from unvaccinated ferrets of group 1 were respectively $10^{2.6}$ and $10^{1.5}$ $TCID_{50}$/ml, while significantly lower ($p<0.01$ for both time points) virus titers were detected in pharyngeal swabs collected 2 and 4 dpi from vaccinated ferrets of group 2 ($10^{1.1}$ and $10^{0.6}$ $TCID_{50}$/ml) (Figure 1D). Mean virus titers in the nasal swabs of unvaccinated ferrets of group 1 were on 2 dpi $10^{3.4}$ and on 4 dpi $10^{1.5}$ $TCID_{50}$/ml, while somewhat lower mean virus titers were detected in nasal swabs collected from vaccinated ferrets of group 2 (respectively $10^{2.8}$ and $10^{0.7}$ $TCID_{50}$/ml on 2 and 4 dpi) , although these differences were not statistically significant (respectively p=0.32 and p=0.14 on 2 and 4 dpi) (Fig 1C). No virus was detected in nasal and pharyngeal swabs collected from H3N2 inoculated ferrets at 7 dpi and in nasal and pharyngeal swabs collected from mock-infected ferrets of groups 3 and 4 (data not shown). In addition, at 28 dpi no histopathological differences were observed in the brains, nasal and lung tissue of ferrets of each group (data not shown).

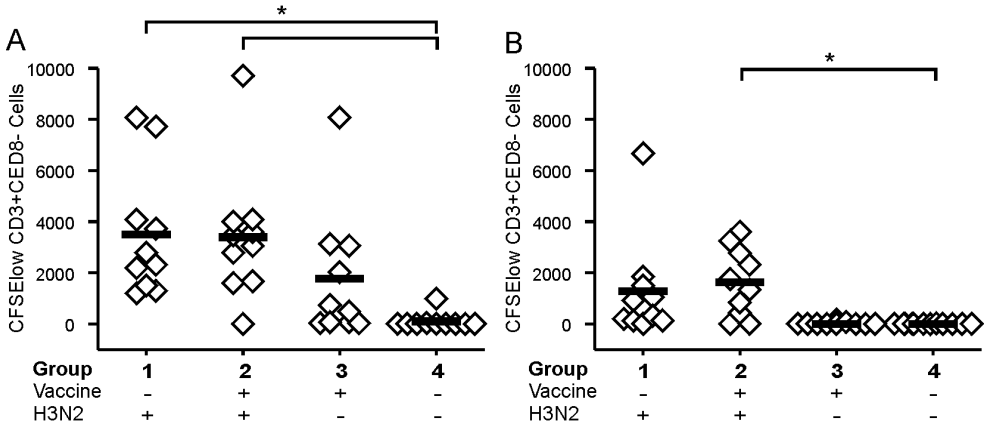
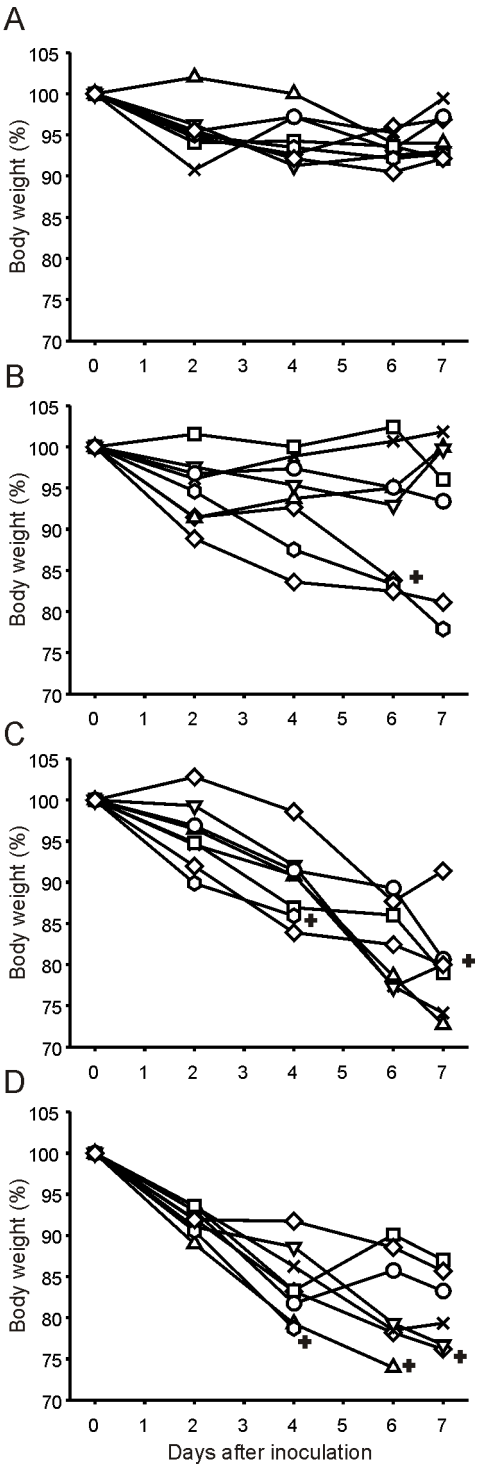


Figure 2. *T cell immune responses 28 days after infection with influenza A/H3N2 virus. Proliferation of CD3+CD8- cells upon stimulation with whole inactivated influenza A/Brisbane/010/07 (H3N2) antigen (A) and upon stimulation with whole inactivated influenza A/Indonesia/5/2005 (H5N1) antigen (B). *, P<0.05 compared with unvaccinated mock-infected animals.*

Virus-specific T cell responses after inoculation with influenza A/H3N2 virus

T cell responses against both influenza A/Brisbane/010/07 (H3N2) and A/Indonesia/5/05 (H5N1) antigens were assessed using PBMC collected from ferrets of all groups 28 days after inoculation with influenza A/H3N2 virus or PBS. Influenza A/H3N2 antigen-specific proliferation of CD3+CD8- cells was observed in only 1 out of 10 mock-vaccinated and mock-infected ferrets of group 4, while influenza A/H3N2 antigen-specific proliferation was observed in all ferrets of groups 1 and 3 and in 9 out of 10 ferrets of group 2 (although only low numbers of CFSElow cells were observed in 4 ferrets of group 3). In Figure 2A, the number of proliferating cells of each individual ferrets and the mean number of proliferating cells of each group are indicated. Differences in influenza A/H3N2 antigen-specific proliferation of CD3+CD8- cells were statistically significant between ferrets of groups 1 and 2 compared to ferrets of group 4 (p-values of respectively 0.01 and 0.02), while differences between groups 3 and 4 approached statistical significance (p=0.05). No significant differences were observed between other groups. Furthermore, similar antigen-specific proliferation of CD3+CD8- cells was observed at 28 days and 56 days after the second vaccination for mock-infected, vaccinated ferrets of group 3 (data not shown). In addition, influenza A/H5N1 antigen-specific proliferation of CD3+CD8- cells was observed in 9 out of 10 ferrets of groups 1 and 2, while H5N1 antigen-specific proliferation was virtually absent in ferrets of group 3 and group 4. The number of proliferating cells of each individual ferrets and the mean number of proliferating cells of each group are indicated in Figure 2B. However, only differences between groups 2 and 4 were statistically significant (p=0.02).

Only very small numbers of viral antigen-specific proliferation of CD3+CD8+ cells was observed in ferrets of all groups, and no significant differences were observed between groups (data not shown).



Outcome of inoculation with H5N1 virus

Twenty-eight days after inoculation with the A/H3N2 virus, ferrets were inoculated with influenza virus A/Ind/5/05 (H5N1). Clinical signs were observed in ferrets from day 1 after inoculation onwards and included anorexia, weight loss, labored breathing, neurological disorders and diarrhea. In figure 3, observed clinical signs are listed per group. Severe clinical signs including weight loss were observed in all mock-vaccinated and mock-infected ferrets of group 4 and the H3N2-vaccinated and mock-infected ferrets of group 3. In contrast, only mild to moderate clinical signs were observed in the ferrets of group 1, that had experienced an H3N2 infection. These animals hardly lost body weight upon infection with influenza virus A/Ind/5/05 and developed less severe clinical signs. Prior vaccination against H3N2 infection partially prevented the protective effect of H3N2 infection and three out of eight animals of group 2 developed severe clinical signs and displayed >15% loss of body weight. In total 6 ferrets of groups 2, 3 and 4 died or had to be euthanized according to animal welfare regulations before all ferrets were euthanized at 7 dpi. No significant differences in weight loss were observed at 2 dpi between groups, while at 4 dpi significant more weight loss was observed in ferrets of group 4 compared to groups 1 and 2 (p-values of respec-

Figure 3. *Weight loss after inoculation with influenza A/Indonesia/5/05 virus. After inoculation with influenza A/H5N1, ferrets were weighed and the relative weight loss compared to the body weight at the day of inoculation was calculated. Indicated is the relative weight loss of all individual ferrets for unvaccinated, H3N2-primed animals of group 1 (A), vaccinated, H3N2-primed animals of group 2 (B), vaccinated, unprimed animals of group 3 (C), and unvaccinated and unprimed animals of group 4 (D). +: indicates ferrets that died or had to be euthanized due to the presence of severe clinical symptoms.*

tively p=0.001 and p=0.01). In addition, at days 6 and 7, significantly more weight loss was observed in ferrets of group 4 compared to group 1 (p-values of respectively 0.009 and 0.006). Differences in body weight loss on these days p.i. between groups 2 and 4 were not statistically significant (p-values of respectively 0.07 and 0.08). The weight loss of all ferrets.

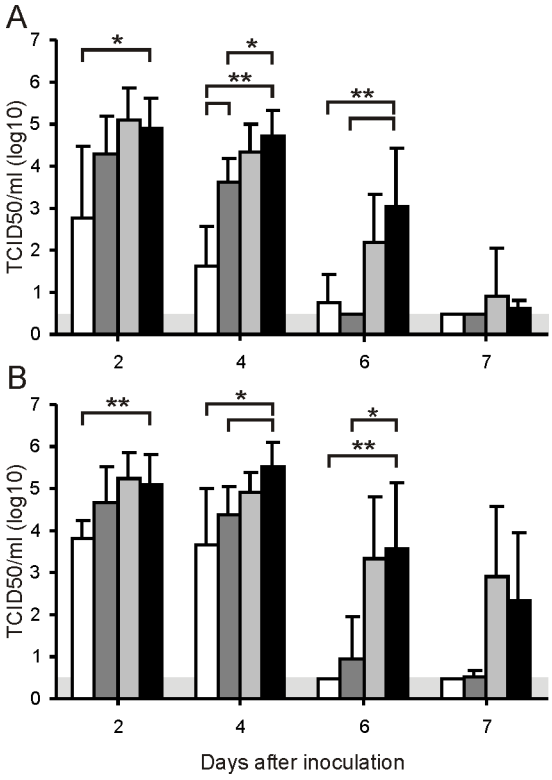


Figure 4. *Virus titers in nasal and pharyngeal swabs collected after inoculation with influenza A/H5N1 virus. Nasal (A) and pharyngeal swabs (B) were collected 2, 4, 6 and 7 dpi with influenza A/H5N1 virus and virus titers were determined. Indicated are mean virus titers ± S.D. for unvaccinated, H3N2-infected ferrets of group 1 (white bars), vaccinated, H3N2-infected ferrets of group 2 (dark shaded bars), vaccinated, mock-infected ferrets of group 3 (light shaded bars) and unvaccinated, mock-infected bars of group 4 (black bars). * indicates a difference of P<0.05 between groups and ** indicates a difference of P<0.01 between groups. The grey bar indicates the detection limit of the assay.*

Virus replication in the upper respiratory tract after inoculation with influenza A/H5N1 virus

Nasal and pharyngeal swabs collected at 2, 4, 6 and 7 dpi with influenza virus A/Ind/5/05 were tested for the presence of infectious virus. Except for two ferrets of group 1, virus was detected in all nasal swabs collected 2 and 4 dpi, with mean virus titers of $10^{2.7}$,$10^{4.3}$, $10^{5.1}$, $10^{4.5}$ $TCID_{50}$/ml at 2 dpi and $10^{1.6}$,$10^{3.6}$,$10^{4.3}$, $10^{4.7}$ $TCID_{50}$/ml at 4 dpi for groups 1, 2, 3 and 4 respectively. The average virus titers in nasal swabs of mock-vaccinated H3N2-infected ferrets of group 1 were significantly lower (p=0.04) than those of mock-infected ferrets of group 4 at 2 dpi. At 4 dpi differences between virus titers of group 1 were significantly lower than those of groups 2, 3 and 4 (p-values below 0.01 for all comparisons). At 6 dpi, virus was undetectable in nasal swabs collected from ferrets of group 2 and in 7 out of 8 of group 1. In contrast, virus was detected in all except one of the nasal swabs of ferrets of groups 3 and 4, which thus had significantly higher titers than those of groups 1 and 2 (both p<0.01). No significant differences in virus titers were observed in nasal swabs collected at 7 dpi. In figure 4A, the mean virus titers in nasal swabs collected from ferrets of each groups are indicated. Similar results were obtained for virus titers in the pharyngeal swabs. Virus was detected

in all pharyngeal swabs collected from H5N1-infected ferrets, with mean virus titers of $10^{3.8}$,$10^{4.7}$, $10^{5.2}$, $10^{5.1}$ $TCID_{50}$/ml at 2 dpi and $10^{3.7}$,$10^{4.4}$,$10^{4.9}$, $10^{5.5}$ $TCID_{50}$/ml at 4 dpi from ferrets of groups 1, 2, 3 and 4 respectively. Differences between virus titers in pharyngeal swabs collected at 2 dpi from ferrets of group 1 and 4 were statistically significant ($p<0.01$), while also differences between ferrets of groups 1 and 4 ($p=0.01$) and 2 and 4 ($p=0.02$) were statistically significant. At 6 and 7 dpi, no virus was detected in ferrets of group 1 and in two respectively one of the ferrets of group 2, while virus was detected in all ferrets except one respectively two of groups 3 and 4. Thus, significantly lower virus titers were detected in groups 1 ($p=0.004$) and 2 ($p=0.01$) compared to group 4, while differences between these groups were not significant at 7 dpi. In Figure 4B, the mean virus titers in pharyngeal swabs collected from ferrets of each groups are indicated.

Table 2. A/Indonesia/5/05 (H5N1) virus detection in tissues by virus isolation and IHC 7 dpi

Group	Treatment		Brain		Lung		Duodenum	
	H3N2 vaccination	H3N2 infection	Virus isolation	IHC	Virus isolation	IHC	Virus isolation	IHC
1	-	+	0/8*	0/8	0/8	0/8	0/8	0/8
2	+	+	0/7	2/7	0/7	1/7	0/7	0/7
3	+	-	3/7	7/7	3/7	4/7	2/7	0/7
4	-	-	0/6	5/6	1/6	2/6	0/6	0/6

*: numbers of animals tested positive/total number of ferrets tested. The number of tested tissues is lower than 8 in some group due to mortality before day 7.

Pathology and immunohistochemistry after A/H5N1 infection

Macroscopically, only focally extensive pulmonary lesions were observed in a number of lungs of ferrets of groups 2, 3 and 4 while no lesions were observed in lungs of ferrets of group 1. Brains, lungs, spleens, liver and duodenum were tested for the presence of histopathological changes caused by infection with influenza A/H5N1 virus A/Ind/5/05. In brains of three out of eight ferrets of group 2, and all ferrets of group 3 and 4, moderate to severe lesions were observed in the brain (Figure 5A), which co-localized with the abundant expression of influenza virus antigen (Figure 5C). In contrast, in the brains of ferrets of group 1 and five ferrets of group 2 no or only a mild lesions were observed (Fig 5B) and influenza virus expression was absent or scarce (Figure 5D). In lungs of some ferrets of groups 2, 3 and 4, a mild to moderate broncho-interstitial pneumonia was observed (Figure 5E), which co-localized with expression of viral antigen (Figure 5G). These lesions were not observed in the lungs of ferrets of group 1 (Figure 5F), and no influenza virus antigen expression was detected (Figure 5H).

In livers of a number of ferrets of groups 2 and all ferrets of groups 3 and 4, there was a diffuse vacuolization of the hepatocellular cytoplasm, consistent with fat, which was not observed in livers of ferrets of group 1 and a number of ferrets of group 2 with heterosubtypic immunity against influenza A/H5N1 virus. These changes were considered to be hepatic lipidosis resulting from anorexia. No lesions were observed in spleens and duodenums of ferrets of all groups.

Presence of virus in tissues 7 days after inoculation

From all ferrets on which necropsy was performed at 7 dpi, olfactory bulbs, brains, lungs, spleens and the duodenum were tested for the presence of infectious virus by virus isolation in MDCK cells. Virus was undetectable in spleens of all ferrets and in all tissues of ferrets of group 1. For all other groups, infectious virus was detected in tissues of one or more ferrets. In general, low virus titers were detected. In addition to the virus titrations, the presence of virus-infected cells in organs of ferrets collected at 7 dpi was tested by immunohistochemistry. For the lungs, spleens and duodenum, the results obtained with immunohistochemistry correlated with virus isolation on MDCK cells. However, by immunohistochemistry, virus was detected in brains of almost all ferrets of groups 2, 3 and 4, while virus was not detected in brains of animals of group 1 (Table 2).

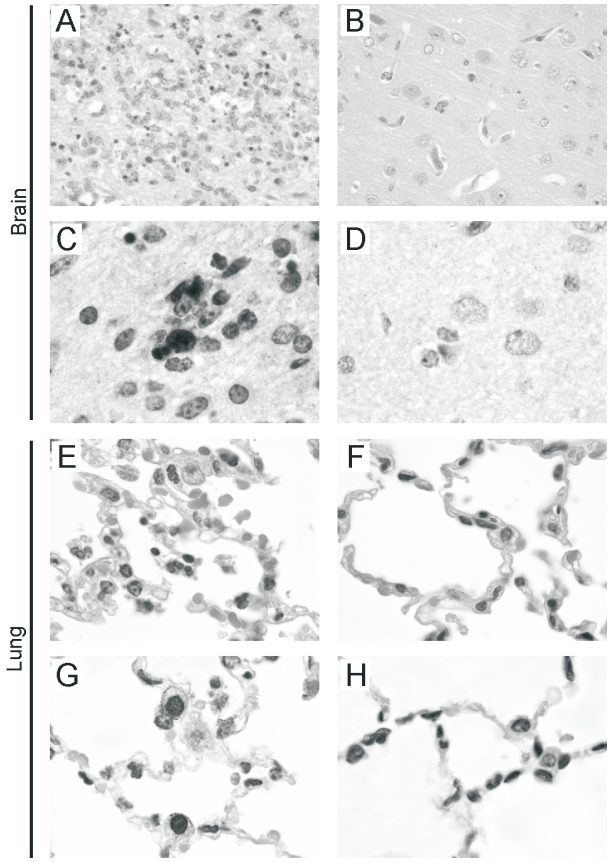


Figure 5. *Histopathological analysis of tissues of ferrets inoculated with influenza A/H5N1. Seven days after inoculation, histopathologic changes associated with the expression of influenza virus antigen are observed in brains and lungs of ferrets without heterosubtypic immunity in three ferrets of group 2, and in all ferrets of group 3 and 4 (A, C, E, G). In the cerebrum, there is necrosis of neurons and infiltration with inflammatory cells (A) associated with expression of influenza virus antigen in neurons (C). In the lungs, cell debris and erythrocytes are present in the alveolar lumina and neutrophils present in the alveolar walls (E). This is associated with the expression of influenza virus antigen in type II pneumocytes (G). Neither histopathological changes (B and F) nor influenza virus expression (D and H) were present in lungs and brains respectively of five ferrets of group 2 and all ferrets of group 1.*

DISCUSSION

In the present study, we have demonstrated that infection with a seasonal influenza A/H3N2 virus induced robust heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. Prior infection with influenza A/Brisbane/010/07 (H3N2) reduced replication of influenza virus A/Ind/5/05 (H5N1) in the upper respiratory tract and the brains and prevented clinical signs and his-

topathological changes associated with their infection. The induction of heterosubtypic immunity by infection with influenza A viruses has been demonstrated already more than 40 years ago in animal models and there is evidence for the presence of heterosubtypic immunity in humans (40, 54). In addition, it has been demonstrated recently that infection with a seasonal influenza virus afforded protection against the pandemic influenza A/H1N1(2009) virus in the ferret model (155, 161). However, the immunological basis of heterosubtypic immunity has not been completely elucidated yet, and mucosal immunity, B cells and both CD4+ and CD8+ T cells may contribute to this type of immunity (40). In concordance with our previous findings in the mouse model, we were unable to detect antibodies cross-reactive with the H5N1 strain after infection with influenza A/H3N2 virus by HI and virus neutralization assay, indicating that serum antibodies did not play a major role in the protection against influenza A/H5N1 virus. In contrast, infection with an influenza A/H3N2 virus induced strong T cell responses that cross-reacted with influenza A/H5N1 viral antigen. To our knowledge, this is the first time that the presence of cross-reactive T cell responses is demonstrated in the ferret model. The cross-reactive in vitro proliferative response of CD3+CD8- cells, most likely CD4+ T helper cells, obtained from A/H3N2 virus infected ferrets correlated to some extent with the presence of heterosubtypic immunity. However, the three ferrets that displayed weight loss after infection with influenza A/H5N1 also had T cell responses against the H5N1 viral antigen indicating that other arms of the immune system also contribute to the development of heterosubtypic immunity. In addition, we were unable to assess CD8+ T cell responses because probably the use of inactivated viral antigens precluded proper antigen processing and presentation to CD8+ T cells. Thus, we cannot exclude or confirm that virus-specific CD8+ T lymphocytes contributed to the heterosubtypic immunity observed after infection with the A/H3N2 influenza virus. Furthermore, the trace amounts of nucleoprotein and matrix protein present in the subunit vaccine of this study could have induced immunodominant CD8+ T cell responses that skewed subsequent CD8+ T cell responses after infection with the influenza A/H3N2 virus, as was observed previously in the mouse model using a whole inactivated influenza A/H3N2 vaccine (157). In contrast to our findings in the mouse model (135), none of the ferrets euthanized 28 dpi after influenza A/H3N2 inoculation developed inducible bronchus associated lymphoid tissue (iBALT) or other histopathological changes. Probably, the infection of ferrets with influenza A/H3N2 virus was restricted to the upper respiratory tract, while infection of mice also involves the lower respiratory tract. In the present study, we also demonstrated that effective vaccination against infection with the seasonal influenza virus A/Brisbane/010/07 (H3N2) hampered the induction of heterosubtypic immunity. The vaccinated ferrets suffered more from the subsequent infection with the highly pathogenic H5N1 influenza virus A/Ind/5/05 than their unvaccinated counterparts. These findings in the ferret model are in concordance with those we have obtained in the mouse model recently (135, 145, 157). Since subunit preparations are poorly immunogenic in mice (135) and ferrets (161), we used an adjuvant (Titermax) to increase the immunogenicity of the A/H3N2 subunit vaccine. Although the use of adjuvanted subunit preparations induced potent antibody responses to the influenza A/H3N2 virus, it failed to induce heterosubtypic immunity and did not afford any protection against infection with the influenza A virus A/Ind/5/05 (H5N1) as expected. Thus, the use of a potent adjuvant did not induce T cell responses against the trace amounts of NP and M1 present in the subunit H3N2 prepa-

ration, strong enough to protect against the influenza A/H5N1 protection. Likewise, adjuvants have been used to increase the immunogenicity of seasonal and pandemic influenza A/H1N1(2009) vaccines (162, 163). Furthermore, the vaccine used in this study contained only influenza A/H3N2 virus antigen, while the currently used seasonal vaccine also contains influenza A/H1N1 and influenza B virus antigen. Especially the addition of the influenza A/H1N1 virus antigen might have changed the results as it has been described in mice that immunization with a seasonal influenza A/H1N1 vaccine induce cross-reactive antibodies against the neuraminidase of the influenza A/H5N1 virus (164). In the present study, the ferrets were inoculated with influenza virus A/Ind/5/05 (H5N1) by the intranasal route. Although unprotected ferrets animals developed severe clinical signs, most of them did not develop severe pneumonia, typically seen after intratracheal inoculation (165) that could account for the loss of body weight. In contrast, these animals developed a moderate to severe meningo-encephalitis sometimes with a fatal outcome before the end of the experiment on day seven post inoculation, which was chosen for ethical reasons and to allow a meaningful comparison of the experimental groups on the same timepoint p.i.. The occurrence of meningo-encephalitis also explains the neurological signs that we and others have observed after intranasal inoculation of ferrets with highly pathogenic avian influenza viruses of the H5N1 subtype (166, 167). The occurrence of severe encephalitis in absence of severe pneumonia suggests that the brains of these ferrets were infected directly from the nasal cavity, as has been shown in influenza A/H5N1 infection in mice (168) . The possible occurrence of this phenomenon will be explored further. Prior infection with a seasonal influenza virus of the H3N2 subtype prevented dissemination of the H5N1 virus and the clinical signs associated with virus replication in the brain, severe weight loss and the development of moderate to severe meningo-encephalitis. This protective effect correlated with a reduction of H5N1 virus replication in the upper respiratory tract and was prevented by vaccination against the H3N2 virus infection in three out of eight ferrets (38%). This scenario resembles the observation in Canada, where subjects vaccinated against seasonal influenza in previous seasons more likely developed severe disease caused by infection with pandemic influenza A/H1N1(2009) influenza viruses (169, 170). However, the lack of heterosubtypic immunity in these patients was not confirmed. Annual vaccination against influenza is beneficial, especially for patients with underlying disease and the elderly, which are at high-risk for developing complications caused by influenza virus infection. In some countries, annual vaccination is also recommended for all healthy children >6 months of age. Although annual vaccination can reduce the burden of disease caused by seasonal influenza, on the long term it may affect the development of heterosubtypic immunity in this age group. Our findings emphasize the need for vaccines that can induce more broadly protective immune responses. In this respect the induction of cross-reactive virus-specific CD8+ T cells to conserved viral proteins like the NP and M1 protein holds promise (83, 171). Also the induction of antibody responses to the M2 protein and the conserved stalk region of the HA molecule may be interesting avenues for the development of more universal vaccines (172, 173).

Acknowledgements

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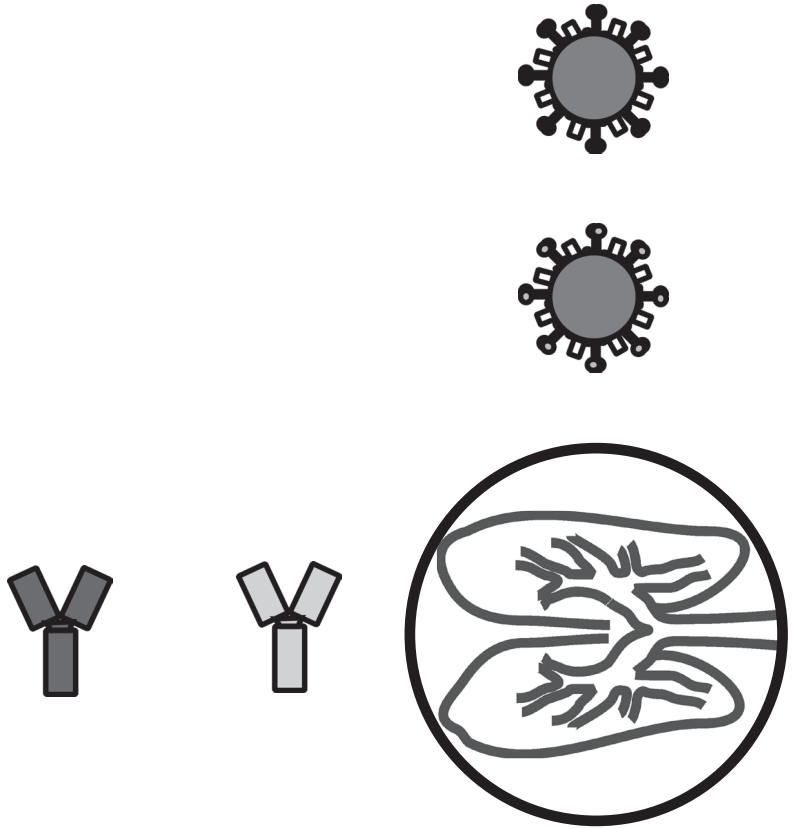

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Prevalence of antibodies against seasonal influenza A and B viruses in children in the Netherlands

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ABSTRACT

To gain insight in the age at which children become infected with influenza viruses for the first time, we analyzed the seroprevalence of antibodies against influenza viruses in children 0-7 years of age in The Netherlands. Serum samples were collected during a cross-sectional population-based study in 2006 and 2007 and were tested for the presence of antibodies against influenza A/H1N1, A/H3N2 and B viruses representative for previous influenza seasons using the hemagglutination inhibition assay. The seroprevalence of antibodies to influenza was higher in children 1-6 months of age than that of children 7-12 months of age, which likely reflects the presence of maternally derived antibodies.

The proportion of study subjects >1 year of age with detectable antibodies against influenza viruses gradually increased with age until they reached the age of six when they all had antibodies to at least one influenza A virus. These findings may have implications for the development of vaccination strategies aiming at the protection of young children against seasonal and/or pandemic influenza virus infection.

INTRODUCTION

Infection with influenza viruses is an important cause of illness in children with estimated annual attack rates in this age group ranging between 20 and 30% during epidemics (174, 175). Especially young children with underlying disease are at risk for severe disease after infection with an influenza virus, but it has been demonstrated that also the hospitalization rates attributable to influenza virus infection of young children without underlying disease are similar to those observed among older adults (59, 176). Furthermore, the importance of influenza as a cause of severe disease was demonstrated during the 2003-2004 influenza season when a newly emerged drift variant caused an unusual high number of severe fatal cases of influenza amongst children (177). In addition, the pandemic caused by the influenza A/H1N1(2009) virus has highlighted the importance of influenza viruses as a cause of morbidity and mortality in infants (178, 179).

Furthermore, since children have a high number of contacts relative to other age groups, and have a tendency to make contacts within their own age group, they may have the highest incidence of infection after the introduction of a newly emerging virus (180). In addition, they also may shed virus for a prolonged period of time and have higher virus loads in the nasopharynx (181, 182). Therefore, children most probably play an important role in the transmission of virus and are considered efficient vectors for spreading the disease.

To prevent morbidity and mortality of children due to infection with influenza viruses, a number of countries, including the USA, have recommended vaccinating all healthy children 6-59 months of age against influenza (60, 61). In various studies, it has been demonstrated that annual vaccination against seasonal influenza is beneficial for children and reduces the transmission of virus (62-66, 183). However, the impact of vaccination will be influenced by the immune status of the vaccinated individuals. Since they will be more at risk to become infected and develop disease, naïve subjects most likely will benefit from vaccination more than children that already have experienced an infection with one or more influenza viruses. In addition, it can be anticipated that with increasing age the chance of having experienced an influenza virus infection also increases. However, at present it is not fully clear at which age children become infected for the first time and develop influenza virus specific immunity and detailed sero-epidemiological studies of this age group are largely lacking (184, 185). Here we report the seroprevalence of antibodies against influenza A/H1N1, A/H3N2 and B viruses in children from one month to seven years of age in The Netherlands. To this end, serum samples were used that were collected during a cross-sectional population-based study designed to represent the population of the Netherlands (186). These serum samples were tested for the presence of antibodies against representative influenza A/H1N1, A/H3N2 and B viruses from multiple influenza seasons using the hemagglutination inhibition (HI) assay, which is the gold standard for the demonstration of antibodies against influenza viruses (187). In addition, we were able to discriminate between antibodies against various antigenically distinct influenza A/H1N1 and influenza A/H3N2 viruses and antibodies to influenza B viruses from B/Victoria/2/87 and B/Yamagata/16/88 lineages. In children >1 year of age, there was a gradual, age-related increase in the seroprevalence of antibodies against all influenza viruses until in all children >6 years of age antibodies against at least one influenza virus were detected. Results obtained in this study give more

insight in the rate of infection of children with influenza viruses during non-pandemic seasons and may aid policy making regarding the implementation of vaccination strategies in this vulnerable age group.

MATERIALS AND METHODS

Collection of serum samples

Serum samples were collected during a nationwide cross-sectional population-based study which was performed in the Netherlands from February 2006 to June 2007 (PIENTER 2 Study) to evaluate the Dutch national immunization program (186). For this purpose, serum samples were collected from in total 6386 individuals (aged 0-79 years, men and women). For our study, 720 serum samples obtained from children 0-7 years of age were used. Fifty-six samples were obtained from children 1-6 months of age and 98 serum samples were obtained from children 7-12 months of age. The number of samples obtained from children that were one, two, three, four, five, six and seven years of age was 57, 80, 93, 91, 72, 75 and 97, respectively.

Selection of representative influenza viruses

Representative influenza A/H3, A/H1 and B viruses were selected that circulated in The Netherlands in seasons 1999/2000 to 2006/2007 based on data collected by the National Influenza Center for the World Health Organization (WHO) in the Netherlands (188-195). For most seasons, vaccine strains were used, but when epidemiological strains could be identified that gave higher antibody titers with reference ferret serum these were included as well (Table 1). Furthermore, influenza B viruses of both B/Victoria/2/87-like and B/Yamagata/16/88-like lineages (Victoria- and Yamagata-lineages) were used for each year, although in some seasons only influenza B viruses belonging to one lineage were detected in clinical specimens in the Netherlands. In addition, data collected by the Dutch National surveillance program was used to assess the severity of the influenza seasons and to evaluate relative dominance of each of the influenza virus types and subtypes.
Before use in the HI assay, vaccine strains were inoculated in the allantoic cavity of 11-days old embryonated chicken eggs while epidemiological strains were propagated in confluent Madin-Darby Canine Kidney (MDCK) cells. Allantoic fluid was harvested after two days and culture supernatant was harvested after cytopathologic changes were complete and both were cleared by low speed centrifugation. Sera of children between one month and 12 months of age were tested for the presence of antibodies against all influenza viruses representative for the six preceding influenza seasons to analyze the presence of maternal antibodies, while serum samples collected from children older than one year were tested for the presence of antibodies against all influenza viruses of seasons that they might have been exposed to according to their age (Table 1).

Serological testing

Serum samples were tested for the presence of antibodies against the hemagglutinin of the respective influenza viruses by HI assay as described previously (107). In brief, serum samples were treated with cholera filtrate and heat inactivated at 56°C for one hour. Duplicate two-fold serial dilutions

of pre-treated serum samples were subsequently incubated with 4 HA units of an influenza virus or phosphate buffered saline (PBS) for 30 minutes at 37°C and subsequently 1% turkey erythrocytes was added. Hemagglutination patterns were read after incubation for one hour at 4°C. The highest dilution of serum that still gave complete inhibition of the hemagglutination was recorded as titer and when duplo results were different, geometric mean titers were calculated.
Serum samples were considered negative when they failed completely to inhibit agglutination of erythrocytes (antibody titer < 10) by any of the selected viruses. Serum samples of ferrets collected before and after infection with each of the respective influenza viruses were used as negative and positive control in the HI assay.

Statistical analysis
Pearson's correlation coefficient was used to calculate correlations between antibody titers detected against multiple variants of influenza A/H3N2, A/H1N1 and B viruses. Furthermore, assuming binominal distribution, the two-sided exact 95% confidence interval (CI) was calculated for seroprevalences of antibodies against influenza A/H3N2, A/H1N1 and B viruses using Stata/SE software version 11.0. Statistical analysis of differences between children one to six months of age and six to 12 months was performed using the chi-square test. The Cochrane-Armitage Trend test was performed to evaluate the presence of a age-related trend in the presence of antibodies against influenza viruses using SAS software version 9.2.

RESULTS

Influenza epidemics from 1999 to 2007 in the Netherlands
Using epidemiological and virological data, we were able to assess the relative severity of the influenza epidemics in the Netherlands from 1999 to 2007 and the causative viruses. During most seasons, influenza viruses caused moderate epidemics, except for the 2004/2005 season that was relatively severe and caused by influenza A/H3N2 viruses predominantly and the 2000/2001 season that was relatively mild and caused by influenza A/H1N1 viruses. Furthermore, most seasons were dominated by influenza A/H3N2 viruses, while during the 2002/2003 and 2005/2006 influenza seasons, both influenza A/H3N2 and B viruses were co-dominant. During most seasons, the majority of isolated influenza B viruses belonged to the Yamagata-lineage. However, during the 2002/2003 influenza seasons, in which influenza B viruses were co-dominant, only viruses from the Victoria-lineage were isolated in The Netherlands. During most epidemics from 1999 to 2007, influenza A/H1N1 viruses caused only low influenza activity, except during the 2000/2001 season (Table 1).

Age-dependent seroprevalence of antibodies against individual influenza virus strains
First, the prevalence of antibodies directed against individual influenza virus strains was assessed using serum samples of children one to seven years of age. Strains were used against which based on their age at the time point of sampling, the study subjects potentially could have developed an antibody response. As shown in figure 1A, an age-dependent increase in the proportion of subjects with antibodies to selected A/H3N2 strains was observed. The highest prevalence of antibodies to

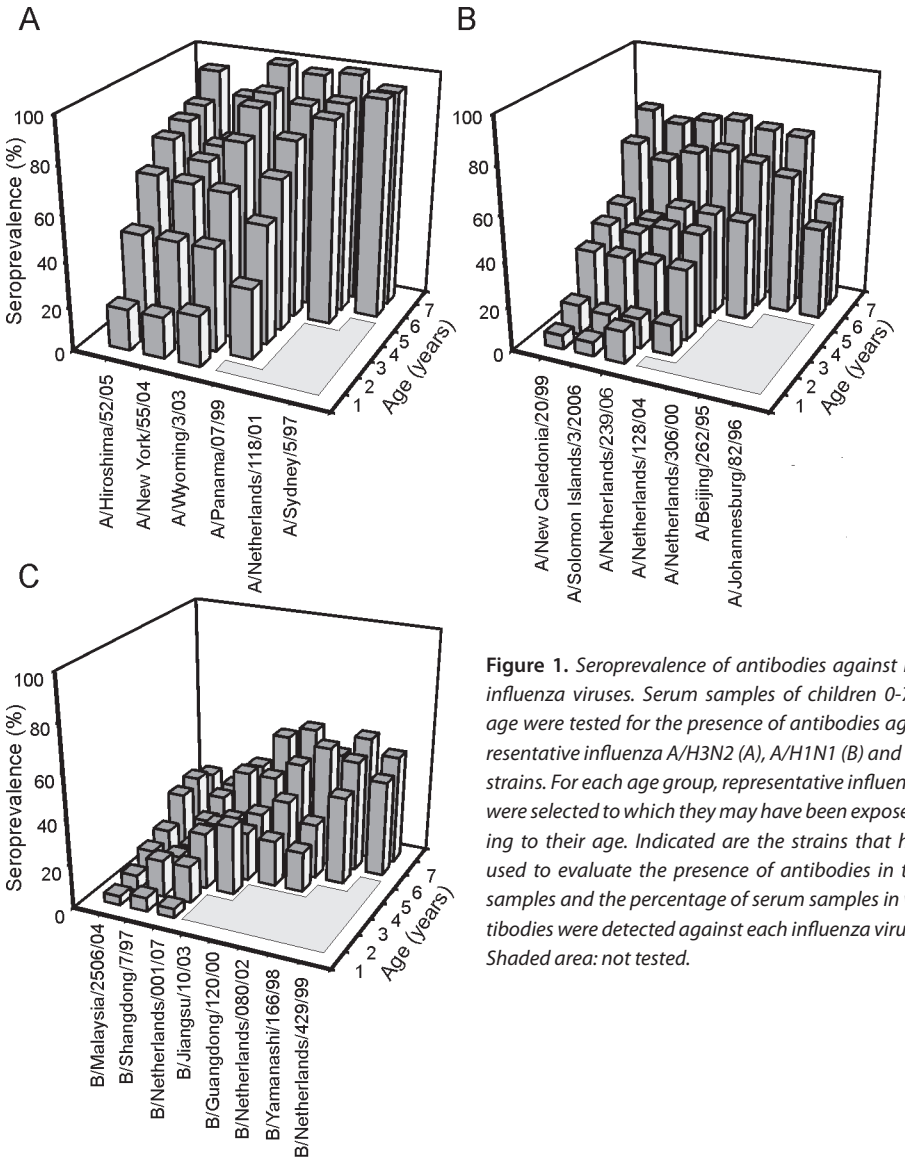

Figure 1. *Seroprevalence of antibodies against individual influenza viruses. Serum samples of children 0-7 years of age were tested for the presence of antibodies against representative influenza A/H3N2 (A), A/H1N1 (B) and B (C) virus strains. For each age group, representative influenza viruses were selected to which they may have been exposed according to their age. Indicated are the strains that have been used to evaluate the presence of antibodies in the serum samples and the percentage of serum samples in which antibodies were detected against each influenza virus antigen. Shaded area: not tested.*

a single strain was observed against influenza viruses A/NL/118/01 and A/Wyoming/3/03 (100%) in subjects of seven years old.

A similar pattern was observed for the prevalence of antibodies to individual influenza A viruses of the H1N1 subtype, although the overall seroprevalence was lower (Figure 1B). The highest seroprevalence to individual strains was observed to influenza viruses A/NL/128/04 (77%) in subjects seven years of age, which was similar to that against most other H1N1 strains.

The seroprevalence of antibodies to individual influenza B virus strains displayed a different pattern and was largely depending on the lineage of the influenza B virus that was used. In general, higher

seroprevalences of antibodies against influenza B viruses of the Yamagata-lineage were detected than those to viruses of the B/Victoria lineage (B/Malaysia/2506/04 and B/Shangdon/7/97) (Figure 1C).

Seroprevalence during first year of life

Serum samples of children one to twelve months of age were not only tested for the presence of antibodies to influenza viruses from the 2006/07 season, but also for those specific for older strains since it was anticipated that these sera also might contain maternally derived antibodies.

In 15% (CI 6-27%) of the children between one and six months of age, antibodies were detected against at least one of the influenza A/H1N1 viruses tested, while only 4% (CI 1-10%) of children between 7 and 12 months of age had antibodies against A/H1N1 viruses (Figure 2A and 2C). In 43% (CI 30-57%) and 36% (CI 23-50%) of the children one to six months of age, antibodies were detected against a least one influenza A/H3N2 or B virus respectively. In the serum samples obtained from children 7-12 months of age the proportion of subjects with antibodies to these viruses was 19% (CI 12-28%) and 5% (CI 2-12%), respectively (Figure 2A). The significant differences in the prevalence of antibodies to A/H3N2 and B viruses between the two age groups could be largely attributed to a difference in the proportion of serum samples containing antibodies to strains from previous influenza seasons like A/Wyoming/3/03, A/Panama/07/99, A/Sydney/5/97 (all A/H3N2) and B/Yamanashi/429/99 (Figures 2B and 2D). This indicates that the relatively high seroprevalence of antibodies in children 1-6 months of age indeed can be attributed to maternally derived antibodies.

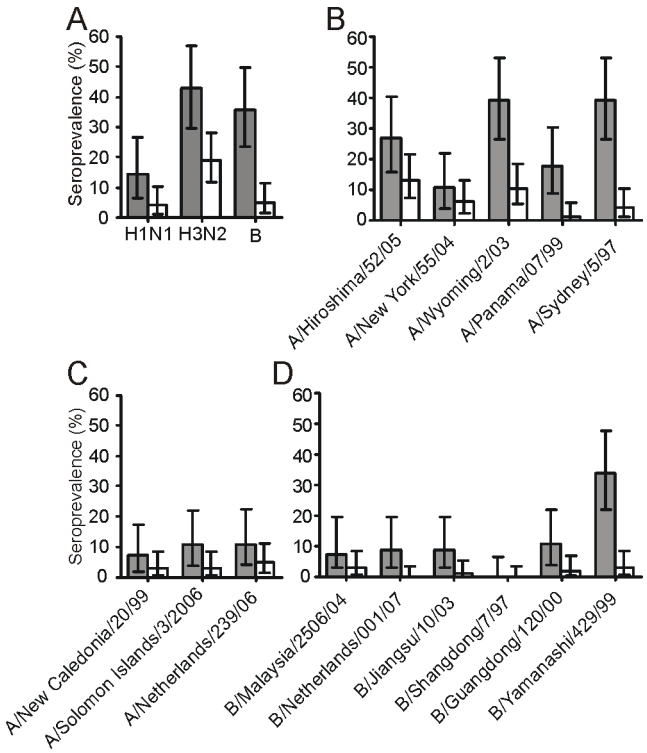

Figure 2. *Seroprevalence of antibodies against influenza viruses in children 1-12 months of age. Seroprevalences of antibodies against influenza A/H3N2, A/H1N1 and B viruses of the 2000-2007 influenza season in children one to six months of age (grey bars) and seven to 12 months of age (white bars) (A). Serum samples were tested for the presence of antibodies against multiple antigens as is indicated for influenza A/H3N2 (B), influenza A/H1N1 (C) and B (D) viruses. Bars indicate the percentage of the serum samples in which antibodies were detected and error bars indicate the 95% confidence intervals.*

Age-dependent seroprevalence of antibodies to any influenza A or B virus

The seroprevalence to individual influenza virus strains was used to calculate the proportion of subjects with antibodies to at least one influenza A or B virus. Within the influenza A viruses the relative contribution of antibodies to influenza A/H3N2 and A/H1N1 viruses was discriminated and within the influenza B viruses, those to the Yamagata and Victoria-lineage.

As shown in Figure 3, the seroprevalence of antibodies to influenza A viruses declined after 6 months of age. Thereafter, with increasing age the proportion of subjects with antibodies to influenza A viruses increased steadily. At the age of six virtually all subjects (99%; CI 93-100) had developed antibodies to an influenza A virus. For subjects >2 years of age, the proportion with antibodies to influenza A/H3N2 viruses was significantly higher than those with antibodies to A/H1N1 viruses.

A similar pattern was observed for the development of antibodies to influenza B viruses. After six months the proportion of subjects with antibodies to an influenza B virus dropped to 5% (CI 2-12%). With increasing age a gradual incline was observed of the proportion of children with antibodies to influenza B virus. At age seven, 72% (CI 61-80%) of the subjects had developed antibodies to at least one influenza B virus. The seroprevalence of antibodies to influenza B viruses of the Yamagata lineage was higher that that of the Victoria lineage. Using the Cochrane-Armitage trend test, the presence of a significant age-related trend in the increase of seroprevalence of antibodies to influenza A/H1N1, A/H3N2 and B viruses was demonstrated ($p<0.01$).

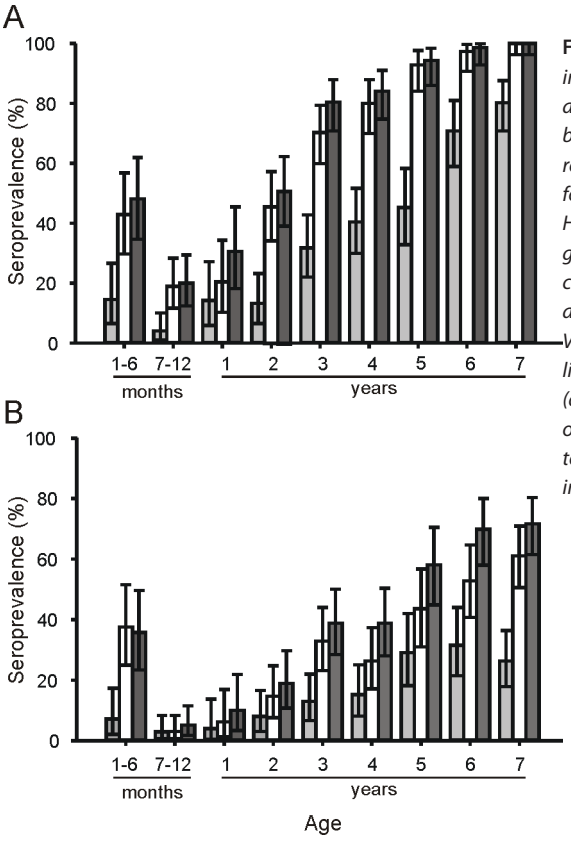

Figure 3. *Seroprevalence of antibodies against influenza A and B viruses depends on age. Percentages of serum samples of children in which antibodies were detected against at least one of the representative influenza viruses were calculated for influenza A/H1N1 (light grey bars), influenza A/H3N2 (white bars) and all influenza A viruses (dark grey bars) (A). The same procedures was used to calculate the seroprevalence of antibodies against at least one of the influenza B viruses from the Victoria-lineage (light grey bars), the Yamagata-lineage (white bars) and all influenza B viruses (dark grey bars (B). Bars indicate the percentage of the serum samples in which antibodies were detected and error bars indicate the 95% confidence intervals.*

Table 1. Influenza virus epidemics in the Netherlands during influenza seasons from 1999-2007

Season	Selected influenza viruses			Severity	Dominance of (sub)type*			Dominance B-lineage	Age at which may be exposed
	A/H3N2	A/H1N1	B		A/H3N2	A/H1N1	B		
06-07	A/Hiroshima/52/05	A/NewCaledonia/20/99 A/Solomon Islands/3/2006	B/Malaysia/2506/04 B/Neth/001/07 B/Jiangsu/10/03	Moderate	D	LA	LA	Yamagata only	0-<8
05-06	A/New York/55/04	A/NewCaledonia/20/99 A/Neth/239/06	B/Malaysia/2506/04 B/Jiangsu/10/03	Moderate	CD	LA	CD	Yamagata+ Victoria	0-<8
04-05	A/Wyoming/3/03	A/NewCaledonia/20/99	B/Malaysia/2506/04 B/Jiangsu/10/03	Relative severe	D	LA	LA	Yamagata only	1-<8
03-04	A/Wyoming/3/03	A/NewCaledonia/20/99 A/Neth/128/04	B/Malaysia/2506/04 B/Jiangsu/10/03	Moderate	D	NI	LA	Yamagata only	2-<8
02-03	A/Panama/07/99	A/NewCaledonia/20/99	B/Shangdong/7/97	Moderate	CD	LA	CD	Victoria only	3-<8
01-02	A/Panama/07/99	A/NewCaledonia/20/99	B/Guangdong/120/00 B/Neth/080/02	Moderate	D	LA	LA	Yamagata+ Victoria	4-<8
00-01	A/Panama/07/99 A/Neth/118/01	A/NewCaledonia/20/99 A/Neth/306/00	B/Yamanashi/166/98 B/Neth/429/99	Mild	LA	D	LA	Yamagata only	5-<8
99-00	rA/Sydney/5/97	A/Beijing/262/95 A/Johannesburg/82/96	B/Yamanashi/166/98 B/Neth/429/99	Moderate	D	LA	LA	Yamagata only	6-<8

**D = dominant, CD= co-dominant, LA = Low activity, NI = no viruses isolated of this subtype*

Estimated attack rates

The differences in seroprevalence of antibodies to the respective influenza viruses at various ages were used to estimate attack rates. The proportion of children with antibodies to influenza A/H3N2 viruses, only increased 1.5% between children 7-12 months of age and one year of age. The highest increase in the seroprevalence of antibodies against influenza A/H3N2 viruses was observed at age two and three. At these ages, the proportion of subjects with antibodies increased with 25% each year. The highest increase in seroprevalence of antibodies to influenza A/H1N1 viruses were observed at age three (18%) and at age six (26%). During the first year of life, only a minority of the subjects acquired antibodies to influenza B viruses (5%). The highest increase in the seroprevalence in antibodies against influenza B viruses was observed at age three (20%) and five (19%). These increases could be attributed largely to the development of antibodies directed to influenza B viruses of the Yamagata lineage. In general, the increase in the seroprevalence of antibodies against viruses from the Victoria-lineage was modest, with the exception of a 14% increase observed in subjects five years of age.

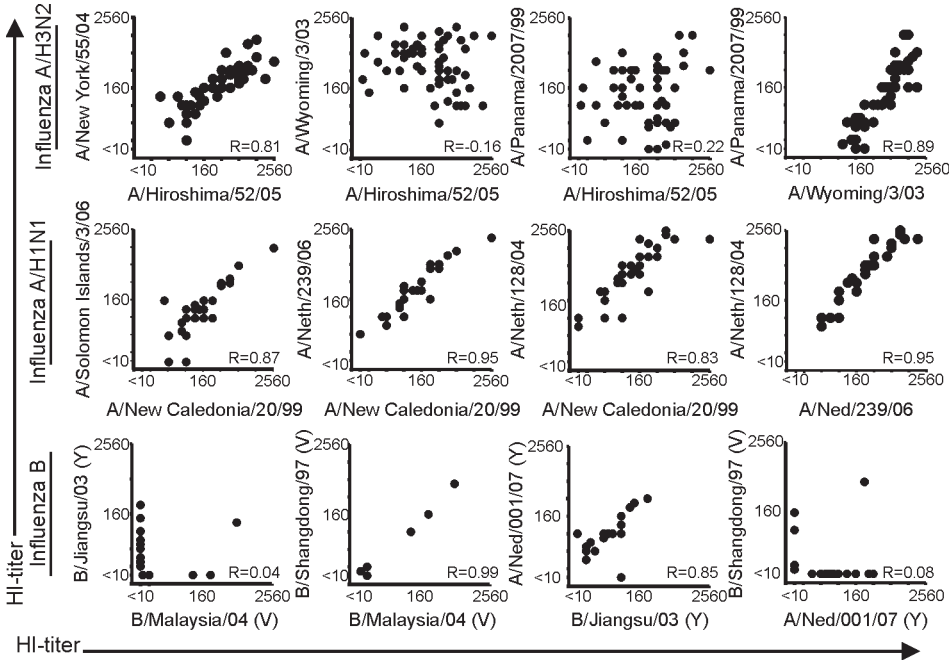

Figure 4. *Correlation of antibody titers against individual influenza A virus strains in four year old children. Correlation between the antibody titers against multiple representative influenza A/H3N2 viruses, influenza A/H1N1 viruses and influenza B viruses. Dots indicate individual serum samples and Pearson correlation coefficient was calculated for all datapoints for which antibodies against at least one influenza virus was detected. For influenza B viruses, the letter behind the name of each strain indicates the lineage to which the virus belongs (V= Victoria-lineage, Y= Yamagata-lineage).*

Correlation between antibody titers against multiple influenza virus strains

As serum samples were tested for antibodies against various influenza viruses, we determined the correlation between antibody titers against different strains within a (sub)type (Figure 4). In

general, antibody titers to various influenza A/H1N1 viruses correlated well (R>0.8), and also those against strains of each of the lineages of influenza B viruses (R>0.8). In contrast, antibody titers against viruses from the two different influenza B lineages correlated poorly (R<0.1), although in some samples antibodies against viruses from both lineages were detected. The correlation of antibody titers against different influenza A/H3N2 viruses was dependent on the year of isolation and most likely on the antigenic match between the two strains that were studied. For example a good correlation was observed between titers against A/New York/55/04 and A/Hiroshima/52/05 and between A/Panama/2007/99 and A/Wyoming/3/03, whereas titers between A/Wyoming/3/03 or A/Panama/2007/99 and A/Hiroshima/52/05 correlated poorly. Figure 4 shows an example of the correlations between antibody titers that were observed with the serum samples obtained from children four years of age.

DISCUSSION

In the present study, the seroprevalence of antibodies against influenza viruses in The Netherlands was investigated in children. Sera were collected from February 2006 to June 2007 in a cross-sectional population-based study and were tested for the presence of antibodies against influenza virus strains representative for viruses that circulated during the life span of the children tested. Since the persistence of maternally derived antibodies is short-lived and probably less than six months (196), sera of children <12 months of age were also tested for antibodies against older influenza viruses that may have infected their mothers. Indeed the seroprevalence of antibodies to influenza viruses was relatively high in children between one and six months of age which could be attributed to the presence of maternally derived antibodies to older influenza virus strains. The seroprevalence was lower in children between six months and one year of age, but showed an age-dependent increase until the age of seven, when all of the children had developed antibodies to at least one influenza A virus and 72% antibodies to at least one influenza B virus. The increase in the seroprevalence was not caused by differences in the geometric mean titer (GMT) against influenza viruses, since GMTs against the respective strains were independent of age. Also high antibody titers were observed in serum samples collected from some children 7-12 months of age, reflecting recent infections with the corresponding viruses. In children of all ages, the seroprevalence of antibodies to influenza A/H3N2 viruses was higher than the seroprevalence of antibodies against influenza A/H1N1 or B viruses. This is in accordance with epidemiological data from the Netherlands collected between 1999 and 2007. During influenza seasons in this period, influenza A/H3N2 viruses were detected predominantly in clinical specimens compared to influenza A/H1N1 and influenza B viruses. In addition, we observed a relative strong increase in the seroprevalence of antibodies against influenza A/H1N1 viruses in children six years of age compared to children of other ages, which could be attributed to the dominant circulation of influenza A viruses of this subtype during the 2000/2001 influenza season. Furthermore, the presence of antibodies to influenza B viruses of the B/Yamagata lineage and the B/Victoria lineage could be discriminated. These two lineages are antigenically distinct and cross-react poorly (197, 198). In addition in young children that most likely had been infected with only one influenza B virus only antibodies were detected

against influenza B viruses of a single lineage. In older children antibodies were detected against influenza B viruses of both lineages, with is in accordance with the possibility that these children have been infected subsequently with both viruses during their life. Overall, the sero-prevalence of antibodies to influenza B viruses of the B/Yamagata-lineage is higher than those specific for viruses of B/Victoria-lineage. This correlates with epidemiological data which indicate that in five out of eight seasons under investigation only viruses from the B/Yamagata-lineage were isolated and in two other seasons viruses of both lineages were co-dominant. Assuming that children that were infected with influenza viruses also developed antibodies against the corresponding virus, we calculated the estimated attack rates based on the sero-conversion rates at the respective ages. Influenza A/H3N2 viruses had the highest attack rates in children that were between two and four years old. However, it can not be excluded that the attack rate of older children was underestimated, since subsequent infection with viruses of the same subtype may have remained undetected due to the presence of antibodies induced by previous infections. The estimated attack rates based on the sero-conversion rates are comparable with the attack rates during inter-pandemic influenza seasons reported by others (174, 175).

Strikingly, in children <2 years of age, the attack rates were relatively low compared to older children. Since the length and severity of the influenza seasons between 2004 and 2006 was not different from most other seasons and antibody titers in seropositive subjects was not age dependent, differences in exposure to influenza viruses may explain the observed differences in attack rates. To account for potential confounding differences in the length and severity of flu seasons experienced between each age group, birth and sample collection dates were used to calculate the duration of flu season time each subject would have experienced. Further, these values were weighted using influenza-like illness data (199) as a measure of epidemic severity during each weekly period. When these values were used to control for differences in circulating flu conditions throughout the lives of the subjects forming each year group, a similar pattern of increases in seroprevalence was still encountered (Figure 5).

In addition, vaccination against seasonal influenza is currently only recommended in The Netherlands for children that are at high risk for developing complications after infection with influenza due to underlying disease, and therefore is considered a minor confounding factor in the present study. Our results regarding the relatively high seroprevalence in infants <7 months of age coincide with those reported for newborns (200, 201) and it is likely that transplacentally acquired maternal antibodies can protect young infants to a certain extent (202, 203). The high seroprevalence in children <7 months of age is explained by the presence of antibodies to older influenza viruses to which their mothers may have been exposed. In addition, since vaccination against influenza is not recommended for (pregnant) mothers in The Netherlands, the proportion of vaccinated mothers is most likely very low. Indeed the titers to these older strains decline rapidly and are not detectable in children 7-12 months of age. The presence and duration of maternal antibodies against influenza has been demonstrated previously (201, 204, 205). It is unlikely that the children <7 months of age had experienced an infection with influenza viruses since the day of birth and day sample collection were in between two influenza seasons for 20 of these children, including 14 with antibodies to various older influenza virus strains. In addition, two children >7 months were sero-negative and

may not have been exposed to influenza viruses for the same reason. The presence of maternal antibodies against various influenza A and B viruses in infants <7 months of age seems in paradox with the high hospitalization rate in this age group (176). However, in a substantial proportion of these infants (30%) antibodies against any influenza virus were not detectable which may constitute the subjects highly susceptible to infection with influenza virus.

As expected, the antibody titers against antigenically related influenza A and B viruses correlated well. In contrast, antibodies to influenza B viruses of the B/Yamagata and B/Victoria lineages did not cross-react. Furthermore, a poor correlation was observed when antibody titers against antigenically distinct A/H3N2 viruses were compared. Apparently, there is heterogeneity in the antibody repertoire of various subjects, which dictates the level of cross reactivity with different influenza viruses. Collectively, in this study we determined the seroprevalence of antibodies against various influenza viruses in children from 0-7 years of age during non-pandemic influenza seasons. We demonstrated that at seven years of age, all children developed antibodies against at least one of the influenza viruses tested. Furthermore, the highest attack rates calculated based on the seroprevalence of antibodies to influenza A viruses was observed in children two and three years of age. These data provide information on the age at which children experience their first infections with influenza viruses and develop immunity to these viruses. This type of information may aid decision making for the implementation of vaccination strategies that aim at achieving optimal protective immunity against seasonal and pandemic influenza. Ideally, in infants vaccines are used that not only induce antibodies to seasonal influenza viruses but also immunity to influenza A viruses of other subtypes (145, 146).

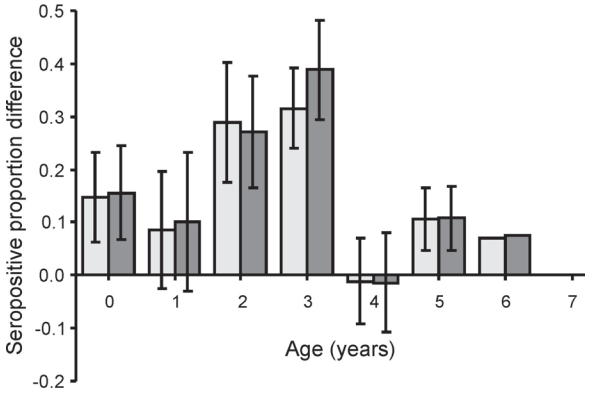

Figure 5. *The difference in proportion of seropositive individuals for each age group compared to the previous age group. Unadjusted (light grey) and adjusted (dark grey) proportions controlled for estimated differences in the severity of flu incidence throughout the life of individuals in each group. For the adjustment, firstly the mean total weighted season time experienced by the individuals of each age group was calculated using information about date of birth, date of sample collection and relevant influenza-like illness data. Next, the differences in this mean for each age group compared to the previous age group were calculated, alongside an overall mean difference between age groups. Finally, the adjustments were made by scaling the value for each age group by the factor by which it differed from the overall mean for the dataset, to account for age groups that had lived through a time of abnormally high or low flu incidence. For age 0, only individuals greater than 220 days old were included to reduce the chance of detecting potential maternal immunity rather than genuine exposure and values for age 0 were plotted assuming a previous seroprevalence of 0%. Error bars indicate the 95% confidence intervals.*

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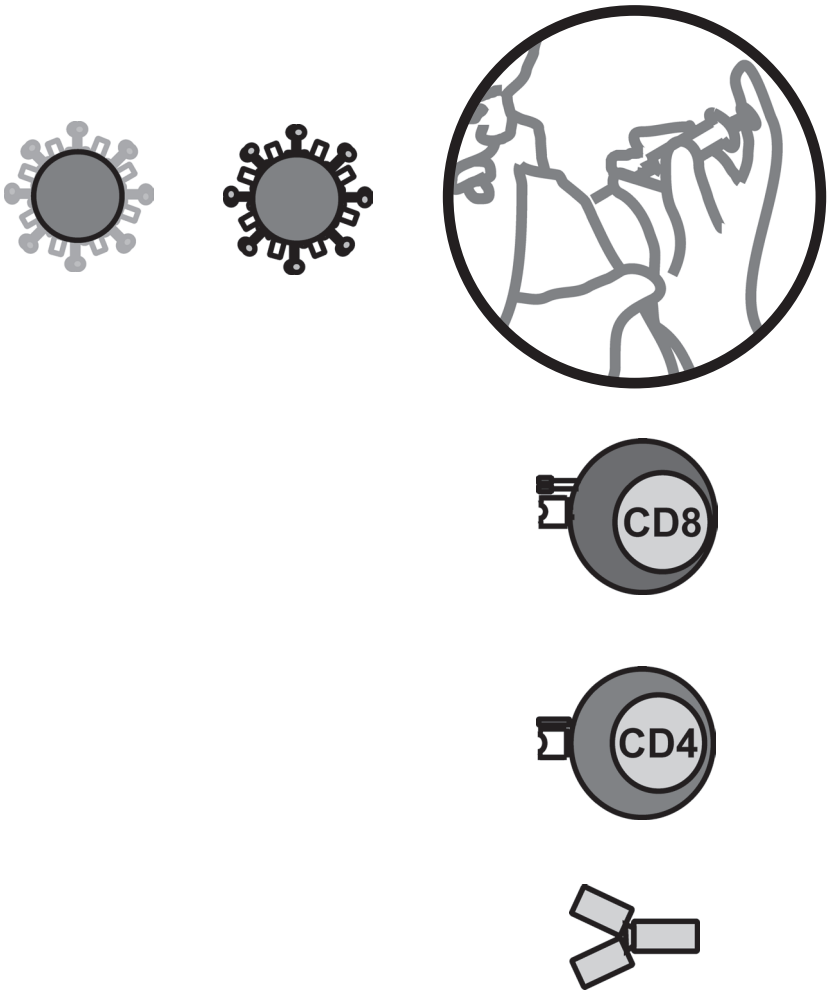
CD8
CD4

7 Annual vaccination against influenza hampers the development of virus-specific CD8+ T cell immunity in children

Submitted for publication

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ABSTRACT

Seasonal vaccination against influenza is recommended for all healthy children in a number of countries. However, we recently demonstrated that vaccination against seasonal influenza prevented the induction of virus-specific CD8+ T cell responses by infection with an influenza A/H3N2 virus and the induction of heterosubtypic immunity against influenza A/H5N1 in mice and ferrets. In the present study, we compared influenza A virus-specific cellular and humoral responses of unvaccinated healthy control children with children with cystic fibrosis (CF) that were vaccinated annually. No differences were observed between groups in the virus-specific CD4+ T cell response, while the antibody profile of the vaccinated children was more broadly cross-reactive. An age-related increase in the virus-specific CD8+ T cell response was observed in unvaccinated healthy control children that was absent in vaccinated CF children. Our results indicate that influenza vaccination is effective against seasonal influenza viruses but hampers the development of virus-specific CD8+ T cell responses. The consequences of these findings are discussed in the light of the development of protective immunity to seasonal and pandemic influenza viruses.

INTRODUCTION

The recent pandemic caused by influenza A/H1N1 virus of swine origin and the ongoing pandemic threat of highly pathogenic avian influenza A/H5N1 viruses highlight the importance of influenza as a cause of disease in humans. Also seasonal influenza viruses are an important cause of morbidity and mortality annually, especially in groups of people that are at risk to develop complications after infection due to underlying disease. The World Health Organization (WHO) has recommended annual influenza vaccination in these subjects to prevent excess morbidity and mortality (12). In addition to this recommendation the public health authorities of a number of countries have recommended to vaccinate all healthy children older than 6 months of age against seasonal influenza (60, 206). Since universal vaccines that protect against various intra subtypic drift variants and multiple subtypes of influenza A viruses are currently unavailable, annual vaccination aims at the induction of immunity to circulating seasonal influenza viruses (A/H3N2, A/H1N1 and B-viruses). Currently used inactivated influenza vaccines generally induce protective antibody responses against the influenza A and B virus antigens but inefficiently induce protective immunity to other influenza A virus subtypes (e.g. H5N1), so called heterosubtypic immunity.

In animal models, infection with influenza A virus reduces the morbidity and mortality after subsequent challenge infection with an influenza A virus of another subtype (40, 42, 54, 77, 155). There is also evidence for the presence of heterosubtypic immunity in humans (56, 207). For example, individuals that experienced an infection with an influenza A/H1N1 virus before 1957 less likely developed flu during the H2N2 pandemic of 1957 (54). Especially influenza virus-specific CD8+ Cytotoxic T lymphocytes (CTL) are thought to contribute to heterosubtypic immunity since the majority of these cells recognize conserved epitopes located in proteins like the nucleoprotein and the matrix protein (208, 209). Virus-infected cells present these epitopes in association with MHC Class I molecules and are eliminated upon recognition by specific CTL (51, 52, 210, 211). Furthermore, it has been demonstrated in humans that the presence of cross-reactive CTLs inversely correlated with the extent of viral shedding in the absence of antibodies specific for the virus used for experimental infection and also in young children it has been demonstrated that cellular immune responses correlated with protection against culture-confirmed influenza virus (50, 212).

The use of inactivated influenza virus vaccines induces virus-specific CD8+ T cell responses inefficiently (105, 157). Furthermore, it can be hypothesized that the use of these vaccines interfere with the induction of virus-specific CD8+ T cell responses otherwise induced by natural infections especially in children who are immunologically naïve to influenza viruses (145). We tested this hypothesis in mice and ferrets recently and confirmed that the use of subunit and whole inactivated A/H3N2 vaccines prevented the induction of heterosubtypic immunity to a lethal challenge infection with influenza A/Indonesia/5/05 (H5N1) otherwise induced by infection with A/H3N2 influenza virus (135, 157, Bodewes et al J Virol in press). The prevention of heterosubtypic immunity by H3N2 vaccination correlated with reduced virus-specific CD8+ T cell responses. Furthermore, epidemiological data obtained during the 2009 pandemic suggests that previous vaccination against seasonal influenza increased the risk of infection with the antigenically distinct influenza A/H1N1 pandemic virus in children and the risk of medically attended illness caused by this virus in adults

(169, 170, 213). However, the reason for this in humans is lacking and therefore we wished to compare the number of influenza virus-specific CD8+ T cells in children that received annually influenza vaccination with that of un-vaccinated children. To this end, we collected peripheral blood mononuclear cells (PBMC) and plasma samples from cystic fibrosis (CF) patients and otherwise healthy children undergoing correctional surgery. Since CF patients are at risk for complications caused by influenza virus infections, annual influenza vaccination is recommended from the time point of CF diagnosis onwards. In the Netherlands, influenza vaccination is not practiced in healthy young children. PBMC of the study subjects were tested for the presence of virus-specific T cells by intracellular IFN-γ staining and plasma samples were tested for the presence of virus-specific antibodies against various influenza A virus strains. The results obtained in the present study give insight in the development of virus-specific CD8+ T cell immunity in young children and the effect that annual vaccination with inactivated influenza A virus antigens has on the induction of this type of immunity.

MATERIALS AND METHODS

Study subjects

Children with cystic fibrosis (CF) that received inactivated influenza virus vaccine annually and unvaccinated healthy children that visited the hospital to undergo correctional surgery were enrolled in this study. Inclusion criteria for CF children were: age between 2 and 9 years first recorded vaccination against seasonal influenza viruses before or at four years of age and subsequently annual vaccination, no clinical signs of acute disease at the moment of blood collection, not chronically treated with immunosuppressive medications, and no laboratory confirmed infection with influenza A/H1N1(2009) before or at the moment of blood collection. Inclusion criteria for healthy control children were: between 2 and 9 years of age, not vaccinated against seasonal influenza, not chronically treated with immunosuppressive medications and no clinical signs of disease at the moment of blood collection. Blood samples were collected during autumn of 2009 and winter of 2009/2010. Written informed consent was obtained from parent or care takers prior to enrolment. The study was approved by the institutional medical ethics committee (Medisch Ethische Toetsings Commissie Erasmus MC (METC)). Protocol registration number MEC-2009-359; ABR-number 29399.

Serology

Plasma samples of children were collected and stored at -20°C until further processing. The presence of antibodies against influenza A viruses was evaluated using the virus neutralization (VN)-assay as described previously (87). Plasma samples were tested for the presence of antibodies against influenza A vaccine viruses from influenza seasons 2000-2010 and the influenza A/H1N1(2009) virus. To this end, influenza A/H3N2 viruses A/Panama/07/1999, A/Wyoming/3/2003, A/New York/55/2004, A/Hiroshima/52/2005, A/Wisconsin/67/2005, A/Brisbane/010/2007, influenza A/H1N1 viruses A/New Caledonia/20/1999, A/Solomon Islands/3/2006, A/Brisbane/059/2007 and the influenza A/H1N1(2009) virus A/Netherlands/602/2009 were inoculated in the allantoic cavity of 11-days old embryonated chicken eggs. Allantoic fluid was harvested after two days, cleared by

low speed centrifugation and stored at -80°C before use in the VN-assay. Sera from ferrets infected with each influenza A virus were used as a positive control. The plasma samples were also tested for the presence of IgG antibodies specific for various bacterial and viral vaccine antigen used in the National Immunization program including mumps, measles, rubella, Tetanus Toxin and Diphteria toxin and a common viral pathogen (Varicella Zoster virus) as described previously (Van Gageldonk et al J Immunol Methods 2008).

Collection of PBMC and intracellular IFN-γ staining of stimulated PBMC

Blood samples (max. 5ml) were collected in EDTA tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and subsequently PBMC were isolated by density gradient centrifugation using lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and then cryopreserved at -135°C until use. Thawed PBMC were resuspended in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) supplemented with 10% Fetal Bovine Serum, 2mM L-Glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. PBMC were seeded in 96 wells U-bottomplates (8x105 cells/well) and infected with influenza A/H3N2 virus Resvir-9 (a reassortant containing the nucleoprotein, hemagglutinin and neuraminidase of A/Nanchang/933/95 and all other genes of A/Puerto Rico/8/34) with a multiplicity of infection of three or left untreated. After 16 hours at 37°C, Brefeldin A (2µg/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the cells. As a positive control, cells from each individual were also incubated with 1µg/ml staphylococcus enterotoxin B (SEB; Sigma-Aldrich, Zwijndrecht, The Netherlands) during incubation with brefeldin A. After another six hours of incubation, cells were washed, stained with fluorescent labelled monoclonal antibodies (moabs) CD4-Pacific Blue (BD, Alphen a/d Rijn, The Netherlands) and CD8-PeCy7 (eBioscience, San Diego, USA). To exclude dead cells in the analysis, cells were also stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, The Netherlands). Subsequently cells were fixed with Facs Lysing Solution (BD) and stored at -80°C until further processing. Cells were permeabilized with Facs permeabilizing solution (BD) and stained with moabs CD3-PerCP, CD69-APC (both BD) and IFN-γ-FITC (eBioscience). Data were acquired using a FACSCanto-II and analysed with FACS Diva software (BD, stad?). For each well, the virus-specific CD8+ and CD4+ T cell response was determined by calculating the percentage of IFN-γ+ cells of the CD69+CD3+CD8+ cell population (IFN-γ+CD8+) or CD69+CD3+CD4+ (IFN-γ+CD4+) cells. For cells incubated with medium or with Resvir-9, the assay was performed in duplicate. Subsequently, the influenza A virus-specific and SEB-specific CD8+ and CD4+ T cell response of each individual was calculated by subtracting the mean percentage of IFN-γ+CD8+ or IFN-γ+CD4+ cells from cells incubated with medium only from the (mean) percentage of IFN-γ+CD8+ or IFN-γ+CD4+ cells incubated with Resvir-9 or SEB.

Statistical analysis

Associations between the age of children and the T cell responses of all groups were calculated using the Pearson's correlation coefficient (r) and the significance was calculated using ANOVA which was also used to assess the difference in slope between groups. Furthermore, assuming binominal distribution, the two-sided exact 95% confidence interval (CI) was calculated for seroprevalences of antibodies against influenza A/H3N2 and A/H1N1 viruses using Stata/SE software version 11.0. The

Mann Whitney test was used to compare T cell responses of groups. Differences were considered significant at $p<0.05$.

RESULTS

Study population

Between October 15, 2009 and February 5, 2010, blood samples were collected from 27 unvaccinated healthy control children and 14 children with CF vaccinated against influenza annually. The mean age of unvaccinated control children was 5.9 years and the median age of this group was 6.0 years (range 2.0-8.8 years), while the mean age of the group of vaccinated children was 6.2 (median 6.6; range 3.1-9.0 years).

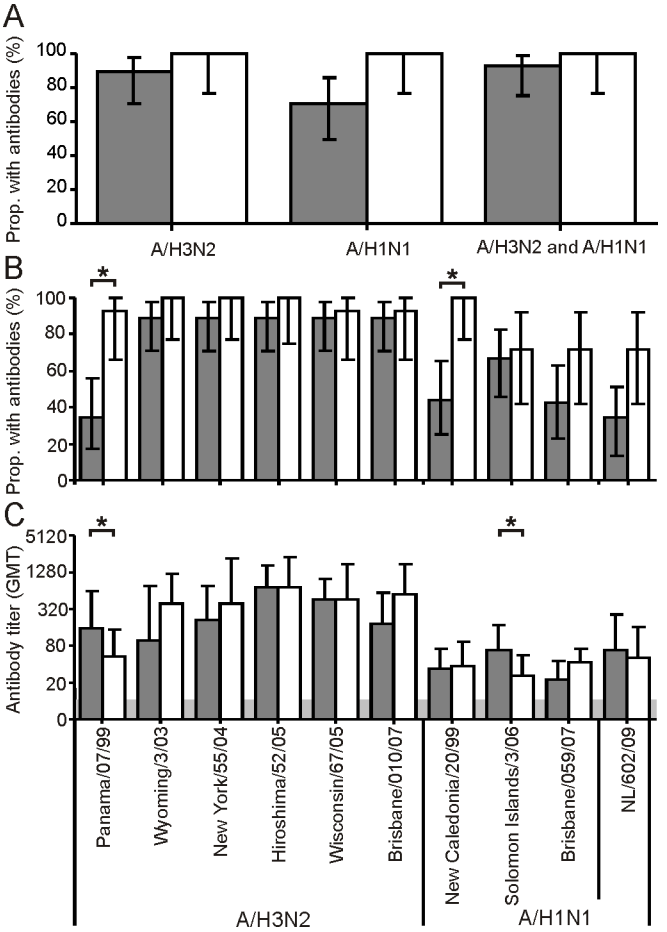

Figure 1. *Influenza A virus-specific humoral immunity. The proportion of children of the healthy unvaccinated control group (grey bars) and the vaccinated group of children with CF (white bars) with antibodies against at least one of the influenza viruses was calculated for all influenza viruses of each subtype (A) or for all indicated viruses individually (B). Bars represent the percentage with 95% confidence intervals. GMTs were calculated for all samples in which antibodies were detected against the indicated viruses (C). Bars represent GMT with standard deviations and the horizontal grey bar indicates the detection limit of the assay. The asterisk indicates a significant differences between the two groups ($p<0.05$).*

Antibody responses to influenza viruses and other selected antigens

Plasma samples were tested for the presence of antibodies against influenza A/H3N2 and influenza A/H1N1 viruses by VN-assay. In 24 out of 27 children (89%) of the unvaccinated control group, an-

tibodies were detected against at least one influenza A/H3N2 virus and in 20 out of 27 children, antibodies were detected against one of the influenza A/H1N1 viruses, including the influenza A/H1N1(2009) virus. In two children of the unvaccinated control group, no antibodies were detected against both influenza A/H3N2 and influenza A/H1N1 viruses.

In all vaccinated children, antibodies were detected against at least one influenza A/H3N2 virus and at least one influenza A/H1N1 virus. In 10 out of 14 (71%) plasma samples of these children antibodies were detected against the influenza A/H1N1(2009) virus against which they were also vaccinated (Fig 1A). The proportion of subjects with antibodies to the relatively old strains A/Panama/07/99 (H3N2) and A/New Caledonia/20/99 was significantly ($p<0.05$) greater in the group of vaccinated children with CF than in the unvaccinated control group (Fig 1B). These differences were not observed with more recent virus strains.

Geometric mean titers (GMT) were calculated for seropositive plasma samples to compare the magnitude of the antibody response of both groups. A significant higher GMT was observed in children of the unvaccinated control group for both influenza A/Panama/07/99 (H3N2) and A/Solomon Islands/3/2006 (H1N1) viruses ($p=0.04$ and $p=0.01$ respectively). No significant differences were observed between groups for GMTs of all other viruses (Fig 1C).

The IgG antibody responses to viral antigens mumps, measles, rubella and Varicella Zoster and the bacterial antigens Tetanus Toxin and Diphteria toxin were similar between the two study groups (Fig 4 A-F).

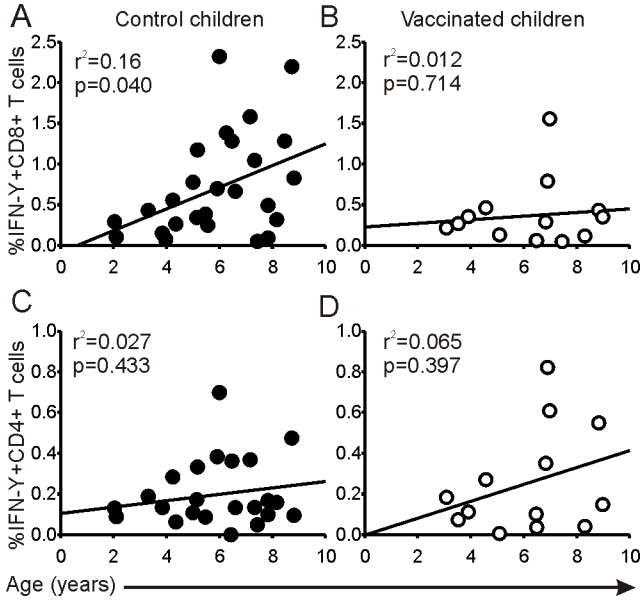

Figure 2. *Correlation between age and influenza virus-specific T cell responses. The percentage of virus-specific CD8+ IFN-γ+ T cells (A and B) and CD4+ IFN-γ+ T cells (C and D) was determined and plotted as a function of age of the individual subjects. Each dot represents the result of an individual subject and the correlation between all subjects of one group was calculated and is indicated by the black line. Both data of unvaccinated control children (A and C) and vaccinated children with CF (B and D) is shown. The correlation between age and the percentage of CD8+ IFN-γ+ T cells was significantly different ($p<0.05$) between the two study groups.*

Influenza A virus-specific T cell immunity

To assess the influenza A virus-specific CD8+ and CD4+ T cell immunity of each study subject, PBMC were stimulated with Resvir-9 and subsequently intracellular IFN-γ staining was performed. The

percentage of virus-specific IFN-γ+CD8+ T cells varied between 0.00 and 2.32 for unvaccinated control children while the percentage of IFN-γ+CD8+ T cells ranged between 0.06 and 1.56 for vaccinated children. An age-dependent increase of the virus-specific CD8+ T cell response was observed in the group of unvaccinated healthy control children (r2=0.16; p=0.040; Fig 2A), which was not observed in the group of vaccinated children (r2=0.012; p=0.714; Fig 2B). In addition, the age-dependent increase of virus-specific CD8+ T cells of the unvaccinated control group was significantly different from that of the vaccinated group (p=0.047) and in children older than 5 years of age, also a significant higher percentage (p=0.038) of IFN-γ+CD8+ T cells was observed in the unvaccinated control group (mean 0.86, s.d. 0.67) compared to the vaccinated group (mean 0.368, s.d. 0.451).
No significant age-dependent increase was observed for influenza A virus-specific CD4+ T cell immunity in both the unvaccinated control groups (r2=0.027; p=0.433; Fig 2C) and the vaccinated group (r2=0.065; p=0.379; Fig 2D). Furthermore, influenza A virus-specific CD4+ T cell responses were similar in both groups; in unvaccinated control group the mean percentage of IFN-γ+CD4+ T cells was 0.187 (s.d. 0.169), while in the vaccinated control group the mean percentage of IFN-γ+CD4+ T cells was 0.202 (s.d. 0.276) (p=1.00).

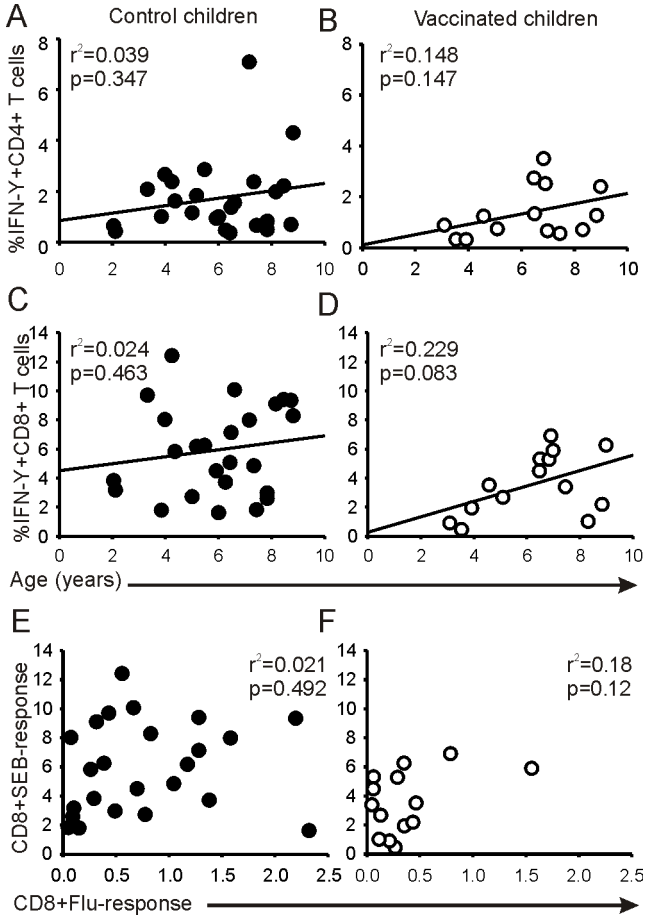

Figure 3. *T cell responses to stimulation with SEB. The percentage of CD4+ IFN-γ+ T cells (A,C) and CD8+ IFN-γ+ T cells (B,D) responding to SEB was determined for each subject of the group of unvaccinated control children (A,C) and the vaccinated children with CF (B,D). The percentage of SEB-specific CD8+ IFN-γ+ T cells was also plotted against the percentage influenza virus-specific CD8+ IFN-γ+ T cells for both the control children (E) and the vaccinated children with CF (F).*

SEB-specific T cell immunity

In addition to the virus-specific T cell response, the response of CD4+ and CD8+ T cells to the superantigen SEB was assessed for each subject. No age-related increase for both the SEB-specific CD4+ (r^2=0.039; p=0.347; Fig 3A) and CD8+ (r^2=0.024; p=0.463; Fig 3C) T cell response was observed in the unvaccinated control group and no significant differences were observed between the vaccinated children (mean 1.72, s.d. 1.47) and the unvaccinated control children (mean 1.37, s.d. 1.01) regarding the SEB-specific CD4+ T cell responses (p=0.558).

In addition, no age-related increase of the SEB-specific CD4+ T cell response was observed in vaccinated children (r^2=0.148, p=0.147; Fig 3B), while the correlation of age-related with the SEB-specific CD8+ T cell response approached statistical significance (r^2=0.229, p=0.083; Fig 3D). This indicates that at young age the percentage of SEB specific CD8+IFN-γ+ T cells was lower in vaccinated children compared to unvaccinated children, while in children older than five years of age no significant differences were present between groups (p=0.134). Furthermore, no correlation was observed between the SEB-specific CD8+ T cell response and the influenza A virus-specific CD8+ T cell response in the group of unvaccinated control children (r^2=0.021, p=0.492; Fig 3E) and vaccinated children (r^2=0.18, p=0.12; Fig 3F).

DISCUSSION

In the present study, we assessed influenza A virus-specific cellular and humoral immune responses in children with CF that had been vaccinated against seasonal influenza annually and in unvaccinated healthy control children. The virus-specific antibody profile was broader in vaccinated children with CF compared to unvaccinated control children. No differences were observed in the development of virus-specific CD4+ T cell responses. However, in healthy control children, an age-related increase in the percentage of virus-specific CD8+ T cells was detected which was not observed in children with CF, vaccinated annually. This is in concordance with our previous results in the mouse model in which we demonstrated that vaccination against seasonal influenza A virus prevented the development of influenza A virus specific CD8+ T cell immunity otherwise induced by infection (135, 157).

The age-dependent increase in the frequency of virus-specific CD8+ T cells in the unvaccinated control group most likely reflexes the increase in the number of subjects that experienced an infection with an influenza virus early in life. Of interest, a similar pattern was observed for the development of antibodies to influenza viruses in a large sero-epidemiological study performed in children age 0-7 years recently (Bodewes et al, Clinical Vaccine Immunology, in press). Indeed two healthy unvaccinated control subjects without detectable antibodies to any of the influenza A viruses also had very low frequencies of virus-specific CD8+ T cells which thus reflects lack of exposure to influenza A virus. In addition, maturation of the immune system may have contributed to increased responsiveness observed in older children. The latter was demonstrated using non-specific stimulation of the lymphocytes resulting in an age-dependent increase in Th1-like cytokines (214-216). However, using SEB, we were not able to demonstrate an age-dependent increase in CD4 and CD8 T cell responses to this super antigen.

In the group of CF patients vaccinated annually the age-dependent increase in virus-specific CD8+ T cell responses was absent. Our interpretation of these findings is that vaccination efficiently induced virus-specific antibodies which protected against infection with seasonal influenza viruses to a great extent, and thereby prevented the induction of specific CD8+ T cell responses. Although, ideally cell-mediated and humoral immune responses of unvaccinated healthy children were compared with those of vaccinated healthy control subjects, it is unlikely that patients with CF responded poorly because of intrinsic immunological defects for various reasons. First, the virus-specific CD4+ T cell response of this group was comparable with that of the unvaccinated healthy control group. This confirms that the use of inactivated vaccines induced CD4+ T cell responses but not virus-specific CD8+ T cell responses. Second, the antibody titers in the seropositive subjects were comparable between the two groups. The vaccinated children with CF responded rather better than the unvaccinated subjects especially to older strains of influenza A virus. This confirms that patients with CF can be vaccinated effectively against seasonal influenza and the complications these infections may cause in this vulnerable group of high-risk patients. In addition, it has been demonstrated in adults that during years with only mild influenza activity also the CTL immunity declined (217). Third, the CD8+ T cell response after stimulation with SEB was not affected in the group of CF patients and comparable to that of unvaccinated control subjects. 218 comparable to that of unvaccinated control subjects. Finally, the antibody responses to various viral and bacterial vaccine antigens used in the Dutch national immunization program were similar for the two study groups, indicating that there were no differences in the functionality of T and B cells between the groups. Furthermore, universal vaccination of healthy children is not practised in the Netherlands, so this study group was not available. In addition, since vaccination of children 6-59 months of age is only recommended and practised since 2007 in other countries, the long term effects of vaccination of healthy children can not be examined at present. Therefore, the results from the present study warrant follow-up studies with a larger cohort of vaccinated and unvaccinated children in the future especially since epidemiological data suggests that previous vaccination against seasonal influenza increased the risk of infection with pandemic influenza A/H1N1 virus in 2009 (207).

Thus, annual vaccination against influenza is effective but may have potential drawbacks that have been underappreciated previously and that are also a matter of debate (145, 146, 170). By no means, we suggest halting annual vaccination of children, especially those at high risk for complications such as the CF patients. A number of studies have demonstrated that annual vaccination reduces morbidity and mortality caused by seasonal influenza in children and is (cost-)effective (63-65, 213). However, long-term annual vaccination using inactivated vaccines may hamper the induction of cross-reactive CD8+ T cell responses by natural infections and thus may affect the induction of heterosubtypic immunity. This may render young children that have not been previously infected with an influenza virus more susceptible to infection with a pandemic influenza virus of a novel subtype. Therefore, we argue for the development and use of vaccines that could induce broadly protective immune responses in children. For example, it has been demonstrated that live attenuated influenza vaccines induce virus-specfic CD8+ T cell responses. The development of broadly protecive vaccines has been on the research agenda for some time and progress has been made (121, 173, 218). Especially young children, whether at high risk for influenza-associated complica-

tions or not, may benefit the most from these vaccines.

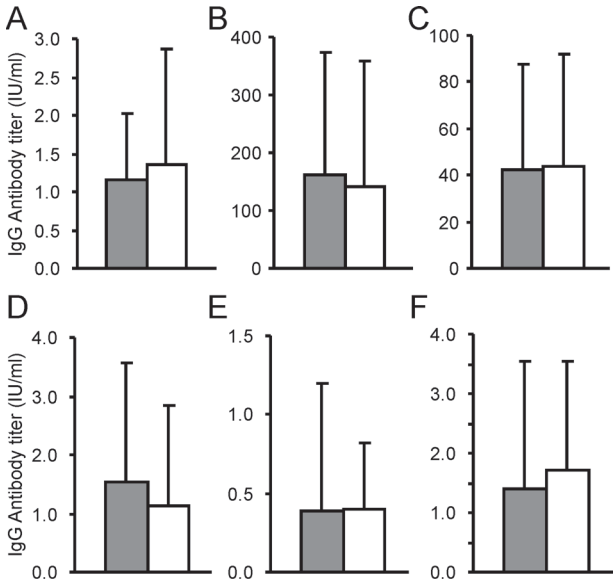

Fig 4. *IgG antibody responses to various viral and bacterial vaccine antigens. Mean IgG antibody response (± s.d.) to various viral and bacterial antigens including those of the Dutch national vaccination program of the healthy unvaccinated control group (grey bars) and the vaccinated group of children with CF (white bars) for Measles (A), Mumps (B), Rubella (C), Varicella Zoster (D), Diphtheriatoxin (E) and Tetanus toxin (F).*

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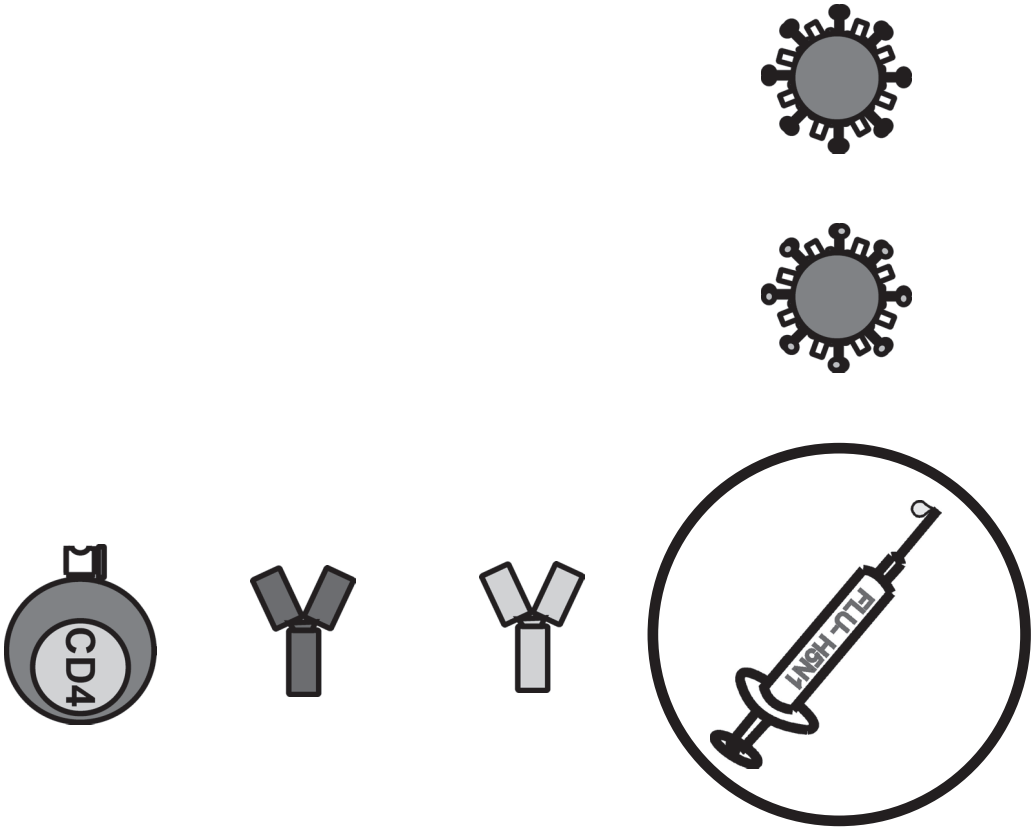
CD4
FLU-H5N1

8 The novel adjuvant CoVaccine HT™ increases the immunogenicity of cell-culture derived influenza A/H5N1 vaccine and induces the maturation of murine and human dendritic cells *in vitro*

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ABSTRACT

A candidate influenza H5N1-vaccine based on cell-culture-derived whole inactivated virus and the novel adjuvant CoVaccine HT™ was evaluated in vitro and in vivo. To this end, mice were vaccinated with the whole inactivated influenza A/H5N1 virus vaccine with and without CoVaccine HT™ and virus-specific antibody and cellular immune responses were assessed. The addition of CoVaccine HT™ increased virus specific primary and secondary antibody responses against the homologous and an antigenically distinct heterologous influenza A/H5N1 strain. The superior antibody responses induced with the CoVaccine HT™-adjuvanted vaccine correlated with the magnitude of the virus-specific CD4+ T helper cell responses. CoVaccine HT™ did not have an effect on the magnitude of the CD8+ T cell response. In vitro, CoVaccine HT™ upregulated the expression of co-stimulatory molecules both on mouse and human dendritic cells and induced the secretion of pro-inflammatory cytokines TNF-α, IL-6, IL-1ß and IL-12p70 in mouse- and IL-6 in human dendritic cells. Inhibition experiments indicated that the effect of CoVaccine HT™ is mediated through TLR4 signaling. These data suggest that CoVaccine HT™ also will increase the immunogenicity of an influenza A/H5N1 vaccine in humans.

INTRODUCTION

Influenza viruses are a major cause of respiratory tract infections and responsible for excess morbidity and mortality annually. In addition to seasonal influenza epidemics, influenza pandemics occur occasionally caused by the introduction of influenza A viruses of novel subtypes into the human population. At present, highly pathogenic avian influenza A viruses of the H5N1 subtype continue to cause outbreaks in domestic birds and are transmitted regularly from infected poultry to humans. Since 2003, more than 400 human cases have been reported of which >60% had a fatal outcome (219). It is feared that these viruses will adapt to their new host, become transmissible from human-to-human and cause a new pandemic (17, 102). Furthermore, a previous undescribed influenza A/H1N1 virus was isolated from humans in Mexico in april 2009, which did subsequently spread all over the world (13, 220, 221). As of july 6 2009, there have been 94512 laboratory confirmed cases of pandemic new influenza A/H1N1, resulting in 429 deaths (221). Therefore, the World Health Organization has issued a pandemic alert phase 6 for these viruses on june 11 2009. To mitigate the impact of a next influenza pandemic, there is a need for safe and effective vaccines (222-225). Key issues for pandemic influenza vaccine development are: the time required that vaccines become available after the start of a pandemic, optimal use of the existing production capacity to produce sufficient vaccine doses for a world wide vaccination campaign and improvement of the efficacy of the vaccine not only against homologous virus strains but also against heterologous viruses derived from antigenically distinct clades. Some of these issues have been addressed and it has been shown that the use of adjuvants increases the immunogenicity of inactivated vaccines that are otherwise poorly immunogenic (226, 227), allows dose sparing, increasing the number of vaccine doses that can be made (226) and broadens the specificity of the antibody responses that are induced (149, 227).

In the present study, we evaluated the immune potentiating effect of the novel adjuvant CoVaccine HT™ on the immune response in mice induced with a whole inactivated influenza A/H5N1 vaccine produced in Madin Darby Canine Kidney (MDCK) cells on a commercial scale. CoVaccine HT™ consists of a sucrose fatty acid sulphate ester (SFASE) immobilized on the oil droplets of a submicron emulsion of squalane-in-water (228). It has been shown that CoVaccine HT™ increased the humoral and cellular immune responses to an experimental classical swine fever vaccine based on recombinant E2 glycoprotein in pigs and it enhanced humoral responses to a Gonadotropin-releasing hormone (GnRH) conjugate vaccine in horses and was well-tolerated in both species (228-230). Furthermore, preliminary data on the immune potentiating properties of CoVaccine HT™ in mice and ferrets also shows improved immunogenicity to an inactivated cell culture derived whole influenza virus vaccine by the addition of CoVaccine HT™ (231).

Here we show that the addition of CoVaccine HT™ to the influenza A/H5N1 vaccine increased the magnitude of the virus specific antibody response in mice considerably. Even after a single immunization sizable antibody response were detected, which were boosted after a second immunization. Furthermore the antibody responses directed to the homologous vaccine strain A/Vietnam/1194/04 (clade 1) cross-reacted to a certain extent with influenza virus A/Indonesia/5/05 derived from an antigenically distinct clade of H5N1 viruses (clade 2.1) (232). The enhanced anti-

body responses observed with the use of CoVaccine HT™ correlated with improved CD4+ T helper cells responses. CoVaccine HT™ did not potentiate CD8+ T cell responses.
To elucidate the potential mechanism underlying the adjuvant effect of CoVaccine HT™, we tested its effect on the maturation of mouse and human dendritic cells. CoVaccine HT™ induced the expression of co-stimulatory molecules CD80 and CD86 and the production of pro-inflammatory cytokines in murine bone marrow derived myeloid dendritic cells (BM-mDCs). A similar effect was observed with human monocyte-derived dendritic cells. Based on these findings we anticipate that CoVaccine HT™ also increases the immunogenicity of whole inactivated H5N1 vaccines in humans.

MATERIALS AND METHODS

Vaccine and adjuvant

The vaccine strain NIBRG-14, based on strain A/Vietnam/1194/04, (H5N1, clade 1) was used to prepare a whole inactivated influenza A virus vaccine. The vaccine was produced in MDCK cells and inactivated using beta-propiolactone on a commercial scale by Nobilon Schering-Plough, Boxmeer, The Netherlands. The hemagglutinin content was assessed by a single radial immunodiffusion assays according to standard methods (233). CoVaccine HT™ was produced and formulated according proprietary technology by Protherics Ltd., London, United Kingdom.

Mice and immunizations

Female specified pathogen free 6-8 weeks old C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Mice were housed in individual ventilated cages and were fed food and water ad libitum. Mice were divided in 5 groups (n=16-17 mice per group) and were immunized twice by the intramuscular route in the hind legs (50 µl per leg) with an interval of four weeks with either PBS (group 1), 5µg HA of whole inactivated influenza A virus vaccine (WIV NIBRG-14) only (group 2), CoVaccine HT™ (1 mg SFASE) only (group 3) or 5 µg HA of WIV NIBRG-14 in combination with CoVaccine HT™ (group 4). Mice of group 5 were infected intranasally twice with an interval of four weeks with 2x 10^4 $TCID_{50}$ in 50µl PBS of a reassortant virus containing the surface glycoproteins of influenza A/VN/1194/04 (H5N1) and the inner proteins of influenza A/PR/8/34, which is a commonly used vaccine backbone strain. This virus was produced by reverse genetics technology as described previously (234) and the basic cleavage site was removed from the HA by site-directed mutagenesis, corresponding the NIBRG-14 vaccine strain. Mice of this group were used as a positive control for the detection of CD8+ Tetramer+ T cells (group 5). Mice of each group were euthanized 8 days after the first vaccination (n=6 per group), 8 days after the second vaccination (n=6 per group) and 21 days after the second vaccination (n=4-5 mice per group). After collection of blood samples for the determination of serum antibody responses and euthanasia by cervical dislocation, spleens and inguinal lymph nodes were resected. Infections and euthanasia were performed under anesthesia with isoflurane (3% in 0_2). An independent animal ethics committee approved the experimental protocol before the start of the experiments.

Serology

Serum samples were collected before and twenty-eight days after the first vaccination and twenty-one days after the second vaccination. Sera were stored at –20°C until use. Sera were tested for the presence of anti-HA antibodies using a hemagglutination inhibition assay (HI-assay) with 1% turkey erythrocytes and for the presence of virus neutralizing antibodies using a micro virus neutralization assay (VN-assay) as described previously (87, 107). Sera were tested for the presence of antibodies reactive with influenza A/H5N1 viruses of two antigenically distinct clades, influenza A/VN/1194/04 (H5N1) and influenza A/IND/5/05 (H5N1). For this purpose, reverse genetics viruses of both viruses were produced. The titers obtained with these viruses were comparable with those against the wild-type strains (data not shown). Positive control serum specific for influenza A/VN/1194/04 was obtained from hyperimmune serum from a rabbit and positive control serum specific for influenza A/IND/5/05 was obtained from a swan immunized twice with inactivated H5N1 influenza virus A/Duck/Potsdam/1402/86 (Intervet, Boxmeer, The Netherlands) (89).

Intracellular cytokine staining of virus-specific CD4+ T cells

Virus-specific CD4+ T cells were detected by intracellular cytokine staining. After single cell suspensions were obtained from spleens of mice and red blood cells were removed using erythrocyte lysis buffer (Roche, Almere, The Netherlands), cells were stained with trypan blue and living cells were counted. Five-hundred-thousand splenocytes were incubated for two hours with or without 1µg HA of WIV NIBRG-14 in Iscove's Modified Dulbecco's Medium (Lonza, Breda, The Netherlands) containing 5% Fetal Calf Serum (FCS), and subsequently Golgistop (Monensin, BD, Alphen a/d Rijn, The Netherlands) was added. After an additional incubation for six hours in the presence of Golgistop, cells were o/n stored at +4°C and subsequently stained for flow cytometry with fluorescently labelled antibodies to detect intracellular IL-2, IFN-γ, IL-4 and IL-5. In brief, cells were washed with PBS containing 2% FCS (P2F) and Golgistop and stained with monoclonal antibodies directed to CD3e-APC-Cy7 and CD4-PerCP (Both from BD, Alphen a/d Rijn, The Netherlands). To exclude dead cells in the analysis, cells were stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, The Netherlands). After staining, cells were fixed and permeabilized with Cytofix and Cytoperm (BD) and stained with monoclonal antibodies specific for IL-2 FITC, IL-5 PE, IL-4 APC and IFN-γ PE-Cy7 (all BD, Alphen a/d Rijn, The Netherlands). Samples were acquired with a FACSCanto and analyzed using FACS Diva software (BD). The mean percentage of cytokine-positive CD4+ T cells of duplicates was calculated.

Detection of virus-specific CD8+ CTL by tetramer staining

After the removal of red blood cells from single cell suspensions of spleens and inguinal lymph nodes, cells were washed with P2F and stained for flow cytometry with antibodies to anti-CD3e PerCP, anti-CD8a APC, the PE labeled H-2Db tetramer with the $NP_{366-374}$ epitope ASNENMETM ($TM_{ASNENMETM}$; Sanquin Research, Amsterdam, The Netherlands) and LIVE/DEAD Aqua Fixable Dead Cell Stain. Samples were acquired with a FACSCanto and analyzed using FACS Diva software (BD).

Collection of murine bone marrow derived myeloid dendritic cells

Bone marrow cells were collected from the femurs and tibiae of six PBS vaccinated C57bl/6J mice

and cultured for nine days in DC culture medium (DC-CM; RPMI 1640 containing GlutaMAX-I; Invitrogen, Breda, The Netherlands) supplemented with 5% (v/v) FCS (HyClone, Etten-Leur, The Netherlands), 50µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 U/ml penicillin, 100 U/ml streptomycin and 20 ng/ml recombinant mouse GM-CSF (a gift from K. Thielemans, Vrije Universiteit Brussel, Brussels, Belgium) essentially as described previously (235). On day 9, 1 x 10^6 cells/ml were stimulated with PBS, three different concentrations of CoVaccine HT (50, 100 and 200 µg SFASE/ml) or 1µg/ml lipopolysaccharide (LPS; positive control). After incubation for 16-18 hours at 37°C, BM-mDCs were harvested and subsequently stained with CD80-FITC and CD86-PE or CD11c-APC, I-A-FITC and CD86-PE (all from BD, Alphen a/d Rijn, The Netherlands). Dead cells were excluded by staining with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, The Netherlands). Samples were acquired on a FACSCanto and subsequently analyzed with Cellquest Pro Software (both BD).

Collection of human monocyte derived dendritic cells

PBMC from healthy blood donors, between 35 and 50 years of age, were isolated from heparinized blood (Sanquin Bloodbank, Rotterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Axis-Shield PoC, Oslo, Norway) and were cryopreserved at
–135°C. Immature human DCs were obtained by isolation of CD14+ cells from thawed PBMC of three different donors by magnetic cell sorting, using human CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. Subsequently, cells were cultured in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) containing 10% FCS (Sigma-Aldrich, Zwijndrecht, The Netherlands), 1000 U/ml recombinant human GM-CSF and 200 U/ml recombinant human IL-4 (both BD Pharmingen, Alphen a/d Rijn, The Netherlands). After culturing for 7 days, cells were stimulated for 16-18 hours with PBS, CoVaccine HT (50 µg SFASE/ml) or LPS (1 µg/ml). After stimulation, cells were stained with CD80-FITC, CD83-APC and CD86-PE or HLA-DR-PerCP and CD11c-APC (all from BD, Alphen a/d Rijn, The Netherlands) and subsequently acquired on a FACSCanto and analysed with Cellquest Pro Software (both BD).

Detection of cytokines in DC culture supernatants

Concentrations of TNF-α, IL-12p70, IL-6, IL-1ß and IL-10 in culture supernatants of murine and human dendritic cells were assessed by enzyme-linked immunosorbent assay (ELISA) in 96-well microtitre plates using Elisa-kits (eBioscience, San Diego, USA) according to the instructions of the manufacturer. Culture supernatants of dendritic cells incubated with LPS were used as a positive control for the release of cytokines while culture supernatants of dendritic cells incubated with PBS were used as negative controls.

Effect of anti-Toll Like Receptor (TLR) antibodies on the secretion of IL-6 and IL-10 by human DCs

Immature human DCs (1x10^6 cells/ml) of three healthy blood donors were incubated with 20µg/ml of either anti-TLR4 MoAb (Clone HTA-125), anti-TLR2 MoAb (Clone TL2.1) (both eBiosciences, San Diego, USA), both monoclonal antibodies, PBS or mouse IgG1 isotype control for half an hour at room temperature. Subsequently CoVaccine HT™ (50 µg SFASE/ml) or LPS (1 µg/ml) was added and

cells were incubated for 16-18 hours at 37°C. After incubation, supernatants were harvested and stored at –70°C until concentrations of IL-6 and IL-10 were determined using Elisa-kits.

Statistical analysis

Differences in the antibody titer, and the percentages of CD4+ Cytokine+ T cells and CD8+ TMAS-NENMETM+ T cells between mice of groups 2 and 4 were analyzed statistically using the Mann-Whitney U test. The differences between mice of groups 2 and 4 regarding the percentages of CD4+ Cytokine+ T cells were calculated after subtracting the mean percentage of CD4+ Cytokine+ T cells stimulated with PBS from the mean percentage of WIV stimulated cells of each individual mouse.

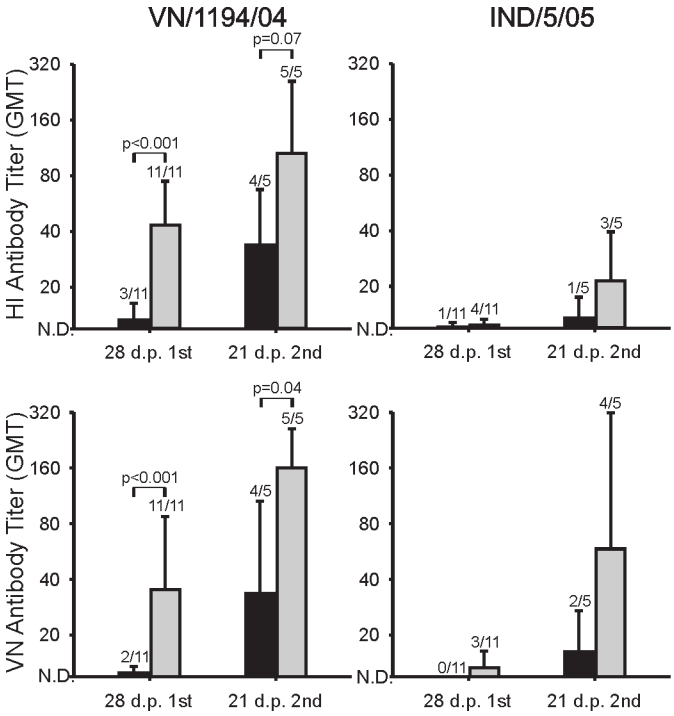

Figure 1. *Geometric mean serum antibody titers of mice 28 days after the first vaccination and 21 days after the second vaccination with 5µg HA of WIV NIBRG-14 with (grey bars) and without (black bars) CoVaccine HT . Sera were tested for the presence of antibodies against influenza A/VN/1194/04 (H5N1) and influenza A/IND/5/05 (H5N1) with the HI assay and the VN assay. Indicated above the bars is the number of mice that developed detectable antibody titers (e.g. 1/11 = 1 out of 11 mice).*

RESULTS

Serology

To assess the immunogenicity of WIV NIBRG-14 and the adjuvant effect of CoVaccine HT™, pre- and post vaccination sera were tested for the presence of virus specific antibodies by HI and VN assay. Mice vaccinated with PBS or CoVaccine HT™ only did not develop detectable antibody titers against homologous and the heterologous influenza A/H5N1 virus (data not shown). After one vaccination, in 3 out of 11 mice vaccinated with WIV NIBRG-14 only low antibody titers against influenza A/VN/1194/04 were detected in the HI assay (Geometric Mean Titer (GMT) = 8). Two of these animals also developed detectable VN antibodies (GMT =6). In contrast, all eleven mice vaccinated with WIV NIBRG-14 and CoVaccine HT™ developed detectable HI and VN antibody titers, with GMTs of 42 and

36 respectively. The differences in the HI and VN antibody titers between mice vaccinated with WIV NIBRG-14 only and WIV NIBRG-14 in combination with CoVaccine HT™ were statistically significant ($p<0.001$ for both assays). Antibodies against influenza A/IND/5/05 were detected in 1 out of 11 mice vaccinated with WIV NIBRG-14 only in the HI-assay and not in the VN-assay, while 4 out of 11 mice vaccinated with CoVaccine HT™ adjuvanted WIV NIBRG-14 had detectable HI antibodies against influenza A/IND/5/05.

Three weeks after the second vaccination, antibody titers were boosted and antibodies against influenza A/VN/1194/04 were detected in the HI and VN assay in four out of five mice vaccinated with WIV NIBRG-14 only (GMTs of 35 in both assays). All mice vaccinated with CoVaccine HT™-adjuvanted WIV NIBRG-14 developed virus specific antibodies detected in HI and VN assays with GMTs of 112 and 160 respectively. The VN antibody titers induced with CoVaccine HT™ adjuvanted vaccine preparation were significantly higher than those induced with the WIV NIBRG-14 only ($p=0.04$). For the HI antibody titers this difference approached statistical significance ($p=0.07$). Twenty-one days after the booster vaccination, also antibodies were detected in the HI and VN assay that cross-reacted with influenza virus A/IND/5/05. The proportion of mice with these cross-reactive antibodies was higher in the group vaccinated with CoVaccine HT™-adjuvanted vaccine than in mice vaccinated with WIV NIBRG-14 only (Fig 1).

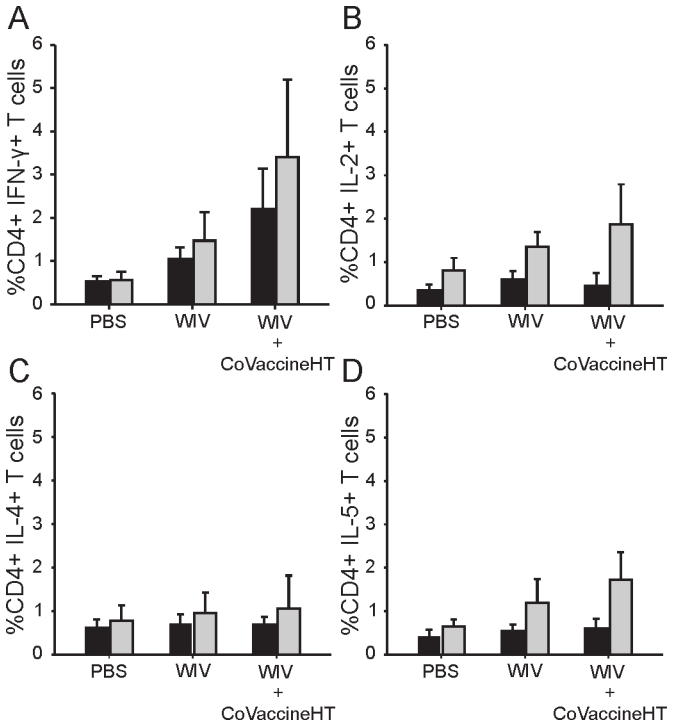

Figure 2. *Intracellular cytokine expression of CD4+ T cells after vaccination with PBS, 5µg HA of WIV NIBRG-14 with and without CoVaccine HT™. Splenocytes of mice obtained 21 days after the second vaccination were incubated with medium with (grey bars) and without (black bars) NIBRG-14. Production of IL-2 (A), IFN-γ (B), IL-4 (C) and IL-5 (D) by CD4 T cells was monitored using intracellular cytokine staining.*

Intracellular cytokine staining of virus-specific CD4+ T cells

Splenocytes of mice euthanized 21 days after the second vaccination were incubated with or with-

out WIV NIBRG-14 and the production of IFN-γ, IL-2, IL-5 and IL-4 by virus specific CD4+ T cells was determined using intracellular cytokine staining. Virtually no cytokine+ cells were found in mice vaccinated with PBS, while vaccination with WIV NIBRG-14 resulted in the production of cytokines IL-2, IFN-Y and IL-5 by CD4+ T cells after re-stimulation in vitro. The use of CoVaccine HT™ in the vaccine increased the percentages of virus specific cytokine+ CD4+ T cells to some extent (p-values of 0.08, 0.25, 0.9 and 0.30 for the difference in percentage of CD4+ Cytokine+ T cells between groups 2 and 4 for IL-2, IFN-γ, IL-4 and IL-5 respectively) (Fig.2).

Detection of virus-specific CTL by tetramerstaining

Inguinal lymph nodes and spleens were collected from vaccinated or infected mice 8 days after the first and 8 and 21 days after the second vaccination and the percentage of CD8+ TMASNENMETM+ T cells was determined. In the spleen, CD8+ TMASNENMETM+ T cells were detected only in mice vaccinated with WIV NIBRG-14 with (group 4) and without CoVaccine HT™ (group 2) and in infected mice (group 5). In mice of groups 2 and 4 and in infected mice (group 5), CD8+ TMASNENMETM+ T cells were observed 21 days after the second immunization, but the percentage of CD8+ TMASNENMETM+ T cells was lower in mice of group 2 compared to mice of group 5 ($p<0.03$) (Fig 3). The addition of CoVaccine HT™ did not significantly alter the CD8+ T cell responses compared to the use of WIV NIBRG-14 alone ($p=0.2$). Only very low percentages of CD8+ TMASNENMETM+ T cells were observed in the inguinal lymph nodes of both vaccinated and unvaccinated mice (data not shown).

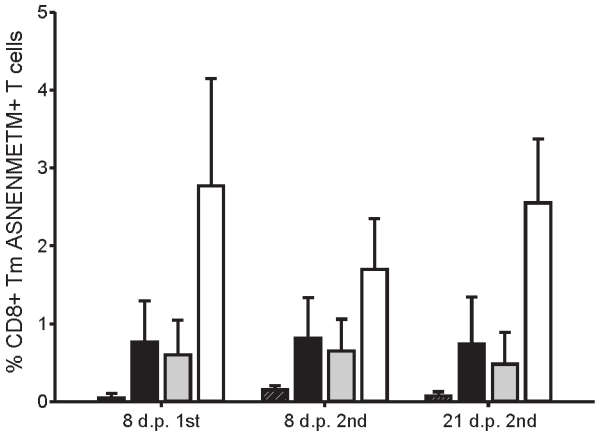

Figure 3. *CD8+ T cell responses to the immunodominant influenza A epitope on the nucleoprotein (NP366-374) of C57bl/6 mice as detected by tetramerstaining 8 days after the first, 8 days after the second and 21 days after the second vaccination with either PBS (hatched bars), WIV NIBRG-14 with (grey bars) and without (black bars) CoVaccine HT™. Infected mice were used as a positive control for the tetramerstaining (white bars).*

Effect of CoVaccine HT™ on murine bone marrow derived myeloid DCs

BM-mDCs of 6 mice were incubated for 16-18 hours with PBS, three different concentrations of CoVaccine HT™ (50, 100, 200 µg SFASE/ml) or LPS. After incubation, the cell surface expression of MHC-II (I-A), CD11c, CD80 and CD86 was analyzed using flow cytometry. DCs incubated with PBS had low expression of MHC-II (data not shown), moderate expression of CD11c (data not shown) and low expression of CD80 and CD86. Incubation with LPS or CoVaccine HT™ induced a marked increase of CD80, CD86 and MHC-II expression on BM-mDCs compared to BM-mDCs incubated with PBS (Fig. 4A), while CD11c expression was not increased (data not shown). The expression of CD80 or CD86 was not dependent on the concentration of CoVaccine HT™ that was used.

Furthermore, culture supernatants of BM-mDCs incubated with PBS, CoVaccine HT™ (50 µg SFASE/ml) or LPS were collected and the concentration of IL-6, IL-10, TNF-α, IL-1ß and IL-12p70 was determined using ELISA. CoVaccine HT™ induced an increase of the secretion of IL-6, TNF-α, IL-1ß and IL-12p70, but not of IL-10 (Table 1).

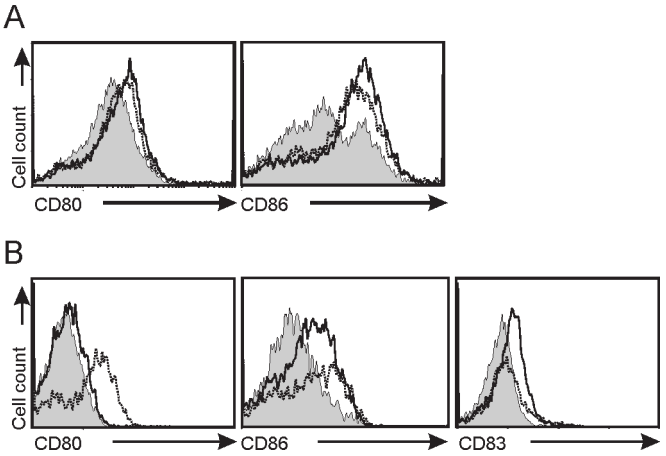

Figure 4. *Effect of CoVaccine HT™ on the phenotype of murine and human DCs. DCs were incubated with either PBS (grey area), CoVaccine HT™ (-) and LPS (---) and CD80 and CD86 expression of was monitored on murine DCs (A) and CD80, CD83 and CD86 expression on human DCs (B). A representative example of at least three experiments with three human subjects or six mice is shown.*

Effect of CoVaccine HT™ on human monocyte derived DCs

Human dendritic cells of three donors were incubated for 16-18 hours with PBS, CoVaccine HT™ (25, 50, 100 and 200µg SFASE/ml) or LPS. After incubation, the expression of MHC-II, CD11c, CD80, CD83 and CD86 on the surface of the cells was analyzed. After incubation with 50µg SFASE/ml of CoVaccine HT™, a marked increase of CD86 expression and a moderate increase of CD83 expression were observed on human DCs of all donors compared to incubation with PBS, while expression of CD80, MHC class II and CD11c was not increased. The use of higher concentrations of CoVaccine HT™ did not further increase the expression of CD83 and CD86. LPS, which was used as a positive control, induced a marked increase in the expression of CD80 and CD86 (Fig 4B).

Culture supernatants of human DCs incubated with PBS, CoVaccine HT™ (50 µg SFASE/ml) or LPS were also collected and the concentration of IL-6, IL-10, TNF-α, IL-1ß and IL-12p70 was determined using ELISA. CoVaccine HT™ increased the secretion of IL-6 and decreased the secretion of IL-10. The secretion of TNF-α, IL-1ß and IL-12p70 was essentially unaffected (Table 1).

Effect of blocking anti-TLR antibodies on the CoVaccine HT™-induced cytokine secretion by humans DCs

To assess the possible involvement of TLR signaling in the adjuvant acitivity of CoVaccine HT™, antibodies directed to TLR4 and TLR2 (both IgG2a) were used that can block the interaction of these TLRs with their respective ligands (236). As shown in figure 5A, treatment of human DCs with TLR4 specific antibodies abrogated the CoVaccine HT™-induced IL-6 secretion almost completely by these cells, whereas TLR2 specific antibodies did not affect the secretion of IL-6 into the culture supernatant. Similarly, the treatment of the DCs with TLR4 specific antibodies reversed the reduction of IL-10 secretion induced by CoVaccine HT™ in two out of three donors (figure 5B), whereas the

use of TLR2 specific antibodies did not. The induction of IL-6 secretion by LPS, which was used as a positive control, was downregulated when the the human DCs were treated with a combination of the TLR4 and TLR2 specific antibodies, but not when these antibodies were used individually. Treatment of DCs with isotype-matched control antibodies did not affect the CoVaccine HT™ induced changes in cytokine secretion (data not shown).

Table 1. Effect of CoVaccine HT™ on the secretion of cytokines by murine and human DCs

	Murine BM-mDCs			Human DCs		
Cytokine	PBS	CoVaccine HT™	LPS	PBS	CoVaccine HT™	LPS
TNF-α	176 (39)	8317 (2614)	1775 (1190)	<4	7 (7)	779 (193)
IL-6	16 (11)	16003 (3822)	13739 (4368)	12 (13)	139 (81)	360 (63)
IL-10	<30	<30	<30	20 (6)	<2	486 (162)
IL-12p70	20 (5)	1859 (376)	2081 (1750)	<4	<4	11 (5)
IL-1ß	<8	207 (67)	135 (16)	<4	6 (2)	18 (13)

Effect of CoVaccine HT™ on the secretion of cytokines by murine bone-marrow derived DCs and human monocyte-derived DCs. The secretion of cytokines was determined by ELISA using the culture supernatants of DCs incubated with PBS, CoVaccine HT™ or LPS. Results were acquired of at least three mice or human subjects. Indicated are mean cytokine concentrations (pg/ml) and in between brackets the standard deviations.

DISCUSSION

In the present study, state of the art cell-culture vaccine production technology and the novel adjuvant CoVaccine HT™ were combined to formulate a whole inactivated virus vaccine preparation against A/H5N1 influenza viruses. The use of CoVaccine HT™ increased the immunogenicity of the WIV vaccine prepared with the vaccine strain NIBRG-14 considerably and improved response rates and GMT antibody titers not only against the homologous strain A/Vietnam/1194/04, but also against the antigenically distinct strain A/Indonesia/5/05 derived from clade 2.1. [18]. The superior antibody responses induced with the aid of CoVaccine HT™ correlated with improved CD4+ T helper cell responses, which in turn, correlated with the stimulating effect of CoVaccine HT™ on professional antigen presenting cells in vitro. CoVaccine HT™ stimulated the expression of co-stimulatory molecules of mouse and human dendritic cells and the production of pro-inflammatory cytokines by these cells.

Early attempts to prepare H5N1 vaccines were based on the production method and formulation of seasonal influenza vaccines (237). However, these vaccines were poorly immunogenic and high doses of antigen were required for the induction of appreciable antibody responses (237, 238). The use of adjuvants MF59 and AS03, but not aluminium hydroxide, improved the immunogenicity of H5N1 vaccines prepared in embryonated chicken eggs and allowed dose-sparing (147-150, 239). For the induction of antibody titers that are considered protective (240, 241), usually two administrations of adjuvanted vaccine is required (147, 149). Of course a single immunization for the induction of protective antibody levels would be more ideal since the time that people are susceptible to infection can be limited which could save lives in case a pandemic occurs. Here we show that a

single dose of 5 µg HA of whole inactivated NIBRG-14 adjuvanted with CoVaccine HT™ induced a HI antibody response in all vaccinated mice with GMT against the homologous strain of >40, sufficient to meet the EMEA criteria for licensing (pre-pandemic) influenza vaccines (240). A second vaccination with the same dose increased the HI and VN antibody levels. The addition of the adjuvant CoVaccine HT™ not only increased the magnitude of the antibody response but also the breadth of the response, since in most vaccinated mice antibodies were detected against the influenza virus A/Indonesia/5/05 obtained from the antigenically distinct clade of A/H5N1 viruses (clade 2.1). This is of importance since it cannot be predicted which viruses ultimately may cause the next pandemic and broadly reactive antibodies may confer protection against a variety of different viruses.
In order to elucidate the mechanisms underlying the potent adjuvant effect of CoVaccine HT™, we also assessed the virus specific T cell responses. The virus-specific CD4+ T cell response induced after vaccination with WIV NIBRG-14 was analysed by intracellular cytokine staining using antibodies against IL-2, IFN-Y, IL-4 and IL-5. Mainly IL-5, IFN-γ and IL-2 producing CD4+ T lymphocytes were detected, suggesting that both T helper 1 and T helper 2 cell responses were induced. Furthermore, the virus-specific CD4+ T cell responses observed in mice vaccinated with WIV and CoVaccine HT™seemed stronger than those observed in mice vaccinated with WIV only, although this difference was not statistically significant.
Vaccination with WIV NIBRG-14 induced a modest virus-specific CD8+ T cell response as assessed by tetramer staining using H-2Db tetramers containing the $NP_{366-374}$ epitope from the vaccine backbone strain A/PR/8/34, which was still detectable 21 days after the second vaccination. Also others have demonstrated that with WIV vaccine preparation virus-specific CD8+ T cell responses can be induced (104, 242), most likely through cross-priming (139). However, compared to infection, vaccination with WIV induced virus-specific CD8+ T cell responses inefficiently and the addition of CoVaccine HT™ to WIV NIBRG-14 did not increase the response of virus specific the CD8+ T cells.

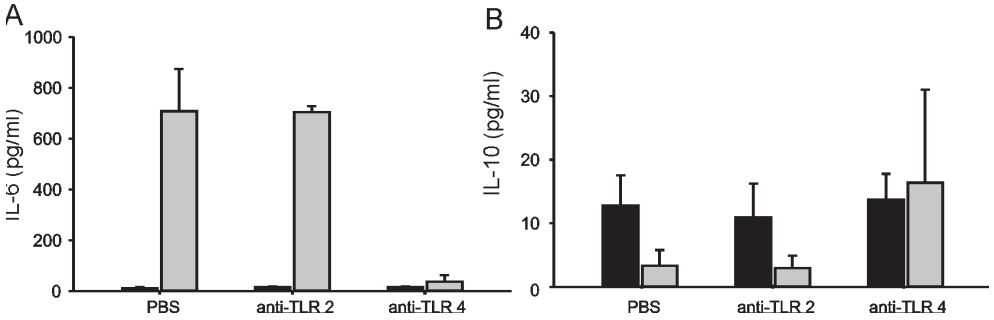

Figure 5. *Effect of blocking anti-TLR antibodies on the CoVaccine HT™-induced cytokine secretion by humans DCs. Human DCs were either incubated with PBS, anti-TLR2 or anti-TLR4 and subsequently incubated o/n with (grey bars) or without (black bars) CoVaccine HT™. The concentration of IL-6 (A) and IL-10 (B) was determined in supernatants. Indicated are mean cytokine concentrations of three human subjects with standard deviations.*

The potentating effect of CoVaccine HT™ on the induction of T helper cell responses could be explained by its effect on dendritic cells in vitro. CoVaccine HT™ induced the upregulation of CD80 and CD86 expression on murine dendritic cells and CD83 and CD86 expression on human dendritic

cells in vitro and an increase in the release of pro-inflammatory cytokine TNF-α, IL-6, IL-10, IL-12p70 and IL-1ß by murine DCs and IL-6 by human DCs. The secretion of IL-10 was reduced in hDCs incubated with CoVaccine HT™. As IL-10 is known as a major anti-inflammatory cytokine (243, 244), CoVaccine HT™ may prevent an anti-inflammatory response induced by the secretion of IL-10 by human DCs. Results of experiments with MoAbs which block the signal transduction through either TLR2 and TLR4 showed that the effect of CoVaccine HT™ on the increased secretion of IL-6 and the reduced secretion of IL-10 by human DCs was blocked by the addition of MoAbs TLR4 and not TLR2. This indicates that the effect of CoVaccine HT™ was mediated by binding to the TLR4. Interestingly, no reduced expression of CD80 and CD86 was observed by the incubation with MoAbs against TLR2 and TLR4. This indicates that the effect of CoVaccine HT on human DCs can not completely attributed to binding to TLR4. The observed release of pro-inflammatory cytokines by both murine and human DCs indicate activation of DCs via a MyD88 or Trif dependent pathway, which is characterized by expression of maturation markers and production of cytokines (245). The upregulation of the co-stimulatory molecules on professional antigen presenting cells implies that this played a role in the induction of the strong T helper cell responses that were observed after vaccination with CoVaccine HT™ adjuvanted WIV NIBRG-14.

Furthermore, intramuscular vaccination of mice with CoVaccine HT™ resulted in the enlargement of the inguinal lymph nodes eight days after vaccination. The macroscopic enlargements was associated with an increase in the numbers of various cell types, including CD4+ T cells, CD8+ T cells, DCs and B-cells as determined by flow cytometry (data not shown). This indicates that CoVaccine HT™ can provoke a pro-inflammatory response in vivo as well. Clinical dose-finding trials need to be performed to obtain information about the safety and optimal doses of CoVaccine HT™ for future use in humans.

In conclusion, the data presented here show that the use of the adjuvant CoVaccine HT™ increases the virus-specific antibody response against a whole inactivated influenza A/H5N1 antigen prepared in cell culture. The addition of CoVaccine HT™ to the vaccine slightly improved the CD4+ T cell-, but not the CD8+ T cell response and up regulated the expression of co-stimulatory molecules and the production of pro-inflammatory cytokines by dendritic cells. Since the effect on dendritic cells, which may be at the basis of the adjuvant effect of CoVaccine HT™ on T helper cell and antibody responses, was also observed with human dendritic cells, it is anticipated that the use of CoVaccine HT™ may also enhance the antibody response to vaccination with a whole inactivated influenza A/H5N1 virus vaccine in humans.

Acknowledgements

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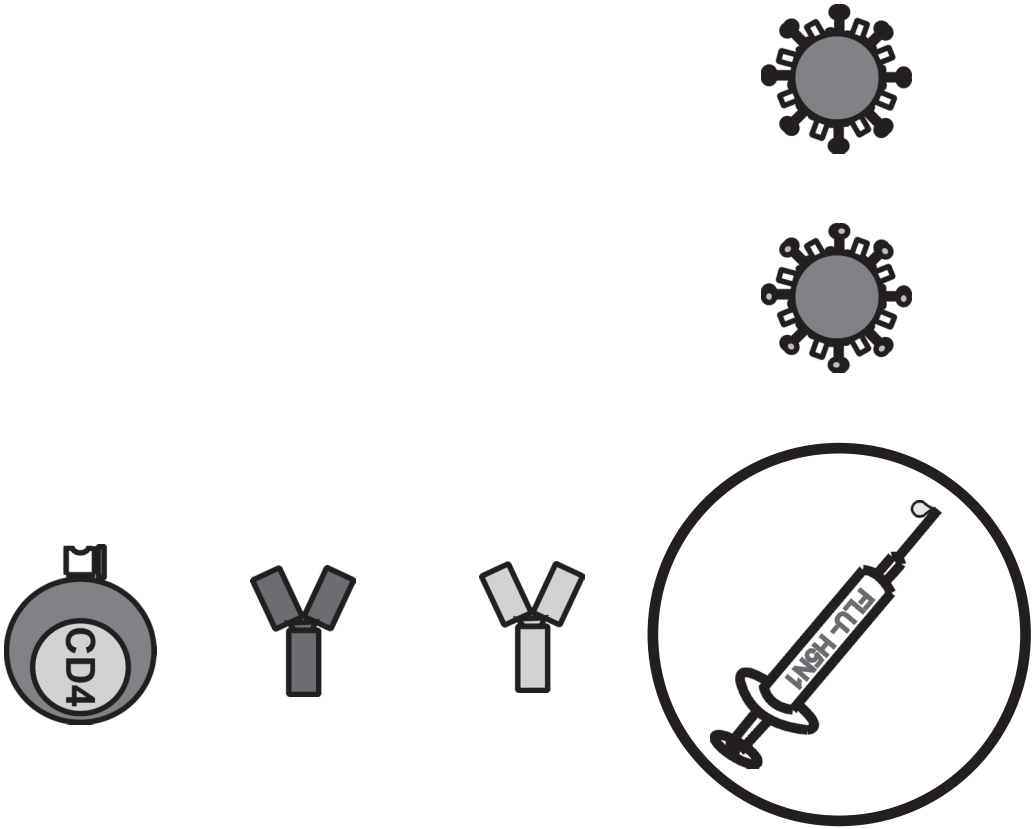
CD4
FLU-H5N1

9 A single immunization with CoVaccine HT™-adjuvanted H5N1 influenza vaccine induces protective cellular and humoral immune responses in ferrets

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ABSTRACT

Highly pathogenic avian influenza A viruses of the H5N1 subtype continue to circulate in poultry and zoonotic transmissions are reported frequently. Since a pandemic caused by these highly pathogenic viruses is still feared, there is interest in the development of influenza A/H5N1 virus vaccines that can protect humans against infection, preferably after a single vaccination with a low dose of antigen. Here we describe the induction of humoral and cellular immune responses in ferrets after vaccination with a cell-culture derived whole inactivated influenza A virus vaccine in combination with the novel adjuvant CoVaccine HT™. The addition of CoVaccine HT™ to the influenza A virus vaccine increased antibody responses to homologous and heterologous influenza A/H5N1 viruses and increased virus-specific cell-mediated immune responses. Ferrets vaccinated once with a whole-virus equivalent of 3.8 µg HA and CoVaccine HT™ were protected against homologous challenge infection with influenza virus A/VN/1194/04. Furthermore, ferrets vaccinated once with the same vaccine/adjuvant combination were partially protected against infection with a heterologous virus derived from clade 2.1 of H5N1 influenza viruses. Thus, the use of the novel adjuvant CoVaccine HT™ with cell culture derived inactivated influenza A/H5N1 antigen is a promising and dose sparing vaccine approach warranting further clinical evaluation.

INTRODUCTION

Since the first human case of infection with a highly pathogenic avian influenza A virus of the H5N1 subtype in 1997 (17, 18, 103) hundreds of zoonotic transmissions have been reported with a high case-fatality rate (17, 246). Since these viruses continue to circulate among domestic birds and human cases are regularly reported, it is feared that they will adapt to their new host or exchange gene segments with other influenza A viruses, become transmissible from human-to-human and cause a new pandemic. Recently, a novel influenza A virus of the H1N1 subtype emerged. This virus, which originated from pigs, was transmitted between humans efficiently, resulting in the first influenza pandemic of the 21st century (247, 248). Although millions of people have been inoculated with the (H1N1) 2009 virus, compared to infections with the H5N1 viruses, the case-fatality rate was relatively low (249, 250). However, the unexpected pandemic caused by influenza A/H1N1(2009) viruses, has further highlighted the importance of rapid availability of safe and effective pandemic influenza vaccines. Other key issues for the development of pandemic influenza A virus vaccines include optimal use of the existing (limited) production capacity of viral antigen and effectiveness against viruses that are antigenically distinct. Ideally, a single administration of a low dose of antigen would be sufficient to induce protective immunity against the homologous strain and heterologous antigenic variant strains. However, since the population at large will be immunologically naïve to a newly introduced virus, high doses of antigen are required to induce protective immunity in un-primed subjects (149, 226). The use of safe and effective adjuvants in pandemic influenza vaccines is considered as a dose-sparing strategy. Clinical trials evaluating candidate inactivated influenza A/H5N1 vaccines showed that the use of adjuvants can increase their immunogenicity and broaden the specificity of the induced antibody responses (147-150, 226, 239, 251). These research efforts have resulted in the licensing of adjuvanted vaccines against seasonal and pandemic influenza (252). The protective efficacy of immune responses induced with candidate influenza A/H5N1 vaccines was demonstrated in ferrets after two immunizations (68, 253-255) or after a single immunization. The latter was achieved with a low dose of antigen in combination with the adjuvant ISCOMATRIX (256).

Recently, a novel adjuvant has been developed that consists of a sucrose fatty acid sulphate ester (SFASE) immobilized on the oil droplets of a submicron emulsion of squalane-in-water (228). It has been demonstrated that the addition of this novel adjuvant, called CoVaccine HT™, to multiple antigens increased the immune response to these antigens in pigs and horses and was well tolerated in both-species (228-230). Furthermore, it was shown that the use of CoVaccine HT™ increased the virus-specific antibody responses in mice and ferrets after vaccination with a cell culture-derived whole inactivated influenza A/H5N1 virus vaccine (231, 257). One of the mode of actions of CoVaccine HT™ is the activation of antigen presenting cells like dendritic cells, most likely through TLR-4 signaling (257).

In the present study, we evaluated the protective potential of CoVaccine HT™-adjuvanted cell-culture derived whole inactivated influenza A/H5N1 (WIV) vaccine in the ferret model, which is considered the most suitable animal model for the evaluation of candidate influenza vaccines (166, 258, 259). To this end, ferrets were vaccinated once or twice with various antigen doses with or without

the adjuvant to test if dose-sparing could be achieved. The use of CoVaccine HT™ increased virus specific antibody responses and T cell responses. A single administration of 3.8µg hemagglutinin of WIV NIBRG-14 vaccine-preparation in combination with CoVaccine HT™ conferred protection against challenge infection with the homologous highly pathogenic A/H5N1 strain A/VN/1194/04 and partial protection against infection with a heterologous, antigenically distinct strain, A/IND/5/05. Therefore, it was concluded that the use of CoVaccine HT™ in inactivated influenza vaccines induced protective virus specific humoral and cell mediated immune responses and could be suitable as adjuvant in (pre)pandemic A/H5N1 vaccines. Further clinical testing of these candidate vaccines seems warranted.

MATERIALS AND METHODS

Vaccine and adjuvant formulations

The vaccine strain NIBRG-14 (H5N1), based on strain A/Vietnam/1194/04 (H5N1, clade 1) was used to prepare a whole inactivated influenza A virus (WIV NIBRG-14) vaccine. The vaccine was produced in Madin-Darby Canine Kidney (MDCK; European Collection of Cell Cultures, London, UK) cells and inactivated using beta-propiolactone on a commercial scale by Nobilon Schering-Plough (Boxmeer, The Netherlands). The hemagglutinin content was assessed by a single radial immunodiffusion assay according to standard methods (233). CoVaccine HT™ is a proprietary adjuvant of Protherics Medicines Development Ltd., a BTG Company London, United Kingdom. The optimal dose of CoVaccine HTTM in ferrets was determined by Nobilon Schering-Plough, Boxmeer, The Netherlands before the start of this experiment (data not shown).

Influenza A viruses

Virus stocks of influenza viruses A/Vietnam/1194/2004 (H5N1, clade 1) and A/Indonesia/5/2005 (H5N1, clade 2.1) were prepared by propagation in confluent MDCK cells. After cytopathologic changes were complete, culture supernatants were cleared by low speed centrifugation and stored at −70°C. Infectious virus titers were determined in MDCK cells as described previously (85). All experiments with these viruses were performed under Bio Safety Level (BSL)-3 conditions.

Ferrets

Healthy young adult outbred female ferrets (Mustela putorius furo; between 6 and 12 months old) were purchased from a commercial breeder. Ferrets were screened for the presence of antibodies against circulating influenza A/H1N1 and A/H3N2 viruses and the influenza A/H1N1(2009) virus by hemagglutination inhibition assay. Ferrets that were tested negative for these viruses were used in this experiment. An independent animal ethics committee (DEC consult) approved the experimental protocol before the start of the experiments.

Immunizations and inoculations

Thirty-six sero-negative ferrets were divided into six groups of 6 ferrets and vaccinated with either one dose of 3.8 µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE;

group S3.8+), one dose of 15µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE; group S15+), two doses of 3.8 µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE; group T3.8+), two doses of 3.8 µg HA-content of the WIV NIBRG-14 only (group T3.8-), two doses of phosphate buffered saline (group PBS), or two doses of CoVaccine HT™ (2 mg SFASE; Cve). Groups are listed in table 1. Vaccinations were performed under anesthesia with ketamine in the quadriceps muscles of the left hindleg in a total volume of 0.5 ml. Ferrets that received two immunizations were vaccinated with an interval of four weeks, while ferrets that received only one immunization were vaccinated with PBS at the moment of the first vaccination of ferrets receiving two vaccinations. During vaccination experiments, ferrets were housed in groups and received food and water ad-libitum. Four weeks after the (last) immunization, the animals were anesthesized with ketamine/medetomidine (reversed with atipamezole), weighed and subsequently inoculated intratracheally with $1x10^5$ $TCID_{50}$ influenza A/VN/1194/04 (H5N1) virus in a total volume of 3 ml PBS. After inoculation, ferrets were monitored three times daily for the development of clinical signs. Before inoculation and two and four days after inoculation, throat swabs of each ferret were collected while the ferrets were anesthesized with ketamine. Four days after inoculation, animals were weighed and subsequently killed by exsanguination while under anesthesia with ketamine and medetomidine. In previous experiments, it has been demonstrated that inoculated ferrets start to lose weight as early as one day after infection and continue to lose weight as disease progresses (166, 167). It was anticipated that also during the course of our experiments the maximum weight loss was at day 4 post inoculation and therefore the difference in body weight between the day of inoculation and 4 days post inoculation was considered to be a good clinical indicator for protection against the development of disease.

Necropsies were performed according to standard procedures. After finishing this experiment, the experiment was repeated following exactly the same procedure (n=6 animals per group) except that ferrets were challenged with $1x10^5$ $TCID_{50}$ of influenza A/IND/5/05 (H5N1) virus. Because no differences were observed between mock-vaccinated ferrets and ferrets vaccinated with CoVaccine HT only (group Cve) in the first experiment, the latter group was omitted in the second experiment to reduce the number of animals. One ferret of the group that was vaccinated twice with 3.8 µg HA of WIV NIBRG-14 (T3.8-) died between the second vaccination and inoculation with influenza virus A/IND/5/05 due to reasons unrelated to the experiment.

Table 1. Overview of vaccination regimens in this study

Group	Number of immunizations	WIV NIBRG-14 (µg HA-content)	CoVaccine HT™ (2mg SFASE)
S3.8+	1	3.8	+
S15+	1	15	+
T3.8+	2	3.8	+
T3.8-	2	3.8	-
PBS	2	-	-
CVe	2	-	+

Serology

Serum samples were collected before, twenty-eight days after the first and twenty-eight days after the second vaccination. Sera were stored at –20°C until use. Sera were tested for the presence of anti-HA antibodies using a hemagglutination inhibition assay (HI-assay) with 1% turkey erythrocytes and for the presence of virus neutralizing antibodies using a micro virus neutralization assay (VN-assay) as described previously (87, 107). Sera were tested for the presence of antibodies reactive with influenza A/H5N1 viruses A/VN/1194/04 and A/IND/5/05. For this purpose, reverse genetics viruses of both viruses were produced. The titers obtained with these viruses were comparable with those against the wild-type strains (data not shown). A hyperimmune rabbit serum to influenza strain A/Tern/South Africa/1/63 and serum obtained from a swan immunized twice with inactivated H5N1 influenza virus A/Duck/Potsdam/1402/86 (Intervet, Boxmeer, The Netherlands) (89) were used as positive control sera specific for influenza viruses A/VN/1194/04 and A/IND/5/05, respectively .

T cell proliferation assay

Blood samples were collected from the jugular vein of ferrets before, twenty-eight days after the first vaccination and twenty-eight days after the second vaccination in EDTA-tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and then cryopreserved at -135°C until use. Thawed PBMC were washed twice with PBS, labeled with 0.3µM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS (Invitrogen, Breda, The Netherlands) for five minutes at 37°C, washed twice and subsequently cells were resuspended in RPMI 1640 (Cambrex, East Rutherford, USA) containing 10% (v/v) fetal calf serum, penicillin (100 µg/ml), streptomycin (100 U/ml), L-glutamine (2mM). Cells were seeded (105 cells per well) in a 96-well round-bottom plate in the presence or absence of WIV NIBRG-14 (200ng HA content) or phytohaemagglutinin (PHA) (1 µg/ml) and incubated at 37°C/5% CO_2 for six days. For each condition duplicate samples were tested. Two days after stimulation, 100µl supernatant of Concanavalin A-stimulated ferret lymph node cells was added, which was prepared essentially as described previously (160). After the remaining four days of incubation, cells were transferred to a 96-wells V-bottom plate, washed and subsequently stained with a monoclonal antibody directed to human CD8 (OKT-8)-Pacific Blue (eBioscience, San Diego, USA). To exclude dead cells in the analysis, cells were also stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, The Netherlands). Cells were subsequently fixed and permeabilized with Cytofix and Cytoperm (BD Pharmingen, Alphen a/d Rijn, The Netherlands) and stained with a Alexa Fluor 647-labelled monoclonal antibody specific for human CD3 (PC3/188A) (Santa Cruz Biotechnology, Santa Cruz, USA). It has been demonstrated that these CD3 and CD8-specific monoclonal antibodies are cross-reactive with ferret CD3 and CD8 (260, 261). Data were acquired using a FACSCanto-II and analysed with FACS Diva software (BD). The proliferation of PBMC from at least 9 ferrets of each group was assessed in this assay, except for group Cve, since this group only contained six ferrets. The proliferation of CD3+CD8- cells was calculated by subtracting the mean number of CD3+CD8-CFSElow cells of the medium only controls from the mean number of CD3+CD8-CFSElow cells stimulated with WIV NIBRG-14. The same calculation was performed for

CD3+CD8+ cells.

Virus titers of lungs and throat swabs

Samples of all lobes of the right lung and the accessory lobe of inoculated ferrets were collected and snap frozen on dry ice with ethanol and stored at –70°C until further processing. Lung samples were weighed and subsequently homogenized with a FastPrep-24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, The Netherlands) and centrifuged briefly.
After collection of throat swabs, swabs were stored at -70°C in the same medium as used to homogenize lung samples. Quadruplicate 10-fold serial dilutions of both throat and lung samples were used to infect MDCK cells as described previously (85). HA activity of the culture supernatants collected 5 days post infection was used as indicator of infection. The titers were calculated according to the Spearman-Karber method and expressed as log $TCID_{50}$ per gram for lung tissue or per milliliter (ml) for swabs (108).

Histopathology and immunohistochemistry

Four days after inoculation with influenza A/VN/1194/04 and A/IND/5/05 virus, ferrets were euthanized and lungs were observed macroscopically and weighed before samples from the right lungs were collected to determine the virus titers. Subsequently left lung lobes were inflated with 10% neutral buffered formalin. After fixation and embedding in paraffin, lungs were sectioned at 4 µm and tissue sections were examined by staining with hematoxylin and eosin (HE). Using an immunoperoxidase method, serial lung tissue sections were also stained with a monoclonal antibody directed against the nucleoprotein of the influenza A virus (110).

Statistical analysis

The presence of overall statistical significant differences between groups regarding weight loss, the number of CFSElow cells, percentage of affected areas in the lungs, viral load in the lungs and throat swab specimens was calculated using the Kruskal-Wallis test. Before individual When calculated P values were less than an α of 0.05, data of each group was individually compared with data from the PBS group using the Games-Howell test. Differences were considered significant when P values were less than an α of 0.05. The number of CFSElow cells were converted to ^{10}log values before statistical analysis was performed.

RESULTS

Antibody responses after vaccination

To assess antibody responses after vaccination with various NIBRG-14 antigen/CoVaccine HT™ combinations, pre- and post-vaccination ferret sera were tested for the presence of antibodies against influenza viruses A/VN/1194/04 and A/IND/5/05 by HI and VN-assay. Like in mock-vaccinated ferrets (group PBS) and ferrets receiving CoVaccine HT™ only (group Cve), virus-specific antibodies were

not detectable by HI or VN assay in ferrets that received two vaccinations with 3.8µg un-adjuvanted WIV NIBRG-14 (group T3.8-) (Table 2). In contrast, a single immunization with as little as 3.8 µg HA of WIV NIBRG-14 adjuvanted with CoVaccine HT™ (group S3.8+) induced HI and VN antibodies against the homologous virus in seven and eight out of twelve animals respectively. The geometric mean antibody titers were low (24 and 9, respectively) and only in a small number of ferrets, antibodies against the heterologous virus A/IND/5/05 were detected. Increasing the antigen dose did not improve the serological outcome of vaccination. Five out of 12 ferrets receiving a single dose of 15 µg WIV NIBRG-14 with CoVaccine HT™ (group S15+), developed antibodies against influenza virus A/VN/1194/04. The serum of one of these animals displayed cross-reactivity with the heterologous influenza virus A/IND/5/05.

All twelve ferrets that received two immunizations of 3.8µg HA of NIBRG-14 with CoVaccine HT™ (group T3.8+) developed antibodies against influenza virus A/VN/1194/04 as detected by HI- (GMT 86) and the VN assay (GMT 65) 28 days after the second immunization. The serum antibodies of these twelve animals cross-reacted with the heterologous influenza virus A/IND/5/05 as detected by the VN assay (GMT 38). By HI assay, cross-reactive antibodies were detected in six out of the twelve serum samples (GMT 30).

Table 2. Antibody responses four weeks after the second immunization

Group	Antibody titers against A/VN/1194/04				Antibody titers against A/IND/5/05			
	HI GMT (sd)	Responders	VN GMT (sd)	Responders	HI GMT (sd)	Responders	VN GMT (sd)	Responders
S3.8+	24 (22)	7/12	9 (4)	8/12	<10	1/12	<10	2/12
S15+	13 (12)	5/12	9 (6)	5/12	<10	1/12	<10	1/12
T3.8+	86 (21)	12/12	65 (23)	12/12	30 (30)	6/12	38 (21)	12/12
T3.8-	<10	0/11	<10	0/11	<10	0/11	<10	0/11
PBS	<10	0/12	<10	0/12	<10	0/12	<10	0/12
Cve	<10	0/6	<10	0/6	<10	0/6	<10	0/6

Vaccine-induced T cell responses

To assess the induction of T cell immunity after vaccination, PBMC of the ferrets collected before the first vaccination, 28 days after the first and 28 days after the second vaccination were labeled with CFSE and subsequently stimulated with WIV NIBRG-14 antigen, PHA or were left untreated (medium control). The number of CSFE low cells was determined in the CD3+CD8- and CD3+CD8+ cell population as shown in figure 1A.

Viral antigen specific proliferation was virtually undetectable with PBMC collected before the immunization of the ferrets. PBMC obtained from mock-vaccinated ferrets or from those vaccinated with CoVaccine HT™ only responded to stimulation with NIBRG-14 antigen very poorly. In contrast, PBMC obtained from ferrets vaccinated with NIBRG-14 proliferated upon in vitro re-stimulation

with viral antigen, whereas CSFElow cells were virtually absent in un-stimulated PBMC obtained from any of the ferrets (medium controls). The latter numbers were substracted from the number of antigen or PHA-stimulated PBMC to asses the number of cells that proliferated upon stimulation. Increased numbers of CFSElow CD3+CD8- cells and CFSElow CD3+CD8+ cells were observed in PBMC of ferrets after stimulation with PHA compared to the stimulation with medium only. Furthermore, the numbers of CFSElow CD3+CD8- and CFSElow CD3+CD8+ cells after PHA stimulation did not differ between ferrets from groups S3.8+, S15+, T3.8+, T3.8- and Cve in mock-vaccinated ferrets (data not shown).

Antigen-specific proliferation of CD3+CD8- cells was observed in PBMC collected from ferrets of groups S3.8+, S15+, T3.8+ and T3.8-. The in vitro proliferative response of ferrets from groups S3.8+, S15+ and T3.8+ was significantly stronger than that of mock-vaccinated animals (p-values below 0.01) for all three groups compared to mock-vaccinated group, while differences between ferrets vaccinated twice with WIV NIBRG-14 without CoVaccine HT™ and mock-vaccinated animals approached statistical significance (p=0.08). In addition, the in vitro proliferative response of CD3+CD8- cells obtained from ferrets that received two immunizations of 3.8 µg HA of WIV NIBRG-14 with CoVaccine HT™ compared to ferrets that received 3.8 µg HA of WIV NIBRG-14 twice without CoVaccine HT™ also approached statistical significance (p=0.06) (Fig 1B). With PBMC obtained from ferrets of groups S15+ and T3.8+, a virus-specific proliferative response of CD3+CD8+ cells was observed (Fig 1C), that was significantly stronger than that of mock-vaccinated ferrets (p-values of respectively 0.03 and 0.01). Furthermore, proliferative responses of CD3+CD8+ cells obtained from ferrets that received two immunizations of 3.8 µg HA of WIV NIBRG-14 with CoVaccine HT™ was significantly stronger than that of ferrets that received 3.8 µg HA of WIV NIBRG-14 twice without CoVaccine HT™ (p=0.03). Like CD3+CD8+ cells obtained from mock-vaccinated ferrets, also those from animals vaccinated with CoVaccine HT™ only responded to NIBRG-14 antigen poorly.

Clinical signs after inoculation with influenza viruses A/VN/1194/04 and A/IND/5/05

From day 3 post inoculation with influenza viruses A/VN/1194/04 and A/IND/5/05 onwards, severe clinical signs were observed in mock-vaccinated ferrets and those vaccinated with CoVaccine HT™ only (group Cve) or with WIV NIBRG-14 only (group T3.8-), and included breathing difficulties, lethargy, decreased appetite and weight loss.

In contrast, only mild clinical signs were observed after inoculation with influenza virus A/VN/1194/04 in ferrets immunized once with 3.8 µg NIBRG-14 and CoVaccine HT™ (S3.8+), once with 15 µg NIBRG-14 and CoVaccine HT™ (S15+) or twice with 3.8 µg NIBRG-14 and CoVaccine HT™ (T3.8+). In these groups moderate clinical signs were observed after inoculation with influenza virus A/IND/5/05 (H5N1). One ferret vaccinated with CoVaccine HT™ died in the night between day three and four post inoculation with influenza virus A/VN/1194/04 and one mock-vaccinated ferret succumbed two days post inoculation with influenza virus A/IND/5/05. No mortality was observed in other groups. The relative loss in body weight four days post inoculation was calculated for each ferret. The mean weight loss of each group was used as a quantative clinical indicator of disease upon inoculation with influenza viruses A/VN/1194/04 and A/IND/5/05. Following inoculation with influenza virus A/VN/1194/04 (Fig 2A), all ferrets lost body weight, except one animal vaccinated

twice with 3.8 µg NIBRG-14 and CoVaccine HT™. The highest mean weight loss was observed in ferrets of groups S15+ (12%), T3.8- (11%), PBS (11%) and Cve (13%), while ferrets of groups S3.8+ and T3.8+ lost 3% and 2% of their body weight respectively, significantly less than mock-vaccinated ferrets (p-values respectively 0.02 and 0.04). After inoculation with influenza virus A/IND/5/05 (Fig 2B), all ferrets lost body weight. The mean weight loss of ferrets in groups PBS (mock-vaccinated), T3.8- , S3.8+, S15+, T3.8+ was 13%, 14%, 9%, 10% and 8%, respectively. No statistically significant differences were observed between groups.

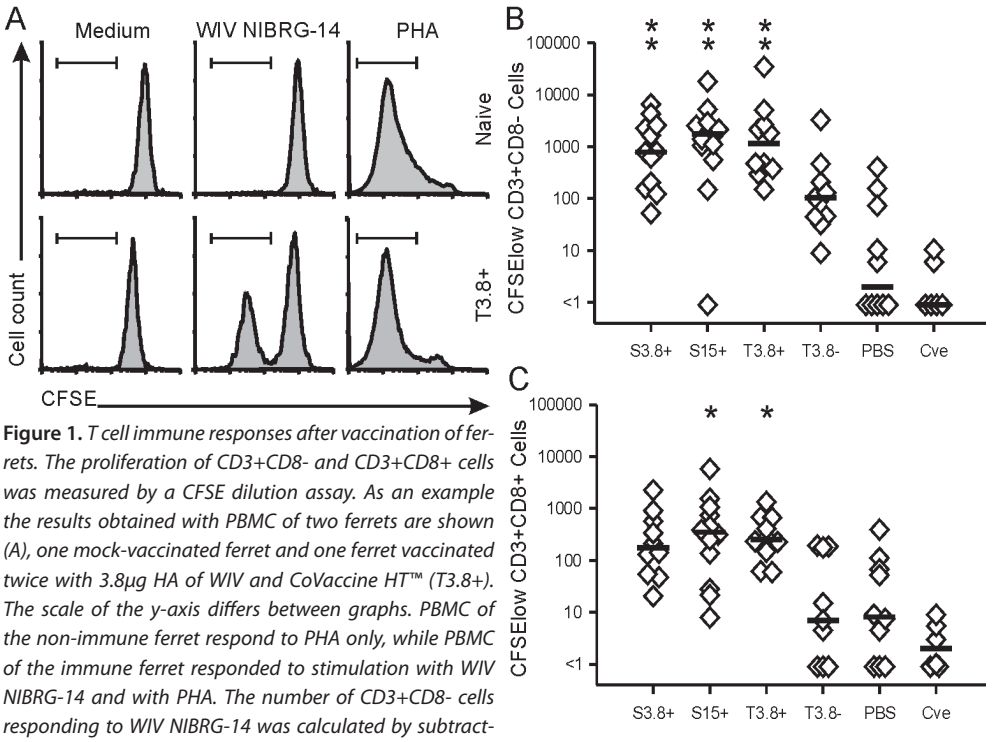

Figure 1. *T cell immune responses after vaccination of ferrets. The proliferation of CD3+CD8- and CD3+CD8+ cells was measured by a CFSE dilution assay. As an example the results obtained with PBMC of two ferrets are shown (A), one mock-vaccinated ferret and one ferret vaccinated twice with 3.8µg HA of WIV and CoVaccine HT™ (T3.8+). The scale of the y-axis differs between graphs. PBMC of the non-immune ferret respond to PHA only, while PBMC of the immune ferret responded to stimulation with WIV NIBRG-14 and with PHA. The number of CD3+CD8- cells responding to WIV NIBRG-14 was calculated by subtracting the mean number of CD3+CD8-CFSElow cells incubated with medium from the mean number of CD3+CD8-CFSElow cells incubated with WIV NIBRG-14 (B). The same procedure was used to calculate the number of responding CD3+CD8+ cells (C). The number of ferrets of each group tested in this assay differed between the respective groups, group Cve (n=6), group T3.8- (n=9), groups T3.8+ and PBS (n=10) and groups S3.8+ and S15+ (n=12). Significant differences compared to the mock-vaccinated group are indicated by one (p<0.05) or two asterisks (p<0.01).*

Virus titers in pharyngeal swabs

To assess the extent of virus replication in the upper respiratory tract after inoculation with both influenza A/H5N1 viruses, pharyngeal swabs were collected before and on day 2 and 4 after inoculation and tested for the presence of virus. No virus was detected in any of the swabs collected before inoculation (data not shown). On day 2 and 4 post inoculation with influenza virus A/VN/1194/04, virus was not detected in pharyngeal swabs obtained from ferrets of groups 3.8+, S15+ and T3.8+, while those obtained from ferrets of T3.8-, PBS and Cve all tested positive at day 2 with mean virus titers of $10^{4.4}$, $10^{3.7}$ and $10^{3.4}$ $TCID_{50}$/ml respectively (Fig 3A). Four days post inoculation these swabs

also tested positive with mean virus titers of $10^{4.0}$, $10^{4.9}$, and $10^{5.1}$ $TCID_{50}$/ml, respectively. Following inoculation with influenza virus A/IND/5/05, similar results were obtained. Two and four days post inoculation, no virus was detected in pharyngeal swabs collected from ferrets of groups S3.8+ and T3.8+, while virus was detected in only one ferret of group S15+ on day 2 and 4 post inoculation. All swabs obtained from ferrets of groups T3.8- and PBS tested positive with mean virus titers of $10^{3.9}$ and $10^{4.3}$ TCID50/ml two days post inoculation and $10^{4.1}$ and $10^{5.5}$ TCID50/ml four days post inoculation, respectively (Fig 3B).

Virus detection in the lungs

Lungs of ferrets euthanized four days after inoculation and the lungs of the mock-vaccinated ferret that died two days after inoculation were tested for the presence of infectious virus. After inoculation with influenza virus A/VN/1194/04, virus was detected in lungs of all ferrets of the T3.8-, PBS and Cve groups with mean titers of $10^{5.5}$, $10^{6.7}$, $10^{6.5}$ $TCID_{50}$/gram lung respectively (Fig 3C). The mean virus titer in lungs of ferrets vaccinated twice with 3.8 µg of WIV NIBRG-14 only were (10-fold) lower than mock-vaccinated ferrets, although differences were not significant (p=0.17). In contrast, after inoculation with influenza virus A/VN/1194/04, no virus was detected in lungs of ferrets vaccinated once or twice with 3.8 µg WIV NIBRG-14 and CoVaccine HT™ (S3.8+ and T3.8+), while in only two out of six ferrets vaccinated with 15 µg WIV NIBRG-14 and CoVaccine HT™ (S15+) low titers were detected in the lungs, which was significantly lower than mock-vaccinated animals ($p<0.01$ for all three groups). After inoculation with influenza virus A/IND/5/05, lungs of all ferrets tested positive by virus isolation, except for one ferret of group T3.8+. The virus titers in lungs of ferrets of group T3.8- were not significantly different from those in lungs of mock-vaccinated ferrets (p=0.99) (titers of $10^{7.4}$ and $10^{7.0}$ TCID50/gram lung respectively). In contrast, the mean lung virus titers of ferrets of groups S3.8+ ($10^{4.7}$ TCID50/gram), S15+ ($10^{5.4}$ TCID50/gram) and T3.8+ ($10^{3.8}$ TCID50/gram) were significantly lower ($p<0.01$, $p<0.01$ and p=0.03 respectively) than those of mock-vaccinated ferrets and ferrets of group S3.8- (Fig 3D).

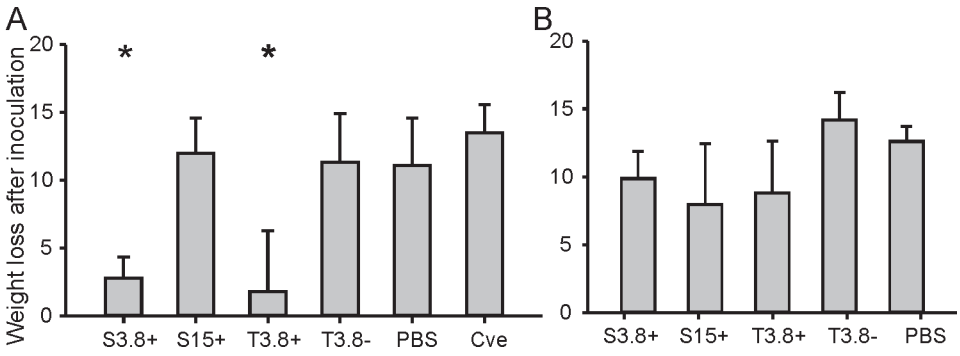

Figure 2. *Weight loss after inoculation with influenza A/VN/1194/04 and A/IND/5/05 viruses. The loss of body weight four days p.i. was determined for each ferret relative to the body weight at the day of inoculation (%). For each group, the mean weight loss ± s.d. after inoculation with influenza A/VN/1194/04 (A) and A/IND/5/05 (B) was calculated. Bars represent mean weight loss of each group with standard deviations. Significant differences compared to the mock-vaccinated group are indicated by an asterisk (p<0.05).*

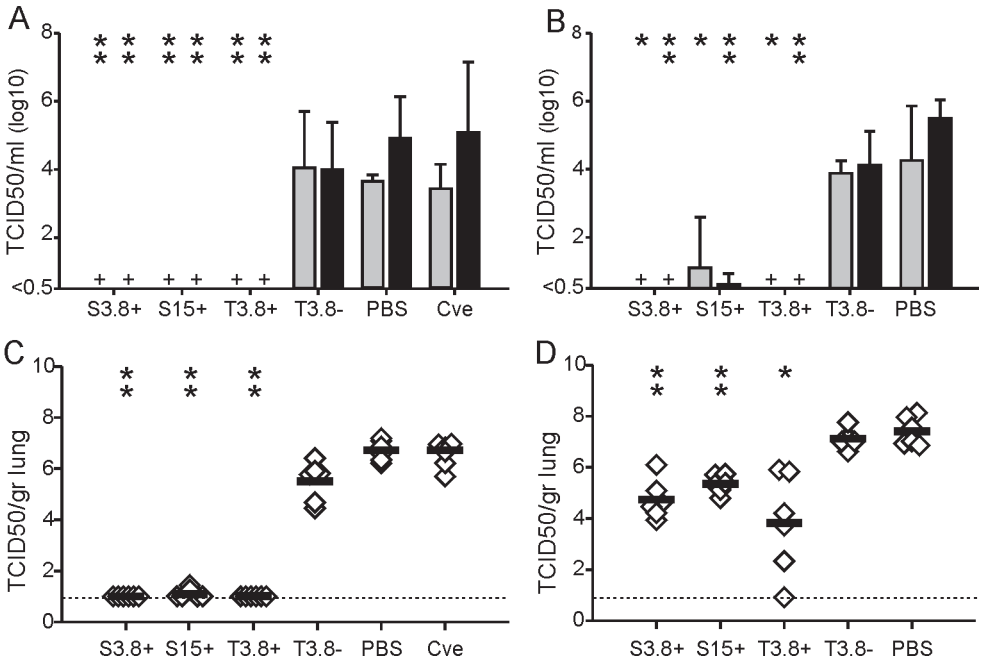

Figure 3. *Virus titers of pharyngeal swabs and lungs after inoculation with influenza A/VN/1194/04 and A/IND/5/05 virus. Virus titers were determined in pharyngeal swabs obtained 2 (grey bars) and 4 days (black bars) after inoculation with influenza A/VN/1194/04 (A) and influenza A/IND/5/05 (B). Virus titers are expressed as TCID50 per milliliter (log10). The plus sign indicates that no virus was detected in pharyngeal swabs obtained from ferrets of groups S3.8+, S15+ and T3.8+ after inoculation with influenza A/VN/1194/04 virus and from ferrets of groups S3.8+ and T3.8+ after inoculation with influenza A/IND/5/05 virus resulting in a mean virus titer below the cut-off value. Lung virus titers were determined four days after inoculation with influenza A/VN/1194/04 (C) and influenza A/IND/5/05 (D) virus. Virus titers are expressed as the TCID50 per gram lung (log10). The dotted line indicates the cut-off value for obtaining a positive result. Significant differences compared to the mock-vaccinated group are indicated by one ($p<0.05$) or two asterisks ($p<0.01$).*

9

Gross pathologic findings in the lungs of inoculated ferrets

Four days after inoculation with influenza viruses A/VN/1194/04 or A/IND/5/05, ferrets were euthanized and lungs were examined macroscopically and weighed before assessing virus replication and histopathological changes. Macroscopically, dark red and firm consolidated areas were present in lungs of inoculated animals. The percentage of affected lung tissue was estimated and varied significantly between different groups of animals. In lungs obtained from ferrets of groups S3.8+, S15+ and T3.8+ affected areas were significantly smaller than in lungs of mock-vaccinated animals (p-values for all groups <0.01) (Fig 4A and 4B). The extent of consolidation was more pronounced in lungs of ferrets from group S15+ than in those from group S3.8+, especially after inoculation with influenza virus A/IND/5/05, although no significant differences were present between these two groups (p=0.09).

After inoculation with influenza A/VN/1194/04 virus, the mean relative lung weight was highest in groups T3.8- (1.6% of body weight ± 0.4), PBS (1.8% ± 0.2), Cve (1.7% ± 0.2), while that of groups S3.8+ (1.0 ± 0.2), S15+ (1.0 ± 0.2) and T3.8+ (1.0 ± 0.1) was significant lower than that of mock-vacci-

nated ferrets (p-values for all groups <0.01) (Fig 4C) Similar results were observed after inoculation with influenza virus A/IND/5/05. Again, the highest relative lung weight was observed in groups T3.8- (2.0 ± 0.3) and PBS (1.7 ± 0.2). The mean relative lung weight of groups S3.8+ (1.1 ± 0.1), S15+ (1.3 ± 0.1) and T3.8+ (1.1 ± 0.1) was significant lower (p-values <0.01, 0.03 and 0.01 respectively) than that of mock-vaccinated ferrets (Fig 4D).

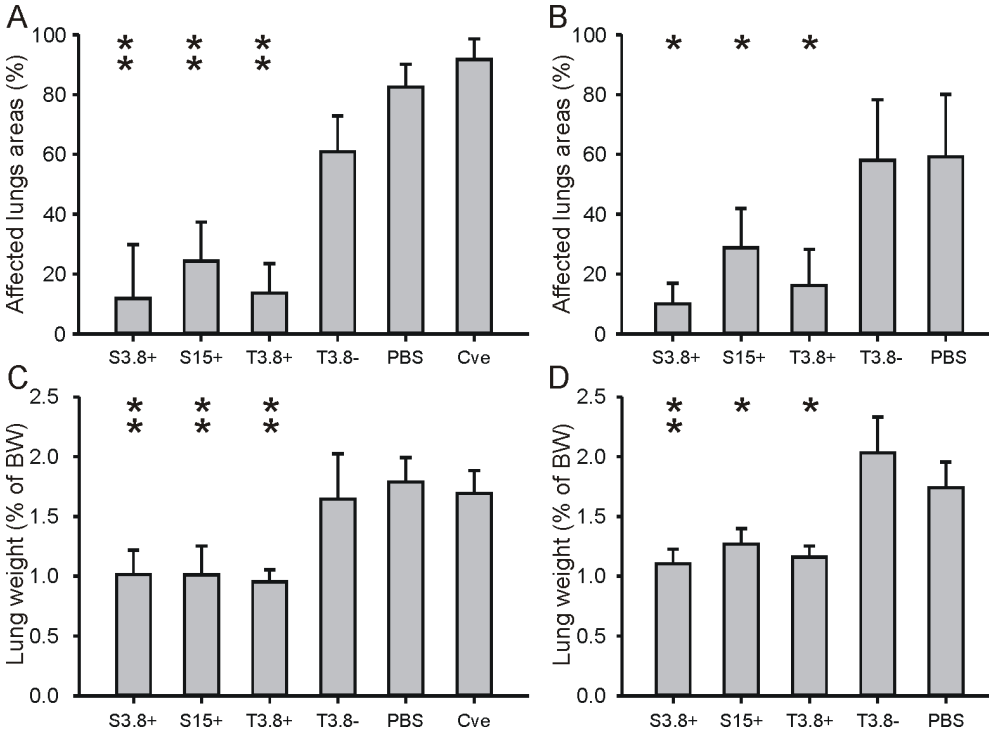

Figure 4. *Gross pathological changes after inoculation with influenza A/H5N1 viruses. The percentage lung tissue displaying consolidation at necropsy of the ferrets was estimated for all ferrets of each group after inoculation with influenza virus A/VN/1194/04 (A) and A/IND/5/05 (B). Lungs of all ferrets of each group were also weighed after necropsy and the weight of the lungs was related to the body weight of the ferrets four days after inoculation which is shown as a percentage for ferrets inoculated with influenza virus A/VN/1194/04 (C) and A/IND/5/05 (D). Bars represent relative lung weight of each group with standard deviations (BW =body weight). Significant differences compared to the mock-vaccinated group are indicated by one (p<0.05) or two asterisks (p<0.01).*

Histopathologic findings in lungs after inoculation

Upon inoculation with either influenza virus A/VN/1194/04 or A/IND/5/05, ferrets of groups T3.8-, PBS and Cve developed a moderate to severe broncho-interstitial pneumonia. Multifocally in the alveoli, the lumina contained many macrophages and neutrophils mixed with variable numbers of erythrocytes, edema fluid and fibrin; the alveolar septa also contained many macrophages and neutrophils and showed moderate hypertrophy and hyperplasia of type II pneumocytes (Fig 5A and B). Multifocally in the bronchioles and bronchi, the walls had loss of epithelium and the lumina contained cell debris. In addition, the walls of bronchioles, and bronchi contained multifocal many macrophages and neutrophils. A few macrophages, lymphocytes, plasmacytes, and occasional

neutrophils were present in perivascular and peribronchiolar tissue (Fig 5C).
In contrast, upon inoculation with either of the two above viruses, ferrets of groups S3.8+, S15+ and T3.8+ developed only a mild to moderate broncho-interstitial pneumonia. Compared to the above lesion, fewer and smaller areas of lung parenchyma were affected, there were fewer inflammatory cells in alveoli and bronchioles, and there was less severe edema (Fig 5D). However, there was more prominent peribronchiolar and perivascular, and also peribronchial infiltration with many lymphocytes and plasmacytes and occasional macrophages and neutrophils. Also, bronchus-associated lymphoid tissue was present in the lungs of these ferrets Fig 5E and F). Upon infection with either of the two viruses, ferrets in the S15+ group had more severe broncho-interstitial pneumonia than ferrets in S3.8+ and T3.8+ groups. This increased severity was characterized by both a greater extent of necrosis and inflammation, and by a higher number of inflammatory cells.

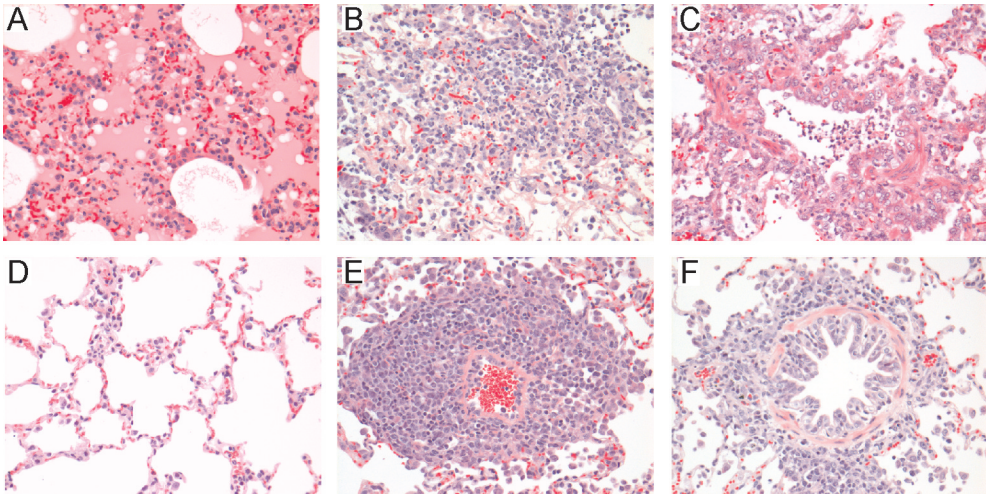

Figure 5. *Examples of histopathologic findings in lungs after inoculation with influenza A/VN/1194/04 and A/IND/5/05. Histopathologic changes in the lungs of ferrets of groups T3.8-, Cve and PBS inoculated with either influenza virus A/VN/1194/04 or influenza A/IND/5/05 included multiple large areas of severe alveolitis with proteinaceous fluid (edema) (A) and the presence of an inflammatory infiltrate consisting mainly of neutrophils and macrophages, cellular debris and erythrocytes (B). In the bronchioles, there was multifocal loss of epithelial cells and cellular debris (C). In lungs of ferrets of groups S3.8+, S15+ and T3.8-, only a mild to moderate alveolitis was observed with inflammatory infiltrate mainly in the alveolar septa (D). Perivascular (E) and peribronchiolar (F), infiltrates were present consisting mainly of lymphocytes and plasma cells and some macrophages and neutrophils. Pictures were made from lungs of ferrets inoculated with influenza A/VN/119/04. H&E staining, magnification 20x.*

Detection of virus-infected cells by immunohistochemistry

By immunohistochemistry, influenza virus antigen expression of cells in the lung tissue was assessed. After inoculation with influenza A/VN/1194/04 virus, numerous virus-infected cells were present in the lungs of mock-vaccinated ferrets or ferrets vaccinated with 3.8µg HA WIV NIBRG-14 only or CoVaccine HT™ only (Fig 6A and B). The infected cells were predominantly observed in affected areas of the lungs. Inoculated cells were only found sporadically in the lungs of ferrets of groups S3.8+, S15+ and T3.8+ (Fig 6C and D). After inoculation with influenza A/IND/5/05 virus, virus-inoculated cells were present widespread in lungs of ferrets of groups PBS and Cve (Fig 6E and

F), while the number of virus-inoculated cells was considerably lower in the lungs of ferrets from groups S3.8+, S15+ and T3.8+ (Fig 6G and H). Influenza virus antigen expression was predominantly observed in alveolar and bronchiolar epithelial cells.

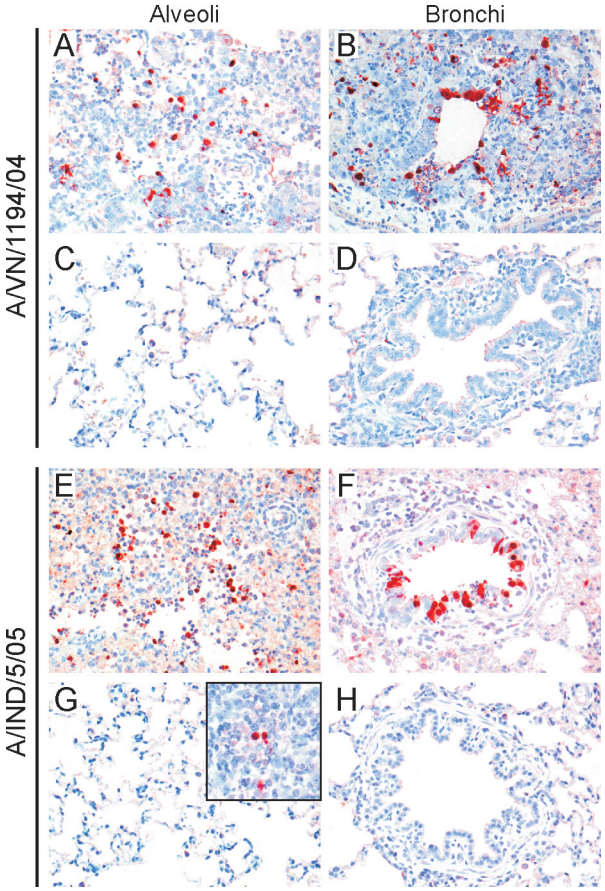

Figure 6. *Detection of virus-infected cells by immunohistochemistry. Cells that are positive for the presence of viral antigen show a deep red staining in the nucleus. Four days after inoculation with influenza A/VN/1194/04 (A+B) virus and influenza A/IND/5/05 (E+F), multiple virus-inoculated cells were present in both alveoli and bronchioles of lungs of ferrets of groups T3.8-, PBS, Cve. Inoculated cells were only found sporadically in the lungs of ferrets of groups S3.8+, S15+ and T3.8+ (C+D). After inoculation with influenza A/IND/5/05 virus, the number of inoculated cells was reduced in the lungs of ferrets from groups S3.8+, S15+ and T3.8+ (G+H), most inoculated cells were present in areas with inflammatory infiltrate (see insert).*

DISCUSSION

In the present study we have demonstrated that the addition of the novel adjuvant CoVaccine HT™ to an inactivated influenza H5N1 vaccine produced by state-of-the-art cell-culture technology makes a promising pandemic influenza vaccine candidate. This vaccine afforded protection against infection with the homologous and an antigenically distinct strain of A(H5N1) virus, even after a single administration of a low antigen dose. The protection of ferrets was associated with the induction of humoral and cell-mediated immune responses in vaccinated animals.

CoVaccine HT™ potentiated the antibody response to the vaccine strain considerably. After two immunizations of 3.8 µg HA of the NIBRG-14 preparation without the adjuvant, virus-specific antibodies were undetectable whereas the addition of CoVaccine HT™ resulted in detectable antibody responses in all ferrets. Even after a single immunization with the adjuvanted vaccine, approximately

50% of the animals developed detectable antibody levels against the homologous vaccine strain. In some ferrets, but especially those vaccinated twice with the CoVaccine HT™-adjuvanted vaccine, antibodies reactive with the heterologous strain A/IND/5/05 were detectable. This is of importance since multiple antigenically distinct A/H5N1 viruses are circulating and it is impossible to predict which clade of these viruses eventually may cause a pandemic. In this light, the induction of broadly reactive antibodies is advantageous and may correlate with protection against infection with heterologous influenza A/H5N1 viruses.

In addition, the induction of T cell responses after vaccination was evaluated. Recently reagents have become available to evaluate T cell responses in ferrets but assays for the detection of virus-specific T-cells have not been described sofar to our knowledge (260 -262). Using monoclonal antibodies cross-reactive with ferret CD3 and CD8 (260, 261) we developed a T cell proliferation assay that allowed discriminating proliferation of CD8+ and CD8- virus-specific T cells. Using this assay, it was shown that the use of CoVaccine HT™ significantly improved the virus-specific T cell response after vaccination of ferrets. Even after a single immunization with the adjuvanted vaccine substantial T cell responses were observed. Strong CD8-, presumably CD4+, T cell response, were detected that could be at the basis for the improved immunogenicity of the vaccine in term of antibody responses. After vaccination with the CoVaccine HT™-adjuvanted vaccines, a virus-specific CD8+ T cell response was observed, which could be the result of cross-priming *in vivo* (139). Previously we were unable to detect virus-specific CD8+ T cell responses in mice after vaccination with CoVaccine HT™-adjuvanted vaccine (257). However, in the mouse model CD8+ T cell responses were assessed *ex vivo* using tetramers detecting the responses to a single epitope only. It is likely that the proliferation and amplification of virus specific T cells in vitro increased the sensitivity of the detection of these cells in the current study. In the mouse model it was demonstrated that, at least partially, the adjuvant effect of CoVaccine HT™ was mediated through TLR-4 signaling (257). At present it is unclear whether this mode of action of the adjuvant also played a role in the improved immunogenicity of the vaccine in the ferret model.

The ultimate goal of vaccination of course is to protect against the development of severe disease upon exposure to the pathogen. Since the breadth of protective immunity is considered an important property of vaccines, vaccinated ferrets were inoculated with two antigenically distinct influenza A/H5N1 viruses, the homologous clade 1 virus A/VN/1194/04 and the heterologous clade 2.1 virus A/IND/5/05. With the vaccines containing CoVaccine HT™, a dramatic reduction of clinical signs, gross pathological and histopathological changes and virus replication in the upper and lower respiratory tract after inoculation with influenza virus A/VN/1194/04 was achieved. In contrast, two immunizations with unadjuvanted NIBRG-14 vaccine failed to induce protective immunity. Even after a single administration of a vaccine containing CoVaccine HT™ and as little as 3.8 µg HA, ferrets were fully protected against the homologous virus A/VN/1194/04. Vaccination was somewhat less effective against the development of clinical signs after infection with the heterologous virus A/IND/5/05, although still a significant reduction of virus replication in the upper and lower respiratory tract was achieved, even after a single administration of a low dose of antigen and CoVaccine HT™. In previous studies, it was demonstrated that with two immunizations with a low vaccine dose in combination with the adjuvant AS03, protection could be achieved against the

virus A/IND/5/05, but this vaccine also failed to induce sterilizing immunity in ferrets against this heterologous challenge virus (68). This indicates that it is inherently difficult to induce sterilizing immunity against viruses that are antigenitically distinct. Our findings are in concordance with the results obtained in other studies that show that immunogenicity of otherwise poorly immunogenic inactivated H5N1 vaccines can be increased dramatically with the use of adjuvants, like MF59 and AS03 (237, 238, 252). It is of interest to note that the use of a higher vaccine dose (15 µg of HA) did not result in the induction of higher antibody titers or increased protection against infection. In contrast, a single administration of the adjuvanted vaccine containing 15 µg seemed less effective in preventing virus replication, weight loss and the development of (histo)pathological changes after inoculation with influenza viruses A/VN/1194/04 or A/IND/5/05 than the use of the vaccine containing only 3.8 µg of HA. The mechanism of this phenomenon is poorly understood, but it suggests that the ratio between the amount of viral antigen and adjuvant can affect the outcome of vaccination and the protective efficacy of the vaccine.

In general, the protection against infection with both challenge viruses correlated with the induction of virus specific HI and VN antibodies. However, a number of animals vaccinated once with adjuvanted-vaccine were protected against infection in the absence of detectable virus-specific antibodies. This discrepancy also has been observed in other studies that evaluated candidate H5N1 vaccines (68, 70, 256, 263). It is possible that lower, undetectable levels of virus specific antibodies are already protective, or that priming of B cells for a secondary antibody response contributes to protective immunity. Alternatively, the induction of virus specific T cell immunity could have contributed to protection against infection. However, in the present study, the induction of virus specific T cells correlated only partially with the protection against infection and the magnitude of the antibody response did not correlate with the CD3+CD8- cell response.

The CoVaccine HT™-adjuvanted vaccines were well tolerated by the ferrets. Only some mild local reactions were observed at the site of vaccination, consisting of local erythema for one day. Data collected from temperature loggers that were placed in the abdominal cavity before the start of the experiment with ferrets that were challenged with influenza A/VN/1194/04 indicated that the CoVaccine HT™ adjuvanted NIBRG-14 vaccine caused a rise of body temperature for 1-2 days. It should be noted however, that these side effects are not uncommon after administration of adjuvanted influenza vaccines (149, 163).

Collectively, we conclude that CoVaccine HT™ is a promising adjuvant for the use in (pandemic) influenza vaccines. In the present study we have shown that its use in ferrets increased the immunogenicity of a cell-culture produced inactivated H5N1 vaccine. It increased both virus-specific T cell and antibody responses, resulting in protection against infection with the homologous and an antigenically distinct virus, even after a single administration of as little as 3.8 µg of HA antigen. These favorable properties justify the further clinical development of this influenza vaccine candidate.

Acknowledgements

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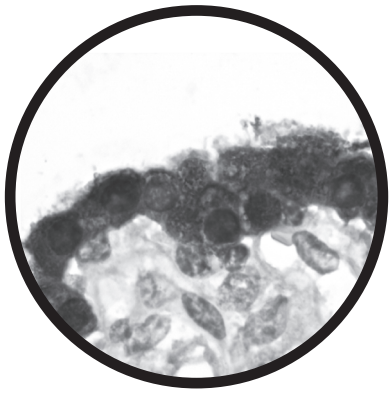

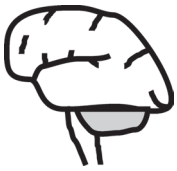

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Pathogenesis of influenza A/H5N1 virus infection in ferrets differs between intranasal and intratracheal routes of inoculation

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ABSTRACT

Most patients infected with highly pathogenic avian influenza A/H5N1 virus suffer from severe pneumonia resulting in acute respiratory distress syndrome, with extra-respiratory disease as an uncommon complication. The ferret is a traditional animal model for influenza in humans. Intranasal inoculation of ferrets with influenza A/H5N1 virus causes lesions in both respiratory tract and extra-respiratory organs, primarily brain. However, the route of spread to extra-respiratory organs and the relative contribution of extra-respiratory disease to pathogenicity are largely unknown. In the present study, we characterized lesions in respiratory tract and central nervous system (CNS) of ferrets (n = 8) inoculated intranasally with influenza A/Indonesia/5/2005 (H5N1) virus. By 7 days after inoculation, only 3 of 8 ferrets had a mild or moderate broncho-interstitial pneumonia. In contrast, all 8 ferrets had moderate or severe CNS lesions, characterized by meningo-encephalitis, choroiditis and ependymitis, and centred on tissues adjoining the cerebrospinal fluid. These findings indicate that influenza A/H5N1 virus spread directly from nasal cavity to brain, and that CNS lesions contributed more than pulmonary lesions to the pathogenicity of influenza A/H5N1 virus infection in ferrets. In comparison, intratracheal inoculation of ferrets with the same virus reproducibly caused severe broncho-interstitial pneumonia. The implications are that the method of virus inoculation needs to be considered carefully when designing ferret experiments as a model for influenza A/H5N1 in humans.

INTRODUCTION

Since the first human cases of infection with highly pathogenic avian influenza A/H5N1 virus were recognized in 1997, infection has been confirmed in more than 500 people, about 60% of whom have died (21). Typically, infected individuals develop a severe pneumonia, which often is fatal (24, 264). Different experimental animal species also develop severe pneumonia after inoculation with highly pathogenic avian influenza A/H5N1 virus (166, 167, 265-267). In addition to the respiratory tract, influenza A/H5N1 virus has been detected in different extra-respiratory organs, particularly the central nervous system (CNS), both in a few human cases and in multiple experimental animal species (24, 110, 166, 266, 268, 269).

The ferret (Mustela putorius furo) is considered a good animal model for influenza virus infection in humans and frequently is used to study the pathogenesis of influenza (156, 270-272). In most pathogenesis studies with influenza A/H5N1 viruses in ferrets, intranasal inoculation is performed (166, 167, 273). After intranasal inoculation, it is assumed that the virus spreads from the nose to the lower respiratory tract and, if pathogenic, causes pneumonia. From there, virus might spread systemically and cause extra-respiratory disease, including encephalitis. However, the route of spread to the CNS and the relative contribution of CNS disease to the pathogenicity of influenza A/H5N1 virus infection for ferrets are largely unknown. Therefore, we wished to determine the character and severity of lesions caused by influenza A/H5N1 virus in the respiratory tract and CNS after intranasal inoculation. To this end, ferrets were inoculated intranasally with highly pathogenic avian influenza A/Indonesia/5/2005 (H5N1) virus and multiple tissues were collected for macroscopic, microscopic, and immunohistochemical evaluation after euthanasia at 7 days post inoculation (dpi), and results were compared with those of ferrets inoculated intratracheally with the same virus.

MATERIALS AND METHODS

Virus preparation

Influenza virus A/Indonesia/5/2005 (H5N1, clade 2.1) was propagated in confluent Madin-Darby canine kidney (MDCK) cells. After cytopathic changes were complete, culture supernatants were harvested and cleared by low speed centrifugation and stored at –80°C. The virus titer was determined in MDCK cells as described previously (85).

Experimental protocol

Healthy outbred female ferrets between 6 and 12 months of age were purchased from a commercial breeder. They tested negative for the presence of antibodies against recent influenza A/H1N1 and A/H3N2 viruses, influenza A/Indonesia/5/05 virus, and Aleutian disease virus. During the experiment, ferrets were housed together and received food and water ad libitum. Eight ferrets were inoculated intranasally with 5×10^6 median tissue culture infectious dose ($TCID_{50}$) influenza virus A/Indonesia/5/2005 in a total volume of 0.5 ml phosphate-buffered saline under anesthesia with ketamine and medetomidine, and reversed with atipamezole. After inoculation, ferrets were checked daily for the presence of clinical signs. Before and 2, 4, 6, and 7 dpi with influenza A/H5N1 virus, fer-

rets were weighed under anesthesia with ketamine. At 7 dpi, or earlier in case ferrets became moribund, animals were weighed and subsequently killed by exsanguination under anesthesia with ketamine and medetomidine. Experimental procedures were approved by an independent animal ethics committee, and were performed under biosafety level 3 conditions.

Pathological examination and immunohistochemistry

Necropsies were performed according to standard procedures. Samples of olfactory bulb, cerebrum, cerebellum, brain stem, lungs (all lobes of the right lung and the accessory lobe; after inflation with 10% neutral-buffered formalin), spleen, liver and duodenum were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4 µm. Tissue sections were stained with hematoxylin and eosin (HE) for histological evaluation or with an immunoperoxidase method using a monoclonal antibody directed against the nucleoprotein of the influenza A virus for detection of virus-infected cells (110). An IgG2a isotype control for each tissue and a positive control tissue were included in each staining procedure.

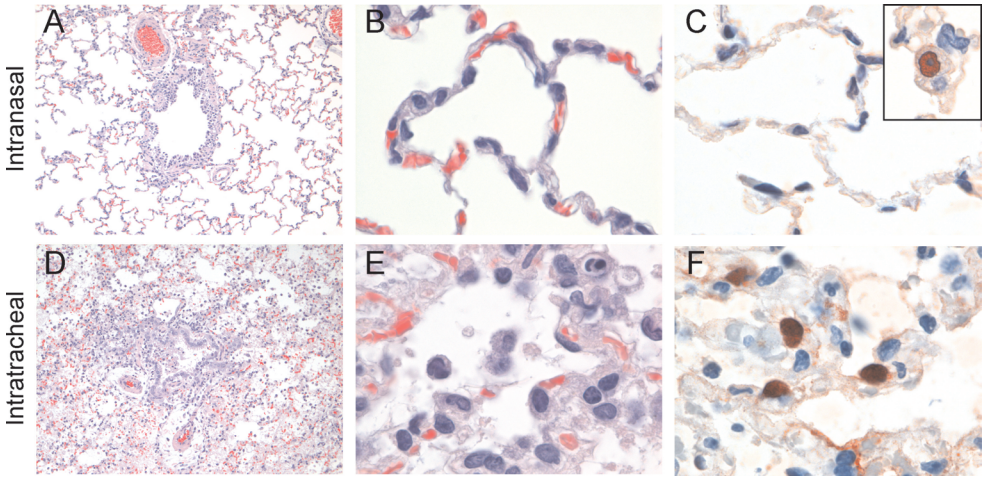

Figure 1. *Comparison of histological lesions and viral antigen expression in lungs of ferrets inoculated intranasally (A to C) or intratracheally (D to F) with influenza A/Indonesia/5/05 (H5N1) virus. A, B: Absence of lesions in the lung of an intranasally inoculated ferret (original magnifications, X200 and X1000). C: Absence of cells expressing virus antigen in alveoli of an intranasally inoculated ferret (original magnification, X1000). Inset: Rare type 2 pneumocyte expressing virus antigen. D: Broncho-interstitial pneumonia, with inflammatory exudate in bronchiolar and alveolar lumina, in the lung of an intratracheally inoculated ferret (original magnification, X200). E: Neutrophils, macrophages, cellular debris and erythrocytes in the alveolus of an intratracheally inoculated ferret (original magnification, X1000. F: Epithelial cells, mainly type 2 pneumocytes, expressing virus antigen in alveoli of an intratracheally inoculated ferret (original magnification, X1000).*

K, L: Influenza viral antigen in neurons and glial cells of the cerebrum (original magnifications, X400 and X1000). M: Infiltration of inflammatory cells, vacuolation and neuronal necrosis in the olfactory bulb (original magnification, X400). N, O: Influenza viral antigen in glial cells and neuronal cells of the olfactory bulb (original magnifications, X400 and X1000).

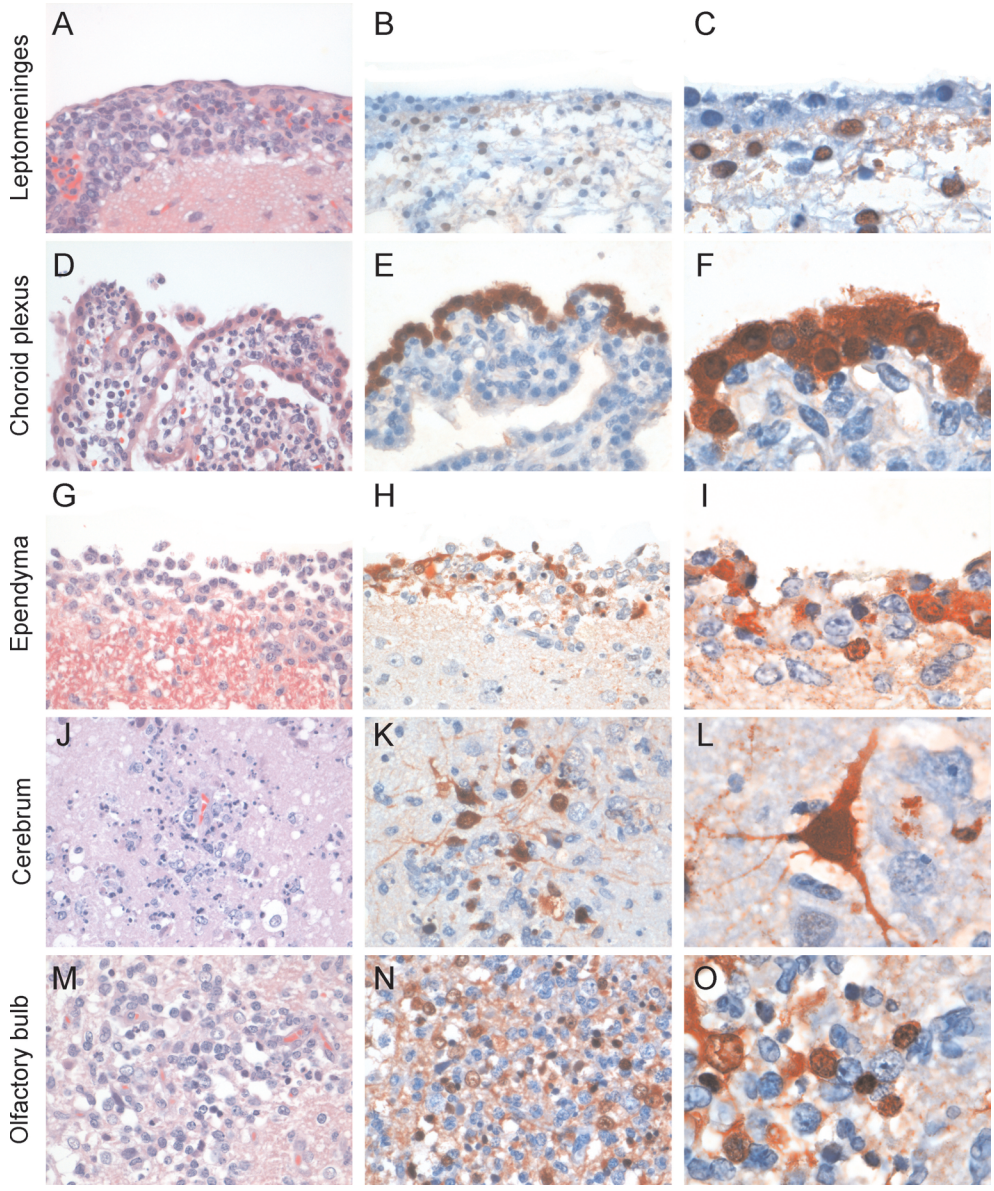

Figure 2. *Histological lesions and viral antigen expression in the central nervous system of ferrets inoculated intranasally with influenza A/Indonesia/5/05 (H5N1) virus. A: Leptomeninges of the cerebrum infiltrated with many inflammatory cells, mainly macrophages (original magnification, X400). B, C: Influenza viral antigen in mesothelial cells of the leptomeninges (original magnifications, X400 and X1000). D: Infiltration of mainly macrophages in the connective tissue of the choroid plexus, and segmental dissociation of the epithelial layer (original magnification, X400). E, F: Influenza viral antigen in epithelial cells of the choroid plexus (original magnifications, X400 and X1000). G: Segmental loss of the epithelial layer of the ependyma, and infiltration of mainly macrophages (original magnification, X400). H, I: Influenza viral antigen in epithelial cells of the ependyma (original magnifications, X400 and X1000). J: Infiltration of inflammatory cells, vacuolation and neuronal necrosis in the cerebrum (original magnification, X400).*

RESULTS

Clinical signs

From 1 dpi onwards, ferrets developed clinical signs including anorexia, diarrhea, neurological signs and lethargy. All ferrets lost between 13% and 24% of their original body weight by 7 dpi. Two ferrets were found dead at 5 and 7 dpi. One ferret was euthanized at 6 dpi due to the presence of severe clinical signs, in accordance with animal welfare regulations.

Macroscopic and microscopic observations

At necropsy, three of eight ferrets had multifocal dark red areas of consolidation in the lungs. The estimated percentage of lung tissue involved per ferret was 8 ± 14 (mean ± s.d.) and the lung weight as a percentage of body weight was 0.9 ± 0.48 (mean ± s.d.) (Table 1). No macroscopic lesions were seen in other organs except for diffuse hepatic pallor in all eight ferrets.

Upon histological examination, three of eight ferrets had a mild or moderate, multifocal, bronchointerstitial pneumonia (Figures 1A and B; Table 2). In the affected areas, alveolar lumina were filled with cellular debris, edema fluid, eyrthrocytes, fibrin and inflammatory cells (many macrophages and some neutrophils); alveolar walls showed epithelial necrosis, moderate hypertrophy and hyperplasia of type 2 pneumocytes, and infiltration with moderate numbers of macrophages and neutrophils. Bronchiolar lumina contained cellular debris, macrophages and neutrophils; bronchiolar walls had loss of epithelium and infiltration with moderate numbers of macrophages and neutrophils. Bronchial lumina contained a few inflammatory cells and fibrin, while bronchial walls contained a few inflammatory cells.

Table 1. Comparison of macroscopic lung lesions between ferrets inoculated intranasally (this study) or intratracheally (159) with influenza A/Indonesia/5/05 (H5N1) virus

Route of inoculation	Inoculation dose ($TCID_{50}$)	Number of animals with macroscopic lung lesions/total number	Area of affected lung tissue (%, mean ± s.d.)	Relative lung weight (mean ± s.d.)
Intranasal	5×10^6	3/8	8 ± 14	0.9 ± 0.5
Intratracheal	1×10^5	6/6	59 ± 21	1.7 ± 0.2

All eight ferrets had a moderate or severe, diffuse, non-suppurative leptomeningitis in cerebrum, cerebellum, brain stem, and olfactory bulb (Figure 2A). It was characterized by an infiltrate in the subarachnoidal space ranging from 4 to 20 cells in thickness and consisting of many large mononuclear cells (macrophages), a few lymphocytes and rare neutrophils. Multifocally, there was dissociation of the superficial mesothelial cells of the arachnoid membrane and endothelial hypertrophy of the meningeal blood vessels.

Five of seven ferrets had a moderate or severe, necrotizing choroiditis (Figure 2D). The connective tissue of affected choroid plexi had necrosis of cells and infiltration with many macrophages, while

cellular debris, inflammatory cells, fibrin and erythrocytes were present on the epithelial surface.
Six of seven ferrets had a moderate or severe, necrotizing ependymitis. This was characterized by segmental loss of the ependymal cell lining and infiltration by many macrophages, although at some locations higher proportions of lymphocytes or neutrophils were present (Figure 2G).
All eight ferrets had a multifocal necrotizing encephalitis, which was moderate in cerebrum and olfactory bulb and mild in the brain stem. Affected areas were characterized by infiltration with neutrophils, necrosis of neurons, gliosis, and vacuolization of neuropil (Figure 2J and M). Affected areas in the cerebrum were superficial and localized adjacent to the inflammatory lesions present in leptomeninges, ependyma, and olfactory bulb. Affected areas in the olfactory bulb were present throughout the tissue.
All eight ferrets had mild diffuse hepatic lipidosis, consistent with anorexia. None of the eight ferrets had microscopic lesions in spleen or duodenum.

Table 2. Presence of histological lesions and viral antigen expression in tissues of ferrets inoculated intranasally with influenza A/Indonesia/5/05 (H5N1) virus (n = 8)

Tissue	No. of ferrets positive	
	Histological lesion	Virus antigen expression
Leptomeninges	8	3
Choroid plexus*	5	4
Ependyma*	6	5
Olfactory bulb	6	6
Cerebrum	8	5
Cerebellum	8	0
Brain stem	0	1
Lung	3	3
Spleen	3	0
Liver	0	0
Duodenum	0	0

**Tissue missing from one ferret.*

Expression of viral antigen

Virus antigen expression was detected in the CNS of all eight ferrets and respiratory tract of three of eight ferrets (Table 2). In the CNS, moderate or abundant virus antigen expression was detected in mesothelial cells of cerebral leptomeninges (Figures 2B and C), epithelial cells of choroid plexus (Figures 2E and F) and ependyma (Figures 2H and I), and neurons and glial cells of cerebrum (Figures 2K and L), brain stem, and olfactory bulb (Figure 2N and O). Interestingly, virus-positive cells in cerebrum and brain stem were located superficially, adjacent to meninges, ependyma and olfactory bulb, while virus-positive cells in the olfactory bulb were located in the central part of this tissue.
In the respiratory tract, scant virus antigen expression was detected in the lungs, mainly in type 2 pneumocytes (Figure 1C). None of the eight ferrets showed virus antigen expression in spleen, liver, or duodenum.

DISCUSSION

Our study shows that all ferrets inoculated intranasally with this influenza A/H5N1 virus developed widespread and often fatal CNS disease, characterized by non-suppurative meningo-encephalitis, choroiditis, and ependymitis. Surprisingly for a highly pathogenic respiratory virus inoculated into the respiratory tract, broncho-interstitial pneumonia developed only in a minor proportion of ferrets, and was milder than the CNS disease.

Comparison of our results with those from other influenza A/H5N1 virus infections in experimental animals indicates the importance of route of inoculation for primary disease presentation and character of CNS lesions. First, route of inoculation is important for primary disease presentation. Intranasal inoculation in our ferrets resulted in primary disease of the CNS rather than of the respiratory tract. These results are consistent with previous studies (274, 275), where intranasal inoculation of influenza A/Vietnam/1203/04 (H5N1) in ferrets resulted in death from severe neurologic disease, but caused only mild lung lesions. The authors suggested that these results were likely due to the low virus inoculation dose—10^1 or 10^2 median egg infectious dose (EID_{50}) per ferret—used in those studies, and indicated that higher doses—10^6 or 10^7 EID_{50}— led to more severe pulmonary lesions (166, 276, 277). However, in our study, we used a relatively high dose—5 x10^6 $TCID_{50}$—without inducing severe pulmonary lesions. A possible explanation is that intranasal inoculation results in virus deposition in the lower respiratory tract of some ferrets but not others. In contrast, when we inoculated a similar dose of the same virus intratracheally rather than intranasally (159), all ferrets developed severe broncho-interstitial pneumonia associated with influenza virus infection (Figure 1D, E and F; Table 1) and had to be euthanized at 4 dpi due to severe respiratory distress. CNS disease was not evaluated pathologically, but was not considered significant based on absence of neurologic signs.

Second, route of inoculation is important for the character of CNS lesions. In our intranasally inoculated ferrets, CNS lesions were centered on tissues adjacent to cerebrospinal fluid in the subarachnoidal space and ventricular system—leptomeninges, choroid plexi, and ependyma—and were characterized by prominent infiltration of mononuclear cells; there was lesser involvement of adjacent brain parenchyma. Other studies also point out the infiltration of mononuclear cells in the meninges of intranasally inoculated ferrets (275, 276). In contrast, intratracheal inoculation of influenza A/H5N1 virus in cats resulted in randomly distributed foci of necrosis and inflammation in the brain parenchyma, with minor involvement of leptomeninges, choroid plexi, and ependyma (267).

A possible explanation for the above disparities in pathological changes between routes of inoculation is a different route of virus entry into the CNS. As suggested previously, intranasal inoculation of influenza A/H5N1 virus in ferrets may lead to direct spread of virus from the nasal cavity, via olfactory nerves through the ethmoidal plate, to the olfactory bulb (166). This route of entry has been shown for influenza A/H5N1 virus in mice (168, 265) and for canine distemper virus in ferrets (278). Because the barrier between olfactory bulb and cerebrospinal fluid is weak (279), virus would have easy and rapid access to tissues around the subarachnoidal space and ventricular system. This route of entry would explain the prominent involvement of the olfactory bulb, leptomeninges, choroid plexi, and ependyma in our intranasally inoculated ferrets. In contrast, there was evidence of

blood-borne spread of influenza A/H5N1 virus in intratracheally inoculated cats (267), which might explain the random distribution of lesions in the brain parenchyma and the lesser involvement of tissues around the subarachnoidal space and ventricular system in those animals.
In conclusion, we here show that choice of route of inoculation is critical in designing ferret models to study the pathogenesis of influenza A/H5N1 virus infection in humans. Based on our results with different routes of inoculation of influenza virus A/Indonesia/5/2005 (H5N1) in ferrets, intranasal inoculation would be the route of choice to study influenza A/H5N1-virus-induced CNS disease, while intratracheal inoculation may be more appropriate to study influenza A/H5N1-virus-induced lower respiratory tract disease.

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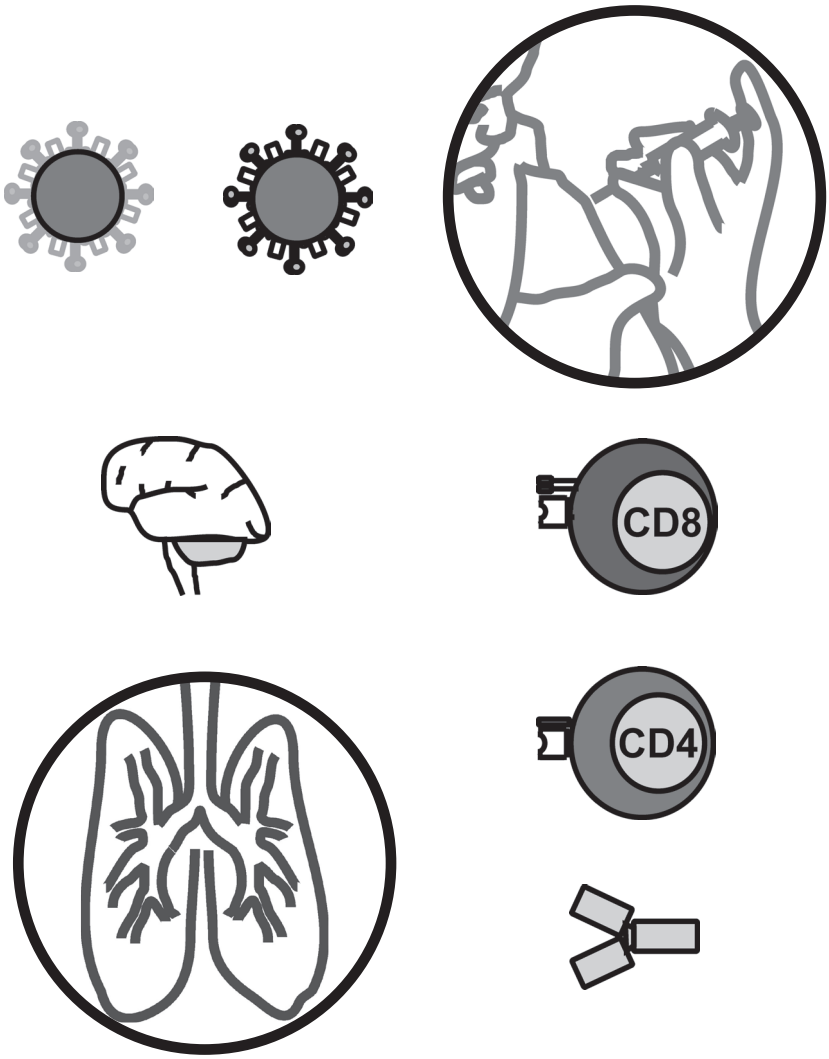
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Summarizing Discussion

Partially based on:

Yearly influenza vaccinations: a double-edged sword?
R. Bodewes, J.H.C.M. Kreijtz and G.F. Rimmelzwaan
Lancet Infectious Diseases 2009; 9: 784-88

The recent pandemic caused by the influenza A/H1N1(2009) virus has highlighted the importance of influenza viruses as a cause of disease in humans. However, seasonal influenza viruses also cause morbidity and mortality and the WHO has estimated that between 250.000 and 500.000 people die due to infection with influenza viruses annually (12). Mainly very young children, the elderly, or immunocompromised people die due to infection with seasonal influenza virus, while during the recent influenza A/H1N1(2009) pandemic also relatively high numbers of young healthy people died due to influenza virus infection (55). In addition, the relatively high mortality rates among young healthy people due to infection with highly pathogenic influenza A/H5N1 viruses indicate that influenza virus infection is not just an infection of the elderly or immunocompromised and that the development of safe and effective vaccines is important to prepare for a future pandemic.
The focus of this thesis is the development of immunity against influenza A/H5N1 viruses. In the first part, the induction of heterosubtypic immunity by infection with a seasonal influenza virus and the effect of vaccination against seasonal influenza on the development of this type of immunity against influenza was investigated. In addition, the seroprevalence of antibodies against influenza viruses in the Netherlands during non-pandemic years was analyzed and influenza virus-specific humoral and cellular immune responses between non-vaccinated healthy children and children with cystic fibrosis that were vaccinated annually against influenza viruses were compared. The focus of the second part is the development of a novel influenza A/H5N1 virus vaccine based on a low dose of whole inactivated influenza A/H5N1 virus antigen in combination with the novel adjuvant CoVaccine HT™.

Heterosubtypic immunity against influenza A/H5N1

As indicated, pandemics are caused by the introduction of influenza A viruses into the human population with HA and NA that are antigenetically distinct from the currently circulating human seasonal influenza A viruses. Therefore antibodies directed to the seasonal influenza viruses are unable to recognize and neutralize the new strains allowing them to replicate to higher titers causing high transmission and attack rates. Under these circumstances the presence of heterosubtypic immunity may tip the balance in favour of the host and afford some level of protection against these new pandemic strains. In chapter 2 of this thesis, it was demonstrated in the mouse model that previous infection with an influenza A/H3N2 virus also induces heterosubtypic immunity against a highly pathogenic influenza A/H5N1 virus. The observed reduction of clinical signs including weight loss and mortality correlated with differences in viral load in the lungs. Furthermore, it was demonstrated that survival of lethal challenge with the influenza A/H5N1 virus correlates with the presence of a strong response of CD8+ T cells against both the immunodominant NP366-374 and PA224-233 epitopes of the influenza A/H5N1, while no cross-reactive antibodies were observed and serum transfer did not provide protection against challenge infection. In addition, previous infection with a respiratory syncytial virus (RSV) did not induce protective immunity against influenza indicating that an influenza A virus-specific component of the immune system is responsible for the observed differences in protection. Essentially the same results were obtained in the ferret model using a recent seasonal influenza A/H3N2 virus and the same highly pathogenic influenza A/H5N1 virus (chapter 5). Ferrets that were primed by infection with an influenza A/H3N2 virus, developed less severe clinical signs including weight loss following challenge infection than unprimed animals. The reduction of clinical signs

correlated with a reduction in viral load in nose and pharyngeal swabs collected at various time points after inoculation and with histopathological changes in the lungs and brain observed 7 days after inoculation. Also in this animal model, no cross-reactive antibodies were observed prior to inoculation with challenge with influenza A/H5N1. Results obtained in both animal models further confirm data from other studies in which it was demonstrated that previous infection with seasonal influenza A viruses can induce immunity against future pandemic influenza A viruses. However, the exact mechanism of the observed infection-induced heterosubtypic immunity is currently matter of debate, but it is generally accepted that various arms of the immune system other than serum-antibodies to hemagglutinin and neuraminidase contribute to heterosubtypic immunity, like CD4+ and CD8+ T cells specific for conserved viral proteins, mucosal antibodies and B cells (40, 43, 47, 123, 136).

Vaccination against seasonal flu and interference with the induction of heterosubtypic immunity against pandemic strains

Since seasonal influenza A viruses of the H3N2 and H1N1 subtypes and influenza B viruses cause epidemics annually associated with excess morbidity and mortality mainly among the elderly, immunocompromised and other high-risk groups, influenza vaccination is recommended for these high-risk groups. Furthermore, due to the higher risk of complications and hospitalizations secondary to influenza in children (58, 59), annual vaccination of all healthy children 6 to 59 months of age was recommended in various countries including the United States since 2007 (60). Also in Europe, vaccination of children is currently considered and a number of countries already recommend vaccination of healthy children (61). In general, influenza vaccines most frequently used are inactivated vaccines, including subunit preparations that consist of the envelope proteins of the influenza viruses, the HA and NA. However, there is a potential downside of annual vaccination against seasonal flu as vaccination may prevent the induction of heterosubtypic immunity otherwise induced by infection. This interference with the induction of heterosubtypic immunity may not be of any consequence under normal circumstances, but in light of the pandemic threat caused by the influenza A/H5N1 viruses, the presence or absence of heterosubtypic immunity might affect the clinical outcome of infection with the new pandemic strain. Since young infants are immunologically naïve to influenza viruses (184), especially in this age group annual vaccination against seasonal influenza may prevent the induction of heterosubtypic immunity and render this age group more susceptible to pandemic strains compared to subjects that have experienced prior infections with seasonal strains. Currently used inactivated influenza vaccines provide protection against the homologous seasonal strains, but fail to induce cross-protective immune responses to strains of alternative subtypes. This poor cross-protective potential correlates with the inefficient induction of virus-specific CD8+ T cell responses induced by these vaccines (104, 105). In chapter 3 of this thesis we tested this hypothesis and investigated the effect of vaccination against a seasonal influenza virus A/H3N2 strain on the induction of heterosubtypic immunity against potentially pandemic highly pathogenic influenza A/H5N1 viruses in a mouse model. Strikingly, mice that were protected from infection with the seasonal A/H3N2 strain developed severe disease and had a fatal outcome of the A/H5N1 infection whereas those that were not vaccinated against a prior infection with the A/H3N2 strain became less ill and did not succumb to infection. The lack of clinical protection in the H3N2-vaccinated mice against

infection with A/H5N1 virus correlated with the lack of control of virus replication in the lungs normally seen in mice that experienced a prior infection with the influenza A/H3N2 virus. The disease was similar to that observed in fully naïve mice infected with the A/H5N1 strain and characterized as a severe necrotizing broncho-interstitial pneumonia. Essentially the same results were obtained in chapter 4, where a whole inactivated influenza A/H3N2 virus vaccine containing all components of the influenza A virus including the relatively conserved inner proteins was used. It was demonstrated that mice immunized with this vaccine developed a response of CD8+ T cells towards an epitope located on the nucleoprotein (NP366-374), however this response was relatively small compared to the response observed after infection. In addition, it was shown that the use of WIV vaccine for the protection against A/H3N2 infection affected the induction of heterosubtypic immunity otherwise afforded by A/H3N2 influenza virus infection. The reduction of protective immunity correlated with changes in the immunodominance patterns of the CD8+ T cell responses directed to the epitopes located in the acid polymerase ($PA_{224-233}$) and the nucleoprotein ($NP_{366-374}$).

Since results obtained in the mouse model for influenza A virus infection are not easily translated to humans, the results were reproduced in ferrets that are more closely related to humans regarding the pathogenesis of influenza virus infections. To this end, in chapter 5 of this thesis the effect of vaccination on the induction of heterosubtypic immunity against influenza A/H5N1 was tested in this animal species. Also in ferrets, vaccination against seasonal influenza virus hampered the induction of heterosubtypic immunity in a proportion of the animals.

It has been suggested that vaccination with a seasonal influenza vaccine in children does not induce sterilizing immunity and only reduces clinical signs and virus replication in the upper respiratory tract (280, 281) which is in contrast to our results in the mouse model as sterilizing immunity was observed after H3N2 infection in vaccinated animals. However, in our experiments with ferrets we observed that vaccination did not induce sterilizing immunity as reduced virus replication was observed in all vaccinated ferrets. Strikingly, a proportion of the vaccinated ferrets did not develop heterosubtypic immunity after infection with influenza A/H3N2 indicating that also reduction of virus replication can prevent the induction of heterosubtypic immunity.

Furthermore, the findings in our animal models coincided with a number of recent observational studies performed in adults in Canada and in children in Hong Kong (169, 170, 207), in which it was demonstrated that prior vaccination against seasonal influenza increased the risk of medically attended, laboratory-confirmed influenza A/H1N1(2009) illness. However, results inconsistent with this study have been published as well (282).

Besides the absence of heterosubtypic immunity another possible explanation for these findings are the presence of high titers of low-avidity, non-protective antibodies and complement activation induced by pulmonary immune complexes in vaccinated subjects (283). In addition, it has been demonstrated that vaccination against seasonal influenza during the previous influenza season had a negative impact on the development of antibodies after vaccination against influenza A/H1N1(2009) virus, although the underlying mechanism is unknown (284).

Seroprevalence of antibodies against influenza in children

Since it was demonstrated in chapters 2, 4 and 5 that experimental infection of laboratory animals

with influenza A virus could provide at least partial protection against challenge with an influenza A virus of an unrelated subtype, it is of importance to know at which age children would be infected with influenza viruses for the first time. As detailed information about the seroprevalence of influenza viruses in children was lacking, serum samples collected during a nationwide cross-sectional population-based study in the Netherlands were tested for the presence of antibodies against various influenza viruses that circulated in recent years in chapter 6. In a relatively high proportion of the serum samples collected from children between one and six months of age antibodies were detected, especially against older influenza virus strains, which could be attributed to the presence of maternally derived antibodies to these strains. Lower seroprevalences of antibodies were observed in children 7-12 months age, while it was observed that the proportion of study subjects >1 year of age with detectable antibodies against influenza viruses gradually increased with age until at age six all children had developed antibodies against at least one influenza A virus. In concordance with epidemiological data collected in the Netherlands, the highest seroprevalences were at each age detected against influenza A/H3N2 viruses. Besides the seroprevalence of antibodies against influenza A viruses, also the presence of antibodies against influenza B viruses from both the Yamagata and Victoria lineage was evaluated. Also for these viruses an increase in the seroprevalence of antibodies was observed from one to seven years of age, with the highest seroprevalence detected against viruses from the Yamagata lineage. In addition, using the increase in the seroprevalence of antibodies for each year, we were able to calculate attack rates. The attack rate observed in this study are similar to observed attack rates in young children in other studies in other countries (174, 175). Interestingly, the observed attack rates were lower than those demonstrated for other respiratory viruses in children (285-288). A possible explanation for this might be that viruses like RSV and human metapneumovirus induce only transient immunity (289, 290), indicating that every year people will become again susceptible to these viruses and can transmit the virus to new hosts, while only a small part of the population will be susceptible for influenza viruses as these viruses induce antibodies that are able to prevent infection for a prolonged period of time.

Strikingly, as it was demonstrated that previous infection with an influenza A virus can provide immunity against influenza A viruses of another subtype, findings of this seroprevalence study indicate that a proportion of the children below 6 six years of age has not been infected with an influenza A virus previously and might therefore not possess heterosubtypic immunity. Especially this age group might be at risk to develop severe disease upon infection with a highly pathogenic influenza A/H5N1 virus or another future pandemic influenza virus.

Effect of annual vaccination on the virus-specific CD8+ T cell immunity in children

Since the extrapolation of data obtained in animal studies to the human situation is a matter of debate since it was demonstrated in mice that the presence of CD8+ T cell immunity correlated with the presence of heterosubtypic immunity, the studies in mice and ferrets were extended to humans by comparing the virus-specific antibody responses and CD4+ and CD8+ T cell responses in vaccinated children with cystic fibrosis (CF) and unvaccinated healthy control children undergoing correctional surgery in chapter 7. A somewhat broadened antibody response was observed in vaccinated children compared to unvaccinated children, while no differences were observed in the virus-specific

CD4+ T cell response. In contrast, an age-related increase of the virus-specific CD8+ T cell response was observed in unvaccinated children that was not present in vaccinated children with CF. These findings confirm observations in the mouse model in which it was demonstrated that 28 days after infection with influenza A/H3N2 virus vaccinated mice had lower virus-specific CD8+ T cell responses than unvaccinated infected animals. In addition, as it has been demonstrated in mice that the presence of virus-specific CD8+ T cells is an important correlate of heterosubtypic immunity against influenza A viruses, these results might indicate that vaccinated children will not have heterosubtypic immunity against future pandemic viruses. These findings are important in the light of the pandemic threat caused by influenza A/H5N1 viruses. Especially for these children vaccines should be used that do induce heterosubtypic immunity. However, ideally our results obtained in this study should be confirmed with larger groups of children without differences in underlying disease, although we observed no differences in the immune response between healthy control children and vaccinated children with CF to other antigens. In the Netherlands, vaccination is only recommended for children with a high risk to develop severe disease upon infection with an influenza virus, so it was not possible to compare young healthy children vaccinated annually with unvaccinated children.

Development of universal vaccines

Findings presented in this thesis highlight the importance of the development of vaccines that do induce heterosubtypic immunity (121). The elucidation of the role of various components of the adaptive immune system in heterosubtypic immunity is important to establish the exact correlates of protection (40). It has been demonstrated both in mice and humans that the number of virus-specific CD8+ T cells correlates with reduced virus shedding and T cell immunity correlates with protection in children (50, 77, 212). Therefore, focussing on the vaccines that induce virus-specific CD8+ T cell immunity might be a valid route for the development vaccines that induce of heterosubtypic immunity (218). It has been shown that live attenuated vaccines induce virus specific CD8+ T cell responses in contrast to the frequently used subunit, split virion or whole inactivated virus vaccines and therefore may confer some degree of protection against heterosubtypic influenza virus strains (105, 120). Indeed in animal models it has been shown that live attenuated vaccines induced broad protective immune responses (134, 291, 292). In addition, novel generations of influenza vaccines, like viral vector vaccines, may be attractive alternatives since they also can induce virus specific CD8+ T cell responses directed to conserved viral proteins like the matrix protein and the nucleoprotein, in addition to antibodies directed against the HA of seasonal influenza virus strains (293-298). Examples of vector vaccine production platforms are the recombinant replication deficient adenoviruses, poxviruses and the Newcastle disease virus vector, which have been shown to induce protective immunity to influenza viruses efficiently (171, 299-304). Another conserved viral protein is the M2 protein that can induce cross-reactive antibodies. Using M2-based candidate vaccines protective immunity could be induced against influenza A viruses of various subtype (96, 172). Furthermore, it has been demonstrated recently that a vaccine that is able to provoke an antibody response against the relatively conserved stalk region of the hemagglutinin can also provide protection against multiple influenza A virus subtypes (173). These vaccine candidates not only may afford protection against seasonal influenza viruses but also against future pandemic strains and can be used even if they

provide only partial protection against these strains as they may reduce the morbidity and mortality during the first months of the pandemic until virus-specific vaccines become available.

Development of a novel influenza A/H5N1 vaccine

An example of a vaccine that has been developed to be used as an adequate future pandemic vaccine against the highly pathogenic influenza A/H5N1 virus, is the MDCK-grown whole inactivated influenza A/H5N1 vaccine in combination with the novel adjuvant CoVaccine HT™. Key issues for the development of pandemic influenza A virus vaccines are the time required that vaccines become available after the start of a pandemic, optimal use of the existing (limited) production capacity of viral antigen and effectiveness against viruses that are antigenetically distinct. Ideally, a single administration of a low dose of antigen would be sufficient to induce protective immunity against the homologous strain and heterologous antigenic variant strains. Therefore, the use of safe and effective adjuvants in the vaccine is considered an efficient strategy to induce dose-sparing and to broaden the specificity of the induced antibody responses. During the last years, multiple adjuvanted vaccines have been tested and licensed against seasonal and pandemic influenza viruses but for all these vaccines it has been demonstrated that two vaccinations are necessary to provide protection against influenza A/H5N1 viruses (68, 252, 305). In chapters 8 and 9 experiments are described in mice and ferrets in which it is demonstrated that a single administration of a low dose of H5N1-antigen in combination with the CoVaccine HT™ induces antibody responses against homologous and heterologous influenza A/H5N1 viruses. In addition, it is demonstrated in ferrets that this vaccine-adjuvant combination is able to provide complete protection against homologous and partial protection against heterologous challenge. Furthermore, it is shown that CoVaccine HT™ induces the maturation and secretion of cytokines by human dendritic cells and that this effect was mediated by binding of CoVaccine HT™ to Toll like receptor-4. The latter most likely is facilitated by the most important component of CoVaccine HT™, the synthetic sucrose fatty acid sulphate ester (SFASE), which may mimic the lipopolysaccharide present on gram negative bacteria. Based on these promising results and data collected during in a study in rabbits and macaques (306), it can be concluded that this vaccine candidate will fulfill a number of the key criteria for the development of an ideal pandemic influenza A/H5N1 vaccine, as low doses of MDCK-grown antigen and a broad-protective potential. Before it can be used and that it was also effective against antigenitically distinct viruses. However, before it can be used in humans, of course this vaccine candidate needs to be tested in clinical trials.

Pathogenesis of influenza A/H5N1 virus in ferrets

The use of an appropriate animal model is crucial for the testing of vaccines and to study the pathogenesis of viruses. For influenza A viruses, ferrets are currently regarded as the golden standard animal model. In chapter 10, the intranasal route (used in chapter 5) and intratracheal route (used in chapter 6) of inoculation of ferrets with influenza A/Indonesia/5/05 (H5N1) were compared. Intratracheal inoculation results primarily in disease of the lower respiratory tract, which is also the most prominent cause of death of the reported human H5N1 influenza cases (24, 264). In contrast, intranasal inoculation induces primarily in disease of the central nervous system and only in a proportion

of the inoculated ferrets in a bronchopneumonia. These findings in combination with the character of the CNS lesions also suggest that intranasal inoculation of influenza A/H5N1 virus in ferrets may lead to direct spread of virus from the nasal cavity, via olfactory nerves through the ethmoidal plate, to the olfactory bulb. Based on the results of chapter 10, intranasal inoculation would be the route of choice to study influenza A/H5N1-virus-induced CNS disease, while intratracheal inoculation may be more appropriate to study influenza A/H5N1-virus-induced lower respiratory tract disease.

Concluding remarks

Studies presented in this thesis provide information which may aid decision making for the implementation of vaccination strategies that aim at achieving optimal protective immunity against seasonal and pandemic influenza. It is demonstrated that the use of inactivated influenza vaccines against seasonal influenza has an negative impact on the development of heterosubtypic immunity, which highlights the importance of the development of vaccines that induce heterosubtypic immunity. By no means, results presented in this thesis suggest that children or other people with underlying disease should not be vaccinated against seasonal influenza virus infection. In addition, the effect of vaccination on the development of heterosubtypic immunity is typically for influenza viruses and our considerations are not intended for all other viruses and vaccines that are currently implemented in the (Dutch) governmental vaccination programs. However, results presented in this thesis can provide information for a balanced discussion if children should be vaccinated against influenza until vaccines that induce heterosubtypic immunity are available or until techniques are used for the production of vaccines that allow the very fast production of hundreds of millions of vaccines when an influenza A virus of a novel subtype is introduced into the human population.

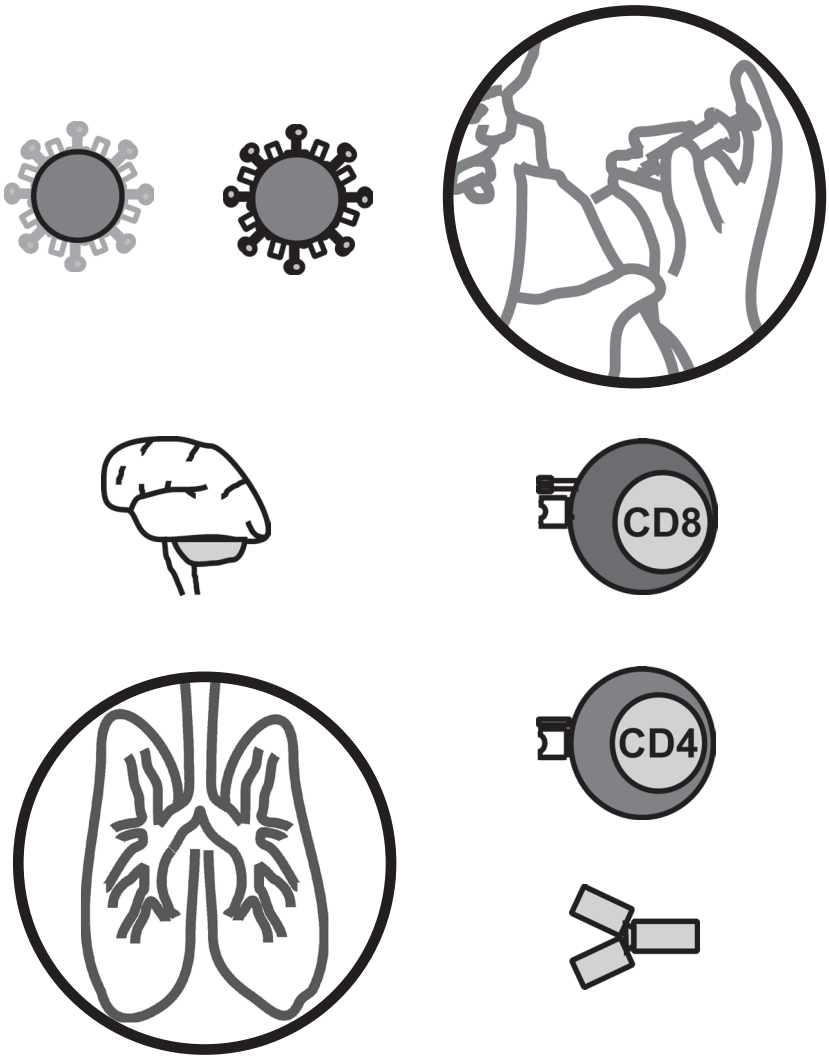
In table 1, possible advantages and possible disadvantages of vaccination of children against seasonal influenza viruses are listed. Especially for children with underlying disease, the risk of developing severe disease upon infection with a seasonal influenza virus is much higher than the risk of reduction of heterosubtypic immunity due to vaccination. Therefore, vaccination is recommended for these children. In addition, children without underlying disease that are older than six years of age will have been infected with an influenza A virus at least once as is demonstrated in chapter 6, so they will most likely have developed virus-specific CD8+ T cell immunity and antibodies as is demonstrated in chapter 7. Reasons to recommend vaccination of these children include the prevention of morbidity and mortality of children and the elderly but also economical aspects (183, 213). For these children, both inactivated and live attenuated influenza virus vaccines can be used. However, it is currently unknown if vaccination with inactivated antigens also reduces the presence of virus-specific CD8+ T cell immunity in children when administered for a number of years but it has been demonstrated in adults that during years with only mild influenza activity also the CTL immunity declined (217). Therefore, vaccination of children of these ages with live attenuated vaccines might be an alternative, because these vaccines do induce CD8+ T cell responses and are effective (62, 281). For children under 6 years of age, the situation is different as a proportion of them has not been infected with an influenza A virus previously. Especially for these children the effect of vaccination on the prevention of sometimes severe disease due to infection with seasonal influenza viruses should be weighed against the possible prevention of the development of heterosubtypic immunity as

demonstrated in this thesis and in a recent epidemiological study (59, 207). In addition, the number of hospitalizations and deaths attributable to influenza virus infection in very young children should be evaluated in the Netherlands before vaccination of these children is recommended as differences in health care systems between countries might influence these results. Furthermore, live attenuated vaccines are licensed recently in Europe, but only for children older than two years of age. Children below two years of age could be vaccinated against seasonal influenza viruses in combination with components that induce immunity against multiple influenza viruses. These vaccines are currently in various stages of development (218). A novel approach would be to decide if children should be vaccinated based on an individual serological profile. Children with virus-specific antibodies in their serum samples have been infected previously with a seasonal influenza virus and can be vaccinated against influenza without affecting the presence of heterosubtypic immunity, while children without antibodies should ideally not be vaccinated or vaccinated with live attenuated influenza virus vaccines. However, another option is testing if these children have antibodies against the predicted epidemic influenza A viruses as vaccination of children that already have antibodies against these strains is of limited value. This service might be of interest to parents as an optional self-pay service that do not want to have their children vaccinated frequently.

Table 1. Possible advantages and possible disadvantages of vaccination of all healthy children with inactivated antigens against seasonal influenza A viruses.

Possible advantages	Possible Disadvantages
• Less morbidity due to infection with seasonal influenza viruses in children. • Lower hospitalization rates of children and elderly. • Lower absenteeism of parents due to sickness of children.	• Prevention of induction of CTL immunity and heterosubtypic immunity against future pandemic strains • Mild side effects induced by vaccination. • Reduced antibody responses after vaccination against pandemic viruses

In the face of an possible pandemic, studies in this thesis demonstrate that a single vaccination with the novel adjuvant CoVaccine HT™ in combination with influenza A/H5N1 viral antigen can protect ferrets against challenge with influenza A/H5N1 viruses. As there is still concern about the continuous spread of the highly pathogenic influenza A/H5N1 viruses among poultry and the occasional spread to humans, a pandemic with this virus is still feared. However, as the first human infections with this virus have already been described more than a decade ago, it is at present unclear if this virus will adapt to humans in the future. However, the unexpected emergence and rapid spread of the influenza A/H1N1(2009) virus has demonstrated that the introduction of a novel influenza A virus into humans has to be expected in the future. The multiple pandemics during the 20th century confirm this. Therefore, screening of birds, pigs and other animal species for the presence of influenza viruses, research to elucidate the pathogenesis of influenza A viruses and the development and adjustment of influenza virus vaccines are of crucial importance to prevent high mortality rates during a future influenza pandemic. In addition, the development of influenza vaccines that are able to protect against influenza viruses of various subtypes, should be considered a high priority.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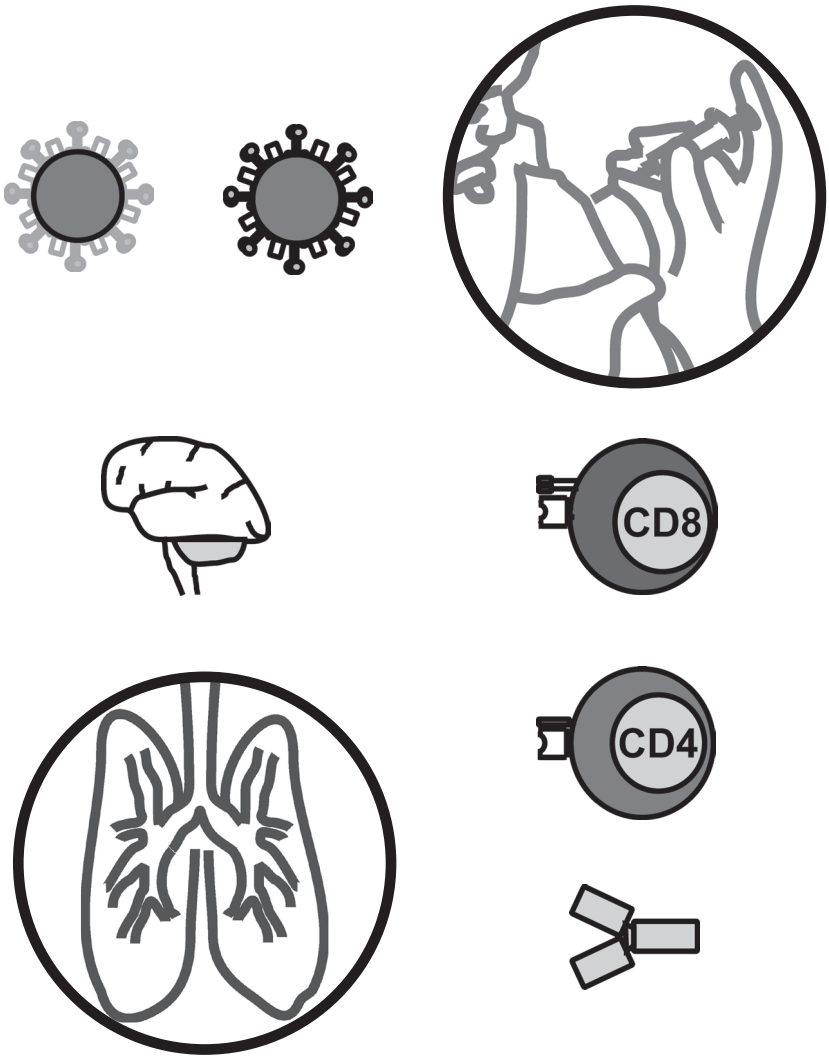
CD8
CD4

13

Nederlandse Samenvatting

Influenza virussen zijn de veroorzakers van influenza, ook wel 'griep' genoemd. Dit is in het algemeen een ziekte van de bovenste luchtwegen, die gedurende ongeveer een week klinische verschijnselen als verkoudheid, hoesten, koorts, spierpijn en hoofdpijn veroorzaakt. Influenza virussen behoren tot de familie van de orthomyxoviridae en worden onderverdeeld in influenza A, B en C virussen. Influenza A virussen worden verder van elkaar onderscheiden op basis van twee eiwitten die aan de buitenkant van het influenza virus partikel zitten, het hemagglutinine (HA) en het neuraminidase (NA). Er zijn 16 verschillende subtypen van het HA en 9 van het NA bekend. Influenza virussen circuleren in het algemeen gedurende de wintermaanden van zowel het noordelijk als het zuidelijk halfrond rond bij mensen. Ondanks dat infectie met influenza virussen bij de meeste mensen met milde klinische verschijnselen gepaard gaat, schat de wereldgezondheidsorganisatie (WHO) dat er jaarlijks tussen de 250.000 en 500.000 mensen overlijden als gevolg van ernstige longontsteking na infectie met het influenza virus. Dit zijn voornamelijk ouderen of mensen met een ernstige onderliggende aandoening.

Naast de jaarlijkse seizoensgriepepidemie, wordt er zo nu en dan een 'nieuw' influenza virus geïntroduceerd in de humane populatie, waartegen het merendeel van de bevolking geen beschermende antistoffen heeft. Zo'n influenza virus kan dan een influenza virus pandemie veroorzaken, het recente influenza A/H1N1(2009) virus dat de Mexicaanse grieppandemie veroorzaakte is hier een voorbeeld van. De introductie van een 'nieuw' influenza A virus in de humane populatie is mogelijk doordat er maar een beperkt aantal verschillende influenza A virus subtypen circuleren in mensen (H1N1 en H3N2), terwijl wilde vogels het reservoir van alle influenza virus subtypes vormen. Door aanpassing aan de mens of door recombinatie met een ander influenza virus afkomstig uit mensen of bijvoorbeeld varkens, kan een influenza virus ontstaan dat zich goed kan verspreiden onder mensen en waartegen het merendeel van de bevolking niet beschermd is. Een voorbeeld van een influenza virus waarvan op dit moment wordt gevreesd dat het zich in de toekomst onder mensen kan verspreiden, is het vogelgriepvirus (influenza A/H5N1 virus). Dit virus circuleert op dit moment voornamelijk in Zuidoost-Azië onder pluimvee en heeft daar al miljoenen kippen gedood sinds 1997. Daarnaast zijn er ook meer dan 500 mensen geïnfecteerd met dit virus waarvan ongeveer 60% is overleden. Echter, dit virus is op dit moment nog niet in staat om zich goed onder mensen te verspreiden, maar de angst bestaat dat dit virus zich in de toekomst zo aanpast dat het wel efficiënt van mens tot mens kan worden overgedragen en op die manier voor miljoenen slachtoffers zorgt. Deze angst heeft er toe geleid dat er veel onderzoek is en wordt gedaan naar dit influenza virus. Door goede surveillering van wilde vogels, het onderzoeken van de wijze waarop mensen ziek worden door infectie met dit virus of een influenza A virus van een ander subtype en door de zoektocht naar nieuwe vaccines en antivirale middelen wordt getracht te voorkomen dat dit virus in de toekomst miljoenen mensen doodt.

In dit proefschrift worden twee onderdelen van het onderzoek naar de mogelijkheden van bescherming van mensen tegen het influenza A/H5N1 virus beschreven. In hoofdstuk 2 wordt aangetoond in muizen dat eerdere infectie met een seizoensgriepvirus (influenza A/H3N2 virus) gedeeltelijke bescherming biedt tegen infectie met een vogelgriepvirus. Naar deze bescherming, die heterosubtypische immuniteit genoemd wordt, is al vele jaren onderzoek gedaan door middel van experimenten met proefdieren, maar ook in mensen zijn er duidelijke aanwijzingen dat deze

bescherming een rol van betekenis speelt. Verder is gebleken dat deze heterosubtypische immuniteit niet leidt tot steriele immuniteit. Dit betekent dat het virus nog wel in staat is om de cellen van de gastheer te infecteren en te repliceren, en dus ziekte niet voorkomen kan worden, maar dat de ziekteverschijnselen wel veel minder ernstig zijn. Voornamelijk van een bepaald celtype van het immuunsysteem, de cytotoxische T cellen (CTL), wordt gedacht dat ze een belangrijke rol spelen in de bescherming tegen dit virus aangezien ze de geconserveerde onderdelen van het influenza virus kunnen herkennen dat gepresenteerd wordt door cellen die door het virus geïnfecteerd zijn. Door deze cellen op te herkennen en op te ruimen kunnen ze voorkomen dat het virus zich verder verspreidt. Ook de in hoofdstuk 2 beschreven heterosubtypische immuniteit correleerde met de aanwezigheid van virus-specifieke CTL, terwijl geen antilichamen tegen het influenza A/H5N1 virus werden gevonden. In grote lijnen werden dezelfde resultaten waargenomen in fretten, een diersoort waarvan wordt gedacht dat de wijze van ziekteverwekken van influenza A virussen vergelijkbaar is met die van de mens. Deze studie staat beschreven in hoofdstuk 5. Naar aanleiding van de bevindingen in hoofdstukken 2 en 5 en studies gedaan door andere onderzoekers is in hoofdstukken 3, 4 en 5 gekeken of vaccinatie met een geïnactiveerd vaccin mogelijk zou interfereren met de heterosubtypische immuniteit opgewerkt door infectie met een seizoensgriepvirus. Dit is van groot belang aangezien het sinds een paar jaar door volksgezondheidsinstituten van een aantal landen (waaronder de Verenigde Staten en een aantal Europese landen) wordt aanbevolen om alle gezonde kinderen te vaccineren tegen seizoensgriep, om te voorkomen dat kinderen ernstig ziek worden als gevolg van infectie met het seizoensgriepvirus. Deze vaccinatie kan een toegevoegde waarde hebben voor de gezondheid van alle kinderen, maar in theorie zou het ook kunnen zijn dat vaccinatie de ontwikkeling van goede CTL reacties en dus heterosubtypische immuniteit voorkomt. Dit zou belangrijke implicaties kunnen hebben met het oog op een toekomstige pandemie van bijvoorbeeld het vogelgriepvirus. In hoofdstuk 3 en 4 worden studies beschreven in het muismodel waarin wordt aangetoond wordt dat vaccineren met een vaccin tegen seizoensgriep de ontwikkeling van heterosubtypische immuniteit tegen influenza A/H5N1 virussen voorkomt. Ook het voorkomen van deze heterosubtypische immuniteit correleerde met de afwezigheid van CTL immuniteit in de niet beschermde dieren. Naar aanleiding van deze studies in muizen is er ook in fretten een vergelijkbare studie gedaan zoals beschreven in hoofdstuk 5. Aangezien fretten gevoelig zijn voor humane influenza virussen en klinische verschijnselen ontwikkelen die lijken op die van de mens na infectie, wordt aan het frettenmodel veel waarde gehecht. Ook in deze diersoort werd aangetoond dat vaccinatie tegen seizoensgriep de ontwikkeling van heterosubtypische immuniteit door infectie met een seizoensgriepvirus in een deel van de dieren voorkwam.

De resultaten beschreven in hoofstukken 2-5 laten zien dat het doormaken van een natuurlijke infectie een grote invloed heeft op de ontwikkeling van heterosubtypische immuniteit. Daarom, en om een goed vaccinatiebeleid voor kinderen te kunnen bepalen, is het van groot belang om te weten op welke leeftijd kinderen een infectie met een influenza virus doormaken. Om dit te onderzoeken, is er gebruik gemaakt van bloedmonsters van kinderen die verzameld zijn tijdens het een landelijke studie van het RIVM, het Pienter2-project. Door middel van het uitvoeren van de hemagglutinatie remmingstest is de aanwezigheid van antilichamen tegen verschillende stammen van het influenza A en B virus in bloedmonsters van kinderen tussen de 0 en 7 jaar oud beoordeeld in

hoofdstuk 6. Een relatief groot gedeelte van de kinderen tussen de 1 en 6 maanden oud bezat antilichamen tegen een of meerdere influenzavirussen, waarschijnlijk antilichamen afkomstig van de moeder aangezien relatief hoge titers waargenomen tegen influenza virusstammen van enkele jaren geleden die circuleerden toen de kinderen nog niet geboren waren. Lagere percentages werden gevonden in kinderen van 7 maanden tot 1 jaar oud. Vanaf een leeftijd van 1 jaar werden geleidelijk hogere seroprevalenties van antilichamen tegen influenza virussen gevonden totdat in alle monsters van kinderen van 6 jaar oud antilichamen werden gevonden tegen minimaal één influenza A virus subtype. Dit geeft aan dat een relatief groot aandeel van de jonge kinderen nog geen infectie met een influenza virus heeft doorgemaakt, wat belangrijke gevolgen kan hebben voor de aanwezigheid van heterosubtypische immuniteit in deze kwetsbare leeftijdsgroep.
Na de verkregen resultaten in proefdieren is het natuurlijk van belang om vast te stellen of hetzelfde effect ook in mensen aanwezig is. Daarom worden in hoofdstuk 7 van dit proefschrift de influenza A virus-specifieke antilichamen en T cel reacties van kinderen met cystic fibrosis (taaislijmziekte) die jaarlijks zijn gevaccineerd tegen influenza vergeleken met die van ongevaccineerde gezonde kinderen. Uit dit onderzoek werd duidelijk dat gevaccineerde kinderen met cystic fibrosis een wat bredere antilichaamrespons hadden en een vergelijkbare respons van T helper cellen. Echter, de leeftijdsafhankelijke stijging van de virus-specifieke CTL respons die waargenomen werd in gezonde kinderen, was niet aanwezig in de gevaccineerde kinderen. Hoewel niet uitgesloten kan worden dat dit veroorzaakt zou kunnen worden door een verschil in het immuunsysteem van deze twee groepen kinderen, is dit onwaarschijnlijk aangezien geen verschillen tussen de T helper cel responsen en de respons van CTL tegen een 'superantigeen' is waargenomen in kinderen ouder dan 5 jaar. De resultaten uit dit onderzoek bevestigen dus eerdere conclusies uit de studies met muizen en tonen aan dat ook in kinderen vaccinatie de ontwikkeling van virus-specifieke CTL reacties remt. Dit betekent dat er een mogelijk nadeel kleeft aan de aanbeveling van een aantal volksgezondheidsinstituten om alle gezonde kinderen te vaccineren tegen seizoensgriep. Echter, voor gesteld kan worden dat vaccinatie daadwerkelijk een negatief effect heeft op de aanwezigheid van heterosubtypische immuniteit zijn grotere epidemiologische studies met twee groepen gezonde kinderen noodzakelijk. Duidelijk is wel dat het belangrijk is dat er onderzoek wordt gedaan naar de ontwikkeling van vaccins die bescherming bieden tegen meerdere subtypen van het influenza A virus, al was het maar om te gebruiken in de tijd tussen de start van een pandemie en het moment dat er adequate vaccines beschikbaar zijn. Omdat deze vaccines (nog) niet beschikbaar zijn, is het gebruik van levend geattenueerde vaccins mogelijk een goed alternatief. Dat dit onderzoek niet bedoeld is om het vaccineren van kinderen met een onderliggende aandoening te af te wijzen mag duidelijk zijn, het risico dat deze kinderen ernstig ziek worden van de seizoensgriep is vele malen groter dan het risico dat deze kinderen minder goed beschermd zijn tegen een nieuw pandemisch virus. Ook zijn onze bevindingen specifiek van toepassing voor het influenza virus en gelden dus niet voor alle andere virussen uit het rijksvaccinatieprogramma. Voor het advies om kinderen te vaccineren is de leeftijd van groot belang, aangezien uit hoofdstuk 6 blijkt dat alle kinderen ouder dan zes jaar antilichamen hebben tegen seizoensgriepvirussen en ook CTL immuniteit hebben ontwikkeld zoals blijkt uit hoofdstuk 7. Vooral voor jonge kinderen, die nog niet eerder een infectie hebben doorgemaakt is het goed afwegen van de voor- en nadelen dus van groot belang.

Naast dit onderzoek naar de rol van vaccinatie op de aanwezigheid van heterosubtypische immuniteit, hebben we ook onderzoek gedaan naar vaccins die immuniteit tegen het influenza A/H5N1 virus induceren. Een aantal zaken zijn erg belangrijk bij de ontwikkeling van vaccins tegen het influenza A/H5N1 virus. Ze moeten in staat zijn om te beschermen tegen meerdere varianten van het influenza A/H5N1 virus bij voorkeur door middel van een enkele vaccinatie, ze moeten veilig zijn en weinig tot geen bijwerkingen geven. Om bescherming door middel van een enkele vaccinatie mogelijk te maken en om de immuunrespons te verbreden, wordt er op dit moment veel onderzoek gedaan naar hulpstoffen die het immuunsysteem aspecifiek stimuleren om toe te voegen aan vaccins, de zogenaamde adjuvans. In hoofdstukken 8 en 9 worden studies beschreven waarin de immunogeniciteit en de beschermende werking van een heel geïnactiveerde influenza A/H5N1 virus vaccin in combinatie met het nieuwe adjuvant CoVaccine HT™ wordt geëvalueerd. Uit deze studies blijkt dat het nieuwe vaccine immunogeen is in zowel muizen als fretten en dat het in staat is om al na één vaccinatie met een lage dosis antigeen fretten te beschermen tegen twee verschillende varianten van het influenza A/H5N1 virus. Verder blijkt ook dat het adjuvans CoVaccine HT™ in vitro bij humane dendritische cellen de secretie van cytokines stimuleert door middel van het binden aan Toll like receptor-4. Aangezien dendritische cellen een belangrijke rol spelen in het immuunsysteem, zou dit er op kunnen wijzen dat dit adjuvant ook in mensen het immuunsysteem zou kunnen stimuleren. Meer onderzoek is echter noodzakelijk om dit aan te tonen.

Onderzoek naar nieuwe vaccins of naar de gevolgen van vaccinatie is onmogelijk zonder proefdieren. Voor onderzoek naar influenza virussen worden verschillende proefdiermodellen gebruikt die ook in dit proefschrift worden beschreven, zoals de muis en de fret. Aangezien fretten gevoelig zijn voor humane influenza virussen en klinische verschijnselen ontwikkelen die lijken op die van de mens na infectie, wordt vooral aan het frettenmodel veel waarde gehecht. In hoofstuk 10 worden twee manieren waarop fretten experimenteel geïnfecteerd kunnen worden met een influenza A/H5N1 virus met elkaar vergeleken. Uit de resultaten van dit onderzoek blijkt dat infectie van fretten met een influenza A/H5N1 virus via de neus voornamelijk tot ziekte van de hersenen leidt, terwijl infectie van fretten met hetzelfde virus in de luchtpijp voornamelijk leidt tot ziekte van de longen. Aangezien influenza A/H5N1 virussen bij mensen vooral ziekte van de longen veroorzaken, is het wellicht beter voor vaccinonderzoek om fretten te infecteren via de luchtpijp dan via de neus.

Samenvattend tonen we in dit proefschrift aan dat vaccinatie tegen seizoensgriep mogelijk een negatief effect heeft op de ontwikkeling van heterosubtypische immuniteit tegen influenza A/H5N1 virussen en dat een enkele vaccinatie met het nieuwe adjuvans CoVaccine HT™ in combinatie met een influenza A/H5N1 vaccin goede bescherming biedt tegen challenge-infectie in fretten. Beide bevindingen zijn van belang voor de voorbereiding op een eventuele nieuwe pandemie veroorzaakt door het influenza A/H5N1 virus of een ander influenza A virus.

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KUNSTHAL
ALS JE NIET KAN VERLIEZEN,
KUN JE OOK NIET WINNEN
Gerrie Knetemann
ZONE
ZONE
P
P
P

About the author
PhD portfolio
Publications

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Rogier Bodewes was born on March 10, 1981 in Delft, the Netherlands. In 1999, he finished high school (VWO) at the I.S. Westland in 's-Gravenzande and started to study Veterinary Medicine at Utrecht University from which he graduated as a doctor in veterinary medicine in 2007. During this study, he did an extended research project at the Department of Virology, ErasmusMC, Rotterdam entitled "Crossprotection mediated by cytototoxic T cells in and influenza-mouse model" and performed an externship at the Virology Division of the Faculty of Veterinary Medicine, Utrecht University and clinical rotations at the Department of Pathology, UC Davis School of Veterinary Medicine, USA. During the last year of his clinical rotations, he focused on companion animals medicine. In 2007 he started as a PhD student at the

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In-depth courses

Course in Molecular Medicine (MolMed)	2008
Course in Virology (MolMed and Department Virology)	2008
Course in Molecular Diagnostics (MolMed)	2010
Internal and external presentations at the Department of Virology twice a week	2007-present

Workshops

Workshop Writing Succesful Grant Proposals (MolMed)	2010
Masterclass Prof. Rino Rappuoli (Novartis Vaccines & Diagnostics) AMC, Amsterdam	2010

(Inter)national conferences

Symposium Post-Infectious Diseases (Oct 4-5)	2007
12th MolMed day, Rotterdam (Feb 6)	2008
Dutch Annual Virology Symposium, Amsterdam (March 7)	2008
3th European Influenza Conference (ESWI), Villamoura, Portugal (Sept 14-17)	2008
13th MolMed day, Rotterdam (Feb 5)	2009
Dutch Annual Virology Symposium, Amsterdam (March 6)	2009
5th Orthomyxovirus Conference, Freiburg, Germany (Sept 9-12)	2009
Vaccine symposium, Utrecht (Nov 19)	2009

Pienter 2 project: resultaten tot nu toe, Bilthoven (March 30)	2010
Vaccine symposium, Utrecht (Nov 26)	2010

Presentations

In vitro analysis of the human immune response to inactivated influenza vaccine and CoVaccine HT™ (EU-project FluVac meeting , London, United Kingdom, 2008)	Oral
Evaluation of WIV influenza A/H5N1 virus vaccine and CoVaccine HT™ *in vitro* and *in vivo* (EU-project FluVac meeting , London, United Kingdom, 2009)	Oral
Primary infection with influenza A/H3N2 induces heterosubtypic immunity against lethal challenge with influenza A/H5N1 in mice (3th European Influenza Conference, 2008)	Poster
Cytotoxic T lymphocytes in heterosubtypic immunity against influenza A viruses (13th MolMed Day, 2009)	Poster
Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus (5th Orthomyxovirus Conference, 2009)	Oral
Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus (Vaccine symposium, 2009)	Oral
Seroprevalentie van influenza in kinderen tijdens niet-pandemische seizoenen (Pienter 2 middag, 2010)	Oral
Impact of vaccination against seasonal influenza on the development of heterosubtypic immunity against influenza A/H5N1 (Masterclass Prof. Rino Rappuoli, 2010)	Oral
Effect of annual vaccination against influenza on the development of virus-specific CD8+ T cell immunity (Vaccine symposium, 2010)	Oral

Travel grants

ESWI Young Scientist travel grant (3th European Influenza Conference)	2008
FEMS Travel grant for Young scientists (5th Orthomyxovirus Conference)	2009

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