Protection Against Influenza A(H5N1) by Primary Infection with Influenza A(H3N2) and MVA-based Vaccination

Bescherming Tegen Influenza A(H5N1) door Primaire Infectie met Influenza A(H3N2) en op MVA gebaseerde Vaccinatie

Proefschrift
ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 3 september 2009 om 13:30 uur
door

Johannes Henricus Cornelus Maria Kreijtz

geboren te Vorstenbosch

Protection Against Influenza A(H_5N_1) by Primary Infection with Influenza A(H_3N_2) and MVA-based Vaccination

Thesis, Erasmus University, Rotterdam, the Netherlands. With summary in Dutch
ISBN: 978-94-9032-001-0

Voor ons pap en mam

Promotie commissie

Promotor:	Prof.dr. A.D.M.E. Osterhaus
Co-promotor:	Dr. G.F. Rimmelzwaan
Overige leden:	Prof.dr. T. Kuiken
	Prof.dr. G. Sutter
	Prof.dr. B.N. Lambrecht

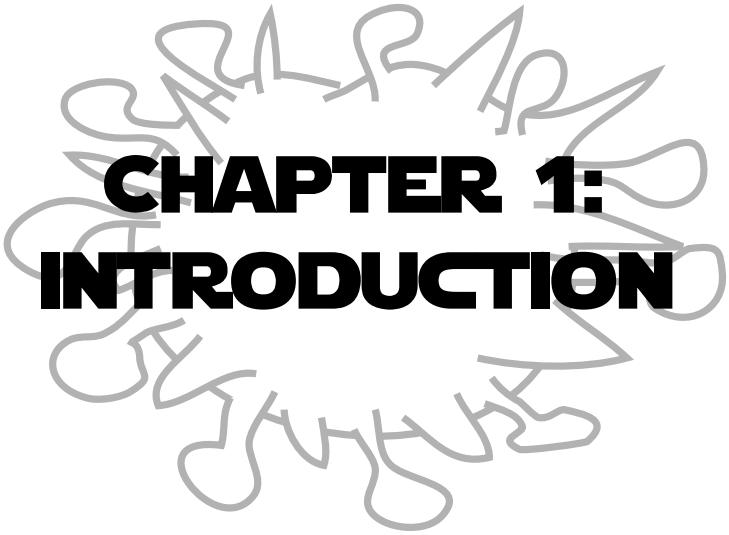
The research described in this thesis was financially supported by ZonMW Grant 91402008 (Netherlands Influenza Vaccine Research Consortium (NIVAREC))

Financial support by the following companies and foundations is gratefully acknowledged:

Viroclinics
Solvay Biologicals
Nobilon International BV
Roche Nederland BV
Clean Air Techniek BV

Cover illustration: Lung john silver by JK

Table of Contents



CHAPTER 1: INTRODUCTION

Partially based on:

Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control

J.H.C.M. Kreijtz, A.D.M.E. Osterhaus, G.F. Rimmelzwaan:

Department of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands

Human Vaccines 2009; 5:3, 126-35

Influenza viruses

Influenza A viruses belong to the family of the orthomyxoviridae that consists of five genera: Thogoto virus, Isavirus and Influenza virus A, B and C. The generae of influenza viruses are distinguished based on their membrane channel protein, genome size and surface glycoprotein(s).[1] Influenza A viruses are classified based on their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). So far 16 HA subtypes and 9 NA subtypes have been identified based on genetic and antigenic analysis.[2] The nomenclature of influenza viruses is based on: subtype, host of origin (except humans), isolation site (geographical), strain number, year of isolation and followed by the description of the antigenic subtype, e.g. A/Chicken/Netherlands/1/03 (H7N7).

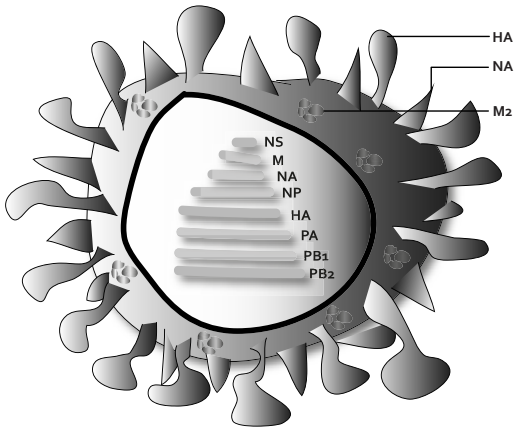


Figure 1 Impression of Influenza A virus particle and gene segments. The PB1 segment encodes also the PB1-F2 protein (present only in infected cells). The M segment encodes the M1 and M2 protein and the NS segment encodes the NS1 (present only in infected cells) and NS2 protein.

Structure of influenza A virus

Influenza A virus particles are enveloped and have a diameter of 80-120nm. Their single stranded negative sense RNA genome is divided over eight gene segments that encode 11 different proteins (Figure 1).[3] The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are located on the viral envelope. The M2 protein, which functions as an ion channel is also incorporated in the viral envelope. The other proteins are located inside the virion: the Matrix protein (M1), the nucleoprotein (NP), polymerases PB1, PB1-F2, PB2 and PA, and the non-structural protein NS2.[3, 4] The M1 proteins form a layer that underlies the lipid bilayer of the viral envelope

that was derived from the infected cell. The M1 protein, in combination with NP and polymerase proteins, is also associated with the ribonucleoproteins (RNPs) that contains the vRNA. The NP is abundant in the nucleocapsid of the RNP whereas the PB1, PB2 and PA proteins are located on one end. The PB1-F2 and non-structural protein NS1 are only found in infected cells and are not incorporated in the virion and the PB1-F2 is not expressed by every influenza A virus. NS2 is a structural component, present at a low copy number and associated with the M1 protein.

Influenza virus replication cycle

The influenza virus attaches to the cell by binding of the HA to galactose-linked sialic acids on the glycoprotein receptors of the host cell membrane (Figure 2).[5] Depending on the HA subtype the virus can either bind to alpha2-3 or alpha2-6 galactose-linked sialic acids. Human influenza viruses prefer the alpha2-6 sialic acid, abundantly present in the upper respiratory tract but not in the lungs, whereas the alpha2-3 linked sialic acid, preferred by avian influenza viruses, is also present in the lower respiratory tract.[6, 7] Binding of the virus to and infection of cells in the upper respiratory tract, in combination with additional mutations that facilitate efficient replication at this site (e.g. PB2-E627K) are required for efficient human-to-human transmission.[3] Human influenza A viruses are able to spread easily among humans whereas the avian influenza A/H5N1 viruses do not replicate in the upper respiratory tract and are not transmitted efficiently from human to human. Mutations that result in amino acid substitutions in the receptor binding pocket of the HA molecule could change its receptor specificity and result in a virus capable of human-to-human transmission.

After binding, the influenza virion enters the cell through receptor-mediated endocytosis and subsequently fuses with the mild acidic primary endosome.[5] The low pH in the endosome triggers the membrane fusion activity of HA by conformational changes in the protein. This results in fusion of the viral and endosomal membranes releasing the RNPs into the cytoplasm. Prior to this event the M2 ion channels, activated by the low pH, allow H^+ ions to enter the virion upon which they detach the M1 protein from the RNPs to prepare it for transport to the nucleus, chaperoned by the NP.[8] In the nucleus the three types of influenza virus RNA are formed.

The template RNA, which consists of full length positive sense copies of vRNA from which new vRNA is transcribed that will constitute the genome of the newly formed

virus particles, and mRNA from which the viral proteins are translated after nuclear export, regulated by NS1. Once the viral RNA is produced, new RNPs are assembled in the nucleus, transported to the cytoplasm and then transported towards the apical membrane, chaperoned by M1. The membrane associated proteins HA, NA and M2 are produced by ribosomes bound to the membrane of the endoplasmatic reticulum (ER). Subsequently they enter the ER for post-translational modification and are transported to the Golgi apparatus where additional modifications take place. From there the HA and NA proteins, that use lipid rafts for transport, and the M2 proteins, are directed to the apical plasma membrane where virus assembly will take place.[9]To allow the newly formed virus particles to be separated from the cell, a process called budding, the cell membrane will bulge outward, most likely mediated by accumulation of M1 under the lipid bilayer. The virus then bulges out of the cell until the cell membrane fuses at the bottom of the virion, hereby closing it. The actual release of the virions from the cell is mediated by the enzymatic activity of NA that catalyses the cleavage of the sialic acids thus preventing binding of newly formed particles to the cell.[10]

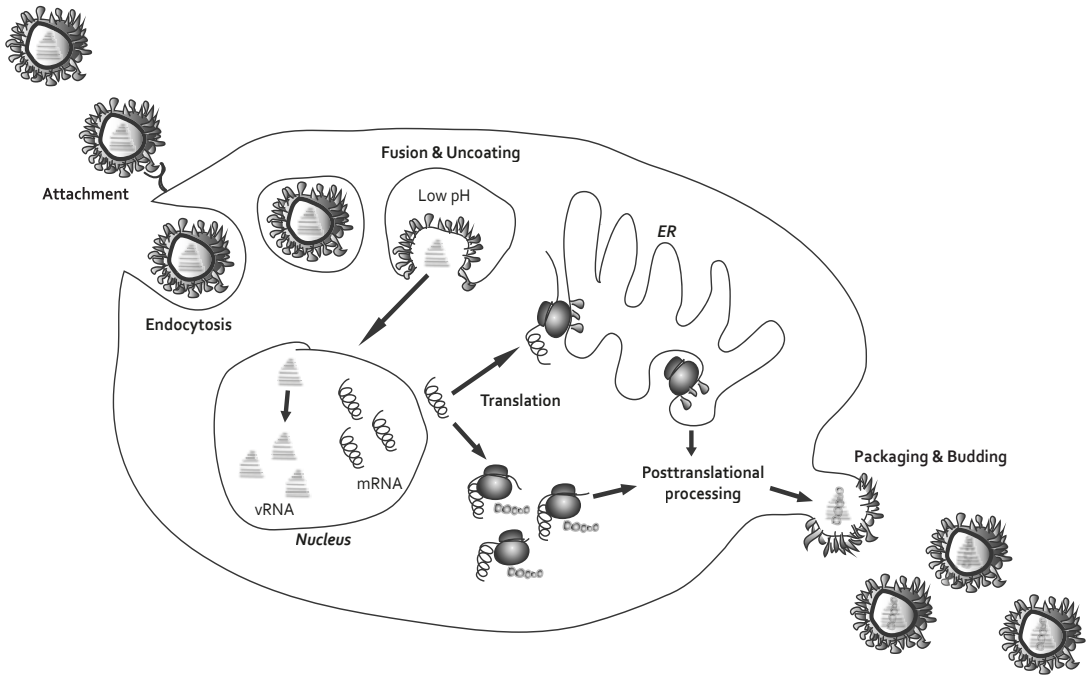


Figure 2 Impression of Influenza virus replication cycle. (ER= Endoplasmatic Reticulum)(vRNA= viral RNA)(mRNA= messenger RNA)

Influenza virus pathogenesis

Human influenza A virus predominantly infects cells in the upper respiratory tract. Fourty-eight hours after infection the peak in virus replication is reached.[11] The virus infects the epithelial cells on the surface of the larynx, trachea and bronchi and can infect type I and II pneumocytes in the alveolar walls.[3, 12] Infected cells vacuolize and die off due to necrosis or apoptosis, the latter induced by a shutdown of cell protein synthesis.[13] As a result of the infection and extensive cell death the tissue becomes inflammated, characterized by flooding of the lumen with proteinacious fluid and the influx of neutrophils and mononuclear cells. Normally, tissue regeneration starts around day 3 post infection. In the beginning cell loss and cell renewal are in balance, nevertheless regeneration will get the overhand and complete resolution of the damage to the epithelium of the respiratory tract is established within a month after infection.

A productive infection typically results in a tracheobronchitis [3] and clinical symptoms that have a rather acute onset: cough, shivers, headache and subsequently fever, total malaise and loss of apetite. The development of fever is the most prominent indication of infection and the highest body temperatures are reached between day 1 and 2 after onset of disease. Normally it will last no longer than six days. By that time viral shedding has also stopped, although it is known that children shed virus for a longer period of time.[3] After fever and viral shedding have resolved the infected individual can still have a cough and feel weak for an additional week. The most common complications caused by influenza A virus infection are primary viral pneumonia, as seen with many H5N1 cases in South East Asia [14, 15], a combination of viral and bacterial pneumonia or secondary bacterial pneumonia. These complications occur more often in risk groups: elderly, immunocompromised subjects, people suffering from cardiovascular disease, respiratory conditions (e.g. asthma) or diabetes, pregnant women and cigarette smokers. In these individuals at risk, also other complications occur as a result of the influenza virus infection such as, pulmonary and cardiac complications, respiratory exacerbations and diabetic ketoacidosis.[16]

Influenza virus infection can thus result in serious illnes and complications and could eventually lead to death. Therefore, treatment of the infection, especially in the high-risk individuals, is desirable. Antiviral drugs are available that can be used prophylactically and therapeutically. There are two types of influenza antiviral drugs.

The M2 inhibitors amantadine and rimantadine that inhibit virus replication at the uncoating step of virus replication.[17] The NA inhibitors zanamivir and oseltamivir block the release of newly formed virions from the cell inhibiting the enzymatic acitivty of NA.[18] Treatment with oseltamivir may result in the emergence of strains resistant to this antiviral drug as was seen in patients infected with influenza A/H5N1 virus.[19, 20] Since the 2007-2008 northern hemisphere influenza season there has been a rise in oseltamivir-resistant influenza A/H1N1 strains, independent of the use of the drug.[21-23] Although it appears that the mutation was not introduced by direct selective pressure due to extensive use of oseltamivir, it is clear that viruses with this mutation can easily spread in the human population. Besides the emergence of resistant strains, long-term prophylactic use of antivirals is unfeasible. That is why for long-term protection it is recommended to vaccinate high-risk individuals against influenza annually.[3]

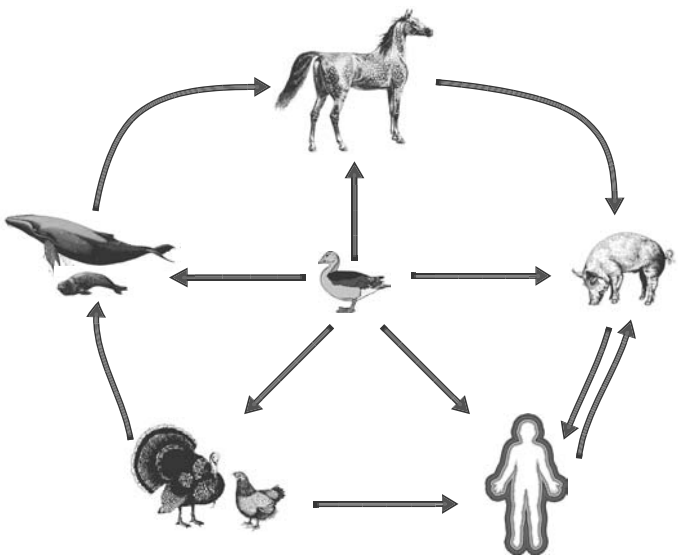


Figure 3 Influenza A virus reservoirs (adapted from [3])

Influenza epidemics and pandemics

Although influenza A viruses of the H1N1 and H3N2 subtype cause annual epidemics amongst humans, aquatic birds are the reservoir of all known subtypes.[2, 24] Occasionally viruses are transmitted from this reservoir to mammals like pigs, horses, marine mammals (seals and whales), dogs, minks, cats, tigers and humans. [3, 25-30]

Seasonal influenza epidemics

Yearly approximately 5-10% of the human population is infected with influenza A viruses of the H1N1 or H3N2 subtype or influenza B virus, globally causing illness in 3-5 million people and 250,000 to 500,000 fatal cases.[31, 32] These epidemics take place in the winter months twice a year on the Northern and Southern hemisphere respectively. The responsible viruses are most likely seeded from East and South-East Asia.[33] During the circulation in humans they gradually accumulate mutations in the RNA genome, resulting in amino acid substitutions preferentially located in the antigenic regions of the surface glycoproteins HA and NA recognized by virus neutralizing antibodies. Because of this antigenic drift, the vaccine composition of seasonal vaccines needs to be updated almost annually.[34]

Pandemics in the 20th century

Antigenic drift is thus based on minor changes in the HA or NA. The introduction of a new HA and/or NA subytpe results is a more drastic change and is called antigenic shift. This can happen by genetic reassortment of a human influenza virus with an avian or swine influenza virus or the introduction of a fully avian or swine influenza virus into the human population. The human population is immunologically naive for the new subtype and therefore the replication of virus is not hampered by specific antibodies and consequently, the new viruses may cause a pandemic: a worldwide outbreak of the new influenza A virus, affecting approximately 30% of the world population. This happened on three occasions in the 20th century: in 1918, 1957, 1968.[3]

In 1918 an influenza A virus of the H1N1 subtype spread worldwide in three waves and within 6 months killed 25-50 million people. The influenza virus responsible for the outbreak is known as the 'Spanish Flu' and, based on phylogenetic analysis, it is thought that it was a fully avian virus that was introduced in the human population.[35] However, conflicting results were obtained by others and the discussion on the exact origin is still ongoing.[36] Four decades after the 'Spanish Flu', in 1957, a new pandemic arose, this time caused by a reassortment of a human influenza A virus and an avian influenza A virus of the H2N2 subtype, resulting in a virus that contained the avian HA, NA and PB1 genes.[37, 38] This 'Asian Influenza' pandemic resulted in more than 1,000,000 deaths worldwide. This influenza A/H2N2 virus was replaced eleven years later by an influenza A/H3N2 virus. This strain originated from Southern China and caused the 'Hong Kong influenza' pandemic with an attack rate

of 40%.[3] Eleven years later the influenza A/H1N1 virus subtype was re-introduced, in the human population, probably by accidental release.[3] Since then, influenza A viruses of the H1N1 and H3N2 subtype 'co-exist'.

Pandemic threats in the 21st century

Since the last pandemic in 1968, single cases and small scale introductions of avian and swine influenza A viruses of various subtypes were reported (for review see De Wit et al).[39-41] A large outbreak of an avian influenza A/H7N7 virus in poultry in the Netherlands in 2003 resulted in 89 bird-to-human transmissions with 1 fatal case.[42, 43] In 1997 in South-East Asia the first human infections with highly pathogenic avian influenza A/H5N1 virus were reported.[44-46] Since 2003, over 400 human cases were reported to the World Health Organization (WHO) and more than 60% of the infected people succumbed to the infection.[47] So far, these viruses do not spread efficiently from human-to-human although sporadic clusters of human-to-human transmissions have been described.[48, 49] Apparantly, these viruses require further adaptation to their new host, as described above, in order to be transmitted and spread in the human population efficiently.

Pandemic in the 21st century

After 41 years since the last influenza pandemic, the introduction of a new influenza A/H1N1 virus that contains gene segments from classical and Eurasian swine influenza viruses and triple reassortants (bird, human, swine) has resulted in the first influenza pandemic of the 21st century.[50, 51] Previously, humans have been infected with swine influenza viruses sporadically.[3, 40, 41] However, since March 2009, the new influenza A/H1N1 virus, originating from swine, spread to 74 countries including the Netherlands and infected over 52,000 people and killed over 230 within three months.[52] The clinical outcome of these infections is relatively benign, although the mortality rate is ~0.5%. This virus continues to spread and is capable of human-to-human transmission.

<u>The immune defense against influenza virus infections</u>

The immune system responds in two different ways to influenza virus infection: the innate response that is quick and forms the first line of defense, and the adaptive immune response that is virus-specific and is based on humoral and cellular immunity.

Innate immunity upon influenza virus infection

Cells infected with influenza virus produce proinflammatory cytokines and chemokines that attract immune cells like alveolar macrophages, natural killer (NK) cells and dendritic cells. These cell types are responsible for opsonization of viruses and cell debri, elimination of virus-infected cells and antigen presentation, respectively. Furthermore they produce cytokines, triggered by recognition of viral components such as single stranded viral RNA by e.g. Toll like receptor (TLR) 7.[53] An important effect of the antiviral cascade is the activation of the interferon pathway that leads to the production of type I interferons: IFNα and IFNβ, key players in the innate immune response.[3] Binding of IFNα to cells in the respiratory tract triggers an antiviral state in which they downregulate their cellular processes, and hereby IFNα contributes to regulation of the infection.

The influenza virus NS1 protein, found only in infected cells, functions as a an IFNa-antagonist and distorts the antiviral state of the cell hereby improving viral pathogenicity.[54, 55] However, IFNα not only acts on the host cell, it also recruits immune cells and plays an important role in initiating the adaptive immune response. IFNa activates dendritic cells resulting in enhancement of antigen presentation to virus-specific CD_4+ T cells and CD8+ cytotoxic T cells (CTL), two key players of the adaptive immune response.[56, 57]

Adaptive immunity: the humoral response

Influenza virus infection results in the induction of virus-specific antibodies that, in the case of influenza A virus, are directed to the HA, NA, NP and M1 proteins predominantly.[58-61] Antibodies against the first two proteins can neutralize the virus and afford protective immunity against influenza virus infection. The two main antibody subtypes that are involved in virus neutralization *in vivo* are mucosal IgA and serum IgG that transudates into the lung. The HA-specific antibodies can prevent binding of the virus to and infection of the cell. They can also facilitate phagocytosis of virus particles by Fc receptor cells and activate the complement pathway. Antibodies directed to the NA can inhibit the enzymatic activity of this protein and prevent the release of newly formed virus particles from the cell surface. [3] The antibody response also contributes to elimination of virus-infected cells through antibody-dependent cell-mediated cytotoxicity (ADCC).[62] Antibodies against the HA and NA can persist for a long time, however the breadth of protection

by these antibodies is limited. They are specific for one subtype of the glycoprotein (e.g. H_3 and N_2) and drift variants that are antigenically too distinct can no longer be recognized.[34] Therefore the components of the seasonal influenza vaccine, that at the induction of HA and NA antibodies, need to be updated frequently.

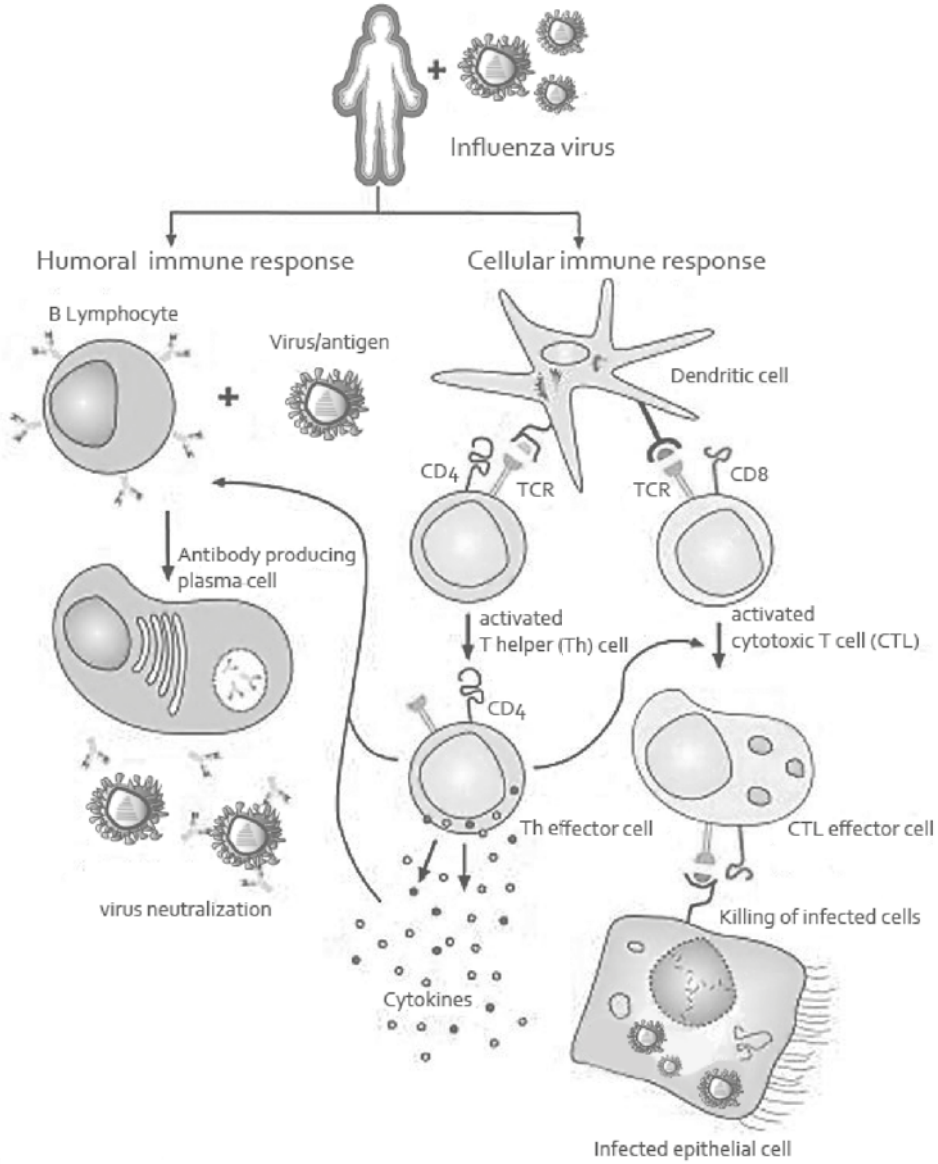


Figure 4 Immune responses to influenza virus infection. Adapted from: Influenza Report

Adaptive immunity: the cellular response

The cellular immune response comprises various T cell subsets. CD4+ T lymphocytes recognize virus-derived peptides in association with Major Histocompatilibity Complex (MHC) class II molecules.

These are present on professional antigen-presenting cells (APC), e.g. dendritic cells (DCs) with a $CD11b^{-}CD8a^{-}$ phenotype, also known as migratory DCs.[63] CD4+ T cells mainly function as T helper cells (type 1 and 2) and to a certain extent display cytolytic activity. They function as catalysts for the production of antibodies by B cells (T helper 2 cells) and the activation of CD8+ CTL (T helper 1 cells). The T helper 2 (Th2) cells produce cytokines that stimulate the proliferation and differentiation of B cells into antibody-producing plasma cells.[64] Furthermore cytokines are produced by T helper 1 (Th1) cells that stimulate the proliferation and differentiation of CD8+ CTL that, upon encounter with MHC class I associated influenza virus-derived peptides (epitopes), presented by infected cells, by APC or via cross-priming, expand to effector cell populations. These CD8+ CTL are able to recognize virus-infected cells and eliminate them by the excretion of perforin and granzyme B that premeabilize the membrane of the infected cell and induce apoptosis respectively, contributing to viral clearance (Figure 6). CTL responses are mainly directed to epitopes derived from the internal proteins of the influenza virus: NP, M1, and the polymerases, that are relatively conserved.[65-70] Therefore, CTL responses are highly cross-reactive in contrast to antibodies and they contribute to heterosubtypic immunity since they are able to recognize and respond to influenza viruses of different subtypes.[67, 71-73] A hallmark of the adaptive immune response is the formation of memory cells that can repond to second infection more rapidly and stronger.

Influenza vaccines

To encounter the seasonal influenza epidemics vaccines are available that are safe and have good efficacy. However new developments are made to improve and optimize vaccine production and improve immunogenicity. Furthermore, to limit a potential outbreak of a new influenza pandemic the availability of safe and effective vaccines is desirable and considered a high priority by the WHO.[74] Major efforts have been made to prepare H5N1 vaccines. However, there were a number of issues that complicated the development of such vaccines, including poor vaccine immunogenicity, long response times, limited production capacity and antigenic variation of circulating strains. Some of these issues have been addressed recently

and are discussed here.

Vaccine immunogenicity: since the population at large is immunologically naïve and not primed for an H5-specific antibody response, conventional inactivated whole virus and subunit preparations are poorly immunogenic and higher doses and/or multiple administrations are required to induce appreciable antibody responses. However, the use of adjuvants that were developed and evaluated recently improves the immunogenicity of the classical vaccine preparations and lowers the amount of antigen required for the induction of protective levels of virus-specific antibodies.

Response time: it is of great importance that vaccines become available as soon as possible after the start of a new pandemic and when the strain responsible for the outbreak has been identified. However, classic technologies to prepare safe and

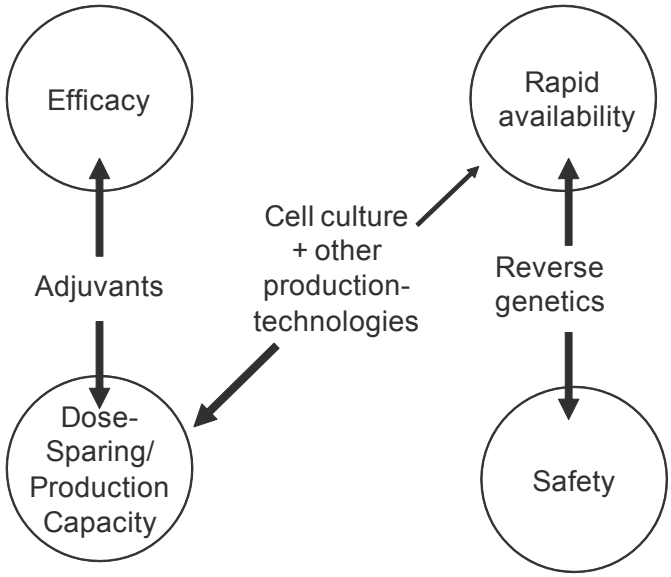


Figure 5: Vaccination strategies and hurdles

effective vaccine strains are time consuming and based on the reassortment of a vaccine backbone strain with the epidemic strain by double infection of embryonated chicken eggs. Over the last years, there have been a number of developments that may contribute to the timely delivery of vaccines. Novel methods have been developed to produce safe vaccine strains more rapidly and with high yields. Recombinant DNA (reverse genetics) technology is now an established procedure to prepare low pathogenic reassortant vaccine strains. Furthermore, cell-culture technology has become available for the flexible production of vaccine antigens.

Antigenic variation: the circulation of multiple antigenically distinct H5N1 viruses complicates the development of effective vaccines. These variants have been divided in clades and subclades.[75] Ideally a vaccine induces broad protective immunity against multiple viruses originating from different clades. With adjuvants that have become available recently it was possible to induce cross-reactive antibody responses in humans and cross-protective immunity in animal models.

Production capacity: at present the combined production capacity in embryonated chicken eggs of all vaccine manufacturers is limited and sufficient doses for a worldwide vaccination campaign cannot be produced in time. Alternative production technologies (e.g. cell culture) have become available to produce more vaccine doses in a flexible manner.

Novel vaccine developments were made during the last decade that overcome some of the difficulties described above and contribute to the rapid availability of sufficient doses of safe and effective vaccines for an influenza pandemic. The four areas of influenza vaccine production described above and the impact of implementation of the new developments are summarized in Figure 5.

Conventional influenza vaccines

Seasonal influenza vaccines are trivalent and contain components of influenza A viruses of the H1N1 and H3N2 subtypes and an influenza B virus, since these viruses are responsible for the annual influenza epidemics.[76]

For the production of vaccines, the growth characteristics of the influenza A vaccine strains and the yields of the viral antigens HA and NA are important issues. To obtain high yields of these antigens reassortant viruses are prepared by infecting embryonated chicken eggs simultaneously with a selected epidemic strain and an egg-adapted laboratory strain, typically influenza virus A/PR/8/34.

Reassortant viruses are selected that carry the hemagglutinin (HA) and neuraminidase (NA) of the epidemic strain and have the high-growth phenotype of the laboratory strain. To prepare effective vaccines, it is of great importance that the vaccine strains match the epidemic strains antigenically. Only then, protective immunity against these viruses will be induced most efficiently. Influenza viruses display a high extent of variation caused by a high mutation rate.[34] By accumulating amino acid substitutions in the antigenic sites that are recognized by the antibodies, variants are selected that can escape from the neutralizing activity of these antibodies that were induced by previous infections or vaccination. This form of variation, found

both in influenza A and influenza B viruses is called antigenic drift and necessitates the update of the vaccine composition when new drift variants emerge which are insufficiently recognized by antibodies induced by the existing vaccine.[76]
In order to assess the antigenic drift of influenza viruses, extensive global surveillance is necessary. Each year during the epidemics large numbers of influenza viruses are isolated and characterized antigenically by national influenza centers and World Health Organisation (WHO) collaborating reference centers. On the basis of this antigenic data and epidemiological data the WHO biannually provides recommendations for the composition of the influenza vaccine for the subsequent influenza season.[76] For example, it was recommended to replace all vaccine strains in the vaccine to be used in the Northern hemisphere 2008/2009 season.

Typically, for the seasonal influenza vaccines, one embryonated chicken egg is needed for the production of one dose that contains 15 micrograms of HA from each vaccine strain. In order to obtain the HA antigens, egg-derived virus is inactivated and used either as whole inactivated vaccine, split virion vaccine or subunit vaccine. For the preparation of whole-inactivated vaccines the virus is inactivated with formaldehyde or b-propiolactone before or after purification. Split virion vaccines are obtained by further treatment with a detergent to disrupt the virus. For the preparation of subunit vaccines, the HA and NA, which are important for the induction of virus-neutralizing antibodies and protective immunity, are further purified by removing other viral proteins and lipids through additional centrifugation steps.
The use of these vaccines has been a cornerstone in preventing influenza-related morbidity and mortality for decades. Patients with certain underlying diseases and the elderly are at high risk for influenza-related complications and benefit especially from vaccination. Therefore, annual vaccination is recommended for these high-risk groups. The vaccine is administered by intramuscular injection before the start of the influenza season. It is recommended also that children who have never been vaccinated and that have not been exposed to influenza viruses should be vaccinated twice with a four week interval. Since the vaccine is prepared with virus derived from embryonated chicken eggs, allergy to egg or egg-products is a contra-indication. Overall, the vaccines provide protection against disease in 70-90% of healthy adults.[77] The efficacy is lower (30-70%) in elderly patients but protection against influenza-related complications and mortality is achieved in 70-90% of the vaccinees in this age group.[77]

The production of these inactivated antigen preparations was also used as a template for the production of candidate H5N1 vaccines. However, it was found that without the use of adjuvants (see below) these antigens were poorly immunogenic in subjects naïve to H5N1 influenza viruses and antibody responses were only induced when high doses of vaccine were used.[78] This is of course not desirable considering the envisaged shortage of vaccines during a pandemic outbreak with these viruses.

Alternative formulations and production technologies

Live attenuated vaccines

The use of live attenuated viruses is another vaccine approach that has been applied successfully for various infectious diseases.[79] Also for vaccination against influenza, live attenuated vaccines have been developed, particularly in Russia and the United States.[80] Influenza viruses can be attenuated by adapting them to replicate at lower temperatures (25-33°C). This way, cold-adapted or temperature-sensitive virus strains can be obtained. To obtain cold-adapted vaccine strains these strains are re-assorted with selected epidemic strains which donate the gene segments encoding the HA and NA.

Instillation of these vaccine strains by the intranasal route does not result in the typical influenza-like symptoms.[81] Only in a small proportion of vaccinees some mild symptoms in the upper respiratory tract have been observed like runny nose and sore throat. These viruses seem sufficiently attenuated, are genetically stable and are not transmitted from vaccinated individuals to other subjects. Therefore, they are considered to be safe and a seasonal influenza vaccine based on this technology has been approved by the United States Food and Drug Administration (FDA) for use in humans 2-49 years of age (Flumist®).[82] Intranasal administration of live attenuated influenza vaccines (LAIV) usually induces good immune responses although the magnitude of the serum antibody response is dependent on the extent of virus replication. Furthermore LAIV induce mucosal IgA responses which are more cross-reactive than IgG responses and provide protection at mucosal sites and in addition, these vaccines induce CTL responses.[83-87] The effectiveness of live attenuated vaccines was found comparable to that of inactivated vaccines in a meta-analysis.[88] However, it has also been reported that, in young children, the effectiveness of LAIV was better than that of inactivated vaccines.[82, 89] The effectiveness of LAIV did not correlate with the magnitude of the serum antibody

response.[86] LAIV cold adapted H5N1 vaccines have also been evaluated in various animal models and it was found that these type of vaccines induced protective immunity against challenge infection with H5N1 viruses from different clades.[83, 90]

Other ways to attenuate influenza viruses are being investigated and include removal of (part of) the non-structural protein-1 (NS1).[54, 91, 92] This protein is known as an antagonist of IFN-a and truncation of NS1 prevents inhibition of IFN-a production because of the loss of the capacity to sequester dsRNA and activation of the NF-κB signaling pathway.[54, 91] Indeed these viruses have an attenuated phenotype in experimental animals.[92] In individuals with defects in their IFN-a response these viruses may gain virulence. Also the possibility that LAIV reassort with a wildtype HPAI virus is considered a risk, although there is no evidence so far that this might occur. The ease of production of these type of vaccines may be attractive when production capacity of inactivated vaccines falls short and does not meet the demand.

Production of vaccine strains by reverse genetics

The classic way to produce reassortant vaccine strains, by double infection of embryonated chicken eggs, is laborious and time consuming and therefore delays the timely availability of vaccine strains suitable for vaccine production. In addition, when highly pathogenic avian influenza viruses are used low yields can be expected since these strains are lethal to the chicken embryo's. Since the advent of reverse genetics technology for the genetic modification of influenza viruses, this procedure is used to generate safe pandemic vaccine strains in a well-defined fashion in the shortest time possible. To this end, cDNA of all viral gene segments is cloned into plasmids. To obtain a safe vaccine strain, the region encoding the basic cleavage site, associated with high virulence, is deleted from the HA gene by site directed mutagenesis. Upon transfection of Vero cells, the viral RNA is transcribed from the plasmids under control of a pol-I promotor and mRNA under control of a pol-II promotor (typically the CMV promotor) and virus is produced and subsequently rescued in a suitable cell line. The rescued virus can then be used for vaccine production. Reverse genetics virus-based vaccines induced protective immunity against homologous and heterologous virus in mice, ferrets and birds.[93-98]

An additional advantage of preparing vaccine strains this way, is that the risk of

extraneous contaminating agents is minimized, since the vaccines are entirely based on PCR amplified genes that are cloned into plasmids. Also the gene constellation is well-defined and can be selected at wish. In the light of pandemic preparedness, the accelerated availability of low pathogenic vaccine strains is undoubtedly the biggest advantage of this approach.[99, 100] Fast-track licensing and registration of a vaccine by using mock-up files will also contribute to shorter response times and faster availability of vaccines.[101]

The use of cell lines for vaccine production

As indicated above, the use of embryonated chicken eggs for influenza vaccine production suffers from a number of disadvantages. In addition, embryonated chicken eggs may be available in limited quantities or not at all when HPAI viruses are circulating widespread. Therefore, the use of egg-independent vaccine production methods is considered as a highly attractive alternative. Recently, cell lines have become available for influenza vaccine production.[102] Cell cultures are easier to handle and can be scaled up in a short period of time. Since this technology does not suffer from some of the logistic problems associated with the use of embryonated chicken eggs, it is more flexible and will reduce the response time to a certain extent, although the down-stream processing of the vaccine production remains essentially unaltered.

Different cell lines are currently in various stages of development and evaluated as influenza vaccine production platform including Madin-Darby-Canine kidney (MDCK) cells, Vero cells and PER.C6 cells.

MDCK cells were isolated in 1958 and have been adapted to grow on micro-carriers and under serum-free conditions.[103] It has been the cell-line of choice for a number of vaccine manufacturers.[102],[104] So far, MDCK-derived influenza vaccines have proven to be safe and immunogenic in clinical trails and one is already licensed for the European market.[105]

Vero cells are kidney fibroblasts from an African monkey and have been used for the production of polio vaccine for more than twenty years.[106, 107] The cells are also adapted to grow on micro carriers under serum-free conditions and cultures can be scaled up to 6000 liters without loss of quality.[106] Vero cells are preferred as the primary cells to be transfected with plasmids for the purpose of generating vaccine strains by reverse genetics technology (see above).[100, 108] Pandemic influenza vaccine candidates were produced by propagating wildtype HPAI H_5N_1 strains in

Vero cells followed by inactivation of the virus with formalin and UV-irradiation. [106] This WIV vaccine induced a protective immune response to homologous and heterologous H5N1 viruses in mice and was immunogenic in men.[106, 109] Surprisingly, this vaccine was immunogenic at a low dose (2x7.5 µg) without the use of an adjuvant.

PER.C6 cells originate from a human fetal retinoblast culture that was immortalized with recombinant DNA technology.[110] The cells are cultured in suspension under serum-free conditions and can be used to propagate influenza viruses or recombinant adenoviruses that express a selected influenza virus antigen.[111, 112] At present no data are available on the immunogenicity of PER.C6 derived influenza vaccines. Another advantages of the use of cell lines for the production of influenza vaccines is that no allergic reactions against egg-derived proteins will be evoked. [113] Furthermore, influenza viruses propagated in cell lines more closely resemble the original virus isolate than viruses propagated in chicken eggs.[114, 115]

Recombinant HA

As an alternative for the conventional production of viral antigen in embryonated chicken eggs or cell culture, the influenza virus hemagglutinin can be produced as a recombinant protein. For this purpose the expression of HA genes by recombinant baculovirus in insect cells has been used.[116-119]

For the generation of recombinant baculoviruses, the gene of interest is inserted into a baculovirus shuttle vector that is transfected into SF9 cells derived from the insect Spodoptera frugiperda.[116] Baculovirus expressed HA has been evaluated as a seasonal influenza vaccine and was shown to effectively induce virus neutralizing antibodies, provided protection against the occurrence of influenza-like illness and a license application has been submitted to the FDA.[120-123] Promising results have also been obtained with rH5-based vaccine although high doses were required for the induction of appreciable antibody response.[124]

Virosomes

Influenza virus antigens can also be incorporated in particles called virosomes. These are spherical unilamellar liposomal vesicles produced by the mixture of purified solubilized influenza virus with phospholipids.[125, 126] Upon vaccination these particles enter the cell in the same way as virions do, resulting in good immunogenicity due to virtually natural antigen-processing and presentation. Since

virosomes facilitate fusion with the endosomal membranes they can induce cellular immunity via the MHC class I pathway.[127, 128] Pre-existing HA-specific antibodies do not interfere with this process.[129] Virosomal vaccines are more immunogenic than conventional influenza vaccines in the elderly.[130-133] Inflexal V® is the first licensed virosomal vaccine and has been administered to more than 40,000,000 subjects.

Virus-like particles

Another particulate antigen formulation of influenza vaccines are virus-like particles (VLPs), which are formed after expression of the HA and NA genes and optionally the M1 gene or other influenza virus genes in insect cells.[134] This can be achieved by infection of SF9 cells with recombinant baculoviruses expressing these influenza virus derived genes. With VLP protective immunity against various influenza virus A subtypes could be induced.[134, 135] Also H5N1 candidate VLP-based vaccines were evaluated and it was shown that these vaccines induced cross-reactive antibodies in mice, providing protective immunity against H5N1 influenza viruses from different clades.[136, 137]

Adjuvants

Whatever technology is used for their production, candidate H5N1 vaccines are poorly immunogenic in naïve individuals. To overcome this problem, higher vaccine doses could be used to induce protective antibody levels (for review see Bridges et al).[138] However this is an undesirable scenario resulting in an even smaller portion of the human population that may be vaccinated in the face of a pandemic outbreak. Therefore, there was an urgent need for adjuvants that could increase the immunogenicity of existing vaccine preparations and that could reduce the dose of antigen required for the induction of protective immune responses (dose-sparing). As a result, more human subjects can be vaccinated with the available amount of antigen that can be produced.

Despite the modest adjuvant effect of aluminum-containing influenza vaccines described in the past four decades, the evaluation of alum as an adjuvant in pandemic H5N1 influenza vaccines has been the subject of several studies probably since it is an already licensed adjuvant free of intellectual property rights and with a known track record in the human influenza vaccine field.[78, 83, 139-143] Again the adjuvant activity in split virion and WIV vaccines proved to be modest and it seems

unlikely that efficient dose-sparing can be achieved with alum.
One concern about the use of alum in combination with formaldehyde-inactivated vaccines is that it could predispose for more severe disease upon subsequent natural infection as was described for alum-adjuvanted formaldehyde-inactivated respiratory syncytial virus and measles virus vaccines.[144, 145] However, with an experimental alum-adjuvanted H5N1 vaccine protective immunity was induced in macaques against challenge infection without any signs of disease exacerbation. [139]
Clearly there is a need for novel adjuvants that improve the immunogenicity and efficacy of influenza vaccines and pandemic influenza vaccines in particular and that would allow dose-sparing. Recently, promising adjuvants, that fulfill these requirements, have been developed (see below).

Oil-in-water emulsions
MF59 is an oil-in-water emulsion consisting of the oil squalene (a terpenoid cholesterol precursor) and the surfactant sorbitan triolate (Span 85) and polyoxyethylenesorbitan monooleate.[153] Addition of the adjuvant to an influenza subunit vaccine improved its immunogenicity.[152] MF59 most likely works through the activation of the macrophages and dendritic cells that phagocytose it.[148, 149, 153, 155] The adjuvant is well-tolerated and improved the immunogenicity of the infuenza vaccine and the breadth and longevity of protection especially in the elderly, one of the largest high-risk groups for influenza virus infection.[146, 147, 150-152] A seasonal MF59-adjuvanted influenza vaccine (Fluad®) has been licensed for use in the elderly in twenty countries and administered to over 20 million individuals.[153, 154] The oil-in-water emulsion was also used in candidate H5N1 vaccines and it was found that with 7.5mg of HA, protective antibodies could be induced compliant with the EU licensing criteria.[152]

More recently, another oil-in-water adjuvant, known as AS03, has been developed. It contains DL-a-tocopherol, squalene and Tween-80.[158] AS03 has been evaluated as an adjuvant in candidate pandemic vaccines. It improved the immunogenicity of H5N1 split virion vaccines in ferrets and humans and was well-tolerated.[156-159] The induced antibody responses were cross-reactive with H5N1 viruses from different clades and an antigen dose of only 3.8mg was sufficient for the induction of serum antibody titers in humans, compliant wit the license criteria.[157]

Table 1: Summary of vaccine formulations, production methods, adjuvants and alternative delivery methods discussed in the introduction

	Development	Example	References
Formulations	Inactivated	(whole virus, split virion, subunit)	[77, 78]
	Live-attenuated	Cold-adapted	[79-90]
		Defective NS1	[54, 91, 92]
	Virosomes		[125-133]
	Virus-like particles		[134-137]
Production	Cell-culture production	antigen	[102-115]
	Reverse genetics	reassortant strain	[93-101]
	Recombinant HA	antigen	[116-124]
Adjuvants	Alum		[78, 83, 139-145]
	Oil-in-water emulsion	MF59	[146-155]
		AS03	[156-159]
	ISCOMs		[160-164]
Alternative antigen delivery	DNA		[165-175]
	viral vector	adenovirus	[112, 176-180]
		MVA	[181-191]

Collectively, it is envisaged that the development of these type of adjuvants will contribute to the production of more efficacious seasonal and pandemic influenza vaccines and will improve the availability of these vaccines.

Immune stimulating complexes (ISCOMs)

ISCOMs are cage-like structures with a diameter of approximately 40nm, consisting of cholesterol, phospholipids and purified saponins of the tree Quillaja Saponaria Molina.[160, 162] The antigen of interest, e.g. the influenza HA or other viral proteins can be incorporated into this structure but this is not essential for its adjuvant effect. ISCOMs are in advanced stage of development and have been tested for a variety of different viruses.[163] As an adjuvant system for influenza vaccines its use resulted in strong antibody responses, Thelper cell responses and cytotoxic T lymphocyte responses in mice, chickens, macaques and humans (for review see Rimmelzwaan et al).[164] Its use improved immunogenicity and effectiveness of a candidate H5N1 vaccine considerably.[161] Over the years the composition of ISCOMs have been

modified and improved to reduce reactogenicity with full retention of its adjuvant effect.

Other adjuvants

Many other adjuvants are in various stages of development (for review see Vogel et al).[192] Their potential in (pandemic) influenza vaccines needs to be assessed but it is likely that some of these will be promising since their adjuvant effects have already been demonstrated in combination with other antigens.[192]

Alternative antigen delivery

DNA vaccines

After it was shown that intramuscular injection of DNA expression vectors resulted in the expression of protein encoded by the DNA this approach has been explored as a vaccination strategy aiming at the induction of immune responses directed to the expressed proteins.[172, 175] Ulmer et al have shown that injection of DNA encoding influenza virus nucleoprotein can confer protection in mice against infection with homologous and heterologous virus strains.[173] This protection correlated with the induction of virus-specific and cross-reactive CTL responses. Numerous studies in various animal species have demonstrated the induction of influenza virus-specific cell-mediated and humoral immune responses by plasmid DNA encoding influenza virus proteins.[165, 167, 170-172, 174] During the last decade a lot of information has become available about how the outcome of DNA vaccination is affected by the dose of DNA used, the route of administration (epidermal delivery), delivery systems (e.g. gene gun), the role of dendritic cells and muscle cells in antigen presentation and the presence of CpG motifs in the DNA.[168] The advantages of the use of DNA expression vectors are obvious. Plasmid DNA can be produced rapidly and at low costs in bacteria and its production is independent of classical vaccine production technology. Although a lot of information has been obtained in animal models, knowledge on the efficacy of this type of vaccines in humans is sparse.[166]

The main reason for this gap of knowledge probably is the concern over the safety of such vaccines.[169] Some of the safety issues that need to be addressed include the risk of integration of the vaccine derived DNA in the host genome, increasing the risk of malignancies and the risk of inducing tolerance rather than immunity.

Viral vectors

Several viruses have been used as recombinant vector vaccines for the delivery of antigens of interest, for example a foreign viral protein. Herpes viruses, measles virus, alpha viruses, lentiviruses, adenoviruses and pox viruses are examples of viruses that have been used for this purpose.[193, 194] The latter two have been tested preclinically as candidate H5N1 vaccines.

Adenovirus

Recombinant adenoviruses have been modified genetically rendering them unable to replicate in normal human cells and enabling them to express one or more genes of interest e.g. influenza virus HA, NA and M1 genes. Recombinant adenoviruses can be produced rapidly and at a large scale in specialized cell lines that support packaging of the otherwise replication deficient viruses.[178] Recently, candidate H5N1 vaccines based on recombinant adenoviruses were tested in animal models.[112, 176-180] It was shown that immunization with an adenoviral vector expressing the HA gene alone or in combination with other genes of H5N1 influenza viruses induced protective immunity against a lethal challenge which was mediated by virus-specific antibodies and CTL.[112, 176-180] The presence of antibodies specific for adenoviruses in the human population may interfere with vaccine efficacy, but the availability of various serotypes of recombinant viral vectors may circumvent this problem and may allow repeated vaccination with this type of vaccine.

Modified Vaccinia virus Ankara

Also replication deficient poxviruses can be used as a vaccine vehicle for the delivery of foreign antigens. Especially the modified vacccinia virus Ankara (MVA) is a promising candidate and has been used for this purpose in many studies. recMVA expressing the HA and NP gene of influenza virus A/PR/8/34 was originally tested as a candidate influenza vaccine and it was shown that in mice it induced HA-specific antibodies and NP-specific CTL and conferred protection against challenge infection.[190]

More recently, recombinant MVA were constructed that express the HA gene of H5N1 influenza viruses. The evaluation of the MVA-HA vaccine is described in this thesis. The use of recombinant MVA vectors has many advantages. First of all, these vectors are safe and well-tolerated in humans, also in immunocompromised subjects.

[183, 187] MVA has already been administered to >120,000 subjects as a smallpox vaccine.[183] It can be produced at large scale under GMP BSL-1 conditions, is stable and can be stored for long periods of time, which may be relevant for stockpiling of vaccines. [181, 184, 186, 188, 189, 191] Furthermore the presence of pre-existing antibodies to the vector does not interfere with vaccine efficacy and so repeated vaccinations are possible. [182, 185]

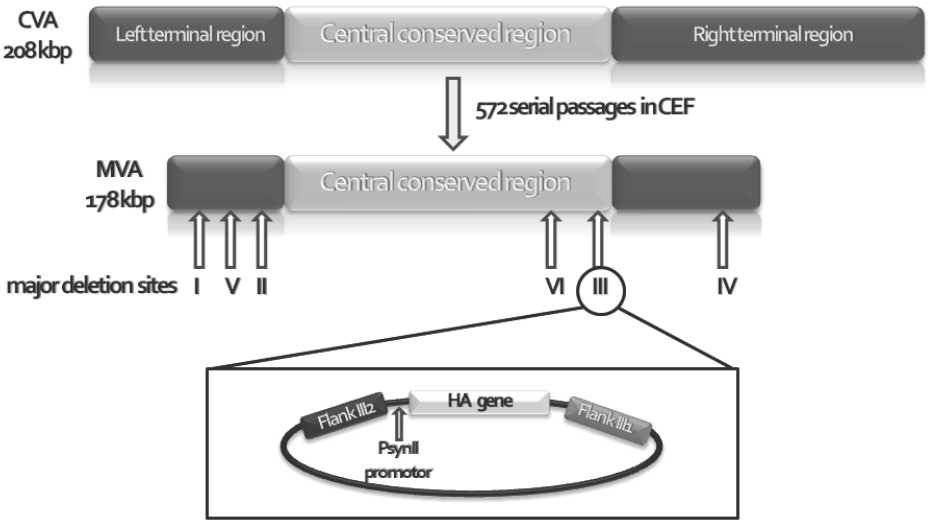


Figure 6 Recombinant MVA expressing the HA-gene (adapted from a figure by G. Sutter)

Alternative vaccine administration methods

The conventional inactivated influenza vaccines are administered by intramuscular injection. Live attenuated influenza vaccines typically are administered by the intranasal route and this way elicit a mucosal immune response, in addition to a systemic immune response. Sublingual administration is another mucosal route and eliminates the risk of virus reaching the olfactory bulbs. Intradermal immunization is being explored as an alternative, needle-free, dose-sparing route of administration. For review of these and other alternative administration routes that are in various stages of development see Girard et al and Weniger et al.[195, 196]

Cross-protective immunity

Intra-subtypic cross-protection

The production of effective vaccines is complicated by the antigenic variation

that influenza viruses display within a certain subtype. This holds true for seasonal influenza virus strains and necessitates the annual update of the vaccine composition to match the epidemic strains as closely as possible. Ideally vaccines confer the broadest possible protection against a variety of viral antigenic variants. The addition of the adjuvant MF59 to conventional vaccine preparations has broadened the cross-reactivity of the vaccine-induced antibodies response to a certain extent. [146, 147] The induction of intra-subtypic cross-reactive antibodies is also relevant for the development of candidate H5N1 vaccines, since multiple antigenically distinct clades of influenza H5N1 viruses have been identified. [75] Again, the use of an adjuvant can broaden the reactivity of the antibody response against H5N1 viruses resulting in the induction of protective immunity against viruses derived from different clades.

Also NA-specific antibodies can be cross-reactive and it has been shown that some humans have serum antibodies directed to the N1 of human A/H1N1 viruses that cross-react with the NA of H5N1 viruses. [197] This may afford some level of protective immunity against H5N1 viruses.

Inter-subtypic cross-protection

Until March 2009 the main pandemic threat came from bird-to-human transmissions of H5N1 viruses. Then, a new influenza A/H1N1 virus emerged in Mexico, spread worldwide within two months and the WHO declared the start of a pandemic on the 11th of June 2009.[51] Avian influenza A virus have also caused infections of humans include H9N2 and H7N7 and it cannot be excluded that other subtypes known to be able to cause a pandemic, like H2N2 viruses, will return. [42, 43, 198] Therefore, there is considerable interest in the development of "universal" vaccines that induce inter-subtypic cross-reactive immunity (or heterosubtypic immunity) and that can afford protection against different subtypes of influenza A virus. [74] To achieve this, immunity should be directed to alternative viral target proteins that are more conserved than HA and NA, like the nucleoprotein (NP) and the matrix proteins M1 and M2. NP and M1 are targets for cellular immunity, in particular CTL responses, and it has been demonstrated that CTL specific for seasonal influenza viruses can cross-react with H5N1 viruses. [73, 199] Furthermore it has been shown that cross-reactive CTL responses also afford heterosubtypic immunity (for review see Kreijtz et al).[67, 200] Vaccines that aim at the induction of cross-reactive CTL responses need to deliver the antigens in such a way that the proteins enter the endogenous

antigen processing and MHC class I presentation pathway, which is important for the efficient induction of CTL responses. Examples of such antigen delivery systems include the use of live attenuated viruses, viral vectors and DNA vaccination.[80, 172, 194]

The M2 protein, an ion channel in the virus replication cycle involved in uncoating of the viral genome, is relatively conserved and a target for antibodies. M2 is a minor antigen on virus particles but is expressed on virus infected cells. The antiviral effect of M2-specific antibodies is most likely based on the induction of antibody dependent cell-mediated cytotoxicity (ADCC) and may contribute to protective immunity.[201-204] Candidate vaccines based on the induction of M2-specific antibodies have been evaluated preclinically and with some of them promising results have been obtained.[201-204]

Vaccination strategies

For the prevention of seasonal influenza, vaccines are typically administered just before the beginning of the influenza season in the winter months. For individuals at high risk for complications of an influenza virus infection, including the elderly, vaccination is recommended. For children under six years of age, which are less likely to be primed for a secondary antibody response, two immunizations with an interval of four weeks are recommended. In the USA, it is also recommended to vaccinate all healthy children from the age of six months, since influenza is an important cause of hospitalization in this age group and children are important vectors for spreading the disease.[205]

For the use of pandemic influenza vaccines there are several options.[206] It has been demonstrated that H5N1 vaccines can be used as pre-pandemic vaccines in order to prime individuals for anamnestic antibody responses against an antigenically variant strain that would match the pandemic strain.[207] This may be an attractive strategy, however it cannot be predicted with certainty whether influenza viruses of the H5N1 subtype will cause the next pandemic and if so, which variant. The H5N1 viruses that have been isolated so far differ considerably genetically and antigenically and subdivision of these viruses in clades and subclades was necessary.[75] As indicated above, also other subtypes of influenza viruses have pandemic potential, H2N2, H7N7 and H9N2. And the newly emerged influenza A/H1N1 virus caused the first influenza pandemic of the 21st century (Mexican flu). Therefore, preparing and using prepandemic vaccines is taking a risk and the resources required to produce the

antigen and in particular an adjuvant, which would be the most expensive component of the vaccine, may be used in a different way. It could be envisaged that as soon as the pandemic strain will be identified, the corresponding vaccine strain will become available quickly (through reverse genetics) and subsequent vaccine production can start immediately. The availability of stockpiled adjuvant would allow the rapid formulation of the final vaccine. The most important advantage of this strategy is that antibody responses of the proper specificity will be induced and that protective immunity is induced which would reduce shedding of virus in the population. This in turn may contribute to optimal control of the outbreak at the population level. Nevertheless, drawbacks of this strategy are the delay in vaccine availability and the need for more than one vaccination to achieve protective antibody levels. In case of the current situation with A/H5N1 viruses, the use of vaccine antigen stockpiled before the start of the pandemic could prime for secondary antibody responses even against a variant pandemic vaccine strain.[207]

During the last decade, big strides have been made in the development of more efficacious and safe influenza vaccines, spurred by the pandemic threat caused by influenza A viruses of the H1N1 and H5N1 subtype. Undoubtedly, the advent of novel procedures (reverse genetics) to prepare vaccine reassortant strains, novel production technologies like the use of cell culture and the development of safe and potent adjuvants will contribute to faster availability of more doses of safe and effective vaccines. Although the emergence of a pandemic cannot be prevented, the fast availability of such vaccines most likely will contribute to a better control of a pandemic outbreak of influenza.

Outline of the thesis

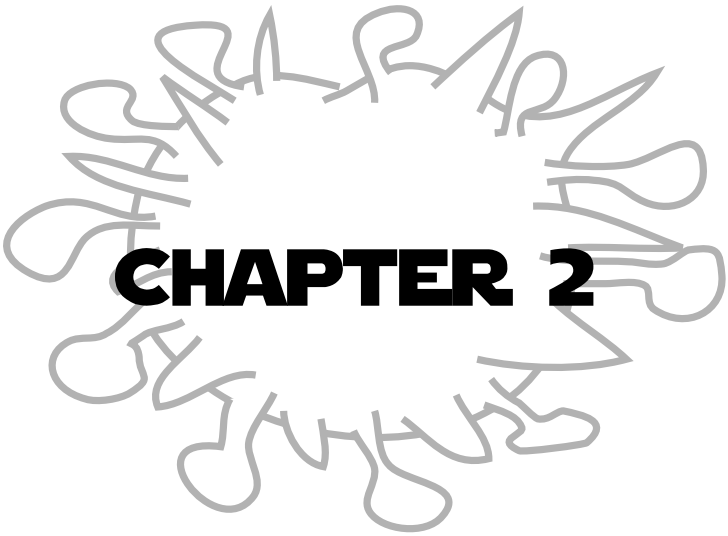
In this thesis, protection against influenza A(H5N1) induced by primary infection with influenza A(H3N2) and MVA-based vaccination was assessed. The first form of protection is based on heterosubtypic immunity which has been studied extensively. CTL are considered to be key players in this type of immune defense. In the light of a new pandemic, a situation in which neutralizing antibodies against the causative agent are virtually absent, CTL, induced by previous infection(s), might play a crucial role in protection against infection.

To assess the protective role of pre-existing immunity we infected mice with influenza A virus X-31 (H3N2) and subsequently challenged them with influenza virus A/Puerto Rico/8/34 (A/PR/8/34) (H1N1). These viruses have identical internal proteins and priming with influenza virus X-31 induced protective immunity against the A/PR/8/34 virus, as described in chapter 2. This proof-of-principle with mouse-adapted strains with fully matched internal proteins warranted a second experiment with a more realistic combination of viruses used for primary and challenge infection. In chapter 3, a human influenza virus of the H3N2 subtype A/HongKong/2/68 (A/HK/2/68) was used for the primary infection, followed by a lethal challenge with HPAI A/H5N1 virus A/Indonesia/5/05 (A/IND/5/05). H3N2-infected animals were protected against the lethal challenge and survived the H5N1 infection whereas naïve animals suffered from severe disease and succumbed. The observed protection correlated with the induction of virus-specific CTL. It was hypothesized that vaccination against the human influenza A/H3N2 virus would interfere with the induction of heterosubtypic immunity by H3N2 infection. To test this hypothesis mice were vaccinated prior to infection with human influenza virus A/HK/2/68, followed by a lethal challenge with influenza virus A/IND/5/05. Indeed, animals effectively vaccinated against the primary infection were not protected against the lethal challenge which correlated with the absence of anamnestic CTL responses after challenge infection. The results of this experiment are described in chapter 4. Thus, cross-reactive virus-specific CTL are associated with protection against H5N1 influenza virus infection. In chapter 5, we assessed the cross-reactivity of human CTL specific for seasonal influenza viruses with H5N1 influenza viruses. Cells that expressed the H5N1-derived NP and cells infected with H5N1 influenza virus were recognized by *in vitro* expanded human CTL and the level of recognition of H5N1 influenza virus was similar to that of human influenza A virus. Thus, pre-existing CTL immunity to influenza viruses in the human

population may provide a certain level of protection against heterosubtypic viruses. Subsequently, the cross-reactive T cells can be targeted by 'universal' vaccines that are based on conserved antigens in the influenza viruses and boost the strength and breadth of the pre-existing T cell immunity.

The role of pre-exisiting immunity to influenza A viruses of the H5N1 subtype and other potentially pandemic strains could be of great importance during a pandemic outbreak. However for the induction of optimal protective immunity against these viruses, there is a need for the availability of safe and effective vaccines that can induce antibody responses of the proper specificity. MVA is a promising candidate vaccine platform that also could be used for the development of pandemic influenza vaccines. This replication deficient poxvirus is relatively easy to produce at large scale, has an excellent safety profile in humans and can be used as a vector for foreign proteins, e.g. HA. MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 (A/VN/1194/04) was evaluated as a (pre)pandemic H5N1 vaccine in two preclinical models (chapter 6 and 7). In chapter 6 it is described that mice vaccinated twice with MVA-HA-VN/04 were protected against infection with the homologous strain and a antigenically distinct strain of H5N1 virus. Since the predictive value of results obtained in the mouse model is limited for humans, this promising vaccine candidate was also tested in a non-human primate model. To this end, cynomolgus macaques were vaccinated twice with a high dose of MVA-HA-VN/04 and challenged with the homologous and a heterologous influenza A/H5N1 virus strain. Vaccinated animals were protected, irrespective of the challenge virus that was used (chapter 7). For application in a (pre)pandemic situation it is desirable that a low dose can be used for the induction of protective immunity which would allow dose-sparing and would maximize the number of subjects that can be protected from infection. Preferentially a single immunization would be sufficient for the induction of protective immunity. In chapter 8, the minimal dose of MVA-HA-VN/04 required for protection was determined in mice. They were vaccinated once or twice using five different doses of MVA-HA and it was found that the vaccine dose could be lowered dramatically without loss of protective efficacy against homologous and heterologous H5N1 viruses. In addition it appeared possible to protect mice with a single immunization of MVA-HA-VN/04.

The results presented in this thesis illustrate the protective efficacy of heterosubtypic immunity against influenza A(H5N1) virus infection and the potential of recombinant MVA as a (pre)pandemic influenza vaccine.



CHAPTER 2

Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice

J.H.C.M. Kreijtz, R. Bodewes, G. van Amerongen, T. Kuiken, R.A.M. Fouchier, A.D.M.E. Osterhaus, G.F. Rimmelzwaan

Department of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands

Vaccine 2007; 25:612-20

Abstract

In order to assess the level of protection against a lethal influenza virus infection provided by a primary infection with a virus strain of another subtype, C57BL/6 mice were infected with the sublethal influenza virus X-31 (H_3N_2) and subsequently challenged with the lethal strain A/PR/8/34 (H_1N_1). The outcome of the challenge infection was compared with that in mice that did not experience an infection with influenza virus X-31 prior to the challenge infection. The X-31 experienced mice cleared the infection with influenza virus A/PR/8/34 in an accelerated fashion, displayed less clinical signs and a reduction of lesions in the lungs resulting in improved survival rates of these mice compared to the naive mice. The improved outcome of the challenge infection with influenza virus A/PR/8/34 in the X-31 experienced mice correlated with priming for anamnestic virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses as was demonstrated by the detection of CTL specific for the $H\text{-}2D^b$ restricted $NP_{366\text{-}374}$ epitope that was shared by the influenza viruses X-31 and A/PR/8/34. Thus previous exposure to influenza A viruses affords partial protection against infection in the absence of virus-neutralizing antibodies specific for the hemagglutinin and the neuraminidase. The implications of these observations are discussed in the light of the current pandemic threat and development of vaccines that aim at the induction of virus-specific CTL.

Introduction

Since 1997 several examples of direct transmission of avian influenza A viruses from birds to humans have been reported [15, 44-46, 208, 209]. Especially infection of humans with the H5N1 subtype caused severe morbidity and mortality: since December of 2003, 218 confirmed cases were reported in Azerbaijan, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Thailand, Turkey and Vietnam; 124 of these cases proved to be fatal [209]. These virulent H5N1 viruses are considered to have the potential to cause a new pandemic and the further global spread of these viruses is feared [210, 211]. If these viruses acquire the capacity to be transmitted from human to human efficiently, then antibodies raised to human influenza A viruses of the H3N2, H1N1, or H2N2 subtypes will provide little or no protection against infection. However, other viral proteins like the nucleoprotein (NP) and the matrix protein are more conserved than the hemagglutinin and the neuraminidase and are major targets for virus specific CD8+ cytotoxic T lymphocytes (CTL) [67, 212, 213]. Therefore, it could be anticipated that T cell immunity induced after infection with human influenza A viruses is cross-reactive with avian influenza viruses and could provide some degree of protection against infection with these highly pathogenic viruses.

Indeed CTL cross-reactive with avian influenza viruses were demonstrated in humans [70, 73]. Furthermore, it was demonstrated in experimentally infected individuals that the extent of viral excretion inversely correlated with CTL activity in the absence of antibodies specific for the virus that was used for infection [67]. Also in various animal models a protective role for CTL against heterotypic viruses was confirmed [174, 214-218]. Since it is not clear which variant of H5N1 virus, or even which influenza virus subtype ultimately will cause the next pandemic, the selection of a vaccine strain that should induce an adequate HA-specific antibody response is still not possible. Therefore there is considerable interest for the development of a universal vaccine that could induce cross-reactive and long-lasting protective immunity against a variety of viruses with a different subtype [219, 220].

In the present study we assessed the extent of protection afforded by exposure to influenza viruses against a secondary infection with a heterosubtypic influenza virus. To this end we took advantage of a mouse model that has been used previously for the detection of heterosubtypic immunity. In contrast to these previous studies, we integrated clinical, virological, immunological and pathological parameters to

evaluate the extent of protection provided by a primary infection. C57BL/6 mice were infected sublethally with influenza virus X-31 (H_3N_2) and subsequently infected with a lethal dose of influenza virus A/Puerto Rico/8/34 (A/PR/8/34) (H_1N_1) [213, 221-223]. Influenza virus X-31 is a reassortant virus containing the internal proteins of influenza virus A/PR/8/34 and the haemagglutinin and neuraminidase glycoproteins of A/Aichi/2/68 (H_3N_2). A/PR/8/34 is a human H_1N_1 influenza virus adapted and lethal to mice [217, 218, 224, 225].

Protective immunity following challenge infection was assessed by measuring survival rates and lung virus titers, and using histopathology and immunohistochemistry. These results were correlated with the detection of virus specific CTL responses before and after challenge infection by tetramerstaining [223, 226, 227]. The integrated approach provided evidence for the induction of cross-protective immunity by primary infection to heterosubtypic influenza A strains. The findings are discussed in the light of the current pandemic threat and the design of candidate vaccines, which aim at the induction of cross-protective CTL responses.

Material and Methods

Mice

Female specified pathogen free 6-8 weeks old C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Mice (n=14) were infected with $2*10^4$ TCID50 of influenza virus X-31 (in 50ml PBS) by the intranasal route (Group I) and five of these animals were sacrificed on day 4, five on day 7 and four on day 28 post infection (p.i.). Group II mice (n=16) were first infected with influenza virus X-31 as described above and were subsequently infected with $5*10^4$ TCID50 of influenza virus A/PR/8/34 on day 28 p.i.. Six of these animals were sacrificed on day 4 post challenge infection, six on day 7 and four on day 28. A third group of twelve naive mice (Group III) was infected with influenza virus A/PR/8/34 only as described above, and were sacrificed on day 4 (n=9) and day 7 (n=3). Mice in Group IV were inoculated with PBS and were included as negative controls and at day 4, 7 and 28 after inoculation two of these mice were sacrificed. According to protocol mice were euthanized when they showed a weightloss of >20% after infection. Mice in all four groups were age-matched at the time point of challenge infection with influenza virus A/PR/8/34.

The experimental protocol was approved by an independent animal ethics committee prior to the start of the experiment. On all timepoints (day 4, day 7 and day 28 p.i.), spleen and lungs were resected from the sacrificed mice. Blood was

drawn by orbita puncture (exsanguination) from mice that were sacrificed on day 28 after infection with influenza virus X-31 and 4, 7 and 28 days after infection with influenza virus A/PR/8/34. Intranasal infections, orbita punctures (and euthanasia) were carried out under anaesthesia with inhalative isoflurane. The animals were housed in filter-top cages and had access to food and water *ad libitum*.

Influenza viruses

Influenza viruses A/PR/8/34 and X-31 (MRC, Cambridge, England) [224] were inoculated in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested after 2 days. Infectious virus titers were determined in Madin-Darby Canine Kideny (MDCK) cells (ATCC: Product CCL-34 (NBL-2)) as described previously [228].

Lung virus titers

Lungs were snap frozen on dry ice with ethanol and stored at -70°C. Lungs were homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection medium (Eagles Minimal Essential Medium (EMEM), Bovine serum albumin (fraction V 7,5%, 1:25), 4mg/ml trypsin, 2mM L-glutamin, 100U/ml penicillin, 100mg/ml streptomycin, 7,5% $NaHCO_3$, 1M Hepes). Quadruplicate ten fold serial dilutions of these samples were used to determine the virus titers in MDCK cells as described previously.[228]

Serology

Sera were obtained on day 28 after infection with influenza virus X-31 and on day 4, 7 and 28 after infection with influenza virus A/PR/8/34 and stored at -20°C until use. 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 X-31 or A/PR/8/34 [229]. Rabbit sera, raised against influenza viruses A/Hongkong/2/68 and A/PR/8/34 were used as positive controls. Sera were also tested for the presence of virus-neutralising antibodies specific for both influenza viruses X-31 and A/PR/8/34 using a micro virus-neutralisation (VN) assay with 100 $TCID_{50}$ of the respective viruses [230]. The same rabbit sera were used as positive controls.

Detection of virus-specific CTL by tetramer-staining

Single cell splenocyte suspensions were obtained and red blood cells were removed using erythrocyte lysis buffer (Roche, Almere, the Netherlands). The cells were washed with 0,5% BSA in PBS and stained for flow cytometry with antibodies: CD3e-PerCP, CD8b.2-FITC (PharMingen, San Diego, United States), ToPro 3-APC (Molecular Probes, Eugene, United States) and PE labeled H-2D^{b}-PE tetramer with the $NP_{366-374}$ epitope ASNENMETM (Sanquin Research, Amsterdam, Netherlands). Cells were analysed on FACSCalibur in combination with Cellquest Pro software (Becton Dickinson, Alphen a/d Rijn, Netherlands).

Histopathology

After sacrificing the mice lungs were resected and inflated with formalin. After fixation in 10% neutral buffered formalin and embedding in paraffin, the lungs were sectioned at 4mm and stained with hematoxylin and eosin for histological evaluation. Furthermore, 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 the secondary antibody. The peroxidase was revealed using diamino-benzidine as a substrate, resulting in a deep red precipitate in the nuclei of influenza A infected cells and a less intense red staining of the cytoplasm. The sections were counterstained with hematoxylin.

Statistical analysis

Data for weight and tetramerstaining were analysed using the two-sided Student's *t* test and differences were considered significant at $P < 0.05$. Survival rate was analysed using a logrank test.

Results

Clinical signs

Weight loss and survival rate were used as clinical parameters to evaluate protective immunity. Four days p.i. with influenza virus A/PR/8/34 the naive mice (Group III) had lost 17.6% (SD=9.2) of their body weight. The loss of body weight in the X-31 experienced mice (Group II) was 12.3% (SD=6.9), which was a statistically significant difference (p=0.013) (Figure 1A). This difference was greater seven days p.i.: the

unprimed animals (Group III) that were still alive on day seven continued to lose body weight.

The average loss of body weight in this group was 15.7% (SD=3.7) whereas the X-31 experienced mice (Group II) gained weight from day 4 p.i. onwards (data not shown). The mean weight loss in this group seven days p.i. was 1.1% (SD=4.3) which was significantly lower than in the naive mice (Group III). Since a loss of >20% of body weight was used as a criterium for euthanizing the animals, we used this criterium as correlate for infection-induced mortality. Indeed mice that lost >20% of their body weight were severely ill, were lethargic, had ruffled fur and were moribund. Seven days p.i. only 33% of the naive mice (Group III) survived infection with influenza virus A/PR/8/34 (Figure 1B). In the group of X-31 experienced mice (Group II) 87.5% survived the challenge infection. This difference in survival rate was statistically significant (p=0.02).

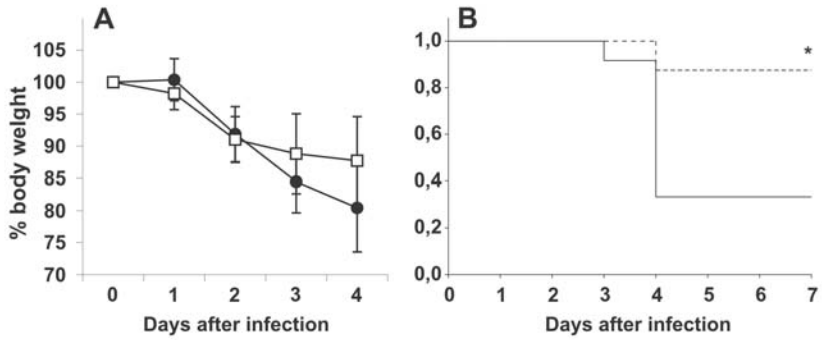


Figure 1 (A) Mean loss in body weight of mice after infection with influenza virus A/PR/8/34 in the X-31 experienced mice (Group II) (□) and the naive mice (Group III) (●). (*) indicates that the difference between the two groups on day 4 is statistically significant (p=0.013). (B) Survival rate of mice after infection with influenza virus A/PR/8/34 in the X-31 experienced mice (Group II) (----) and the naive mice (Group III) (——). The proportion of survival was determined based on euthanasia-criteria. Animals that lost >20% of their bodyweight were considered moribund. At day 3 one naive animal had to be euthanized. At day 4 eight naïve animals were euthanized based a weightloss of more than 20% and six X-31 experienced animals with less than 20% weightloss were sacrificed according to the study-protocol At day 7 three naive animals and ten X-31 experienced mice were alive. (*) Indicates that the difference in survival rate is statistically significant (p=0.02).

Lung virus titers

The virus titers in the lung at day 4 and day 7 p.i. were compared between the groups of mice infected with influenza virus A/PR/8/34 with or without a history

of infection with influenza virus X-31. The influenza virus A/PR/8/34 titers (Figure 2) in X-31 experienced mice and naive mice (Groups II and III respectively) at day 4 after challenge infection did not differ significantly (Figure 3). However, seven days p.i. the average virus titers in the lungs of naive mice (Group III) was still $1.9*10^6$ ($SD=2.9*10^3$) $TCID_{50}$ where as in none of the X-31 experienced mice (Group II) virus was detected. This indicated that a prior infection with a heterosubtypic virus resulted in the induction of immunity that was responsible for accelerated clearance of the infection with influenza virus A/PR/8/34 by day 7 p.i..

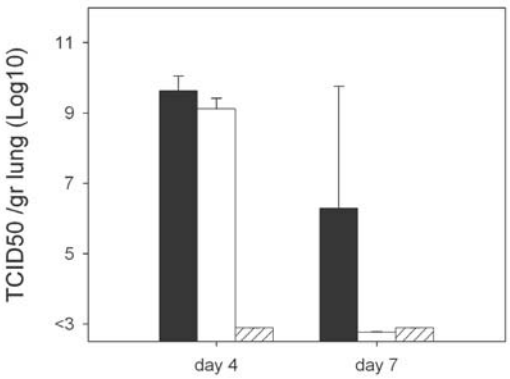


Figure 2 Virus titers in the lungs of mice after infection with influenza virus A/PR/8/34 at day 4 and day 7 post infection. The results are shown for the naive group (Group III) (■) the X-31 experienced group (Group II) (▨) and the PBS-inoculated group (□). (<3= cut-off value)

Serology

After infection with influenza virus X-31, mice developed virus-specific antibodies that were detected by HI- and VN assay that did not crossreact with influenza virus A/PR/8/34. Twenty-eight days after infection with influenza virus X-31 the HI- and VN GMT were 1076 and 67 respectively. X-31 experienced mice (Group II) that were infected with influenza virus A/PR/8/34 developed antibodies against this virus. The mean titer was 190 in the HI assay and 20 in the VN-assay as soon as seven days p.i.. In the X-31 experienced mice (Group II), infection with influenza virus A/PR/8/34 induced similar titers as those observed in the naive animals (Group III). In the X-31 experienced mice (Group II) 28 days after challenge infection these influenza virus A/PR/8/34 titers had increased up to 640 in the HI- and 67 in the VN-assay. Antibodies induced after infection with influenza virus A/PR/8/34 did not crossreact with influenza virus X-31 and vice versa.

Detection of virus-specific CTL by H-2D^b-NP$_{366-374}$ tetramerstaining

Seven days post infection with influenza virus X-31 NP$_{366-374}$ specific T cells (Tm+ cells) were readily detectable and constituted 5.4% (SD=2.3) of the CD8+ T lymphocyte population in the spleen of X-31 experienced mice (Group II). In PBS-inoculated mice (Group IV) these cells were virtually absent (Figure 3A). On day 28 p.i. the percentage of Tm+ cells in X-31 experienced mice (Group II) had declined to 1.9% (SD=0.5). Subsequently mice were challenged with influenza virus A/PR/8/34, which shares the NP$_{366-374}$ epitope with influenza virus X-31.

The percentage of NP$_{366-374}$ specific CD8+ lymphocytes in the spleen increased to 2.7% (SD=1.3) on day 4 post challenge infection, 10.3% (SD=4.9) on day 7 and 18.1% (SD=8.7) on day 28 (Figure 4). Comparison of the frequencies of NP$_{366-374}$ specific CD8+ T cells after infection with influenza virus A/PR/8/34 in X-31 experienced (Group II) and naive mice (Group III) showed that heterosubtypic priming predisposed for stronger virus specific CTL responses. On day 7 post challenge infection, the NP$_{366-374}$ specific CTL response was significantly stronger (P=0.046) in X-31 experienced mice (Group II) (Figure 4).

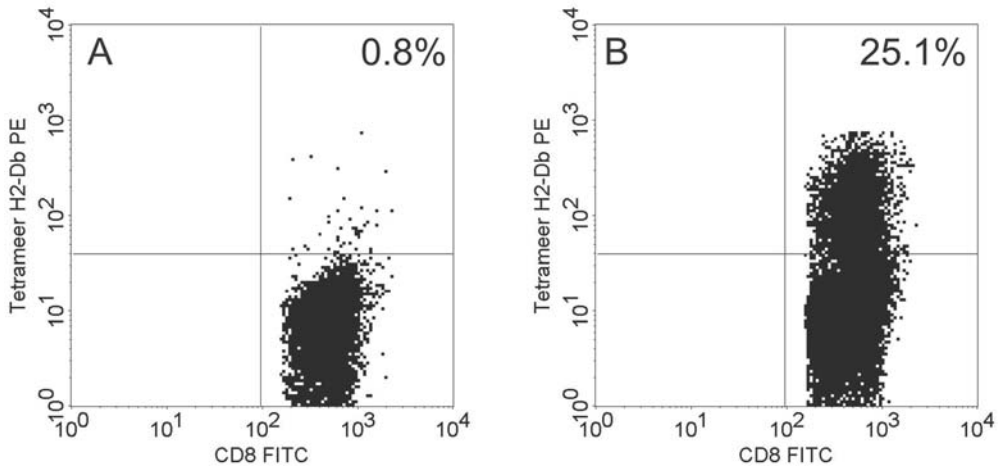


Figure 3 Detection of virus specific CD8+ T lymphocytes in mouse spleen by H-2D^b NP$_{366-374}$ tetramer staining. The proportion of TM+ cells in the CD3+ CD8+ population was determined in a PBS-inoculated mouse (A) and a X-31 experienced mouse 28 days after challenge with influenza virus A/PR/8/34 (B). For the calculation of the percentage of TM+ cells the mean percentage in PBS-inoculated mice was used as background and was subtracted from the percentage measured in other groups.

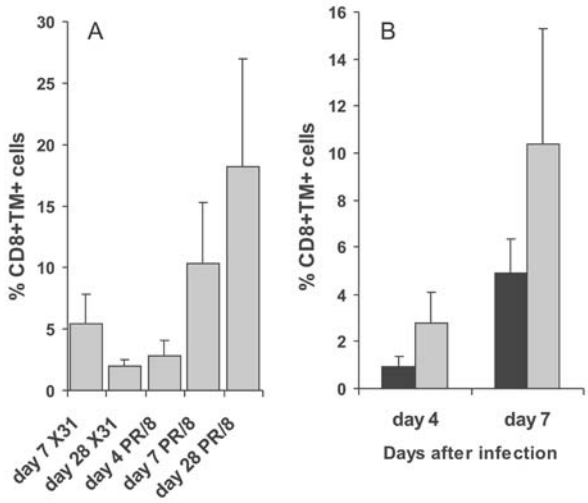


Figure 4 Detection of virus specific CD8+ cells in mouse spleens by H-2D^{b} NP$_{366-374}$ tetramerstaining. *(A)* The population of CD8+ Tm+ cells in the X-31 experienced mice (Group II) (▭) after influenza virus X-31 and influenza virus A/PR/8/34 infection. *(B)* The populations of CD8+ Tm+ cells in the naive mice (Group III) (▬) and the X-31 experienced group (Group II) (▭).

Histopathology

Lungs of mice were examined histologically four or seven days after infection with influenza virus A/PR/8/34. At four days after infection, naive mice (Group III) had severe multifocal broncho-interstitial pneumonia, centred on the bronchioles and characterized by loss of alveolar epithelium and flooding of the alveolar lumina predominantly with cell debris, neutrophils, and oedema fluid. There was a mild infiltration of lymphocytes around pulmonary blood vessels, bronchi, and bronchioles (Fig 5A+B). A small number of type II pneumocytes was present in the alveoli. In contrast, the lungs of X-31 experienced mice (Group II) at 4 days after infection with influenza virus A/PR/8/34 had only mild broncho-interstitial pneumonia, whereas the lymphoid infiltration around blood vessels and airways was more extensive (Fig 5C+D). The lungs of the naive animals (Group III) at seven days after infection with influenza virus A/PR/8/34 differed from those at four days by a more extensive peribroncheal and –vascular lymphoid infiltrate and prominent type II pneumocyte hyperplasia. (Fig 5E+F). At seven days after infection with influenza virus A/PR/8/34, the animals in the X-31 experienced group (Group II) had less evidence of pneumonia, whereas marked lymphoid infiltrates around blood

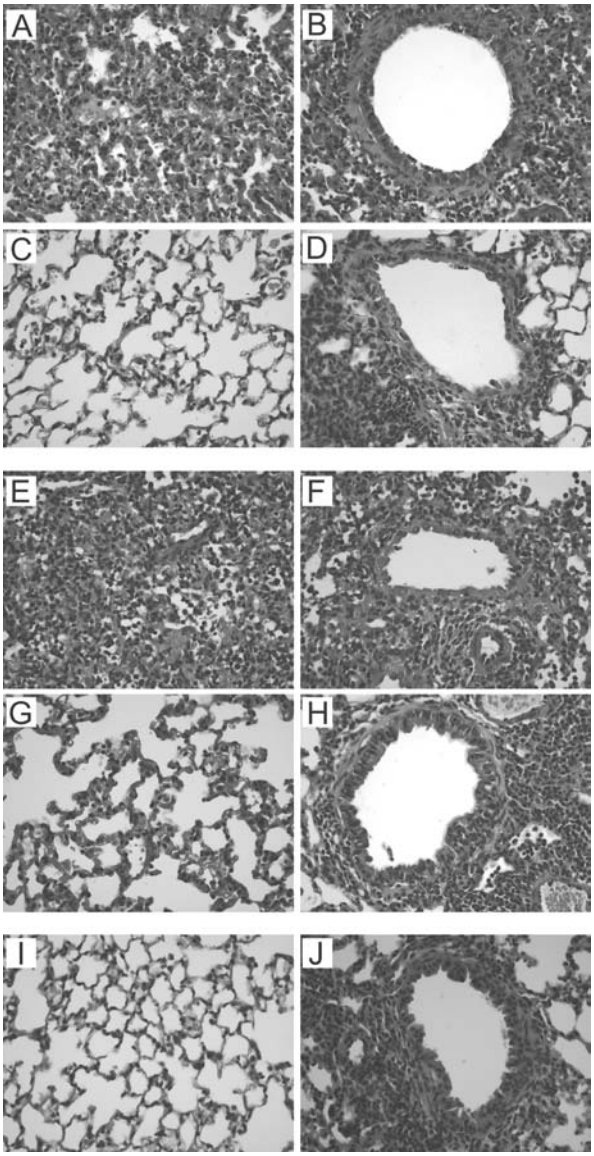


Figure 5 Histology of the lungs of mice infected with either influenza virus X-31, influenza virus A/PR/8/34 or both sequentially. A+B: lung of a naive mouse 4 days after PR/8 infection with flooding of the alveoli (A) and peri-broncheolar and –vascular lymphoid infiltrate (B). (C+D) lung of an X-31 experienced mouse 4 days after PR/8 challenge lacking flooding of the alveoli (C) and stronger peri-brocheolar and –vascular lymphoid infiltrate (D). *(E+F)* lung of a naive mouse 7 days after PR/8 infection, The alveoli are filled with cell debri and fluid, and type II pneumocyte hyperplasia is seen in the alveolar walls (E) combined with strong peri-brocheolar and –vascular lymphoid infiltrate (F). *(G+H)* lung of an X-31 experienced mouse 7 days after PR/8 challenge, with mild type II pneumocyte hyperplasia (G) and marked peri-brocheolar and –vascular lymphoid infiltrate (H). *(I+J)* lung of an X-31 experienced mouse on day 28 after PR/8 challenge with normal looking alveoli (I) and a peri-brocheolar and –vascular lymphoid cuff (J). (full colour figure: APPENDIX I)

vessels and airways were still present (Fig 5G+H). 28 days after infection with influenza virus A/PR/8/34 lungs of mice in the X-31 experienced group (Group II) had minimal evidence of pneumonia. The lymphoid infiltrate remained present, but was less prominent (Fig 5I+J).

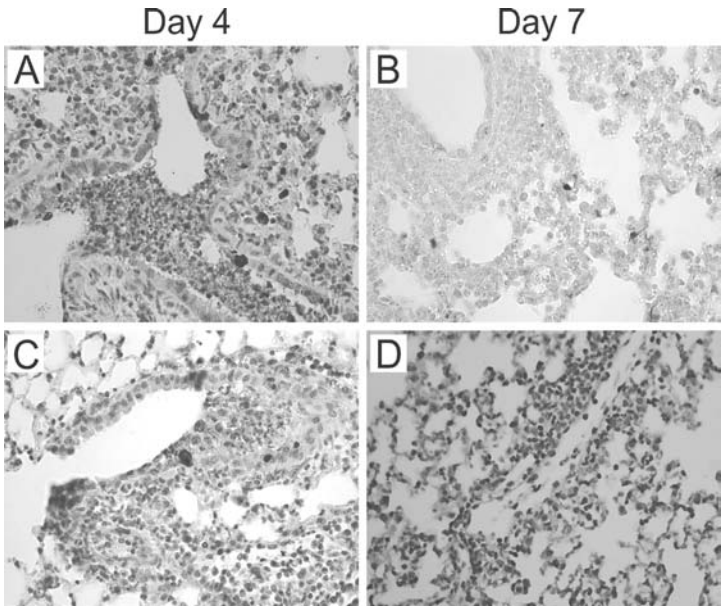


Figure 6 Mouse lung sections stained for influenza virus A NP. Cytoplasm of influenza A virus infected cells stains red, the nuclei stain deep red (A) naive mouse on day 4 after infection with influenza A virus A/PR/8/34 with virus antigen positive epithelial cells alining the bronchiole, and positive cells in the alveoli (B) naïve mouse on day 7 p.i.. with virus antigen positive cells in the alveoli (C) X-31 experienced mouse on day 4 after infection with Influenza virus A/PR/8/34 with virus antigen positive cells around the bronchioles (D) X-31 experienced mouse on day 7 p.i. lacking virus antigen positive cells. (full colour figure: APPENDIX II)

Immunohistochemistry

Mouse lung sections were stained for influenza virus A NP. The cytoplasms of influenza A virus infected cells stained red, the nuclei stain deep red (Figure 6). Virus antigen was detected in naive mice (Group III) on day 4 and day 7 after infection with influenza virus A/PR/8/34 (Figure 6A+B). Infected cells were located at the bronchioles (epithelial cells) and in the alveolar walls (type I pneumocytes) at multiple sites in the lung. On day 7 the number of virus infected cells was lower. Four days p.i. with influenza virus A/PR/8/34 the X-31 experienced mice (Group II) had influenza A virus positive cells predominantly around the bronchioles (Figure 6C). On day 7 no virus-infected cells were detectable in lungs of these mice (Figure 6D).

Discussion

In the present study the extent of protective immunity against a lethal influenza A virus infection induced by a primary infection with an influenza A virus of another subtype was examined. To this end, C57BL/6J mice were infected with influenza A virus X-31and subsequently with influenza virus A/PR/8/34 which is lethal to mice. These viruses share the NP-gene, containing the immunodominant CTL epitope $NP_{366-374}$ that is restricted by $H2\text{-}D^b$. CTL responses against this epitope were monitored using $H2\text{-}D^b/NP_{366-374}$ tetramerstaining and it was found that the CTL responses correlated with protection in the absence of virus neutralising antibodies. Protective immunity was assessed by scoring clinical outcome of disease measured by loss of body weight and mortality, measuring virus titers in the lungs and evaluating the histopathological changes in the lungs. The outcome of this integrated approach supported the notion that virus specific CTL contributed to protective immunity. Upon infection with influenza virus A/PR/8/34 both naive and X-31 experienced mice (Group II) lost weight. Whereas the naive mice became moribund and kept losing weight up until day 7, the X-31 experienced mice (Group II) regained weight from day 4 onwards.

The development of clinical signs in these animals correlated with virus replication in the lungs. In both groups of mice high lung-virus titers were observed on day 4 p.i. with influenza virus A/PR/8/34. In the naive mice that survived the challenge infection more than four days high virus titers were still observed in the lungs on day 7 p.i.. Of course there is a bias in the observations: only mice that were relatively resistant to the infection could be followed up post day 4 after infection. Despite this bias a striking difference was observed with mice from group II that experienced an infection with influenza virus X-31. In none of these mice, virus was detectable in the lungs indicating that the resolvement of clinical disease correlated with an accelerated clearance of virus from the lungs.

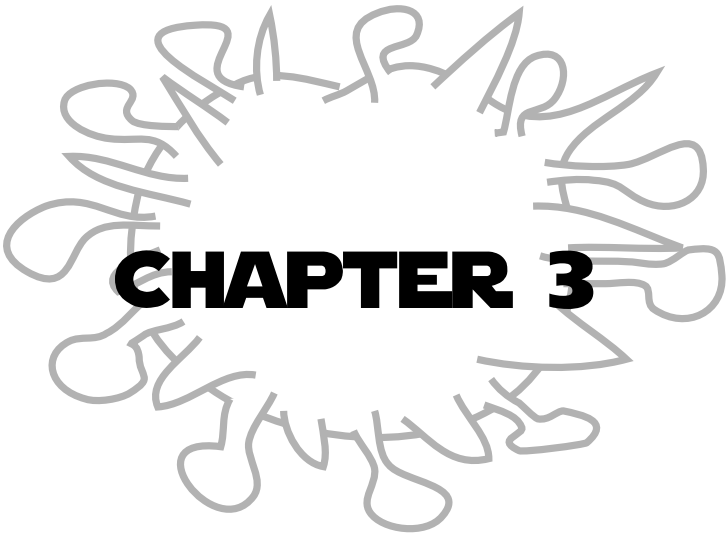
To correlate the protective effect of a priming infection with influenza virus X-31 with immunity to these viruses, humoral and cell-mediated immunity was assessed by serology and the detection of virus specific CD8+ T lymphocytes by $H2\text{-}D^b/NP_{366-374}$ tetramerstaining. Antibodies induced by infection with influenza virus X-31 failed to neutralize influenza virus A/PR/8/34 and vice versa. The reduced clinical manifestations of influenza virus A/PR/8/34-induced disease and the accelerated clearance of virus from the lungs did correlate with the anamnestic CTL responses in X-31 experienced mice (Group II).

The CTL response to the $NP_{366-374}$ epitope is immunodominant, especially during secondary infections, which explains the magnitude of this response; ~20% of all CD8+ T lymphocytes in the spleen were specific for this epitope [223, 231]. Recently it has been demonstrated that memory CTL can also reside in the lungs [232-234]. This would allow an immediate recall response upon a secondary infection. This also could have contributed to the protective effect induced by a primary infection with influenza virus X-31 [67, 213]. It could be argued that antibodies directed to the M2 protein contributed to the heterosubtypic protection that was observed. This protein is relatively conserved and shared between influenza viruses X-31 and A/PR/8/34. However, M2 is known as a minor antigen, and antibody responses induced after infection are very low [235] and cannot be detected in HI or VN assays. A protective effect of M2-specific antibodies only has been demonstrated after hyperimmunization with this antigen or the use of monoclonal antibodies. Its neutralizing effect is dependent on antibody dependent cell mediated cytotoxicity [201, 202, 236, 237]. In addition to reducing disease severity and reducing virus replication in the lungs, the cross-reactive CTL responses also correlated with the prevention of severe alveolar damage. Seven days post infection with influenza virus A/PR/8/34, naive mice developed a severe interstitial pneumonia which correlated with the moribund state of these mice. Primary infection with influenza virus X-31 prevented these severe pathological changes and in these mice the presence of type II pneumocyte hyperplasia was indicative for recovery from disease. The presence of virus infected cells in the lungs by immunohistochemistry on day 4 and day 7 after infection with influenza virus A/PR/8/34 correlated with the virus titers in the lungs and the degree of alveolar damage. These observations may have some important implicationsFirst, most patients infected with highly pathogenic avian influenza viruses (HPAI) of the H5N1 subtype developed severe interstitial pneumonia. Post mortem necropsies confirmed this [208, 238, 239].

In addition most of the human H5N1 cases that were recorded involved children and young adults. We speculate that these individuals might not have been exposed to human influenza A viruses and were unable to mount a secondary cross-reactive and cross-protective CTL response. Indeed CTL cross-reactive with avian influenza viruses have been demonstrated in subjects immune to human influenza viruses [70, 73]. As a consequence of the widespread presence of human influenza A viruses and the high attack rate with these viruses it is likely that a majority of the human population has developed cross-reactive CTL responses and may be protected

against severe morbidity and mortality caused by infection with HPAI H5N1 viruses. The second implication is, that it may be worthwhile to investigate possibilities of the development of universal vaccines which aim at the induction of cross-reactive CTL responses. In the light of the pandemic threat and the long production time of conventional HA based vaccines that might be a favourable strategy [219, 220].

Acknowledgements
The authors wish to thank Nella Nieuwkoop, Chantal Baas and Debby van Riel for technical advice and assistance.



CHAPTER 3

Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus

J.H.C.M. Kreijtz*, R. Bodewes*, J.M.A. van den Brand, G. de Mutsert, C. Baas, G. van Amerongen, R.A.M. Fouchier, A.D.M.E. Osterhaus and G.F. Rimmelzwaan

Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands

Vaccine 2009; Accepted for publication

*authors equally contributed to this study

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 $PA_{224-232}$ 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 [47]. 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 [240] and has been demonstrated in various animal models including mice [200, 216], pigs [241, 242], ferrets [243], chickens [244] and cotton rats [245], 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 [71].

Several lines of evidence indicate that cell-mediated immunity and in particular CD8+ cytotoxic T lymphocytes (CTL) contribute to heterosubtypic immunity [246, 247]. The majority of CTL is directed to conserved epitopes located within the relatively conserved proteins of the virus [214] like the nucleoprotein (NP) and the matrix (M1) protein, which implies a role for CTL in heterosubtypic immunity. 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 [72, 73, 199]. 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 (for review see Rimmelzwaan 2007)[243, 247, 248]. 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 [67].

Recently we confirmed in a mouse model that a prior infection with influenza A virus X-31 (H_3N_2) protected against a lethal challenge infection with influenza virus A/Puerto Rico/8/34 (A/PR/8/34) (H_1N_1), which correlated with anamnestic CTL responses [200]. 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 H_5N_1 subtype to mimic the natural situation and the order of infections more closely. For this purpose, influenza viruses A/Hong Kong/2/68 (H_3N_2) and A/Indonesia/5/05 (H_5N_1) were used.

Again, prior infection with a heterosubtypic strain (H_3N_2) had a beneficial effect on the clinical outcome of the H_5N_1 challenge infection and control of virus replication. The CTL response against the H-2D^b restricted CTL epitopes $NP_{366-374}$ and $PA_{224-232}$ primed for an anamnestic CTL response to the H_5N_1-derived peptide variants that correlated with the observed protection.

Material & Methods

Influenza viruses

Influenza viruses A/Hong Kong/2/68 (A/HK/2/68) (H_3N_2) and A/Indonesia/5/05 (A/IND/5/05) (H_5N_1) were propagated in Madin-Darby Canine Kidney (MDCK) cells. Infectious titers of the virus stocks were determined in MDCK cells as described previously [228].

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 (n=34) were infected intranasally with $5x10^2$ $TCID_{50}$ of influenza virus A/HK/2/68 in a volume of 50µl PBS. This virus was chosen since it replicates well in mice without the need for adaptation.

Control mice were mock-infected with PBS (n=16) or were infected with $5x10^6$ $TCID_{50}$ of respiratory syncytial virus (RSV) (n=28) in a volume of 50ml. 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 [229]. 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 [230]. Influenza virus A/HK/2/68 specific serum was obtained by injecting a rabbit with sucrose gradient purified virus [249]. 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 [250].

Lung virus titers

Lungs (from 6 animals of each group) 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, 100mg/ml streptomycin,

polymyxin B, Nystatin, Gentamicin, 7,5% $NaHCO_3$, 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 [228].

Virus-specific T cells

Tetramer staining

Single-cell splenocyte suspensions were obtained using 100µm cell strainers (BD, Alphen a/d Rijn, The Netherlands) (from 6 animals of each group). 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-2D^b tetramer with the $NP_{366-374}$ epitope ASNENMEVM or an PE labelled H-2D^b 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 $PA_{224-232}$ (SSLENFRAYV) were purchased as synthetic peptides (immunograde, >70% purity) (from Sanquin Research, Amsterdam, The Netherlands and Eurogentec, Seraing, Belgium, respectively) [213, 223]. 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).

After euthanasia, the lungs of the mice were inflated with 10% neutral buffered

formalin. After fixation the lungs were embedded in paraffin, sectioned at 4mm 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.

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 (Fig 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).

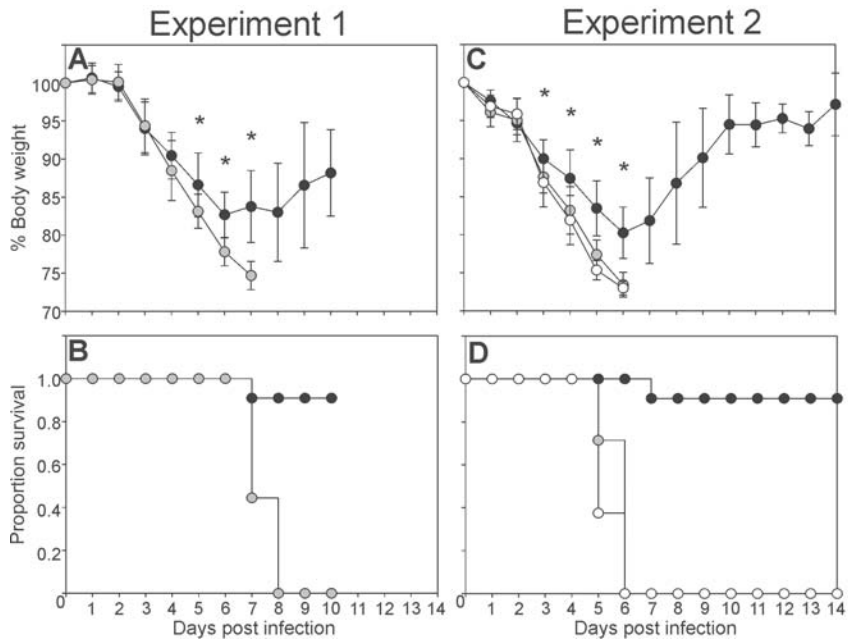


Figure 1 Bodyweight after challenge infection with influenza virus A/IND/5/05 in H3N2-primed mice (●) and mock-infected control mice (◍) or RSV-primed mice (○) *(A,C)*. Survival of these animals after infection with influenza virus A/ IND/5/05 *(B,D)*, which was significantly higher after day 6 for the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

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.

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.

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: $10^{8.1}$ TCID50 (SD=$10^{0.4}$) than for the naïve mice of the PBS group: $10^{9.0}$ TCID50 (SD=$10^{0.4}$) ($p<0.05$) and RSV group: $10^{8.8}$ TCID50 (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 $10^{4.6}$ TCID50 (SD=$10^{0.7}$). Fourteen days p.i. infectious virus was no longer detectable in the lungs of these animals.

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 Tetramer positive (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.

Table 2: $NP_{366-374}$ specific CD8+ T lymphocytes on day 7 post infection with influenza virus A/IND/5/05

epitope	Infection	Experiment 1		Experiment 2	
		IFNgamma+	**Tm+**	**IFNgamma+**	**Tm+**
$NP_{366-374}$ ASNENMEVM	**HK/2/68**	2.0 ± 1.1[1]	5.3 ± 5.7[1]	1.8 ± 0.8	6.2 ± 4.6
	Mock	-0.5 ± 0.2	0.9 ± 0.7	†	†
	RSV	n.d.[2]	n.d.	†	†
$PA_{224-232}$ SSLENFRAYV	**HK/2/68**	3.0 ± 1.4[1]	n.d.	n.d.	n.d.
	Mock	0.2 ± 0.3	n.d.	†	n.d.
	RSV	n.d.	n.d.	†	n.d.

[1] significantly higher than the mock-infected mice ($p<0.05$)

[2] n.d.= not done

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 $PA_{224-232}$ epitope (SSLENFRAYV) was significantly higher in H3N2-primed mice than in mock-infected animals (Table 2).

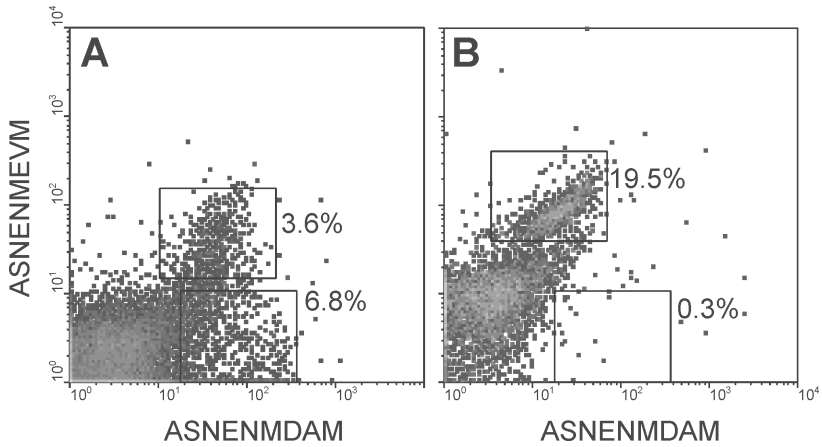


Figure 2 Expansion of a cross-reactive CD8+ T cell population, induced by primary infection, after subsequent infection with influenza virus A/IND/5/05 (H5N1). On day 12 after infection with influenza virus A/HK/2/68 (H3N2) a CD8+ T cell population was detected that recognized both the A/HK/2/68 derived $NP_{366-374}$ epitope (ASNENMDAM) and the A/IND/5/05 derived analog (ASNENMEVM) *(A)*. This cross-reactive population was expanded constituting the majority of $NP_{366-374}$ specific CTL on day 7 after infection with influenza virus A/IND/5/05 (H5N1) *(B)*.

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

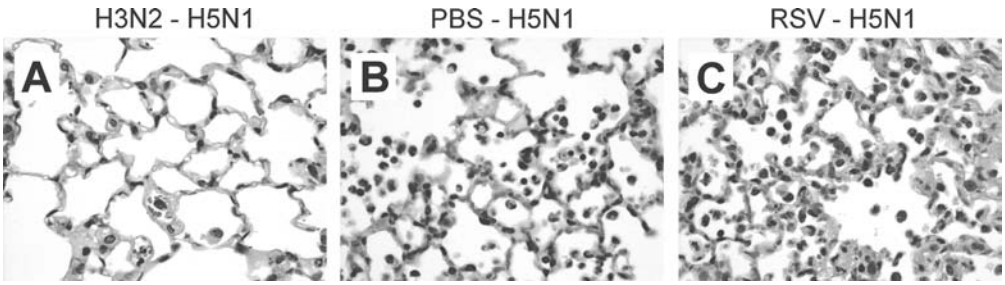


Figure 3 Histopathology was examined in the lungs of mice after infection with influenza virus A/IND/5/05. Four days after infection the lungs of H_3N_2-primed animals showed a multifocal mild broncho-interstitial pneumonia with mild inflammatory infiltrates consisting of predominantly lymphocytes and 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. (full colour figure: APPENDIX III)

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).

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.

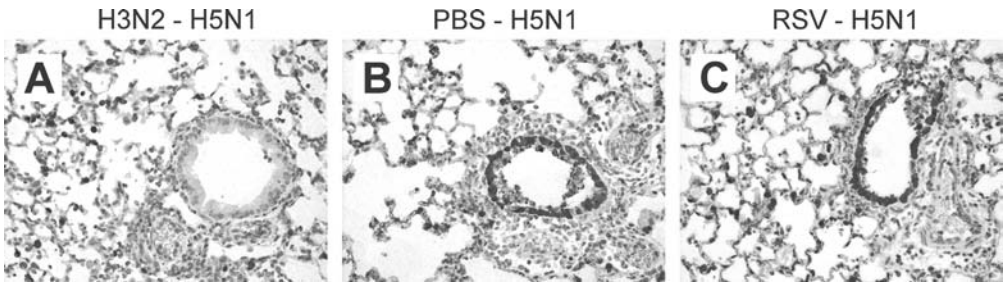


Figure 4 Detection of virus-infected cells by immunohistochemistry in the lungs of influenza virus A/IND/5/05 (H5N1)-infected mice on day 4 p.i. in mice primed by infection with influenza virus A/HK/2/68 (H3N2) *(A)*, mock-infected mice *(B)*, or RSV-primed mice *(C)*. (full colour figure: APPENDIX III)

Inoculation of naive C57BL/6J mice with influenza virus A/IND/5/05 (H5N1), which was chosen for its high virulence in mice [251] 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 correlated with the induction of anamnestic CTL responses specific for the $NP_{366-374}$ (ASNENMEVM) and $PA_{224-232}$ (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) [226].
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 [252].

Although it has been described that the $NP_{366-374}$ epitope is more immunodominant than the $PA_{224-232}$ epitope in a secondary CTL response [253], a stronger response was observed against the $PA_{224-232}$ 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 $PA_{224-232}$. 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).
Furthermore, cross-reactive T cells are also induced to various other (unknown) epitopes located in all viral proteins. Also human CTL directed against seasonal influenza A viruses are highly cross-reactive with influenza A/H5N1 viruses [72, 73, 199].

Additionally, 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 [60] or the M2 protein can have a protective effect against challenge infection [201, 203, 204]. 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 [59, 201]. In addition, transfer of post-infection serum to naïve recipient mice failed to protect the animals from infection with a heterosubtypic strain [254] 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. We used a time interval of four weeks between the two infections but previous studies have shown that heterosubtypic immunity is long-lived, up to 5 and 18 months in mice and ferrets, respectively [243, 255].

Protection of mice against lethal influenza A/H5N1 virus infection induced by primary infection with a heterosubtypic influenza virus has been demonstrated before [216]. 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 [256]. 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 [200]. 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. It can be expected that this type of immunity is also effective against less virulent H5N1 strains. The observed protection against the development of severe disease correlated with the induction of cross-reactive CTL responses in the spleen. In addition, lymphocytic infiltrates were still present in the lungs 28 days post priming infection. The persistence of virus-specific T cells in the lungs was demonstrated previously [232, 257] and may contribute to protection against a subsequent infection [233]. Interestingly, also in humans virus specific T cells have been found in lung tissue, which indicates that also in humans similar protective mechanisms are at work [258].

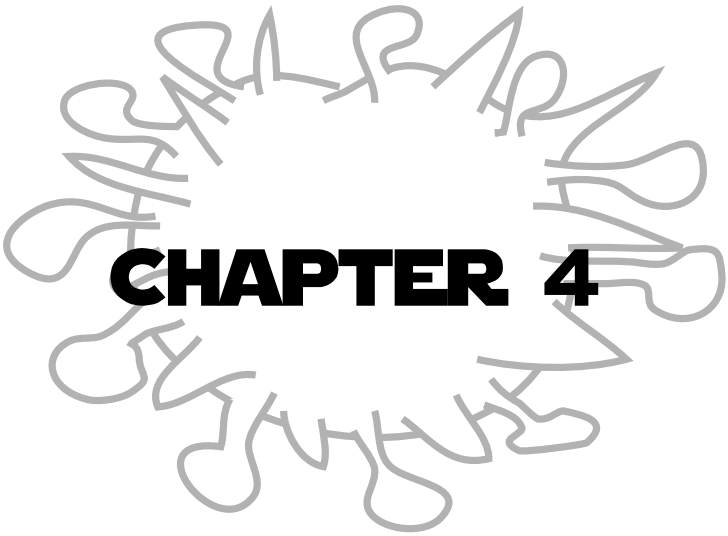
Since human CTL raised against seasonal influenza virus strains of the H1N1 and H3N2 subtypes are highly cross-reactive with highly pathogenic avian influenza

A/H5N1 viruses it is anticipated that they will contribute to protective immunity against these viruses, when they might become pandemic [73, 199]. 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 [71]. 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 [259]. 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.

Acknowledgements

The authors would like to thank Theo Bestebroer, Peter van Run, Tiny Geelhoed-Mieras, Stella van Trierum for excellent technical support.


KAMPIOEN VAN NEDERLAND
KNVB
2007 2008
EREDIVISIE
DON DIABLO
ROOC
LUCIEN FOORT
CHUCKIE
SIDNEY SAMS
SKITZOFRENIX
MISS MONICA
HANS DULFER
MAASSILO
Glenfiddich
12
BLACK BOTTLE
APHROAIC
NIEUW



CHAPTER 4

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

R. Bodewes, J.H.C.M. Kreijtz, C. Baas, M.M. Geelhoed-Mieras, G. de Mutsert, G. van Amerongen, J.M.A. van den Brand, R.A.M. Fouchier, A.D.M.E. Osterhaus, G.F. Rimmelzwaan

Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands

PLoS ONE 2009; 4(5): e5538

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[260]. 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[44, 46, 261]. 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[262]). Also in humans there is evidence that infection with IAV can induce heterosubtypic immunity[71]. Individuals that had experienced an infection with an H1N1 IAV before 1957 less likely developed influenza during the H2N2 pandemic of 1957[71]. 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[67]. It is well documented that seasonal human IAVs and avian IAVs share CTL epitopes located in the internal viral proteins like the nucleoprotein[72, 73, 199]. 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[263]. 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[264, 265], annual vaccination of all healthy children 6 to 59 months of age was recommended

in various countries including the United States since 2007[266].
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[267, 268]. 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 virus. 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[228].

Vaccine preparation

Influenza subunit antigen derived from IAV X-31 (H3N2) was essentially prepared as described previously[98]. 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	-	+	+	+

Mice were divided over seven groups and were either vaccinated twice with subunit vaccine with or without adjuvant (Alum), PBS, or adjuvant only as indicated. Four weeks after the second vaccination, mice were infected with IAV HK/68 (H_3N_2) or mock-infected. Twenty-nine days after the infection with IAV HK/68, mice were challenged with IAV IND/05 (H_5N_1).

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[280] and virus neutralising (VN) antibodies using the VN assay[230].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[249]. 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[250].

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[228]. HA activity of the culture supernatants collected 5 days post inoculation was used as indicator of infection. The titers were calculated according Spearman-Karber[281].

Flow cytometry of virus-specific CD8+ T cells

Peptides and intracellular IFN-γ staining

Single cell suspensions of spleens were prepared as described previously[200]. 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 $NP_{366-374}$ [221, 223]. 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[282].

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 Logrank test. Differences were considered significant at $P<0.05$.

Results

Antibody responses against IAV HK/68 (H_3N_2) 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 antibodies 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).

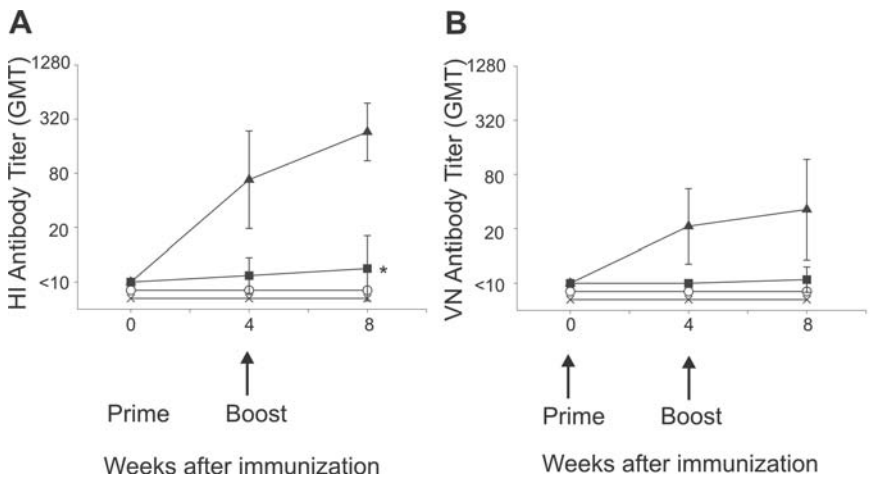


Figure 1 Induction of serum antibodies against IAV HK/68 (H_3N_2) by vaccination. Serum antibody levels were determined before and at the indicated time points after vaccination of mice with PBS (groups 1, 3 and 4; O), subunit vaccine with alum (groups 2 and 5; ▲), subunit vaccine only (group 6; ■) and alum only (group 7; ×) by HI assay (A) and VN assay (B).

Outcome of infection with IAV HK/68 (H_3N_2)

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.

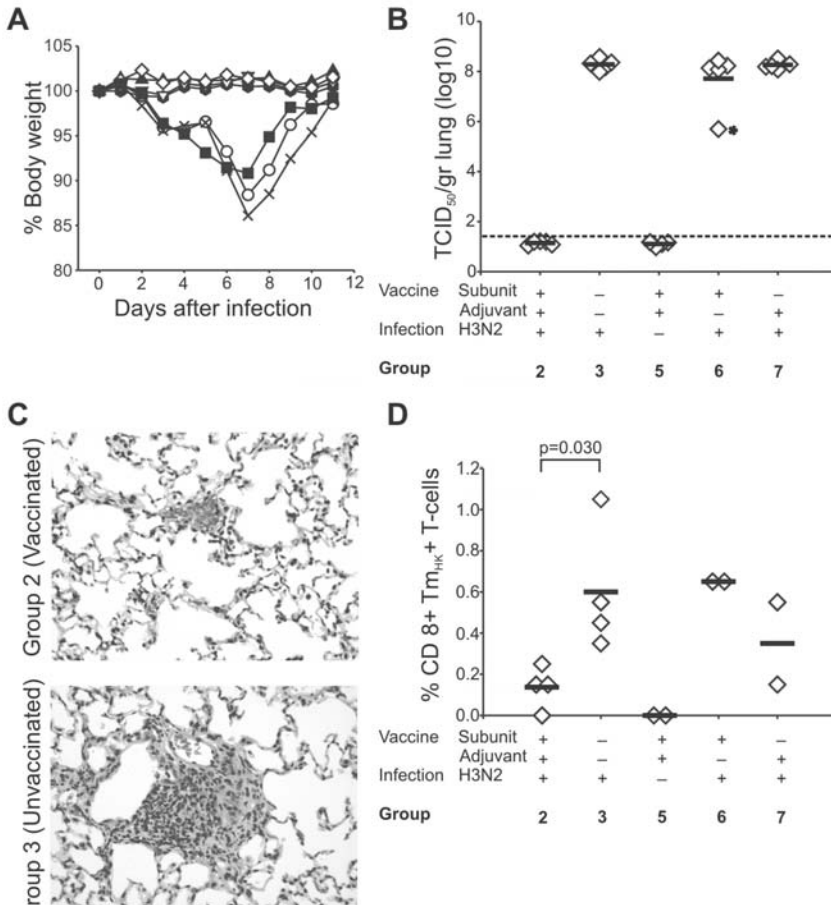


Figure 2 Outcome of infection with IAV HK/68 (H_3N_2). Mice were inoculated with IAV HK/68 (groups 2 (▲), 3 (O), 6 (■) and 7 (×)) or PBS (groups 1 (●), 4 (▽) and 5 (◇)). (**A**) Body weight after infection was determined daily and expressed as the percentage of the original body weight before infection. (**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). (full colour figure: APPENDIX IV)

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).

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 $NP_{366-274}$ 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.

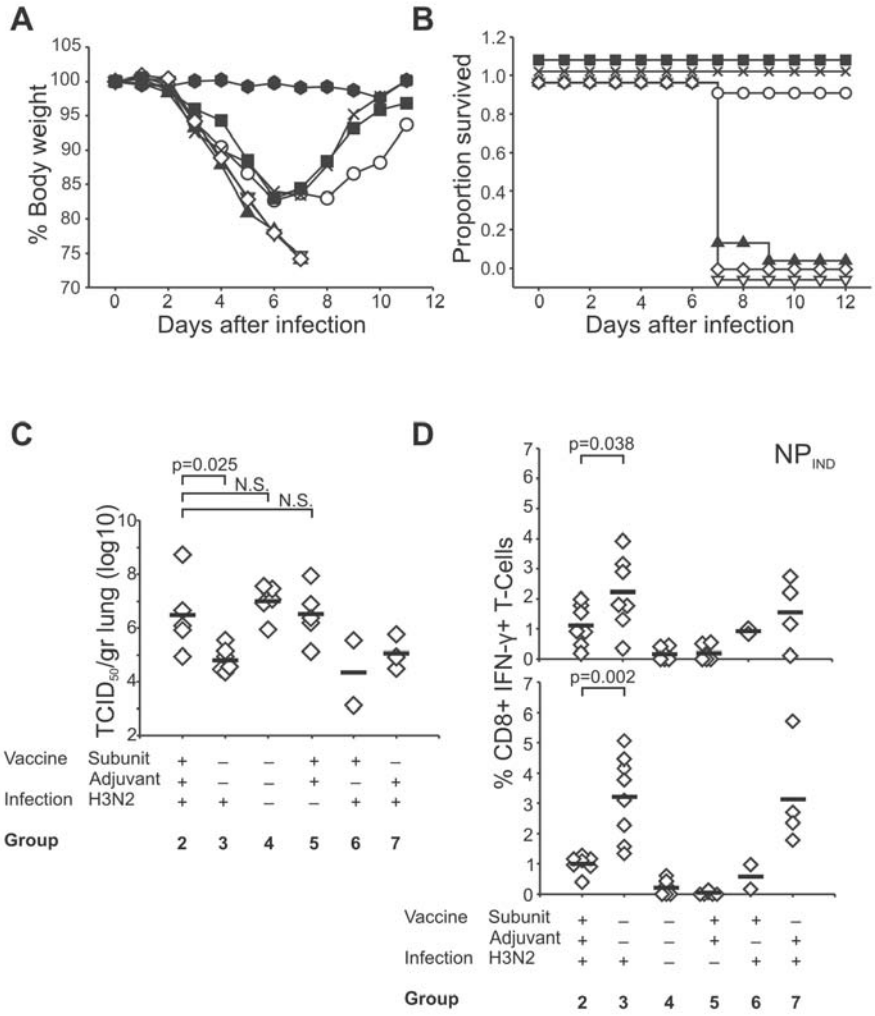


Figure 3 Outcome of infection with IAV IND/05 (H5N1). Mice were inoculated with IAV IND/05 (groups 2 (▲), 3 (O), 4 (▽), 5 (◇), 6 (■) and 7 (×)) or PBS (group 1 (●)). (**A**) Body weight after infection was determined daily and expressed as the percentage of the original body weight before infection. (**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.

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).

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-g 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-g+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.

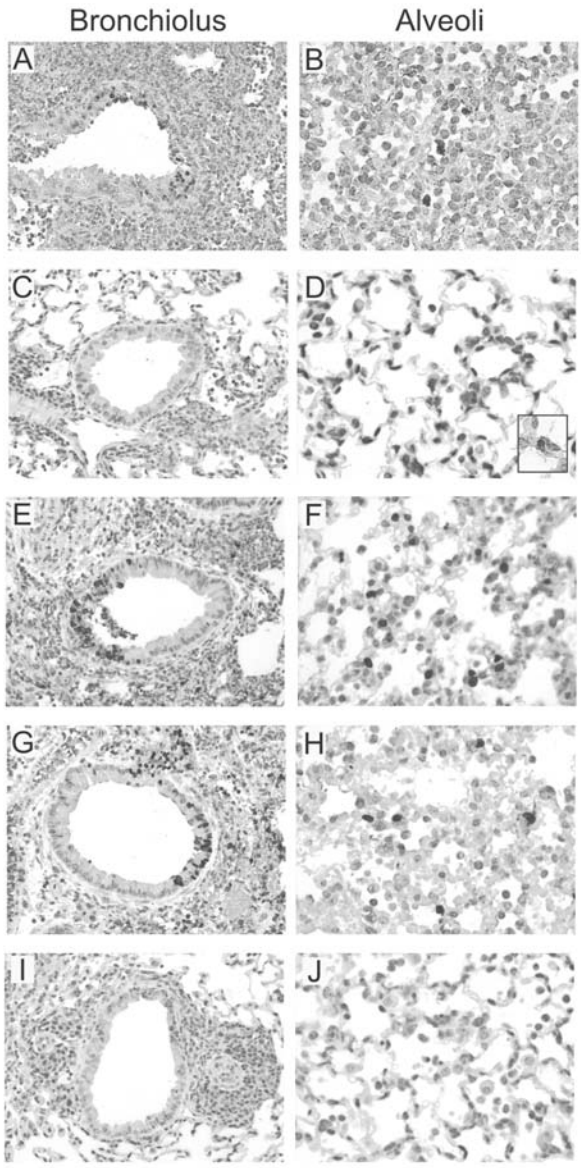


Figure 4 Histopathological analysis and immunohistochemistry of the lungs of mice infected with IAV IND/05. Mouse lung sections were stained for influenza A virus nucleoprotein. Cytoplasm of infected cells stain red, the nuclei of infected cells stain deep red. In the groups without a history of productive A/H_3N_2 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). For more information please see text. (full colour figure: APPENDIX V)

Discussion

Here we demonstrate that successful vaccination of mice against human IAV HK/68 (H_3N_2) prevented the induction of heterosubtypic immunity against a lethal challenge with IAV IND/05 (H_5N_1). As a result, H_3N_2 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, which was already recognized more than 40 years ago[240]. This so-called heterosubtypic immunity was not only demonstrated in animal models[216, 234, 240, 244] but there is also direct and indirect evidence that it exists in humans[67, 71] and that cell-mediated immune responses contribute to this type of immunity (for review see[247]).

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/H_3N_2 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).

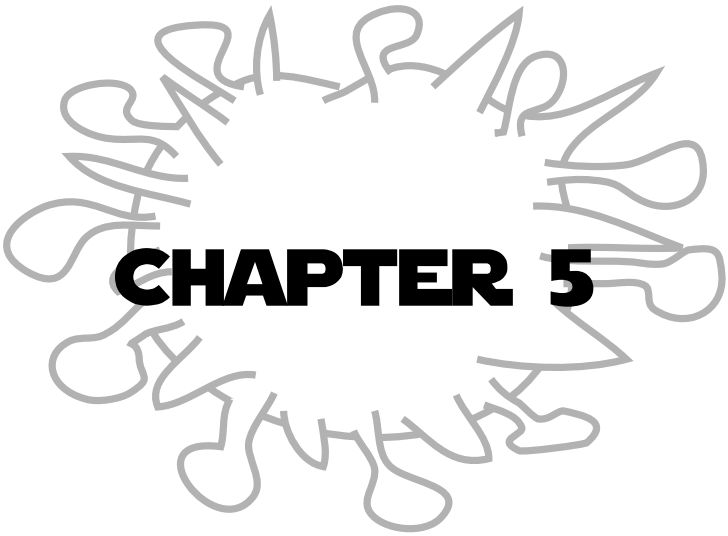
Four weeks after infection with IAV HK/68 virus, the number of virus-specific CD8+ T cells in the spleen was significantly lower in mice vaccinated against IAV HK/68 than in unvaccinated mice. The differences were not observed at earlier time points p.i.. Further evaluation of the CD8+ Tm_{HK}+ T cells indicated that the numbers of $CD62L_{high}$ and CD127+ 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[269]. 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 (H_3N_2) 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[223], 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[257, 270]. Since the frequency of virus-specific CD8+ T cells in the spleen reflected that in the lymph nodes[232, 271] , 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[272]. Also in humans, T cells specific for viral respiratory pathogens have been detected in lung tissue and may play a protective role against subsequent infections in this species as well[258]. 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[237, 254].

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. At present vaccination against seasonal influenza is recommended for all healthy children 6-59 months of age in a number of countries, including the USA[266]. Also in Europe vaccination of children is currently considered and a number of countries already decided to recommend vaccination of healthy children[273]. Although vaccination is (cost-) effective in this age group[274-278], 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[279]. Thus during a next pandemic, especially children that received the annual flu-shot would be at higher risk to develop severe illness and a fatal outcome of the disease than those that experienced an infection with a seasonal IAV strain. This of course, would be of great concern and is supported by the data obtained in our mouse model. Ideally, seasonal influenza vaccines are used that also induce heterosubtypic immunity[219, 268]. 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[195].

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



CHAPTER 5

Cross-recognition of avian H5N1 influenza virus by human cytotoxic T lymphocyte populations directed to human influenza A virus

J.H.C.M. Kreijtz, G. de Mutsert, C.A. van Baalen, R.A.M. Fouchier, A.D.M.E. Osterhaus, G.F. Rimmelzwaan

Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands

Journal of Virology 2008; 82:5161-6

Abstract

Since the number of human cases of infection with avian H5N1 influenza viruses is ever increasing, a pandemic outbreak caused by these viruses is feared. Therefore, in addition to virus-specific antibodies, there is considerable interest in immune correlates of protection against these viruses, which could be a target for the development of more universal vaccines. After infection with seasonal influenza A viruses of the H3N2 and H1N1 subtypes, individuals develop virus-specific cytotoxic T lymphocyte responses, which are mainly directed against the relatively conserved internal proteins of the virus, like the nucleoprotein (NP). Virus-specific cytotoxic T lymphocytes (CTL) are known to contribute to protective immunity against infection but knowledge about the extent of cross-reactivity with avian H5N1 influenza viruses is sparse. In the present study, we evaluated the cross-reactivity with H5N1 influenza viruses of polyclonal CTL obtained from a group of well-defined HLA-typed study subjects. To this end, the recognition of synthetic peptides representing H5N1 analogues of known CTL epitopes was studied. In addition, the ability of CTL specific for seasonal H3N2 influenza virus to recognize the NP of H5N1 influenza virus or H5N1 virus-infected cells was tested. It was concluded that, apart from some individual epitopes that displayed amino acid variation between H3N2 and H5N1 influenza viruses, considerable cross-reactivity exists with H5N1 viruses. This pre-existing cross-reactive T cell immunity in the human population may dampen the impact of a next pandemic.

Introduction

Since the first documentation of bird-to-human transmissions of highly pathogenic avian H5N1 influenza viruses these viruses have spread from South East Asia to other regions of the world [44-46, 283]. Since 2003 the number of human cases continues to increase, as of December 18 2007, 340 human cases have been reported of which 209 were fatal [284]. It is feared that the H5N1 virus may cause the next influenza pandemic when they are able to replicate in mammalian species by adaptation through genetic reassortment or accumulation of point mutations in relevant gene segments [285]. Although neuraminidase subtype 1 crossreactive antibodies have been demonstrated in human subjects, antibodies to H5 molecules are hardly existent in the human population as a result of limited exposure to H5N1 viruses which contributes to a scenario for these viruses to become pandemic [197]. In general, the exposure history and the immune status of the human population will influence the size and the severity of pandemics [67, 70, 71, 73]. The presence of T cell immunity induced by infection with human influenza virus strains may provide some degree of cross-protective immunity against the H5N1 viruses. CTL responses are predominantly directed to internal viral proteins, the nucleoprotein (NP) in particular [65, 286], which is much more conserved than the surface glycoproteins HA and NA [65, 212, 287, 288]. It has been suggested that cross-reactive CD8+ T cells may temper the impact on the human population a pandemic potentially could have [67, 73, 289]. In humans, the presence of cross-reactive CTL responses inversely correlated with the amount of shedding of a heterosubtypic strain that was used for experimental infection of study subjects [67]. Although pre-existing CTL immunity against influenza virus may be of importance in the face of the current H5N1 pandemic threat, our knowledge on the cross-reactive nature of the human CTL response is limited [73].

In the present study we tested the cross-reactivity of polyclonal virus specific CD8+ T cell populations obtained from well-defined HLA-typed study subjects with H5N1 virus. The recognition of target cells pulsed with peptide variants, transfected with the nucleoprotein gene from a human or an avian influenza virus, or infected with viruses of the H3N2 or H5N1 subtype was tested, respectively.

It was concluded that the human CTL response displays a high degree of cross-reactivity with avian H5N1 influenza viruses and could reduce morbidity and mortality during a pandemic caused by these H5N1 strains.

Material & Methods

Cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from fifteen HLA-typed healthy blood donors (Sanquin Bloodbank, Rotterdam, the Netherlands) by density gradient centrifugation using lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and then cryopreserved at -135°C. Genetic subtyping was performed in the laboratory for Histocompatibility and Immunogenetics at the Sanquin Bloodbank using a commercial typing system (Genovision, Vienna, Austria). Three groups of study subjects were selected on basis of their MHC class I alleles, for which influenza CTL epitopes were identified. Within groups the subjects shared identical HLA-A and B alleles, between groups one, or two alleles differed: group I: HLA A*0101, A*0201, B*0801, B*3501, group II: HLA A*0101, A*0201, B*0801, B*2705 (2702) and group III: HLA A*0101, A*0301, B*0801, B*3501 (3503) [68]. Subject #15 was not tested since PBMC of this donor were no longer available.

Peptides

Amino acid sequences of all known human influenza A virus CTL epitopes were compared with their counterparts in H5N1 influenza viruses, isolated since 2003, which were obtained from the influenza sequence database [290]. All possible variants that could be identified in the H5N1 sequences are listed in table 1. A set of immunograde peptides, representing immunodominant CTL epitopes and the most prevalent analogues in H5N1 strains were synthesized, analyzed by mass spectrometry and were >70% pure (Eurogentec, Seraing, Belgium). Variant peptide analogues from the NP, which is the main target for CTL responses, were synthesized when they had a prevalence in H5N1 strains of >0.25%, with the exception of the NP383-391 epitope since the G384K mutation observed in H5N1 viruses was known to abrogate recognition by specific CTL completely [291]. For the remaining viral proteins all variants with a prevalence of >2.25% were synthesized and tested. Only peptides were considered that match the HLA-alleles of the study subjects.

Target cells

B-lymphoblastoid cell lines (BLCL) were established as described previously [162] and used as target or stimulator cells. Thirty thousand cells were incubated in the absence or presence of 10mM peptide for one hour at 37°C, washed once

and resuspended in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) containing antibiotics, L-glutamine and 10% FCS (R10F). Cells of the BLCL were also infected at a multiplicity of infection (MOI) of five TCID50/cell [228] with influenza viruses A/Netherlands/18/94 (A/NL/18/94)(H3N2) or A/Vietnam/1194/04 (A/VN/1194/04)(H5N1) which were propagated and titrated in MDCK cells using standard procedures. After an incubation period of one hour at 37°C, the cells were washed and resuspended in R10F and incubated for 16-18 hours at 37°C prior to their use for the stimulation of CD8+ T cells. Infection rates were determined by an immunofluorescence assay and were similar for both viruses (data not shown). The human influenza virus A/Netherlands/18/94 (H3N2) was used as a representative of seasonal influenza viruses, whilst influenza virus A/Vietnam/1194/04 was used as an example for H5N1 influenza virus.

T cell clones

CD8+ T cell clones directed against the HLA-A1 restricted NP_{44-52} CTELKLSDY epitope, HLA-A3 restricted $NP_{265-273}$ ILRGSVAHK epitope, HLA-B27 restricted $NP_{174-184}$ RRSGAAGAAVK epitope and the HLA-B*3501 restricted $NP_{418-426}$ LPFEKSTVM epitope were generated as described previously [291].

In vitro expansion of influenza A virus specific T cell populations

PBMC were stimulated with influenza virus A/NL/18/94 infected cells as previously described [68]. Eight days after stimulation cells were harvested and used as effector cells in the ELIspot- or FATT-CTL assays. For the ELIspot assays CD8+ T cells were purified from the *in vitro* expanded PBMC by MACSÒ bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Typically a purity of >96% was obtained.

Fluorescent-antigen-transfected target cell (FATT) – CTL assay

The NP genes of influenza viruses A/NL/18/94 and A/VN/1194/04 without their stopcodons were cloned into the plasmid pEGFP-N1 (Becton Dickinson, Alphen a/d Rijn, the Netherlands) in frame with the ORF of the green fluorescent protein (GFP) as previously described [292]. Plasmid DNA was purified using the Genopure plasmid midi kit (Roche, Woerden, the Netherlands). Nucleotide sequences of the recombinant plasmids were confirmed using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primer and plasmid sequences are available

on request.

The plasmids were used in the FATT-CTL assay for the detection of lytic activity of virus specific CTL as described previously [292]. In brief, BLCL were nucleofectedâ using cell line nucleofector® kit V (Amaxa Biosystems, Cologne, Germany) with program T16 and subsequently incubated in R10F for four hours at 37°C. Then they were co-cultured for another four hous in triplicate with PBMC cultures, *in vitro* expanded after stimulation with influenza virus A/NL/18/94, at various E:T ratios. The number of viable GFP positive cells was measured using a FACSCalibur (Becton Dickinson). The % nucleoprotein specific lysis was then calculated by the following formula: 100*((viable GFP-positive cells in sample without effector – viable GFP-positive cells in sample with effector)/viable GFP+ cells in sample without effector).

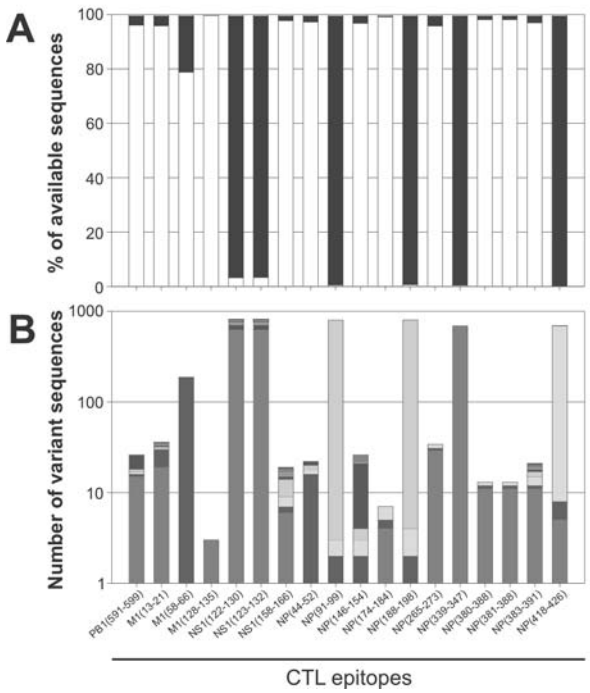


Figure 1 The presence of known CTL epitopes in H5N1 strains. The percentage of H5N1 viruses with an epitope sequence identical to human influenza viruses (white bars) is shown in Figure 1. The black bars indicate the percentage of H5N1 viruses with one or more amino acid substitutions in the epitope sequence. The absolute numbers of each variant of an epitope are shown in Figure 1B, each color represents a single variant (sequences can be found in table 1). For this analysis almost 900 H5N1 viruses were analyzed for which sequence information was available in the influenza sequence database [290]. (full colour figure: APPENDIX VI)

IFN-gamma assay

ELIspot assays were performed with in vitro expanded CD8+ T cells as effector cells and peptide-pulsed or virus-infected HLA-matched BLCL as stimulator cells as described previously [68]. The number of spots was determined using an ELIspot reader and image analysis software (Aelvis, Sanquin Reagents, Amsterdam, the Netherlands) and the average number was calculated of triplicate wells.

Results

Comparison of amino acid sequences of known influenza A virus CTL epitopes

The amino acid sequences of known human influenza A virus CTL epitopes were compared with the corresponding sequences in approximately 900 H5N1 viruses obtained from the influenza sequence database [290]. As shown in Figure 1A, the epitope sequences were identical in >95% of the H5N1 viruses for the majority of the known epitopes analyzed including $PB1_{591-599}$, $M1_{13-21}$, $M1_{128-135}$, $NS1_{158-160}$, NP_{44-52}, $NP_{146-154}$, $NP_{174-184}$, $NP_{265-273}$, $NP_{380-388}$, $NP_{381-388}$ and $NP_{383-391}$. For some of the other epitopes the percentage of H5N1 viruses with identical sequences was variable and ranged from 79% for epitope $M1_{58-66}$, to 4% for epitope $NS1_{122-130}$. For the epitopes NP_{91-99}, $NP_{188-198}$, $NP_{339-347}$ and $NP_{418-426}$, no identical sequences were found in the H5N1 viruses. In order to identify the most prevalent variant sequences in H5N1 viruses the number of individual variants was analyzed (Figure 1B).

In some cases a single variant was identified that accounted for almost all variant sequences observed in H5N1 viruses (Table 1, Figure 1B). For other epitopes multiple variants were identified, although for some of these the number was low and the number of major variants was limited [290].

The recognition of known CTL epitopes and their avian analogues

All subjects in group I (HLA A*0101, A*0201, B*0801, B*3501) displayed T cell reactivity with the epitopes NP_{44-52}, $NS1_{122-130}$, $NP_{418-426}$ and $M1_{58-66}$ as they are present in human influenza A viruses, although the frequency of specific CTL varied between study subjects and the peptides tested (Figure 2A). In none of the subjects of this group reactivity was observed with the peptide variants of epitopes NP_{44-52} and $NS1_{122-130}$, obtained from H5N1 influenza viruses. Three out of four subjects responded to the $NP_{418-426}$ variant LPFERSTIM and all subjects responded to the $M1_{58-66}$ variant GMLGFVFTL. Of group II (HLA A*0101, A*0201, B*0801, B*2705 (2702)) most subjects responded to the peptides representing epitopes from

human influenza A viruses (Figure 2B), although the magnitudes of the responses varied considerably. Only one subject in this group had an appreciable response to the H5N1 analogue sequence of the $NS1_{122-130}$ epitope. Four out of five subjects responded to the H5N1 variants of the HLA B*2705 restricted $NP_{174-184}$ epitope whereas all five of them responded to the $M1_{58-66}$ variant. The subjects of group III (HLA A*0101, A*0301, B*0801, B*3501 (3503)) responded to the original epitopes to variable extent.

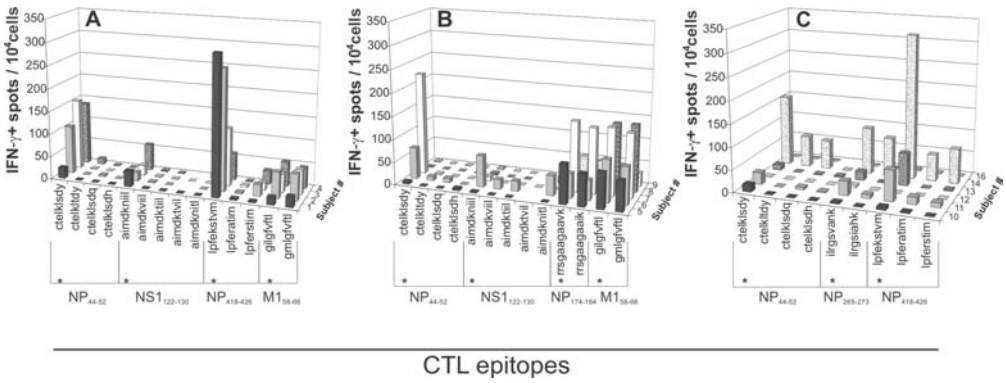


Figure 2 Epitope-specific IFN-gamma production by CTL after stimulation with peptide-pulsed BLCL. The number of IFN-gamma producing cells per 10,000 CD8+ T cells (5,000 cells for subject #3) from subjects from group I: HLA A*0101, A*0201, B*0801, B*3501 (A), group II: HLA A*0101, A*0201, B*0801, B*2705 (2702) (B) and group III: HLA A*0101, A*0301, B*0801, B*3501 (3503) (C) were measured by ELIspot assay. CD8+ T cells were isolated from PBMC populations expanded *in vitro* with influenza virus A/NL/18/94 and subsequently stimulated with peptide variants as indicated. (*= peptide sequence of the known human influenza virus CTL epitopes).

Some subjects were poor responders and hardly displayed CTL reactivity with some of these epitopes (Figure 2C). However the *in vitro* expanded PBMC of subject #14 responded strongly to the $NP_{418-426}$ and also reacted with both epitope variants from H5N1 viruses indicating that at least a fraction of the CTL population was capable of cross-recognizing these analogues. The same holds true for the NP_{44-52}- and $NP_{265-273}$-specific CTL response in this study subject. Clones were used as positive and negative controls and the clonal responses supported the results obtained with the polyclonal populations (data not shown).

Cross-recognition of the NP derived from influenza virus A/VN/1194/04

The capacity of polyclonal T cell populations directed to the human influenza virus A/NL/18/94 to cross-react with the NP of influenza virus A/VN/1194/04 was assessed

in the FATT-CTL assay. PBMC from all study subjects were stimulated with influenza virus A/NL/18/94 and allowed to proliferate. As shown in Figure 3, two out of the fifteen subjects tested (#5 and #8) were low- or non-responders (Figure 3), since no NP-specific lytic activity could be demonstrated. In the remaining thirteen subjects lytic activity was observed against the homologous NP. In most cases the PBMC cross-reacted with the NP of influenza virus A/VN/1194/04 to a considerable extend (Figure3).

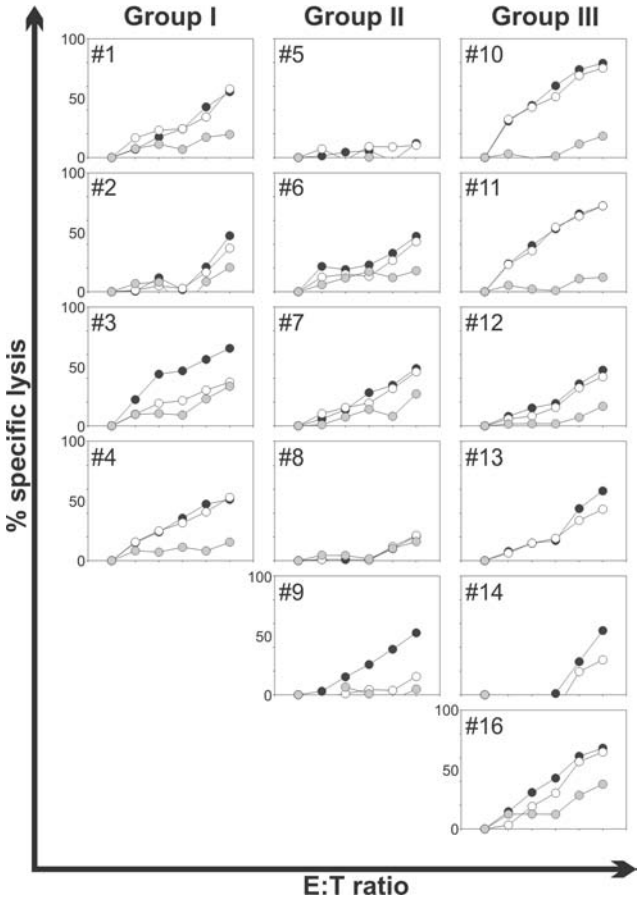


Figure 3 Recognition of NP derived from H3N2 and H5N1 influenza virus by *in vitro* expanded PBMC specific for influenza virus A/NL/18/94 (H3N2). The lytic activity of *in vitro* expanded PBMC was tested with MHC class I matched BLCL nucleofectedÒ with NP-GFP coding plasmid (NP of either influenza virus A/NL/18/94 (black dots) or A/VN/1194/04 (white dots)) or empty GFP plasmid (grey dots). This was tested for the subjects from group I (subjects 1-4), II (subjects 5-9) and III (subjects 10-14 and 16). E:T ratios were 0, 3.125, 6.25, 12.5, 25, and 50 respectively. For subject #13 and #14 the E:T ratios were 0, 0.3, 1, 3, 10 and 30 respectively. The lytic activity against control-plasmid transfected target cells is not visible for subjects 13 and 14 as a result of negative values for the percentage of specific lysis, which was caused by a slight increase of GFP+ viable cells. Standard deviation of the means was 10%.

Only for subject #3 (group I) and subject #9 (group III) the influenza virus A/NL/18/94 NP-specific CTL failed to recognize the NP of influenza virus A/VN/1194/04 (Figure 3).

Cross-recognition of BLCL infected with influenza virus A/VN/1194/04

Next we wished to assess the cross-reactive nature of the whole repertoire of CD8+ T lymphocytes specific for the human influenza virus A/NL/18/94. To this end, PBMC were stimulated with this virus and after eight days the CD8+ cells were isolated to obtain virus-specific polyclonal CTL populations. These cells were used as effector cells in an IFN-gamma ELIspot assay using MHC class I-matched BLCL infected with influenza virus A/NL/18/94 or A/VN/1194/04 as stimulator cells.

As shown in Figure 4, the *in vitro* expanded PBMC population that recognized cells infected with influenza virus A/NL/18/94, also recognized cells infected with influenza virus A/VN/1194/04 . The average number of IFN-gamma spots per 10^4 cells observed after stimulation with A/NL/18/94 infected cells was 151 (SD=58) and the number observed after stimulation with A/VN/1194/04 was even slightly higher at 192 (SD=65) , although this difference was not statistically significant.

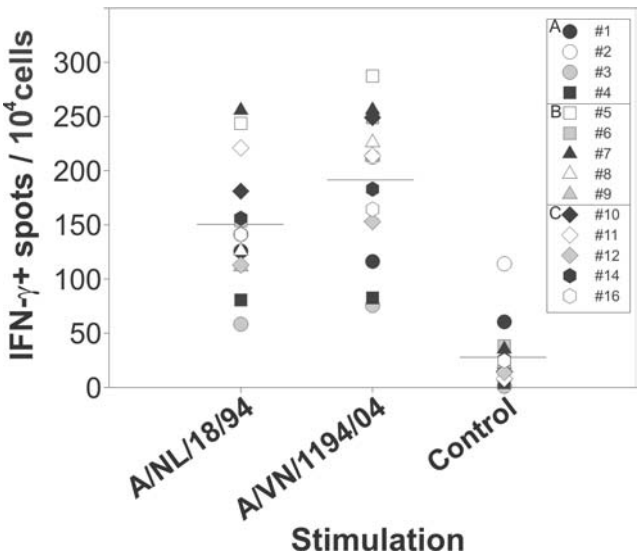


Figure 4 Recognition of influenza virus infected BLCL by CTL. The number of IFN-gamma producing cells per 10,000 CD8+ T cells was measured by ELIspot assay after stimulation with BLCL infected with influenza virus A/NL/18/94 or A/VN/1194/04. Each symbol represents an individual subject either from group I (A), II (B) or III (C). Uninfected BLCL were used as negative controls. The horizontal bars represent the average response of all study subjects in group I, II and III.

Discussion

In the present paper the cross-reactive nature of the human influenza virus-specific CTL response was investigated. It was concluded that a considerable portion of CTL populations specific for the H3N2 influenza virus A/NL/18/94 cross-reacted with the H5N1 strain A/VN/1194/04. For most CTL epitopes, it was found that a vast majority of the H5N1 strains contained identical epitope sequences as those present in human influenza A viruses. This conservation of epitopes is responsible for the cross-reactive nature of CTL responses in humans against seasonal influenza A viruses of the H3N2 and H1N1 subtypes. However, some variation in these epitopes was observed also and for a number of CTL epitopes the H5N1 strains did not contain identical sequences. Apart from the $NP_{174\text{-}184}$ and $M1_{58\text{-}66}$ epitope restricted by HLA-B*2705 and HLA-A*0201 respectively, very little cross-reaction was observed of polyclonal CTL populations with variant peptides derived from H5N1 viruses.

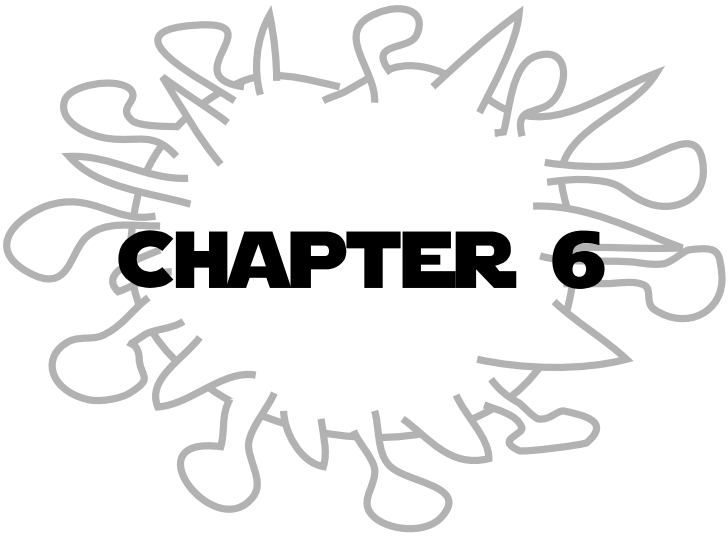
However, as indicated above most epitopes are relatively conserved including those located in the NP which contributed to the cross-reactive nature of the NP-specific CTL response. Most of the study subjects that responded to NP derived from seasonal H3N2 influenza viruses also responded to the NP derived from influenza virus A/VN/1194/04 (H5N1). The polyclonal virus-specific T cell populations of two of these subjects failed however to cross-react with the NP of influenza virus A/VN/1194/04 for unclear reasons. Possibly the most immunodominant responses in these subjects were directed to CTL epitopes in the NP that were not conserved.

To account for the full repertoire of virus-specific CD8+ T lymphocytes also the reactivity with MHC class I-matched cells infected with influenza virus A/NL/18/94 or A/VN/1194/04 was analyzed. In all cases A/VN/1194/04 infected target cells were recognized to a similar extent as A/NL/18/94-infected cells, indicating that the level of cross-reactivity of human CTL responses to seasonal H3N2 influenza viruses with H5N1 strains is substantial.

Thus apart from some individual epitopes that display amino acid sequence variation between H3N2 and H5N1 influenza A viruses, the level of cross-reactivity is considerable and does not seem to be influenced by the HLA-phenotype of the study subjects. Although it is unknown to what extent pre-existing T cell-immunity can dampen the impact of a next influenza pandemic, it is speculated that the protective effect of cross-reactive CTL responses has a beneficial effect on the outcome of infection with new pandemic influenza virus strains. This speculation is supported by a number of different observations. First, in animal models it has been shown that

virus-specific CTL contribute to heterosubtypic immunity [200, 216, 232], secondly it was found in 1957 that individuals that had experienced documented infections with H1N1 influenza A viruses, less likely developed severe disease or succumbed to infection with the pandemic strain of the H2N2 subtype [71]. In this respect it is of interest to note that during the current outbreak of H5N1 infections in humans especially younger individuals are at risk for severe disease and a fatal outcome of infection [259]. It can be hypothesized that younger individuals are less likely to have been exposed to seasonal influenza A viruses of the H3N2 and H1N1 subtype and thus have not mounted a (cross-reactive) CTL response to an alternative subtype. However, it cannot be excluded that confounding factors play a role in the observed disproportionate age distribution of severe H5N1 human cases. Last but not least, the human CTL response against epidemic strains is largely cross-reactive with H5N1 influenza virus strains as was demonstrated in the present study. Although these cross-reactive CTL populations may not prevent infection with pandemic strains, they may contribute to a certain degree of heterosubtypic immunity and facilitate a more rapid clearance of the infection than in immunologically naïve individuals who lack cross-reactive T cell populations. This may determine the difference between life and death during a pandemic outbreak. In addition, the induction of cross-reactive CTL responses may be an attractive target for the development of universal vaccines that could confer broadly protective immunity against influenza viruses of various subtypes.

Acknowledgements
The authors would like to thank Theo Bestebroer and Chantal Baas for outstanding technical assistance.



CHAPTER 6

Recombinant modified vaccinia virus Ankara (MVA)-based vaccine induces protective immunity in mice against infection with influenza virus H5N1

J.H.C.M. Kreijtz[1], Y. Suezer[2], G. van Amerongen[1], G. de Mutsert[1], B. S. Schnierle[2], J.M. Wood[3], T. Kuiken[1], R.A.M. Fouchier[1], J. Löwer[2], A.D.M.E. Osterhaus[1], G. Sutter[2], G.F. Rimmelzwaan[1]

[1] *Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands,* [2] *Paul-Ehrlich-Institut, Langen, Germany,* [3] *NIBSC, Potters Bar, United Kingdom*

Journal of Infectious Diseases 2007; 195:1598-606

Abstract

Since 2003, the number of human cases of infections with highly pathogenic avian influenza viruses of the H5N1-subtype is still increasing and therefore the development of safe and effective vaccines is considered a priority. However, the global production capacity of conventional vaccines is limited and insufficient for a worldwide vaccination campaign. In the present study an alternative H5N1 vaccine candidate, based on the replication deficient modified vaccinia virus Ankara (MVA) was evaluated. C57BL/6J mice were immunized twice with MVA expressing the HA-gene from influenza virus A/Hongkong/156/97 (MVA-HA-HK/97) or A/Vietnam/1194/04 (MVA-HA-VN/04). Subsequently, recombinant MVA-induced protective immunity was assessed after challenge infection with three antigenically distinct strains of H5N1 influenza viruses: A/Hong Kong/156/97, A/Vietnam/1194/04 and A/Indonesia/5/05. Our data suggest that recombinant MVA expressing the HA of influenza virus A/Vietnam/1194/04 is a promising alternative vaccine candidate that could be used for the induction of protective immunity against various H5N1 influenza strains.

Introduction

Since the first human cases of H5N1-infections in 1997, influenza viruses of this subtype caused outbreaks of avian influenza worldwide associated with an accumulating number of bird-to-human transmissions. As of November 19th, 258 human cases were recorded of which 154 proved to be fatal [293]. In addition, the H5N1 virus infections have spread from South-East Asia to other continents [294].

Since these viruses not only infect avian species but also various mammalian species [25-27, 295] including humans [15] there is a risk of the emergence of a new pandemic strain, either through adaptation of the avian viruses to replication in mammalian species or through the exchange of gene segments with normal epidemic influenza A viruses. For these reasons the development of effective and safe H5N1 vaccines is considered a priority [296].

However, the development of such vaccines and the production of sufficient quantities of vaccine doses is not straight forward: at present the combined vaccine production capacity of all manufacturers is not sufficient to timely provide for a worldwide vaccination campaign. There is a clear need for alternative vaccine delivery systems and production technologies that could help to overcome this problem.

Since different antigenically distinct clades of H5N1 viruses have been identified recently [296], an ideal vaccine would also induce cross-protective immunity against these antigenic variants. Recently, conventional inactivated vaccine preparations have been evaluated, such as whole-inactivated virus (WIV) and split-virion vaccines [78, 143, 152]. In addition, vaccines based on recombinant HA expressed by baculoviruses have been tested [116, 117, 124]. In immunologically naïve individuals these vaccines were poorly immunogenic and appreciable antibody responses were only induced when high doses, or a combination with an adjuvant such as alum was used [78, 143, 152]. Clearly, additional development efforts are urgently needed to overcome a catastrophic shortage of vaccine in the case of a H5N1 influenza pandemic. New promising influenza vaccine candidates include recombinant DNA-based vaccines and adenoviral vector vaccines [167, 176, 177, 297]. However, the efficacy of these experimental vaccines in humans still needs to be confirmed, and at present, they are not considered widely acceptable for use in human populations [297].

In the present study we evaluated another candidate vector vaccine based on a replication deficient poxvirus vaccine strain: modified vaccinia virus Ankara (MVA).

MVA has been tested originally in >120,000 individuals and proved to be a safe and effective vaccine against human smallpox [183]. More recently, recombinant MVA expressing foreign genes proved successful in evoking immune responses and providing protection against diseases caused by viruses, bacteria, parasites or tumors from which the antigens were derived [182, 188, 191, 298-301].
The advantages of using MVA vector vaccines include their established safety profile in humans, their efficacy upon delivery of heterologous antigens in clinical trials, and the availability of technologies for large scale production under the requirements of Good Manufacturing Practice (GMP) [189, 191, 300]. Other properties are: good immunogenicity, extreme host-range restriction, possibility of long term storage (stockpiling) and easy production at BSL-1 conditions in chicken embryo fibroblasts (CEF) and baby hamster kidney cells [181, 184, 186, 188, 191, 302].
Here we describe the construction and evaluation of two different recombinant MVA viruses expressing the HA-genes of H5N1 influenza viruses A/Hongkong/156/97 (A/HK/156/97) or A/Vietnam/1194/04 (A/VN/1194/04). These recombinant viruses were evaluated as vaccines in a mouse model to assess the induction of protective immunity against three different H5N1 viruses. A two-dose immunization regimen induced strong antibody responses that partially cross-reacted with heterologous H5N1-strains. The elicited antibody responses correlated with protection against challenge infection with homologous and heterologous influenza virus strains. Thus, MVA can be considered as a promising alternative vaccine candidate for the induction of protective immunity against H5N1 influenza viruses.

Material and Methods

Vaccine preparation

The influenza H5N1 viruses A/HK/156/97 and A/VN/1194/04 were propagated in Madin Darby Canine Kidney (MDCK) cells and the viral RNA was extracted from the culture supernatants using a RNA-isolation kit (Roche, Almere, the Netherlands). Subsequently, cDNA was synthesized from the vRNA using Superscript reverse-transcriptase (Invitrogen, Carlsbad, California, USA) and the AGCAAAAGCAGG oligonucleotide (Eurogentec, Seraing, Belgium) as primer. Next, the HA-genes were amplified by PCR using Pfu (Stratagene, La Jolla, California, USA) as heat-stable DNA polymerase. Primer sequences were extended with the NotI and XhoI restriction sites to facilitate directional cloning into the plasmid pBluescriptSK+ (Stratagene, La Jolla, California, USA). Primer sequences are available on request.

Subsequently, HA gene sequences were excised from these plasmids by NotI /XhoI digestion, treated with Klenow polymerase to generate blunt ends and cloned into the PmeI site of MVA expression plasmid pIIIdHR-PsynII to generate the MVA vector plasmids pIII-HA-HK/97 and pIII-HA-VN/04. Upon transfection in MVA-infected cells these plasmids direct insertion of foreign genes into the site of deletion III within the MVA genome (31) and allow transcription of the HA target genes under control of the vaccinia virus-specific promoter PsynII [302]. Recombinant viruses MVA-HA-HK/97 and MVA-HA-VN/04 were generated in primary CEF upon transfection with 1 µg plasmid DNA, infection with 0.05 infectious units / cell MVA isolate F6 (32), and by plaque selection on RK-13 cells (29). The recombinant MVA genomes were analyzed by PCR to verify HA gene insertion and genetic stability. The production of HA antigens by the MVA vector viruses was confirmed by Western blot analysis of CEF cell lysates harvested at various time points after infection with MVA-HA-HK/97 or MVA-HA-VN/04 (data not shown). One-step and multiple-step growth analysis in CEF demonstrated that the replication capacities of MVA-HA-HK/97 and MVA-HA-VN/04 were comparable to non-recombinant MVA (data not shown). To generate vaccine preparations the viruses were amplified in CEF, purified by ultra-centrifugation through sucrose and reconstituted in 1 mM Tris/HCl pH 9.0. MVA vaccines were used at a dose of 10^8 PFU diluted in 100µl PBS. Whole-inactivated NIBRG-14 virus, a re-assortant vaccine strain based on influenza virus A/Vietnam/1194/04 made by reverse genetics was used as positive control. The lyophilised whole-virus antigen was reconstituted in distilled water at a concentration of 2µg HA/50µl and mixed 1:1 with the adjuvant Stimune® (Specol, Cedi-Diagnostics, Lelystad, the Netherlands) [303]. Control mice were inoculated with PBS.

Influenza viruses

Influenza viruses A/HK/156/97, A/VN/1194/04 and A/Indonesia/5/05 (A/IND/5/05) were grown on MDCK cells. The supernatant was harvested after 3 days. Infectious virus titers were determined in Madin Darby Canine Kidney (MDCK) cells as described previously [228].

Mice

Female specified pathogen-free 6-8 weeks old C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Animals were divided in five groups of 18 mice and immunized with PBS, MVA-HA-HK/97, MVA-HA-VN/04, wtMVA, or Stimune®-

adjuvanted NIBRG-14. Immunizations were performed intra-muscularly, 50ml in the left hind leg and 50ml in the right. Four weeks later, blood samples were collected and animals were immunized again as described above. After another four weeks, again blood samples were collected and each of the five vaccine groups was divided into three sub-groups of six animals each. The sub-groups of each vaccine group were inoculated with 10^3 $TCID_{50}$ of influenza virus A/HK/156/97, A/VN/1194/04 or A/IND/5/05 in 50ml PBS by the intranasal route. A challenge dose of 10^3 $TCID_{50}$ of the respective H5N1 viruses was used since this resulted in the infection and significant loss of body weight in > 90% of the mice reproducibly. Six non-immunized animals were used as negative controls and were inoculated with 50ml PBS. Animals were weighed every day until day 4 after infection and then euthanized by exsanguinations. After euthanasia, brain, lungs (inflated with formalin), spleen and intestines were taken out..

Animals in all groups were properly age-matched at the time point of challenge infection. The experimental protocol was approved by an independent Animal ethics committee prior to the start of the experiment. Intra-muscular immunizations, intranasal infections, blood sampling and euthanasia were carried out under anesthesia with inhalative isoflurane. The animals were housed in filter-top cages and had access to food and water *ad libitum*. During the 5 days of infection with the H5N1 influenza virus, animals were placed in filter-top cages in bio-safety level 3 containment facilities. One BSL-3 isolator unit was used per virus.

Virus titers in organ tissues

Organs were snap frozen using a dry ice/ethanol bath and stored at -70°C. Organs were homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in transport medium (Hanks medium (MEM)), Glycerol, 100U/ml penicillin, 100mg/ml streptomycin, polymyxin B, Nystatin, Gentamicin). Quintuplicate ten-fold serial dilutions of these samples were used to determine the virus titers on confluent layers of MDCK cells.

Serology

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/156/97, A/VN/1194/04

or A/IND/5/05 [229]. For this purpose reverse genetics viruses were produced from which the basic cleavage site was removed. The use of these reverse genetics viruses was validated and titers obtained were comparable with those against the wild type strains (data not shown). Sera were also tested for the presence of virus neutralizing antibodies specific for the three influenza viruses using a micro virus neutralization (VN) assay with 100 $TCID_{50}$ of the respective viruses that were produced by reverse genetics as described above [230]. 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 the 3 different influenza A viruses.

Histopathology

Formalin-inflated lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4mm and stained with hematoxylin and eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method 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 the secondary antibody. The peroxidase was revealed using diamino-benzidine as a substrate, resulting in a deep red precipitate in the nuclei of influenza A virus-infected cells and a less intense red-staining of the cytoplasm. The sections were counterstained with hematoxylin.

Statistical analysis

Data for viral titers and antibody titers were analyzed using the two-sided Student's *t* test and differences were considered significant at $P < 0.05$.

Results

Serology

Upon a single vaccination with MVA-HA-HK/97 mice developed antibody responses against the homologous virus strain with geometric mean titers (GMTs) of 1629 and 239 measured in HI- and VN-assays, respectively. These antibodies however, did not cross-react with the influenza virus strains A/VN/1194/04 or A/IND/5/05 (Figure 1). Four weeks after the booster vaccination the homologous antibody GMTs in the HI- and VN-assays were 1370 and 744, respectively.

Again no cross-reaction was observed with the other H5N1 strains. The MVA-HA-VN/04 vaccine preparation was less immunogenic, since after the first vaccination none of the mice developed HI antibodies against the homologous strain and only one animal developed VN antibodies. After a second dose all animals responded and the GMT increased to 20 and 64 as measured by HI- and VN-assays, respectively. The antibodies induced by MVA-HA-VN/04 vaccination cross-reacted with the H5N1 strain A/HK/156/97 and to a limited extent with the strain A/IND/5/05. The adjuvanted NIBRG-14 vaccine preparation, which was included in the experiments as a positive control induced robust antibody responses against the homologous A/VN/1194/04, which cross-reacted with the strain A/HK/156/97 and A/IND/5/05 both in the HI- and VN-assays.

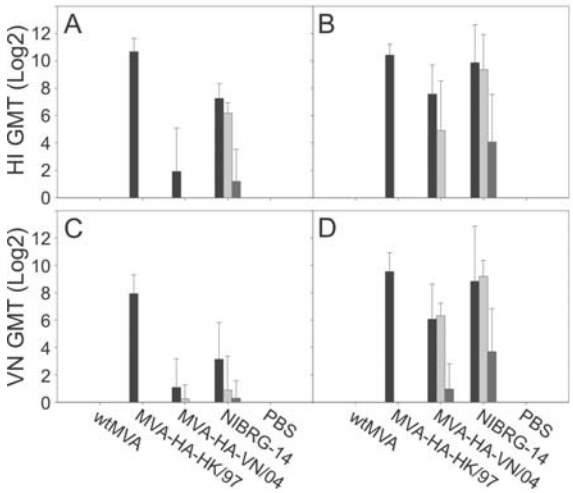


Figure 1 Antibody responses induced by vaccination. Antibody titers against the three challenge viruses: influenza virus A/HK156/97 (■), A/VN/1194/04 (▭) and A/IND/5/05 (▭) were measured by HI-assay (A+B). 28 days after the first immunization (A) and 28 days after the second immunization (B). Titers are presented as GMT (Log 2). Antibody titers against the three different challenge-viruses were measured by VN-assay (C+D). 28 days after the first immunization (C) and second immunization (D). Titers are presented as GMT (Log 2).

Clinical signs

From day two post-infection onwards, mice immunized with PBS or wtMVA developed clinical signs like hunched posture, rapid breathing, ruffled fur and decreased muscle strength irrespective of the influenza H5N1 virus that was used for infection. These clinical signs were not observed in mice infected with influenza virus A/HK/156/97 or A/VN/1194/04 after vaccination with MVA-HA-HK/97 or MVA-

HA-VN/04. MVA-HA-VN/04 vaccination also prevented the development of clinical signs caused by infection with influenza virus A/IND/5/05. The observed protection against clinical signs correlated with reduced loss of bodyweight after infection (Figure 2). In PBS and wtMVA immunized mice an average loss of bodyweight of 16.2% and 11.5% was observed post infection with influenza virus A/HK/156/97 (Figure 2A) or 16.9% and 10.4% post infection with influenza virus A/VN/1194/04 (Figure 2B), respectively. This was largely prevented by vaccination with MVA-HA-HK/97 or MVA-HA-VN/04 (Figure 2). Also infection with influenza virus A/IND/5/05 (Figure 2C) caused severe loss of bodyweight in PBS- or wtMVA-immunized control mice (16.9% and 18.6% respectively), which was significantly reduced by vaccination with MVA-HA-VN/04 but not by vaccination with MVA-HA-HK/97.

Table 1. Positive virus isolation from tissues and weight loss in individual mice after challenge infection

	Viruses used for challenge infection														
	A/HK/156/97					A/VN/1194/04					A/IND/5/05				
Vaccine preparations	brain	intestines	lung	spleen	>10% weight loss	brain	intestines	lung	spleen	>10% weight loss	brain	intestines	lung	spleen	10% weight loss
wtMVA	1/6	1/6	6/6	4/6	5/6	2/6	0/6	6/6	3/6	4/6	2/6	2/6	6/6	4/6	5/6
MVA-HA-HK/97	0/4	0/4	0/4	0/4	0/4	0/6	1/6	6/6	1/6	1/6	0/6	0/6	6/6	0/6	4/6
MVA-HA-VN/04	2/6	0/6	2/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/6	0/6	0/6
NIBRG-14	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
PBS	0/5	0/5	5/5	3/5	4/5	3/5	2/5	5/5	3/5	5/5	0/6	3/6	6/6	4/6	6/6

Group numbers < 6 are caused by fatalities due to causes not related to the experiment.

Virus replication in organs

Infectious virus titers were determined in brains, intestines, lungs and spleens on day 4 post-infection with influenza viruses A/HK/156/97 (Figure 3A), A/VN/1194/04 (Figure 3B) or A/IND/5/05 (Figure 3C). After infection, the highest virus replication was observed in the lungs with average lung virus titers of $10^{7.9}$, $10^{7.8}$ and $10^{8.9}$ TCID50/gram tissue for PBS control mice infected with influenza viruses A/HK/156/97, A/VN/1194/04 or A/IND/5/05 respectively. Mice vaccinated with wtMVA were not protected and similar average virus titers were found in the lungs of infected mice. In some animals of both groups virus replication could be demonstrated in extra-respiratory tissues including brain, intestines and spleen (Table 1). Vaccination with MVA-HA-HK/97 prevented replication of influenza virus A/HK/156/97 in the lungs and other organs completely whereas with MVA-HA-VN/04 vaccination a reduction

of virus replication in the lungs was observed in four out of six mice.
After challenge infection with influenza virus A/VN/1194/04 it was the other way around: vaccination with MVA-HA-VN/04 prevented replication completely, whereas vaccination with MVA-HA-HK/97 only partially reduced virus replication. This reduction was statistically significant compared to PBS-inoculated mice ($p < 0.05$).

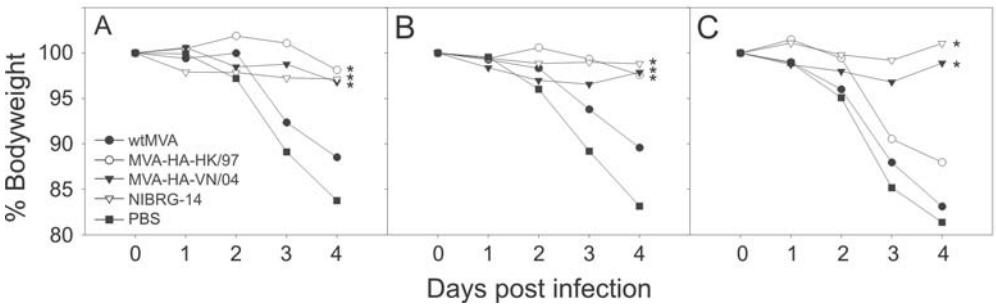


Figure 2 Weight loss of mice intra-nasally infected with 10^3 TCID50 of influenza virus A/HK156/97 (A), A/VN/1194/04 (B), or A/ IND/5/05 (C). Mean weight loss is expressed as the percentage of the original weight before infection. (*) Indicates a statistically significant difference with ($p < 0.05$).

Vaccination with MVA-HA-VN/04 also prevented replication of influenza virus A/IND/5/05 in the lungs of four out of six mice resulting in reduced average lungs titers compared to PBS- and wtMVA-immunized control mice. Vaccination with MVA-HA-HK/97 did not prevent replication of influenza virus A/IND/5/05 and all six mice tested positive (Table 1). Vaccination with the inactivated whole-virus NIBRG-14 adjuvanted with Stimune® not only prevented replication of the homologous strain influenza virus A/VN/1194/04 but also that of A/HK/156/97 and A/IND/5/05.

Pathologic changes and virus replication in the lungs
Four days after infection with each of the three HPAI viruses, the mice were sacrificed and their lungs were inflated with formalin and examined by immunohistochemistry and histology. For all three viruses, viral antigen expression and lesions depended on type of prior immunization. In mice immunized with adjuvanted NIBRG-14, neither viral antigen expression nor lesions were observed after infection with any of the three viruses (Figure 4D, I, N). After infection with influenza virus A/HK/156/97, PBS- and wtMVA-immunized mice had multifocal expression of viral antigen in bronchiolar and alveolar epithelial cells.

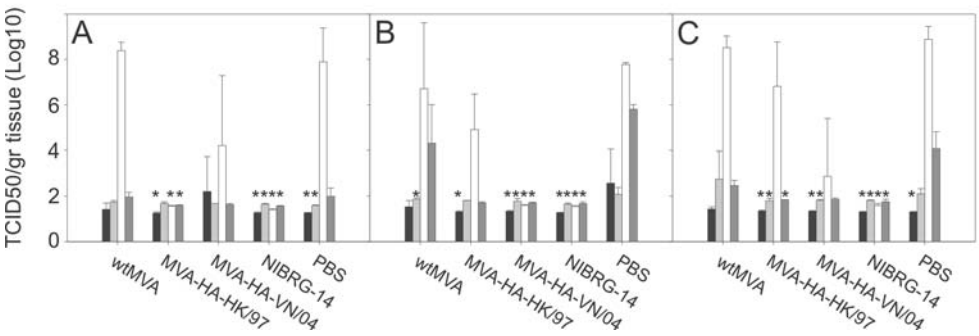


Figure 3 Virus titers in organ tissue at day 4 after infection with either influenza virus A/HK156/97 (A), A/VN/1194/04 (B), or A/ IND/5/05 (C). Results are shown for the wtMVA, MVA-HA-HK/97, MVA-HA-VN/04, Stimune®-adjuvanted NIBRG-14 and PBS immunized mice. Titers were measured in: brain (■), intestines (▭), lungs (□) and spleen (▬) and presented as $TCID_{50}$ per gram tissue (Log10). (*) Indicates an average virus titer below the cut-off value and that all animals tested negative by virus isolation.

This was associated with mild necrotizing bronchiolitis, characterized by necrosis of bronchiolar epithelial cells and peribronchiolar infiltration by inflammatory cells, mainly lymphocytes (Figure 4A, E).

In contrast, MVA-HA-HK/97 and MVA-HA-VN/04 immunized mice showed neither viral antigen expression nor lesions. After infection with influenza virus A/VN/1194/04, PBS- and wtMVA-immunized mice had more widespread expression of viral antigen in bronchiolar and alveolar epithelium than in A/HK/156/97-inoculated mice (Figure 4F, J). This was associated with moderate bronchiolitis and mild interstitial pneumonia, characterized by loss of alveolar epithelium and the presence of edema fluid and inflammatory cells (mainly neutrophils) in alveolar lumina. MVA-HA-HK/97-immunized mice appeared to have less viral antigen expression in alveolar epithelium than wtMVA- and PBS-immunized mice, but the extent of interstitial pneumonia was comparable (Figure 4G). Again, MVA-HA-VN/04 immunized mice showed neither viral antigen expression nor lesions (Figure 4H).

After infection with influenza virus A/IND/5/05, not only PBS- and wtMVA-immunized mice but also MVA-HA-HK/97-immunized mice had widespread expression of viral antigen in bronchiolar and alveolar epithelium (Figure 4K, O, L), associated with moderate bronchiolitis and moderate interstitial pneumonia. In contrast, MVA-HA-VN/04 immunized mice only had viral antigen expression in a few bronchiolar epithelial cells, associated with moderate bronchiolitis (Figure 4M).

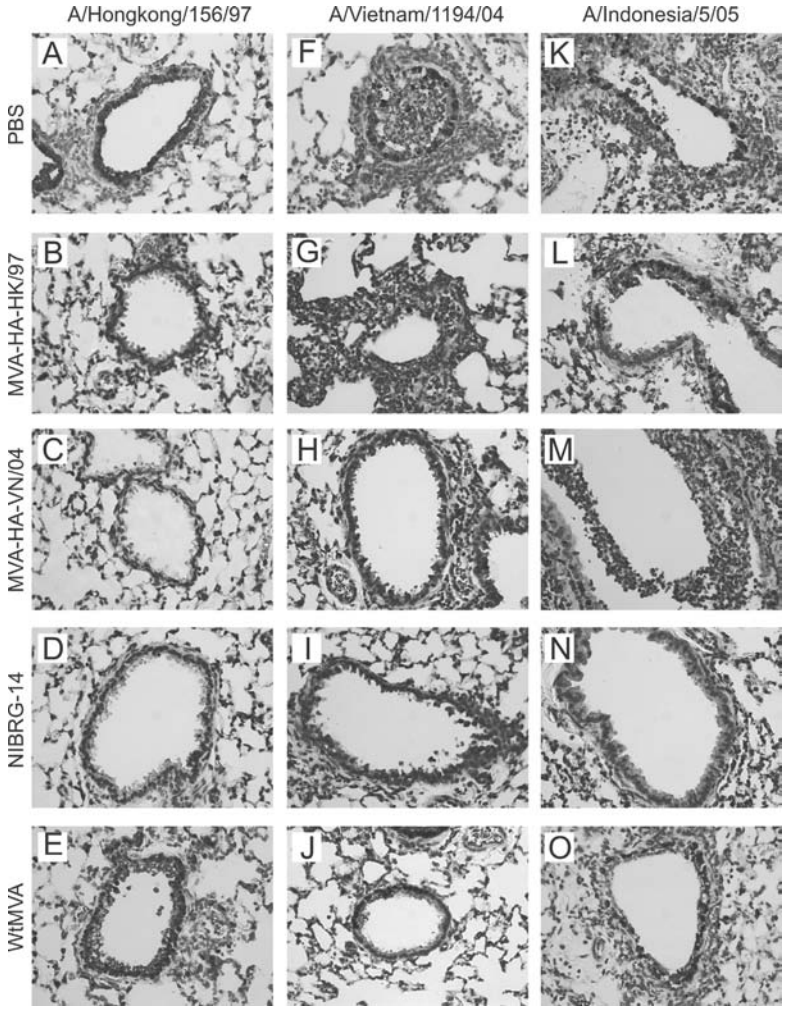


Figure 4 Histopathology and immunohistochemistry of the bronchioles and alveoli in lungs of mice infected with either influenza virus A/HK/157/97, A/VN/1194/04 or A/ IND/5/05 as indicated. Influenza virus A/HK/156/97 infection led to viral antigen expression in cells of the bronchiolar wall of PBS (A) and wtMVA immunized mice (E), combined with mild peribronchiolar inflammatory infiltrate, while in the lungs of MVA-HA-HK/97 (B), MVA-HA-VN/04 (C) and Stimune®-adjuvanted NIBRG-14 (D) immunized mice no viral antigen was detected. Infection with influenza virus A/VN/1194/04 resulted in expression of viral antigen in cells of the bronchiolar walls of PBS (F), MVA-HA-HK/97 (G) and wtMVA (J) immunized mice, also combined with moderate peribronchiolar infiltrate (except for the wtMVA immunized mice). No viral antigen expression or morphological changes were detected in MVA-HA-VN/04 (H) and Stimune®-adjuvanted NIBRG-14 immunized mice (I). Infection with influenza virus A/IND/5/05 resulted in abundant viral antigen expression in the bronchioles of PBS (K), MVA-HA-HK/97 (L) and wtMVA (O) immunized mice, combined with moderate peribronchiolar infiltrate. Only minimal viral antigen expression was detected in the bronchiolar wall of MVA-HA-VN/04 (M) immunized mice, combined with moderate inflammatory infiltrate. No viral antigen was detected in the lungs of Stimune®-adjuvanted NIBRG-14 (N) immunized mice after infection with influenza virus A/Indonesia/5/05. (full colour figure: APPENDIX VII)

Discussion

In the light of the pandemic threat caused by influenza H5N1 viruses, the availability of sufficient doses of safe and effective vaccines is considered a priority [293, 296]. In the present study we have evaluated recombinant MVA expressing the HA genes of two different influenza H5N1 viruses for the induction of protective immunity against three different influenza H5N1 viruses belonging to two different clades [211] in a mouse model. Vaccination with MVA expressing the HA of influenza H5N1 viruses induced potent antibody responses, which correlated with protection against homologous and heterologous challenge infection. For the generation of the MVA recombinants, the HA genes were derived from influenza viruses A/HK/156/97 and A/VN/1194/04. The co-circulation of antigenically different influenza virus strains complicates the development of effective vaccines considerably. Usually protective immunity is only induced with vaccines that closely match the circulating strains. The viruses used in the present study belong to distinct clades of H5N1 viruses [94, 211] and are antigenically different [211]. This allowed the assessment of the level of cross-protective immunity induced by vaccination against these two viruses. Furthermore a third H5N1 variant strain was used for challenge infection of the mice: A/IND/5/05, which was antigenically distinct from the other two viruses [296]. The recombinant MVA-HA-HK/97 was highly immunogenic. A single immunization already induced antibody responses against influenza virus A/HK/156/97, which were further boosted by a second immunization. These antibodies were not cross-reactive in HI- and VN-assays with A/VN/1194/04 or A/IND/5/05. MVA-HA-VN/04 was less immunogenic, but after two immunizations good antibody responses were observed, not only against the homologous virus but also to A/HK/156/97 and to a lesser extent to A/IND/5/05. The observed antibody reactivity pattern is similar to that observed with post-infection ferret sera [296]. Thus this asymmetry in antibody recognition pattern observed with antibodies induced by MVA-HA vaccination resembled that observed with antibodies induced after infection with the original influenza viruses [296].The NIBRG-14 vaccine preparation was included in the experiments as a positive control and was highly immunogenic in combination with the Stimune® adjuvant. This combination not only induced strong antibody responses to the homologous influenza virus A/VN/1194/04 but also to the other two H5N1 strains.

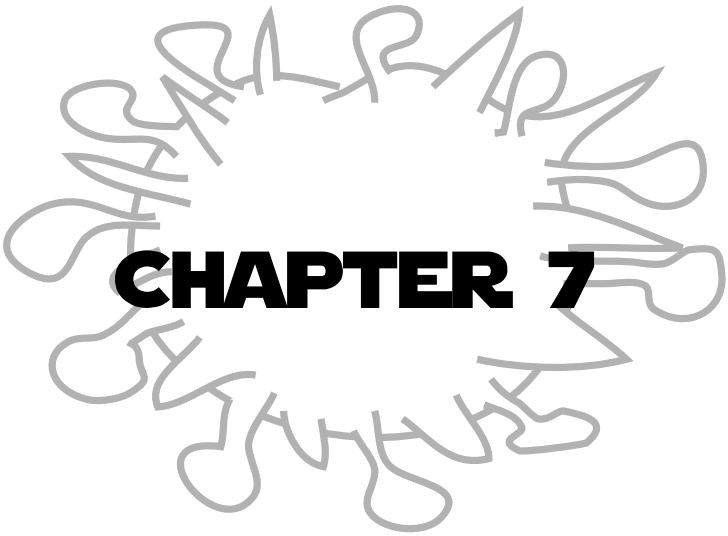
The HI- and VN-antibody titers measured against the three H5N1 strains correlated with protection against challenge infection. The MVA-HA-HK/97 immunized

mice were only protected against a homologous challenge infection. Vaccination prevented virus replication completely and as a result neither histo-pathological changes nor clinical signs were observed in these mice. Although the MVA-HA-HK/97 induced antibodies did not cross-react with influenza virus A/VN/1194/04, replication of this virus was reduced and the immunized animals were protected from clinical signs (Table 1). In contrast, no protective effects were seen upon challenge infection with influenza virus A/IND/5/05. Although it is known that MVA-vaccination can induce strong CTL responses which could have contributed to protection [304], it is unknown at present whether H-2b restricted cross-reactive CTL epitopes exist on the HA molecule of influenza H5N1 viruses. Immunization with MVA-HA-VN/04 induced sterilizing immunity against the homologous strain. In addition, strong protective effects were observed against the antigenically distinct influenza viruses A/HK/156/97 and A/IND/5/05. The replication of these viruses was largely reduced in most immunized animals, which correlated with the absence of infected cells in the respiratory tract and the lack of clinical signs.

The protection is most likely based on virus-neutralizing HA-specific serum antibodies that pass from the circulation into the alveolar epithelium [305]. Thus the use of MVA-HA as a candidate vaccine against emerging pandemic H5N1 strains has the potential to induce a broad immune response that protects individuals from severe clinical signs and histopathological changes in the respiratory tract even when the strains causing the infections do not fully match the vaccine antigen. In addition, MVA-based vaccines have a number of properties that make them favorable vaccine candidates for use in humans. First, recombinant MVA can be considered as extremely safe viral vectors because of their distinct replication deficiency in mammalian cells and their well established avirulence *in vivo* [188, 299, 300, 306-308] including the safety of MVA in immune-suppressed macaques [187] or the innocuous application of high doses of recombinant MVA to HIV-infected individuals [300, 309, 310]. Second industrial scale manufacturing of MVA vaccines appears feasible in recognition of the efforts undertaken to develop MVA as a third generation vaccine against orthopoxvirus-related biothreat [311]. Third, MVA vector vaccines can deliver multiple heterologous antigens and allow for simultaneous induction of high level humoral and cellular immunity [190, 191, 300] providing the possibility to develop multivalent vaccines. Since the production of these MVA-based vaccines is independent of existing production capacity for conventional influenza vaccines, it may help to reduce the envisaged shortage of vaccine doses in the time of an

emerging pandemic. Another advantage is that the excellent immunogenicity of these vaccines is independent of the use of adjuvants. We acknowledge that the use of a safe and effective adjuvant could improve the immunogenicity of conventional vaccines and may reduce the antigen quantity required to induce adequate antibody responses (dose sparing). Our results with the Stimune®-adjuvanted NIBRG-14 whole-inactivated virus underscores this possibility. However, at present such potent adjuvant formulations are not considered suitable for use in humans. We conclude that MVA-based H5N1 vaccines are promising vaccine candidates with favorable properties regarding safety, effectiveness and the potential of rapid large-scale production, which are important in the face of an emerging pandemic.

Acknowledgements
The authors wish to thank Chantal Baas, Theo Bestebroer, Nella Nieuwkoop and Josanne Verhagen for excellent technical assistance.



CHAPTER 7

Recombinant modified vaccinia virus Ankara expressing HA confers protection against homologous and heterologous H5N1 influenza virus infections in macaques

J.H.C.M. Kreijtz[1], Y. Suezer[2], G. de Mutsert[1], J.M.A. van den Brand[1], G. van Amerongen[1], B. S. Schnierle[2], T. Kuiken[1], R.A.M. Fouchier[1], J. Löwer[2], A.D.M.E. Osterhaus[1], G. Sutter[2], G.F. Rimmelzwaan[1]

[1] *Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands.* [2] *Paul-Ehrlich-Institut, Langen, Germany*

Journal of Infectious Diseases 2009; 199:405-13

Abstract

Highly pathogenic avian influenza viruses of the H5N1 subtype are responsible for an increasing number of infections in humans since 2003. More than 60% of the infected individuals succumb and new infections are reported frequently. In the light of the pandemic threat caused by these events the rapid availability of safe and effective vaccines is desirable. Modified Vaccinia virus Ankara (MVA) expressing the HA gene of H5N1 viruses is a promising candidate vaccine that induced protective immunity against infection with homologous and heterologous H5N1 influenza virus in mice. In the present study we evaluated a recombinant MVA vector expressing the HA of H5N1 influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) in non-human primates. Cynomolgus macaques were immunized twice and then challenged with influenza virus A/Vietnam/1194/04 (clade 1) or A/Indonesia/5/05 (clade 2.1) to assess the level of protective immunity. Immunization with MVA-HA-VN/04 induced (cross-reactive) antibodies and prevented virus replication in the upper and lower respiratory tract and the development of severe necrotizing broncho-interstitial pneumonia. Therefore MVA-HA-VN/04 is a promising vaccine candidate for the induction of protective immunity against highly pathogenic H5N1 avian influenza viruses in man.

Introduction

Since 2003 the number of bird-to-human transmissions of H5N1 influenza viruses is increasing and as of the 28nd of February 2008, 369 human cases were reported to the World Health Organization of which 234 proved to be fatal [312]. Thus there is a risk for the emergence of a pandemic H5N1 strain either through adaption of the avian viruses to replication in humans or through the exchange of gene segments with seasonal influenza A viruses. To limit the impact of a pandemic outbreak caused by these viruses the expedite availability of safe and effective H5N1 vaccines is highly desirable [313]. However, the development of such vaccines and the production of sufficient vaccine doses for a global vaccination campaign is a challenge: the combined vaccine production capacity of all seasonal influenza vaccine manufacturers is limited and not sufficient to timely provide sufficient doses for a worldwide vaccination campaign. Therefore, there is considerable interest in dose-sparing vaccination strategies. For example, the use of potent adjuvants may result in a reduction of the amount of hemagglutinin (HA) antigen, required for the induction of protective antibody responses. At present, various adjuvants are being evaluated in combination with conventional inactivated vaccine preparations and these are in various stages of development [141, 142, 156, 158, 314].

Alternatively, novel vaccine production technologies are under development to overcome the shortage of vaccines in the case of an H5N1-influenza pandemic. Cell lines have become available for the production of vaccines as alternative for the conventional production in embryonated chicken eggs [100, 106, 113, 315-317]. The use of reverse genetics for the generation of vaccine strains will further contribute to faster availability of vaccines after the onset of a influenza pandemic [318, 319]. Other novel production technologies include the use of recombinant baculoviruses for the production of H5 in insect cells [116, 117, 124]. In general, these protein-based vaccines are poorly immunogenic in immunologically naïve individuals and appreciable antibody responses were only induced when a high dose or a combination with an adjuvant was used [78, 143, 152]. Since 1997, H5N1 viruses have diverged considerably and are now classified into clades and subclades to reflect their phylogenetic and antigenic differences [313]. These differences complicate vaccine strain selection and ideally vaccines induce cross-clade protective immunity. New promising influenza vaccine candidates that may fulfil the described requirements include DNA vaccines and viral vectors that express the HA gene of H5N1 influenza viruses [176, 177, 297]. We recently have shown that the replication

deficient poxvirus modified vaccinia virus Ankara (MVA) expressing the HA gene of influenza virus A/Vietnam/1194/04 (H5N1) is highly immunogenic in mice [251]. Two immunizations without an adjuvant induced strong antibody responses and immunized mice were protected from infection with the homologous virus and the heterologous H5N1 virus A/Indonesia/5/05 [251]. MVA was tested originally in >120,000 individuals and proved to be a safe and effective vaccine against small pox [183]. The use of recombinant MVA expressing foreign genes as vaccine candidates induced protective immunity against diseases caused by viruses, bacteria, parasites or tumors from which the antigens were derived. Thus, MVA has an excellent safety profile in humans, can be used for the delivery of foreign antigens and can be produced at large scale under the requirements of good manufacturing practice [189, 191]. Other properties are extreme host-range restriction, easy production at biosafety level 1 (BSL-1) conditions in chicken embryo fibroblasts (CEF) and baby hamster kidney cells and the possibility of long-term storage (stock piling) [181, 184, 186, 188, 191].

For reasons outlined above, MVA expressing the HA gene of an H5N1 influenza virus is an attractive and promising pandemic vaccine candidate. However, the immunogenicity and protective efficacy of such vaccines has been demonstrated in mice and chickens only [190, 251, 320]. Since the predictive value of these models for immunogenicity in humans is limited [320, 321], we wished to evaluate a recombinant MVA-H5 vaccine candidate in a non-human primate model to assess the induction of protective immunity against H5N1 viruses from two different clades. Therefore, we used cynomolgus macaques that develop severe interstitial necrotizing pneumonia after infection with H5N1 influenza virus comparable to the pathogenesis observed in humans [282, 322]. To this end, a recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 was used to immunize cynomolgus macaques twice, which resulted in strong influenza H5 specific, virus neutralizing antibody responses.

Thus-induced immunity provided protection against challenge infection with the homologous influenza H5N1 strain from clade 1 and the heterologous strain A/Indonesia/5/05 from clade 2.1. It was concluded that recombinant MVA is a safe and effective vaccine candidate for the induction of protective immunity against H5N1 influenza viruses and warrants further clinical development.

Material & Methods

Vaccine preparation

Recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) was prepared as described previously [251]. As parent MVA strain MVA clonal isolate F6 was used. To generate final vaccine preparations, the virus was amplified in CEF, purified by ultracentrifugation through sucrose, reconstituted in 1mM Tris-HCL pH 9.0 and diluted in PBS.

Influenza viruses

Influenza viruses A/Vietnam/1194/04 (A/VN/1194/04) and A/Indonesia/5/05 (A/IND/5/05) were cultured in Madin Darby Canine Kidney (MDCK) cells. Infectious virus titers were determined in MDCK cells as described previously [228].

Macaques

Colony-bred sero-negative cynomolgus macaques (*Macaca fascicularis*), 3 years of age, were obtained from Grand Forest Scientific Primate Company LTD (Guangxi, China). Two weeks prior to the start of the experiment animals were anesthesized using a cocktail of ketamin® (Nimatek, Eurovet Animal Health BV, Bladel, the Netherlands) and domitor® (Orion Pharma, Espoo, Finland) and a temperature logger (DST micro-T ultra small temperature logger, Star-Oddi, Reykjavik, Iceland) was placed in the peritoneal cavity. This device recorded the body temperature of the animals every 15 minutes. Changes in body temperature were calculated by substracting the mean day (4 hours) and night (4 hours) temperature measured on 4 successive days in the period prior to the challenge from the mean day (4 hours) and night temperatures (4 hours) post infection. This was done for each individual animal.

The macaques were immunized twice with a four week interval and received $10^{8.5}$ pfu MVA-HA-VN/04 (n=12), $10^{8.5}$ pfu wtMVA (empty vector control)(n=12) or PBS (n=10) intramuscularly in a volume of one ml divided over both legs. Blood samples were collected before immunization and four weeks after the first and second immunizations. Four weeks after the second immunization each vaccine group was divided into two groups of six animals (except for the PBS group that was divided in groups of four and six animals respectively) and placed into BSL-3 isolator units. The animals were anesthesized and inoculated intratracheally with influenza virus

A/VN/1194/04 or A/IND/5/05 at a dose of $1*10^6$ $TCID_{50}$ in 3 ml PBS. After infection, the animals were monitored for the development of clinical signs. Before and on days 2 and 4 post infection throat and nose swabs were collected under anesthesia. Four days post infection the animals were sacrificed by exsanguination under anesthesia with ketamin® and dormitor® and necropsies were performed according to standard procedures. This time point for euthanasia was chosen since it allowed the assessment of gross pathology and histopathology, and the extent of virus replication. It was also chosen for ethical reasons since the development of severe disease in the non-protected animals was avoided. The experimental protocol was approved by an independent animal ethics committee before the start of the experiments and performed compliant with National and European legislation.

Serology

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/VN/1194/04 or A/IND/5/05 [229]. For this purpose viruses were produced from which the basic cleavage site in HA, associated with high virulence, was deleted by reverse genetics. The use of these reverse genetics viruses in the HI assay was validated and the obtained antibody titers were comparable with those against the wild type strains (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 the viruses that were produced by reverse genetics as described above [230].

In brief, 50 microliter volumes of serial diluted serum samples were incubated with 100 $TCID_{50}$ of the viruses for one hour at 37°C and then the mixture was added to MDCK cells. After one hour, the cells were washed and subsequently cultured in Eagles Minimal Essential Medium containing bovine serum albumin (BSA, fraction V 0.3%), 4mg/ml trypsin, L-glutamin 2mM, penicillin 100U/ml, streptomycin 100mg/ml $NaHCO_3$ 0.15%, Hepes 20mM, non-essential amino acids 0.1 mM. After five days, residual virus replication was assessed by measuring HA activity in the culture supernatants. Hyper-immune serum obtained from a swan immunized twice with inactivated H5N2 influenza virus A/Duck/Potsdam/1402/86 (Nobilis influenza® H5N2 Intervet International, Boxmeer, the Netherlands) was used as a positive

control against the two influenza viruses. For calculation purposes serum samples with an antibody titer of <10 were arbitrarily assigned a titer of 5.

Virus titers in organ tissues

Tissue samples were snap-frozen using a dry ice/ethanol bath and stored at -70°C. The tissues were homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in transport medium (Hanks medium (MEM) containing: 10% Glycerol, 100U/ml penicillin, 100mg/ml streptomycin, polymyxin B, Nystatin, Gentamicin, 7,5% $NaHCO_3$, 1M Hepes). Quintuplicate ten-fold serial dilutions of these samples were used to determine the virus titers in confluent layers of MDCK cells.

Histopathology and immunohistochemistry

Formalin-inflated lungs were fixed in 10% neutral buffered formalin and then cross-sections were made and embedded in paraffin, sectioned at 4 mm and stained with hematoxylin and eosin for histologic 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 nuclei of influenza virus-infected cells and a less intense red-staining of the cytoplasm. The sections were counterstained with hematoxylin.

Results

Vaccine induced antibody responses

In order to assess the ability of the MVA-HA-VN/04 vaccine to induce antibody responses, HI and VN serum antibody titers were measured after one and two immunizations. After the first immunization the MVA-HA-VN/04 immunized animals developed antibody responses against influenza virus A/VN/1194/04 with geometric mean titers of 20.9 (seven out of twelve animals) and 8.5 (four out of twelve animals) measured in the HI and VN assay respectively (Fig 1A, B). All animals (n=12) had developed an antibody response after the second immunization and the mean HI titer to this virus increased to 207.6 and the mean VN titer to 156.2 (Fig 1A, B). Antibody responses directed to influenza virus A/IND/5/05 HA were detected

in two out of twelve animals with a geometric mean titer of 6.9 in the HI assay, four weeks after the first immunization (Fig 1A). After the boost immunization four animals had detectable antibody responses with geometric mean HI and VN titers of 8.9 and 8.2 respectively (Fig 1A, B). None of the PBS or wtMVA immunized animals developed antibody responses against influenza virus A/VN/1194/04 or A/IND/5/05.

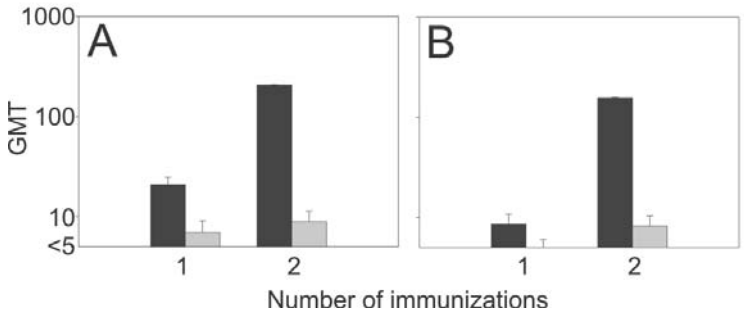


Figure 1 Antibody responses induced by immunization with MVA-HA-VN/04. Antibody titers against two H5N1 influenza viruses: A/Vietnam/1194/04 (black bars) and A/Indonesia/5/05 (grey bars) were measured in a hemagglutination inhibition (HI) assay (A) and virus neutralization (VN) assay (B) 28 days after the first and second immunization. Data are geometric mean titers (GMTs).

Clinical signs after infection

Before the start of the experiment a telemetric transponder was implanted in the abdominal cavity of every animal to record body temperature during the course of the experiment. The body temperature of every animal followed a strict day-night cycle during the experiment. Administration of the MVA-HA-VN/04 vaccine preparation did not affect the cycle and the mean day and night body temperatures (data not shown). After infection with influenza virus A/VN/1194/04 all animals in the PBS group and 5 out of six animals in the wtMVA group developed a fever within the first day (Figure 2A, B, G, H, J, K) with average body temperatures of 38.6°C and 38.8°C for the PBS and wtMVA immunized animals respectively during the subsequent twenty-four hours. The mean body temperature did not decline to the base level on day 3 and 4 in the PBS and wtMVA immunized group respectively (Fig G, H). Following infection with influenza virus A/IND/5/05 the animals in the PBS and wtMVA immunized group developed average fevers of 39.1°C and 38.9°C respectively (Figure 2D, E, M, N, P, Q).

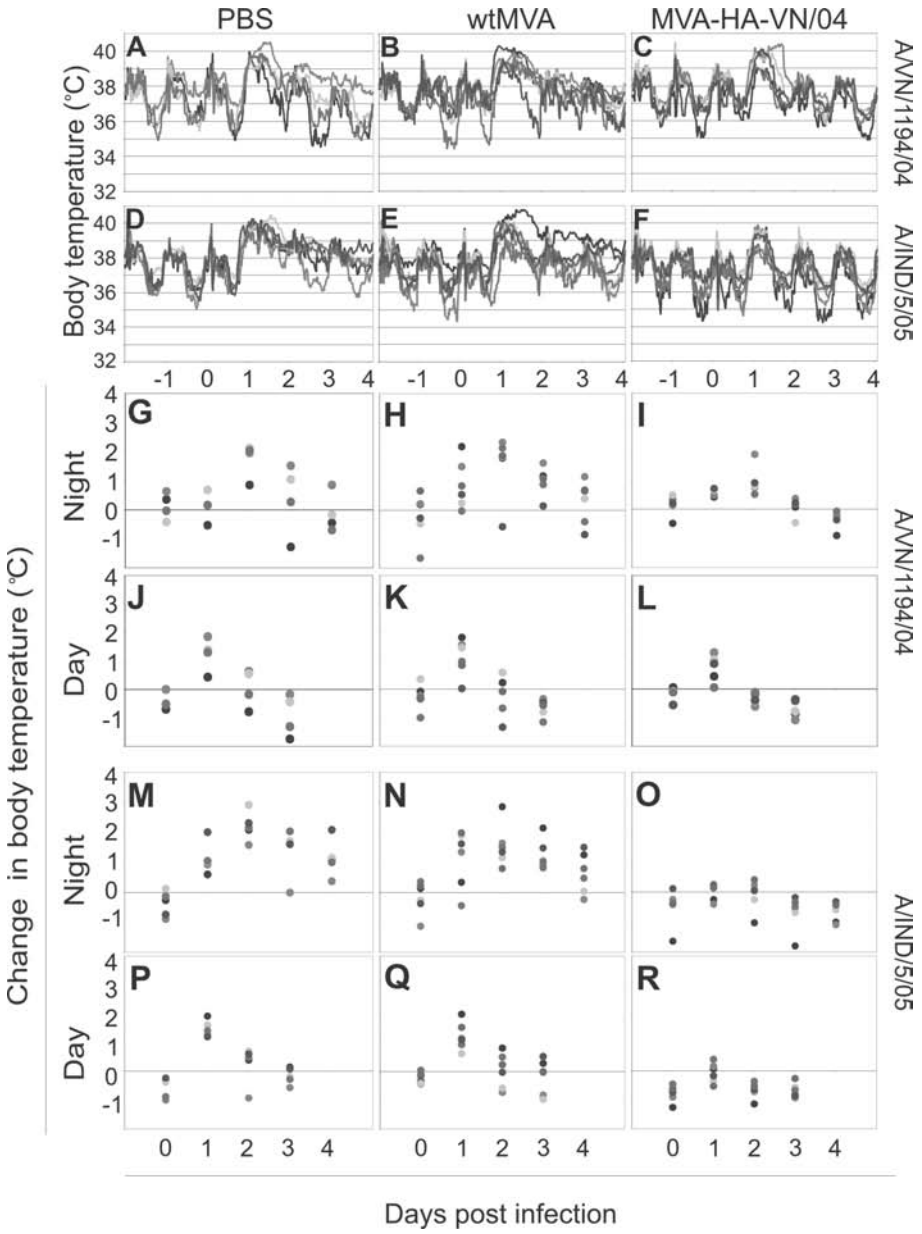


Figure 2 Body temperature recorded before and after infection with influenza virus A/Vietnam/1194/04 (A-C) or A/Indonesia/5/05 (D-F). The animals were immunized with PBS, wtMVA or MVA-HA-VN/04 as indicated. Changes in body temperature of individual animals after infection with influenza virus A/Vietnam/1194/04 (G-L) or A/Indonesia/5/05 (M-R) were calculated for each individual animal. Each dot represents an individual animal. Line colors in Figure 2A-C correspond with dot colors in figure 2G-L. Line colors in Figure 2D-F correspond with dot colors in figure 2M-R. (full colour figure: APPENDIX VIII)

The mean body temperature of these animals remained elevated, mainly during the night, in the four-day-period after infection (Fig 2M, N). The body temperature in the MVA-HA-VN/04 immunized animals maintained its day-night cycle and remained normal after infection with influenza virus A/VN/1194/04 (Fig 2C) except for a small rise during day 1 and the following night (Fig 2I, L).After infection with influenza virus A/IND/5/05 body temperature and day-night cycle were normal (Fig 2F, O, R).

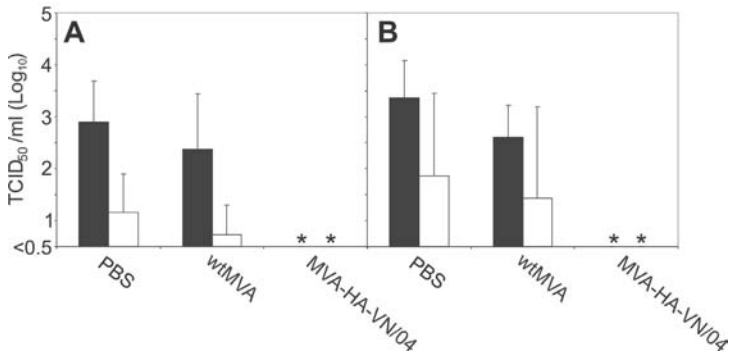


Figure 3 Virus titers in the throat after infection with an H5N1 influenza virus. Virus titers were determined in throat swaps that were taken on day 2 (black bars) and 4 (white bars) after infection with influenza virus A/Vietnam/1194/04 (A) or A/Indonesia/5/05 (B). Titers are presented as $TCID_{50}$ per ml (log_{10}). (*All animals tested negative by virus isolation resulting in an average virus titers below the cut-off value)

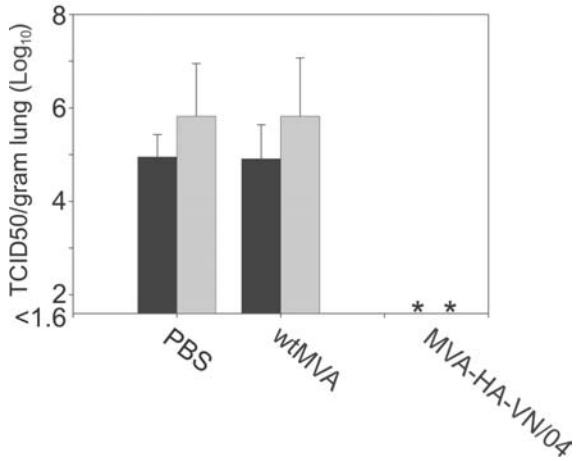


Figure 4 Virus titers in the lungs after infection with H5N1 influenza viruses. Virus titers were determined on day 4 after infection in the lungs of animals that were infected with either A/Vietnam/1194/04 (black bars) or A/Indonesia/5/05 (grey bars). Virus titers are presented as $TCID_{50}$ per gram lung (log_{10}). (*All animals tested negative by virus isolation resulting an average virus titers below the cut-off value.)

Virus detection in the upper respiratory tract

In order to determine the virus titers in the upper respiratory tract, nose and throat swabs were collected on day 0, 2 and 4 after infection. On day 2 after infection with influenza virus A/VN/1194/04 throat swaps were positive for all animals in the PBS group and five out of six animals in the wtMVA group with mean titers of $10^{2.9}$ (SD=$10^{0.8}$) and $10^{2.4}$ (SD=$10^{1.1}$) respectively (Fig 3A). The titers were lower on day 4 in both the PBS group (two out of four animals were positive) and in the wtMVA group (one positive animal) with geometric mean titers of $10^{1.2}$ (SD=$10^{0.8}$) and $10^{0.7}$ (SD=$10^{0.6}$) respectively. After infection with influenza virus A/IND/5/05 the titers were slightly higher for all animals in the PBS and wtMVA group with mean titers on day 2 of $10^{3.4}$ (SD=$10^{0.7}$) and $10^{2.6}$ (SD=$10^{0.2}$) respectively (Fig 3B). On day 4 the titers had declined to $10^{1.9}$ (SD=$10^{1.6}$) (three animals positive) in the PBS group and $10^{1.4}$ (SD=$10^{1.8}$)(two animals positive) in the wtMVA group. Virus was not detectable in the throat of the MVA-HA-VN/04 immunized animals neither on day 2 or 4 after infection with either the homologous or heterologous virus (Fig 3A, B). The nose swaps were negative for all animals in both challenge groups and on both days (data not shown).

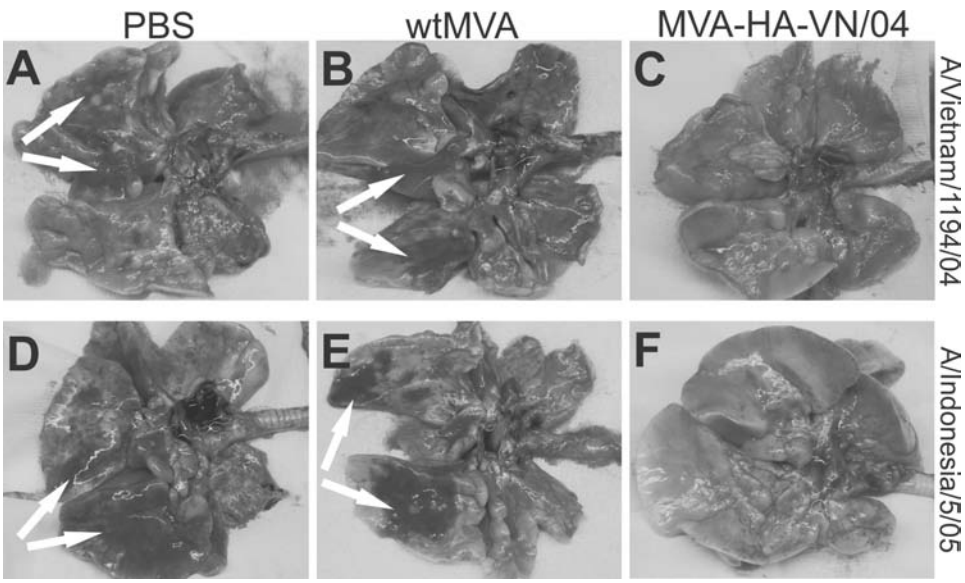


Figure 5 Macroscopic lesions of the lungs after infection with H5N1 influenza virus. The lungs of animals immunized with PBS (A, D), wtMVA (B, E) or MVA-HA-VN/04 (C, F) were fixed in formalin on day 4 after infection with influenza virus A/Vietnam/1194/04 (A-C) or A/Indonesia/5/05 (D-F). The arrows indicate consolidated areas present in the lungs of PBS and wtMVA immunized animals after infection (A, B, D, E). Lungs from the MVA-HA-VN/04 immunized animals had no macroscopical lesions (C, F). (full colour figure: APPENDIX IX)

Virus detection in organs

Lungs, brain and spleen were tested for the presence of infectious virus on day 4 after infection. Virus was detectable in the lungs of all animals in the PBS and wtMVA groups infected with influenza virus A/VN/1194/04 (Fig 4) with mean titers of $10^{5.0}$ (SD=$10^{0.5}$) and $10^{4.9}$ (SD=$10^{0.7}$) $TCID_{50}$/gram tissue respectively. After infection with influenza virus A/IND/5/05 higher infectious virus titers were detected in the animals that received PBS or wtMVA with mean titers of $10^{5.8}$ (SD=$10^{1.1}$) and $10^{5.8}$ (SD=$10^{1.2}$) TCID50/gram tissue respectively. In the lungs of MVA-HA-VN/04 immunized animals no virus was detected regardless the virus that was used for infection (Fig 4). No virus was detected in the brain or spleen obtained from any of the animals.

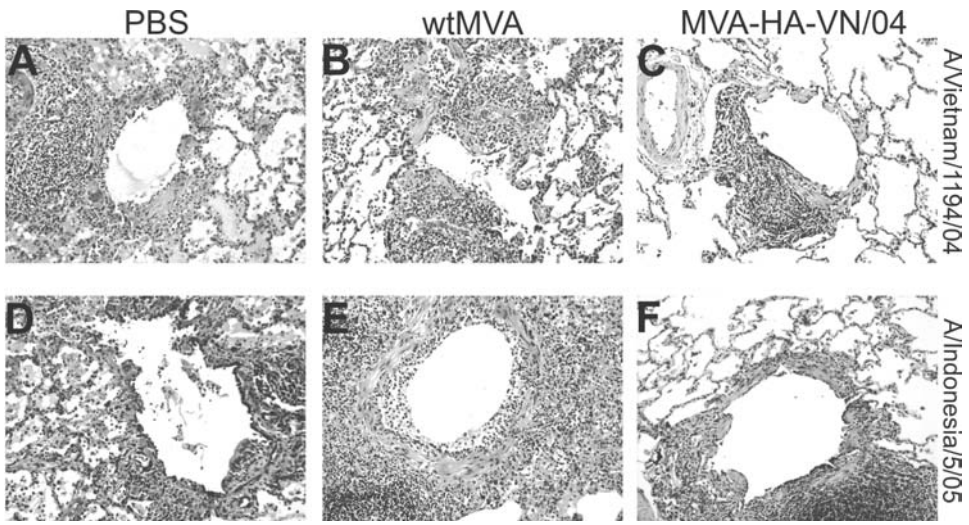


Figure 6 Histopathologic analysis of the lungs on day 4 after infection with influenza virus A/Vietnam/1194/04 (A, B, C) or A/Indonesia/5/05 (D, E, F). Histopathological changes were comparable in PBS and wtMVA inoculated animals with extensive lesions in the lungs of these animals. There was mild necrosis, edema, hyperthropy and hyperplasia of type II pneumocytes combined with peribronchiolar and –vascular infiltration. The epithelium of some bronchioles is denuded due to necrosis of the epithelial cells. (A, B, D, E). In the lungs of the MVA-HA-VN/04 immunized animals no histopathological changes were observed (C,F). (full colour figure: APPENDIX IX)

Pathology in the lungs

Lungs were dissected and inflated with formalin on day 4 after infection to examine pathology. Macroscopically multifocal to coalescing consolidation characterized by depressed, dark red and firm areas was seen in the PBS and wtMVA immunized animals after infection with either of the two H5N1 influenza viruses (Figure 5A, B,

D, E), with a range of 45-90% affected tissue. These lesions were far less extensive (± 5%) or even absent in the MVA-HA-VN/04 immunized groups (Figure 5C, F).
In order to examine the lungs in more detail cross-sections were made and used for histological analysis. By histopathology, the main lesions were seen in the alveoli and bronchioli. The PBS and wtMVA immunized animals infected with influenza virus A/VN/1194/04 had a multifocal, moderate to severe, necrotizing broncho-interstitial pneumonia.

Infection of the PBS and wtMVA immunized animals with the A/IND/5/05 strain resulted in an over all more severe pathology than seen with the first virus. The pneumonia was characterized by variable intra-alveolar amounts of proteinaceous fluid (edema) and eosinophilic fibrillar material (fibrin), cellular debris, moderate numbers of alveolar macrophages and few neutrophils and eosinophils (Figure 6A, B, D, E). In the alveolar septa, there was multifocal karyorrhexis, karyolysis and loss of detail of epithelial cells (necrosis), infiltration with few neutrophils and eosinophils, and mild hypertrophy and hyperplasia of type II pneumocytes. In the bronchioles, there was multifocal loss of epithelial cells and intraluminal edema fluid and cellular debris. There was perivascular and peribronchioar infiltration with many lymphocytes and plasma cells, and few macrophages, neutrophils and eosinophils (Figure 6A, B, D, E). In the MVA-HA-VN/04 immunized animals both after infection with influenza virus A/VN/1194/04 and A/IND/5/05, there was a multifocal mild broncho-interstitial pneumonia characterized by few intra-alveolar macrophages, neutrophils and eosinophils, and few lymphocytes and plasma cells in the alveolar septa, perivascular and peribronchiolar (Figure 6C, F). The bronchus-associated lymphoid tissue (BALT) was hyperplastic and appeared to be activated when compared to the PBS and wtMVA inoculated animals.

Detection of virus-infected cells by immunohistochemistry
After infection with influenza virus A/VN/1194/04 the lungs from the PBS and wtMVA immunized animals showed viral antigen expression (Fig 7A, B). Particularly alveolar epithelial cells showed antigen expression as well as a few alveolar macrophages. The infected cells were predominantly associated with the pulmonary lesions. In the lungs of the PBS and wtMVA immunized animals after infection with influenza virus A/IND/5/05 (Fig 7D, E), the viral antigen expression involved the same cell types and location as seen after infection with influenza virus A/VN/1194/04 but was more

extensive in these animals. No virus-infected cells were detectable in the lungs of MVA-HA-VN/04 immunized animals infected with influenza virus A/VN/1194/04 or A/IND/5/05 (Fig 7C, F).

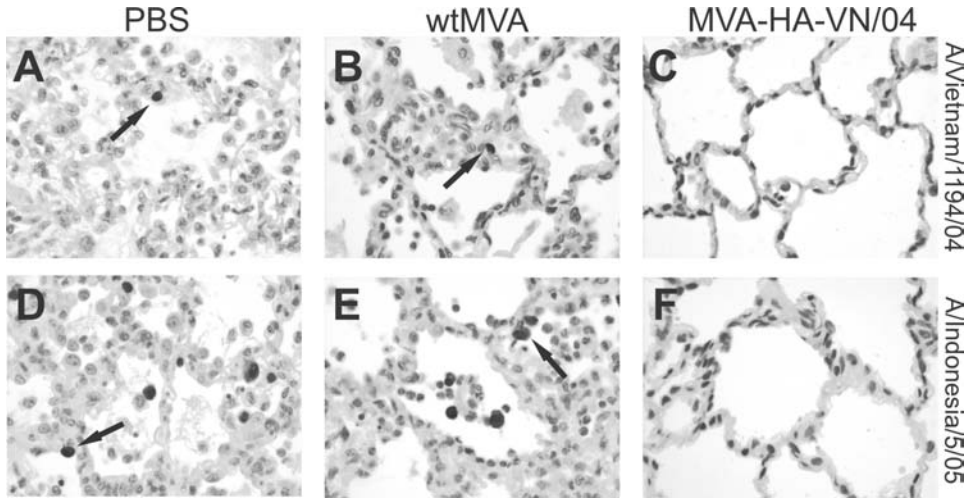


Figure 7 Detection of virus-infected cells in the lungs four days post infection with H5N1 influenza viruses. Immunohistochemistry was used to stain cells that are positive for the presence of viral antigen showing a deep red staining in the nucleus. Influenza viruses A/Vietnam/1194/04 (arrows indicate single infected cells) or A/Indonesia/5/05 antigen expression was seen in alveolar epithelial cells and some alveolar macrophages of PBS (A, D) and wtMVA (B, E) inoculated animals. No viral antigen was observed in the lungs of MVA-HA-VN/04 immunized animals (C, F). (full colour figure: APPENDIX X)

Discussion

The aim of this study was to evaluate the capacity of the MVA-HA-VN/04 vaccine to induce protective immunity against highly pathogenic H5N1 influenza viruses in a non-human primate model. Cynomolgus macaques were immunized twice with MVA-HA-VN/04 and then challenged with influenza virus A/VN/1194/04 or A/IND/5/05, strains from clade 1 and 2.1 respectively [313]. Immunization of cynomolgus macaques with MVA-HA-VN/04 induced virus-specific HI and VN antibodies that in the majority of the animals cross-reacted with the heterologous strain A/IND/5/05 from clade 2.1. Despite the absence of cross-reactive antibodies in some of the animals they were all protected against infection with the homologous and the heterologous strain. A similar result was obtained in the mouse model [251]. Apparently, the induction of cross-reactive antibody responses that are below the detection limit were sufficient for protection against infection with the heterologous strain. This observation is in concordance with results obtained in other H5N1

vaccination-challenge experiments in mice and ferrets [156, 251].

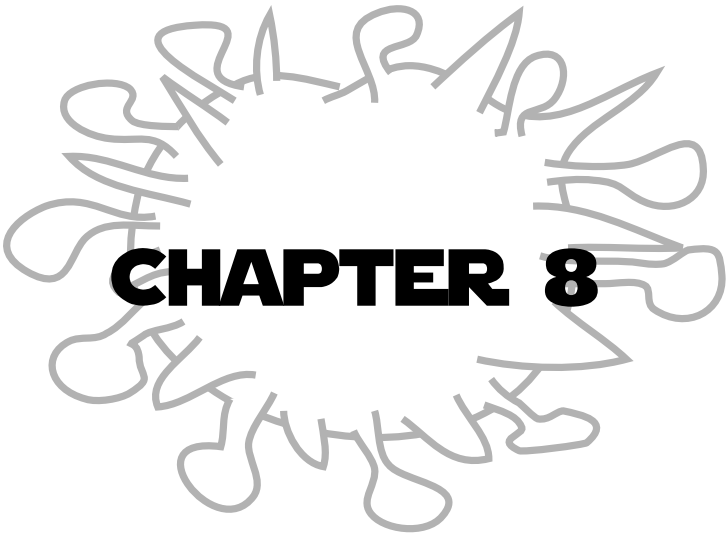
Our data show that vaccination with MVA-HA-VN/04 prevented virus replication and the development of fever and severe interstitial pneumonia after challenge infection. The pathogenesis of infection with influenza viruses A/VN/1194/04 and A/IND/5/05 in cynomolgus macaques was characterized by infection of predominantly pneumocytes resulting in a necrotizing broncho-interstitial pneumonia. This resembled the pathogenesis observed after infection of macaques with influenza virus A/HK/156/97 [282, 322] or that seen after infection of man with H5N1 highly pathogenic avian influenza viruses [323, 324]. Apparently, the infection of pneumocytes is sufficient for the development of interstitial pneumonia in primates, regardless the type of pneumocytes (type I or type II) that are infected [15, 323, 325]. H5N1 viruses bound to epithelial cells of the lower respiratory tract of cynomolgus macaques, most likely through the preferential usage of the (SA)-α2,3-Gal receptor. Collectively, the MVA-HA-VN/04 vaccine preparation proved to be highly effective in inducing protective immunity in primates against homologous and heterologous H5N1 influenza viruses. The reduction of viral shedding from the lower and upper respiratory tract may reduce the risk of human-to-human transmission and may therefore limit the viral spread in the population if this vaccine is used in humans to reduce the impact of the pandemic [326, 327].

The MVA-HA-VN/04 immunization was very well tolerated. Measurement of the body temperature of the animals was not only used as a clinical outcome of the challenge infection, it also allowed monitoring systemic reactions upon vaccination. After MVA-HA-VN/04 or wtMVA vaccination no rise in body temperature was observed in any of the vaccinated animals. This is in concordance with the excellent safety profile of (recombinant) MVA in humans [183]. This safety profile also extends to immune compromised subjects since it has been shown that MVA does not replicate in severly immuno-suppressed macaques [187].

The presence of pre-existing anti-vector immunity may not be a major concern for the efficacy of MVA-HA-VN/04 vaccine although this remains to be demonstrated. In mice, MVA vaccine efficacy was hardly affected by pre-existing immunity [185]. More importantly, repeated administrations of a recombinant MVA vaccine to humans boosted specific immune responses directed against the recombinant antigen 5T4 [182]. Therefore, it can be envisaged that immunization of individuals that received smallpox vaccination in the past, and repeated applications of MVA-HA-VN/04 should be feasible.

In combination with other favourable properties such as good stability, which would allow stock-piling of the vaccine, rapid and easy production at large scale under BSL-1 conditions makes recombinant MVA-HA an attractive and promising candidate as a pandemic influenza virus vaccine. Based on the data presented here, further clinical development of MVA-HA vaccines seems warranted.

Acknowledgements
The authors wish to thank Wim Vos, Jan Staal, Nico Schmidt, Rogier Bodewes, Chantal Baas and Theo Bestebroer for outstanding technical assistance.



CHAPTER 8

MVA-based H5N1 vaccine affords cross-clade protection against influenza A/H5N1 viruses at low doses and after single immunization

J.H.C.M. Kreijtz[1], Y. Suezer[2], G. de Mutsert[1], G. van Amerongen[1], A. Schwantes[2], J.M.A. van den Brand[1], R.A.M. Fouchier[1], J. Löwer[2], A.D.M.E. Osterhaus[1], G. Sutter[2,3], G.F. Rimmelzwaan[1]

[1]Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands. [2]Paul-Ehrlich-Institut, Langen, Germany. [3]Ludwig-Maximilians-Universität, München, Germany

Submitted for publication

Abstract

Human infections with highly pathogenic avian influenza viruses of the H5N1 subtype, frequently reported since 2003, result in high morbidity and mortality. It is feared that these viruses become pandemic, therefore the development of safe and effective vaccines is desirable. MVA-based H5N1 vaccines already proved to be effective when two immunizations with high doses were used.

Dose-sparing strategies would increase the number of people that can be vaccinated when the amount of vaccine preparations that can be produced is limited. Furthermore, protective immunity is induced ideally after a single immunization. Therefore the minimal requirements for induction of protective immunity with a MVA-based H5N1 vaccine were assessed in mice. To this end, mice were vaccinated once or twice with descending doses of a recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04. The protective efficacy was determined after challenge infection with the homologous clade 1 virus and a heterologous virus derived from clade 2.1.

It was concluded that MVA-based vaccines allowed significant dose-sparing and afford cross-clade protection, also after a single immunization, which are favorable properties for an H5N1 vaccine candidate.

Introduction

Over 400 human cases of infections with highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype have been reported since 2003. More than 60% of these cases had a fatal outcome and new cases continue to be reported frequently.[32] Once these viruses become transmittable from human-to-human by adaption to their new host, a new influenza pandemic is imminent. Neutralizing antibodies against H5N1 viruses are virtually absent in the human population and already nine different clades of antigenically distinct viruses have been identified.[52] Therefore, the development of safe and effective vaccines that, ideally, induce cross-clade immunity has high priority.[52, 318, 319] The implementation of reverse genetics for the generation of vaccine strains and cell culture technology contribute to the rapid availability of pandemic influenza vaccines.[98, 100, 106, 113, 116, 117, 124, 315-317] In addition, the use of adjuvants can increase the immunogenicity of seasonal and pandemic influenza vaccines and may lower the amount of antigen needed for the induction of protective antibody responses.[78, 139, 142, 152, 158]

The development of alternative novel generations of influenza vaccines may mitigate the envisaged shortage of vaccine doses in the future. For example, vector vaccines based on recombinant adenovirus or poxvirus expressing selected influenza virus genes have been shown to be immunogenic and to afford protection against infection with H5N1 virus in animal models.[167, 176, 177, 190, 251, 297, 328] Especially the replication-deficient modified Vaccinia virus Ankara (MVA), constitutes an attractive vaccine production platform. This virus was originally developed as a vaccine against small pox and has been administered to >120.000 humans without significant side effects.[183] In addition, administration of MVA to immunocompromised individuals is safe and does not lead to systemic disease often associated with the application of replicating vaccinia virus.[187, 300] Its potential as vaccine candidate has been demonstrated for a number of infectious pathogens (for review see Rimmelzwaan and Sutter).[329] Recently, we have demonstrated that immunization with a recombinant MVA expressing the HA gene of influenza H5N1 virus A/Vietnam/1194/04 (MVA-HA-VN/04) induced protective immunity against infection with the homologous and a heterologous antigenically distinct virus in mice and macaques.[251, 328] In these studies animals were immunized twice with relative high doses (>10^8 pfu) of recombinant MVA. However, to stretch the number of individuals that can be vaccinated with any given amount of vaccine preparation that can be produced it would be desirable if dose-sparing can be

achieved. Furthermore, when a pandemic is imminent, there might not be enough time to induce protective immunity with a two-dose immunization regimen. Thus, ideally, protective immunity is induced after immunization with lower doses and preferable after a single immunization, which are key elements in the development of pandemic influenza vaccines. In the present study, we determined the minimal requirements for the induction of protective immunity with MVA-HA-VN/04 against the homologous virus and against an antigenically distinct H5N1 strain.

Two immunizations with MVA-HA-VN/04 at doses 10,000-fold lower than used previously [251] significantly reduced weight loss and mortality caused by challenge infection with influenza viruses A/Vietnam/1194/04 (clade 1) and A/Indonesia/5/05 (clade 2.1). Strikingly, also protection against the development of clinical signs and mortality was achieved with a single immunization with 10^5 pfu of MVA-HA-VN/04. The clinical protection correlated with a reduction of virus replication and lung pathology.

Thus, apart from the favorable properties already attributed to recombinant MVA [329], the possibilities of dose sparing and single shot immunization regimens makes this vector even more attractive as a pandemic influenza vaccine candidate.

Material & Methods

Vaccine preparation

Recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) was prepared as described previously.[251] MVA clonal isolate F6 served as the parental MVA virus. To generate final vaccine preparations, the virus was amplified in chicken embryo fibroblasts (CEF), purified by ultracentrifugation through sucrose, reconstituted in 1mM Tris-HCL pH 9.0 and diluted in PBS.

Viruses

Influenza viruses A/Vietnam/1194/04 (A/VN/1194/04) and A/Indonesia/5/05 (A/IND/5/05) were cultured in Madin Darby Canine Kidney (MDCK) cells. Infectious virus titers were determined in MDCK cells as described previously.[228]

Animals

Female specified pathogen free 6-8 weeks old C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany) and were age-matched at the time point of the first immunization. Mice were immunized once with MVA-HA-VN/04 at a dose of

10^3, 10^4, 10^5, 10^6, or 10^8 pfu in a volume of 100ml intramuscularly in the hind legs (20 mice per dose). A second group of animals was immunized twice with MVA-HA-VN/04 at a dose of 10^3, 10^4, 10^5, or 10^6 pfu (20 mice per dose). For the control groups mice were immunized with wildtype MVA (wtMVA) (10^6 (two shot) or 10^8 pfu (single shot)) (n=60) or PBS (n=56).

Four weeks after the last immunization blood was drawn from the animals and they were infected with 10^3 $TCID_{50}$ of the homologous influenza virus A/VN/1194/04 or 10^3 $TCID_{50}$ of the heterologous strain influenza virus A/IND/05/05. Virus was inoculated intranasally in a volume of 50ml and the challenge dose was chosen since it resulted in a lethal infection in >90% mice reproducibly (data not shown). Four and fourteen days after challenge infection mice were euthanized and their lungs were resected. Blood sampling, the intranasal infection, and euthanasia were carried out under anesthesia with inhalative isoflurane. The animals were housed in individual ventilated cage units (IVC-units) and had access to food and water *ad libitum*. During the infection with the influenza A/H5N1 viruses, animals were housed in type 3 cages placed in bio-safety level 3 containment facilities. The experimental protocol was approved by an independent animal ethics committee and the experiments were conducted according to national and international guidelines.

Serology

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 (HI) assay was used following a standard protocol using 1% turkey erythrocytes and four HA-units of influenza virus A/VN/1194/04 and A/IND/5/05. [229] For this purpose reverse genetics viruses were produced from which the basic cleavage site in the HA molecule was deleted. The antibody titers obtained with these viruses were comparable with those obtained with the wild type strains (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 the viruses that were produced by reverse genetics as described above [230]. In brief, 50µl volumes of serial diluted serum samples were incubated with 100 $TCID_{50}$ of the viruses for one hour at 37°C and then the mixture was added to MDCK cells. After one hour, the cells were washed and subsequently cultured in Eagles Minimal Essential Medium containing bovine serum albumin (BSA, fraction V 0.3%), 4mg/ml trypsin, L-glutamin 2mM, penicillin 100U/ml, streptomycin 100mg/ml $NaHCO_3$

0.15%, Hepes 20mM and non-essential amino acids 0.1 mM. After five days, residual virus replication was assessed by measuring HA activity in the culture supernatants. Hyper-immune serum obtained from a swan immunized twice with inactivated H5N2 influenza virus A/Duck/Potsdam/1402/86 (Nobilis influenza® H5N2 Intervet International, Boxmeer, the Netherlands) was used as a positive control against the two influenza viruses. For calculation purposes serum samples with an antibody titer of <10 were arbitrarily assigned a titer of 5.

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), lactalbumin, glycerol, penicillin, streptomycin, polymyxin B, nystatin, gentamicin) 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.[228]

Histopathology and immunohistochemistry

Formalin-inflated lungs were fixed in 10% neutral buffered formalin and then cross-sections were made and embedded in paraffin, sectioned at 4 mm 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 nuclei of influenza A virus-infected cells and a less intense red-staining of their cytoplasm. The sections were counterstained with hematoxylin.

Statistical analysis

Data for weight loss and viral titers were analyzed using the two-sided Student's *t* test and differences were considered significant at $P<0.05$.

Results

Antibody responses induced by immunization with MVA-HA-VN/04

After a single immunization with MVA-HA-VN/04, only mice that received a dose of 10^6 or 10^8 pfu developed detectable antibody titers (Table 1). Four weeks after immunization these animals had HI geometric mean titers (GMT) of 6.8 (SD=1.8) and 15.7 (SD=2.6) against the homologous virus (A/VN/1194/04) and 5.5 (SD=1.4) and 6.1 (SD=1.8) against the heterologous virus (A/IND/5/05), respectively. As shown in table 1, also virus-neutralizing antibodies were detected after a single immunization with 10^6 or 10^8 pfu with GMT 5.4 (SD=1.4) and 5.7 (SD=1.5) against the homologous strain respectively. Only mice immunized with 10^8 pfu of MVA-HA-VN/04 developed virus neutralizing antibody titers against the heterologous strain. Mice that received two immunizations with 10^3, 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04 developed HI GMT of 6.3 (SD=2.1), 16.2 (SD=3.8), 77.1 (SD=4.2) and 71.9 (3.3) respectively against the homologous strain. Those that received 10^5 and 10^6 pfu also developed detectable HI antibodies against the influenza virus A/IND/5/05 with GMT 7.7 (SD=2.6) and 7.7 (SD=2.3). In the VN assay, antibodies against the homologous strain were detected with GMT of 5.5 (SD=1.6), 18.0 (SD=4.4) and 15.2 (SD=3.9) in mice immunized with 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04, respectively. The mice that were immunized twice with 10^5 and 10^6 pfu developed virus-neutralizing antibodies against the heterologous strain with GMT of 7.5 (SD=2.9) and 6.2 (SD=2.0).

Table 1: Antibody titers[1] after immunization with MVA-HA-VN/04

HI	single shot		two shot			
	10^6	10^8	10^3	10^4	10^5	10^6
A/VN/1194/04	6.8 (1.8)	15.7 (2.6)	6.3 (2.1)	16.2 (3.8)	77.1 (4.2)	71.9 (3.3)
A/IND/5/05	5.5 (1.4)	6.1 (1.8)	-	-	7.7 (2.6)	7.7 (2.3)

VN	single shot		two shot			
	10^6	10^8	10^3	10^4	10^5	10^6
A/VN/1194/04	5.4 (1.4)	5.7 (1.5)	-	5.5 (1.6)	18.0 (4.4)	15.2 (3.9)
A/IND/5/05	-	5.4 (1.4)	-	-	7.5 (2.9)	6.2 (2.0)

[1]titers are expressed as GMT (SD)

Protection against clinical signs after infection with influenza A/H5N1 virus

From two to three days p.i. onwards, unprotected control animals started to develop clinical signs, irrespective of the challenge virus that was used, although infection with influenza A/IND/5/05 caused more severe disease. Mice that received PBS or wtMVA

once or twice displayed reduced muscle strength, and around day 4 p.i. hunched back posture and heavy breathing. A similar clinical presentation was observed in mice that received one or two immunizations with 10^3 pfu MVA-HA-VN/04 and that were subsequently infected with influenza virus A/IND/5/05. These mice eventually succumbed from infection or had to be taken out of the experiment because they reached humane endpoints. Mice that received a single immunization with 10^4 or 10^5 pfu MVA-HA-VN/04, and those vaccinated twice with 10^3 pfu, developed mild clinical signs after infection with the homologous influenza virus A/VN/1194/04. Also mice vaccinated twice with 10^4 pfu but infected with the heterologous strain A/IND/5/05 had a mild clinical outcome of infection and recovered from infection. Mice vaccinated once with 10^6 or 10^8 pfu and those vaccinated twice with 10^5 or 10^6 pfu did not show any clinical signs after infection regardless the virus that was used for infection. In general, the severity of the clinical signs correlated with the extent of weight loss. Mice vaccinated once with doses > 10^5 pfu did not loose weight after infection with influenza virus A/VN/04 and fully recovered (Figure 1). After challenge infection with A/IND/5/05 some weight loss was observed, but it was limited considerably compared to control mice or those vaccinated with doses of

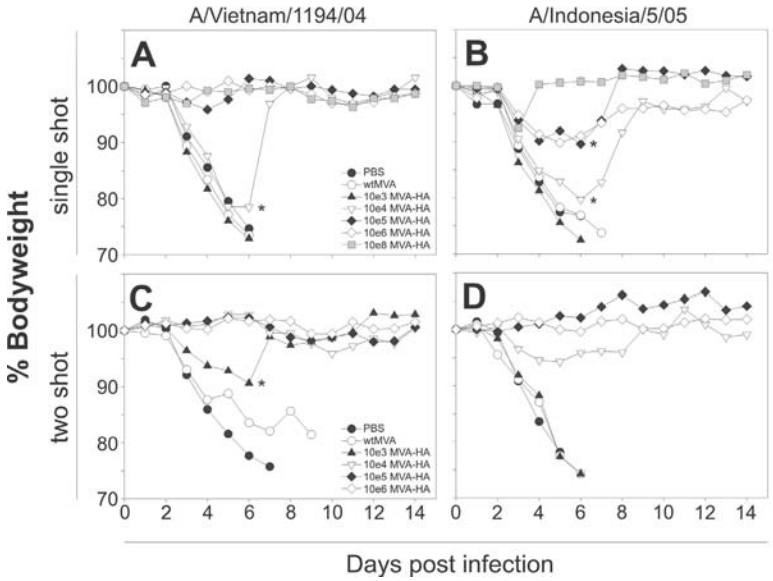


Figure 1 Bodyweight after infection with influenza virus A/Vietnam/1194/04 (A, C) and influenza virus A/Indonesia/5/05 (B, D). Animals were infected four weeks after a single immunization (A, B) with: PBS, wtMVA, 10^3, 10^4, 10^5, 10^6 or 10^8 pfu of MVA-HA-VN/04. A second group of animals was infected four weeks after two immunizations (C, D) with PBS, wtMVA, 10^3, 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04. (*Indicates weight loss of the proportion of animals in this group that survived post day 6 infection)

<10^5 pfu MVA-HA-VN/04. Two vaccinations with doses as low as 10^4 pfu of MVA-HA-VN/04 fully protected mice from weight loss after infection with the homologous strain. Even two vaccinations with 10^3 pfu prevented severe weight loss observed in PBS control mice and those vaccinated with 10^8 pfu of the empty vector. Two vaccinations with doses >10^4 pfu also protected mice from severe weight loss after infection with influenza virus A/IND/5/05

Table 2: Survival after influenza A/H5N1 infection in single shot immunized mice

		controls		MVA-HA-VN/04[1]				
		PBS	wtMVA	10^3	10^4	10^5	10^6	10^8
single shot	A/Vietnam/1194/04	0/4	0/4	0/4	1/4	4/4	4/4	4/4
	A/Indonesia/5/05	0/4	0/4	0/4	1/4	2/4	4/4	4/4

		controls		MVA-HA-VN/04[1]			
		PBS	wtMVA	10^3	10^4	10^5	10^6
two shot	A/Vietnam/1194/04	0/4	0/4	2/3[2]	4/4	4/4	4/4
	A/Indonesia/5/05	0/4	0/4	0/4	4/4	4/4	4/4

[1]Dose of MVA-HA-VN/04 in pfu (immunization in a total volume of 100µl)
[2]One animal had to be euthanized before infection due to a complication unrelated to the experiment

Survival after infection with influenza A/H5N1 virus

Mice that developed severe clinical signs after H5N1 infection and that showed weight loss of more than 20% were euthanized for ethical reasons. Mice that received a single shot of PBS, wtMVA or the lowest dose of MVA-HA-VN/04 (10^3 pfu) did not survive past day 7 p.i. with influenza virus A/VN/1194/04 and A/IND/5/05, and most of them reached humane endpoints six days p.i. (Table 2). Mice immunized once with 10^4 pfu MVA-HA-VN/04 had a survival rate of 25% after infection with both the homologous and heterologous virus. A single vaccination with 10^5 pfu MVA-HA-VN/04 resulted in 100% survival after infection with the homologous virus and 50% after infection with influenza virus A/IND/5/05. A single vaccination with a dose of >10^6 pfu MVA-HA-VN/04 prevented mortality caused by infection with both viruses. Two immunizations with 10^3 pfu MVA-HA-VN/04 protected 67% of mice from death caused by infection with A/VN/04, but not that caused by infection with the heterologous strain A/IND/5/05 (Table 2). Two immunizations with a dose >10^4 pfu of MVA-HA-VN/04, protected mice completely against mortality caused by infection with both influenza viruses.

MVA-HA-VN/04 vaccination reduces virus replication in the lungs

After one vaccination

Lungs were tested for the presence of infectious virus on day 4 post infection (p.i.). After infection with influenza virus A/VN/1194/04 of mice vaccinated with PBS or wtMV, the mean virus titers were $10^{8.3}$ (SD=$10^{0.2}$) and $10^{7.9}$ (SD=$10^{0.5}$), respectively (Figure 2A). These titers were significantly higher than that of mice that were vaccinated with MVA-HA-VN/04 at a doses of 10^4, 10^5, 10^6 or 10^8 pfu ($p<0.05$). The mean virus titer in mice immunized once with 10^3 pfu of MVA-HA-VN/04 was $10^{7.5}$ (SD=$10^{0.2}$) which was still significantly lower than that of mice that received PBS ($p<0.05$). Four days post infection with influenza virus A/IND/5/05 the mean virus titers in the PBS vaccinated and wtMVA vaccinated mice were $10^{8.5}$ (SD=$10^{0.6}$) and $10^{8.7}$ (SD=$10^{0.1}$) respectively (Figure 2B). Mice vaccinated with a dose of 10^3 pfu MVA-HA-VN/04 had similar mean virus titer of $10^{8.5}$ (SD=$10^{0.8}$). Vaccination with higher doses of MVA-HA-VN/04 resulted in lower lung virus titers. The extent of virus replication was vaccine dose dependent. The mean A/IND/5/05 virus titers for mice vaccinated with 10^4, 10^5, 10^6 or 10^8 pfu were $10^{7.6}$ (SD=$10^{0.6}$), $10^{6.7}$ (SD=$10^{1.4}$), $10^{7.1}$ (SD=$10^{0.6}$) and $10^{3.7}$ (SD=$10^{2.8}$), respectively.

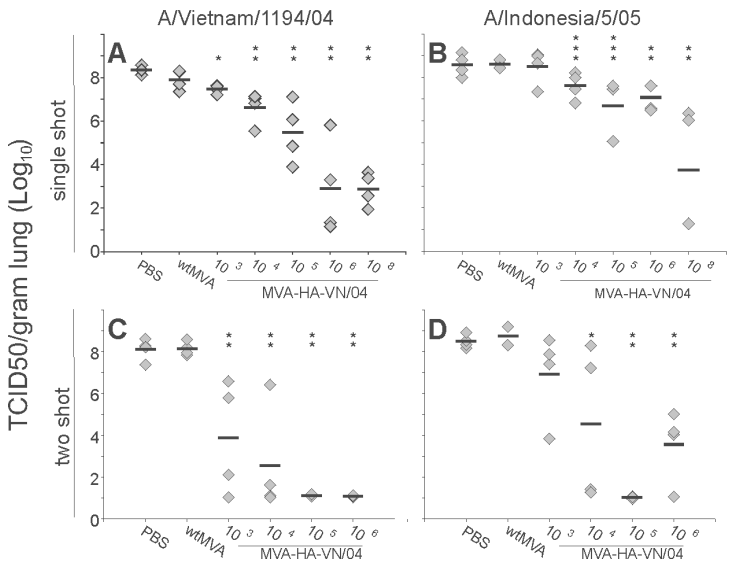


Figure 2 Virus replication in the lungs on day 4 post infection with influenza virus A/Vietnam/1194/04 and A/Indonesia/5/05 in mice that received one (A, B) or two (C, D) immunization(s) of: PBS, wtMVA or 10^3, 10^4, 10^5, 10^6 or 10^8 (single shot only) pfu of MVA-HA-VN/04.(* indicates a statistical significant difference with the PBS immunized group ($p<0.05$) (** indicates a significant difference with both the PBS and wtMVA immunized group ($p<0.05$)(*** indicates a statistical significant difference with the wtMVA immunized group).

After two vaccinations

As shown in figure 2C, two immunizations with MVA-HA-VN/04 resulted in significant lower lung virus titers four days after infection with the homologous virus A/VN/1194/04 compared to the PBS or empty vector inoculated mice, regardless the vaccine dose that was used. In mice vaccinated with vaccine doses > 10^5 , infectious virus was not detected. Four days p.i. with influenza virus A/IND/5/05 infectious virus could not be detected in the lungs of mice that were vaccinated twice with 10^5 pfu of MVA-HA-VN/04. In mice vaccinated twice with 10^6 pfu the mean virus titer in the lungs was $10^{3.6}$ (SD=$10^{1.7}$), which was significantly lower than that in the PBS and wtMVA immunized control mice which had mean titers of $10^{8.5}$ (SD=$10^{0.3}$) and $10^{8.8}$ (SD=$10^{0.6}$) respectively (Figure 2D). Also vaccination with a dose of 10^4 pfu of MVA-HA-VN/04 significantly reduced the virus titers of A/IND/05 compared to PBS control mice.

Vaccination prevents histopathological changes in the lungs after influenza A/H5N1 infection

Upon infection with influenza viruses A/VN/1194/04 and A/IND/5/05, unprotected control mice inoculated with PBS or empty vector developed a moderate to severe broncho-interstitial pneumonia within four days (Figure 3). Histopathological changes were located in multifocal to coalescing lesions with more than 50% of the lungs affected. The lesions were characterized by marked inflammatory peribronchiolar lymphocytic infiltrates and occasionally proteinaceous fluid. There was necrosis in the bronchiolar epithelium resulting in cellular debri in the lumen. All these histopathological changes were located in multifocal to coalescing lesions with more than 50% of the lung affected. Similar lesions were observed in mice vaccinated with 10^3 pfu of MVA-HA-VN/04. Mice that were immunized once with 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04, or twice with 10^4 pfu were partially protected against homologous and heterologous challenge infection. They displayed moderate changes in the lung: moderate peribronchiolar lymphocytic infiltrate and mild necrosis in the bronchiolar walls (Figure 3). Fourteen days p.i. normal architecture of the lung was restored in animals from these groups, apart from some residual peribronchiolar lymphocytic infiltrate and mild hyperplasia and hypertrophy of the bronchiolar and alveolar epithelium, consistent with regeneration. Mice vaccinated once with 10^8 pfu of MVA-HA-VN/04 or twice with >10^5 pfu displayed virtually no histopathological changes in the lung four days p.i. (Figure 3) or at later time points

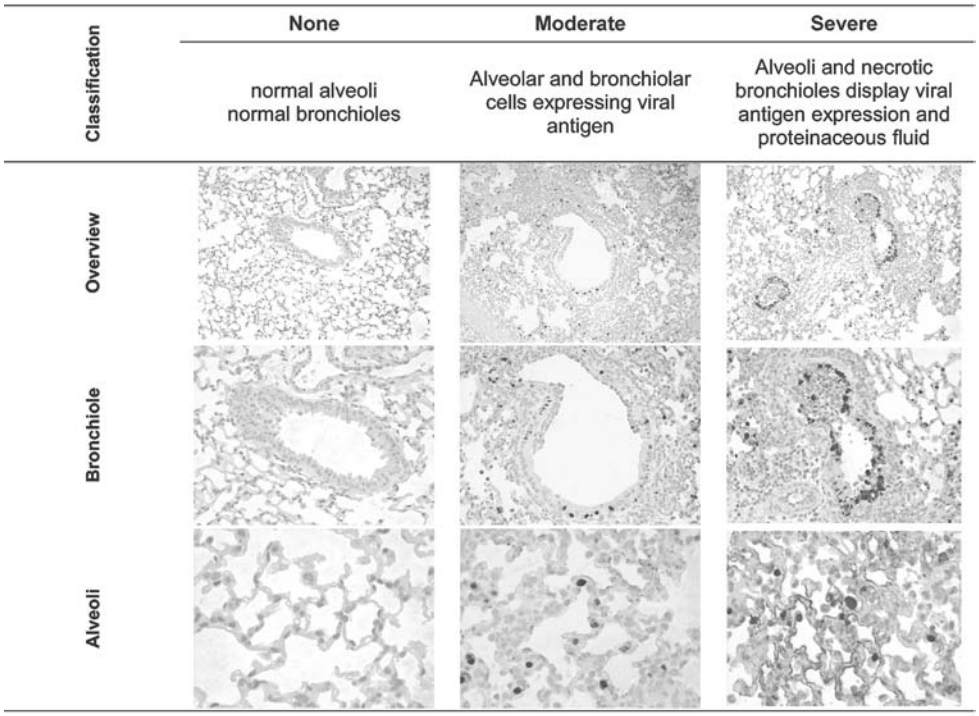
p.i. with influenza viruses A/VN/1194/04 or A/IND/5/05.

Detection of virus-infected cells by immuno-histochemistry

The presence of influenza virus-infected cells in the lungs was detected using a monoclonal antibody against the viral nucleoprotein, resulting in a red-brown precipitate in the nucleus and less in the cytoplasm. Four days p.i. with influenza virus A/VN/1194/04 infected cells were abundantly present in the lungs of control mice that received PBS or empty vector once or twice or mice vaccinated with 10^3 pfu of MVA-HA-VN/04. Also after infection with influenza virus A/IND/5/05, virus-infected cells were abundantly present in the lungs of the control mice. A single vaccination with 10^3, 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04 did not prevent replication of influenza virus A/IND/05. The antigen-expressing cells were epithelial cells in the alveolar wall (type I and type II like pneumocytes) and bronchiolar epithelial cells in most of the bronchiolar walls (Figure 3). Mice vaccinated with 10^4, 10^5 or 10^6 pfu of MVA-HA-VN/04 once or twice with 10^3 pfu were partially protected against the homologous virus and had virus-infected cells, predominantly type II like pneumocytes, at multiple foci in their lungs (Figure 3). A few single infected cells were detected in the lungs of animals that had received a single immunization with 10^8 pfu MVA-HA-VN/04 and virus-infected cells were virtually absent in animals that had received two immunizations with $>10^5$ pfu of MVA-HA-VN/04 (Figure 3). No virus was detectable in any of the animals fourteen days after infection.

Discussion

In the present study, the minimal requirements were assessed for the induction of protective immunity in mice against antigenically distinct influenza A/H5N1 viruses with a recombinant MVA expressing the HA gene of a clade 1 influenza A/H5N1virus. Two immunizations with a dose as low as 10^4 pfu of MVA-HA-VN/04 were sufficient for the induction of protective immunity not only against the homologous strain but also against the antigenically distinct strain A/IND/5/05 from clade 2.1.1. A dose of 10^4 pfu is 10.000 fold lower than the dose of 10^8 pfu that was used in previous studies that demonstrated the protective potential of MVA-HA-VN/04 vaccine candidate in mice and macaques.[251, 328] Thus, substantial less vaccine preparation is needed for the induction of protective immunity in mice. The possibility of dose-sparing would increase the number of individuals that can be vaccinated, with any amount of vaccine preparation, considerably.



		None		Moderate		Severe	
	Challenge virus	Vaccine	Dose	Vaccine	Dose	Vaccine	Dose
One immunization	VN/04	MVA-HA-VN/04	10^8	MVA-HA-VN/04	10^4 10^5 10^6	PBS wtMVA MVA-HA-VN/04	n.a. 10^8 10^3
	IND/05	MVA-HA-VN/04	10^8	MVA-HA-VN/04	10^4 10^5 10^6	PBS wtMVA MVA-HA-VN/04	n.a. 10^8 10^3
Two immunizations	VN/04	MVA-HA-VN/04	10^5 10^6	MVA-HA-VN/04	10^4	PBS wtMVA MVA-HA-VN/04	n.a. 10^6 10^3
	IND/05	MVA-HA-VN/04	10^5 10^6	MVA-HA-VN/04	10^4	PBS wtMVA MVA-HA-VN/04	n.a 10^6 10^3

Figure 3 Histopathological changes and immunohistochemistry of the lungs after infection with influenza A/H5N1 virus. Representative pictures were selected for the different classifications. Magnification: overview (10x), bronchiole (20x), alveoli (40x). (full colour figure: APPENDIX XI)

It was indicated on the website of a manufacturer of MVA based vaccines (www.bavarian-nordic.com) that the production capacity would range in tens of millions of doses, assuming a dose of 10^8 pfu. If it can be confirmed that also in humans a dose of 10^4 pfu is still effective, enough vaccine doses can be made for a global vaccination campaign. Thus the problem of the envisaged shortage of pandemic

influenza vaccines could be addressed with the use of the recombinant MVA technology. Another important issue that complicates the development of effective H5N1 vaccines is the co-circulation of antigenically distinct viruses.

At present, nine different clades of A/H5N1 viruses have been identified and ideally vaccines will induce protective immunity against multiple clades of these viruses. Two immunizations with MVA-HA-VN/04 afforded cross-clade protection. It should be noted that also protective effects were observed with low doses MVA-HA-VN/04 in the absence of detectable antibody responses specific for the two viruses used for challenge infection. It is possible that when low doses of vaccine are used antibody responses are induced below the detection limit, but which still afford some level of protection. Alternatively, it is possible that vaccination with low doses MVA-HA-VN/04 primed for secondary antibody responses. With higher doses of MVA-HA-VN/04 detectable antibodies were induced which indicated that the magnitude of the antibody responses is dependent on the vaccine dose.
In our mouse model, vaccination with two doses of $\geq 10^4$ pfu of MVA-HA-VN/04 reduced virus replication upon challenge infection significantly, which correlated with a reduction of histopathological changes in the lung and a reduction in the presentation of clinical signs, such as weight loss. Only with a high dose of MVA-HA-VN/04 ($\geq 10^5$ pfu) sterilizing immunity was induced.
When a pandemic is imminent the rapid induction of protective immunity by vaccination is desirable and may prevent morbidity and mortality in selected population groups like health care workers or those at high risk for complications associated with infection with influenza viruses. Under these circumstances the instant induction of protective virus-specific immune responses by a single immunization without the need for a booster vaccination would be ideal. In the present study, we showed that a single immunization with MVA-HA-VN/04 protected mice from severe disease caused by infection with the homologous strain or the A/Indonesia/5/05 strain, especially when a high dose (10^8 pfu) was used. However, also vaccination with lower doses in the range of 10^5-10^6 pfu afforded clinical protection, in particular against the homologous strain. In contrast to two immunizations, it was not possible to induce sterilizing immunity after a single immunization with MVA-HA-VN/04. Collectively, we conclude that in addition to well established favorable properties of MVA based vaccines such as superior safety, its good stability allowing stock-piling, high expression of genes of interest and good immunogenicity

without the use of adjuvants, they also allow dose sparing. Two immunizations with relatively low doses of MVA-HA-VN/04 induced protective immunity against H5N1 viruses derived from different antigenically distinct clades. This vaccination strategy would be attractive for prepandemic vaccination, when there is still enough time for prime-boost regimens. Since there could be uncertainty about the strain that ultimately would cause a pandemic, the possibility to induce cross-clade immunity may afford broad protective immunity against a variety of different strains. When the induction of protective immunity becomes more urgent, a single immunization with a high dose might afford rapid protection against infection with the emerging pandemic strain. Of course the minimal requirements for the induction of protective immunity by MVA-HA-VN/04 vaccination need to be confirmed in humans. However, the potential of recombinant MVA-H5 vaccine was confirmed in non-human primates [328] and therefore we anticipate that also in humans dose sparing and single shot regimens are feasible. In this respect the presence of anti-vector immunity is considered to be a potential draw back of MVA based vaccines. However, since MVA is fully replication deficient, pre-existing immunity is unlikely to affect the immunogenicity of these vector vaccines.[182, 185] Thus recombinant MVA is promising as a H5N1 vaccine candidate, but of course this technology can be applied to other subtypes of influenza viruses as well. For example, it would be of interest to evaluate its potential as candidate vaccine against the pandemic influenza A/H1N1 virus that spread worldwide within two months, causing more than 52,000 reported cases, including over 231 deaths as of June 22nd 2009.[52]

Acknowledgements

The authors would like to thank Rogier Bodewes, Eefje Schrauwen, Vincent Munster, Peter van Run, Yolanda Martinez-Fernandez and Stella van Trierum for outstanding technical assistance.


SUMMARIZING
DISCUSSION

Part I: CTL and heterosubtypic immunity

Seasonal influenza A viruses of the H1N1 and H3N2 subtype cause epidemic outbreaks annually. In addition, new subtypes originating from other species are introduced in the human population sporadically, which may spark a pandemic. Currently there are two novel viruses, emerging from birds and swine, that form a threat to public health. First, highly pathogenic avian influenza A/H5N1 viruses are transmitted from birds frequently since 2003, predominantly in South-East Asia. Second, swine influenza A/H1N1 virus that originated from Mexico spread worldwide within two months after the first human cases were reported in April 2009 and resulted in the first influenza pandemic of the 21st century.[51] Antibodies against these two viruses are virtually absent in the human population but cross-reactive pre-existing virus-specific T cells may contribute to protective heterosubtypic immunity.

Heterosubtypic immunity is induced after previous infection(s) with influenza A virus of the H1N1 or H3N2 subtype and can provide a certain level of protection against subsequent infection with an influenza virus of an alternative subtype. In chapter 2, we demonstrated this in a mouse model using two viruses of different subtypes that have identical internal proteins and thus also share the NP-gene, containing the immunodominant CTL epitope $NP_{366-374}$, restricted by H2-D^b. CTL responses against this epitope were monitored using H2-D^b/$NP_{366-374}$ tetramerstaining. C57BL/6J mice (H2-D^b) were infected with influenza A virus X-31 (H3N2) and subsequently with influenza virus A/PR/8/34 (H1N1) that is lethal to mice. X-31 primed animals were protected against the A/PR/8/34 infection, in the absence of virus-neutralizing antibodies. Protection correlated with a significant increase in tetramer-positive CD8+ CTL after challenge infection. To assess the effectiveness of heterosubtypic immunity induced by infection with seasonal influenza virus against highly pathogenic A/H5N1 viruses a second study was performed using more relevant virus strains. In this study, mice were primed with human influenza virus A/HK/2/68 (H3N2) and four weeks later they were challenged with a lethal dose of influenza virus A/IND/5/05 (H5N1) as was described in chapter 3. Although primed animals developed clinical signs, the virus was cleared from their lungs and they survived the H5N1 infection. An expansion of $NP_{366-374}$ epitope specific CD8+ CTL that cross-reacted with the H5N1 $NP_{366-374}$ epitope variant was seen in the protected animals after challenge infection with the H5N1 strain. Adoptive transfer of serum from H3N2-infected animals to naïve mice did not confer protection against subsequent H5N1 infection (data not shown).

Because effective vaccination prevents influenza virus infection it might also interfere with the induction of virus-specific T cells and heterosubtypic immunity. In chapter 4 we demonstrated in a mouse model that vaccination against human influenza A/H3N2 virus prevented the induction of (cross-reactive) virus-specific CTL otherwise induced after infection with that virus. Prevention of the induction of a heterosubtypic immune response by prior vaccination against the H3N2 virus resulted in the loss of protection against secondary infection with HPAI A/H5N1 virus. The three studies described above demonstrate that protection based on heterosubtypic immunity correlates with the expansion of cross-reactive virus-specific CTL. In chapter 5 it was shown that human virus-specific T cells, induced by previous infection(s) with seasonal influenza A virus cross-reacted with HPAI A/H5N1 virus to a great extent. Thus, humans previously infected with seasonal influenza A viruses possess influenza specific CTL that cross-react with heterosubtypic influenza virus strains like H5N1 [72, 73], which may afford a certain degree of protection against infection with these viruses. Vaccination against seasonal influenza A viruses may interfere with the induction of heterosubtypic immunity as we demonstrated in chapter 4. Therefore, the recommendation to vaccinate all healthy children 6-59 months of age in the USA and some other countries should be reconsidered in the light of these findings. Annual vaccination against seasonal influenza may render subjects in this age group immunological naïve with regard to their T cell immunity and more susceptible to infection with pandemic viruses of other subtypes.

In humans, the correlation of pre-existing CTL responses with heterosubtypic immunity was demonstrated by McMichael et al.[67] More circumstantial evidence was provided by Epstein et al who demonstrated that subjects who experienced an H1N1 infection before 1957 less likely developed severe influenza during the 1957 H2N2 pandemic.[71] The disproportionate age distribution of HPAI A/H5N1 and new influenza A/H1N1 cases also suggests that pre-existing immunity to influenza viruses may contribute to protection against infection with an influenza A virus of a novel subtype.[259, 330] Young subjects were more at risk to develop severe disease, which may be attributed to the fact that this age group less likely has been exposed to seasonal influenza A viruses and thus have not mounted a (cross-reactive) CTL response to a novel subtype, although other confounding factors can not be excluded.

Ideally, also vaccines induce this type of broad protective heterosubtypic immunity. Thus, the development of vaccines that aim at the induction of cross-reactive CTL

responses against conserved viral antigens may be an attractive approach for the induction of protective immunity against a variety of influenza A virus subtypes as discussed in chapter 1.[247, 331, 332]

Part II: MVA-based H5N1 influenza vaccines

In the light of the current pandemic threats there is a need for safe and effective vaccines that can be produced rapidly at large scale. MVA-based vaccines fulfil all these criteria and are attractive pandemic influenza vaccine candidates.[329] Therefore, we evaluated recombinant MVA expressing the HA genes of HPAI H5N1 virus A/Hongkong/156/97 (MVA-HA-HK/97) and A/Vietnam/1194/04 (MVA-HA-VN/04) in mice first (chapter 6). Mice were immunized and subsequently challenged with HPAI H5N1 viruses derived from antigenically distinct clades: A/Hongkong/156/97 (clade 0), A/Vietnam/1194/04 (clade 1) or A/Indonesia/5/05 (clade 2.1). Vaccination with MVA-HA-HK/97 afforded protection against the homologous virus only, whereas immunization with MVA-HA-VN/04 provided protection against all three H5N1 viruses.

Since promising results were obtained in mice it was decided to further evaluate the most promising vaccine candidate, MVA-HA-VN/04, in non-human primates. To this end, cynomolgus macaques were immunized and subsequently challenged with HPAI H5N1 viruses A/Vietnam/1194/04 or A/Indonesia/5/05. As described in chapter 7, immunization with MVA-HA-VN/04 induced protective immunity against infection with the homologous and the heterologous virus.

For the studies described in chapters 6 and 7, high vaccine doses (10^8 pfu) were used for immunization. The feasibility of dose-sparing and single shot vaccination regimens was explored in chapter 8. Dose-finding indicated that the administration of two doses of 10^4 pfu of MVA-HA-VN/04 was immunogenic and afforded protection against infection with the homologous strain and the antigenically distinct A/IND/5/05 strain. In addition, similar results were obtained after a single immunization with 10^8 pfu. Thus, the use of recombinant MVA-HA is a promising approach in situations in which urgent induction of protective immunity is needed e.g. during a pandemic outbreak. However, when insufficient vaccine doses are available for a global vaccination campaign with a high dose single shot regimen, it may be decided to give two immunizations with a low dose, owing that there is enough time to allow protective immunity to develop. The minimal requirements for the induction of protective immunity in humans with MVA-HA-VN/04 need to

be assessed in clinical trials. The seed vaccine strain lots then need to be prepared in cells that are certified for vaccine production under GMP conditions. The vaccine production in CEF cells also has to be optimized or be adapted to a continuous cell line.[329] Thus the use of MVA as a platform for the production of pandemic influenza vaccines is promising as was demonstrated for H5N1 viruses.

With the influenza A/H1N1 pandemic we anticipate that MVA-based vaccines expressing the H1 gene of these viruses also could be promising vaccine candidates. Since it cannot be predicted which influenza A subtypes will cause future pandemics it may be an attractive strategy to clone the HA genes of a number of different influenza A subtypes, which has been shown to be transmitted to humans including H1N1, H2N2, H5N1, H7N7 and H9N2. This way, seed vaccine strains are readily available before the start of a pandemic with one of these viruses. Since it was demonstrated that cross-clade protection could be induced with one MVA-HA-VN/04 vaccine, but not with MVA-HA-HK/97, there is a risk that the HA genes of choice may not induce the antibodies of the proper specificity. In contrast, the pandemic influenza A/H1N1 viruses are antigenically homogeneous and a single vaccine strain (A/California/04/2009 or A/California/05/2009) most likely could protect against all currently circulating new influenza A/H1N1 strains.[50] Since it has been shown that MVA-based vaccines also induce cell-mediated immunity, recombinant MVA expressing the influenza virus NP or M1 genes may be attractive vaccine candidates for the induction of cross-protective CTL responses. The addition of gene(s) encoding the relatively conserved internal proteins or other influenza virus proteins, like NA and M2, may broaden the protective potential of MVA vaccines expressing the HA gene.

A potential problem with the use of MVA is interference with the induction of specific immunity to the target antigen by pre-existing anti-vector immunity induced by vaccination against small pox or the use of other recombinant pox viral vectors. This is especially relevant for vaccination against influenza since these vaccines are administered repeatedly on an annual basis, which may reduce vaccine efficacy over time. However, it has been demonstrated that pre-existing immunity to the vector did not affect the immune response to the target protein to a great extent.[182, 185] Nevertheless, this issue should be addressed in clinical trials evaluating MVA-based influenza vaccines.

Concluding remarks

The work described in this thesis focused on the protective potential of heterosubtypic immunity against potentially pandemic influenza viruses and the preclinical evaluation of MVA-based pandemic influenza vaccine candidates. The results from these studies were discussed in the light of the current pandemic threats. The basis for heterosubtypic immunity is not fully understood, however virus specific CTL recognizing conserved epitopes most likely play an important role in this type of immunity. Since the presence of pre-existing memory CTL contributes to accelerated clearance of the virus infection, it may afford protection against severe disease and mortality. In addition to the beneficial effects of heterosubtypic immunity in individual patients, it may also have a favorable effect at the population level since it can reduce the extent and duration of viral shedding and thus the spread of virus. This way, the severity of pandemic outbreaks may be dampened. The current outbreak of new influenza A/H1N1 viruses provides a unique opportunity to investigate the protective effect of heterosubtypic immunity against these potentially pandemic viruses and to elucidate the correlates of protection underlying this type of immunity. Once these correlates of protection have been identified, it opens the possibilities for the development of vaccines that can induce broadly protective immunity. At present, antibodies directed to the hemagglutinin, is the only accepted correlate of protection and vaccines are licensed based on serological outcome of immunization. Therefore, the induction of antibodies specific for the virus causing the (pandemic) influenza outbreak is a minimal requirement, but the induction of heterosubtypic immunity, whatever the correlates may be, will broaden the protective potential of influenza vaccines. The co-circulation of various antigenically distinct clades of A/H5N1 viruses complicates the selection of a single vaccine strain.

We have demonstrated that with the use of a MVA-based vaccine expressing the HA gene of one H5N1 influenza virus cross-clade protective immunity could be induced. Other favorable properties of MVA based vaccines like excellent safety profile and good immunogenicity, warrants further evaluation of these vaccine in clinical trials. Since protective immunity could be induced at very low doses it may be the vaccine preparation of choice for the vaccination of large populations. Facing a pandemic outbreak, ideally protective immunity is induced after a single administration of vaccine, which was possible with a high dose of MVA-based vaccine.

Collectively, the results obtained in the present studies confirmed the protective potential of heterosubtypic immunity, which may be at the basis for the development of novel vaccines and intervention strategies. In addition, the evaluation of MVA-based vaccines indicated that it might be a promising novel generation of influenza vaccines. Eventually, these developments will help limiting the impact of future influenza pandemics.

KNVB
EREDIVISIE
22 may


REFERENCES

[1] Ruigrok RWH. Structure of Influenza A, B and C viruses. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Oxford: Blackwell Science, 1998: 29-42.
[2] Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005 Mar;79(5):2814-22.
[3] Wright PF, Neumann G, Kawaoka Y. Orthomyxoviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, editors. Fields Virology, 5th edition: Lippincott Williams and Wilkins, 2007.
[4] Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 2001 Dec;7(12):1306-12.
[5] Hay AJ. The Virus Genome and its Replication. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Oxford: Blackwell Science, 1998: 43-53.
[6] Baigent SJ, McCauley JW. Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays 2003 Jul;25(7):657-71.
[7] Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: influenza virus receptors in the human airway. Nature 2006 Mar 23;440(7083):435-6.
[8] Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell 1992 May 1;69(3):517-28.
[9] Nayak DP, Hui EK, Barman S. Assembly and budding of influenza virus. Virus Res 2004 Dec;106(2):147-65.
[10] Colman PM. Structure and Function of the Neuraminidase. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Oxford: Blackwell Science, 1998: 65-73.
[11] Richman DD, Murphy BR, Baron S, Uhlendorf C. Three strains of influenza A virus (H_3N_2): interferon sensitivity in vitro and interferon production in volunteers. J Clin Microbiol 1976 Mar;3(3):223-6.
[12] Guarner J, Shieh WJ, Dawson J, Subbarao K, Shaw M, Ferebee T, et al. Immunohistochemical and in situ hybridization studies of influenza A virus infection in human lungs. Am J Clin Pathol 2000 Aug;114(2):227-33.
[13] Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J Virol 1994 Jun;68(6):3667-73.
[14] de Jong MD, Hien TT. Avian influenza A (H5N1). J Clin Virol 2006 Jan;35(1):2-13.
[15] Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, et al. Avian influenza A (H5N1) infection in humans. N Engl J Med 2005 Sep 29;353(13):1374-85.
[16] Nicholson KG. Human Influenza. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Oxford: Blackwell Science, 1998: 219-64.
[17] Wang C, Takeuchi K, Pinto LH, Lamb RA. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J Virol 1993 Sep;67(9):5585-94.
[18] Colman PM. Influenza virus neuraminidase: structure, antibodies, and inhibitors. Protein Sci 1994 Oct;3(10):1687-96.
[19] Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, et al. Avian flu: isolation of drug-resistant H5N1 virus. Nature 2005 Oct 20;437(7062):1108.
[20] de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, et al. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 2005 Dec 22;353(25):2667-72.
[21] Besselaar TG, Naidoo D, Buys A, Gregory V, McAnerney J, Manamela JM, et al. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. Emerg Infect Dis 2008 Nov;14(11):1809-10.
[22] Hauge SH, Dudman S, Borgen K, Lackenby A, Hungnes O. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007-08. Emerg Infect Dis 2009 Feb;15(2):155-62.
[23] Rimmelzwaan GF, de Jong JC, Donker GA, Meijer A, Fouchier RA, Osterhaus AD. [Influenza season 2007/'08 in the Netherlands: antigenic variation, oseltamivir resistance and vaccine composition for the 2008/'09 season]
Het influenzaseizoen 2007/'08 in Nederland: antigene variatie, resistentie tegen oseltamivir en de vaccinsamenstelling voor het seizoen 2008/'09. Ned Tijdschr Geneeskd 2008 Sep 27;152(39):2138-44.
[24] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992 Mar;56(1):152-79.
[25] Kuiken T, Fouchier R, Rimmelzwaan G, Osterhaus A, Roeder P. Feline friend or potential foe? Nature 2006 Apr 6;440(7085):741-2.
[26] Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, et al. Avian H5N1 influenza in cats. Science 2004 Oct 8;306(5694):241.
[27] Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, et al. Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 2006 Jan;168(1):176-83; quiz 364.
[28] Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, et al. Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis 2004 Dec;10(12):2189-91.
[29] Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, et al. Transmission of equine influenza virus to dogs. Science 2005 Oct 21;310(5747):482-5.
[30] Bodewes R, Egberink HF. An update on viral diseases of the dog and cat. Tijdschr v diergeneeskunde 2009 1 april 2009;134(7):NOG AANPASSEN.
[31] Stohr K. Influenza--WHO cares. Lancet Infect Dis 2002 Sep;2(9):517.
[32] WHO. Influenza Fact sheet N°211, Revised March 2003
http://www.who.int/mediacentre/factsheets/2003/fs211/en/. accessed May 08, 2009, 2009.
[33] Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, et al. The global circulation of seasonal

influenza A (H_3N_2) viruses. Science 2008 Apr 18;320(5874):340-6.
[34] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004 Jul 16;305(5682):371-6.
[35] Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. Nature 2005 Oct 6;437(7060):889-93.
[36] Gibbs MJ, Gibbs AJ. Molecular virology: was the 1918 pandemic caused by a bird flu? Nature 2006 Apr 27;440(7088):E8; discussion E9-10.
[37] Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 1989 Nov;63(11):4603-8.
[38] Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H_2N_2 and H_3N_2. Virology 1978 Jun 1;87(1):13-20.
[39] de Wit E, Kawaoka Y, de Jong MD, Fouchier RA. Pathogenicity of highly pathogenic avian influenza virus in mammals. Vaccine 2008 Sep 12;26 Suppl 4:D54-8.
[40] Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, et al. Triple-Reassortant Swine Influenza A (H1) in Humans in the United States, 2005-2009. N Engl J Med 2009 May 7.
[41] Rimmelzwaan GF, de Jong JC, Bestebroer TM, van Loon AM, Claas EC, Fouchier RA, et al. Antigenic and genetic characterization of swine influenza A (H_1N_1) viruses isolated from pneumonia patients in The Netherlands. Virology 2001 Apr 10;282(2):301-6.
[42] Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus (H_7N_7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A 2004 Feb 3;101(5):1356-61.
[43] Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, et al. Transmission of H_7N_7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 2004 Feb 21;363(9409):587-93.
[44] de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. A pandemic warning? Nature 1997 Oct 9;389(6651):554.
[45] Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H_5N_1) virus isolated from a child with a fatal respiratory illness. Science 1998 Jan 16;279(5349):393-6.
[46] Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H_5N_1 virus related to a highly pathogenic avian influenza virus. Lancet 1998 Feb 14;351(9101):472-7.
[47] WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H_5N_1) Reported to WHO http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_02/en/index.html. accessed March 06, 2009, 2009.
[48] Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H_5N_1). N Engl J Med 2005 Jan 27;352(4):333-40.
[49] Yang Y, Halloran ME, Sugimoto JD, Longini IM, Jr. Detecting human-to-human transmission of avian influenza A (H_5N_1). Emerg Infect Dis 2007 Sep;13(9):1348-53.
[50] Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H_1N_1) Influenza Viruses Circulating in Humans. Science 2009 May 22.
[51] WHO. World now at the start of 2009 influenza pandemic http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html. accessed June 11, 2009, 2009.
[52] WHO. New Influenza A(H_1N_1), Number of laboratory confirmed cases and deaths as reported to WHO, status as of 22 June 2009 http://www.who.int/csr/don/Map_20090622_0800.png. accessed June 23, 2009, 2009.
[53] Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 2004 Apr 13;101(15):5598-603.
[54] Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, et al. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 1998 Dec 20;252(2):324-30.
[55] Katze MG, He Y, Gale M, Jr. Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2002 Sep;2(9):675-87.
[56] Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol 2005;23:307-36.
[57] Barchet W, Krug A, Cella M, Newby C, Fischer JA, Dzionek A, et al. Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways. Eur J Immunol 2005 Jan;35(1):236-42.
[58] Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. Br Med Bull 1979 Jan;35(1):69-75.
[59] Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol 1990 Mar;64(3):1375-7.
[60] Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J Immunol 2008 Sep 15;181(6):4168-76.
[61] Roose K, Fiers W, Saelens X. Pandemic preparedness: Toward a universal influenza vaccine. Drug News Perspect 2009 Mar;22(2):80-92.
[62] Wilschut J, McElhaney JE, Palache AM. The immune response to influenza virus infection. In: Wilschut J, McElhaney JE, Palache AM, editors. Influenza, second edition. Philadelphia: Mosby Elsevier, 2006.
[63] GeurtsvanKessel CH, Willart MA, van Rijt LS, Muskens F, Kool M, Baas C, et al. Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. J Exp Med 2008

Jul 7;205(7):1621-34.
[64] Lamb JR, Woody JN, Hartzman RJ, Eckels DD. In vitro influenza virus-specific antibody production in man: antigen-specific and HLA-restricted induction of helper activity mediated by cloned human T lymphocytes. J Immunol 1982 Oct;129(4):1465-70.
[65] Yewdell JW, Bennink JR, Smith GL, Moss B. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 1985 Mar;82(6):1785-9.
[66] Wang M, Lamberth K, Harndahl M, Roder G, Stryhn A, Larsen MV, et al. CTL epitopes for influenza A including the H5N1 bird flu; genome-, pathogen-, and HLA-wide screening. Vaccine 2007 Apr 12;25(15):2823-31.
[67] McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. N Engl J Med 1983 Jul 7;309(1):13-7.
[68] Boon AC, de Mutsert G, Graus YM, Fouchier RA, Sintnicolaas K, Osterhaus AD, et al. The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype. J Virol 2002 Jan;76(2):582-90.
[69] Gotch F, McMichael A, Smith G, Moss B. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. J Exp Med 1987 Feb 1;165(2):408-16.
[70] Jameson J, Cruz J, Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J Virol 1998 Nov;72(11):8682-9.
[71] Epstein SL. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. J Infect Dis 2006 Jan 1;193(1):49-53.
[72] Lee LY, Ha DL, Simmons C, de Jong MD, Chau NV, Schumacher R, et al. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J Clin Invest 2008 Sep 18.
[73] Jameson J, Cruz J, Terajima M, Ennis FA. Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species. J Immunol 1999 Jun 15;162(12):7578-83.
[74] WHO. Options for the use of human H5N1 influenza vaccines and the WHO H5N1 vaccine stockpile: WHO; 2008 1-3 October 2007.
[75] WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as human vaccines - http://www.who.int/csr/disease/avian_influenza/guidelines/200809_H5VaccineVirusUpdate.pdf. 2008.
[76] WHO. Recommended composition of influenza virus vaccines for use in the 2008–2009 influenza season http://www.who.int/csr/disease/influenza/recommended_compositionFebo8FullReport.pdf. 2008.
[77] Nichol KL. Efficacy/clinical effectiveness of inactivated influenza virus vaccines in adults. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of influenza: Oxford: Blackwell Science, 1998: 358-72.
[78] Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 2006 May 20;367(9523):1657-64.
[79] Ada G. Overview of vaccines and vaccination. Mol Biotechnol 2005 Mar;29(3):255-72.
[80] Belshe RB, Walker R, Stoddard JJ, Kemble G, Maassab HF, Mendelman PM. influenza vaccine-live. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Saunders Elsevier, 2008: 291-309.
[81] Keitel W, Piedra P. Live cold-adapted, reassortant influenza vaccines (USA). In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Oxford: Blackwell Science, 1998: 373-90.
[82] Belshe RB, Ambrose CS, Yi T. Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age. Vaccine 2008 Jul 9.
[83] Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin A, Stephenson I, et al. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 2006 Nov 10;24(44-46):6588-93.
[84] Clements ML, Murphy BR. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. J Clin Microbiol 1986 Jan;23(1):66-72.
[85] Belshe RB, Gruber WC, Mendelman PM, Cho I, Reisinger K, Block SL, et al. Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. J Pediatr 2000 Feb;136(2):168-75.
[86] Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis 2000 Mar;181(3):1133-7.
[87] Treanor JJ, Kotloff K, Betts RF, Belshe R, Newman F, Iacuzio D, et al. Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. Vaccine 1999 Dec 10;18(9-10):899-906.
[88] Beyer WE, Palache AM, de Jong JC, Osterhaus AD. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. Vaccine 2002 Jan 31;20(9-10):1340-53.
[89] Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007 Feb 15;356(7):685-96.
[90] Suguitan AL, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, et al. Live, Attenuated Influenza A H5N1 Candidate Vaccines Provide Broad Cross-Protection in Mice and Ferrets. PLoS Med 2006 Sep 12;3(9).
[91] Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, et al. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol 2000 Dec;74(24):11566-73.
[92] Donelan NR, Basler CF, Garcia-Sastre A. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J Virol 2003

Dec;77(24):13257-66.
[93] Govorkova EA, Webby RJ, Humberd J, Seiler JP, Webster RG. Immunization with Reverse-Genetics-Produced H5N1 Influenza Vaccine Protects Ferrets against Homologous and Heterologous Challenge. J Infect Dis 2006 Jul 15;194(2):159-67.
[94] Lipatov AS, Webby RJ, Govorkova EA, Krauss S, Webster RG. Efficacy of H5 influenza vaccines produced by reverse genetics in a lethal mouse model. J Infect Dis 2005 Apr 15;191(8):1216-20.
[95] Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, et al. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology 2005 Oct 10;341(1):153-62.
[96] Webster RG, Webby RJ, Hoffmann E, Rodenberg J, Kumar M, Chu HJ, et al. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology 2006 May 11.
[97] Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, et al. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003 Jan 5;305(1):192-200.
[98] de Wit E, Munster VJ, Spronken MI, Bestebroer TM, Baas C, Beyer WE, et al. Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. J Virol 2005 Oct;79(19):12401-7.
[99] Wood JM, Robertson JS. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2004 Oct;2(10):842-7.
[100] Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005 Apr 22;23(22):2943-52.
[101] EMEA. Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application http://www.emea.europa.eu/pdfs/human/vwp/471703en.pdf. London: EMEA, 2004.
[102] Patriarca PA. Use of Cell Lines for the Production of Influenza Virus Vaccines: An Appraisal of Technical, Manufacturing, and Regulatory Considerations
http://www.who.int/vaccine_research/diseases/influenza/WHO_Flu_Cell_Substrate_Version3.pdf. Geneva, Switzerland: World Health Organization; 2007.
[103] Genzel Y, Olmer RM, Schafer B, Reichl U. Wave microcarrier cultivation of MDCK cells for influenza virus production in serum containing and serum-free media. Vaccine 2006 Aug 28;24(35-36):6074-87.
[104] Palache AM, Scheepers HS, de Regt V, van Ewijk P, Baljet M, Brands R, et al. Safety, reactogenicity and immunogenicity of Madin Darby Canine Kidney cell-derived inactivated influenza subunit vaccine. A meta-analysis of clinical studies. Dev Biol Stand 1999;98:115-25; discussion 33-4.
[105] Gregersen JP. A quantitative risk assessment of exposure to adventitious agents in a cell culture-derived subunit influenza vaccine. Vaccine 2008 Jun 19;26(26):3332-40.
[106] Kistner O, Howard MK, Spruth M, Wodal W, Bruhl P, Gerencer M, et al. Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses. Vaccine 2007 Aug 10;25(32):6028-36.
[107] Vidor E, Meschievitz C, Plotkin S. Fifteen years of experience with Vero-produced enhanced potency inactivated poliovirus vaccine. Pediatr Infect Dis J 1997 Mar;16(3):312-22.
[108] Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombinant DNA. J Virol 1999 Nov;73(11):9679-82.
[109] Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 2008 Jun 12;358(24):2573-84.
[110] Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, et al. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 1998 Sep 1;9(13):1909-17.
[111] Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. Vaccine 2001 Mar 21;19(17-19):2716-21.
[112] Toro H, Tang DC, Suarez DL, Zhang J, Shi Z. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. Vaccine 2008 May 19;26(21):2640-6.
[113] Govorkova EA, Kodihalli S, Alymova IV, Fanget B, Webster RG. Growth and immunogenicity of influenza viruses cultivated in Vero or MDCK cells and in embryonated chicken eggs. Dev Biol Stand 1999;98:39-51; discussion 73-4.
[114] Ghendon YZ, Markushin SG, Akopova, II, Koptiaeva IB, Nechaeva EA, Mazurkova LA, et al. Development of cell culture (MDCK) live cold-adapted (CA) attenuated influenza vaccine. Vaccine 2005 Sep 7;23(38):4678-84.
[115] Schild GC, Oxford JS, de Jong JC, Webster RG. Evidence for host-cell selection of influenza virus antigenic variants. Nature 1983 Jun 23-29;303(5919):706-9.
[116] Nwe N, He Q, Damrongwatanapokin S, Du Q, Manopo I, Limlamthong Y, et al. Expression of hemagglutinin protein from the avian influenza virus H5N1 in a baculovirus/insect cell system significantly enhanced by suspension culture. BMC Microbiol 2006;6:16.
[117] Crawford J, Wilkinson B, Vosnesensky A, Smith G, Garcia M, Stone H, et al. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 1999 May 4;17(18):2265-74.
[118] Possee RD. Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. Virus Res 1986 Jul;5(1):43-59.
[119] Johansson BE. Immunization with influenza A virus hemagglutinin and neuraminidase produced in

recombinant baculovirus results in a balanced and broadened immune response superior to conventional vaccine. Vaccine 1999 Apr 9;17(15-16):2073-80.
[120] Brett IC, Johansson BE. Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 2005 Sep 1;339(2):273-80.
[121] Huber VC, McCullers JA. FluBlok, a recombinant influenza vaccine. Curr Opin Mol Ther 2008 Feb;10(1):75-85.
[122] Cox MM. Progress on baculovirus-derived influenza vaccines. Curr Opin Mol Ther 2008 Feb;10(1):56-61.
[123] Powers DC, Smith GE, Anderson EL, Kennedy DJ, Hackett CS, Wilkinson BE, et al. Influenza A virus vaccines containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults. J Infect Dis 1995 Jun;171(6):1595-9.
[124] Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, et al. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 2001 Feb 8;19(13-14):1732-7.
[125] Almeida JD, Edwards DC, Brand CM, Heath TD. Formation of virosomes from influenza subunits and liposomes. Lancet 1975 Nov 8;2(7941):899-901.
[126] Bron R, Ortiz A, Dijkstra J, Stegmann T, Wilschut J. Preparation, properties, and applications of reconstituted influenza virus envelopes (virosomes). Methods Enzymol 1993;220:313-31.
[127] Bungener L, Huckriede A, de Mare A, de Vries-Idema J, Wilschut J, Daemen T. Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity. Vaccine 2005 Jan 26;23(10):1232-41.
[128] Bungener L, Serre K, Bijl L, Leserman L, Wilschut J, Daemen T, et al. Virosome-mediated delivery of protein antigens to dendritic cells. Vaccine 2002 May 22;20(17-18):2287-95.
[129] de Mare A, Bungener LB, Regts J, de Vries-Idema J, van der Zee AG, Wilschut J, et al. The effect of pre-existing immunity on the capacity of influenza virosomes to induce cytotoxic T lymphocyte activity. Vaccine 2008 May 2;26(19):2314-21.
[130] Gluck R, Mischler R, Finkel B, Que JU, Scarpa B, Cryz SJ, Jr. Immunogenicity of new virosome influenza vaccine in elderly people. Lancet 1994 Jul 16;344(8916):160-3.
[131] Conne P, Gauthey L, Vernet P, Althaus B, Que JU, Finkel B, et al. Immunogenicity of trivalent subunit versus virosome-formulated influenza vaccines in geriatric patients. Vaccine 1997 Oct;15(15):1675-9.
[132] Pregliasco F, Mensi C, Serpilli W, Specchier L, Masella P, Belloni A. Immunogenicity and safety of three commercial influenza vaccines in institutionalized elderly. Aging (Milano) 2001 Feb;13(1):38-43.
[133] de Bruijn IA, Nauta J, Gerez L, Palache AM. The virosomal influenza vaccine Invivac: immunogenicity and tolerability compared to an adjuvanted influenza vaccine (Fluad in elderly subjects. Vaccine 2006 Nov 10;24(44-46):6629-31.
[134] Galarza JM, Latham T, Cupo A. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 2005;18(1):244-51.
[135] Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 2007 Apr;81(7):3514-24.
[136] Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrilov V, et al. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. Vaccine 2007 May 10;25(19):3871-8.
[137] Bright RA, Carter DM, Crevar CJ, Toapanta FR, Steckbeck JD, Cole KS, et al. Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. PLoS ONE 2008;3(1):e1501.
[138] Bridges CB, Katz J, Levandowski R, Cox N. Inactivated influenza vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Saunders Elsevier, 2008: 259-90.
[139] Ruat C, Caillet C, Bidaut A, Simon J, Osterhaus AD. Vaccination of macaques with adjuvanted formalin-inactivated influenza A virus (H5N1) vaccines: protection against H5N1 challenge without disease enhancement. J Virol 2008 Mar;82(5):2565-9.
[140] Davenport FM, Hennessy AV, Askin FB. Lack of adjuvant effect of $A1PO_4$ on purified influenza virus hemagglutinins in man. J Immunol 1968 May;100(5):1139-40.
[141] Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007 May 4;25(18):3554-60.
[142] Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006 Sep 16;368(9540):991-7.
[143] Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol 1999 Jul;73(7):5903-11.
[144] De Swart RL, Kuiken T, Timmerman HH, van Amerongen G, Van Den Hoogen BG, Vos HW, et al. Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. J Virol 2002 Nov;76(22):11561-9.
[145] Polack FP. Atypical measles and enhanced respiratory syncytial virus disease (ERD) made simple. Pediatr Res 2007 Jul;62(1):111-5.
[146] Ansaldi F, Bacilieri S, Durando P, Sticchi L, Valle L, Montomoli E, et al. Cross-protection by MF59-adjuvanted influenza vaccine: neutralizing and haemagglutination-inhibiting antibody activity against A(H3N2) drifted influenza viruses. Vaccine 2008 Mar 17;26(12):1525-9.
[147] De Donato S, Granoff D, Minutello M, Lecchi G, Faccini M, Agnello M, et al. Safety and immunogenicity

of MF59-adjuvanted influenza vaccine in the elderly. Vaccine 1999 Aug 6;17(23-24):3094-101.
[148] Dupuis M, Denis-Mize K, LaBarbara A, Peters W, Charo IF, McDonald DM, et al. Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. Eur J Immunol 2001 Oct;31(10):2910-8.
[149] Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, van Nest G, Ott G, et al. Dendritic cells internalize vaccine adjuvant after intramuscular injection. Cell Immunol 1998 May 25;186(1):18-27.
[150] Gasparini R, Pozzi T, Montomoli E, Fragapane E, Senatore F, Minutello M, et al. Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects. Eur J Epidemiol 2001;17(2):135-40.
[151] Iob A, Brianti G, Zamparo E, Gallo T. Evidence of increased clinical protection of an MF59-adjuvant influenza vaccine compared to a non-adjuvant vaccine among elderly residents of long-term care facilities in Italy. Epidemiol Infect 2005 Aug;133(4):687-93.
[152] Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001 Jun 16;357(9272):1937-43.
[153] O'Hagan DT, Ott GS, Van Nest G. Recent advances in vaccine adjuvants: the development of MF59 emulsion and polymeric microparticles. Mol Med Today 1997 Feb;3(2):69-75.
[154] Podda A, Del Giudice G. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. Expert Rev Vaccines 2003 Apr;2(2):197-203.
[155] Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J Immunol 2008 Apr 15;180(8):5402-12.
[156] Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistoor FH, et al. Cross-Protection against Lethal H5N1 Challenge in Ferrets with an Adjuvanted Pandemic Influenza Vaccine. PLoS ONE 2008;3(1):e1401.
[157] Leroux-Roels I, Bernhard R, Gerard P, Drame M, Hanon E, Leroux-Roels G. Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine. PLoS ONE 2008;3(2):e1665.
[158] Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 2007 Aug 18;370(9587):580-9.
[159] Rumke HC, Bayas JM, de Juanes JR, Caso C, Richardus JH, Campins M, et al. Safety and reactogenicity profile of an adjuvanted H5N1 pandemic candidate vaccine in adults within a phase III safety trial. Vaccine 2008 May 2;26(19):2378-88.
[160] Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 1984 Mar 29-Apr 4;308(5958):457-60.
[161] Rimmelzwaan GF, Claas EC, van Amerongen G, de Jong JC, Osterhaus AD. ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. Vaccine 1999 Mar 17;17(11-12):1355-8.
[162] Rimmelzwaan GF, Nieuwkoop N, Brandenburg A, Sutter G, Beyer WE, Maher D, et al. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine 2000 Dec 8;19(9-10):1180-7.
[163] Rimmelzwaan GF, Osterhaus ADME. ISCOMs: Materials, preparation, antigen delivery and immune response. In: Gander B, Merkle HP, Corradin G, editors. Antigen delivery systems: Harwood Academic Publishers, 1997: 123-38.
[164] Rimmelzwaan GF, Osterhaust AD. Influenza vaccines: new developments. Curr Opin Pharmacol 2001 Oct;1(5):491-6.
[165] Donnelly JJ, Friedman A, Martinez D, Montgomery DL, Shiver JW, Motzel SL, et al. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. Nat Med 1995 Jun;1(6):583-7.
[166] Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. Epidermal DNA vaccine for influenza is immunogenic in humans. Vaccine 2006 May 22;24(21):4475-81.
[167] Epstein SL, Tumpey TM, Misplon JA, Lo CY, Cooper LA, Subbarao K, et al. DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. Emerg Infect Dis 2002 Aug;8(8):796-801.
[168] Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. Annu Rev Immunol 2000;18:927-74.
[169] Klinman DM, Takeno M, Ichino M, Gu M, Yamshchikov G, Mor G, et al. DNA vaccines: safety and efficacy issues. Springer Semin Immunopathol 1997;19(2):245-56.
[170] Lalor PA, Webby RJ, Morrow J, Rusalov D, Kaslow DC, Rolland A, et al. Plasmid DNA-Based Vaccines Protect Mice and Ferrets against Lethal Challenge with A/Vietnam/1203/04 (H5N1) Influenza Virus. J Infect Dis 2008 Jun 15;197(12):1643-52.
[171] Robinson HL, Hunt LA, Webster RG. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine 1993;11(9):957-60.
[172] Ulmer JB. Influenza DNA vaccines. Vaccine 2002 May 15;20 Suppl 2:S74-6.
[173] Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993 Mar 19;259(5102):1745-9.
[174] Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, et al. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J Virol 1998 Jul;72(7):5648-53.
[175] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1990 Mar 23;247(4949 Pt 1):1465-8.
[176] Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, et al. Protection of mice and poultry

from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol 2006 Feb;80(4):1959-64.
[177] Hoelscher MA, Garg S, Bangari DS, Belser JA, Lu X, Stephenson I, et al. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet 2006 Feb 11;367(9509):475-81.
[178] Hoelscher MA, Jayashankar L, Garg S, Veguilla V, Lu X, Singh N, et al. New pre-pandemic influenza vaccines: an egg- and adjuvant-independent human adenoviral vector strategy induces long-lasting protective immune responses in mice. Clin Pharmacol Ther 2007 Dec;82(6):665-71.
[179] Hoelscher MA, Singh N, Garg S, Jayashankar L, Veguilla V, Pandey A, et al. A broadly protective vaccine against globally dispersed clade 1 and clade 2 H5N1 influenza viruses. J Infect Dis 2008 Apr 15;197(8):1185-8.
[180] Holman DH, Wang D, Raja NU, Luo M, Moore KM, Worarattanadharm J, et al. Multi-antigen vaccines based on complex adenovirus vectors induce protective immune responses against H5N1 avian influenza viruses. Vaccine 2008 May 19;26(21):2627-39.
[181] Drexler I, Heller K, Wahren B, Erfle V, Sutter G. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. J Gen Virol 1998 Feb;79 (Pt 2):347-52.
[182] Harrop R, Connolly N, Redchenko I, Valle J, Saunders M, Ryan MG, et al. Vaccination of colorectal cancer patients with modified vaccinia Ankara delivering the tumor antigen 5T4 (TroVax) induces immune responses which correlate with disease control: a phase I/II trial. Clin Cancer Res 2006 Jun 1;12(11 Pt 1):3416-24.
[183] Mayr A, Danner K. Vaccination against pox diseases under immunosuppressive conditions. Dev Biol Stand 1978;41:225-34.
[184] Moss B, Carroll MW, Wyatt LS, Bennink JR, Hirsch VM, Goldstein S, et al. Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. Adv Exp Med Biol 1996;397:7-13.
[185] Ramirez JC, Gherardi MM, Rodriguez D, Esteban M. Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector. J Virol 2000 Aug;74(16):7651-5.
[186] Staib C, Drexler I, Sutter G. Construction and isolation of recombinant MVA. Methods Mol Biol 2004;269:77-100.
[187] Stittelaar KJ, Kuiken T, de Swart RL, van Amerongen G, Vos HW, Niesters HG, et al. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. Vaccine 2001 Jun 14;19(27):3700-9.
[188] Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci U S A 1992 Nov 15;89(22):10847-51.
[189] Sutter G, Staib C. Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery. Curr Drug Targets Infect Disord 2003 Sep;3(3):263-71.
[190] Sutter G, Wyatt LS, Foley PL, Bennink JR, Moss B. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 1994 Aug;12(11):1032-40.
[191] Drexler I, Staib C, Sutter G. Modified vaccinia virus Ankara as antigen delivery system: how can we best use its potential? Curr Opin Biotechnol 2004 Dec;15(6):506-12.
[192] Vogel FR, Hem SL. Immunologic adjuvants. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Saunders Elsevier, 2008: 59-81.
[193] Dudek T, Knipe DM. Replication-defective viruses as vaccines and vaccine vectors. Virology 2006 Jan 5;344(1):230-9.
[194] Liniger M, Zuniga A, Naim HY. Use of viral vectors for the development of vaccines. Expert Rev Vaccines 2007 Apr;6(2):255-66.
[195] Girard MP, Osterhaus A, Pervikov Y, Palkonyay L, Kieny MP. Report of the third meeting on "influenza vaccines that induce broad spectrum and long-lasting immune responses", World Health Organization, Geneva, Switzerland, 3-4 December 2007. Vaccine 2008 May 12;26(20):2443-50.
[196] Weniger BG, Papania MJ. Alternative vaccine delivery methods. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Saunders Elsevier, 2008: 1357-92.
[197] Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med 2007 Feb;4(2):e59.
[198] Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. Lancet 1999 Sep 11;354(9182):916-7.
[199] Kreijtz JH, de Mutsert G, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 2008 Jun;82(11):5161-6.
[200] Kreijtz JH, Bodewes R, van Amerongen G, Kuiken T, Fouchier RA, Osterhaus AD, et al. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine 2007 Jan 8;25(4):612-20.
[201] Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999 Oct;5(10):1157-63.
[202] Jegerlehner A, Schmitz N, Storni T, Bachmann MF. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. J Immunol 2004 May 1;172(9):5598-605.
[203] Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, et al. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine 2004 Aug 13;22(23-24):2993-3003.
[204] Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, et al. Matrix protein 2 vaccination and protection

against influenza viruses, including subtype H5N1. Emerg Infect Dis 2007 Mar;13(3):426-35.
[205] American Academy of Pediatrics Committee on Infectious D. Prevention of influenza: recommendations for influenza immunization of children, 2007-2008. Pediatrics 2008 Apr;121(4):e1016-31.
[206] Osterhaus AD. Pre- or post-pandemic influenza vaccine? Vaccine 2007 Jun 28;25(27):4983-4.
[207] Goji NA, Nolan C, Hill H, Wolff M, Noah DL, Williams TB, et al. Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. J Infect Dis 2008 Sep 1;198(5):635-41.
[208] Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004 Mar 18;350(12):1179-88.
[209] WHO. WHO disease alert 2006. Confirmed human cases of avian influenza H5N1 - http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_04_19/en/index.html. accessed April 12, 2006, 2006.
[210] WHO. WHO intercountry-consultation. Influenza A/H5N1 in humans in Asia. - http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_7/en/. Manila, Philippines, 2006.
[211] Evolution of H5N1 avian influenza viruses in Asia. Emerg Infect Dis 2005 Oct;11(10):1515-21.
[212] McMichael AJ, Michie CA, Gotch FM, Smith GL, Moss B. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. J Gen Virol 1986 Apr;67 (Pt 4):719-26.
[213] Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 1986 Mar 28;44(6):959-68.
[214] Kees U, Krammer PH. Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants. J Exp Med 1984 Feb 1;159(2):365-77.
[215] Liang S, Mozdzanowska K, Palladino G, Gerhard W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J Immunol 1994 Feb 15;152(4):1653-61.
[216] O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL. Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. J Gen Virol 2000 Nov;81(Pt 11):2689-96.
[217] Saelens X, Vanlandschoot P, Martinet W, Maras M, Neirynck S, Contreras R, et al. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. Eur J Biochem 1999 Feb;260(1):166-75.
[218] Coleclough C, Sealy R, Surman S, Marshall DR, Hurwitz JL. Respiratory vaccination of mice against influenza virus: dissection of T- and B-cell priming functions. Scand J Immunol 2005 Jul;62 Suppl 1:73-83.
[219] Cassetti MC, Couch R, Wood J, Pervikov Y. Report of meeting on the development of influenza vaccines with broad spectrum and long-lasting immune responses, World Health Organization, Geneva, Switzerland, 26-27 February 2004. Vaccine 2005 Feb 18;23(13):1529-33.
[220] Cassetti MC, Katz JM, Wood J. Report of a consultation on role of immunological assays to evaluate efficacy of influenza vaccines. Initiative for Vaccine Research and Global Influenza Programme, World Health Organization, Geneva, Switzerland, 25 January 2005. Vaccine 2006 Jan 30;24(5):541-3.
[221] Doherty PC, Biddison WE, Bennink JR, Knowles BB. Cytotoxic T-cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. J Exp Med 1978 Aug 1;148(2):534-43.
[222] Vitiello A, Yuan L, Chesnut RW, Sidney J, Southwood S, Farness P, et al. Immunodominance analysis of CTL responses to influenza PR8 virus reveals two new dominant and subdominant Kb-restricted epitopes. J Immunol 1996 Dec 15;157(12):5555-62.
[223] Belz GT, Xie W, Altman JD, Doherty PC. A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. J Virol 2000 Apr;74(8):3486-93.
[224] Baez M, Palese P, Kilbourne ED. Gene composition of high-yielding influenza vaccine strains obtained by recombination. J Infect Dis 1980 Mar;141(3):362-5.
[225] Kilbourne ED. Future influenza vaccines and the use of genetic recombinants. Bull World Health Organ 1969;41(3):643-5.
[226] Haanen JB, Wolkers MC, Kruisbeek AM, Schumacher TN. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. J Exp Med 1999 Nov 1;190(9):1319-28.
[227] Turner SJ, Kedzierska K, La Gruta NL, Webby R, Doherty PC. Characterization of CD8+ T cell repertoire diversity and persistence in the influenza A virus model of localized, transient infection. Semin Immunol 2004 Jun;16(3):179-84.
[228] Rimmelzwaan GF, Baars M, Claas EC, Osterhaus AD. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J Virol Methods 1998 Sep;74(1):57-66.
[229] Palmer D, et al. Haemagglutination inhibition test. Advanced laboratory techniques for influenza diagnosis Procedural Guide. Atlanta: US Dept. Hlth. ED., 1975: 25-62.
[230] Frank AL, Puck J, Hughes BJ, Cate TR. Microneutralization test for influenza A and B and parainfluenza 1 and 2 viruses that uses continuous cell lines and fresh serum enhancement. J Clin Microbiol 1980 Sep;12(3):426-32.
[231] Belz GT, Xie W, Doherty PC. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. J Immunol 2001 Apr 1;166(7):4627-33.
[232] Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity 1998 Jun;8(6):683-91.
[233] Woodland DL, Scott I. T cell memory in the lung airways. Proc Am Thorac Soc 2005;2(2):126-31.
[234] Seo SH, Peiris M, Webster RG. Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD8(+) T cells

expressing gamma interferon. J Virol 2002 May;76(10):4886-90.
[235] Liu W, Li H, Chen YH. N-terminus of M2 protein could induce antibodies with inhibitory activity against influenza virus replication. FEMS Immunol Med Microbiol 2003 Mar 20;35(2):141-6.
[236] Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis 2005 Aug;58(4):195-207.
[237] Liu W, Zou P, Chen YH. Monoclonal antibodies recognizing EVETPIRN epitope of influenza A virus M2 protein could protect mice from lethal influenza A virus challenge. Immunol Lett 2004 May 15;93(2-3):131-6.
[238] Yuen KY, Chan PK, Peiris M, Tsang DN, Que TL, Shortridge KF, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 1998 Feb 14;351(9101):467-71.
[239] Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, Chunsuthiwat S, Sawanpanyalert P, Kijphati R, et al. Human disease from influenza A (H5N1), Thailand, 2004. Emerg Infect Dis 2005 Feb;11(2):201-9.
[240] Schulman JL, Kilbourne ED. Induction of Partial Specific Heterotypic Immunity in Mice by a Single Infection with Influenza a Virus. J Bacteriol 1965 Jan;89:170-4.
[241] Heinen PP, de Boer-Luijtze EA, Bianchi AT. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J Gen Virol 2001 Nov;82(Pt 11):2697-707.
[242] Reeth KV, Brown I, Essen S, Pensaert M. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res 2004 Jul;103(1-2):115-24.
[243] Yetter RA, Barber WH, Small PA, Jr. Heterotypic immunity to influenza in ferrets. Infect Immun 1980 Aug;29(2):650-3.
[244] Seo SH, Webster RG. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. J Virol 2001 Mar;75(6):2516-25.
[245] Straight TM, Ottolini MG, Prince GA, Eichelberger MC. Evidence of a cross-protective immune response to influenza A in the cotton rat model. Vaccine 2006 Sep 11;24(37-39):6264-71.
[246] Grebe KM, Yewdell JW, Bennink JR. Heterosubtypic immunity to influenza A virus: where do we stand? Microbes Infect 2008 Jul;10(9):1024-9.
[247] Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol 2007 Dec;18(6):529-36.
[248] Benton KA, Misplon JA, Lo CY, Brutkiewicz RR, Prasad SA, Epstein SL. Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gamma delta T cells. J Immunol 2001 Jun 15;166(12):7437-45.
[249] Voeten JT, Brands R, Palache AM, van Scharrenburg GJ, Rimmelzwaan GF, Osterhaus AD, et al. Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. Vaccine 1999 Apr 9;17(15-16):1942-50.
[250] Philippa J, Baas C, Beyer W, Bestebroer T, Fouchier R, Smith D, et al. Vaccination against highly pathogenic avian influenza H5N1 virus in 2005 using an adjuvanted inactivated H5N2 vaccine. Vaccine 2007 May 10;25(19):3800-8.
[251] Kreijtz JH, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, et al. Recombinant Modified Vaccinia Virus Ankara-Based Vaccine Induces Protective Immunity in Mice against Infection with Influenza Virus H5N1. J Infect Dis 2007 Jun 1;195(11):1598-606.
[252] Boon AC, de Mutsert G, van Baarle D, Smith DJ, Lapedes AS, Fouchier RA, et al. Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. J Immunol 2004 Feb 15;172(4):2453-60.
[253] Marshall DR, Turner SJ, Belz GT, Wingo S, Andreansky S, Sangster MY, et al. Measuring the diaspora for virus-specific CD8+ T cells. Proc Natl Acad Sci U S A 2001 May 22;98(11):6313-8.
[254] Epstein SL, Lo CY, Misplon JA, Lawson CM, Hendrickson BA, Max EE, et al. Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, beta2-microglobulin-deficient, and J chain-deficient mice. J Immunol 1997 Feb 1;158(3):1222-30.
[255] Epstein SL, Kong WP, Misplon JA, Lo CY, Tumpey TM, Xu L, et al. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 2005 Nov 16;23(46-47):5404-10.
[256] Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A 1999 Aug 3;96(16):9363-7.
[257] Hogan RJ, Usherwood EJ, Zhong W, Roberts AA, Dutton RW, Harmsen AG, et al. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. J Immunol 2001 Feb 1;166(3):1813-22.
[258] de Bree GJ, van Leeuwen EM, Out TA, Jansen HM, Jonkers RE, van Lier RA. Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. J Exp Med 2005 Nov 21;202(10):1433-42.
[259] Smallman-Raynor M, Cliff AD. Avian influenza A (H5N1) age distribution in humans Emerg Infect Dis 2007 march 2007;13(3):510-2.
[260] Organization WH. Confirmed human cases of avian influenza A H5N1 Available at http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_12_12/en/index.html, 2008.
[261] Virus WCotSWHOCoCAoHIwAIA. Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med, 2008: 261-73.
[262] Grebe KM, Yewdell JW, Bennink JR. Heterosubtypic immunity to influenza A virus: where do we stand? Microbes Infect 2008 Jul 9.
[263] Smallman-Raynor M, Cliff AD. Avian influenza A (H5N1) age distribution in humans. Emerg Infect Dis

2007 Mar;13(3):510-2.
[264] Neuzil KM, Zhu Y, Griffin MR, Edwards KM, Thompson JM, Tollefson SJ, et al. Burden of interpandemic influenza in children younger than 5 years: a 25-year prospective study. J Infect Dis 2002 Jan 15;185(2):147-52.
[265] Izurieta HS, Thompson WW, Kramarz P, Shay DK, Davis RL, DeStefano F, et al. Influenza and the rates of hospitalization for respiratory disease among infants and young children. N Engl J Med 2000 Jan 27;342(4):232-9.
[266] Fiore AE, Shay DK, Broder K, Iskander JK, Uyeki TM, Mootrey G, et al. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2008. MMWR Recomm Rep 2008 Aug 8;57(RR-7):1-60.
[267] Webster RG, Askonas BA. Cross-protection and cross-reactive cytotoxic T cells induced by influenza virus vaccines in mice. Eur J Immunol 1980 May;10(5):396-401.
[268] He XS, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, et al. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. J Virol 2006 Dec;80(23):11756-66.
[269] Williams MA, Bevan MJ. Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells. J Immunol 2004 Dec 1;173(11):6694-702.
[270] Kohlmeier JE, Miller SC, Woodland DL. Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways. J Immunol 2007 Apr 15;178(8):4721-5.
[271] Haanen JB, Toebes M, Cordaro TA, Wolkers MC, Kruisbeek AM, Schumacher TN. Systemic T cell expansion during localized viral infection. Eur J Immunol 1999 Apr;29(4):1168-74.
[272] Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, Hartson L, Sprague F, Goodrich S, et al. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat Med 2004 Sep;10(9):927-34.
[273] Heikkinen T, Booy R, Campins M, Finn A, Olcen P, Peltola H, et al. Should healthy children be vaccinated against influenza? A consensus report of the Summits of Independent European Vaccination Experts. Eur J Pediatr 2006 Apr;165(4):223-8.
[274] Luce BR, Nichol KL, Belshe RB, Frick KD, Li SX, Boscoe A, et al. Cost-effectiveness of live attenuated influenza vaccine versus inactivated influenza vaccine among children aged 24-59 months in the United States. Vaccine 2008 Jun 2;26(23):2841-8.
[275] Salo H, Kilpi T, Sintonen H, Linna M, Peltola V, Heikkinen T. Cost-effectiveness of influenza vaccination of healthy children. Vaccine 2006 Jun 5;24(23):4934-41.
[276] Ritzwoller DP, Bridges CB, Shetterly S, Yamasaki K, Kolczak M, France EK. Effectiveness of the 2003-2004 influenza vaccine among children 6 months to 8 years of age, with 1 vs 2 doses. Pediatrics 2005 Jul;116(1):153-9.
[277] Shuler CM, Iwamoto M, Bridges CB, Marin M, Neeman R, Gargiullo P, et al. Vaccine effectiveness against medically attended, laboratory-confirmed influenza among children aged 6 to 59 months, 2003-2004. Pediatrics 2007 Mar;119(3):e587-95.
[278] Zangwill KM, Belshe RB. Safety and efficacy of trivalent inactivated influenza vaccine in young children: a summary for the new era of routine vaccination. Pediatr Infect Dis J 2004 Mar;23(3):189-97.
[279] Vesikari T, Karvonen A, Pellegrini M, Borkowski A, Groth N. SS04-3: MF59T adjuvanted influenza vaccine (Fluad) in children: safety and immunogenicity following a second year seasonal vaccination. Third European Influenza Conference; 2008; Villamoura, Portugal; 2008.
[280] Palmer D, Dowle W, Coleman M, Schild G. Haemagglutination inhibition test. In: Advances laboratory techniques for influenza diagnosis: procedural guide: Atlanta: US Department of Health, Education and Welfare, 1975: 25-62.
[281] Karber G. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche Exp Pathol Pharmakol 1931;162:480-3.
[282] Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD. Pathogenesis of influenza A (H5N1) virus infection in a primate model. J Virol 2001 Jul;75(14):6687-91.
[283] WHO. Affected areas with confirmed cases of H5N1 avian influenza since 2003, status as of 17.10.2007 http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_FIMS_20071017.png. accessed October 26, 2007 2007.
[284] WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO http://www.who.int/csr/disease/avian_influenza/country/cases_table_2007_12_18/en/index.html. accessed December 18, 2007, 2007.
[285] Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT. Host species barriers to influenza virus infections. Science 2006 Apr 21;312(5772):394-7.
[286] Townsend AR, Skehel JJ. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. J Exp Med 1984 Aug 1;160(2):552-63.
[287] Fleischer B, Becht H, Rott R. Recognition of viral antigens by human influenza A virus-specific T lymphocyte clones. J Immunol 1985 Oct;135(4):2800-4.
[288] McMichael A. Cytotoxic T lymphocytes specific for influenza virus. Curr Top Microbiol Immunol 1994;189:75-91.
[289] Rimmelzwaan GF, Osterhaus AD. Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza? Vaccine 1995;13(8):703-5.
[290] Macken C, Lu H, Goodman J, Boykin L. The value of a database in surveillance and vaccine selection. Amsterdam: Elsevier Science, 2001.
[291] Voeten JT, Bestebroer TM, Nieuwkoop NJ, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J Virol 2000 Aug;74(15):6800-7.
[292] van Baalen CA, Kwa D, Verschuren EJ, Reedijk ML, Boon AC, de Mutsert G, et al. Fluorescent antigen-

transfected target cell cytotoxic T lymphocyte assay for ex vivo detection of antigen-specific cell-mediated cytotoxicity. J Infect Dis 2005 Oct 1;192(7):1183-90.
[293] WHO. WHO disease alert 2006. Confirmed human cases of avian influenza H5N1 - http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_11_13/en/index.html. accessed November 14th, 2006, 2006.
[294] WHO. Affected areas with confirmed cases of H5N1 avian influenza since 2003, status as of 23.08.2006 - http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_20060823.png. 2006.
[295] Vahlenkamp TW, Harder TC. Influenza virus infections in mammals. Berl Munch Tierarztl Wochenschr 2006 Mar-Apr;119(3-4):123-31.
[296] WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines - http://who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf. 2006.
[297] Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y, Webster RG. DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J Virol 1999 Mar;73(3):2094-8.
[298] Mayr A, Munz E. [Changes in the vaccinia virus through continuing passages in chick embryo fibroblast cultures]
Veranderung von Vaccinevirus durch Dauerpassagen in Huhnerembryofibroblasten-Kulturen. Zentralbl Bakteriol [Orig] 1964 Dec;195(1):24-35.
[299] Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J Gen Virol 1991 May;72 (Pt 5):1031-8.
[300] Cosma A, Nagaraj R, Buhler S, Hinkula J, Busch DH, Sutter G, et al. Therapeutic vaccination with MVA-HIV-1 nef elicits Nef-specific T-helper cell responses in chronically HIV-1 infected individuals. Vaccine 2003 Dec 8;22(1):21-9.
[301] Garcia-Hernandez E, Gonzalez-Sanchez JL, Andrade-Manzano A, Contreras ML, Padilla S, Guzman CC, et al. Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with MVA E2 recombinant vaccine. Cancer Gene Ther 2006 Jun;13(6):592-7.
[302] Wyatt LS, Earl PL, Liu JY, Smith JM, Montefiori DC, Robinson HL, et al. Multiprotein HIV type 1 clade B DNA and MVA vaccines: construction, expression, and immunogenicity in rodents of the MVA component. AIDS Res Hum Retroviruses 2004 Jun;20(6):645-53.
[303] Leenaars PP, Hendriksen CF, Koedam MA, Claassen I, Claassen E. Comparison of adjuvants for immune potentiating properties and side effects in mice. Vet Immunol Immunopathol 1995 Sep;48(1-2):123-38.
[304] Bennink JR, Yewdell JW, Smith GL, Moller C, Moss B. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. Nature 1984 Oct 11-17;311(5986):578-9.
[305] Ito R, Ozaki YA, Yoshikawa T, Hasegawa H, Sato Y, Suzuki Y, et al. Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. Vaccine 2003 Jun 2;21(19-20):2362-71.
[306] Mayr A, Stickl H, Muller HK, Danner K, Singer H. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]
Der Pockenimpfstamm MVA: Marker, genetische Struktur, Erfahrungen mit der parenteralen Schutzimpfung und Verhalten im abwehrgeschwachten Organismus. Zentralbl Bakteriol [B] 1978 Dec;167(5-6):375-90.
[307] Stickl H, Hochstein-Mintzel V. [Intracutaneous smallpox vaccination with a weak pathogenic vaccinia virus ("MVA virus")]
Die intrakutane Pockenimpfung mit einem schwach virulenten Vakzinia-Virus ("MVA-Virus". Munch Med Wochenschr 1971 Aug 27;113(35):1149-53.
[308] Werner GT, Jentzsch U, Metzger E, Simon J. Studies on poxvirus infections in irradiated animals. Arch Virol 1980;64(3):247-56.
[309] Harrer E, Bauerle M, Ferstl B, Chaplin P, Petzold B, Mateo L, et al. Therapeutic vaccination of HIV-1-infected patients on HAART with a recombinant HIV-1 nef-expressing MVA: safety, immunogenicity and influence on viral load during treatment interruption. Antivir Ther 2005;10(2):285-300.
[310] Dorrell L, Yang H, Ondondo B, Dong T, di Gleria K, Suttill A, et al. Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant modified vaccinia virus Ankara/HIV-1 Gag vaccine. J Virol 2006 May;80(10):4705-16.
[311] Vollmar J, Arndtz N, Eckl KM, Thomsen T, Petzold B, Mateo L, et al. Safety and immunogenicity of IMVAMUNE, a promising candidate as a third generation smallpox vaccine. Vaccine 2006 Mar 15;24(12):2065-70.
[312] WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_02_28/en/index.html. accessed March 3, 2008, 2008.
[313] WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic Vaccines - http://who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf. 2007.
[314] Ruat C, Caillet C, Bidaut A, Simon J, Osterhaus AD. Vaccination of macaques with adjuvanted formalin-inactivated influenza A (H5N1) vaccines: protection against H5N1 challenge without disease enhancement. J Virol 2007 Dec 19.
[315] Kalbfuss B, Genzel Y, Wolff M, Zimmermann A, Morenweiser R, Reichl U. Harvesting and concentration of human influenza A virus produced in serum-free mammalian cell culture for the production of vaccines. Biotechnol Bioeng 2007 May 1;97(1):73-85.
[316] Nerome K, Kumihashi H, Nerome R, Hiromoto Y, Yokota Y, Ueda R, et al. Evaluation of immune

responses to inactivated influenza vaccines prepared in embryonated chicken eggs and MDCK cells in a mouse model. Dev Biol Stand 1999;98:53-63; discussion 73-4.
[317] Katz JM, Webster RG. Efficacy of inactivated influenza A virus (H_3N_2) vaccines grown in mammalian cells or embryonated eggs. J Infect Dis 1989 Aug;160(2):191-8.
[318] Webby RJ, Webster RG. Are we ready for pandemic influenza? Science 2003 Nov 28;302(5650):1519-22.
[319] Palese P. Making better influenza virus vaccines? Emerg Infect Dis 2006 Jan;12(1):61-5.
[320] Veits J, Romer-Oberdorfer A, Helferich D, Durban M, Suezer Y, Sutter G, et al. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine 2008 Feb 4.
[321] Sidwell RW. The Mouse Model of Influenza Virus Infection. In: MA ZOaS, editor. Handbook of Animal Models of Infection: Experimental Models in Antimicrobial Therapy. San Diego, CA: Academic Press, 1999: 981-7.
[322] Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). Vet Pathol 2003 May;40(3):304-10.
[323] Uiprasertkul M, Kitphati R, Puthavathana P, Kriwong R, Kongchanagul A, Ungchusak K, et al. Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. Emerg Infect Dis 2007 May;13(5):708-12.
[324] Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, et al. Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005 Jul;11(7):1036-41.
[325] van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. H5N1 Virus Attachment to Lower Respiratory Tract. Science 2006 Apr 21;312(5772):399.
[326] Mathews JD, McCaw CT, McVernon J, McBryde ES, McCaw JM. A biological model for influenza transmission: pandemic planning implications of asymptomatic infection and immunity. PLoS ONE 2007;2(11):e1220.
[327] Tellier R. Review of aerosol transmission of influenza A virus. Emerg Infect Dis 2006 Nov;12(11):1657-62.
[328] Kreijtz JH, Suezer Y, de Mutsert G, van den Brand JM, van Amerongen G, Schnierle BS, et al. Recombinant Modified Vaccinia Virus Ankara Expressing the Hemagglutinin Gene Confers Protection against Homologous and Heterologous H5N1 Influenza Virus Infections in Macaques. J Infect Dis 2009 Feb 1;199(3):405-13.
[329] Rimmelzwaan GF, Sutter G. Candidate influenza vaccines based on recombinant modified vaccinia virus Ankara. Expert Rev Vaccines 2009 Apr;8(4):447-54.
[330] Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, et al. Pandemic Potential of a Strain of Influenza A (H1N1) : Early Findings. Science 2009 May 14.
[331] Kreijtz JHCM, Osterhaus ADME, Rimmelzwaan GF. Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control. Hum Vaccin 2009 May;5(5).
[332] Rimmelzwaan GF, McElhaney JE. Correlates of protection: novel generations of influenza vaccines. Vaccine 2008 Sep 12;26 Suppl 4:D41-4.

SAMENVATTING

Het influenza A virus wordt getypeerd op basis van zijn twee oppervlakte eiwitten: hemagglutinine (HA) waarvan er 16 subtypes zijn, en neuraminidase (NA) waarvan er 9 subtypes zijn. Voorbeelden van influenza A virus subtypes zijn: H1N1, H3N2 en H5N1. Influenza A virussen, oorspronkelijk afkomstig uit watervogels, kunnen mensen infecteren en veroorzaken "de griep". De griepepidemie is een jaarlijks terugkerend fenomeen dat plaats heeft gedurende de winter maanden. Hierbij wordt in Nederland gemiddeld 1% van de bevolking ziek en uiteindelijk overlijden er in ons land elk jaar ongeveer 1000 mensen aan de gevolgen van griep, met name ouderen. Het virus infecteert de cellen van de bovenste luchtwegen (neus, keel en luchtpijp) en veroorzaakt hier een ontsteking die zich onder andere uit in de vorm van luchtwegklachten en veelal gepaard gaat met koorts en een gevoel van algehele malaise. Ouderen en risicogroepen (patiënten met hart- en vaatziekten, diabetes, etc) wordt aangeraden jaarlijks de griepprik te halen, een vaccin dat bestaat uit representatieve humane influenza A/H1N1 en A/H3N2 virussen en een influenza B virus. De componenten van het vaccin dienen jaarlijks onder de loep genomen te worden om na te gaan of ze afdoende matchen met de circulerende influenza virussen. Indien ze teveel verschillen dienen er nieuwe kandidaat-virussen geselecteerd te worden die in het nieuwe vaccin komen.

Op het moment dat een nieuw influenza virus geïntroduceerd wordt in de bevolking door overdracht van dier (bijv vogel of varken) op mens hebben we te maken met een virus dat potentieel pandemisch is. De wereldbevolking heeft geen immuniteit opgebouwd tegen een dergelijk virus en is daardoor extra kwetsbaar voor tijdens een dergelijke uitbraak. In de 20ste eeuw hebben 3 pandemieën plaatsgevonden, in 1918 (Spaanse griep), 1957 (Aziatisch griep) en 1968 (Hong Kong griep). De eerstgenoemde was verantwoordelijk voor meer dan 25-50 miljoen doden, en eiste meer slachtoffers dan de Eerste Wereldoorlog.
In de 21ste eeuw zijn reeds meerdere introducties van nieuwe influenza virus subtypes geregistreerd. Vanaf 2003 zijn er continu introducties van hoog pathogene influenza A/H5N1 virussen in de humane populatie, deze variant wordt ook wel vogelgriep genoemd en is hoofdzakelijk terug te vinden in Zuidoost Azië. Vooralsnog zijn deze virussen niet in staat om effectief van mens op mens over te gaan. In 2003 zorgde een uitbraak van aviaire influenza A/H7N7 virussen in pluimvee voor 89 infecties in mensen waaronder een dierenarts die uiteindelijk is overleden aan de gevolgen hiervan.

De meest recente introductie van een nieuw influenza A virus vond plaats in het voorjaar van 2009 in Mexico. Het nieuwe influenza A/H1N1 virus, een reassortant van 3 virussen, is overgedragen van varken op mens en heeft zich binnen korte tijd aangepast aan zijn nieuwe gastheer. Binnen 3 maanden zijn meer dan 52.000 mensen geïnfecteerd geraakt waarvan er meer dan 230 zijn overleden. Het virus is in staat om effectief van mens op mens over gedragen te worden en op 11 juni 2009 kondigde de Wereldgezondheids Organisatie fase 6 af, wat inhoudt dat de eerste influenza pandemie van de 21ste eeuw een feit is.

Heterosubtypische immuniteit

De humane bevolking heeft geen antistoffen opgebouwd tegen deze nieuwe influenza stammen zoals hierboven beschreven en is daardoor meer vatbaar voor infectie met deze virussen. Eerdere infectie(s) met influenza A virussen tijdens de normale griepepidemieën kan een zekere mate van bescherming bieden tegen een vervolg infectie met een nieuw influenza virus zoals de vogelgriep of de Mexicaanse griep. Deze vorm van immuniteit wordt ook wel heterosubtypisch immuniteit genoemd. Het is bekend dat T cellen, op basis van herkenning van geconserveerde delen van het influenza virus in staat zijn om te kruisreageren met virussen van bijvoorbeeld het H5N1 subtype.

In hoofdstuk 2 hebben we heterosubtypische immuniteit gedemonstreerd in een model met muizen die geïnfecteerd worden met twee verschillende subtypes griepvirussen waarvan de interne eiwitten hetzelfde zijn. Ook het nucleoproteine (NP) van deze virussen is identiek en bevat het immunodominante cytotoxische T cel (CTL) epitoop NP366-374, gerestricteerd door H2-Db. CTL responsen tegen dit epitoop werden uitgelezen met behulp van tetrameren gebaseerd op een MHC – NP366-374 complex. C57Bl/6J (H2-Db) muizen werden geïnfecteerd met influenza A virus X-31 (H3N2) en vervolgens met influenza virus A/Puerto Rico/8/34 (H1N1) dat lethaal is voor muizen. De X-31 geïnfecteerde dieren waren beschermd tegen de secundaire infectie in de afwezigheid van kruisreagerende antilichamen. Bescherming correleerde met een significant sterkere inductie van virus-specifieke CTL na challenge infectie. De effectiviteit van een heterosubtypische immuun respons geïnduceerd door infectie met een humaan influenza A virus (H3N2) tegen een hoog pathogeen influenza A/H5N1 virus hebben we uitgezocht zoals beschreven in hoofdstuk 3. Daarin wordt beschreven hoe infectie met het humane influenza virus

A/Hong Kong/2/68 muizen beschermd tegen sterfte als gevolg van een secundaire infectie met het lethale influenza H5N1 virus A/Indonesia/5/05. Ondanks dat de dieren wel ziek werden waren ze in staat het virus te klaren en te overleven. Expansie van virus-specifieke CTL (o.a. uitgelezen met tetrameren), kruisreagerend met het H5N1 virus, was aantoonbaar in de overlevende dieren. Transfusie van serum van een beschermde muis naar een naïeve muis beschermde het dier niet tegen infectie. Effectieve vaccinatie tegen humaan influenza A virus voorkomt infectie en zou daarom ook kunnen interfereren met de inductie van virus-specifieke T cellen en heterosubtypische immuniteit. In hoofdstuk 4 wordt een muis model beschreven waarin we aantonen dat vaccinatie tegen humaan influenza A/H3N2 virus interfereert met de inductie van (kruisreagerende) virus-specifieke CTL. Met het wegvallen van deze cellulaire immuniteit waren de dieren niet meer beschermd tegen een secundaire infectie met het H5N1 virus.
Bovenstaande studies demonstreren dat bescherming op basis van heterosubtypische immuniteit correleert met de expansie van kruisreagerende virus-specifieke CTL. In het 5e hoofdstuk wordt beschreven dat humane CTL, gericht tegen en gestimuleerd met humaan influenza A virus in staat zijn om, cellen die eiwitten van hoog pathogeen influenza A/H5N1 virus tot expressie brengen, te herkennen en te elimineren en ze kunnen cellen geïnfecteerd met het H5N1 virus herkennen. Het is dus goedwel mogelijk dat mensen met een geschiedenis van humane influenza A virus infectie(s) over een bepaalde mate van T cel immuniteit beschikken die tot op zekere hoogte bescherming zou kunnen bieden tegen infectie met heterosubtypische influenza virussen (bijv. H1N1 of H5N1).

Vaccinatie tegen de griep zou kunnen interfereren met de inductie van heterosubtypische immuniteit zoals is beschreven in hoofdstuk 4. Daarom is het wenselijk om de aanbeveling, om alle kinderen in de leeftijd van 6-59 maanden te vaccineren, zoals die er ligt in de VS en in verschillende Europese landen te herzien. Jaarlijkse vaccinatie tegen humane influenza virussen zou namelijk kunnen resulteren in een populatie die volledig naïef is voor influenza virussen en daarmee extra vatbaar wordt voor nieuwe influenza stammen die worden geïntroduceerd in de humane populatie. Idealiter bevatten vaccins ook componenten die heterosubtypische immuniteit induceren waarmee ze een bredere bescherming zouden kunnen bieden tegen nieuw (pandemische) influenza virussen.

Influenza A(H5N1) vaccins gebaseerd op MVA

Met het oog op de Mexicaanse grieppandemie en de dreiging uitgaande van andere potentieel pandemische virussen zoals H5N1 is het duidelijk dat, om de wereld te beschermen tegen dit soort virussen, de ontwikkeling van veilige en effectieve vaccins die snel en op grote schaal geproduceerd kunnen worden hoge prioriteit heeft. Vaccins gebaseerd op het MVA-platform zijn aantrekkelijk pandemische influenza vaccin kandidaten. MVA staat voor Modified Vaccinia virus Ankara, een gemodificeerd replicatie-deficiënt pokkenvirus dat gebruikt kan worden als vehikel voor 'vreemde' antigenen.

In hoofdstuk 6 is de evaluatie van de MVA-H5N1 kandidaat vaccins: MVA-HA-Hong Kong/156/97 (MVA-HA-HK/156) en MVA-HA-Vietnam/1194/04 (MVA-HA-VN/04) beschreven. Deze vaccins zijn gebaseerd op een MVA vector die het HA-gen van de verschillende H5N1 virussen tot expressie brengt. C57Bl/6J muizen ontvingen twee vaccinaties en werden vervolgens, 4 weken na de 2e vaccinatie geïnfecteerd met antigeen-verschillende H5N1 virussen: A/Hong Kong/156/97 (clade 0), A/Vietnam/1194/04 (clade 1) of A/Indonesia/5/05 (clade 2.1). Vaccinatie met MVA-HA-HK/97 induceerde alleen homologe bescherming terwijl de dieren die twee shots met MVA-HA-VN/04 hadden gehad beschermd waren tegen alle drie de virussen. Deze veelbelovende resultaten waren de aanleiding voor een vervolg experiment met het beste kandidaat vaccin, MVA-HA-VN/04, in cynomolgus makaken. Deze dieren kregen ook twee vaccinaties waarna ze geïnfecteerd werden homoloog of heteroloog H5N1 virus zoals beschreven in hoofdstuk 7. De gevaccineerde dieren waren beschermd tegen de infectie met beide virussen.

De doses van MVA-HA-VN/04 zoals deze gebruikt zijn in hoofdstuk 6 en 7 zijn relatief hoog ($\geq 10^8$ pfu). De haalbaarheid van dosisverlaging en de inductie van immuniteit na één vaccinatie staat beschreven in hoofdstuk 8. Twee vaccinaties met een dosis 10.000x lager dan zoals gebruikt in hoofdstuk 6 en 7 waren nog steeds effectief in de inductie van bescherming dit gold tevens voor een enkele vaccinatie met een hoge dosis (10^8 pfu). Het gebruik van MVA-HA is dus een veelbelovende optie op momenten dat dringend beschermende immuniteit verworven dient te worden zoals bijvoorbeeld tijdens een pandemie. Desalniettemin, mocht er te weinig vaccin zijn voor een wereldwijde vaccinatie campagne gebaseerd op één vaccinatie met een hoge dosis dan is er de mogelijkheid om twee keer te vaccineren met een la-

gere dosis, tenminste als er genoeg tijd is voor de ontwikkeling van beschermende immuniteit. De minimale eisen voor MVA-HA vaccins in mensen dienen uitgezocht te worden in klinische trials. De vaccins dienen dan geproduceerd te worden in cellijnen die geschikt zijn voor het maken van vaccins onder GMP condities. De productie zoals die nu plaatsvindt in CEF cellen dient geoptimaliseerd te worden of vervangen te worden door productie in een continue cellijn. Samenvattend, MVA is een veelbelovend platform voor pandemische influenza vaccins zoals we hebben gedemonstreerd voor influenza A/H5N1 virus.

Voor het nieuwe pandemische influenza A/H1N1 virus achten we het aannemelijk dat op MVA-gebaseerde vaccins die het HA-gen van dit virus tot expressie brengen ook veelbelovend zijn. Aangezien het schier onmogelijk is om te voorspellen welke influenza A virus subtypes verantwoordelijk zullen zijn voor toekomstige pandemieën is het een aantrekkelijke strategie om de HA genen van meerdere pandemische kandidaten, influenza A virussen die aantoonbaar op de mens zijn overgedragen zoals: H2N2, H5N1, H7N7, H9N2, te kloneren. Zo kunnen we beschikken over een databank met subtype-specifieke vaccins voordat deze virussen pandemisch worden. Echter, we hebben aangetoond dat niet met elk MVA-H5 vaccin kruis-reagerende immuniteit kan worden geïnduceerd en er bestaat dus het risico dat een vaccin uit de databank niet de antilichamen met de juiste specificiteit induceert. Daarentegen, de pandemische influenza A/H1N1 virussen zijn antigeen homogeen en een enkele vaccin-stam (A/California/04/2009 of A/California/05/2009) zal naar alle waarschijnlijkheid in staat zijn om bescherming te induceren tegen alle circulerende influenza A/H1N1 stammen.

Het is aangetoond dat MVA-vaccins ook in staat zijn om cellulaire immuniteit te induceren. Daarom zou het opnemen van genen coderend voor interne eiwitten zoals NP en M1 van het influenza virus in een recombinant MVA vaccin een attractieve optie zijn voor een vaccin dat kruis-beschermende CTL moet induceren. De toevoeging van de relatief geconserveerde interne eiwitten of andere eiwitten zoals het NA en M2 kan de beschermende capaciteit van een MVA-HA vaccin verbeteren.

Een potentieel probleem met het gebruik van MVA-vaccins is de interferentie van aanwezige anti-vector immuniteit, geinduceerd door vaccinatie tegen pokken of eerder gebruik van recombinante pokken-vectoren, met de inductie van antigeenspecifieke immuniteit. Dit is met name relevant voor vaccinatie tegen humane influenza virussen aangezien deze jaarlijks worden toegepast en uiteindelijk eventuele interferentie zou kunnen leiden tot verminderde vaccin doeltreffendheid. Eerdere

studies hebben echter aangetoond dat de aanwezigheid van anti-vector immuniteit niet teveel stoort met de inductie van immuniteit tegen het recombinante antigeen dat de vector tot expressie brengt. Toch is dit een kwestie die in ogenschouw moet worden genomen in klinische trials met MVA influenza vaccines.

Conclusie

Het werk dat hier besproken is beschrijft de potentie van heterosubtypische immuniteit om te beschermen tegen potentieel pandemische influenza virussen en de preklinische evaluatie van op MVA gebaseerde pandemische influenza vaccin kandidaten. De resultaten van de studies zijn besproken in het context van de huidige pandemie en pandemische dreigingen.

Hoewel de mechanismen van heterosubtypische immuniteit nog niet volledig helder zijn is het zeer aannemelijk dat virus-specifieke CTL gericht op geconserveerde epitopen een belangrijk aandeel hebben in deze immuun respons. Gezien het feit dat aanwezigheid van memory T cellen correleert met versnelde klaring van het virus zou het ook bij kunnen dragen aan de bescherming tegen ernstige ziekte en sterfte bij influenza virus infecties. Naast het positieve effect voor individuele patiënten kan T cel immuniteit ook op populatie niveau een belangrijke rol van betekenis spelen omdat heterosubtypische immuniteit de mate en duur van virus-uitscheiding kan reduceren en hiermee de verspreiding van het virus kan indammen. Op deze manier zou de ernst van een pandemische uitbraak beperkt kunnen worden. De huidige pandemie met het Mexicaanse griepvirus voorziet ons van een unieke gelegenheid om het beschermende effect van heterosubtypische immuniteit tegen een daadwerkelijk pandemisch virus te onderzoeken en daarnaast de onderliggende mechanismes te verduidelijken. Als deze eenmaal uitgekristalliseerd zijn kunnen deze gebruikt worden als aangrijpingspunt voor de ontwikkeling van vaccins die een bredere immune respons induceren. Tot op heden zijn antistoffen tegen het HA de enige maat voor de immunogeniteit die geaccepteerd wordt voor de registratie van vaccins. Het induceren van serologische responsen gemeten aan de hand van deze antistoffen is dan ook de minimale vereiste voor nieuwe pandemische vaccins. Daar staat tegenover dat, wat de onderliggende werkingsmechanismen ook mogen zijn, de inductie van heterosubtypische immuniteit de breedte van de immuunrespons zal vergroten.

Het co-circuleren en parallele introducties van verschillende antigeen verschillende influenza A/H5N1 virussen bemoeilijkt de selectie van één vaccin stam. We hebben

aangetoond dat met behulp van een MVA vaccin dat het HA van één H5N1 virus tot expressie brengt kruisreagerende beschermende immuniteit geïnduceerd kan worden. Tezamen met de overige kwaliteiten van op MVA-gebaseerde vaccins zoals het excellente trackrecord wat betreft veiligheid in de mens en de goede immunogeniteit, rechtvaardigen verdere evaluatie van deze vaccins in klinische trials. Aangezien beschermende immuniteit kon worden verkregen met lage doses zou het wel eens een vaccin kandidaat kunnen zijn voor massale vaccinatie campagnes. Oog in oog met een pandemische uitbraak zou beschermende immuniteit idealiter geïnduceerd worden met één enkele vaccinatie en we hebben aangetoond dat dit mogelijk is met het MVA-HA vaccin.

Heterosubtypische immuniteit zou aan de basis kunnen staan van de ontwikkeling van nieuwe vaccins en interventie strategieën en MVA is een veelbelovend influenza vaccin-platform. In de toekomst zullen deze ontwikkelingen bijdragen aan het inperken van het aantal mensen dat ziek wordt en sterft als gevolg van (pandemische) influenza.


Vaccine
Celebrating 25 Years of Publication
TICKET
BIG AIR 7 OKT '07
ZON. 27 MEI 2007
Pinkster Par-t
XFACTOR
WASA COMPLOT
Zijn de foto's van de maanlanding van de Apollo vervalst?
GRATIS Verzame band
THE BRO CODE
how I met your mother
STAR WARS
RKVV ERP-PSV
DJBROADCAST
Quentin Tarantino Presents
dogs
J. K. ROWLING
HARRY POTTER
AND THE DEATHLY HALLOWS
Pocket Guide

ABOUT THE AUTHOR

Curriculum Vitae

The author of this thesis was born on the 26th of June 1982 in a little town called Vorstenbosch and after a few years moved with his family to Uden. In 2000 he finished his high school (Kruisheren Kollege, Uden) and started his study in Biomedical Sciences at the Radboud University in Nijmegen. He specialized in pathobiology and during his bachelor internship his research focussed on the characterization of DNA coating for titanium bone-implants at the department of Biomaterials at the University Medical Centre Radboud in Nijmegen. During his master phase he did an extra internship on tissue-engineering at the department of Matrix Biology at the Nijmegen Center for Molecular Life Sciences. There he worked on the incorporation of growth factors in collagen skin grafts and the development of collagen films. His master research project he performed at Organon Biosciences (Merck, Oss, the Netherlands) where he studied the effect of glucocorticoids on human lymphocytes. He graduated in 2005 and immediately started as a PhD student at the department of Virology in the Erasmus Medical Center in Rotterdam under supervision of Prof. Dr. Ab Osterhaus and Dr. Guus Rimmelzwaan. His project, focussing on the protective role of heterosubtypic immunity and influenza vaccines against influenza H5N1 virus has resulted in the present thesis. He will continu his work at the department of virology, focussing on the elucidation of heterosubtypic immunity, MVA-based influenza vaccines and the new, pandemic influenza A/H1N1 virus.

Publications

1. **Kreijtz JHCM**, Bodewes R, van Amerongen G, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine 2007; 25:612-20.

2. **Kreijtz JHCM**, de Mutsert G, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 2008; 82:5161-6.

3. **Kreijtz JHCM**, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF. Recombinant Modified Vaccinia Virus Ankara-Based Vaccine Induces Protective Immunity in Mice against Infection with Influenza Virus H5N1. J Infect Dis 2007; 195:1598-606.

4. **Kreijtz JHCM**, Suezer Y, de Mutsert G, van den Brand JMA, van Amerongen G, Schnierle BS, Kuiken T, Fouchier RAM, Löwer J, Osterhaus ADME, Sutter G, Rimmelzwaan GF. Recombinant modified vaccinia virus Ankara expressing HA confers protection against homologous and heterologous H5N1 influenza virus infections in macaques. J Infect Dis 2009; 199:405-13.

5. **Kreijtz JHCM**, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, Kuiken T, Fouchier RAM, Löwer J, Osterhaus ADME, Sutter G, Rimmelzwaan GF. Induction of Protective Immunity in Mice Against Antigenically Distinct Influenza Virus H5N1 Strains With Recombinant MVA-based Vaccine. Options for the Control of Influenza VI. Toronto, Ontario, Canada: Elsevier Science, 2008:195-8.

6. **Kreijtz JHCM**, Osterhaus ADME, Rimmelzwaan GF. Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control. Hum Vaccine 2009; 5:3, 126-35.

7. Rimmelzwaan GF, **Kreijtz JHCM**, Bodewes R, Fouchier R, Osterhaus ADME. Influenza virus CTL epitopes, remarkably conserved and remarkably variable. Vaccine 2009

8. **Kreijtz JHCM**, Suezer Y, de Mutsert G, van den Brand JMA, van Amerongen G, Schnierle BS, Kuiken T, Fouchier RAM, Löwer J, Osterhaus ADME, Sutter G, Rimmelzwaan GF. Preclinical evaluation of a modified vaccinia virus Ankara (MVA)-based vaccine against influenza A/H5N1 viruses. Vaccine 2009

9. Bodewes R, **Kreijtz JHCM**, Baas C, Geelhoed-Mieras MM, de Mutsert G, van Amerongen G, van den Brand JMA, Fouchier RAM, Osterhaus ADME, Rimmelzwaan GF.et al. Vaccination against human influenza virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus. PlosOne 2009; 4(5):e5538

10. **Kreijtz JHCM**, Bodewes R, van den Brand JMA, de Mutsert G, Baas C, van Amerongen G, Osterhaus ADME and Rimmelzwaan GF, Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus. Vaccine 2009

11. **Kreijtz JHCM**, Suezer Y, de Mutsert G, van den Brand JMA, van Amerongen G, Schnierle BS, Kuiken T, Fouchier RAM, Löwer J, Osterhaus ADME, Sutter G, Rimmelzwaan GF, I Single shot MVA-H5 induces protection against influenza A/H5N1 virus infection in mice. Submitted for publication

PhD portfolio

Joost Kreijtz

Research group:	Department of Virology
Research school:	Post-graduate Molecular Medicine
PhD period:	2005-2009
Promotor:	Prof.dr. A.D.M.E. Osterhaus
Co-promotor:	Dr. G.F. Rimmelzwaan

Education

2005-2009	PhD program, Erasmus Medical Center, Rotterdam, the Netherlands. PhD thesis: Protection against influenza A(H5N1) by primary infection with influenza A(H3N2) and MVA-based vaccination
2000-2005	Master of Science, Radboud University, Nijmegen, the Netherlands. Study: Biomedical Sciences: Pathobiology
1994-2000	General High School (Gymnasium), Kruisheren Kollege, Uden, the Netherlands

In-depth courses

- Course in English Biomedical writing and communication. Four-months course provided to PhD students in the Erasmus MC, Rotterdam, the Netherlands (Jan – April 2008)
- Course in Immunology. Two-week international post-doctoral training course in Immunology, provided by the Leiden Institute for Immunology, Leiden, the Netherlands (October 2007)
- Course in Molecular Medicine. International training course in molecular medicine provided by the Post-graduate School Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands (June 2007)
- Course in Virology. One-week international training course in general virology provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC, Rotterdam, the Netherlands (March 2006)
- Presentations by internal and external speakers at the department of Virology (Erasmus Medical Center, Rotterdam, the Netherlands) twice a week (2005-present)

Poster presentations

February 2009	Molecular Medicine day (Rotterdam, the Netherlands)
February 2008	Molecular Medicine day (Rotterdam, the Netherlands)

Oral Presentations

April 2009	Protection against influenza A/H5N1 virus based on heterosubtypic immunity.Wissenschaftliches Kolloquium (Paul-Ehrlich Institut, Langen, Germany)(On invitation of Dr. Y. Süzer)
April 2009	H5N1: Promoveren tussen de vaccins en de vogelgriep virussen. Symposium infectious diseases (UMCG Groningen, the Netherlands)(On invitation of Dr. A. Huckriede)
April 2009	Protection against highly pathogenic avian influenza A/H5N1 virus based on heterosubtypic immunity. Seminar in Virology (UMCG Groningen, the Netherlands)(On invitation of Dr. A. Huckriede)
February 2009	Recombinant MVA-HA confers protection against H5N1 influenza virus infections in macaques. 13th Molecular Medicine day (Groothandelsgebouw, Rotterdam, the Netherlands)(Mol Med school)
September 2008	Recombinant MVA expressing HA confers protection against homologous and heterologous H5N1 infections in macaques. 3rd European Influenza Conference (Villamoura, Portugal)(ESWI)
September 2007	Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. 4th Orthomyxovirus Research Conference (MBL, Woods Hole, Massachusetts, USA)
September 2007	Influenza: Antibodies, CTL and Vaccines. Organon Research meeting (Organon Research Center, Boston, Massachusetts, USA)(On invitation of Dr. A. van Elsas)
April 2007	Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. Joint vaccine meeting(University Library, Utrecht, the Netherlands) (NOW WG Vaccines & Eijkman graduate school)

Grants & Awards

2009	Award for best oral presentation, Molecular Medicine day 2009
2008	ESWI young scientist grant (3rd European Influenza Conference)
2007	4th Orthomyxovirus Research Conference travel grant

Attended

2009

- Dutch Annual Virology Symposium, Amsterdam, the Netherlands (March)
- Molecular Medicine day, Rotterdam, the Netherlands (February)

2008

- Joint vaccine meeting (NOW WG Vaccines & Eijkman graduate school) (November)
- 3rd European Influenza Conference (Villamoura, Portugal)(ESWI) (September)
- Joint vaccine meeting (NOW WG Vaccines & Eijkman graduate school) (April)
- Dutch Annual Virology Symposium, Amsterdam, the Netherlands (March)
- Molecular Medicine day, Rotterdam, the Netherlands (February)

2007

- 4th Orthomyxovirus Research Conference, MBL, Woods Hole, Massachusetts, USA (September)
- Joint vaccine meeting (NOW WG Vaccines & Eijkman graduate school) (April)
- Dutch Annual Virology Symposium, Amsterdam, the Netherlands (March)
- Molecular Medicine day, Rotterdam, the Netherlands (February)

2006

- Dutch Annual Virology Symposium, Amsterdam, the Netherlands (March)
- Molecular Medicine day, Rotterdam, the Netherlands (February)

2005-present

- International seminar series in Virology, Immunology, Cell Biology and Molecular Medicine, provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC

National and international collaborations

- NIVAREC consortium: UMCG, Department of Virology, the Netherlands and Solvay Pharmaceuticals, Weesp, the Netherlands. Collaboration on the development and evaluation of influenza vaccines.
- Prof. Dr. G.A. Sutter and Dr. Süzer, Division of Virology, Paul-Ehrlich Institut, Langen, Germany. Collaboration on the development of MVA-based influenza vaccines

Miscellaneous

- Frequent reviewer for Vaccine
- Inventor on patent application PCT/EP2007/062446
- Supervision of technicians and MSc students during the course of my PhD project


KAMPIOEN
ERLAND
KNVB
2007 - 2008
EREDIVISIE
PSV
PHILIPS


DANKWOORD

Dames en heren, vrienden, kennissen en collega's. Promoveren is een mooie zaak, en al helemaal als het gaat om een onderwerp als influenza. Een onderwerp dat zo breed benaderd kan worden dat er dan ook vele mensen, binnen en buiten ons lab, aan werken en een bijdrage hebben geleverd aan dit boekje. Dat maakt ook dat er op de kaft van dit proefschrift eigenlijk nog wat extra namen zouden mogen staan want zoals het welbekende cliché luidt: promoveren doe je niet alleen, en dus zie hier: het dankwoord.

Om te beginnen wil ik graag mijn promotor Prof. Dr. A. Osterhaus en co-promotor Dr. G. Rimmelzwaan bedanken. Beste Ab, voor mijn sollicitatie-gesprek werd mij al op het hart gedrukt dat ik me moest voorbereiden op een pittig gesprek. Niets was minder waar, tijdens mijn gesprek met jou werd me nog maar weer eens duidelijk dat het een goede zaak was geweest dat ik had gereageerd op de baan van promovendus aan de afdeling Virologie van het Erasmus MC. Je scherpe blik en altijd kritische visie maakt dat je als AIO nooit de context uit het oog verliest en pragmatisch en toepassingsgericht blijft werken. Daarnaast was er de eeuwige strijd tussen Amsterdam en Brabant die, gebaseerd op het aantal kampioensschalen in de afgelopen 4 jaar, duidelijk in het voordeel is voor de laatstgenoemde.
Beste Guus, als echte Ajacied een PSV'er aannemen getuigd van lef. En dan ook nog eens iemand die voorheen bar weinig met virussen had gedaan. Door jou tomeloze enthousiasme en inzicht heb ik het promoveren tussen de influenza virussen als een waar feest ervaren. Altijd hadden we meerdere projecten tegelijk draaien en als het ene paper weg was lag de draft van de volgende alweer klaar. Op maandagmorgen de wedstrijden doorspreken samen met Ron (volgens mij heb ik nog pils tegoed van het seizoen 2006-2007 J) en natuurlijk die legendarische CL wedstrijd Arsenal-PSV met een glansrol voor Alex die we in Bergschenhoek hebben bekeken. Ook wil ik jou, Janet en jullie meiden bedanken voor de gastvrijheid waarmee jullie me altijd hebben ontvangen. Het schrijven van artikelen was en is voor mij een groot leerproces. Langzaam maar zeker blijft er steeds meer overeind en jou onvermoeibare hulp bij het schrijven heeft hiertoe een onmisbare bijdrage geleverd. Bedankt voor alle hulp, adviezen en wijze raad en gezellige pils- en whiskyuurtjes en we gaan het komend jaar vrolijk verder.

Dan mijn paranimfen, Gerrie en Rogier, of moet ik zeggen, de 3 handen op 1 buik. Gerrie, vanaf het begin ben je betrokken geweest bij een aantal van mijn projecten.

Af en toe waren 't mega-titraties en ander stampwerk dat we dan met z'n tweeen doorwerkten. Dan waren het weer FATT-assays en ELIspots die uiteindelijk tot een schitterend paper hebben geleid. Jouw energie en onverschrokkenheid waarmee je grote essays aanpakt zijn bewonderenswaardig, net als je vermogen tot relativeren en het genieten van je welverdiende vakanties. Ook je telefoongesprekken met Charles (alsof je een pasverliefd stelletje hoort) zijn geliefd binnen onze kamer. Bedankt voor al je energie, kennis en kunde en binnenkort kunnen je gaan genieten van een heerlijke lange vakantie J

Rogier, maat, op 1 september 2005 begon ik als AIO en hoorde ik dat twee weken later mijn eerste stagiaire zou beginnen, een dierenarts, werd er duidelijk bijgezegd. De slechte reputatie van dierenartsen op het lab is op jou niet van toepassing. Je pikte alles gruwelijk snel op en met wat pipetteer-lesjes maakte je je 't labwerk snel eigen. Dat je een veelbelovend onderzoeker bent bleek al snel en ik twijfelde daarom ook geen moment toen gevraagd werd of je geschikt zou zijn als AIO. Onze goede samenwerking in het najaar van 2005 konden we dan ook vervolgen vanaf mei 2007 en met veel plezier en succes tot gevolg. We vullen elkaar prima aan en daar waar nodig ondersteunen we elkander. Naast professionele samenwerkingen vormen we ook een prima duo waar het gaat om slappe klets, rare fratsen, het bouwen van mooie feestjes en het wegwerken van onnoemelijke hoeveelheden chips! Bedankt voor je bijdrage aan dit proefschrift en dat we nog maar veel mogen samenwerken.

Geert, ook jij verdient een eigen alinea. Op het gebied van dier-experimenten heb jij me ontzettend veel bijgebracht, zowel qua kennis als kunde. Daarnaast was je altijd gezond kritisch over de opzet en uitvoering van de experimenten. We hebben samen een aantal zeer grote experimenten gedaan waaronder het MVA-experiment in Bilthoven waarvoor ik ook je collega's wil bedanken. Ondanks alle drukte en stress was er altijd ruimte voor een goed gesprek of een vrolijke, al dan niet schunnige noot, wat het werken tot een waar feest maakte.

Dan de vrienden en vriendinnen van de Virologie en de 19e: Robbie, Willem, Koert, Robin, Hans, Stella I, Stella II, Lisette, David, Erhard, Joyce, Maarten, Gijs, Rory, Monique, Werner, Josanne (na je stage bij mij kom je binnenkort weer terug naar de 17^{e}) en natuurlijk alle anderen. Werken met collega's zoals op de 3^{e}, 17^{e} en 19^{e} maakt dat je niet anders dan fluitend naar je werk kan gaan.

Tiny en Nella, als kamer- en groepsgenoten wil ik jullie bedanken voor de goede samenwerking, alle hulp en adviezen en de hele gezellige tijd in de gaafste kamer van het faculteitsgebouw: Ee1771.

Een aantal mensen ontbreken nog en deze wil ik dan ook even persoonlijk bedanken:

Eefje, als Brabantse matties hebben we het vanaf het begin prima met elkaar kunnen vinden. Allebei ADHD, allebei Carnavals-addicts en voor velen misschien iets te aanwezig. Maar niemand kon zeggen dat het saai was als 1 van ons in de buurt was. Hoewel we niet vaak direct hebben samengewerkt was je nooit te beroerd om ff te helpen met sequenties en dergelijke. Ik ben erg blij dat je mijn buurvrouw bent geworden.
Emmie en Vincent, Meneer en mevrouw Munster-de Wit, heb ontzettend veel van jullie geleerd en vele mooie dingen met jullie beleefd, ff een kort resumé: battle for the JID-cover, Mojito's sushi en bubbelen in Villamoura, Full Throttle, Spiritualien-reizen en de altijd mooie verjaardagen. Bedankt en alle succes van de wereld voor jullie USAvontuur.
Na het ene stel nu het andere, Sander en Miranda. Sander, maat van het eerste uur, vele pilsjes, platen en feestjes hebben we samen versleten. En tussendoor nog ff tijd vinden om het over werk te hebben. Ik weet dat je nogal emotioneel bent aangelegd dus ik zal niet te ontroerend worden want ik weet niet of het papier water bestendig is. Miranda, als je niet op Sanders' schoot zat zat je wel in je Mac. Je vermogen om door te blijven gaan ondanks nachtenlang doorhalen en werken heb ik altijd intrigerend gevonden. Af en toe weer een slaap-refill en dan was je weer de oude.
Patrick (uit te spreken als Petturrriiiiik!), altijd bezig met van alles en altijd met van alles bezig. Je huis, timmeren, nieuwe SOPs, je perfectionisme is bewonderenswaardig. Ook met jou vele pilsjes, platen en feestjes versleten. Samen met Sander vormen we een prima House-uitvoering van de Drie Musketiers.
Martin (alter Befhäse), altijd enthousiast en alles over voor anderen. Heb nog nooit iemand zichzelf zo snel een taal eigen zien maken. En de trip naar Keulen was legendarisch.

Imke, onvermoeibaar en altijd enthousiast. Ik herken veel van mezelf in jou en denk dat de afdeling Virologie blij mag zijn met een collega zoals jij.
Corine, onze eerste experimenten hadden veel overlap en daarna waren het vooral

de sociale gelegenheden waar we elkaar troffen met als hoogtepunten onze duo-presentatie op tv (gaaf!) en de trip naar Dakar. Khaossou, merci aussi!
Debby, Lonneke, Judith, Parafine Peter en Thijs,. Ik heb heel veel van jullie mogen leren op het gebied van pathologie. Jullie waren altijd in voor een mooie discussie en een goeie buurt. De nieuwste bekroning op onze samenwerking is de cover op JID.

(Oud)Sinterkerstcommissie-leden, ik wil jullie bedanken voor alle schitterende uren en dagen die we in de filmpjes en feesten hebben gestopt. Was vereerd dat ik het stokje mocht overnemen van Rocco (Willem, bedankt!) en heb het met heel veel plezier gedaan.

De flugroep: Theo, Salin, Juthatip, Pascal, Oanh, Monique, Björn, Kim, Chantal (how are you mate) en natuurlijk Ron. Bedankt voor alle behulpzaamheid, wijze raad, discussies en gezelligheid op Flu-I en II.
Daarnaast wil ik natuurlijk iedereen van de afdeling virologie (3e en 17e) en de bedrijven op de 19e bedanken voor hun bijdrage, hoe groot of hoe klein ook, aan dit proefschrift en aan de schittterende tijd die mijn promotie-periode heeft mogen zijn. Simone, Loubna, Sabine, Wim, Sumeira, Carola, Fernanda en natuurlijk "kan ik niet, hebben we niet, krijg je niet, maar misschien toch wel" – Robert. Ook wil ik de mensen van het EDC en in het speciaal, Ed en Dennis bedanken voor hun ondersteuning bij een aantal van onze experimenten. Graag wil ik ook mijn voorgangers: Theo, Jacco en Femke bedanken voor de erfenis die ze voor me hebben achtergelaten.

Gerd und Yasemin. Vielen Dank für alles was sie getan haben für die MVA-Studien. Es war super in Langen (Dagmar, Melanie, Tobias, Astrid und Yolanda, Danke Sehr) und Ich hoffe das wir nog viel zusammenarbeiten sollen.
Dank ook aan alle leden van het NIVAREC-consortium: de groep van Jan Wilschut aan het UMCG en Solvay Biologicals.

Zonder diploma geen promotie dus wil ik ook graag even wat mensen uit mijn studie-tijd bedanken. Allereerst Andrea, Hans, Hans, Eric en Judith en alle anderen bij de afdeling TDO die mijn afstudeerstage tot een zeer mooie en leerzame periode hebben gemaakt. En dan natuurlijk de vrienden en vriendinnen van de welzeer edele Budblaffers: Anke, Anne, Dennis, Elles, Femke, Fons, Harald, Hub, Jasper, Jentjes, Koos, Lars, Marcel, Rene, Rob en Zairah. De feestjes, Risoul en Serre Chevalier,

brakke college's, milk&fruits, potjes voetbal voor de faculteit, Hou-je en Hoogeveld (alwaar de vonk oversprong tussen mijn lief en mij). In 1 woord: legendarisch.

Ik wil de familie Bongers en Kreijtz bedanken voor hun belangstelling en interesse voor mijn onderzoek, ik ga nu dan eindelijk afstuderen/promoveren. Juul, Joep, Wout en Stijn, bedankt voor de altijd mooie stapavondjes/uitstapjes in Rotterdam.

Uje, Zeland en Volkel de gekste! Roel & Rianne, Evelien & Joost, Marieke & Harben, Maryono & Charlotte, Erik & Kristel, bedankt voor alle interesse de afgelopen jaren. Het was altijd goed om weer omringd te zijn door goede vrienden na een periode hard werken.

Peter, Lenie, Marieke, Marloes, Paul, Paul en Roy. Ik wil jullie bedanken voor alles wat jullie voor Suzanne en mij hebben gedaan en ook ik ben nu van Vorstenbosche Boy, via Udenaar en Rotterdammer dan toch ook een Volkelnaar geworden.

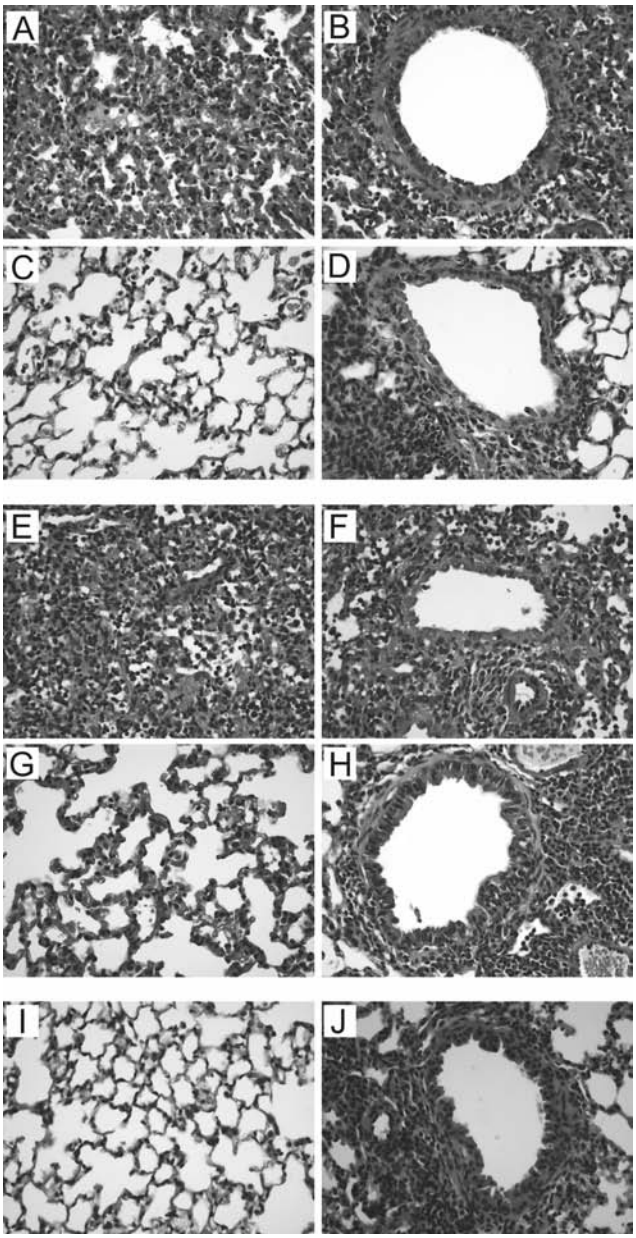
En dan nu de allerbelangrijkste alineas uit dit epistel.
Lieve Floor en Janko, zusje en schoonbroeder. Jullie zijn een heel belangrijk onderdeel van mijn leven. We hebben 't allemaal ontzettend druk en zien elkaar eigenlijk veel te weinig. Maar als we bij elkaar zijn is het altijd één groot feest. Jullie interesse en vrolijkheid hebben mij altijd gestimuleerd in mijn werk en ik ben trots op de manier waarop jullie je carrières zo mooi aan het opbouwen zijn en daarnaast gewoon blijven genieten van het leven. En ons volgende feestje wordt er vast één met een ja-woord.

Lieve Papa en Mama, ons pap en mam. Zonder jullie was er nooit een letter van dit proefschrift op papier gekomen. Jullie hebben mij gemaakt tot wie ik nu ben. Een warm nest en onvoorwaardelijke steun voor alles wat jullie kinderen ondernemen. Jullie hadden de wetenschapper in mij al vroeg ontdekt en hebben deze altijd geprikkeld met bezoekjes aan musea en dierentuinen, tripjes naar Maastricht om fossielen te zoeken en altijd maar zeulen met alle stenen, schelpen en 'dinosauruseieren' die ik weer in het bos had gevonden. Honderden kilometers voor ons op en neer gereden, bulten was verwerkt en altijd een luisterend oor en een helpende hand. Later, op zondagavond nog ff kletsen met ons mam op de bank en dan toch weer te laat te bed. Op vrijdagmiddag gezellig bijbuurten met ons pap in

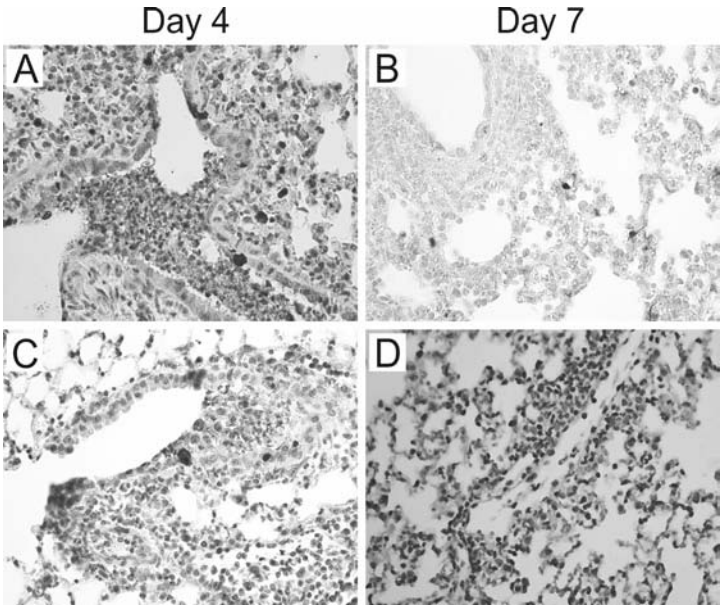
de auto vanuit Rotterdam om dit vervolgens nog eens dunnetjes en lichtelijk luider over te doen aan de keukentafel als Floor, Janko, Suzanne en ik weer eens met z'n allen bij jullie in Uden zijn.
Zoals jullie samen genieten van zowel de grote maar vooral ook kleine dingen in 't leven is inspirerend voor me. Lekker wandelen, een heerlijk stuk chocolade, gewoon een vogeltje dat even op de schutting komt zitten of gewoonweg met z'n zesjes wat eten en buurten. Het is altijd zo vanzelfsprekend en fijn dat jullie er voor ons zijn maar ik wil het bij deze dan toch voor altijd op schrift vastleggen: ik hou van jullie.

Lieve Suzanne, mijn dank voor jou is met geen pen te beschrijven. Ze zeggen dat promoveren zwaar is, en dat zal ook best, maar met jou aan mijn zijde was alle drukte met gemak te relativeren. Altijd had je interesse in m'n nieuwste fratsen op 't lab, lichtelijke bezorgheid was er bij jou als ik wat teveel werkte en me niet helemaal hield aan de (bami)schijf van vijf. Maar bovenal laat je me vaak weten trots op me te zijn en dat voelt ontzettend goed. Ik ben ook ontzettend trots op jou: een nieuwe opleiding die je met zoveel stress begon en met zoveel klasse hebt afgerond om vervolgens meteen een droombaan in de wacht te slepen, gevolgd door nog maar weer een extra opleiding. Heb ontzettend veel respect voor de manier waarop je dit hebt volbracht. Naast al je studies en je werk was 't feit dat we zo ver uit elkaar zaten en allebei druk waren niet altijd even makkelijk: ik in Rotterdam, jij in Volkel, jij vrij, ik werken, of andersom. Maar samen hebben we 't allemaal prima-deluxe volbracht en nu zijn we dan eindelijk samen in ons eigen stulpje. Als jij uit je avonddienst komt nog ff heerlijk samen op de bank en na je nachtdienst zorg ik dat je in een warm bedje terechtkomt. Ik heb het ontzettend fijn met jou en we hebben samen al zoveel gave dingen gezien, gedaan en meegemaakt. Ons nieuwe avontuur begint nu in Volkel en wie weet waar het ons allemaal gaat brengen. Eén ding weet ik zeker: ons avontuur kent geen grenzen want samen kunnen wij de hele wereld aan. Ik hou van je kleine...

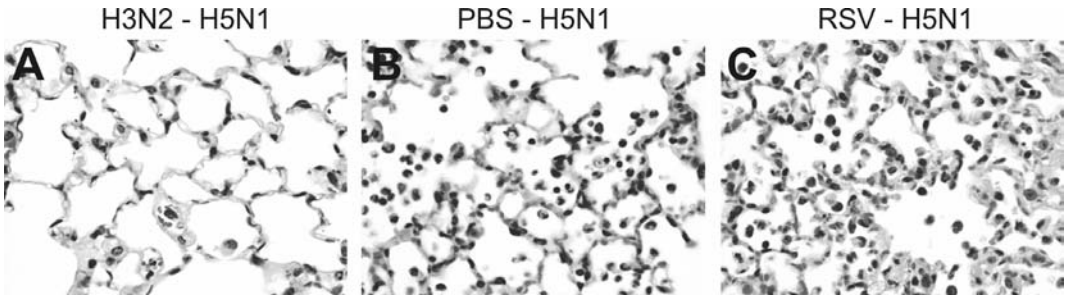

APPENDIX



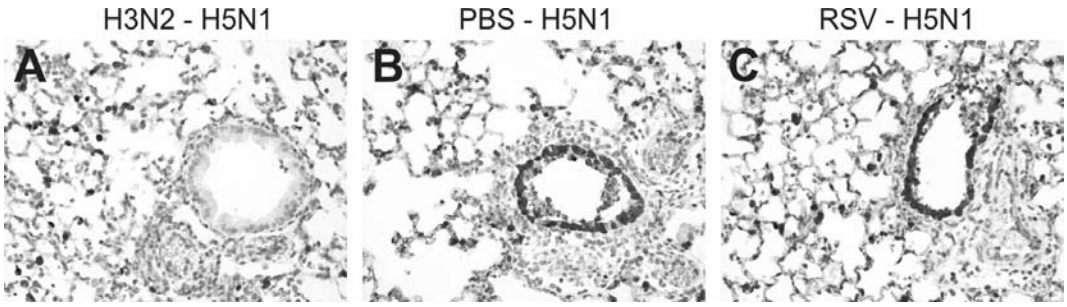
Chapter 2 - Figure 5 Histology of the lungs of mice infected with either influenza virus X-31, influenza virus A/PR/8/34 or both sequentially. A+B: lung of a naive mouse 4 days after PR/8 infection with flooding of the alveoli (A) and peri-broncheolar and –vascular lymphoid infiltrate (B). (C+D) lung of an X-31 experienced mouse 4 days after PR/8 challenge lacking flooding of the alveoli (C) and stronger peri-brocheolar and –vascular lymphoid infiltrate (D). *(E+F)* lung of a naive mouse 7 days after PR/8 infection, The alveoli are filled with cell debri and fluid, and type II pneumocyte hyperplasia is seen in the alveolar walls (E) combined with strong peri-brocheolar and –vascular lymphoid infiltrate (F). *(G+H)* lung of an X-31 experienced mouse 7 days after PR/8 challenge, with mild type II pneumocyte hyperplasia (G) and marked peri-brocheolar and –vascular lymphoid infiltrate (H). *(I+J)* lung of an X-31 experienced mouse on day 28 after PR/8 challenge with normal looking alveoli (I) and a peri-brocheolar and –vascular lymphoid cuff (J).



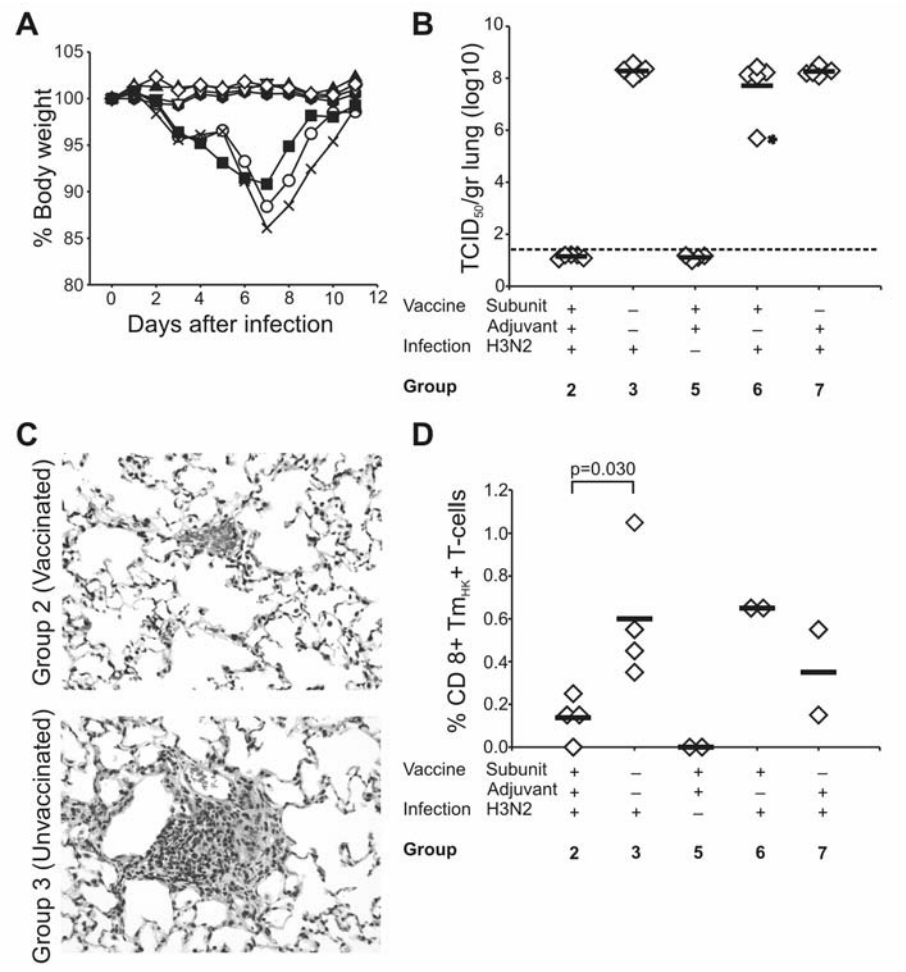
Chapter 2 - Figure 6 Mouse lung sections stained for influenza virus A NP. Cytoplasm of influenza A virus infected cells stains red, the nuclei stain deep red (A) naive mouse on day 4 after infection with influenza A virus A/PR/8/34 with virus antigen positive epithelial cells alining the bronchiole, and positive cells in the alveoli (B) naïve mouse on day 7 p.i.. with virus antigen positive cells in the alveoli (C) X-31 experienced mouse on day 4 after infection with Influenza virus A/PR/8/34 with virus antigen positive cells around the bronchioles (D) X-31 experienced mouse on day 7 p.i. lacking virus antigen positive cells.



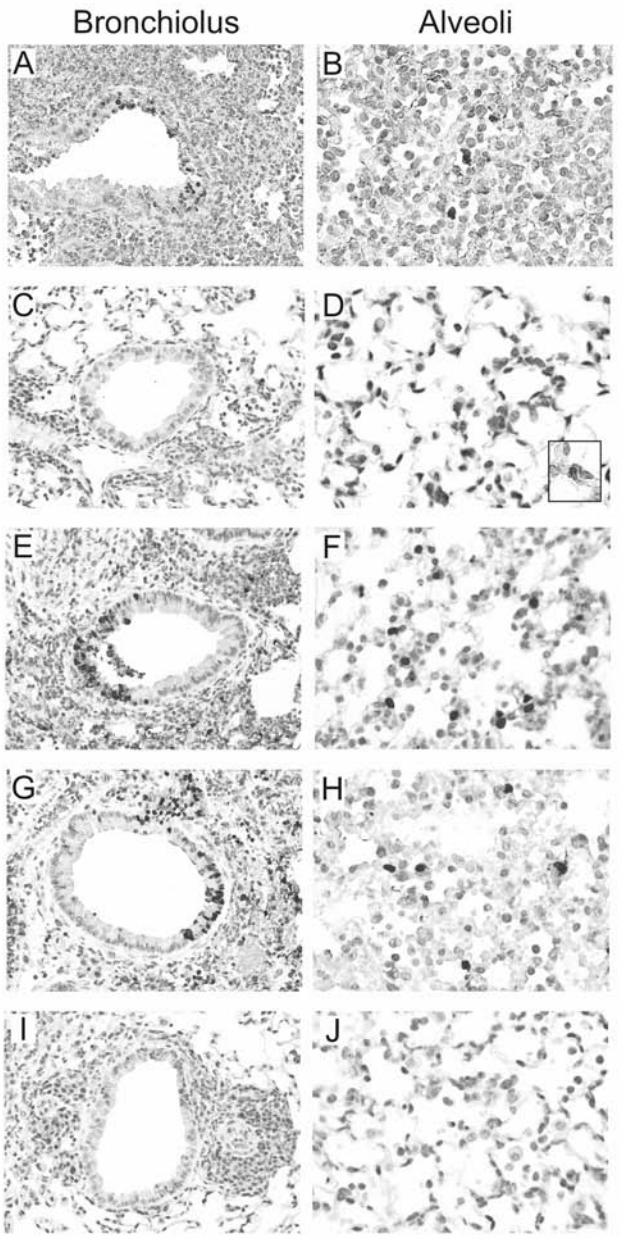
Chapter 3 - Figure 3 Histopathology was examined in the lungs of mice after infection with influenza virus A/IND/5/05. Four days after infection the lungs of H_3N_2-primed animals showed a multifocal mild broncho-interstitial pneumonia with mild inflammatory infiltrates consisting of predominantly lymphocytes 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.



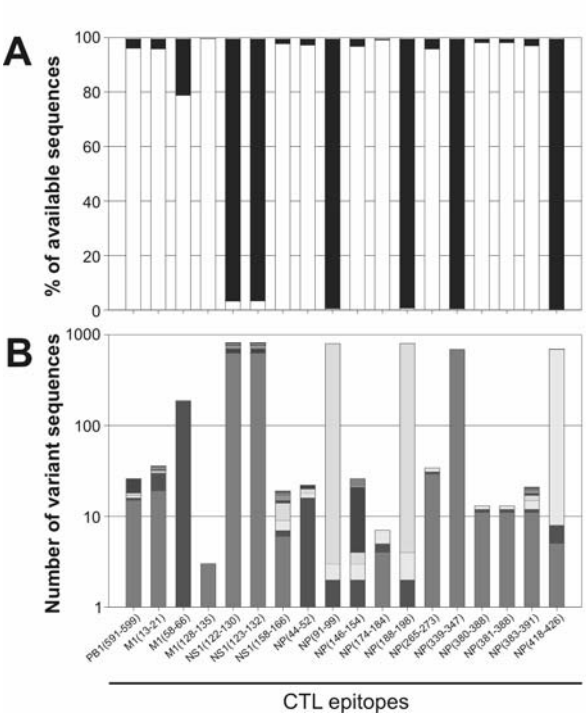
Chapter 3 - Figure 4 Detection of virus-infected cells by immunohistochemistry in the lungs of influenza virus A/IND/5/05 (H_5N_1)-infected mice on day 4 p.i. in mice primed by infection with influenza virus A/HK/2/68 (H_3N_2) *(A)*, mock-infected mice *(B)*, or RSV-primed mice *(C)*.



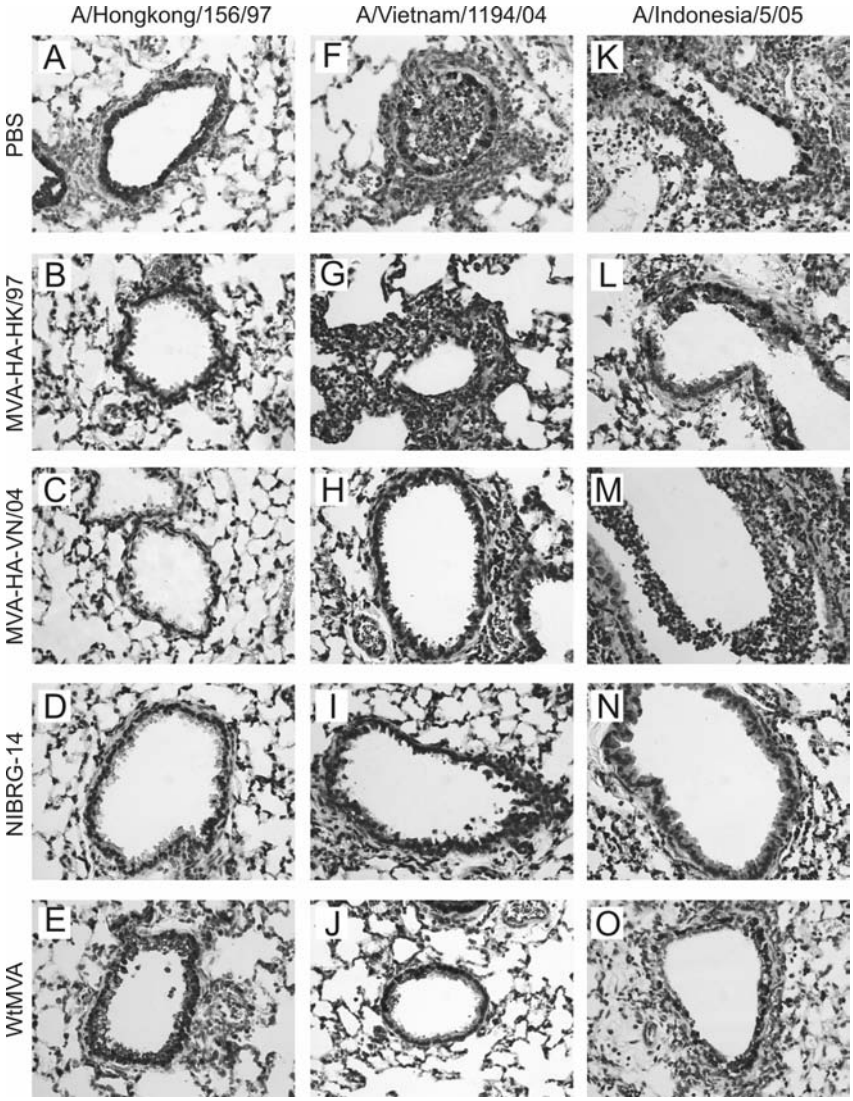
Chapter 4 - Figure 2 Outcome of infection with IAV HK/68 (H_3N_2). Mice were inoculated with IAV HK/68 (groups 2 (▲), 3 (O), 6 (■) and 7 (×)) or PBS (groups 1 (●), 4 (▽) and 5 (◇)). (**A**) Body weight after infection was determined daily and expressed as the percentage of the original body weight before 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 H_2-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).



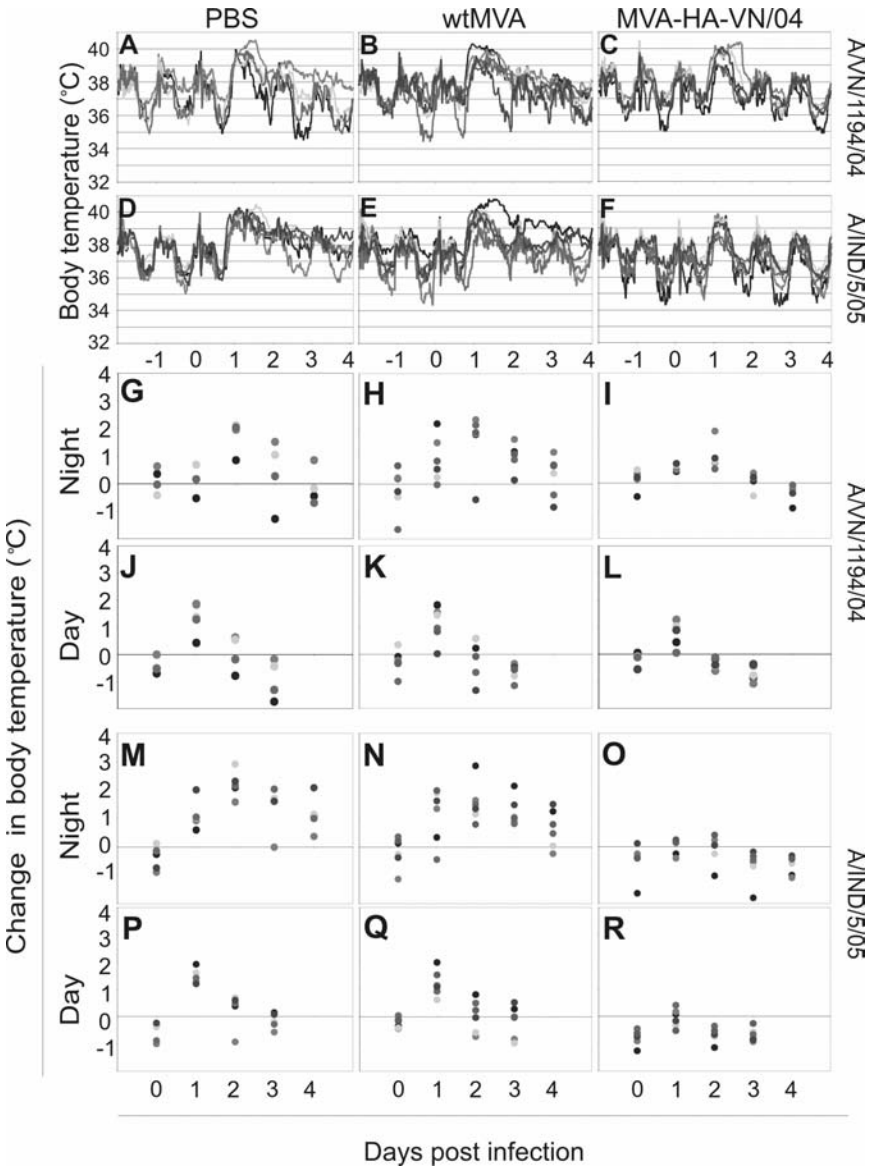
Chapter 4 - Figure 4 Histopathological analysis and immunohistochemistry of the lungs of mice infected with IAV IND/05. Mouse lung sections were stained for influenza A virus nucleoprotein. Cytoplasm of infected cells stain red, the nuclei of infected cells stain deep red. In the groups without a history of productive A/H_3N_2 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). For more information please see text.



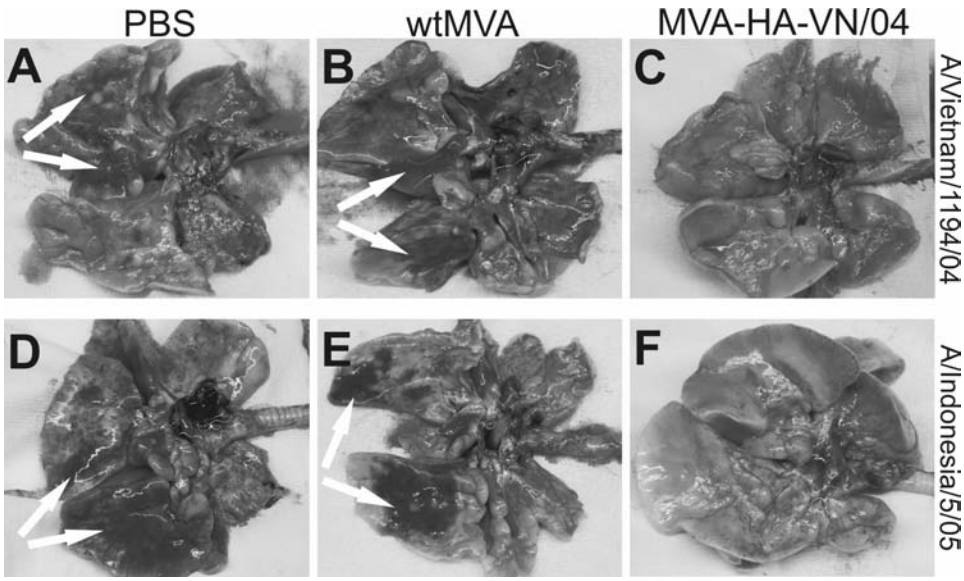
Chapter 5 - Figure 1 The presence of known CTL epitopes in H5N1 strains. The percentage of H5N1 viruses with an epitope sequence identical to human influenza viruses (white bars) is shown in Figure 1. The black bars indicate the percentage of H5N1 viruses with one or more amino acid substitutions in the epitope sequence. The absolute numbers of each variant of an epitope are shown in Figure 1B, each color represents a single variant (sequences can be found in table 1). For this analysis almost 900 H5N1 viruses were analyzed for which sequence information was available in the influenza sequence database [290].



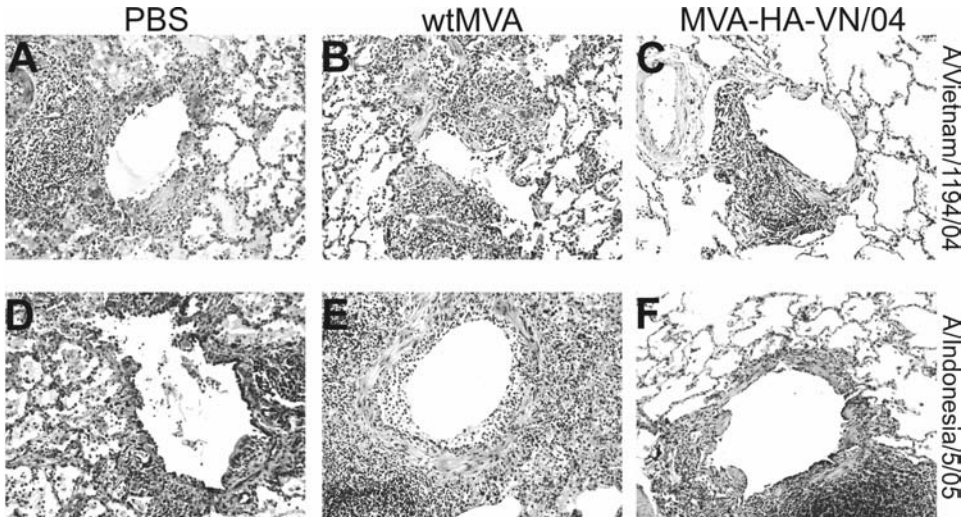
Chapter 6 - Figure 4 Histopathology and immunohistochemistry of the bronchioles and alveoli in lungs of mice infected with either influenza virus A/HK/157/97, A/VN/1194/04 or A/ IND/5/05 as indicated. Influenza virus A/HK/156/97 infection led to viral antigen expression in cells of the bronchiolar wall of PBS (A) and wtMVA immunized mice (E), combined with mild peribronchiolar inflammatory infiltrate, while in the lungs of MVA-HA-HK/97 (B), MVA-HA-VN/04 (C) and Stimune®-adjuvanted NIBRG-14 (D) immunized mice no viral antigen was detected. Infection with influenza virus A/VN/1194/04 resulted in expression of viral antigen in cells of the bronchiolar walls of PBS (F), MVA-HA-HK/97 (G) and wtMVA (J) immunized mice, also combined with moderate peribronchiolar infiltrate (except for the wtMVA immunized mice). No viral antigen expression or morphological changes were detected in MVA-HA-VN/04 (H) and Stimune®-adjuvanted NIBRG-14 immunized mice (I). Infection with influenza virus A/IND/5/05 resulted in abundant viral antigen expression in the bronchioles of PBS (K), MVA-HA-HK/97 (L) and wtMVA (O) immunized mice, combined with moderate peribronchiolar infiltrate. Only minimal viral antigen expression was detected in the bronchiolar wall of MVA-HA-VN/04 (M) immunized mice, combined with moderate inflammatory infiltrate. No viral antigen was detected in the lungs of Stimune®-adjuvanted NIBRG-14 (N) immunized mice after infection with influenza virus A/Indonesia/5/05.



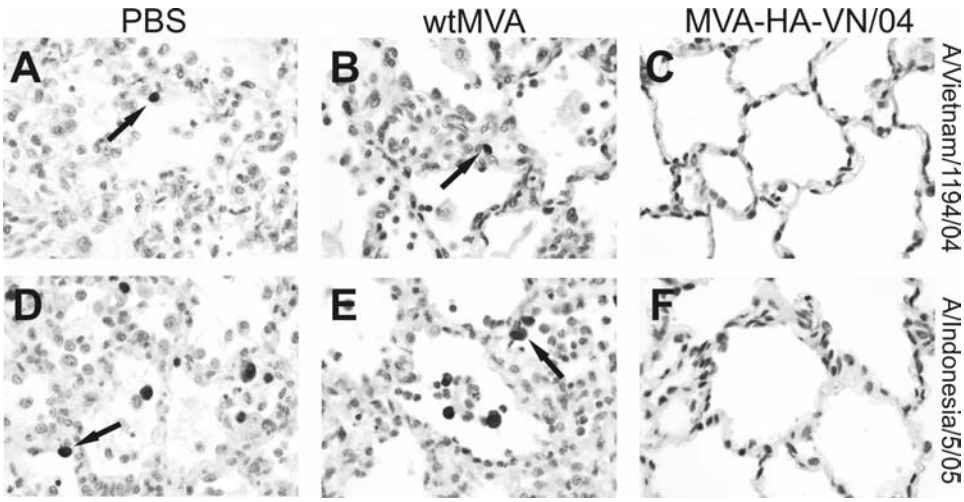
Chapter 7 - Figure 2 Body temperature recorded before and after infection with influenza virus A/Vietnam/1194/04 (A-C) or A/Indonesia/5/05 (D-F). The animals were immunized with PBS, wtMVA or MVA-HA-VN/04 as indicated. Changes in body temperature of individual animals after infection with influenza virus A/Vietnam/1194/04 (G-L) or A/Indonesia/5/05 (M-R) were calculated for each individual animal. Each dot represents an individual animal. Line colors in Figure 2A-C correspond with dot colors in figure 2G-L. Line colors in Figure 2D-F correspond with dot colors in figure 2M-R.



Chapter 7 - Figure 5 Macroscopic lesions of the lungs after infection with H5N1 influenza virus. The lungs of animals immunized with PBS (A, D), wtMVA (B, E) or MVA-HA-VN/04 (C, F) were fixed in formalin on day 4 after infection with influenza virus A/Vietnam/1194/04 (A-C) or A/Indonesia/5/05 (D-F). The arrows indicate consolidated areas present in the lungs of PBS and wtMVA immunized animals after infection (A, B, D, E). Lungs from the MVA-HA-VN/04 immunized animals had no macroscopical lesions (C, F).

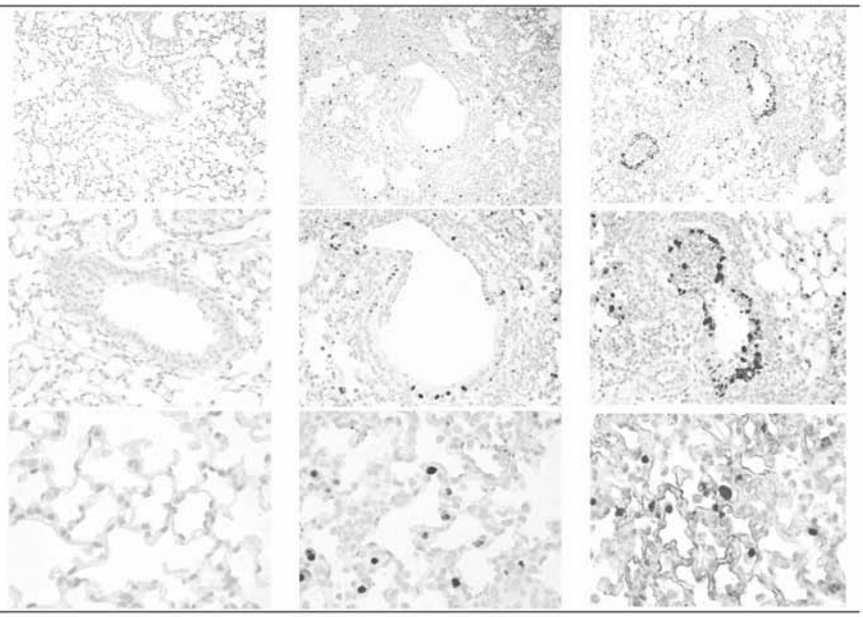


Chapter 7 - Figure 6 Histopathologic analysis of the lungs on day 4 after infection with influenza virus A/Vietnam/1194/04 (A, B, C) or A/Indonesia/5/05 (D, E, F). Histopathological changes were comparable in PBS and wtMVA inoculated animals with extensive lesions in the lungs of these animals. There was mild necrosis, edema, hyperthropy and hyperplasia of type II pneumocytes combined with peribronchiolar and –vascular infiltration. The epithelium of some bronchioles is denuded due to necrosis of the epithelial cells. (A, B, D, E). In the lungs of the MVA-HA-VN/04 immunized animals no histopathological changes were observed (C,F).



Chapter 7 - Figure 7 Detection of virus-infected cells in the lungs four days post infection with H5N1 influenza viruses. Immunohistochemistry was used to stain cells that are positive for the presence of viral antigen showing a deep red staining in the nucleus. Influenza viruses A/Vietnam/1194/04 (arrows indicate single infected cells) or A/Indonesia/5/05 antigen expression was seen in alveolar epithelial cells and some alveolar macrophages of PBS (A, D) and wtMVA (B, E) inoculated animals. No viral antigen was observed in the lungs of MVA-HA-VN/04 immunized animals (C, F).

Classification	None	Moderate	Severe
	normal alveoli normal bronchioles	Alveolar and bronchiolar cells expressing viral antigen	Alveoli and necrotic bronchioles display viral antigen expression and proteinaceous fluid



		None		Moderate		Severe	
	Challenge virus	Vaccine	Dose	Vaccine	Dose	Vaccine	Dose
One immunization	VN/04	MVA-HA-VN/04	10^8	MVA-HA-VN/04	10^4 10^5 10^6	PBS wtMVA MVA-HA-VN/04	n.a. 10^8 10^3
	IND/05	MVA-HA-VN/04	10^8	MVA-HA-VN/04	10^4 10^5 10^6	PBS wtMVA MVA-HA-VN/04	n.a. 10^8 10^3
Two immunizations	VN/04	MVA-HA-VN/04	10^5 10^6	MVA-HA-VN/04	10^4	PBS wtMVA MVA-HA-VN/04	n.a. 10^6 10^3
	IND/05	MVA-HA-VN/04	10^5 10^6	MVA-HA-VN/04	10^4	PBS wtMVA MVA-HA-VN/04	n.a 10^6 10^3

Chapter 8 - Figure 3 Histopathological changes and immunohistochemistry of the lungs after infection with influenza A/H5N1 virus. Representative pictures were selected for the different classifications. Magnification: overview (10x), bronchiole (20x), alveoli (40x).


THE END