Design and validation of novel cross-reactive Influenza B vaccines

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List of abbreviations

A	AA	amino acid
	ADCC	antibody-dependent cellular cytotoxicity
	AF	Alexa Fluor
	APC	antigen presenting cell
B	BM2e	BM2 ectodomain
	B/HA0	Influenza B virus hemagglutinin cleavage loop
C	CDC	Centers for Disease Control
	CFSE	carboxyfluorescein diacetate succinimidyl ester
	CTL	cytotoxic T Lymphocyte
D	DC	dendritic cell
	dpc	days post-challenge
	dpi	days post-infection
E	ELISA	enzyme-linked immunosorbent assay
F	FDA	Food and Drug Administration
G	GCN4	general control non-derepressible 4
H	HA	hemagglutinin
	HBc	Hepatitis B core
	HEK	human embryonal kidney
	HI	hemagglutination inhibition
	HLA	Human leukocyte antigen
I	IAV	Influenza A virus
	IBV	Influenza B virus
	ICS	intracellular cytokine staining
	IFN	interferon
	iBALT	inducible bronchus-associated lymphoid tissue
	IEDB	immune epitope database
	IFA	incomplete Freund's adjuvant
	IL	interleukin
	ILN	inguinal lymph node
	im.	intramuscular
	in.	intranasal
	ip.	Intraperitoneal
	ISG	interferon-stimulated gene

	iv.	intravenous
K	kDa	kilodalton
L	LAH	long α-helix
	LAIV	live attenuated influenza virus
	LPS	lipopolysaccharide
M	M2e	M2 ectodomain
	MDCK	Madin-Darby canine kidney
	MHC	major histocompatibility complex
	MLN	mediastinal lymph node
	MPL	monophosphoryl lipid A
	MW	molecular weight
N	NA	neuraminidase
	NBe	NB ectodomain
	NEP	nuclear export protein
	NK	natural killer
	NP	nucleoprotein
	NS	non-structural protein
P	PA	polymerase acidic
	PAGE	polyacrylamide gel electrophoresis
	PB	polymerase basic
	PBS	phosphate-buffered saline
	pfu	plaque-forming units
	PKR	protein kinase R
R	RBC	red blood cell
	RNA	ribonucleic acid
S	sc.	Subcutaneous
T	TAA	tumor-associated antigen
	TCR	T cell receptor
	tGCN4	tetrameric GCN4
	Th	T helper cell
	TIV	trivalent inactivated vaccine
	TLR	Toll-like receptor
W	WHO	World Health Organization
	WIV	whole-inactivated vaccine

Part 1.

Design and validation of novel cross-protective influenza B vaccines

I. Introduction

Chapter 1

Innovative and novel designs of broad influenza vaccines

1.1. Introduction

Yearly, almost half a million people die and three to five million people become severely ill after an influenza virus infection [1]. Furthermore, infection with influenza A viruses can occur as pandemic outbreaks, which spread rapidly worldwide and are usually more severe and lethal [2]. Despite global surveillance efforts to trace circulating influenza viruses, the predictability of seasonal influenza outbreaks during winter seasons and the availability of fairly effective vaccines, influenza has a huge social and economic impact on society. Influenza vaccination is undoubtedly a cost-effective intervention, and the global influenza vaccine market is a multibillion dollar business [3,4]. Hence, there are strong economical as well as medical incentives for improving currently licensed influenza vaccines. In addition, since their discovery in 1933, influenza viruses and their interactions with the vertebrate host have continued to attract the interest of researchers in virology, immunology and vaccinology [5].

Hemagglutinin (HA) and neuraminidase (NA) are the two membrane glycoproteins of influenza A and B viruses. Both HA and NA are expressed abundantly on the surface of infected cells. On the surface of influenza A and B virions they form spikes that can easily be discerned in electron micrographs [6]. HA allows the virions to bind to human airway epithelial cells *via* surface receptors containing sialic acid residues. Because of their abundance and accessibility, HA and NA draw most of the immunological attention in response to an infection. In accordance with Jenner's approach, licensed influenza vaccines (in use since the 1940s) aim to induce HA-specific and to a lesser extent NA-specific humoral responses in vaccines as occurs during the recovery from natural infection. However, the continuous antigenic variations in HA and NA, known as drift, allow the virus to bypass humoral immunity built up against these antigenic determinants during previous infections or in response to prior influenza vaccination. This antigenic drift makes annual vaccinations necessary for protection against influenza disease. Vaccination is effective when the vaccine antigenically matches the circulating virus strains. Accurate predictions of the epidemic strains that will circulate the next influenza season are sometimes difficult to make, and inaccurate matching might lead to failure of a vaccination campaign. When a new virus subtype emerges, a phenomenon called antigenic shift, or a virus with an HA that is antigenical profoundly different from recent seasonal strains (e.g. the H1N1 2009 pandemic virus), little or no preexisting cross-reactive humoral immunity against HA or NA will be present in the majority of the population and the virus can spread worldwide. Yearly updates of influenza vaccines also challenge the vaccine-industry because it is difficult to produce enough vaccines quickly, and stockpiling is not an option. As a result, only a small part of the population can be actively vaccinated. Vaccines that protect against multiple strains or even subtypes of influenza could avoid several problems: such vaccines could be produced in advance, used in the general population, stockpiled, and they could provide prolonged protection. For these reasons, there is considerable interest in cross-protective or universal vaccines.

1.2. Natural pandemic immunity in humans: lessons from the past

Four major pandemics have plagued man during the past century (Figure 1.1). The Spanish Flu (H1N1) in 1918 was by far the severest, causing 40-50 million deaths worldwide [7]. One should take into account the lack of adequate medical supplies and the lack of antibiotics to treat the frequent secondary bacterial infections, but nonetheless experimental infections of mice, ferrets and cynomolgus macaques with reconstructed 1918 H1N1 influenza virus confirm the unusual virulence of this virus strain. The pandemic of 1957 (H2N2; Asian Flu) and 1968 (H3N2; Hong Kong Flu) were less severe, but still responsible for 1.5 and 0.5 million deaths worldwide, respectively, within a single flu season. Importantly, the latter two pandemics occurred after the identification of influenza virus as the causative agent of influenza, at which time the field of virology was already largely developed, and so valuable immunological and epidemiological data are available about them. For instance, the Cleveland Family Study performed before and during the 1957 pandemic showed that adults, who had been infected with the previously circulating H1N1 virus, were unlikely to exhibit H2N2 influenza symptoms. By contrast, children who had contracted H1N1 in previous years were far less protected against H2N2 infection than adults [8]. This suggests that natural, recurrent pre-exposure to an H1N1 virus over a long period of time can provide protection against a heterosubtypic strain. Epidemiological data from the 1968 pandemic also revealed an interesting aspect of natural heterosubtypic immunity. In Europe and Asia, influenza-related mortality was relatively infrequent during the 1968 pandemic year, but was much more frequent during the second year. It turned out that the 1968 H3N2 pandemic virus had retained the N2 gene of the H2N2 ascendant. The increased mortality observed during the 1969-1970 influenza season coincided with antigenic drift of NA of the H3N2 virus, making it more antigenically distinct from the NA of H2N2 compared to the prior (pandemic) season [9]. This epidemiological observation indicates that also pre-existing anti-NA immunity can temper the severity of a pandemic [10].

The latest establishment of a human-transmissible shifted influenza strain, the 2009 H1N1 pandemic (H1N1/09), provided more detailed information about possible natural cross-protective mechanisms. The most remarkable fact was the lack of intrasubtypical immunity, potentially provided by recurrent seasonal H1N1 vaccination or infection. It soon became clear that trivalent seasonal vaccines did not provide protection against pandemic H1N1/09 infection, based on classic criteria of haemagglutination inhibition (HI) titers [11]. The same study also showed that people over 60 years old contain much higher HI titers compared to people under 30 years old, results which were corroborated by numerous other studies [12-15]. These observations can be linked to the circulation of early 20th century H1 strains, structurally and antigenically closer related to the pandemic virus then more recent strains [16]. Likewise, the contested vaccination campaign aimed at preventing a potential swine flu pandemic in 1976 did offer some protection against the H1N1/09 pandemic [17].

So it seems that recurrent infections or vaccinations during interpandemic periods may provide some degree of heterosubtypic immunity that can mitigate the clinical outcome of infection with a subsequent pandemic influenza strain. However, there needs to be a certain degree of relation between seasonal/vaccination strains and the pandemic virus, be it based on conservation of HA and/or NA epitopes or on shared internal epitopes. However, the nature of the NA subtype of the influenza virus that will cause the next pandemic remains as much an enigma as that of HA, although

apparently NA has a slightly lower drift rate than HA [18]. Are there influenza vaccine approaches that could mimic the level of natural cross-protective immunity induced after recurrent natural exposure? Are there other approaches that could lead to a pandemic influenza vaccine? The answers to these questions require a closer look at the conserved antigens of the influenza virus and finding ways to make them immunoprotective in a safe and effective way.

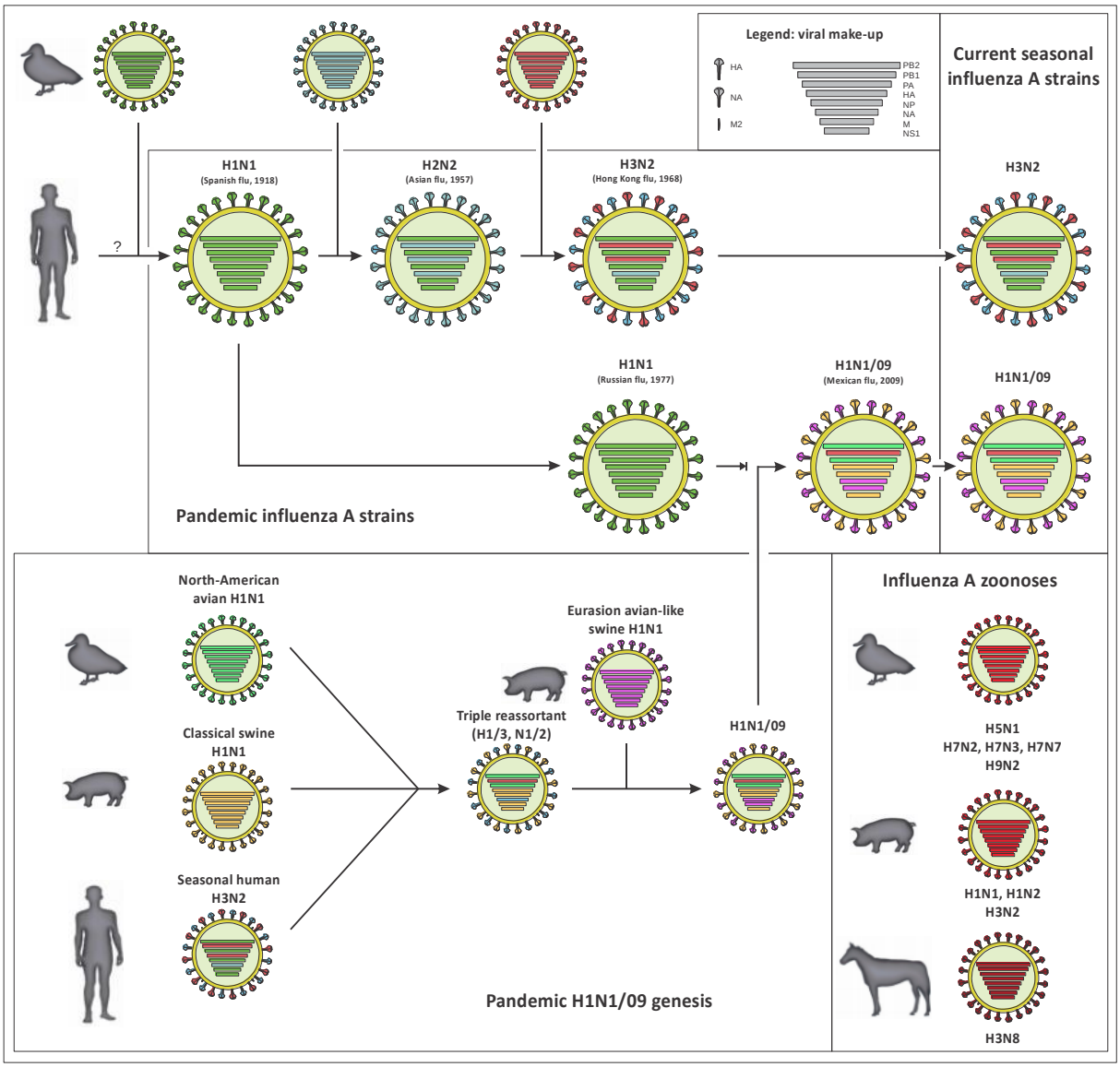


Figure 1.1. Seasonal, pandemic and zoonotic human influenza of the past 100 years. Since 1900, five pandemics have plagued the human population: Spanish flu (H1N1) in 1918, Asian flu (H2N2) in 1957, Hong Kong flu (H3N2) in 1958, Russian flu (H1N1) in 1977 and most recently Mexican flu (H1N1) in 2009. Of these, H3N2 strains related to the Hong Kong flu and H1N1 strains related to Mexican flu currently circulate as seasonal IAV strains in the human population. The Mexican flu originated from reassortment of the triple reassortant swine H1N1 strain and the Eurasian avian-like swine H1N1; the generated strain crossed the species barrier, establishing itself in the human population. Likewise, a number of other swine, equine and avian viruses have infected individuals, but these have been restricted to small-scale zoonotic scares.

1.3. Heterosubtypic immunity and classical vaccines: formulation is critical

Currently, licensed inactivated influenza vaccines contain three different viral strains: influenza A/H1N1, A/H3N2 and one influenza B strain. Vaccines are produced by growing viral seed strains, issued by the WHO, in embryonated chicken eggs; however, alternative production methods based on tissue culture methods are being explored by a number of vaccine manufacturers and research groups [19,20]. Three main methods are available for inactivation of produced viruses. In a first method, virions are partially purified by zonal gradient centrifugation followed by inactivation with formalin or β-propiolactone. Untill the 1970s, whole inactivated virus (WIV) manufactured this way was the main formulation of influenza vaccines. However, although they are generally regarded as more immunogenic [21], WIV are also more reactogenic, particularly in children [22-24]. Therefore, more pure formulations have been produced. In split vaccine formulations, purified virions are treated with detergents or ethyl ethers, leading to inactivation of the virus and disruption of the virionic integrity. This allows further partial purification by mainly centrifugal techniques, leading to a less reactogenic vaccine. If the viral surface antigens HA and NA are purified further to obtain a formulation which is mainly consisting of these proteins, a subunit vaccine is obtained. In all cases, each dose of vaccine should be normalized to contain 15µg of HA for each viral strain used. Main vaccines used today in the Belgian market are the split vaccines Alpharix® (GSK) and Vaxigrip® (Sanofi-Pasteur) and the subunit vaccine Influvac® (Abbott Biologicals).

Despite the notion that immune protection by conventional inactivated influenza vaccines (including split and subunit vaccines) is highly strain-specific, these vaccines can provide more broad protection. A critical parameter is the preservation of the virion structure during the inactivation process, and whole virus vaccines, which are not often used anymore, can provide a considerable degree of heterosubtypic immunity, at least in animal models of human influenza [25]. Formalin-inactivated virus, when administered intranasally without adjuvant, was superior in providing cross-protection than an ether-split vaccine, and even protected mice against an H5N1 challenge (A/HongKong/483/97). The mechanism responsible for this cross-protection is unknown but a role for mucosal IgA antibodies was suggested. The particulate nature of formalin-inactivated vaccines, which have a liposome-like structure, is more stable than ether-split vaccines, less prone to degradation, and has intrinsic adjuvant properties. By contrast, split vaccines are less immunogenic, especially when no adjuvant is included [25]. A more recently evaluated procedure for virus inactivation makes use of the non-toxic photoactivatable alkylating membrane probe, 1,5-iodonaphtylazide (INA). This hydrophobic chemical targets transmembrane domains of proteins and accumulates there. Upon UV-irradiation, membrane proteins and lipids are covalently bound by INA. This leads to inactivation of the targeted transmembrane protein, but the overall structure is preserved and the virion can initiate membrane fusion [26]. Remarkably, INA-inactivated X31 (H3N2) virus elicited protective humoral and cellular responses in mouse comparable to live X31 virus infection and, moreover, was protective against heterosubtypic (H1N1) challenge [27]. These results suggest that application of whole virus vaccines should be considered, perhaps following improvement of currently used chemical inactivation procedures. Whole virus influenza vaccines, however, are not only less pure, but also usually more reactogenic, confirming the saying that a good immune response has its price.

Immunization with inactivated viruses does not elicit strong protective immunity, and high dosing is usually required for an effective response. Particularly, mucosal administration of non-replicating antigens is associated with poor immune responses. A solution to this problem could come from bacterial holotoxins. These represent a bacterial protein family, of which the members have been known since a long time as strong mucosal adjuvants. As their name implies, toxicity has been the main impediment to clinical application, but recent developments might have overcome this problem. Two different molecular approaches have been used to remove toxicity without affecting mucosal adjuvant activity. Bacterial holotoxins are composed of an ADP-ribosylating A subunit that is associated with a pentameric B-domain that binds to gangliosides present on the outer membrane of almost all mammalian cells. Most of the approaches that reportedly prevent toxicity of these bacterial holotoxins were aimed at inactivating the enzyme. For example, an *E. coli* heat-labile enterotoxin detoxified in this way, LT(R192G), was combined with inactivated H3N2 vaccine. When given intranasally to mice, this vaccine induced substantial heterosubtypic protection, even against challenge with the A/HongKong/483/97 virus (H5N1) [28]. Other adjuvants that have been used mainly for parenteral vaccination may also function as mucosal adjuvants. For example, the synthetic toll-like receptor 3 agonist, poly(I):poly(C(12)U), also augments immune responses directed against intranasally administered inactivated vaccine and provides heterosubtypic immunity in mice [29].

From these and other studies it has become clear that the route of administration is a key factor for inducing heterosubtypic immunity, and the same vaccine formulation given parenterally usually does not give the same protection as mucosal delivery [25,28,29]. Since airway mucosal sites are the entry port of influenza virus, protection is most likely achieved by providing a frontline defense in the respiratory tract in the form of local IgG and IgA antibody induction and/or cross-protective T cell responses. Intranasal, oral or even sublingual administration of classical vaccines as well as new, recombinant vaccines, with or without specific adjuvant, could become realistic alternatives for vaccination against influenza and other pathogens [30,31].

Without novel generation adjuvantation systems however, the protection induced by inactivated viruses remains strictly strain-specific. This has been very clearly shown by the Bodewes *et al.* in the group of Guus Rimmelzwaan. Based on the premises that there is evidence that recurrent infection with seasonal influenza can induce heterosubtypic immunity based on cellular immunity [8,32,33] and that vaccination with inactivated viruses does not elicit cellular responses effectively [34], they designed experiments showing that inactivated virus vaccination abrogates induction of natural cross-immunity. Naive mice were immunized with split vaccine [35] or whole inactivated virions (WIV) [36] based on seasonal A/H3N2 virus, or mock-immunized. Following a non-lethal A/H3N2 infection, vaccinated mice were found to be strongly protected from morbidity, while mock-immunized animals developed clear disease systems and lung virus titers. With a subsequent challenge using a potential lethal dose of A/H5N1 virus, previously protected mice succumbed to the infection, while initial mock-immunized mice showing morbidity after the A/H3N2 infection survived with reduced signs of morbidity. This observation could be attributed to the induction of cross-reactive cytotoxic T cell (CTL) responses and shifts in immunodominance of elicited responses. Together with the aforementioned epidemiological data, these results favor the induction of cross-reactive CTL responses to achieve broad protection against influenza infection. The non-neutralizing nature of such immunity does imply infection cannot be blocked; however, it may subside to subclinical level. Moreover, each infection could further boost or broaden cellular immunity to combat antigenically distinct strains.

1.4. The influenza hemagglutinin: revisiting an old foe

Vaccination with inactivated virions evokes strong humoral responses that protect against homologous, *i.e*. serologically matching, virus strains. In the case of influenza, this protection is very narrow, and protection is lost or severely compromised when an antigenically drifted influenza virus infects the vaccinated host. Nevertheless, currently licensed influenza vaccines use inactivated virions or HA-containing subunit preparations with an antigenic profile based on strains which according to WHO guidelines are most likely to cause the next epidemic. Such antigenically matching immunity is largely confined to the viral HA. Also natural humoral immunity against influenza A and B viruses predominantly targets the so-called major antigenic determinants in HA [37-39]. HA-specific antibodies can neutralize the virus by preventing it from attaching to the cell membrane [40]; in addition, these antibodies might interfere with membrane fusion (figure 1) [41]. Such immunity is often referred to as sterilizing immunity, based on extrapolating the *in vitro* activity of HA-specific neutralizing antibodies to influenza virus infection *in vivo*. Indeed, neutralizing antibodies can be detected and measured by a hemagglutination inhibition (HI) or a microneutralization assay. Under such immunological pressure, there is a strong selection of mutant viruses that avoid the virus-neutralizing effect of HA-specific antibodies. The high error frequency of influenza virus RNA polymerase, along with the high degree of antigenic freedom within the immunological determinants of HA, allows seemingly endless cycles of such escape from immune protection.

Though HA is an elusive antigen, it remains a relevant target of vaccination. HA-specific antibodies can act as gatekeepers to prevent influenza infection, because of its pivotal role in the influenza life cycle: attachment of the virus to the host cell, endocytosis of the virus, and fusion of the viral envelope with the cell membrane (figure 1) [39,42]. This has been acknowledged by many, and a number of different strategies have been tested to exploit the HA antigen in a more structured manner (Figure 1.2.). But one critical aspect that should be taken into account is the presence of mainly conformational epitopes within the protein. This was illustrated by Green *et al.*, who showed, using a HA-based peptide library, that although anti-peptide antibodies can recognize HA, anti-HA antibodies do not bind to the linear peptides [43]. This apparent contradiction exemplifies the complexity of the HA molecule and the resulting difficulties to design novel HA-based vaccines with broader efficacy.

1.4.1 Broadly reactive anti-HA responses: deconstructing the antigen

Early studies, based on primary sequence analysis of the HA gene, showed that there are conserved regions in HA. For example, the HA2 subunit, which provides the peptide needed for membrane fusion and release of the viral genetic material into the cytosol [39,44-46], shows sequence and structural conservations even between influenza A and B [47-49]. The group of Peter Palese took advantage of this structural conservation to investigate whether the HA2 subunit can induce protective responses. However, HA1 and HA2 associate tightly within the trimeric molecule. Therefore, to unmask HA2 and present it to the immune system, the membrane-distal HA1 subunit of HA has to be removed. In an

initial study [50], acid treatment combined with chemical reduction or enzymatic cleavage to release the HA1 part from intact virions was used to create 'subviral particles' containing only HA2. When these subviral particles were tested as vaccine antigens in rabbit, the immune sera reacted with the HA2 protein and subviral particles. Moreover, cross-reactivity was observed between HA2 subunits of the H1 and H3 subtypes. However, the antisera did not bind to intact HA-bearing viruses. In accordance with this, the subviral particles did not induce protective immunity against a viral challenge when used to vaccinate mice, indicating that reactivity against a conserved domain or part of HA does not guarantee immune protection.

The value of the membrane-proximal part of HA as the basis for a vaccine might not have been established in early experiments, but a decade later monoclonal antibody (mAb) screenings led to a re-evaluation of its use. Okuno *et al.* set out to identify virus-specific antibodies that recognize different HA subtypes [51]. Using a screening method designed to score *in vitro* inhibition of virus replication [52], they identified a mAb (C179) that neutralized both H1 and H2 viruses. Remarkably, although mAb C179 could neutralize the virus, it did not show any of the HI activity that is generally associated with reactivity towards the globular head of HA. Since neutralization of influenza virus by monoclonal antibodies can only occur in two ways, namely by blocking viral attachment to the host cell or by inhibition of membrane fusion [53], the lack of HI activity indicates that C179 acted by inhibiting membrane fusion. In mapping the binding domains of C179 mAb, Okuno *et al*. identified conserved parts in the stalk region of the protein responsible for its broad binding profile. This finding, along with the protective effect of passive immunization with C179 mAb against viral challenge [54,55], stimulated the search for a vaccine candidate that can be used for active immunization.

More elegant than the chemical approach used to release HA1 [50], Sagawa *et al.* made a genetic HA deletion mutant lacking the immunodominant globular head but retaining the complete HA2 domain and HA1-associated stem regions [56]. Using an H2-derived head-less HA, they were able to induce protection against an H1 virus by using transfected cells as vaccine. Since heat-treated cells did not lead to comparable protection levels, this was the first description of a conformational epitope in headless HA constructs that was capable of inducing broad humoral protection. More recently, the group of Peter Palese also constructed similar 'headless HA' constructs based on direct linking of two cysteine residues in HA1 [57]. Applying their construct directly as a DNA vaccine in combination with purified HIV Gag-based virus-like particles (VLPs), the group demonstrated that an H1-based vaccine can induce cross-reactive immune sera and protection against a homologous infection. The first true soluble HA2-based immunogen was designed by Bommakanti *et al.* [58]. This recombinant molecule, derived from A/Hong Kong/68, mimics the membrane-proximal HA part at neutral pH and can be solubilized after expression in *E. coli*. Intrasubtypic reactivity was seen against heterologous H3 viruses, demonstrating the accessibility of the stalk of HA to neutralizing antibodies.

1.4.2 Digging deeper: antibodies will lead the way

When discussing broad anti-HA responses, one has to keep in mind the phylogenetic grouping of the HA protein. Sixteen different HA subtypes have been identified so far, the large majority only present in bird populations [59]. These subtypes can be discriminated by the binding of immune sera to the HA globular head in double radial immunodiffusion assays, using a set of reference sera. When basing vaccine design on the stalk of the protein, however, other classifications should be applied. The presence of a single glycosylation site at position N38 of subtypes H3, H7, H10 and H15, identified when scrutinizing the binding profile of another stalk-specific neutralizing antibody (CR6261) [60], along with other structural differences, distinguishes two groups of hemagglutinins with differing stem-structures [61]. Group 1 contains the H1-2, H5-6, H8-9, H11-13 and H16 subtypes, while group 2 consists of subtypes H3-4, H7, H10 and H14-15. For most of the known monoclonal antibodies that can bind the stalk region (and deduced vaccine constructs), specificity is restricted to one of the two groups. This has been documented for the group 1 specific mAbs C197 [51,54-56], CR6261 [60,62,63] as well as for other murine and human mAbs [61,64,65].

Similarly, mAbs specific for only group 2 viruses have been isolated. Serial infection of mice with antigenically distinct H3 viruses induced an antibody repertoire with binding profiles restricted to heterologous group 2 viruses [66]. In this study by Wang et al., a set of H3 HA-specific mAbs that were isolated did not show HI activity but could prevent HA-induced cell fusion, which is reminiscent of HA2-specific antibodies [41]. Of particular interest is mAb 12D1, which mapped to a very short region within the HA2 part of the protein, making it seemingly less conformationally restricted than other described HA stalk-specific mAbs. The epitope was reduced to the membrane-distal part of the so-called long α-helix (LAH). The strong conservation of this region, even between the two evolutionarily distinct HA groups with distinct stalk structures, generated a great deal of interest in further validation. Even more so, the apparent inability to isolate escape viruses after 16 passages of virus in the presence of 12D1 is very promising from a vaccine-design point of view.

The minimal sequence needed for optimal binding of the 12D1 mAb was pinpointed to the region of amino acids 76–130 of HA2, encompassing the entire long alpha helix (LAH) of HA [67]. In an attempt to induce 12D1-like IgG responses, the LAH was synthesized, acetylated to expand the *in vivo* half-life, and coupled to keyhole limpet hemocyanin (KLH) using a tagged sequence as spacer arm. This prototype vaccine design proved to be effective: the vaccine was very immunogenic when used in a vaccination-boost scheme in mice together with Complete Freund as adjuvant, and it elicited antibody responses with reactivity towards the HA2 domains of H1, H2, H3, H5 and H7 subtypes. Challenging immunized mice with a lethal dose of H1, H3 or H5 viruses revealed a fair degree of protection compared to controls. Remarkably, the universal character of the LAH domain surpassed that of the HA2 domain when antisera raised to both were probed against a wide range of HA proteins. This shows that even the HA2 domain, which is antigenically subdominant to HA1, can mask more conserved regions within its own structure.

1.4.3 Prevalence of cross-reactive humoral anti-HA memory in the population

Licensed influenza vaccines are poor inducers of cross-reacting antibodies. The emergence of the pandemic H1N1/09 provided a unique opportunity to quantify the induction of cross-reactive immunity by seasonal vaccination or by previous infection to this strain, antigenically new for the majority of the human population. Based on studies on human sera performed by the Centers for Disease Control and Prevention, it deemed unlikely that vaccination with seasonal trivalent inactivated vaccines (TIV), both adjuvanted and non-adjuvanted, or live attenuated vaccines (LAIV), used during the flu seasons 2006-2009, had induced a protective cross-reactive antibody response against H1N1/09 virus [68]. Using HI and microneutralization assays, which allow determination of the standard correlates of protection for human influenza vaccines, it became clear that most elderly people (but not children or young adults) had fairly high titers of serum antibodies reacting with the H1N1/09 virus, regardless of their vaccination status [14,69,70]. This finding may explain why fewer cases of laboratory-confirmed pandemic H1N1 infections were reported for this age group. Presumably, these cross-protective antibodies were generated during infections in early live with a virus that was genetically similar.

Based on a detailed analysis of plasmablasts and monoclonal antibodies isolated from nine adult patients who had been infected with H1N1/09, there is some evidence that antibodies directed against conserved regions of HA are dominant following infection with pandemic H1N1 virus [71]. These findings support the idea of constructing a “pan-influenza” or even “universal” vaccine that protects against diverse viral strains by means of neutralizing antibodies directed against conserved regions in HA. In this regard, it would be possible to score their effectiveness in the same way that is used now for the TIV, *i.e*. using HI and microneutralization. For neutralizing antibodies, HI is still the gold standard correlate of protection [72]. Universal HA-based vaccines would simplify administration and acceptance by the FDA and would allow the pharmaceutical industry to evaluate a new vaccine in the existing system of scoring protective efficacy.

1.4.4 The hemagglutinin fusion peptide: the weak spot of the virus

The HA of influenza is a homotrimer, which binds to sialic acid receptors on the host cells, followed by virion internalization and acidification of the vesicle. This leads to a conformational change, resulting in membrane fusion and entry of the viral core into the cytosol. For this to occur, the mature HA (HA_0) needs to be cleaved into two subunits, HA_1 and HA_2. The N-terminal HA_1 subunit is larger and bears the hypervariable immunodominant regions, which are targeted by virus-neutralizing antibodies. The C-terminal HA_2 component anchors the protein in the viral or cellular membrane. Upon cleavage of HA_0, the N-terminal part of HA_2 (the fusion peptide) is freed and becomes available for fusion of the viral and endosomal membranes under low pH conditions [73].

To be accessible for proteases, the HA cleavage peptide has to be exposed [74]. Sequence comparison of this posttranslational maturation site of HA reveals that it is strongly conserved in all influenza A subtypes. For influenza B, which is not subdivided in subtypes but in two antigenically distinct

lineages (the B/Victoria/2/87 and B/Yamagata/16/88 lineage), the cleavage site has a high state of conservation across both lineages [75]. The fusion peptide appears to be conserved even across influenza types A and B, since there are only two amino acid differences between the consensus sequence of types A and B [75]. Two studies focused on the protective potential of interference with the cleavage site or fusion peptide of influenza A [75,76] or influenza B [75]. In the latter study, it was shown that antibodies against the influenza B cleavage peptide could lead to broad-spectrum protection against influenza B viruses of both lineages, while partial heterosubtypic antibody-based protection could also be obtained with the A/H3-based vaccine against influenza A/H1 infection. Furthermore, partial protection was even obtained against influenza B challenge by using the A/H3 vaccine. Of note, infection of humans or mice does not significantly elicit antibodies directed to the fusion peptide or cleavage site [75], reminiscent of the absence of natural M2e-specific humoral immunity. The structural constraints of the fusion peptide make it unlikely that immune pressure induced by vaccination would lead to escape mutants, as indicated by mutational studies [77]. These considerations suggest that the fusion peptide could be a valid component for development of a broad-spectrum vaccine, possibly with a true cross-typic universal character.

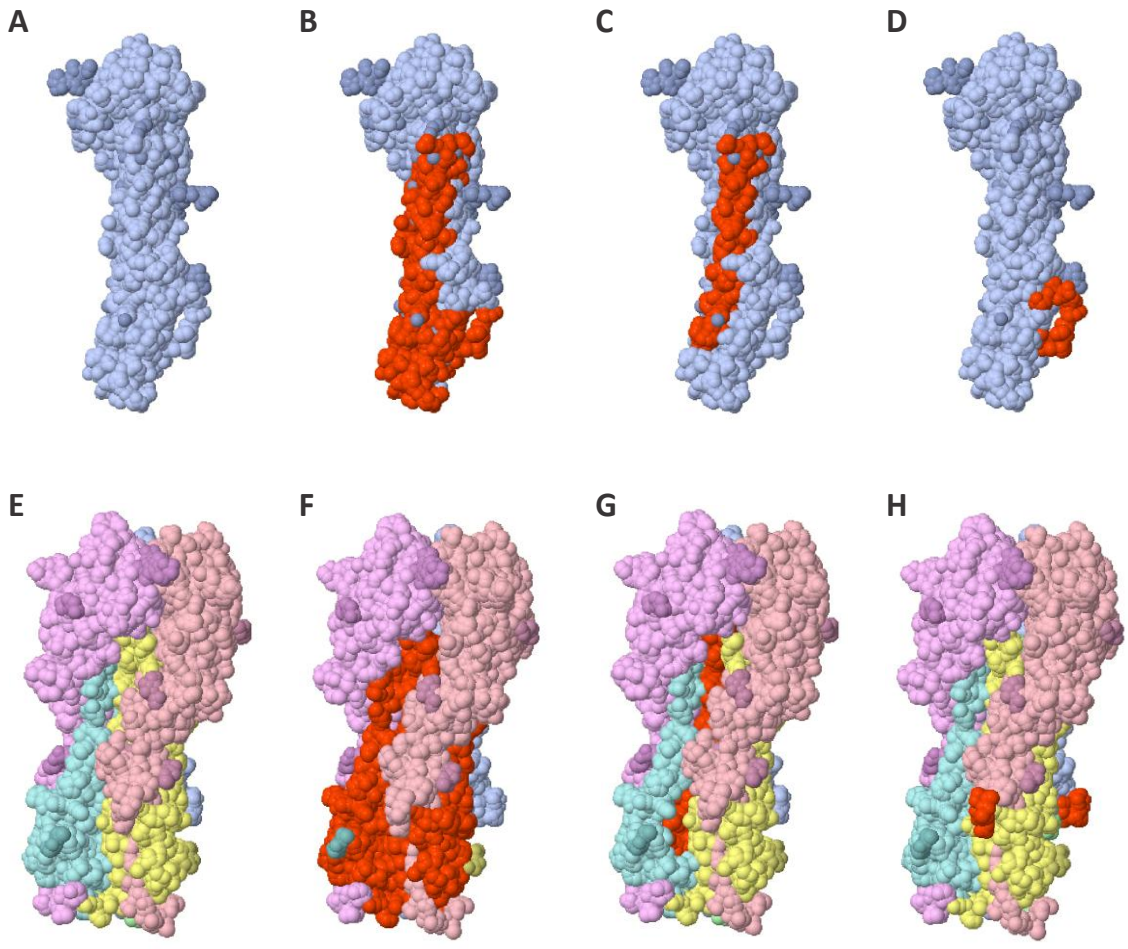


Figure 1.2. Graphical display of monomeric and trimeric HA with highlighting of cross-protective regions. Depicted are HA monomers (A-D) and trimers (E-H), with in orange the HA2-domain (B, F), the LAH (C, G) or the cleavage loop (D, H). Structures were made using the modeling software provided by the Influenza Research Database (www.fludb.org).

1.5. The M2 protein as vaccine target: broad protection derived from a small protein

In the previous section, we described examples of broad immunity afforded by conformational parts of the HA protein. Although deducing the minimal sequence needed for such conformation-dependent antigens by using reverse vaccinology can be tedious and time-consuming, it can lead to the identification of novel vaccine targets. When assessing the influenza A virus structure as an entire antigen, similar deductions can be made. The focus for humoral protection against infection is restricted to the three transmembrane proteins: HA, neuraminidase (NA) and M2. Of these, HA and NA have intricate structures and are prone to constant immunological pressure, which complicates the identification of cross-protective epitopes. The intravirionic dominance of HA and NA over M2 might have safeguarded the latter from antigenic stress. Moreover, not only is the extracellular part of M2 much smaller than that of HA or NA, it is also much less abundant on the viral particle. The negligible role of M2e-specific antibodies in protection or recovery from infection is exemplified by the weak antibody responses against M2e following natural infection or vaccination with currently licensed vaccines, both in humans and animals [78-80]. Nevertheless, the smallest extracellular protein of influenza A virus has become one of the biggest targets in the past two decades for the generation of a “universal” influenza vaccine.

1.5.1 Physical and functional characteristics of the influenza A M2 protein

Since its original description in 1981 [81], several characteristics of the M2 protein have made it an alluring target for vaccine design. At the genetic level, M2 is expressed from RNA segment 7 along with the M1 matrix protein. The reading frame of M2 overlaps partially with that of M1: the latter is translated from the linear mRNA and the former from a spliced mRNA variant. As translation of both proteins is initiated at the same codon, they share the first stretch of eight amino acids. Hence, it is not surprising that specifically these amino acids show strong conservation. The M2 protein is further modified by phosphorylation and palmitoylation. The latter modification of M2 contributes to influenza virus virulence *in vivo* [82,83]. M2 assembles into a membrane-spanning homotetramer utilizing four parallel disulfide-linked monomers [84]. M2 is abundant at the surface of the infected cell, reaching densities close to that of HA [85], but it is scarce in virions for unknown reasons [86,87]**.** In its native conformation, M2 serves many important functions in the influenza A life cycle.

The transmembrane portion of M2 contains an HXXXW motif, which in its tetrameric conformation can support the transfer of protons from a low pH environment to more basic compartments [88,89]. By allowing such passive proton transport, M2 can modulate the pH of the viral interior when it is present in the endosomal compartment and of the trans-Golgi network, respectively promoting or protecting the fusion capacity of HA [90-92]. The cytoplasmic domain of M2 assists in the process of viral budding by interacting directly with the M1 protein [93]. In conjunction with M1, M2 governs the general viral morphology. By association with cholesterol within the membrane, M2 can modulate the formation of filamentous virus particles instead of spherical particles [94,95]. Also, the cholesterol-binding M2 protein was recently shown to mediate viral budding in a manner that is independent on

endosomal sorting complex required for transport (ESCRT), and that the intracellular, membrane-proximal amphipathic alpha-helix is responsible for membrane scission [96]. Moreover, direct and indirect interactions of M2 with cellular proteins suggest additional functions for the M2 viroporin in regulating the influenza A virus infection cycle or modulating the anti-viral response of the host [97-99].

Mutational studies have shown that C-terminal alterations [93-95], mutation of the transmembrane section [100] or removal of the M2 ectodomain [101] can have deleterious effects on M2 activity. In addition, deletion of the entire protein severely affects virus assembly and infectivity [94,102]. This led to the use of M2-negative viruses rescued from M2-expressing cell lines and with a strong attenuated phenotype as experimental live vaccine strains [103]. Thus, with the functional constraints imposed on the protein, M2 shows remarkable sequence conservation. The 23-amino-acid ectodomain of M2, termed M2e, is particularly conserved (Figure 1.3) and has drawn major attention as a vaccine target. The low level of sequence variation along with its high expression level and accessibility at the surface of infected cells define an excellent basis for cross-protective vaccine designs based on M2e.

1.5.1 Strategies to render M2e immunogenic

Numerous research groups have endeavored to develop an M2e-based vaccine that evokes broad protective responses towards influenza A viruses. Although anti-M2e responses induced by infection or vaccination appear to be weak in humans, different types of carriers have been used to present the peptide in a more immunogenic fashion to the adaptive immune system of the vertebrate host. Here, we will highlight a few successful examples of such M2e-vaccines.

Initial immunogenicity studies were based on solubilization of intact full-length M2-protein [104]. These enriched membrane preparations were tested in mice and induced a significant anti-M2e antibody titer. The larger part of the humoral response however was directed to the intracellular part of M2. When vaccinated mice were challenged with a lethal dose of a homologous or heterologous influenza strain, protection was observed. Since the M2-induced immunity could not be transmitted by transfer of serum, protection was believed to be based on cellular immunity. In a further development, the transmembrane section of M2 was deleted and fused to glutathione-S-transferase in order to obtain a less hydrophilic protein [105]. Immunization of mice with these constructs led to high antibody titers specific for M2e, leading to enhanced viral clearance following infection with influenza strains of different subtypes.

Given the small length of the M2e epitope, combined with a better understanding of how immune cells can be triggered, densely arrayed presentation of the antigen has been investigated rigorously. Fusion to certain capsid proteins derived from viruses can lead to self-assembly of uniform virus-like particles (VLPs), which present the antigen in an arranged fashion at high density. Neirynck *et al.* (1999) evaluated the Hepatitis B core (HBc) protein as carrier, by genetically fusing M2e to the N-terminus of HBc [106]. Immunization of mice led to long-lasting protection against influenza A virus infection, and this protection could be transferred to naive mice by using anti-M2e antisera. By adding one or two more M2e copies at the N-terminus, or inserting the epitope into the immunodominant loop of HBc protruding as spikes from the VLP, immunogenicity was further

enhanced [107]. Other groups followed the reasoning of high epitope-density presentation of M2e using capsid proteins from animal or plant viruses or bacteriophages [108-112]. Besides the antigenic

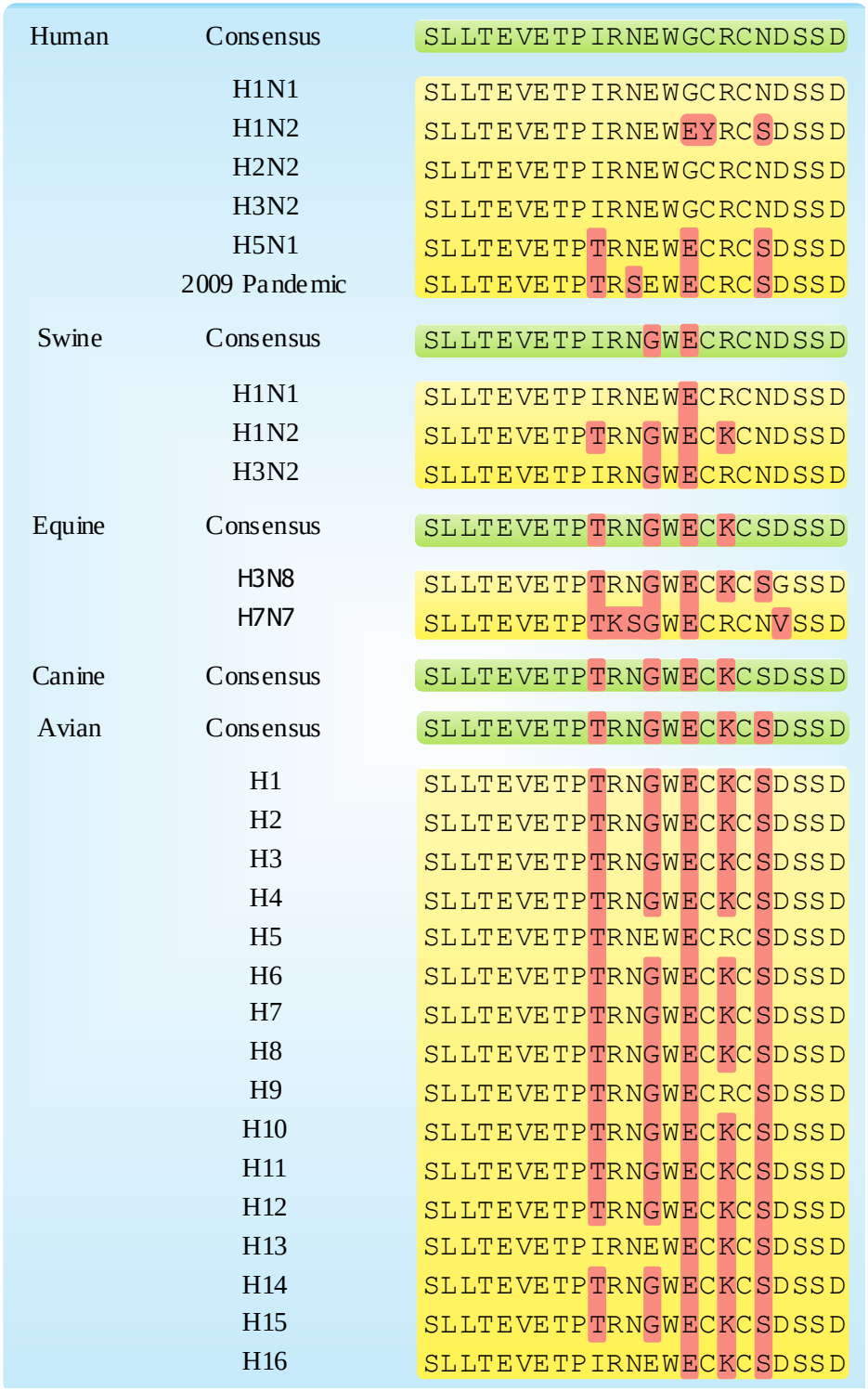


Figure 1.3. Alignment of M2e sequences avialable in the influenza sequence database (http://www.fludb.org) before February 2011. In green the consensus sequences are shown for the indicated host, in yellow the consensus sequences for the indicated viral subtypes. Residues marked in red deviate from the human consensus sequence. For the alignments, 8316 human, 4856 avian, 526 swine, 131 equine and 178 canine sequences were aligned.

display benefits of VLPs, their ordered and rigid three-dimensional structure and, in some cases, the potential to encapsulate nucleic acids, may bypass the need for adjuvants. Moreover, these VLPs not only induce strong humoral responses, but also lead to T-cell-based cellular immunity [113].

The encouraging results obtained with M2e-based vaccines, showing broad protection against influenza-induced morbidity and mortality, enticed several other research groups to further explore M2e vaccine design. In more classical chimeric products, M2e has been chemically fused to well-known carrier proteins, such as KLH or bovine serum albumin. Testing such constructs in animal models generally requires the addition of adjuvants, such as incomplete Freund's adjuvant or alum, to elicit substantial antibody responses. Therefore, more elegant and easier to produce carriers for M2e have been tested, some of which even have been evaluated in phase I clinical trials. With the dawn of new-generation adjuvants, the concepts of vaccine design have changed. Recombinant production of antigens and immunomodulatory components allows direct linking of both for optimal formulations. Coupling of M2 antigens to microbial proteins [114-119], innate immune components [120] or synthetic lipids [121] has been proven effective in preclinical studies against a wide range of influenza A subtypes. The antigen-sparing qualities of such vanguard formulations further meet the requirements for novel cross-protective vaccines.

Some research groups avoided the use of natural scaffold proteins and have developed instead synthetic constructs for presentation of the M2e [122-125]. Mozdzanowska *et al*. (2003) coupled M2e peptides, along with defined T-helper epitopes, to a scaffold. The absence of a potential immunodominant carrier protein provided a clean model for initial dissection of the anti-M2e immune response. In their model of intranasal immunization, this group demonstrated a clear difference between the upper and lower respiratory tract with regard to the presence of anti-M2e antibodies. In a follow-up study [123], this was elucidated further. While parenteral immunization through the subcutaneous route induced protection that correlated with the presence of M2e antibodies in the serum, intranasal vaccination did not show such a correlation, even though it was superior in inhibiting viral replication in the lung. Moreover, the correlations only were valid when comparing antibody titers reactive with native M2 expressed on infected cells, and not with peptide-specific titers.

The results obtained by Mozdzanowska *et al*. indicated that protection elicited by a given vaccine can vary considerably with the route of administration, and this obscures or at least complicates the detection of protective correlates. Indeed, the additional effects of local immunity induced by intranasal immunization, both antibody and T-cell-based, are not easily quantifiable before infection. A second conclusion to be drawn from the study is the pivotal role of reactivity towards native M2e species. Titers purely based on recognition of peptidic M2e might not be indicative of a prechallenge protected state, since only antibodies capable of binding native M2 expressed on virions or infected cells can convey immunity. Based on these premises, De Fillette *et al*. (2008) designed a tetrameric presentation model closely mimicking the native M2 structure [126]. M2e was fused to a tetramerizing form of the leucine zipper of the GCN4 transcription factor of yeast [127]. Immune sera raised with this chimeric protein showed high specificity for cell-expressed M2, which could not be completely competed away using free M2e peptide. This result substantiated the merit of a quaternary structured vaccine. Other similar native presentation strategies are based on simultaneous expression of M2 and M1, with or without NP, which leads to formation of enveloped VLPs carrying

membrane-inserted full-length M2 [128,129]. Application of such VLPs not only induces anti-M2e immunity, but also builds a degree of cellular immunity provided by the internal proteins M1 and NP.

1.5.2 Dissecting the anti-M2e response

While the objective of M2e immunization has been by and large induction of antibodies, and vaccine efficacy has mainly been evaluated as such, the anti-M2e response is broader. The early studies with M2e-based vaccines showed that protection was transferrable by serum, and hence was largely based on induction of M2e-specific humoral immunity [106,128-131]. Furthermore, both murine and human monoclonal antibodies can protect against lethal influenza infection [132-138]. Using these mAbs, two immunogenic regions were mapped in M2e: they comprised the middle EVETPIRN sequence and the N-terminal SLLTEVET part [132,133,136]. The latter is of particular interest, since this epitope is also present in the M1 protein and therefore highly conserved. However, although the former epitope can induce protective antibodies when presented with classical carrier proteins [139], this is not true for the M1-M2 common epitope [140]. Likewise, certain mAbs and polyclonal sera show specificity for only native M2 on infected cells [123,126,138]. Thus, not only the tetrameric presentation of M2e can form the basis of conformational epitopes, but also the monomeric M2e sequence seems to be more structured than might be expected.

Clearly, antibodies directed to M2e can thwart influenza infection, but how do they function? Although they can have an effect on viral replication *in vitro* (e.g. plaque size reduction assays for some influenza A virus strains) [86], they do not display neutralizing activity. *In vivo*, this translates into immune mechanisms dependent on opsonization of infected cells and possibly virions. Downstream of this, effecter cells, such as natural killer cells and alveolar macrophages, kill and remove the antibody-opsonized infected cells by the process of antibody-dependent cell cytotoxicity (ADCC) [131,141].

The role of M2-protein in protection may be broader than mere humoral immunity. Also M2e-specific T-cell-responses contribute to viral clearance in the BALB/c mouse model and most likely form the basis of local immunity in the lung [122,123,142,143]. Furthermore, the infection-permissive nature of M2e antibodies may allow other anti-influenza immune mechanisms to be activated, thereby enforcing the induction of broad influenza immunity.

Stand-alone M2e vaccines can provide defense against viral infection, but some groups have also considered a mixture of a classical vaccine with M2e antigen. This is in accordance with the concept of intravirionic dominance, based on the overwhelming effect of HA on the humoral immune response compared to other viral antigens. The dominance of HA was acknowledged by Johansson *et al*. (1987), which led to redefinition of classical vaccine concepts [144]. It was found that intravirionic competition between dominant and subdominant antigens could be diminished when they are administered as separated (i.e. molecularly non-associated) antigens in one vaccine preparation [145,146]. Does this hold true for M2e-based vaccines? Wu *et al*. (2009) addressed this issue by combining a traditional split vaccine with M2e peptide [147]. Using a combination vaccine together with aluminum salt, they could induce stronger humoral and cellular M2e responses than those obtained

with the classical inactivated viral vaccine alone, and immune sera were protective in passive transfer experiments. This enhanced M2e response also conveyed heterosubtypic immunity surpassing that of the classical split vaccine on its own. Similarly, Song *et al*. (2011) enhanced the interstrain protective efficacy of inactivated virus by adding M1-M2 VLPs; protection was transferable with serum and apparently dependent on dendritic cells and macrophages [128]. Hence, apart from its own protective potential, under certain conditions the M2 ectodomain can improve the immunity provided by classical vaccines. However, in non-optimal formulations, strong epitopes of HA and NA antigens may still dominate the immune response, abrogating the induction of cross-protective M2e-based immunity.

1.5.3 M2e immunity: one size fits all?

M2e can be regarded as universal, but this claim should only be seen as valid for a particular host species. Variations in M2e-sequence appear to coincide with host restriction, and mutations in the M-gene is one of the genes believed to be involved in adaptation of influenza virus to new hosts [148]. When comparing sequences of different influenza subtypes within one host, it becomes clear that consensus sequences can be compiled. Even within the short M2e sequence, a number of key changes occur (Figure 1.2). On the one hand, this endorses the cross-protective potential of M2e-based constructs for a specified host, but on the other hand interspecies variation can complicate rendition of reactivity towards deviating viruses [114,149], as present in any epidemic or pandemic. However, differences are limited and vaccines encompassing the major antigenically distinct sequences could overcome this hurdle. Moreover, while zoonoses of avian influenza strains are still of great concern, these viruses appear to be in evolutionary stasis [150]. Swine viruses pose another potential threat, as shown by the 2009 Mexican flu pandemic. Surprisingly, the M2e sequence of this pandemic virus digressed to a large extent from other human-to-human transmissible strains, but vaccine studies utilizing 2009 pandemic strains as challenge virus have proven protection based on classical human or avian M2e sequences [124,128,129].

As M2e is a very poor immunogen in natural setting, this lack of antigenic pressure probably means that there was no need for mutational escape. With the use of M2e-directed vaccines, the problem of escape might occur, but can we predict the likelihood of such an event? This issue was addressed by Zharikova *et al*. (2005) [151]. Under controlled conditions, they serially infected SCID mice in the presence or absence of M2e-directed or control mAbs. Somewhat expected, they could select a large number of viruses with mutated M2e sequences. However, the identity of changes was limited to only one residue in the epitope, namely proline at position 10, and only two variants emerged in this model: proline 10 was changed to histidine or leucine. Both mutations also occur naturally, mainly in avian strains. The narrow window of mutational freedom points to strong conservation mechanisms, most likely related to the linkage of the M1 and M2 reading frames and the functional importance of M2. Importantly, the selection for escape mutants was not successful in in vivo experiments using immunity-deficient mice. In active immunizations however, the broad recognition by elicited polyclonal antisera would in all likeliness not be hindered by such localized mutations.

The mouse is the influenza animal model that is easiest to handle, and preclinical studies are predominantly performed in mice. How do results obtained using mice translate to other animal models and to humans? A number of groups have used other animals for preclinical testing of M2e vaccines. In a large collaborative study, Fan *et al*. (2004) compared their conjugate vaccine in mice, ferrets and rhesus monkeys [114]. Protection against human influenza viruses was evident in mice and ferrets, and monkey sera could protect mice in passive transfer experiments. Even with failed recognition of avian virus M2e-peptides *in vitro* by mouse sera, these results are encouraging in translational views. However, to some extent, the choice of carrier protein can influence the overall immunogenicity of M2e [152], but this might not be reflected in overall anti-viral activity. In a model of respiratory stress in cotton rats, M2e antibodies could reduce tachypnea when transferred before challenge [153]. Additional protective effects of M2 immunity were also seen in pigs [154]. Protection with M2e vaccines has also been tested in birds. However, few convincing results have so far been obtained in avian challenge models. Fusion of M2e to the chicken complement factor C3d proved effective in mice, but protection of SPF chickens remained unsatisfying [120]. In a related study, immunization with recombinant Newcastle Disease Viruses expressing HA, NA and M2 showed that although M2 was immunogenic, it had a negative effect on immunogenicity in general in a combined vaccine and showed no M2-related protection [155]. These results demonstrate the need for caution when evaluating efficacy of novel vaccine designs, and they clearly show possible barriers in the compatibility of immunogens and hosts, even in inbred mouse strains [143].

1.6. Can isotypic NA responses provide pandemic protection?

Influenza NA is the second major glycoprotein and is targeted by immune responses induced by vaccination or natural infection. Like HA, NA is prone to drift and shift in influenza A viruses as a consequence of the natural occurrence of immunological pressure in the host [18]. *In vitro*, anti-NA antibodies cannot prevent infection, but they can interfere with the enzymatic activity of NA and severely reduce virus progeny production [156]. Anti-NA antibodies thwart the infection process sufficiently to slow down disease progression while allowing other immune mechanisms to become activated, resulting finally in viral clearance. During infection, NA on influenza virions probably also plays an essential role at the portal site of entry in the host by hydrolyzing structures that contain sialic acid in the mucus layer lining the respiratory tract [157]. Therefore, it is conceivable that anti-NA antibodies, in particular secretory IgA, could also play a role during the early phase of infection.

Only two NA subtypes, N1 and N2, have been introduced in the human population for a prolonged time. H1N1 and H3N2 human viruses have been cocirculating since 1977. Therefore, one could expect that the presence of only two serotypes in humans, which have been circulating for presumably 90 years or more, has resulted in considerable anti-NA herd immunity. A downside, however, is the immune dominance of HA, *i.e*. the skewing of the anti-influenza immune response towards HA during homotypic recall infections [158]. Nonetheless, as mentioned above, the 1968 H3N2 pandemic provided some evidence supporting the possibility that NA afforded heterosubtypic protection [9]. Could a homosubtypic anti-NA response provide some defense against a pandemic strain? The recent threats of avian H5N1 strains as a potential pandemic precursor, for instance, make N1 an interesting vaccine candidate. Sandbulte *et al*. (2007) showed that despite the difference

between human and avian N1, the NA derived from human H1N1 (A/New Caledonia/20/99), delivered as a DNA vaccine, could partially protect mice against a lethal H5N1 challenge [159]. This protection was apparently antibody-mediated, since passive transfer of immune serum resulted in a similar level of protection. This study also demonstrated that up to 20% of people have serum IgG titers against avian N1 that can inhibit the enzyme *in vitro*. This could imply that prevailing anti-N1 immunity provides baseline protection against H5N1 infections. Unfortunately, there is no reliable standardization for the neuraminidase content in currently licensed inactivated influenza virus vaccines. Supplementation of classical inactivated vaccines with NA, or immunization with purified NA or DNA vectored NA, may elicit more balanced anti-NA responses and heterosubtypic protection [160-163]. These observations warrant further investigation of possible protection against pandemic influenza strains with an N1 or N2 make-up by vaccination with NA-vaccines.

The introduction of the pandemic H1N1/09 virus in the human population provided a contemporary situation to analyze the influence of NA-immunity. The group of Richard Webby examined the effect that repetitive immunization with or previous exposure to seasonal influenza A/ H1N1 viruses has on susceptibility to H1N1/09 infection [164]. Using a panel of recombinant viruses, only differing in their NA gene, they showed induction of anti-NA antibodies cross-reactive with H1N1/09. Moreover, sera taken from mice after challenge with these recombinant strains could protect naive recipient mice in passive transfer experiments against a potential lethal H1N1/09 infection, despite the lack of cross-reactive HI titers. In addition, they showed that exposure to multiple seasonal H1N1 strains elicited H1N1/09 NA cross-reactive titers. Using convalescent sera from elderly humans immunized with conventional TIV vaccines, they could also detect anti-NA responses reactive with H1N1/09 [164,165].

Clearly, pandemic settings of the past do provide evidence for the merit of anti-NA responses for broad protection. This has instigated quite some interest in the development of vaccination strategies augmenting NA-specific responses. Indeed, without a clear definition of the NA content in conventional vaccines and the immunodominance of HA towards NA, novel formulations are warranted. A potential successful approach is the combination of soluble trimeric HA and tetrameric NA, produced by eukaryotic expression systems, to be combined as vaccine antigens. Bosch *et al*. followed this strategy in assessing protection against H1N1/09 infection in a ferret model [166]. The addition of NA in the vaccine preparation augmented the induction of viral neutralizing titers and had a profound impact on antiviral immunity compared to HA-only vaccination. Specifically, NA inclusion decreased morbidity and lung pathology quite significantly. Moreover, the induced anti-NA responses were shown to be cross-reactive with other N1NA molecules, neutralizing their NA activity. Overall, these epidemiological and experimental data underscore the value of anti-NA responses in broad anti-influenza immunity.

1.7. Influenza-specific CTLs: help from the inside

The major structural proteins nucleoprotein (NP) and matrix protein 1 (M1) are the main candidate antigens to contain conserved regions. But also M2, basic polymerase 1 (PB1) and non-structural protein 1 (NS1), and even HA bear epitopes that are conserved across subtypes. Of note, among the described immune epitopes of influenza, the sequence conservation of T cell epitopes seems to be 4-

5 times greater than that of the dominant B cell epitopes [167]. The reason is that the majority of identified antibody-interacting antigens are located in HA and NA, while most known T cell epitopes are present in conserved internal gene products. Many of these are cytotoxic T cell epitopes, i.e. linear peptides presented by MHCI molecules and recognized by a matching T cell receptor. Cellular immunity directed against several of these T-cell epitopes has been proven to be protective (Table 1.1.).

Table 1.1. Overview of identified MHCI and MHCII binding influenza epitopes capable of inducing cytotoxic T cells.

Virus	CTL type	Epitope	Sequence	restriction	ref
IAV	CD8	PB1 (591-599)	VSDGGPNLY	A1	177
		M1 (58-66)	GILGFVFTL	A*0201	178-181
		M1 (128-135)	ASCMGLIY	B*3501	182
		M2 (7-15)	VETPIRNEW	B*44	180,181
		NS1 (122-130)	AIMDKNIIL	A*0201	180,181,183
		NP (44-52)	CTELKLSDY	A1	177
		NP (146-154)	TTYQRTRAL	B*1402	184
		NP (174-184)	RRSGAAGAAVK	B*2705	180,181
		NP (265-273)	ILRGSVAH	A3	177
		NP (339-347)	EDLRVLSFI	B*3701	185,186
		NP (380-388)	ELRSRYWAI	B*0801	187-190
		NP (381-388)	LRSRYWAI	B*2702	187
		NP (383-391)	SRYWAIRTR	B*2705	180,181,189-192
		NP (418-426)	LPFEKSTVM	B*3501	189,193,194
	CD4	M1 (17-31)	SGPLKAEIAQRLEDV	DR1	180,181
		NS1 (34-42)	DRLRRDQKS	DR3	180,181
		NP (254-262)	EDLTFLARSAL	-	180,181
IBV	CD8	NP (30-38)	RPIIRPATL	B8	195
		NP (85-93)	KLGEFYNQM	A*0201	196
		NP (263-271)	ADRGLLRDI	B8	195
		NP (413-421)	ALKCKGFHV	B8	195
	CD4	HA (308-320)	PYYTGEHAKAIGN	DR1	195

The first evidence of heterosubtypic immunity induced by influenza infection dates back to the mid 1960s, when Schulman and Kilbourne reported that infection of mice with one influenza A virus could induce protective immunity against an unrelated influenza A virus but not against a B virus [168]. By

contrast, parenteral inoculation with inactivated virus did not have the same effect, implying that such cross-protection requires viral replication. A decade later, it was demonstrated that cytotoxic activity of T cells derived from infected mice could kill infected syngeneic cells, that this cytotoxic activity could also target heterosubtypically infected cells and could be boosted in mice by using antigenically distinct viruses [169-172]. It thus seemed that cellular cytotoxicity was cross-subtype-specific, a first indication of the broad protective potential of this immune compartment. These influenza-specific CTLs apparently played a role *in vivo*, because they are found in lungs and draining lymph nodes after intranasal challenge of mice, and because adoptive transfer of primed CTLs protects naive mice [173-176]. Following these initial studies, the field of CTL-mediated anti-influenza immunity expanded substantially.

1.7.1 Influenza-specific CTL epitopes

Influenza virus NP is involved in coating of the viral RNA and assembly of the ribonucleoprotein complexes. These complexes play a role in nuclear transport, and possibly in transcription of viral genes and in replication (reviewed in [197]). This multitude of functions imposes considerable structural constraints on the protein and leaves little room for sequence variation. Consequently, immune responses directed against NP epitopes are often heterosubtypic. The first evidence that heterosubtypic cellular cytotoxicity was directed against NP came from Townsend and Skehel, who showed that CTL activity coincided with the presence of NP [198]. The protective potential of NP as a vaccine was demonstrated by using both purified NP and adoptively transferred NP-specific CTLs [199,200]. Although cytotoxic cellular anti-NP activity does not confer sterilizing immunity – in fact, it is infection-permissive – NP-specific CTL activity in the mouse has a beneficial effect on survival and lowers viral titers. Recently, a more surprising protective role for NP was reported. Carragher et al. used recombinant NP adjuvanted with LPS to show that humoral responses raised against NP can protect mice against influenza infection, as reflected in reduced morbidity and lung viral titers [201]. This protection was antibody-dependent, since immunization of mice unable to produce antibodies (*AID/μS* mice) did not lead to reduction in lung viral titer. However, protection was also T cell-dependent, as passive transfer of immune serum to B cell-deficient *μMT* mice protected the mice, whereas B and T cell-deficient *Rag1*$^{-/-}$ mice were not protected by passive immunization. These results show that non-neutralizing anti-NP antibodies, in conjunction with a responsive T cell compartment, can provide protection against influenza infection.

It is important to note that influenza CTL epitopes are characterized by a hierarchy of immune dominance based on the magnitude of the T cell responses. This particular hierarchy depends on the HLA/MHC genetic background [202,203] and consequently differs between different animal models and between individual humans. On the other hand, this hierarchy implies that depending on the individual, several potentially useful viral CTL epitopes can be masked by the presence of immunodominant epitopes [204]. Careful selection of strong CTL-inducing epitopes and their presentation in the absence of viral immune dominance, *e.g.* by using peptide-based vaccines, is therefore crucial for the design of a broad-spectrum vaccine based on CTL induction. A prerequisite for efficient cognate $CD8^+$ T cell induction is productive processing of the epitope during infection or vaccination followed by MHCI-associated presentation [203,205]. Besides immune dominance, HLA

restriction is another feature of CTL epitopes that might pose a problem for development of a broad-spectrum CTL-based vaccine for the entire human population [202,206]. Therefore, for human use, a CTL-based vaccine should preferably contain or code for multiple epitopes covering most of the HLA pool.

Noteworthy, *in silico* screening for human CTL-inducing sequences revealed several putative epitopes that are highly conserved and are present in a diverse range of human and avian influenza isolates [207]. Some are genuine CTL-inducing T-cell epitopes *in vitro* and cover a wide range of human HLA haplotypes, providing direct evidence for the natural occurrence of cross-reactive cytotoxic T cell-inducing determinants, even directed against a potential pandemic (avian) strain. H5N1 cross-reactive CTLs appear to be present in healthy humans as a consequence of repeated H1N1 and H3N2 infections, as reported by Kreijtz *et al* [208]. The authors showed that known human CTL epitopes are present in a set of 900 different H5N1 viruses, although several mutations were detected. In addition, isolated PBMCs from healthy Dutch blood donors produced a cytotoxic reaction against cells presenting NP-epitopes of H5N1 (A/Vietnam/1194/04) and against H5N1-infected cells. This suggests that at least some humans possess memory CTLs that can cross-react with (heterosubtypical) divergent influenza strains. The contribution of NP-specific CTL responses to natural protection in humans is supported by the evolution of human influenza virus strains with escape mutations in NP CTL epitopes [209]. However, not everyone in a population will have a frontline CTL memory response, because repeated viral encounters during epidemic seasons seem to be necessary. The above-mentioned scientific, historical and even anecdotal evidence indicates that CTLs could play a role in surviving a pandemic. But one important question remains: how to induce cross-reactive, influenza-specific CTL responses in a safe, effective and reproducible manner?

1.7.2 CTL-inducing vaccines: a practical overview

A vaccine inducing effective CTL responses has to cover a wide range of HLA-binding epitopes. But another necessary property is topological: for efficient uploading of peptides onto MHCI molecules, the antigen has to be processed in the cytosol of the presenting cell. This implies that the CTL peptides or their precursors must be delivered into the cytosol, and so the vaccine has to be formulated with this in mind. Four main options are available for this: the use of live, attenuated influenza vaccines (LAIV), recombinant viral or bacterial vectors, gene vaccination, and protein vaccines formulated with particular adjuvants.

LAIV have been used in Russia for over 40 years and in the USA since 2003. They can be divided in two classes: cold-adapted viruses and genetically engineered viruses with attenuated features. The former are derived from virus that was passaged *in vitro* at low temperatures (25-33°C) [210]. Influenza virus isolates selected under these conditions replicate poorly at temperatures over 35°C, and when they are administered to humans, they only replicate in the upper respiratory tract, without causing illness. Cold-adapted viruses have acquired mutations in PB1, PB2 and possibly NP and NS [211,212]. Other mutations in the influenza genome can also attenuate the virus, and several rationally designed candidate live attenuated vaccine strains have been generated in this way [213-215]. Use of these cold-adapted viruses as intranasal live vaccines provokes an immune response that provides

strain-matched humoral immunity (mainly HA-directed) and some CTL responses. In the mouse, these T-cell mediated responses provide heterosubtypic protection [216]. Also for humans, cross-reactive responses could be shown *in vitro* after immunization with cold-adapted viruses [217]. Some evidence for enhanced cross-protection by live attenuated influenza vaccines was provided during the 2003-2004 season, during which the circulating H3N2 strain did not antigenically match the vaccine strain [218,219]. A word of caution is that efficacy of attenuated viruses as vaccines might be counteracted by pre-existing (heterosubtypic) immunity, be it cell-mediated or antibody-mediated. However, non-sterilizing heterosubtypic immunity in children has only a limited effect on the efficacy of vaccination with LAIV [220,221]. In adults and the elderly, however, natural infections are usually numerous, and pre-existing memory might pose a barrier for vaccine efficacy [222].

Besides LAIV, other replication-competent viruses have been employed as vectors for influenza antigens, including Fowlpox virus, Newcastle disease virus (NDV), human and non-human Adenovirus, and Vaccinia virus. Initial, proof-of-concept findings were established mainly by using HA expressing viral vectors. Replication-deficient Adenoviral vectors offer immune protection against highly pathogenic H5N1 virus challenge in mice and poultry, including protection against antigenically distinct H5N1 virus challenge [223,224]. Such vaccines, and in particular live recombinant NDV and Fowlpox vectors, could be useful to protect against potential pandemic strains in animal reservoirs of influenza, such as poultry [225]. However, hurdles that have to be overcome are cost-effectiveness, biocontainment, pre-existing vector immunity and regulation of the use of genetically modified organisms.

A surprisingly simple way to present processed antigens to the T cell receptor in the context of MHCI is the use of DNA vaccines. The first evidence that a DNA-vaccine strategy can provide protective immunity was reported by Ulmer *et al.*, who showed that intramuscular delivery of an NP-expression plasmid could induce CTLs and protect mice against homo- as well as heterosubtypic influenza virus challenge [226]. This fascinating finding became the starting point for many studies investigating the protective potential of DNA vaccines, including their efficacy against H5N1 challenge [227]. Although the protective potential of DNA vaccines cannot be disputed, and clinical trials have been done, their immunogenicity in humans remains feeble. Factors that determine the immunogenicity of DNA vaccines include the route and mode of administration, the CpG content of the plasmid backbone, codon optimization of the DNA sequence of the vaccine antigen, and formulation with adjuvants [228]. Despite many scientific and technological advances in the use of DNA vaccines, the potential for genomic integration and disruption of normal cellular homeostasis remain an important concern in consideration of clinical application of this method.

Recombinant protein vaccines are in principle safe, easy to handle, and can be formulated accurately and reproducibly. Compared to conventional influenza vaccines, which are based on virus growth, the process of producing recombinant influenza antigens requires less strict biocontainment. On the downside, injected protein antigens engage mainly the extracellular face of the immune compartment, and only some adjuvants or formulations can promote their uptake into the cytosolic route, with subsequent processing by the proteasome and uploading on MHCI molecules. One such example is the use of ISCOMs, or immune-stimulatory complexes, consisting mainly of Quil A, that form micelles in solution; Quil A is derived from the tree *Quillaja saponaria* [229]. ISCOMs are hydrophobic, and the presence of a hydrophobic domain in antigens, such as the transmembrane domain of HA, promotes their incorporation into the ISCOM particle [230]. ISCOM-formulated vaccines

can elicit high titers of serum antibodies and induce CTLs, both in the systemic and in the mucosal compartments of the immune system [231-235]. A second example is ASO4 (Adjuvant System 04), developed by GSK, which is based on the addition of the LPS derivative MPL (3-*O*-desacyl-4-monophosphoryl lipid A) to particulate aluminium salt adjuvant [236]. The specific stimulation of TLR4 by MPL activated the Th1-arm of the immune response, evoking broad humoral and cellular responses to soluble exogenous antigen.

In general, natural and vaccine-induced CTL responses against influenza have shown protection in several animal models. However, evidence for CTL-based protection by influenza vaccines in humans is still sparse. Several vaccine types that induce CTLs are in phase I clinical trials [237], and the results may provide more insight into cellular immunity and help define correlates of protection for CTL-inducing vaccines. Quantifying CTL responses in a clinical laboratory is technically demanding and poses yet another hurdle for implementation of CTL-based vaccines. However, future technical developments could make CTL tests fast and cost-effective. In the following part of this review, we will focus on approaches that rely on antibody-mediated protection to induce heterosubtypic protection.

1.8. Concluding remarks

With conventional vaccines aiming at inducing homotypic anti-HA responses, induced immunity will be very strong against homologous strains. However, drifted epidemic strains could easily evade this immunity, bringing down vaccine effectiveness and heightening the disbelief in its efficacy. Pandemic strains will completely circumvent seasonal vaccine induced immunity, requiring timely responses on the spot. However, a great number of novel strategies have been explored the past few decades in eliciting broad anti-influenza responses; a general overview of these is given in Figure 1.4. Many of these are scrutinized in clinical trials [237]; time will tell which strategy proves the most efficient.

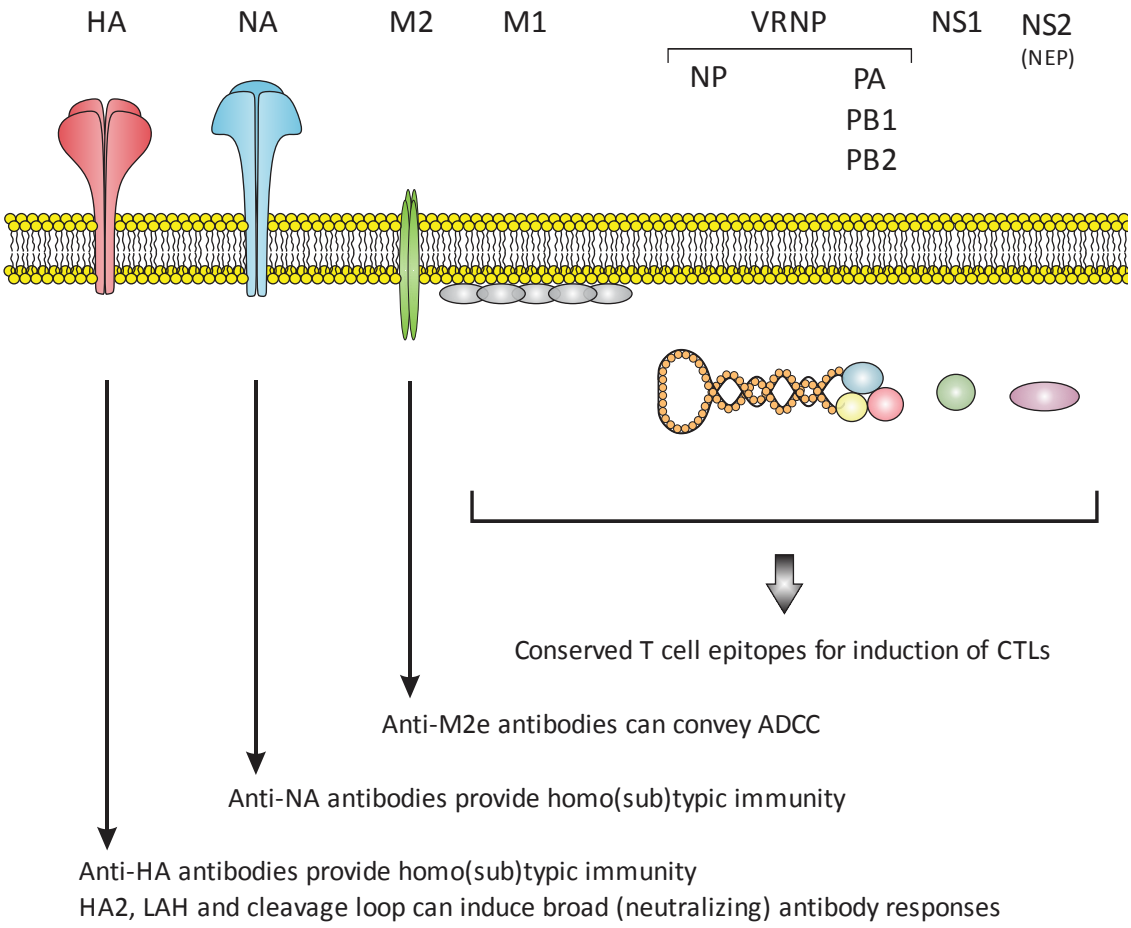


Figure 1.4. Concise overview of novel strategies for broad influenza vaccine development.

1.9. References

1. WHO. http://www.who.int/mediacentre/factsheets/fs211/en/index.html. (2011).
2. Russell, C.J. & Webster, R.G. The genesis of a pandemic influenza virus. *Cell* **123**, 368 (2005).
3. Kresse, H. & Rovini, H. Influenza vaccine market dynamics. *Nat Rev Drug Discov* **8**, 841-842 (2009).
4. Fedson, D.S. Evaluating the impact of influenza vaccination. A North American perspective. *Pharmacoeconomics* **9**, 54 (1996).
5. Doherty, P.C., Turner, S.J., Webby, R.G. & Thomas, P.G. Influenza and the challenge for immunology. *Nature immunology* **7**, 449 (2006).
6. Harris, A.*, et al.* Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19123 (2006).
7. Johnson, N.P. & Mueller, J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull Hist Med* **76**, 105-115 (2002).
8. Epstein, S.L. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. *The Journal of infectious diseases* **193**, 49-53 (2006).
9. Viboud, C., Grais, R.F., Lafont, B.A., Miller, M.A. & Simonsen, L. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. *J Infect Dis* **192**, 233-248 (2005).
10. Monto, A.S. & Kendal, A.P. Effect of neuraminidase antibody on Hong Kong influenza. *Lancet* **1**, 623-625 (1973).
11. Hancock, K.*, et al.* Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *New England Journal of Medicine* **361**, 1945-1952 (2009).
12. Gilbert, G.L.*, et al.* Influenza A (H1N1) 2009 antibodies in residents of New South Wales, Australia, after the first pandemic wave in the 2009 southern hemisphere winter. *PLoS One* **5**, e12562 (2010).
13. Ikonen, N.*, et al.* High frequency of cross-reacting antibodies against 2009 pandemic influenza A (H1N1) virus among the elderly in Finland. *Euro Surveill* **15**, 19478 (2010).
14. Miller, E.*, et al.* Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. *Lancet* **375**, 1100-1108 (2010).
15. Kelly, H.*, et al.* The age-specific cumulative incidence of infection with pandemic influenza H1N1 2009 was similar in various countries prior to vaccination. *PLoS One* **6**, e21828 (2011).
16. Xu, R.*, et al.* Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science* **328**, 357 (2010).
17. McCullers, J.A.*, et al.* Recipients of vaccine against the 1976 “swine flu” have enhanced neutralization responses to the 2009 novel H1N1 influenza virus. *Clinical Infectious Diseases* **50**, 1487 (2010).
18. Kilbourne, E.D., Johansson, B.E. & Grajower, B. Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins. *Proc Natl Acad Sci U S A* **87**, 786-790 (1990).
19. Gregersen, J.P., Schmitt, H.J., Trusheim, H. & Bröker, M. Safety of MDCK cell culture-based influenza vaccines. *Future Microbiology* **6**, 143-152 (2011).
20. Feng, S.Z., Jiao, P.R., Qi, W.B., Fan, H.Y. & Liao, M. Development and strategies of cell-culture technology for influenza vaccine. *Applied microbiology and biotechnology* **89**, 893 (2011).
21. Geeraedts, F.*, et al.* Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling. *PLoS pathogens* **4**, e1000138 (2008).
22. Nicholson, K.G.*, et al.* Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. *Journal of biological standardization* **7**, 123-136 (1979).
23. Wright, P.F.*, et al.* Trials of influenza A/New Jersey/76 virus vaccine in normal children: an overview of age-related antigenicity and reactogenicity. *Journal of Infectious Diseases* **136**, S731 (1977).
24. Stephenson, I., Nicholson, K.G., Wood, J.M., Zambon, M.C. & Katz, J.M. Confronting the avian influenza threat: vaccine development for a potential pandemic. *The Lancet infectious diseases* **4**, 499-509 (2004).
25. Takada, A., Matsushita, S., Ninomiya, A., Kawaoka, Y. & Kida, H. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. *Vaccine* **21**, 3212-3218 (2003).
26. Raviv, Y., Bercovici, T., Gitler, C. & Salomon, Y. Selective photoinduced uncoupling of the response of adenylate cyclase to gonadotropins by 5-iodonaphthyl 1-azide. *Biochemistry* **23**, 503-508 (1984).

27. Raviv, Y.*, et al.* Hydrophobic inactivation of influenza viruses confers preservation of viral structure with enhanced immunogenicity. *Journal of virology* **82**, 4612-4619 (2008).
28. Tumpey, T.M., Renshaw, M., Clements, J.D. & Katz, J.M. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. *Journal of virology* **75**, 5141-5150 (2001).
29. Ichinohe, T.*, et al.* Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. *J Infect Dis* **196**, 1313-1320 (2007).
30. Lidbury, B.A., Grissell, T.V., Sizer, P.J., Clancy, R. & Cripps, A.W. Studies on the IgA-independent immunological responses in mice to influenza virus challenge after oral vaccination with irradiated whole virus and an erythrocyte complex. *Immunol Cell Biol* **78**, 149-155 (2000).
31. Song, J.H.*, et al.* Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proc Natl Acad Sci U S A* **105**, 1644-1649 (2008).
32. McMichael, A.J., Gotch, F.M., Noble, G.R. & Beare, P.A.S. Cytotoxic T-cell immunity to influenza. *New England Journal of Medicine* **309**, 13-17 (1983).
33. McMichael, A.J., Michie, C.A., Gotch, F.M., Smith, G.L. & Moss, B. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *Journal of general virology* **67**, 719 (1986).
34. He, X.S.*, et al.* Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *Journal of virology* **80**, 11756 (2006).
35. Bodewes, R.*, et al.* Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus. *PLoS One* **4**, e5538 (2009).
36. Bodewes, R., Kreijtz, J.H.C.M. & Hillaire, M. Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8+ T-cell responses in mice. *Journal of general virology* **91**, 1743 (2010).
37. Wiley, D.C., Wilson, I.A. & Skehel, J.J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* **289**, 373 (1981).
38. Gerhard, W., Yewdell, J., Frankel, M.E. & Webster, R. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. (1981).
39. Wiley, D.C. & Skehel, J.J. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual Review of Biochemistry* **56**, 365-394 (1987).
40. Knossow, M. & Skehel, J.J. Variation and infectivity neutralization in influenza. *Immunology* **119**, 1-7 (2006).
41. Vareçková, E., Mucha, V., Wharton, S.A. & Kostolanský, F. Inhibition of fusion activity of influenza A haemagglutinin mediated by HA2-specific monoclonal antibodies. *Archives of virology* **148**, 469-486 (2003).
42. Skehel, J.J. & Wiley, D.C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual Review of Biochemistry* **69**, 531-569 (2000).
43. Green, N.*, et al.* Immunogenic structure of the influenza virus hemagglutinin. *Cell* **28**, 477-487 (1982).
44. Verhoeyen, M.*, et al.* Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature* **286**, 771 (1980).
45. White, J., Kartenbeck, J. & Helenius, A. Membrane fusion activity of influenza virus. *The EMBO journal* **1**, 217 (1982).
46. Min Jou, W.*, et al.* Complete structure of the hemagglutinin gene from the human influenza A/Victoria/3/75 (H3N2) strain as determined from cloned DNA. *Cell* **19**, 683 (1980).
47. Waterfield, M.D., Espelie, K., Elder, K. & Skehel, J.J. Structure of the haemagglutinin of influenza virus. *British Medical Bulletin* **35**, 57-63 (1979).
48. Skehel, J.J., Waterfield, M.D., McCauley, J.W., Elder, K. & Wiley, D.C. Studies on the Structure of the Haemagglutinin. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **288**, 335-339 (1980).
49. Krystal, M., Elliott, R.M., Benz, E.W., Young, J.F. & Palese, P. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4800 (1982).
50. Graves, P.N., Schulman, J.L., Young, J.F. & Palese, P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology* **126**, 106-116 (1983).
51. Okuno, Y., Isegawa, Y., Sasao, F. & Ueda, S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *Journal of virology* **67**, 2552 (1993).

52. Okuno, Y*., et al.* Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *Journal of clinical microbiology* **28**, 1308 (1990).
53. Martinez, O., Tsibane, T. & Basler, C.F. Neutralizing anti-influenza virus monoclonal antibodies: therapeutics and tools for discovery. *International Reviews of Immunology* **28**, 69-92 (2009).
54. Okuno, Y., Matsumoto, K., Isegawa, Y. & Ueda, S. Protection against the mouse-adapted A/FM/1/47 strain of influenza A virus in mice by a monoclonal antibody with cross-neutralizing activity among H1 and H2 strains. *Journal of virology* **68**, 517 (1994).
55. Smirnov, Y.A., Lipatov, A.S., Gitelman, A.K., Claas, E.C.J. & Osterhaus, A. Prevention and treatment of bronchopneumonia in mice caused by mouse-adapted variant of avian H5N2 influenza A virus using monoclonal antibody against conserved epitope in the HA stem region. *Archives of virology* **145**, 1733-1741 (2000).
56. Sagawa, H., Ohshima, A., Kato, I., Okuno, Y. & Isegawa, Y. The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region. *Journal of General Virology* **77**, 1483 (1996).
57. Steel, J*., et al.* Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain. *mBio* **1**, e00018 (2010).
58. Bommakanti, G*., et al.* Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. *Proceedings of the National Academy of Sciences* **107**, 13701 (2010).
59. Fouchier, R.A.M*., et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of virology* **79**, 2814 (2005).
60. Ekiert, D.C*., et al.* Antibody recognition of a highly conserved influenza virus epitope. *Science* **324**, 246 (2009).
61. Sui, J*., et al.* Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature structural & molecular biology* **16**, 265-273 (2009).
62. Throsby, M*., et al.* Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One* **3**, 3942 (2008).
63. Friesen, R.H.E*., et al.* New Class of Monoclonal Antibodies against Severe Influenza: Prophylactic and Therapeutic Efficacy in Ferrets. *PLoS One* **5**, e9106 (2010).
64. Kashyap, A.K*., et al.* Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proceedings of the National Academy of Sciences* **105**, 5986 (2008).
65. Corti, D*., et al.* Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *The Journal of clinical investigation* **120**, 1663-1673 (2010).
66. Wang, T.T*., et al.* Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog* **6**, e100796 (2010).
67. Wang, T.T*., et al.* Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proceedings of the National Academy of Sciences* **107**, 18979-18984 (2010).
68. Hancock, K*., et al.* Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *The New England journal of medicine* **361**, 1945 (2009).
69. Ikonen, N*., et al.* High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland. *Euro Surveill* **15**(2010).
70. McVernon, J*., et al.* Absence of cross-reactive antibodies to influenza A (H1N1) 2009 before and after vaccination with 2009 Southern Hemisphere seasonal trivalent influenza vaccine in children aged 6 months-9 years: a prospective study. *Influenza Other Respi Viruses* **5**, 7-11 (2011).
71. Wrammert, J*., et al.* Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *The Journal of Experimental Medicine* **208**, 181 (2011).
72. Hobson, D., Curry, R.L., Beare, A.S. & Ward-Gardner, A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *The Journal of Hygiene* **70**, 767 (1972).
73. Skehel, J.J. & Wiley, D.C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**, 531-569 (2000).
74. Kido, H., Murakami, M., Oba, K., Chen, Y. & Towatari, T. Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. *Mol Cells* **9**, 235-244 (1999).
75. Bianchi, E*., et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380-7388 (2005).

76. Horvath, A.*, et al.* A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza A virus infection. *Immunol Lett* **60**, 127-136 (1998).
77. Steinhauer, D.A., Wharton, S.A., Skehel, J.J. & Wiley, D.C. Studies of the membrane fusion activities of fusion peptide mutants of influenza virus hemagglutinin. *Journal of virology* **69**, 6643-6651 (1995).
78. Black, R.A., Rota, P.A., Gorodkova, N., Klenk, H.D. & Kendal, A.P. Antibody response to the M2 protein of influenza A virus expressed in insect cells. *Journal of General Virology* **74**, 143 (1993).
79. Feng, J.Q.*, et al.* Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. *Virology Journal* **3**, 102 (2006).
80. Kitikoon, P., Strait, E.L. & Thacker, E.L. The antibody responses to swine influenza virus (SIV) recombinant matrix 1 (rM1), matrix 2 (M2), and hemagglutinin (HA) proteins in pigs with different SIV exposure. *Veterinary microbiology* **126**, 51 (2008).
81. Lamb, R.A. & Choppin, P.W. Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. *Virology* **112**, 729-737 (1981).
82. Holsinger, L.J., Shaughnessy, M.A., Micko, A., Pinto, L.H. & Lamb, R.A. Analysis of the posttranslational modifications of the influenza virus M2 protein. *Journal of virology* **69**, 1219 (1995).
83. Grantham, M.L.*, et al.* Palmitoylation of the Influenza A Virus M2 Protein Is Not Required for Virus Replication In Vitro but Contributes to Virus Virulence. *Journal of virology* **83**, 8655 (2009).
84. Holsinger, L.J. & Lamb, R.A. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology* **183**, 32 (1991).
85. Lamb, R.A., Zebedee, S.L. & Richardson, C.D. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell* **40**, 627-633 (1985).
86. Zebedee, S.L. & Lamb, R.A. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *Journal of virology* **62**, 2762 (1988).
87. Leser, G.P. & Lamb, R.A. Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins. *Virology* **342**, 215-227 (2005).
88. Okada, A., Miura, T. & Takeuchi, H. Protonation of Histidine and Histidine- Tryptophan Interaction in the Activation of the M2 Ion Channel from Influenza A Virus†. *Biochemistry* **40**, 6053-6060 (2001).
89. Venkataraman, P., Lamb, R.A. & Pinto, L.H. Chemical rescue of histidine selectivity filter mutants of the M2 ion channel of influenza A virus. *Journal of Biological Chemistry* **280**, 21463 (2005).
90. Bron, R., Kendal, A.P., Klenk, H.D. & Wilschut, J. Role of the M2 protein in influenza virus membrane fusion: effects of amantadine and monensin on fusion kinetics. *Virology* **195**, 808-811 (1993).
91. Takeuchi, K. & Lamb, R.A. Influenza virus M2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. *Journal of virology* **68**, 911 (1994).
92. Wharton, S.A., Belshe, R.B., Skehel, J.J. & Hay, A.J. Role of virion M2 protein in influenza virus uncoating: specific reduction in the rate of membrane fusion between virus and liposomes by amantadine. *Journal of General Virology* **75**, 945 (1994).
93. Chen, B.J., Leser, G.P., Jackson, D. & Lamb, R.A. The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding. *Journal of virology* **82**, 10059 (2008).
94. Iwatsuki-Horimoto, K.*, et al.* The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. *Journal of virology* **80**, 5233 (2006).
95. Rossman, J.S.*, et al.* Influenza virus M2 ion channel protein is necessary for filamentous virion formation. *Journal of virology* **84**, 5078-5088 (2010).
96. Rossman, J.S., Jing, X., Leser, G.P. & Lamb, R.A. Influenza Virus M2 Protein Mediates ESCRT-Independent Membrane Scission. *Cell* **142**, 902-913 (2010).
97. Gannagé, M.*, et al.* Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. *Cell Host & Microbe* **6**, 367-380 (2009).
98. Mi, S.F., Li, Y. & Yan, J.H. Na+/K+-ATPase 1 subunit interacts with M2 proteins of influenza A and B viruses and affects the virus replication. *Sci China Life Sci* **53**, 1098-1105 (2010).
99. Guan, Z.*, et al.* Interaction of Hsp40 with influenza virus M2 protein: implications for PKR signaling pathway. *Protein & Cell* **1**, 944-955 (2010).
100. Holsinger, L.J., Nichani, D., Pinto, L.H. & Lamb, R.A. Influenza A virus M2 ion channel protein: a structure-function analysis. *Journal of virology* **68**, 1551 (1994).
101. Park, E.K., Castrucci, M.R., Portner, A. & Kawaoka, Y. The M2 ectodomain is important for its incorporation into influenza A virions. *Journal of virology* **72**, 2449 (1998).

102. Watanabe, T., Watanabe, S., Ito, H., Kida, H. & Kawaoka, Y. Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. *Journal of virology* **75**, 5656 (2001).
103. Watanabe, S., Watanabe, T. & Kawaoka, Y. Influenza A virus lacking M2 protein as a live attenuated vaccine. *Journal of virology* **83**, 5947 (2009).
104. Slepushkin, V.A.*, et al.* Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine* **13**, 1399-1402 (1995).
105. Frace, A.M., Klimov, A.I., Rowe, T., Black, R.A. & Katz, J.M. Modified M2 proteins produce heterotypic immunity against influenza A virus. *Vaccine* **17**, 2237-2244 (1999).
106. Neirynck, S.*, et al.* A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nature medicine* **5**, 1157-1163 (1999).
107. De Filette, M.*, et al.* Universal influenza A vaccine: optimization of M2-based constructs. *Virology* **337**, 149-161 (2005).
108. Nemchinov, L.G. & Natilla, A. Transient expression of the ectodomain of matrix protein 2 (M2e) of avian influenza A virus in plants. *Protein expression and purification* **56**, 153-159 (2007).
109. Bessa, J.*, et al.* Efficient induction of mucosal and systemic immune responses by virus like particles administered intranasally: implications for vaccine design. *European journal of immunology* **38**, 114-126 (2008).
110. Denis, J.*, et al.* Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. *Vaccine* **26**, 3395-3403 (2008).
111. Tissot, A.C.*, et al.* Versatile virus-like particle carrier for epitope based vaccines. *PLoS One* **5**, 54-60 (2010).
112. Ameiss, K.*, et al.* Delivery of woodchuck hepatitis virus-like particle presented influenza M2e by recombinant attenuated Salmonella displaying a delayed lysis phenotype. *Vaccine* **28**, 6704-6713 (2010).
113. Ludwig, C. & Wagner, R. Virus-like particles--universal molecular toolboxes. *Current opinion in biotechnology* **18**, 537-545 (2007).
114. Fan, J.*, et al.* Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine* **22**, 2993-3003 (2004).
115. Huleatt, J.W.*, et al.* Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* **26**, 201-214 (2008).
116. Eliasson, D.G.*, et al.* CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. *Vaccine* **26**, 1243-1252 (2008).
117. Zhao, G.*, et al.* Induction of protection against divergent H5N1 influenza viruses using a recombinant fusion protein linking influenza M2e to Onchocerca volvulus activation associated protein-1 (ASP-1) adjuvant. *Vaccine* **28**, 7233-7240 (2010).
118. Ebrahimi, S.M., Tebianian, M., Toghyani, H., Memarnejadian, A. & Attaran, H.R. Cloning, expression and purification of the influenza A (H9N2) virus M2e antigen and truncated Mycobacterium tuberculosis HSP70 as a fusion protein in Pichia pastoris. *Protein expression and purification* **70**, 7-12 (2010).
119. Zhang, G.G., Li, D.X., Zhang, H.H., Zeng, Y.M. & Chen, L. Enhancement of mucosal immune response against the M2eHBc+ antigen in mice with the fusion expression products of LTB and M2eHBc+ through mucosal immunization route. *Veterinary research communications* **33**, 735 (2009).
120. Zhang, Z.*, et al.* Fusion to chicken C 3 d enhances the immunogenicity of the M 2 protein of avian influenza virus. *Virology Journal* **7**, 89 (2010).
121. Pejoski, D., Zeng, W., Rockman, S., Brown, L.E. & Jackson, D.C. A lipopeptide based on the M2 and HA proteins of influenza A viruses induces protective antibody. *Immunology and cell biology* **88**, 605-611 (2010).
122. Mozdzanowska, K.*, et al.* Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. *Vaccine* **21**, 2616-2626 (2003).
123. Mozdzanowska, K., Zharikova, D., Cudic, M., Otvos, L. & Gerhard, W. Roles of adjuvant and route of vaccination in antibody response and protection engendered by a synthetic matrix protein 2-based influenza A virus vaccine in the mouse. *Virology Journal* **4**, 118 (2007).
124. Zhao, G.*, et al.* An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus. *Virology Journal* **7**, 151 (2010).

125. Zhao, G.*, et al.* An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. *Virology Journal* **7**, 9 (2010).
126. De Filette, M.*, et al.* An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. *Journal of Biological Chemistry* **283**, 11382 (2008).
127. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science (New York, NY)* **262**, 1401 (1993).
128. Song, J.M., Van Rooijen, N., Bozja, J., Compans, R.W. & Kang, S.M. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. *Proc Natl Acad Sci U S A* **108**, 757-761 (2011).
129. Song, J.M.*, et al.* Influenza Virus-Like Particles Containing M2 Induce Broadly Cross Protective Immunity. *PLoS One* **6**, 1921-1925 (2011).
130. Tompkins, S.M.*, et al.* Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. *Emerging infectious diseases* **13**, 426 (2007).
131. Jegerlehner, A., Schmitz, N., Storni, T. & Bachmann, M.F. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *Journal of immunology (Baltimore, Md.: 1950)* **172**, 5598 (2004).
132. Treanor, J.J., Tierney, E.L., Zebedee, S.L., Lamb, R.A. & Murphy, B.R. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *Journal of virology* **64**, 1375 (1990).
133. Liu, W., Zou, P. & Chen, Y.H. Monoclonal antibodies recognizing EVETPIRN epitope of influenza A virus M2 protein could protect mice from lethal influenza A virus challenge. *Immunology letters* **93**, 131 (2004).
134. Wang, R.*, et al.* Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. *Antiviral research* **80**, 168 (2008).
135. Fu, T.M.*, et al.* Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. *Virology* **385**, 218 (2009).
136. Wang, Y.*, et al.* Monoclonal antibody recognizing SLLTEVET epitope of M2 protein potently inhibited the replication of influenza A viruses in MDCK cells. *Biochemical and biophysical research communications* **385**, 118 (2009).
137. Beerli, R.R.*, et al.* Prophylactic and therapeutic activity of fully human monoclonal antibodies directed against Influenza A M2 protein. *Virology Journal* **6**, 224 (2009).
138. Grandea, A.G.*, et al.* Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza A viruses. *Proceedings of the National Academy of Sciences* **107**, 12658-12663 (2010).
139. Zou, P., Liu, W. & Chen, Y.H. The epitope recognized by a monoclonal antibody in influenza A virus M2 protein is immunogenic and confers immune protection. *International immunopharmacology* **5**, 631 (2005).
140. De Filette, M.*, et al.* Antiserum against the conserved nine amino acid N-terminal peptide of influenza A virus matrix protein 2 is not immunoprotective. *Journal of General Virology* **92**, 301 (2011).
141. El Bakkouri, K.*, et al.* Universal Vaccine Based on Ectodomain of Matrix Protein 2 of Influenza A: Fc Receptors and Alveolar Macrophages Mediate Protection. *The Journal of Immunology* **186**, 1022-1031 (2011).
142. Wu, F., Huang, J.H., Yuan, X.Y., Huang, W.S. & Chen, Y.H. Characterization of immunity induced by M2e of influenza virus. *Vaccine* **25**, 8868-8873 (2007).
143. Misplon, J.A., Lo, C.Y., Gabbard, J.D., Tompkins, S.M. & Epstein, S.L. Genetic control of immune responses to influenza A matrix 2 protein (M2). *Vaccine* **28**, 5817-5827 (2010).
144. Johansson, B.E., Moran, T.M. & Kilbourne, E.D. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 6869 (1987).
145. Johansson, B.E. & Kilbourne, E.D. Dissociation of influenza virus hemagglutinin and neuraminidase eliminates their intravirionic antigenic competition. *Journal of virology* **67**, 5721 (1993).
146. Johansson, B.E. & Kilbourne, E.D. Immunization with dissociated neuraminidase, matrix, and nucleoproteins from influenza A virus eliminates cognate help and antigenic competition. *Virology* **225**, 136 (1996).
147. Wu, F., Yuan, X.Y., Huang, W.S. & Chen, Y.H. Heterosubtypic protection conferred by combined vaccination with M2e peptide and split influenza vaccine. *Vaccine* **27**, 6095 (2009).

148. Furuse, Y., Suzuki, A., Kamigaki, T. & Oshitani, H. Evolution of the M gene of the influenza A virus in different host species: large-scale sequence analysis. *Virology Journal* **6**, 67 (2009).
149. Liu, W., Zou, P., Ding, J., Lu, Y. & Chen, Y.H. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. *Microbes and Infection* **7**, 171-177 (2005).
150. Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. & Kawaoka, Y. Evolution and ecology of influenza A viruses. *Microbiology and Molecular Biology Reviews* **56**, 152 (1992).
151. Zharikova, D., Mozdzanowska, K., Feng, J., Zhang, M. & Gerhard, W. Influenza Type A Virus Escape Mutants Emerge In Vivo in the Presence of Antibodies to the Ectodomain of Matrix Protein 2. *Journal of virology* **79**, 6644 (2005).
152. Fu, T.M.*, et al.* Comparative immunogenicity evaluations of influenza A virus M2 peptide as recombinant virus like particle or conjugate vaccines in mice and monkeys. *Vaccine* **27**, 1440 (2009).
153. Straight, T.M., Ottolini, M.G., Prince, G.A. & Eichelberger, M.C. Antibody contributes to heterosubtypic protection against influenza A-induced tachypnea in cotton rats. *Virology Journal* **5**, 44 (2008).
154. Kitikoon, P.*, et al.* Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates. *Vaccine* **28**, 523-531 (2009).
155. Nayak, B.*, et al.* Contributions of the Avian Influenza Virus HA, NA, and M2 Surface Proteins to the Induction of Neutralizing Antibodies and Protective Immunity. *Journal of virology* **84**, 2408-2420 (2010).
156. Johansson, B.E., Bucher, D.J. & Kilbourne, E.D. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. *Journal of virology* **63**, 1239-1246 (1989).
157. Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A. & Klenk, H.D. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *Journal of virology* **78**, 12665-12667 (2004).
158. Johansson, B.E., Moran, T.M., Bona, C.A., Popple, S.W. & Kilbourne, E.D. Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. II. Sequential infection of mice simulates human experience. *J Immunol* **139**, 2010-2014 (1987).
159. Sandbulte, M.R.*, et al.* Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. *PLoS Med* **4**, e59 (2007).
160. Brett, I.C. & Johansson, B.E. 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* **339**, 273-280 (2005).
161. Deroo, T., Jou, W.M. & Fiers, W. Recombinant neuraminidase vaccine protects against lethal influenza. *Vaccine* **14**, 561-569 (1996).
162. Johansson, B.E. & Kilbourne, E.D. Immunization with purified N1 and N2 influenza virus neuraminidases demonstrates cross-reactivity without antigenic competition. *Proc Natl Acad Sci U S A* **91**, 2358-2361 (1994).
163. Johansson, B.E., Pokorny, B.A. & Tiso, V.A. Supplementation of conventional trivalent influenza vaccine with purified viral N1 and N2 neuraminidases induces a balanced immune response without antigenic competition. *Vaccine* **20**, 1670-1674 (2002).
164. Marcelin, G.*, et al.* A Contributing Role for Anti-Neuraminidase Antibodies on Immunity to Pandemic H1N1 2009 Influenza A Virus. *PLoS One* **6**, e26335 (2010).
165. Marcelin, G.*, et al.* Inactivated seasonal influenza vaccines increase serum antibodies to the neuraminidase of pandemic influenza A (H1N1) 2009 virus in an age-dependent manner. *Journal of Infectious Diseases* **202**, 1634 (2010).
166. Bosch, B.J.*, et al.* Recombinant soluble, multimeric HA and NA exhibit distinctive types of protection against pandemic swine-origin 2009 A (H1N1) influenza virus infection in ferrets. *Journal of virology* **84**, 10366 (2010).
167. Bui, H.H., Peters, B., Assarsson, E., Mbawuike, I. & Sette, A. Ab and T cell epitopes of influenza A virus, knowledge and opportunities. *Proc Natl Acad Sci U S A* **104**, 246-251 (2007).
168. Schulman, J.L. & Kilbourne, E.D. Induction of Partial Specific Heterotypic Immunity in Mice by a Single Infection with Influenza a Virus. *J Bacteriol* **89**, 170-174 (1965).
169. Braciale, T.J. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus-strain specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell Immunol* **33**, 423-436 (1977).

170. Doherty, P.C., Effros, R.B. & Bennink, J. Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. *Proc Natl Acad Sci U S A* **74**, 1209-1213 (1977).
171. Effros, R.B., Doherty, P.C., Gerhard, W. & Bennink, J. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J Exp Med* **145**, 557-568 (1977).
172. Zweerink, H.J., Courtneidge, S.A., Skehel, J.J., Crumpton, M.J. & Askonas, B.A. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature* **267**, 354-356 (1977).
173. Yap, K.L. & Ada, G.L. The recovery of mice from influenza A virus infection: adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand J Immunol* **8**, 413-420 (1978).
174. Yap, K.L., Ada, G.L. & McKenzie, I.F. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* **273**, 238-239 (1978).
175. Bennink, J., Effros, R.B. & Doherty, P.C. Influenzal pneumonia: early appearance of cross-reactive T cells in lungs of mice primed with heterologous type A viruses. *Immunology* **35**, 503-509 (1978).
176. Ennis, F.A., Wells, M.A., Butchko, G.M. & Albrecht, P. Evidence that cytotoxic T cells are part of the host's response to influenza pneumonia. *J Exp Med* **148**, 1241-1250 (1978).
177. DiBrino, M.*, et al.* HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. *The Journal of Immunology* **151**, 5930 (1993).
178. Berkhoff, E.G.M.*, et al.* Functional constraints of influenza A virus epitopes limit escape from cytotoxic T lymphocytes. *Journal of virology* **79**, 11239 (2005).
179. Gotch, F., Rothbard, J., Howland, K., Townsend, A. & McMichael, A. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. (1987).
180. Jameson, J., Cruz, J. & Ennis, F.A. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *Journal of virology* **72**, 8682 (1998).
181. Jameson, J., Cruz, J., Terajima, M. & Ennis, F.A. Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species. *The Journal of Immunology* **162**, 7578 (1999).
182. Dong, T.*, et al.* An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. *European journal of immunology* **26**, 335-339 (1996).
183. Man, S.*, et al.* Definition of a human T cell epitope from influenza A non-structural protein 1 using HLA-A2. 1 transgenic mice. *International immunology* **7**, 597 (1995).
184. DiBrino, M.*, et al.* The HLA-B14 peptide binding site can accommodate peptides with different combinations of anchor residues. *Journal of Biological Chemistry* **269**, 32426 (1994).
185. Martinon, F., Gomard, E., Hannoun, C. & Lévy, J.P. In vitro human cytotoxic T cell responses against influenza A virus can be induced and selected by synthetic peptides. *European journal of immunology* **20**, 2171-2176 (1990).
186. McMichael, A.J., Gotch, F.M. & Rothbard, J. HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. *The Journal of experimental medicine* **164**, 1397 (1986).
187. Tussey, L.G.*, et al.* Different MHC class I alleles compete for presentation of overlapping viral epitopes. *Immunity* **3**, 65-77 (1995).
188. Suhrbier, A., Schmidt, C. & Fernan, A. Prediction of an HLA B8-restricted influenza epitope by motif. *Immunology* **79**, 171 (1993).
189. Rimmelzwaan, G.F.*, et al.* Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus research* **103**, 97-100 (2004).
190. Rimmelzwaan, G.F.*, et al.* Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants. *Journal of General Virology* **86**, 1801 (2005).
191. Voeten, J.T.M.*, et al.* Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *Journal of virology* **74**, 6800 (2000).
192. Berkhoff, E.G.M.*, et al.* A mutation in the HLA-B* 2705-restricted NP383-391 epitope affects the human influenza A virus-specific cytotoxic T-lymphocyte response in vitro. *Journal of virology* **78**, 5216 (2004).
193. Boon, A., De Mutsert, G., Fouchier, R.A.M., Osterhaus, A.D.M.E. & Rimmelzwaan, G.F. The hypervariable immunodominant NP418-426 epitope from the influenza A virus nucleoprotein is recognized by cytotoxic T lymphocytes with high functional avidity. *Journal of virology* **80**, 6024 (2006).

194. Boon, A.*, et al.* Recognition of homo-and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. *The Journal of Immunology* **172**, 2453 (2004).
195. Robbins, P.A., Rota, P.A. & Shapiro, S.Z. A broad cytotoxic T lymphocyte response to influenza type B virus presented by multiple HLA molecules. *International immunology* **9**, 815 (1997).
196. Robbins, P.A., Garboczi, D.N. & Strominger, J.L. HLA-A* 0201 complexes with two 10-Mer peptides differing at the P2 anchor residue have distinct refolding kinetics. *The Journal of Immunology* **154**, 703 (1995).
197. Portela, A. & Digard, P. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* **83**, 723-734 (2002).
198. Townsend, A.R. & Skehel, J.J. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. *J Exp Med* **160**, 552-563 (1984).
199. Taylor, P.M. & Askonas, B.A. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. *Immunology* **58**, 417-420 (1986).
200. Wraith, D.C., Vessey, A.E. & Askonas, B.A. Purified influenza virus nucleoprotein protects mice from lethal infection. *The Journal of general virology* **68**, 433 (1987).
201. Carragher, D.M., Kaminski, D.A., Moquin, A., Hartson, L. & Randall, T.D. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *The Journal of Immunology* **181**, 4168 (2008).
202. Belz, G.T., Stevenson, P.G. & Doherty, P.C. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. *The Journal of Immunology* **165**, 2404 (2000).
203. Yewdell, J.W. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity* **25**, 533-543 (2006).
204. Zhong, W., Reche, P.A., Lai, C.C., Reinhold, B. & Reinherz, E.L. Genome-wide characterization of a viral cytotoxic T lymphocyte epitope repertoire. *J Biol Chem* **278**, 45135-45144 (2003).
205. Crowe, S.R.*, et al.* Differential antigen presentation regulates the changing patterns of CD8+ T cell immunodominance in primary and secondary influenza virus infections. *J Exp Med* **198**, 399-410 (2003).
206. Boon, A.C.*, 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. *Journal of virology* **76**, 582-590 (2002).
207. Wang, M.*, et al.* CTL epitopes for influenza A including the H5N1 bird flu; genome-, pathogen-, and HLA-wide screening. *Vaccine* **25**, 2823-2831 (2007).
208. Kreijtz, J.H.*, et al.* Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. *Journal of virology* **82**, 5161-5166 (2008).
209. Rimmelzwaan, G.F.*, et al.* Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus Res* **103**, 97-100 (2004).
210. Maassab, H.F. Adaptation and growth characteristics of influenza virus at 25 degrees c. *Nature* **213**, 612-614 (1967).
211. Jin, H., Zhou, H., Lu, B. & Kemble, G. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. *Journal of virology* **78**, 995-998 (2004).
212. Tsfasman, T.M., Markushin, S.G., Akopova, II & Ghendon, Y.Z. Molecular mechanisms of reversion to the ts+ (non-temperature-sensitive) phenotype of influenza A cold-adapted (ca) virus strains. *J Gen Virol* **88**, 2724-2729 (2007).
213. Gabriel, G.*, et al.* The potential of a protease activation mutant of a highly pathogenic avian influenza virus for a pandemic live vaccine. *Vaccine* **26**, 956-965 (2008).
214. Horimoto, T., Takada, A., Iwatsuki-Horimoto, K. & Kawaoka, Y. A protective immune response in mice to viral components other than hemagglutinin in a live influenza A virus vaccine model. *Vaccine* **22**, 2244-2247 (2004).
215. Vincent, A.L.*, et al.* Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. *Vaccine* **25**, 7999-8009 (2007).
216. Powell, T.J.*, et al.* Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. *J Immunol* **178**, 1030-1038 (2007).
217. Gorse, G.J.*, et al.* Immunity to influenza in older adults with chronic obstructive pulmonary disease. *J Infect Dis* **190**, 11-19 (2004).

218. Mendelman, P.M., *et al.* Live attenuated influenza vaccine induces cross-reactive antibody responses in children against an a/Fujian/411/2002-like H3N2 antigenic variant strain. *Pediatr Infect Dis J* **23**, 1053-1055 (2004).
219. Halloran, M.E., *et al.* Efficacy of trivalent, cold-adapted, influenza virus vaccine against influenza A (Fujian), a drift variant, during 2003-2004. *Vaccine* **25**, 4038-4045 (2007).
220. Burlington, D.B., *et al.* Development of subtype-specific and heterosubtypic antibodies to the influenza A virus hemagglutinin after primary infection in children. *J Clin Microbiol* **21**, 847-849 (1985).
221. Steinhoff, M.C., Fries, L.F., Karron, R.A., Clements, M.L. & Murphy, B.R. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. *J Clin Microbiol* **31**, 836-838 (1993).
222. He, X.S., *et al.* Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *Journal of virology* **80**, 11756-11766 (2006).
223. Gao, W., *et al.* Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *Journal of virology* **80**, 1959-1964 (2006).
224. Hoelscher, M.A., *et al.* Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet* **367**, 475-481 (2006).
225. Veits, J., *et al.* Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. *Vaccine* **26**, 1688-1696 (2008).
226. Ulmer, J.B., *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745 (1993).
227. Lalor, P.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* **197**, 1643-1652 (2008).
228. Lu, S., Wang, S. & Grimes-Serrano, J.M. Current progress of DNA vaccine studies in humans. *Expert Rev Vaccines* **7**, 175-191 (2008).
229. Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. & Osterhaus, A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* **308**, 457-460 (1984).
230. Voeten, J.T., Rimmelzwaan, G.F., Nieuwkoop, N.J., Lovgren-Bengtsson, K. & Osterhaus, A.D. Introduction of the haemagglutinin transmembrane region in the influenza virus matrix protein facilitates its incorporation into ISCOM and activation of specific CD8(+) cytotoxic T lymphocytes. *Vaccine* **19**, 514-522 (2000).
231. Rimmelzwaan, G.F., *et al.* Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. *Journal of General Virology* **78**, 757 (1997).
232. Paillot, R., Grimmett, H., Elton, D. & Daly, J.M. Protection, systemic IFNgamma, and antibody responses induced by an ISCOM-based vaccine against a recent equine influenza virus in its natural host. *Vet Res* **39**, 21 (2008).
233. Rimmelzwaan, G.F., Claas, E.C., van Amerongen, G., de Jong, J.C. & Osterhaus, A.D. ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. *Vaccine* **17**, 1355-1358 (1999).
234. Sambhara, S., *et al.* Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. *Cell Immunol* **211**, 143-153 (2001).
235. Sambhara, S., *et al.* Heterotypic protection against influenza by immunostimulating complexes is associated with the induction of cross-reactive cytotoxic T lymphocytes. *J Infect Dis* **177**, 1266-1274 (1998).
236. Didierlaurent, A.M., *et al.* AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *The Journal of Immunology* **183**, 6186 (2009).
237. WHO, I.f.V.R.I. Table on Clinical trials of pandemic and potentially pandemic influenza vaccines. (2011).

Chapter 2

Influenza B virus

Biology, etiology and epidemiology

2.1. Introduction

The *Orthomyxoviridae* family of RNA viruses comprises at least five different genera [1]. Next to Isavirus and Thogotovirus, these include the influenza A, B and C viruses. They are all characterized by a negative-stranded, segmented single-stranded RNA genome. The influenza viruses are subdivided in types A, B and C based on the degree of antigenic similarity of their nucleoprotein (NP) and matrix protein 1 (M1), which show no cross-reactivity across the three types. Influenza B (IBV) and influenza C viruses are human pathogens, although in rare cases infections have been identified in other animals [2,3]. In contrast, influenza A viruses (IAV) circulate in a vast animal reservoir. IAV are subdivided based on the similarity of the major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, 16 different HA subtypes and nine NA subtypes have been identified [4]. All of these can be found back in the avian reservoir of IAV strains. Additionally, certain strains can be readily detected in equine (H3N8, H7N7), swine (H1N1, H1N2, H3N1, H3N2 and H2N3) and occasionally canine, feline and other mammalian species. This large group of hosts, combined with the segmented genome, can result in co-infection of an individual host, with mixing of gene segments as a consequence. In rare cases, this will yield a gene constellation with intragenic compatibility, resulting in a replication-competent virus with dramatically altered antigenic characteristics compared to these of influenza viruses that circulate in human. If this does occur, it can lead to a pandemic or panzootic with profound effects [5]. In recent human history, five major pandemics have occurred: the 1918 Spanish flu (H1N1), the 1957 Asian flu (H2N2), the 1968 Hong Kong flu (H3N2), the 1977 Russian flu (H1N1) and most recently the 2009 Mexican flu (H1N1). During interpandemic periods, strains related to the previous pandemic circulate further as epidemic strains, diversifying under immunological pressure. Currently, the descendants of the Hong Kong and Mexican flu roam in the human population. These not only continue to drift antigenically, but could also feed future pandemic strains. Indeed, molecular characterization of the Mexican flu virus has shown that it is a reassortant virus composed of genes derived from both the Spanish flu and the Hong Kong flu [6].

With a constant threat imposed by zoonotic transfers of IAV, or emergence of pandemic strains, a major focus has been put on broad IAV vaccine development [7]. However, IBV also causes epidemics. Even with a lack of a comprehensive animal reservoir, and no distinction of HA and NA subtypes, the situation is getting precarious. Since the 1970s, two antigenically distinct lineages have been recognized, prototyped by B/Victoria/02/1987 and B/Yamagata/16/1988 [8]. This has imposed a great amount of pressure on conventional trivalent vaccination strategies that are based on inactivated viruses (TIV or trivalent inactivated vaccine) of A/H1N1, A/H3N2 and a single IBV strain. Over the past decade, frequent mismatches have occurred between vaccine and circulating IBV strain with regard to lineage selection (Table 2.1). In fact, in five out of eleven seasons, the selected vaccine strain was of the opposite lineage as the epidemic strain. Clearly, novel strategies need to be addressed to avoid future problems. In this chapter, the biological and epidemiological properties of IBV will be discussed, leading to front-line approaches based on these characteristics for broad influenza B vaccine development.

Table 2.1. Overview of European circulating strains and IBV vaccine strains, illustrating recurrent mismatches of IBV lineage selection. Data is based on information from the European Influenza Surveillance Network (www.ecdc.europa.eu), complete until the 2009-2010 season. *, pandemic H1N1 / seasonal H1N1; [+], pandemic H1N1 only, replacing seasonal H1N1 circulating before 2009.

Season	Prevalence (%)				IBV lineage prevalence			IBV vaccine strain		Dominating circulating IBV strain		Mismatch
	A (Untyped)	A/H1	A/H3	B	Untyped	Yam	Vic	Prototypical strain	Lineage	Prototypical strain	Lineage	
2000-2001	48,9	32,9	0,8	17,4	-	-	-	B/Beijing/184/93	Yam	B/Beijing/184/93	Yam	No
2001-2002	47,9	4,0	26,8	21,3	91,8	1,7	6,5	B/Sichuan/379/99	Yam	B/Hong Kong/330/01	Vic	Yes
2002-2003	24,0	7,0	25,0	44,0	51,5	5,3	43,2	B/Hong Kong/330/2001	Vic	B/Hong Kong/330/01	Vic	No
2003-2004	56,4	0,4	42,3	0,9	84,5	9,5	6,03	B/Hong Kong/330/2001	Vic	B/Shanghai/361/2002	Yam	Yes
2004-2005	39,3	6,6	37,1	17,0	69,8	17,1	13,0	B/Shanghai/361/2002	Yam	B/Shanghai/361/2002	Yam	No
2005-2006	28,9	7,2	5,9	58,0	69,2	3,1	27,7	B/Shanghai/361/2002	Yam	B/Malaysia/2506/2004	Vic	Yes
2006-2007	48,3	3,6	45,3	2,8	65,7	5,8	28,5	B/Malaysia/2506/2004	Vic	B/Malaysia/2506/2004	Vic	No
2007-2008	26,5	32,1	1,4	40,2	75,6	24,1	0,3	B/Malaysia/2506/2004	Vic	B/Florida/4/2006	Yam	Yes
2008-2009	23,0	3,0	55,0	19,0	34,5	2,3	63,2	B/Florida/4/2006	Yam	B/Brisbane/60/2008	Vic	Yes
2009-2010	5,0	93,8 / 0,2*	0,05	1,0	87,9	3,0	9,1	B/Brisbane/60/2008	Vic	B/Brisbane/60/2008	Vic	No
2010-2011		(80)[+]		(20)				B/Brisbane/60/2008	Vic	B/Brisbane/60/2008	Vic	No
2011-2012	-	-	-	-	-	-	-	B/Brisbane/60/2008	Vic	-	-	-

2.2. The biology of influenza B virus

Like IAV viruses, the genome of IBV is divided in eight segments. However, despite considerable biological similarities, a number of features differentiate the two viruses. On the whole, the length of the genome of IBV is appr. 1kb larger than that of IAV (14.6kB vs. 13.6kb). This is largely due to the much longer 3' and 5' non-coding regions flanking the IBV coding sequences [9,10]. These non-coding regions contain complementary sequences, forming a basis for viral RNA (vRNA) secondary structure formation [11,12], promoter regions essential for replication and transcription [13,14] and packaging signals for virion formation [15]. For IAV, the eight segments code for up to 12 different proteins; in the case of IBV, 11 open reading frames (ORF) have been identified (Table 2.2). IAV has two genus-specific proteins, which have only recently been identified: PB1-F2 [16,17] and N40 [18], both encoded by the PB1 segment. IBV contains one protein with no known homologue in IAV: the tetrameric membrane protein NB, encoded by segment 6 [19]. In the next part of this review, proteins with a distinctive biological role for IBV as compared to IAV will be discussed.

Table 2.2. Overview of the IAV and IBV encoded proteins. The coding strategy used to express multiple proteins from a single genomic segment is indicated.

Segment	Virus	Proteins	Coding strategy
1	IAV/IBV	PB2	-
2	IAV	PB1 / PB1-F2 / N40	Overlapping reading frames
	IBV	PB1	-
3	IAV/IBV	PA	-
4	IAV/IBV	HA	-
5	IAV/IBV	NP	-
6	IAV	NA	-
	IBV	NA/NB	Overlapping reading frames
7	IAV	M1/M2	M1 colinear mRNA; M2 spliced mRNA
	IBV	BM1/BM2	Biscistronic mRNA; start-stop pentanucleotide sequence (UA**A**UG)
8	IAV/IBV	NS1/NEP (NS2)	NS1 colinear mRNA; NEP spliced mRNA

2.2.1. The NB protein

The presence of the coding sequence for the NB protein was first described by Shaw *et al.* in 1983, by careful examination of the nucleotide sequence of segment 6 of IBV [19]. NB had not been previously detected in radiolabeling experiments using ^{35}S-Met, since NB only contains one methionine residue : the initiator methionine, which is posttranslationally removed. In a later study, Williams and Lamb showed that NB was a membrane associated disulfide linked oligomeric protein, with an N-terminal ectodomain containing two glycosylation sites used for the addition of complex-type poly-N-lactosaminoglycans [20,21]. Later, two groups independently reported that NB is present in virions as an oligomer, adopting a similar N_{out}-C_{in} orientation as in infected cells [22,23].

The NB ORF, initiated by the first AUG codon in the segment, overlaps almost completely with that of NA. The NA ORF starts at the eighth NB-coding nucleotide (AUGAACA**AUG**, with the NB start codon underlined and the NA start codon bold) [19]. Although ribosomal control of viral gene expression is often used to control relative protein expression levels, NB and NA are expressed in equal amounts in infected cells, suggesting an equal preference of ribosomes to initiate translation from either AUG-codon [24]. However, in purified IBV virions, much lower amounts of NB than NA are found back, reminiscent of the relative distribution of M2 in IAV virus particles versus infected cells [22,23,25].

The strong similarities with the M2 ion channel of influenza A virus, *i.e*. membrane association, orientation and oligomeric structure [26,27], led to the hypothesis that NB was the biological counterpart of M2 for IBV, serving as a passive proton channel. Initial data using viral expressed NB or synthesized NB transmembrane peptides reconstituted in lipid bilayers revealed cation gating activity, although proton-selectivity could not be certified [28-30]. Moreover, this activity could be blocked with amantadine, also capable of blocking M2 activity [31]. However, amantadine could not inhibit IBV infectivity, contradicting the functional equivalence of M2 and NB. Indeed, construction of recombinant IBV strains lacking NB expression showed no need for NB activity in *in vitro* systems, although the protein does promote efficient replication *in vivo* in mice [32]. As of yet, no exact ion-selectivity or function has been attributed to this IBV specific protein. However, since circulating IBV retain NB expression the protein is most likely important in the viral life cycle.

2.2.2. The BM2 protein

Currently, the BM2 protein, encoded by RNA segment 7 alongside the matrix M1 protein, is the accepted biological counterpart of the M2 ion channel of IAV. Surprisingly, the ORF for the protein was already described in 1982, *i.e.* before the identification of the NB ORF [33]. Immediately it was clear that it represented a conserved feature of the virus, since alignment revealed high homology between different IBV strains [33-35]. It was not until 1990 that a protein could be assigned to the ORF, which was designated BM2 [36]. Using antiserum raised with bacterial expressed BM2, Odagiri *et al.* could show that BM2 is a phosphorylated membrane-associated protein which is incorporated into virions [37]. Later studies identified BM2 as an oligomeric transmembrane protein with N_{out}-C_{in}

orientation [38-40]. This entails that BM2, like M2 and NB, is a type III transmembrane protein lacking a cleavable signal sequence.

On the functional level, BM2 fulfills the role of M2 in IAV replication, although some dissimilarities are observed [41]. Paterson *et al*. first recognized the similarity in the transmembrane section of M2 and BM2, in that both contain the motif HXXXW, essential for M2 proton conductance [38,42,43]. The assumption that BM2 was the pH-modulating channel of IBV was confirmed by electrophysical measurements in cellular systems [44]. Precise modeling of the pore of the BM2 ion channel explained the inability of amantadine to block IBV replication: while the hydrophobic pore of the M2 transmembrane section allows association with amantadine, the hydrophilic BM2 pore does not accommodate binding of the drug [41,45-47]. An additional but essential role in IBV virion formation was attributed to BM2. The cytoplasmic tail of BM2 ensures membrane association of M1 and incorporation of vRNP in viruses, leading to virion formation; truncation of this domain yields improper formed virus particles [48-50]. A similar function can be assigned to the M2 protein for IAV, which has been shown to fulfill a protagonistic role in the budding process by executing membrane scission of newly formed virions [51-53]. The non-redundant function of BM2 in the IBV life cycle is exemplified by the fact that BM2-negative viruses are not viable and can only be rescued by complementation of the BM2 functions e.g. by growth of BM-deficient IBV in stable BM2 expressing cells [54].

The specific coding strategy employed by IBV RNA segment 7 is quite intriguing. It utilizes a stop-start pentanucleotide (UAAUG), in which the start codon of the BM2 ORF is downstream of but overlaps with the stop codon (UAA) of the M1 ORF. Expression of BM2 is assured by termination/re-initiation events, circumventing direct recruitment of ribosomes and the need of sequences to do so. The biological significance of such a coding strategy is often related to expression levels. Generally, the second ORF will only be translated to a level of 10% compared to the first ORF, as indeed is the case for BM2 (reviewed in [55]). Molecular analysis has revealed that the minimal upstream nucleotide sequences that are needed for efficient BM2 expression include the 3'-end 45 nucleotides of the M1 ORF [56]. Such sequences have been termed TURBS (termination upstream ribosome binding site) and they interact directly with ribosomal RNA and eukaryotic initiating factor eIF3 [55,57]. The precise and compact organization of coding sequences demonstrated by such an assembly of ORFs emphasizes the highly evolved viral strategies used for genomic condensation and protein expression regulation.

2.2.3. The BNS1 protein

The influenza NS1 proteins serve as antagonists of innate immunity to give the virus the upper hand at the early stage of infection. As for AM2 and BM2, the gross function of ANS1 and BNS1 is similar. Both proteins possess an N-terminal dsRNA binding domain and can interact with PKR to limit dsRNA related immune responses [58]. However, both proteins also exert distinct functions within their respective viral life cycle. ANS1 can specifically retain poly(A)-mRNA in the nucleus and prevent pre-mRNA splicing to create a nuclear pool of capped mRNA by inhibiting CPSF30 and PABPII function [59]. BNS1 in its turn binds ISG15 (interferon stimulated gene 15), an antiviral ubiquitin-like protein upregulated by IFN type I induction, thus prohibiting its conjugation to target proteins [60]. ISGylation is

a modification with broad implications in immunity and cellular processes, and related research has only scratched the surface of its biological and anti-infectious role [61]. Most interestingly, two recent publications have identified the specificity of BNS1 to be restricted to human and non-human primate ISGylation [62,63]. The domain of ISG15 bound by BNS1 shows species-specific conservation motifs, forming the basis for the restricted antagonism [64]. This specific interaction is one of the first indications for the confined host range of IBV.

2.3. Epidemiological characteristics of influenza B virus

Since the 1970s, two antigenically distinct IBV lineages are acknowledged, denoted by their prototypical strains B/Victoria/02/1987 and B/Yamagata/16/1988 [8,65,66]. During the 1980s, B/Victoria strains dominated, after which strains of both lineages co-circulated in the human population [8]. This has led to the creation of reassortant viruses, bearing genetic constellations containing gene segments of both lineages. Such viruses possess adaptive benefits and can be detected all over the globe [8,67-74]. Specifically, recombinant viruses bearing a Victoria HA gene and a Yamagata NA gene appear to have quite some epidemic power, pointing to an important role for NA in creating IBV diversity [69-74].

The HA of IBV is characterized by four antigenic sites: the 120-loop, the 150-loop, the 160-loop and the 190-helix, all of which show positive selection of specific residues [75-77]. This selective pressure is mainly induced by antibody responses evoked by infection or vaccination, similar to IAV. Specific sites can be attributed to either lineage: the 150-loop appears to contain a conserved neutralizing epitope for Yamagata strains [78], while the 160-loop is more specific for Victoria strains [79]. The latter site is characterized by amino acid deletions in the Yamagata lineage, and as such it has been postulated that immunological pressure on this site in Victoria strains initiated the emergence of the Yamagata lineage [78,79]. The evolutionary path followed by either lineage nowadays is dictated by infection and vaccination-induced herd immunity, exerting differential pressure on both lineages, and co-circulation with IAV [75,80]. Additional to antigenic drift and insertion-deletion mechanisms, reassortment between both lineages can benefit IBV greatly by creating genetic diversity, often related to NS1 gene usage [81,82]. Accordingly, groups of strains can be differentiated within each lineage, differing in gene constellation [80]. The precise effect of specific IBV genes on replication efficiency and transmissibility remains unknown and could provide a wealth of information and could lead to formulation of novel IBV vaccines or therapeutics.

The epidemiological significance of the antigenic distinctiveness of the two influenza B lineages has been illustrated by a number of studies. Two concomitant studies by the same research group using conventional TIV vaccinated children aimed to investigate the effect of spacing between a prime and booster immunization [83,84] (summarized in Table 2.3). The timing of their study combined the 2003/2004 and 2004/2005 influenza seasons, which were characterized by a dual change in composition. The A/H3N2 strain was changed from A/Panama/2007/1999 to A/Wyoming/03/2003 (a A/Fujian/411/2002-like strain) and the IBV strain was adapted from B/Hong Kong/330/2001-like viruses (belonging to the Victoria-lineage) to B/Jiangsu/10/2003 (a B/Shanghai/361/2002-like virus, belonging to the Yamagata-lineage). In both studies one group of children was immunized according to a conventional scheme, *i.e.* twice one-month apart, with the 2004/2005 TIV vaccine. A second

group of children was primed with the 2003/2004 vaccine either in the fall [83] or in the spring [84] of that season and boosted the next fall with the 2004/2005 vaccine. Quite striking results were seen when evaluating the serological responses of the two groups (scored by hemagglutination inhibition titers; HAI). Titers for A/H1N1 were similar in both groups, as would be expected from the inclusion of the same strain in both vaccines. The deviating A/H3N2 strains also elicited protective titers (HAI≥32) in a high percentage of both groups (70% and 84% for differential immunized children, 83% and 91% for identically immunized children). For IBV, the situation was completely different. Giving the same vaccine twice (in a short period of time) yielded protective titers in 88% and 86% of vaccinated children in both studies. However, the distinct vaccines could only induce protective titers against the novel B/Shanghai/361/2002-like virus in 39% or 27% of vaccinees, underlining the antigenic distinctiveness of strains belonging to the Victoria- or Yamagata-lineage. In a study utilizing MF59 adjuvanted TIV vaccines for immunization of elderly people in the 2007/2008, similar observations were made: a mismatch between the B/Malaysia/2506/2004-like vaccine strain (Victoria lineage) and the circulating B/Florida/04/2006-like strain (Yamagata lineage) could not prevent an outbreak of IBV in vaccinated people [85].

Table 2.3. Overview of the results obtained by Englund *et al.* (2006) and Walter *et al.* (2006), scrutinizing the boosting effect of distinct IBV strains in children. Groups of children were either primed with the 2003/2004 inactivated influenza vaccine, containing A/Panama/2007/1999 (H3N2) and B/Hong Kong/1434/2002 (Victoria-lineage) or with the 2004/2005 vaccine, containing A/Wyoming/03/2003 (H3N2) and B/Jiangsu/10/2003 (Yamagata-lineage), and boosted with the 2004/2005 vaccine. Boosting efficacy was evaluated by the induction of hemagglutination inhibition (HAI) titers ≥32.

Virus	Priming strain	Booster strain	HAI titer ≥32	Ref
A/H3N2	A/Panama/2007/1999	A/Wyoming/03/2003	84%	83
			70%	84
	A/Wyoming/03/2003	A/Wyoming/03/2003	91%	83
			83%	84
IBV	B/Hong Kong/1434/2002 (Vic)	B/Jiangsu/10/2003 (Yam)	27%	83
			39%	84
	B/Jiangsu/10/2003 (Yam)	B/Jiangsu/10/2003 (Yam)	86%	83
			88%	84

Very clear evidence for antigenic individuality of the Victoria and Yamagata lineage has come from clinical trials using live attenuated influenza viruses (LAIV), formulated as trivalent vaccines. Belshe *et al*. organized all available data from such trials performed using successive LAIV immunization in children aged 0.5-6 years [86]. In strain-matching situations, the effectiveness of vaccination reached 86%. However, when circulating strains drifted from the vaccine strain (but both of the same lineage), this diminished to 55%. In situations where the vaccine and epidemic strain were from opposite lineages, effectiveness of vaccination was further reduced to 31%, leading to a lack of protection in vaccinees. Similar observations were made by Ohmit *et al*., who performed a study in

adults using LAIV in the 2004/2005 season when vaccine and epidemic strains did not match [87], and by Skowronski *et al.*, who determined vaccine effectiveness in the 2005/2006 season in Canada when both the A/H3N2 and IBV vaccine strain did not match the circulating strain [88].

Overall, the biological and epidemiological data discussed here indicate the need for better tactics to combat IBV epidemics. Indeed, in instances a mismatch occurs between vaccine strain and circulating strain, excess morbidity can be observed in vaccinated populations. This has been evidenced by a number of reports describing IBV outbreaks in closed environments during the 2007/2008 season [85,89]. This is especially worrying for high-risk groups, such as the elderly and people with underlying medical conditions. Moreover, it should be noted that IBV induces higher morbidity in children as compared to adults, and is more often associated with severe complications as compared to IAV [82,90-95]. Although very little data is available comparing influenza A and B infections directly in children [96], one study showed influenza B infection predisposes infected children to develop myositis six times more than influenza A infection does [97].

2.4. Novel influenza B vaccination strategies

Conventional TIV vaccines, containing a single IBV strain from one selected lineage, are not effective against IBV infection. This is clearly illustrated by the mismatch of 5 out of eleven selected vaccine strains with circulating strains (Table 2.1). Additionally, for reasons not quite clear yet, the immunogenicity of IBV in TIV vaccines is reproducibly lower than for IAV, inducing lower geometric mean titers (GMT) and HAI titers [85,98]. Without a doubt, novel frontline strategies for inducing IBV immunity are clearly warranted. The most straightforward answer to the problem is inclusion of strains of both lineages in vaccine preparations, thus creating quadrivalent vaccines. However, this would impose a tremendous amount of pressure on manufacturing capacity, which would require re-organization of production systems [99]. Alternatively, frontline adjuvantation system might broaden the antibody responses to B/HA antigens in such a way that they become cross-reactive for strains of both lineages [100]. Such strategies are supported by data showing that immunization with TIV vaccines can induce cross-lineage reactive antibodies if the subject has been primed by infection or TIV vaccination, although these generally do not reach theoretical protective levels [101-104].

It is widely accepted that inactivated virus vaccines elicit a more restricted immune response as opposed to infectious virus. Hence, a great deal of effort has been put in development of live attenuated influenza vaccines (LAIV). One such approach is based on cultivation of viruses at decreasing temperatures, leading to the accumulation of mutations restricting efficient growth at higher temperature. Such cold-adapted strains display a temperature-sensitive attenuated growth phenotype *in vivo* [105-108]. Recently, the group of Jonathan McCullers performed a direct comparison of inactivated IBV and LAIV in inducing (homotypical) protection in mice and ferrets [109]. They concluded that LAIV is superior to inactivated virus in the animal models tested. However, clinical use of LAIV has shown that lineage mismatch is still an issue [86,87].

Other LAIV formats for IBV have been developed preclinically. Based on a strategy employed for IAV, deletion, mutation or truncation of the BNS1 gene induces severe attenuation of viral growth in IFN-

competent cells and mice [110-113]. Infection of mice with such strains induces an immune response that is protective against a subsequent lethal challenge with homologous or heterologous IBV [112]. Whether this also translates into broad protection for humans still needs to be addressed.

A third method of inducing attenuation is by mutating the maturational cleavage site of the HA. By changing the basic residue responsible for cleavage of HA0 into HA1 and HA2 to Ala or Val, the specificity for trypsin-like proteases is changed to elastase-dependent cleavage [114,115]. Recombinant viruses can be grown efficiently *in vitro* to similar titers as wild type virus, by inclusion of elastase instead of trypsin in culture media, but these viruses display a very low infectivity *in vivo*, restricted to a single or at most a few replication cycles. By administering high viral doses, protective immune responses can be evoked in homologous settings [114,115].

A last format used for attenuation of influenza is chimerization by mixing IAV and IBV genes. Introduction of the B/HA gene in the IAV genetic background rendered the virus crippled *in vitro* and *in vivo*, but allowed the induction of very broad effective immunity active against lethal IAV [116] and IBV [117] infection. Analysis of the breadth and type of immunity induced by such attenuated strains, or LAIV in general, is warranted to optimally induce antiviral activity. Additionally, alternative approaches inducing attenuation, such as deletion or modification of the BM2 or NB protein [32,49,54], also deserve further investigation to obtain broader immunity inducing LAIV.

A select number of research groups have endeavored to develop a recombinant protein approach to combat IBV infection. Most noteworthy, Bianchi *et al*. fused the maturational cleavage sequence of HA of IBV (B/HA0) to OMPC (outer membrane protein complex) as immunogen for immunization of mice [118]. A potential lethal infection with B/Ann Arbor/04/55 (pre-dating lineage separation), B/Hong Kong/330/01 (Victoria lineage) or B/Jamanashi/166/98 (Yamagata lineage) showed induction of protective responses with the B/HA0-vaccine. This immunity was Fc-mediated, showing no direct neutralization effect on IBV replication. Quite strikingly, because of conservation of the maturational cleavage site across IAV and IBV, immunization with a similar vaccine based on the AH3/HA0 cleavage site also could afford protection against A/H1N1, A/H3N2 and IBV infection [118], showing an ultimate universal profile of protection. Most recently, Wang *et al*. reported on the development of BM2e-specific antibodies [119]. These are able to protect MDCK cells from cytopathic effects induced by IBV to a certain extent. Additionally, they are able to reduce but not neutralize replication of IBV in a plaque size reduction assay, reminiscent of M2-specific antibodies and IAV replication [25].

2.5. Concluding remarks

The antigenic situation of IBV is troublesome, to say the least. The constant co-circulation and reassortment of the Yamagata and Victoria lineage of IBV hinder effective vaccination with conventional TIV. Indeed, a mismatch in 5 out of the last eleven seasons highlights a major problem in the current vaccination policy. The easiest and fasted way of avoiding future problems is upgrading current vaccines (be it inactivated or live attenuated) from trivalent to quadrivalent, although this might create logistic and manufacturing problems. Alternative approaches should aim at inducing cross-lineages responses, which can be achieved by certain LAIV strains inducing cellular immunity or

cross-reactive antibody responses, novel adjuvantation systems, or consensus IBV sequences such as the maturation cleavage site of HA and BM2e. Of these, only temperature-sensitive cold-adapted LAIV viruses have been tested clinically and are used currently in the human population (Flumist, MedImmune Inc.). Clinical data from seasons with a vaccine mismatch indicate a lack of high cross-lineage protection induced by these [86]. Further investigation in immune responses elicited by (live) vaccination and infection might avoid this and afford the broad IBV immunity so desperately needed through identification of cross-protective viral features.

2.6. References

1. Presti, R.M.*, et al.* Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae. *Journal of virology* **83**, 11599 (2009).
2. Osterhaus, A., Rimmelzwaan, G.F., Martina, B.E.E., Bestebroer, T.M. & Fouchier, R.A.M. Influenza B virus in seals. *Science* **288**, 1051 (2000).
3. Kawano, J., Onta, T., Kida, H. & Yanagawa, R. Distribution of antibodies in animals against influenza B and C viruses. *Japanese Journal of Veterinary Research* **26**, 74-80 (1978).
4. Fouchier, R.A.M.*, et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of virology* **79**, 2814 (2005).
5. Morens, D.M. & Taubenberger, J.K. Original Article: Historical thoughts on influenza viral ecosystems, or behold a pale horse, dead dogs, failing fowl, and sick swine. *Influenza and Other Respiratory Viruses* **4**, 327-337 (2010).
6. Smith, G.J.D.*, et al.* Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* **459**, 1122-1125 (2009).
7. Roose, K., Fiers, W. & Saelens, X. Pandemic preparedness: toward a universal influenza vaccine. *Drug News Perspect* **22**, 80-92 (2009).
8. McCullers, J.A., Saito, T. & Iverson, A.R. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *Journal of virology* **78**, 12817 (2004).
9. Stoeckle, M.Y., Shaw, M.W. & Choppin, P.W. Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. *Proceedings of the National Academy of Sciences* **84**, 2703 (1987).
10. Hoffmann, E.*, et al.* Rescue of influenza B virus from eight plasmids. *Proceedings of the National Academy of Sciences* **99**, 11411 (2002).
11. Desselberger, U., Racaniello, V.R., Zazra, J.J. & Palese, P. The 3'and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* **8**, 315-328 (1980).
12. Hsu, M.T., Parvin, J.D., Gupta, S., Krystal, M. & Palese, P. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proceedings of the National Academy of Sciences* **84**, 8140 (1987).
13. Barclay, W.S. & Palese, P. Influenza B viruses with site-specific mutations introduced into the HA gene. *Journal of virology* **69**, 1275 (1995).
14. Zheng, H., Palese, P. & García-Sastre, A. Nonconserved nucleotides at the 3'and 5'ends of an influenza A virus RNA play an important role in viral RNA replication. *Virology* **217**, 242-251 (1996).
15. Fujii, Y., Goto, H., Watanabe, T., Yoshida, T. & Kawaoka, Y. Selective incorporation of influenza virus RNA segments into virions. *Proceedings of the National Academy of Sciences* **100**, 2002 (2003).
16. Chen, W.*, et al.* A novel influenza A virus mitochondrial protein that induces cell death. *Nature medicine* **7**, 1306-1312 (2001).
17. Krumbholz, A.*, et al.* Current knowledge on PB1-F2 of influenza A viruses. *Medical microbiology and immunology* **200**, 69-75 (2011).
18. Wise, H.M.*, et al.* A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *Journal of virology* **83**, 8021 (2009).
19. Shaw, M.W., Choppin, P.W. & Lamb, R.A. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proceedings of the National Academy of Sciences* **80**, 4879 (1983).
20. Williams, M.A. & Lamb, R.A. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Molecular and Cellular Biology* **6**, 4317 (1986).
21. Williams, M.A. & Lamb, R.A. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Molecular and Cellular Biology* **8**, 1186 (1988).
22. Betakova, T., Nermut, M.V. & Hay, A.J. The NB protein is an integral component of the membrane of influenza B virus. *Journal of general virology* **77**, 2689 (1996).
23. Brassard, D.L., Leser, G.P. & Lamb, R.A. Influenza B virus NB glycoprotein is a component of the virion. *Virology* **220**, 350-360 (1996).
24. Williams, M.A. & Lamb, R.A. Effect of mutations and deletions in a bicistronic mRNA on the synthesis of influenza B virus NB and NA glycoproteins. *Journal of virology* **63**, 28 (1989).

25. Zebedee, S.L. & Lamb, R.A. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *Journal of virology* **62**, 2762 (1988).
26. Lamb, R.A., Zebedee, S.L. & Richardson, C.D. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell* **40**, 627-633 (1985).
27. Sugrue, R.J. & Hay, A.J. Structural characteristics of the M2 protein of influenza a viruses: Evidence that it forms a tetrameric channe. *Virology* **180**, 617-624 (1991).
28. Sunstrom, N.A*., et al.* Ion channels formed by NB, an influenza B virus protein. *Journal of Membrane Biology* **150**, 127-132 (1996).
29. Fischer, W.B*., et al.* Transmembrane peptide NB of influenza B: a simulation, structure, and conductance study. *Biochemistry* **39**, 12708-12716 (2000).
30. Premkumar, A., Ewart, G.D., Cox, G.B. & Gage, P.W. An amino-acid substitution in the influenza-B NB protein affects ion-channel gating. *Journal of Membrane Biology* **197**, 135-143 (2004).
31. Fischer, W.B., Pitkeathly, M. & Sansom, M.S.P. Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B. *European Biophysics Journal* **30**, 416-420 (2001).
32. Hatta, M. & Kawaoka, Y. The NB protein of influenza B virus is not necessary for virus replication in vitro. *Journal of virology* **77**, 6050 (2003).
33. Briedis, D.J., Lamb, R.A. & Choppin, P.W. Sequence of RNA segment 7 of the influenza B virus genome: partial amino acid homology between the membrane proteins (M1) of influenza A and B viruses and conservation of a second open reading frame. *Virology* **116**, 581-588 (1982).
34. Hiebert, S.W., Williams, M.A. & Lamb, R.A. Nucleotide sequence of RNA segment 7 of influenza B/Singapore/222/79: maintenance of a second large open reading frame. *Virology* **155**, 747-751 (1986).
35. DeBorde, D.C., Donabedian, A.M., Herlocher, M.L., Naeve, C.W. & Maassab, H.F. Sequence comparison of wild-type and cold-adapted B/Ann Arbor/1/66 influenza virus genes. *Virology* **163**, 429-443 (1988).
36. Horvath, C.M., Williams, M.A. & Lamb, R.A. Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. *The EMBO Journal* **9**, 2639 (1990).
37. Odagiri, T., Hong, J. & Ohara, Y. The BM2 protein of influenza B virus is synthesized in the late phase of infection and incorporated into virions as a subviral component. *Journal of General Virology* **80**, 2573 (1999).
38. Paterson, R.G., Takeda, M., Ohigashi, Y., Pinto, L.H. & Lamb, R.A. Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology* **306**, 7-17 (2003).
39. Watanabe, S., Imai, M., Ohara, Y. & Odagiri, T. Influenza B virus BM2 protein is transported through the trans-Golgi network as an integral membrane protein. *Journal of virology* **77**, 10630 (2003).
40. Balannik, V., Lamb, R.A. & Pinto, L.H. The oligomeric state of the active BM2 ion channel protein of influenza B virus. *Journal of Biological Chemistry* **283**, 4895 (2008).
41. Wang, J., Pielak, R.M., McClintock, M.A. & Chou, J.J. Solution structure and functional analysis of the influenza B proton channel. *Nature Structural & Molecular Biology* **16**, 1267-1271 (2009).
42. Wang, C., Lamb, R.A. & Pinto, L.H. Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue. *Biophysical journal* **69**, 1363-1371 (1995).
43. Tang, Y., Zaitseva, F., Lamb, R.A. & Pinto, L.H. The gate of the influenza virus M2 proton channel is formed by a single tryptophan residue. *Journal of Biological Chemistry* **277**, 39880 (2002).
44. Mould, J.A*., et al.* Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. *Developmental cell* **5**, 175-184 (2003).
45. Ma, C*., et al.* Identification of the pore-lining residues of the BM2 ion channel protein of influenza B virus. *Journal of Biological Chemistry* **283**, 15921 (2008).
46. Leonov, H., Astrahan, P., Krugliak, M. & Arkin, I.T. How do aminoadamantanes block the influenza M2 channel and how does resistance develop? *Journal of the American Chemical Society* **133**, 9903-9911 (2011).
47. Cady SD, W.J., Wu Y, DeGrado WF, Hong M. Specific binding of adamantane drugs and direction of their polar amines in the pore of the influenza M2 transmembrane domain in lipid bilayers and dodecylphosphocholine micelles determined by NMR spectroscopy. *J Am Chem Soc* **133**, 4274-4284 (2011).
48. Imai, M., Watanabe, S., Ninomiya, A., Obuchi, M. & Odagiri, T. Influenza B virus BM2 protein is a crucial component for incorporation of viral ribonucleoprotein complex into virions during virus assembly. *Journal of virology* **78**, 11007 (2004).

49. Jackson, D., Zurcher, T. & Barclay, W. Reduced incorporation of the influenza B virus BM2 protein in virus particles decreases infectivity. *Virology* **322**, 276-285 (2004).
50. Imai, M., Kawasaki, K. & Odagiri, T. Cytoplasmic domain of influenza B virus BM2 protein plays critical roles in production of infectious virus. *Journal of virology* **82**, 728 (2008).
51. Iwatsuki-Horimoto, K.*, et al.* The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. *Journal of virology* **80**, 5233 (2006).
52. Chen, B.J., Leser, G.P., Jackson, D. & Lamb, R.A. The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding. *Journal of virology* **82**, 10059 (2008).
53. Rossman, J.S., Jing, X., Leser, G.P. & Lamb, R.A. Influenza Virus M2 Protein Mediates ESCRT-Independent Membrane Scission. *Cell* **142**, 902-913 (2010).
54. Hatta, M., Goto, H. & Kawaoka, Y. Influenza B virus requires BM2 protein for replication. *Journal of virology* **78**, 5576 (2004).
55. Powell, M.L., Brown, T.D. & Brierley, I. Translational termination-re-initiation in viral systems. *Biochemical Society Transactions* **36**, 717 (2008).
56. Hatta, M., Kohlmeier, C.K., Hatta, Y., Ozawa, M. & Kawaoka, Y. Region required for protein expression from the stop-start pentanucleotide in the M gene of influenza B virus. *Journal of virology* **83**, 5939 (2009).
57. Powell, M.L.*, et al.* Further Characterisation of the Translational Termination-Reinitiation Signal of the Influenza B Virus Segment 7 RNA. *PLoS One* **6**, e16822 (2011).
58. Wang, W. & Krug, R.M. The RNA-binding and effector domains of the viral NS1 protein are conserved to different extents among influenza A and B viruses. *Virology* **223**, 41-50 (1996).
59. Chen, Z. & Krug, R.M. Selective nuclear export of viral mRNAs in influenza-virus-infected cells. *Trends in microbiology* **8**, 376-383 (2000).
60. Yuan, W., Aramini, J.M., Montelione, G.T. & Krug, R.M. Structural basis for ubiquitin-like ISG 15 protein binding to the NS1 protein of influenza B virus: a protein-protein interaction function that is not shared by the corresponding N-terminal domain of the NS1 protein of influenza A virus. *Virology* **304**, 291-301 (2002).
61. Zhang D, Z.D. Interferon-stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res* **31**, 119-130 (2011).
62. Sridharan, H., Zhao, C. & Krug, R.M. Species Specificity of the NS1 Protein of Influenza B Virus. *Journal of Biological Chemistry* **285**, 7852 (2010).
63. Versteeg, G.A.*, et al.* Species-specific antagonism of host ISGylation by the influenza B virus NS1 protein. *Journal of virology* **84**, 5423 (2010).
64. Li, L.*, et al.* Crystal Structure of Human ISG15 Protein in Complex with Influenza B Virus NS1B. *Journal of Biological Chemistry* **286**, 30258-30262 (2011).
65. Rota, P.A.*, et al.* Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59-68 (1990).
66. Chen, J.M.*, et al.* Exploration of the emergence of the Victoria lineage of influenza B virus. *Archives of virology* **152**, 415-422 (2007).
67. Lindstrom, S.E.*, et al.* Comparative analysis of evolutionary mechanisms of the hemagglutinin and three internal protein genes of influenza B virus: multiple cocirculating lineages and frequent reassortment of the NP, M, and NS genes. *Journal of virology* **73**, 4413 (1999).
68. Chi, X.S., Bolar, T.V., Zhao, P., Rappaport, R. & Cheng, S.M. Cocirculation and evolution of two lineages of influenza B viruses in Europe and Israel in the 2001-2002 season. *Journal of clinical microbiology* **41**, 5770 (2003).
69. Abed, Y., Coulthart, M.B., Li, Y. & Boivin, G. Evolution of surface and nonstructural-1 genes of influenza B viruses isolated in the Province of Quebec, Canada, during the 1998-2001 period. *Virus genes* **27**, 125-135 (2003).
70. Barr, I.G.*, et al.* Reassortants in recent human influenza A and B isolates from South East Asia and Oceania. *Virus research* **98**, 35-44 (2003).
71. Ikonen, N., Pyhälä, R., Axelin, T., Kleemola, M. & Korpela, H. Reappearance of influenza B/Victoria/2/87-lineage viruses: epidemic activity, genetic diversity and vaccination efficacy in the Finnish Defence Forces. *Epidemiology and infection* **133**, 263-271 (2005).
72. Chi, X.S.*, et al.* Detection and characterization of new influenza B virus variants in 2002. *Journal of clinical microbiology* **43**, 2345 (2005).

73. Motta, F.C*., et al.* The reappearance of Victoria lineage influenza B virus in Brazil, antigenic and molecular analysis. *Journal of clinical virology* **36**, 208-214 (2006).
74. Jian, J.W*., et al.* Genetic analysis and evaluation of the reassortment of influenza B viruses isolated in Taiwan during the 2004-2005 and 2006-2007 epidemics. *Virus research* **131**, 243-249 (2008).
75. Pechirra, P*., et al.* Molecular characterization of the HA gene of influenza type B viruses. *Journal of medical virology* **77**, 541-549 (2005).
76. Wang, Q., Cheng, F., Lu, M., Tian, X. & Ma, J. Crystal structure of unliganded influenza B virus hemagglutinin. *Journal of virology* **82**, 3011 (2008).
77. Shen, J., Kirk, B.D., Ma, J. & Wang, Q. Diversifying selective pressure on influenza B virus hemagglutinin. *Journal of medical virology* **81**, 114-124 (2009).
78. Nakagawa, N., Kubota, R., Nakagawa, T. & Okuno, Y. Neutralizing epitopes specific for influenza B virus Yamagata group strains are in the 'loop'. *Journal of General Virology* **84**, 769 (2003).
79. Nakagawa, N., Kubota, R., Nakagawa, T. & Okuno, Y. Antigenic variants with amino acid deletions clarify a neutralizing epitope specific for influenza B virus Victoria group strains. *Journal of General Virology* **82**, 2169 (2001).
80. Chen, R. & Holmes, E.C. The evolutionary dynamics of human influenza B virus. *Journal of Molecular Evolution* **66**, 655-663 (2008).
81. McCullers, J.A., Wang, G.C., He, S. & Webster, R.G. Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *Journal of virology* **73**, 7343 (1999).
82. Li, W.C*., et al.* Clinical and genetic characterization of severe influenza B-associated diseases during an outbreak in Taiwan. *Journal of clinical virology* **42**, 45-51 (2008).
83. Englund, J.A*., et al.* Immunization with trivalent inactivated influenza vaccine in partially immunized toddlers. *Pediatrics* **118**, e579 (2006).
84. Walter, E.B*., et al.* Influenza vaccine immunogenicity in 6-to 23-month-old children: are identical antigens necessary for priming? *Pediatrics* **118**, e570 (2006).
85. Camilloni, B*., et al.* An influenza B outbreak during the 2007/2008 winter among appropriately immunized elderly people living in a nursing home. *Vaccine* **28**, 7536-7541 (2010).
86. Belshe, R.B., Coelingh, K., Ambrose, C.S., Woo, J.C. & Wu, X. Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. *Vaccine* **28**, 2149-2156 (2009).
87. Ohmit, S.E*., et al.* Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines. *New England Journal of Medicine* **355**, 2513-2522 (2006).
88. Skowronski, D.M*., et al.* Estimating vaccine effectiveness against laboratory-confirmed influenza using a sentinel physician network: results from the 2005-2006 season of dual A and B vaccine mismatch in Canada. *Vaccine* **25**, 2842-2851 (2007).
89. Win, M.K*., et al.* Influenza B Outbreak among Influenza-vaccinated Welfare Home Residents in Singapore. *Annals of the Academy of Medicine, Singapore* **39**, 448-452 (2010).
90. Glezen, W*., et al.* Epidemiologic observations of influenza B virus infections in Houston, Texas, 1976-1977. *American journal of epidemiology* **111**, 13 (1980).
91. Goni, N*., et al.* Influenza B viruses isolated in Uruguay during the 2002-2005 seasons: Genetic relations and vaccine strain match. *Virus research* **123**, 100-104 (2007).
92. Olson, D.R*., et al.* Monitoring the impact of influenza by age: emergency department fever and respiratory complaint surveillance in New York City. *PLoS Medicine* **4**, e247 (2007).
93. Hite, L.K., Glezen, W.P., Demmler, G.J. & Munoz, F.M. Medically attended pediatric influenza during the resurgence of the Victoria lineage of influenza B virus. *International journal of infectious diseases* **11**, 40-47 (2007).
94. Landi, K.K. & Coleman, A.T. Sudden Death in Toddlers Caused By Influenza B Infection: A Report of Two Cases and a Review of the Literature*. *Journal of forensic sciences* **53**, 213-215 (2008).
95. Baine, W.B., Luby, J.P. & Martin, S.M. Severe illness with influenza B. *The American journal of medicine* **68**, 181-189 (1980).
96. Carrat, F*., et al.* Time lines of infection and disease in human influenza: a review of volunteer challenge studies. *American journal of epidemiology* **167**, 775 (2008).
97. Hu, J*., et al.* Clinical features of influenza A and B in children and association with myositis. *Journal of Microbiology Immunology and Infection* **37**, 95 (2004).
98. Iorio, A.M*., et al.* Effects of repeated annual influenza vaccination on antibody responses against unchanged vaccine antigens in elderly frail institutionalized volunteers. *Gerontology* **53**, 411 (2007).
99. Belshe, R.B. The need for quadrivalent vaccine against seasonal influenza. *Vaccine* **28**, D45-D53 (2010).

100. Camilloni, B., Neri, M., Lepri, E. & Iorio, A.M. Cross-reactive antibodies in middle-aged and elderly volunteers after MF59-adjuvanted subunit trivalent influenza vaccine against B viruses of the B/Victoria or B/Yamagata lineages. *Vaccine* **27**, 4099-4103 (2009).
101. Zuckerman, M.A*., et al.* Serological responses in volunteers to inactivated trivalent subunit influenza vaccine: antibody reactivity with epidemic influenza A and B strains and evidence of a rapid immune response. *Journal of medical virology* **33**, 133-137 (1991).
102. Levandowski, R.A*., et al.* Antibody responses to influenza B viruses in immunologically unprimed children. *Pediatrics* **88**, 1031 (1991).
103. Levandowski, R.A*., et al.* Cross-reactive antibodies induced by a monovalent influenza B virus vaccine. *Journal of clinical microbiology* **29**, 1530 (1991).
104. Pyhälä, R*., et al.* Immune response to inactivated influenza virus vaccine: antibody reactivity with epidemic influenza B viruses of two highly distinct evolutionary lineages. *Vaccine* **10**, 631-636 (1992).
105. Maassab, H.F. Adaptation and growth characteristics of influenza virus at 25 C. *Nature* **213**, 612-614 (1967).
106. Maassab, H.F. & DeBorde, D.C. Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine* **3**, 355-369 (1985).
107. Cox, N.J., Kitame, F., Klimov, A., Koennecke, I. & Kendal, A.P. Comparative studies of wild-type and cold-mutant (temperature-sensitive) influenza virus: detection of mutations in all genes of the A/Ann Arbor/6/60 (H2N2) mutant vaccine donor strain. *Microbial Pathogenesis* **1**, 387-397 (1986).
108. Seo, S.U*., et al.* Development and characterization of a live attenuated influenza B virus vaccine candidate. *Vaccine* **26**, 874-881 (2008).
109. Huber, V.C., Kleimeyer, L.H. & McCullers, J.A. Live, attenuated influenza virus (LAIV) vehicles are strong inducers of immunity toward influenza B virus. *Vaccine* **26**, 5381-5388 (2008).
110. Talon, J*., et al.* Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. *Proceedings of the National Academy of Sciences* **97**, 4309 (2000).
111. Dauber, B., Heins, G. & Wolff, T. The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. *Journal of virology* **78**, 1865 (2004).
112. Hai, R*., et al.* Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. *Journal of virology* **82**, 10580 (2008).
113. Wressnigg, N*., et al.* Development of a live-attenuated influenza B [Delta] NS1 intranasal vaccine candidate. *Vaccine* **27**, 2851-2857 (2009).
114. Stech, J., Garn, H., Wegmann, M., Wagner, R. & Klenk, H.D. A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. *Nature medicine* **11**, 683-689 (2005).
115. Stech, J*., et al.* Influenza B Virus With Modified Hemagglutinin Cleavage Site as a Novel Attenuated Live Vaccine. *Journal of Infectious Diseases* **204**, 1483-1490 (2011).
116. Horimoto, T., Takada, A., Iwatsuki-Horimoto, K. & Kawaoka, Y. A protective immune response in mice to viral components other than hemagglutinin in a live influenza A virus vaccine model. *Vaccine* **22**, 2244-2247 (2004).
117. Horimoto, T*., et al.* Generation of influenza A viruses with chimeric (type A/B) hemagglutinins. *Journal of virology* **77**, 8031 (2003).
118. Bianchi, E*., et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
119. Wang, Y*., et al.* Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity. *New Microbiologica* **33**, 311-317 (2010).

II. Aim and objectives

Aims and objectives

As discussed in Chapter 2, the current situation of IBV vaccination and circulation does not fulfill the requirements needed for a reliable immunization strategy. Since the 70s, IBV has evolved into two antigenically distinct lineages (Victoria and Yamagata lineage) showing little or no cross-immunity [1,2]. This entails that vaccination with trivalent inactivated vaccines, containing a single IBV strain belonging to one of the two lineages, does not induce protective responses against strains of the opposite lineage. Consequently, in the recent past a mismatch has occurred between vaccine and circulation strain in no less than 5 out of eleven influenza seasons. This inevitably resulted in a lack of protection of vaccinated people [3-5], elevating influenza-associated disease in vaccinated people, limiting herd immunity for non-vaccinated people and bringing discredit to the merit of general influenza vaccination. Novel strategies to induce cross-reactive IBV immunity, encompassing strains of both lineages, are needed. A small number of research groups have endeavored to do so, although little *in vivo* evidence for cross-lineage protection has been generated as yet [5-10]. In light of this, we set out to develop next-generation cross-reactive IBV vaccine designs.

In a first part of the research, the immunogenic and protective potential of a number of conserved features of IBV was assessed. Alignment of the sequence of selected extravirionic antigens, namely the NB and BM2 ectodomain (termed NBe and BM2e, respectively) and the maturational cleavage site of HA (termed B/HA0), instigated the development of chimeric proteins based on consensus sequences of these three antigens. A number of different protein carriers were chosen to render the small antigens immunogenic, and were combined with different expression systems. The rationale for this was to present the selected antigens in a manner mimicking their natural structure, adopted during infection, as much as possible. To assure such a conformational mimickry, and to evaluate the assembly of the proteins in general, *in vitro* experiments were designed to identify the molecular characteristics of the chimeric proteins. In a next phase, the *in vivo* immunogenicity and protective nature of the induced response of the cross-reactive IBV vaccine candidates was tested. To this end, the vaccine candidates were combined with different adjuvants and used to immunize mice. Serological analysis of evoked immune sera was done in several manners to tax the recognition of viral proteins. Finally, challenge of immunized mice with mouse-adapted IBV strains of both lineages formed the ultimate test to determine the merit of the selected antigens in general and the design of our cross-reactive vaccine candidates specifically.

Performing experiments with NBe-based proteins led to the serendipitous identification of an NBe-encoded T cell epitope, specific for the $H2^b$-background of C57Bl/6 mice. In a second part of the research, the induction and antiviral activity of such cellular responses was examined in-depth. Specifically, the relation between proteinaceous and viral induction of NBe-specific T cells was scrutinized, as a model for boosting primed cellular immunity by natural IBV infection.

Overall, the main objective of this research, scrutinizing conserved features of IBV in eliciting cross-reactive protection, was to contribute to the strategic development of novel frontline influenza vaccines. Additionally, we hope to obtain further insight in influenza immunity and the cross-talk between immunization and infection in inducing cross-reactive immunity to IBV. Eventually, this might lead to the adaptation of new antiviral tactics to prevent or treat influenza infection.

References

1. Rota, P.A.*, et al.* Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59-68 (1990).
2. Chen, J.M.*, et al.* Exploration of the emergence of the Victoria lineage of influenza B virus. *Archives of virology* **152**, 415-422 (2007).
3. Camilloni, B.*, et al.* An influenza B outbreak during the 2007/2008 winter among appropriately immunized elderly people living in a nursing home. *Vaccine* **28**, 7536-7541 (2010).
4. Goni, N.*, et al.* Influenza B viruses isolated in Uruguay during the 2002-2005 seasons: Genetic relations and vaccine strain match. *Virus research* **123**, 100-104 (2007).
5. Belshe, R.B., Coelingh, K., Ambrose, C.S., Woo, J.C. & Wu, X. Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. *Vaccine* **28**, 2149-2156 (2009).
6. Camilloni, B., Neri, M., Lepri, E. & Iorio, A.M. Cross-reactive antibodies in middle-aged and elderly volunteers after MF59-adjuvanted subunit trivalent influenza vaccine against B viruses of the B/Victoria or B/Yamagata lineages. *Vaccine* **27**, 4099-4103 (2009).
7. Bianchi, E.*, et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
8. Wang, Y.*, et al.* Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity. *New Microbiologica* **33**, 311-317 (2010).
9. Stech, J.*, et al.* Influenza B Virus With Modified Hemagglutinin Cleavage Site as a Novel Attenuated Live Vaccine. *Journal of Infectious Diseases* **204**, 1483-1490 (2011).
10. Horimoto, T., Iwatsuki-Horimoto, K., Hatta, M. & Kawaoka, Y. Influenza A viruses possessing type B hemagglutinin and neuraminidase: potential as vaccine components. *Microbes and infection* **6**, 579-583 (2004).

III. Results

Chapter 3

Design and *in vitro* characterization of cross-reactive influenza B vaccine candidates

3.1. Introduction

With the appearance of two antigenically distinct lineages of influenza B virus in the past three to four decades, classic trivalent vaccines have been shown to be inappropriate for optimal protection against influenza B. Numerous mismatches between the selected vaccine strain and epidemic circulating strain have already occurred, leading to excessive infection rates and associated morbidity in the (vaccinated) population [1]. Most of these mismatches are the mere consequence of predicting circulation of a B virus strain belonging to the opposite lineage, or of co-circulation of viruses belonging to both lineages. Hence, a number of authors have already pushed the idea forward of including viruses of both lineages in tetravalent influenza vaccine preparations [1,2]. Alternatively, attention has been drawn towards new prophylactic treatments of influenza B virus infection in humans, using novel immunization strategies or conserved viral features. In this chapter, we describe the first in-depth analysis of the potential merit of the ectodomains of the NB and BM2 transmembrane protein of influenza B in vaccination strategies. Here, we will focus on the construction, expression, purification and *in vitro* characterization of proteins based on selected conserved features fused to appropriate carrier proteins.

3.2. Results and discussion

3.2.1 Vaccine basis, construction and expression

a) Selected influenza B derived epitopes for cross-protective vaccine development

Influenza B viruses contain a number of conserved viral features which can serve as a basis for a broad vaccine design. In this PhD study, three epitopes were selected to form the basis of a broadly protective influenza B vaccine (Fig. 3.1).The first of these received the most attention, namely the ectodomain (NBe) of the NB ion channel. NB is a tetrameric transmembrane protein, encoded by segment 6 by a open reading frame overlapping with that of neuraminidase (NA). NB serves an as yet unidentified but seemingly indispensable *in vivo* role in the influenza B virus life cycle [3,4]. NBe forms the N-terminal region of the protein, comprising a total of 18 amino acids. It shows a high degree of conservation, based on the alignment of 854 NBe-sequences obtained from influenza B strains isolated between 1940 and April 2011 (Fig. 3.1A). Of note, this short sequence contains two sites (N3 and N7) for the addition of N-linked glycosyl-structures, which are used in NB protein expressed by infected mammalian cells [5,6].

A second epitope that was studied, is the ectodomain (BM2e) of the selective proton channel BM2. This protein is the biological counterpart of the M2 proton channel of influenza A virus and shares a number of features illustrating this. Like the NB protein, both M2 and BM2 are type III transmembrane proteins, with an N-terminal ectodomain and lacking a secretion signal [5,7]. The transmembrane section of both proteins contains the conserved HXXXW motif, responsible for

selective passive transport of protons across lipid membranes [7,8]. BM2 or M2 is necessary for efficient *in vivo* replication of influenza B or A virus, respectively [9,10]. The BM2e, however, is significantly shorter than M2e, comprising only 8 amino acids compared to 23 for M2e. These however are highly conserved among all sequenced influenza B isolates (Fig. 3.1B). Quite recently, Wang *et al.* described the isolation of BM2e-specific monoclonal antibodies and their successful use in *in vitro* inhibition of influenza B infection [11], enforcing its potential use in cross-protective influenza B vaccine development.

A third epitope scrutinized here is the cleavage peptide of the influenza B hemagglutinin (HA). For HA to obtain its membrane fusion activity, the protein needs to be cleaved at residue R329. This transforms the precursor HA, termed HA0, into two chains, covalently linked by cysteine bridges, termed HA1 and HA2. HA1 comprises the globular head of the protein, bearing the main antigenic sites responsible for the induction of anti-HA neutralizing humoral responses. The HA2 segment contains the stem region, in recent years shown to be minor immunogenic in natural infectious settings but an interesting candidate for a broadly protective vaccine for influenza A viruses [12-15]. In addition, the N-terminus of HA2 forms the fusion peptide, responsible for membrane insertion and fusion of the viral and host cell membranes, a step that is essential for delivery of the influenza genetic material into the cytoplasm [16]. To be accessible for proteases, the cleavage peptide forms a loop protruding from the stem structure [17]. This loop is likely also exposed to the host's adaptive immune system. Moreover, the structural and biological constraints impose a strong conservational pressure on the primary sequence of the exposed loop (Fig. 3.1C). On these premises, Bianchi *et al.* explored the natural immunogenicity of the influenza A and B HA cleavage peptide (termed A/HA0 and B/HA0) and demonstrated protection against viral challenge following immunization of mice with conjugate vaccines comprising the maturational cleavage loop of HA [18]. With conservation of the cleavage loop sequence even crossing the A/HA – B/HA barrier, they were able to show protection against influenza A/H1, A/H3 and B viruses when using a fusion loop conjugate derived from A/HA3 based vaccine.

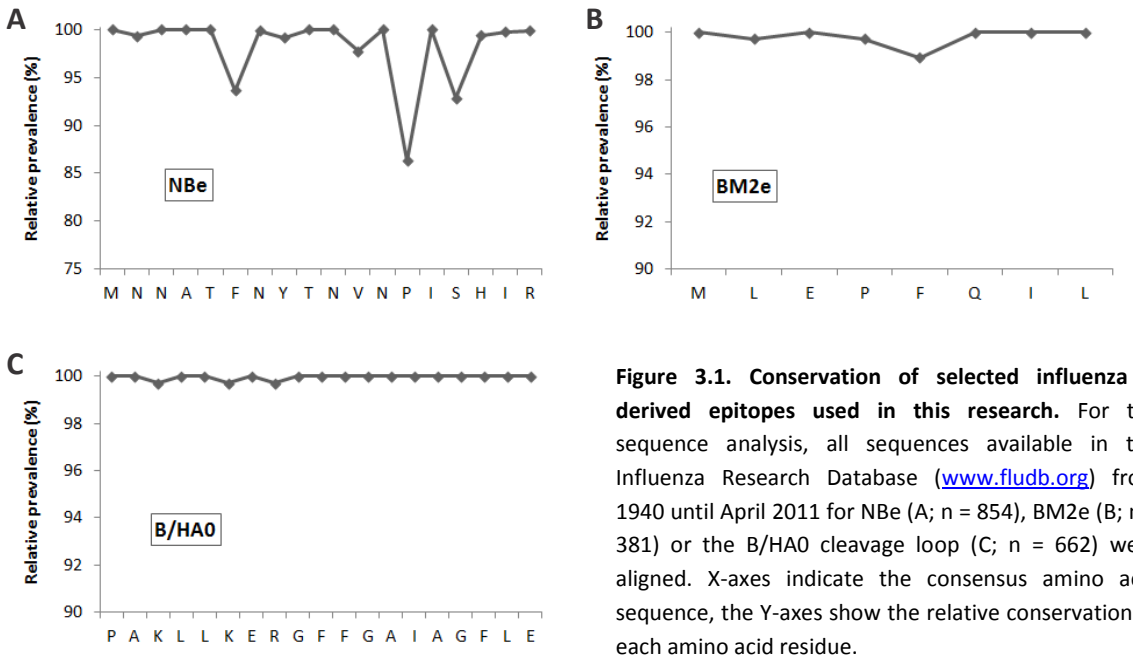


Figure 3.1. Conservation of selected influenza B derived epitopes used in this research. For the sequence analysis, all sequences available in the Influenza Research Database (www.fludb.org) from 1940 until April 2011 for NBe (A; n = 854), BM2e (B; n = 381) or the B/HA0 cleavage loop (C; n = 662) were aligned. X-axes indicate the consensus amino acid sequence, the Y-axes show the relative conservation of each amino acid residue.

b) Selected carrier proteins

To render small linear polypeptides immunogenic, they need to be linked to a scaffold that facilitates their presentation to the immune system. In most cases, these carriers are protein-based, with a potential adjuvant function and or helper T cell epitopes. The fusion of an immunogen to a carrier can also be designed in such a way that it provides conformational mimicry of the epitope that is naturally produced. In their natural conformation, NBe and BM2e are presented on the infected cell or viral surface in a tetrameric fashion. As a whole, the tetrameric assembly might form a conformational epitope able to elicit tetramer-specific antibodies. Assuming this, a second carrier chosen for fusion with NBe and BM2e is a tetramerizing mutant form (tGCN4) of the leucin zipper of the GCN4 transcription factor of yeast [19]. It has been successfully used in combination with M2e, showing induction of tetrameric M2e-specific antibodies and protection against a potential lethal influenza A infection [20]. The construction and assembly of tGCN4-based immunogens used in this research is depicted in Figure 3.2. For the BM2 ectodomain, two different constructs were made differing in the length of BM2e (Fig. 3.2A and C). In a first, only the 5 first amino acids of BM2e were used, linked to tGCN4 by a Gly-Gly linker. A second BM2e-based tetramer used the full 8 amino acids of the BM2 ectodomain fused directly to tGCN4, mimicking the natural BM2 structure as close as possible regarding implantation of the ectodomain on the tetramer [21].

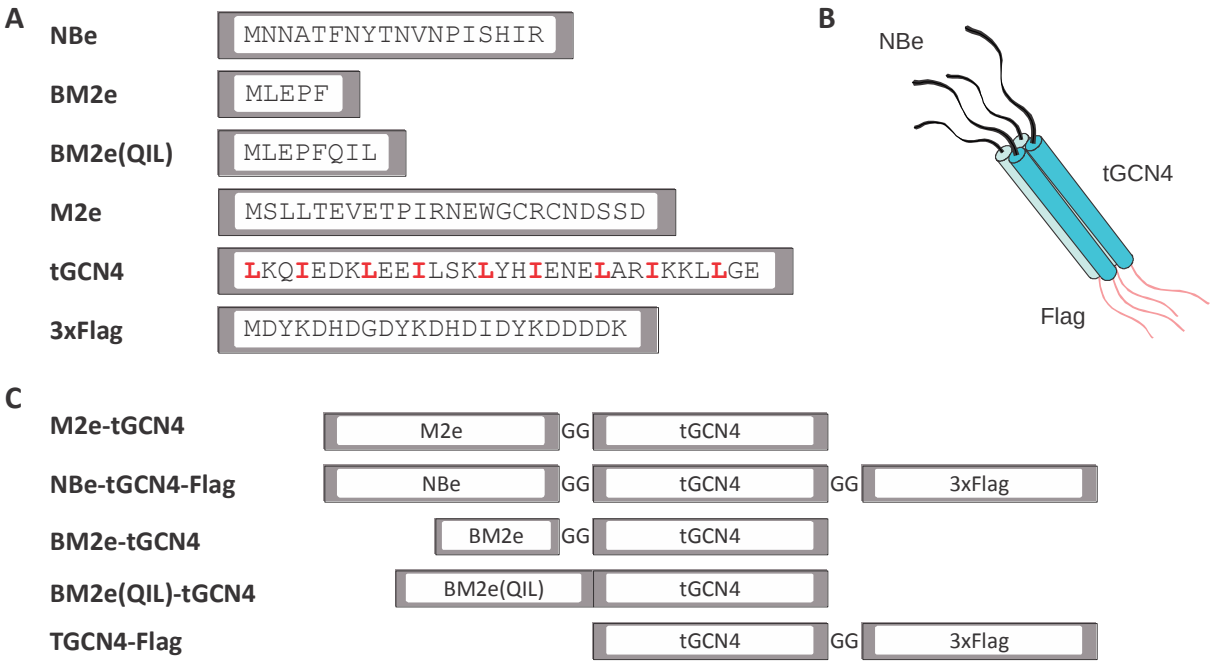


Figure 3.2. Construction and make-up of tetrameric tGCN4-based chimeric vaccines. A. Amino acid sequences of the different ectodomains used in this research (top four), the tGCN4 scaffold and the triple Flag-tag added for solubility of NBe-tGCN4. For the tGCN4-sequence, residues forming the hydrophobic interface responsible for tetramerization are bold in red. **B.** Schematic representation of the tetrameric NBe-tGCN4-Flag chimeric protein. **C.** Build-up of all bacterial expressed tGCN4-based proteins used in this research.

For presenting NBe, the ectodomain is linked to tGCN4 by a Gly-Gly linker (Fig. 3.2C). This fusion however turned out to be almost completely insoluble (see further). The addition of a triple Flag-tag improved solubility tremendously, allowing successful production and purification of the resulting chimeric protein NBe-tGCN4-Flag. As control for tGCN4-based immunizations, M2e-tGCN4 was used. For detection of tGCN4-specific antibody titers in immunization experiment, tGCN4-Flag without an additional N-terminal sequence was produced.

For a stable, repetitive and high-density presentation of immune epitopes, we used the core protein (HBc) of Hepatitis B virus [22-24]. It is a well-evaluated carrier protein, that has already been successfully used with a wide variety of antigens [23,24]. Of these, the fusion of M2e of influenza A virus with HBc has been extensively evaluated by our research group [25-28]. As scaffold protein, it is used in different conformations, based on the presence or absence of the protamine-like nucleic acid binding C-terminal regions. It has been shown that the presence of trace amounts of nucleic acids inside HBc-particles (derived from the organism used for production [29]) adds on to the adjuvant effect of the protein [30]. Here, however, the truncated form of HBc was used (149 amino acids in length) to avoid issues with complexity of the vaccine preparations. A C-terminal cysteine-residue was added to increase stability of particle formation. This approach allows properly folded HBc-monomers to dimerize and self-assemble into particles containing 180 or 240 monomers upon expression (Fig. 3.3B) [31].

Two of the selected influenza B epitopes were combined with C-terminally truncated HBc particles (HBc149) lacking the nucleic acid binding domain (Fig. 3.3A). The NBe-epitope was fused N-terminally to HBc-sequence (replacing amino acids 1-4) or inserted into the immunodominant loop (replacing amino acids 78-82), protruding as spikes from assembled particles formed by two adjacent α-helices in the dimer [24]. The N-terminal fusion yielded particle 1651; the particle with NBe inserted into the loop was termed 1646. A third construct combined the presence of NBe and M2e into one particle. In this construct, NBe was fused N-terminally to particle 1817 containing 2 M2e-copies [26]. In this particle, the most HBc-proximal M2e-copy contains its natural cysteine-residues, while these are removed in the more distal copy. It is expected that the remaining cysteines within M2e stabilize particle formation additionally [26]. The resulting construct was termed NM_2H (NBe-2M2e-HBc), which showed improved solubility over other NBe-HBc fusions tested (data not shown) and would display NBe N-terminally (as in natural expressed NB) extending from the particle. Moreover, with the combination of NBe and M2e, this vaccine could be protective against influenza A and B viruses. All of these construct contain an additional cysteine-residue C-terminally of HBc (position 150), mimicking the cysteine-residue in full-length HBc at position 183, which should further assist in particle formation by cross-linking HBc-dimers to each other [26,32].

The second epitope used in combination with HBc is the B/HA0 cleavage sequence (for structural information, see Fig. 1.2D and H, p16). Conformationally, this is a loop sequence, and hence was introduced in the immunodominant loop of HBc. To overcome problems with solubility, this chimeric protein was produced by a dual expression system combining unsubstituted HBc and B/HA0-HBc, yielding particles with mixed free and substituted loops termed 3004. An overview of all HBc-constructs used in this thesis is given in Table 3.1.

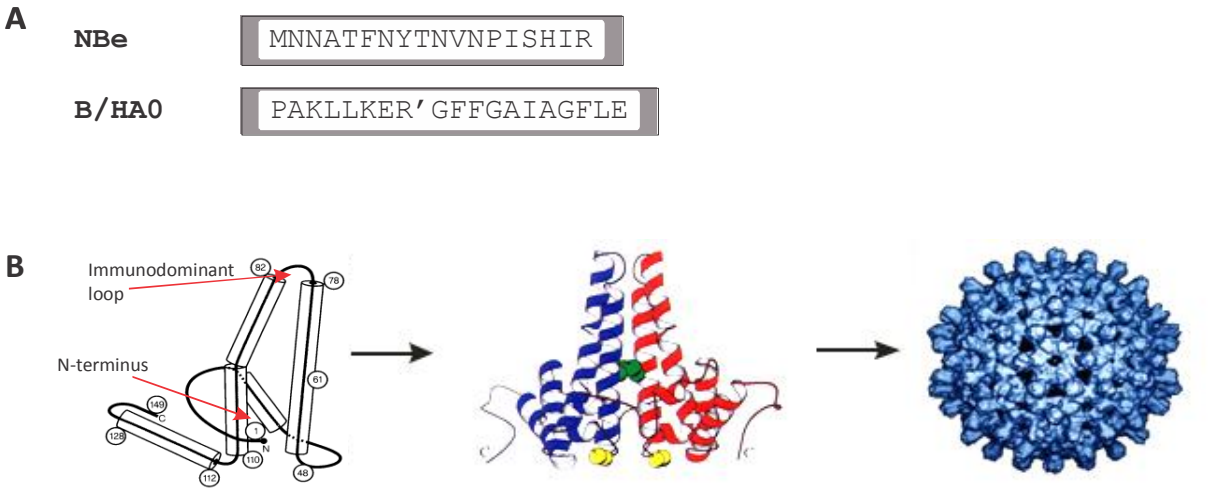


Figure 3.3. Construction and make-up of Hepatitis B core (HBc) protein based vaccines. A. Amino acid sequences of the influenza B epitopes used for fusion to HBc, NBe (top) and the B/HA0 cleavage loop (bottom). The apostrophe in the B/HA0 sequence indicates the cleavage site. **B.** Assembly of HBc-particles. Properly folded HBc monomers combine into dimers, which assemble into particles consisting of 180 or 240 monomeric building blocks. The spikes in the particle are formed by two adjacent α-helices of monomers in the HBc-dimer. Specific sites in monomers used for insertion of antigens are indicated by the red arrows. Pictures based on [33]

Table 3.1. Overview and description of HBc-based IBV vaccine candidates used throughout this thesis.

Shortname	Construct	Specifications
1646	NBe-HBc(149)	NBe sequence inserted in the immunodominant loop of HBc(149)
1651	NBe-HBc(149)	NBe fused N-terminally to HBc(149)
1817	$M2e_2$-HBc(149)	Two copies of M2e fused N-terminally to HBc(149)
NM_2H	NBe-$M2e_2$-HBc(149)	One copy of NBe fused N-terminally to 1817
3004	B/HA0-HBc(149)	B/HA0 cleavage loop inserted in HBc(149), produced as mixed substituted/unsubstituted HBc-particles

During infection, the NB protein is expressed as a glycoprotein, with addition of complex-type poly-N-lactosaminoglycan structures on two sites in NBe [6]. To assess whether glycosylated NBe-based vaccines can offer protection against influenza B infection, two different strategies were employed. In a first approach, NBe-tGCN4 was produced in a yeast expression system. In this instance, the protein was soluble without the Flag-tag (see further).

In a second approach, cellular expressed and membrane-associated NBe-based proteins were utilized. In our hands, full-length NB protein expression by transfection of mammalian cells is not high-yielding, both by transient transfection or stable transduction, when analyzed by western blot

(data not shown). Full-length M2 and BM2 proteins however were efficiently expressed in mammalian cells that had been transfected with the corresponding expression plasmids (see further). For these reasons, ectodomain swaps were performed, in which the BM2- or M2-ectodomain was replaced by the NB-ectodomain and *vice versa* (Fig. 3.4). The influence of glycosylation on expression and immunogenicity was taxed through mutation of these (N3Q and N7Q), either separately or together (Fig. 3.4C). To ease detection, a single Flag-tag was added C-terminally of these chimeric constructs.

For all constructs, a silent mutation was introduced in the NBe-sequence, deleting the startcodon of the downstream open reading frame of NA (ATGAACAATGCT → ATGAACAACGCT). This was done to avoid potential problems with translation initiating at this second AUG-codon.

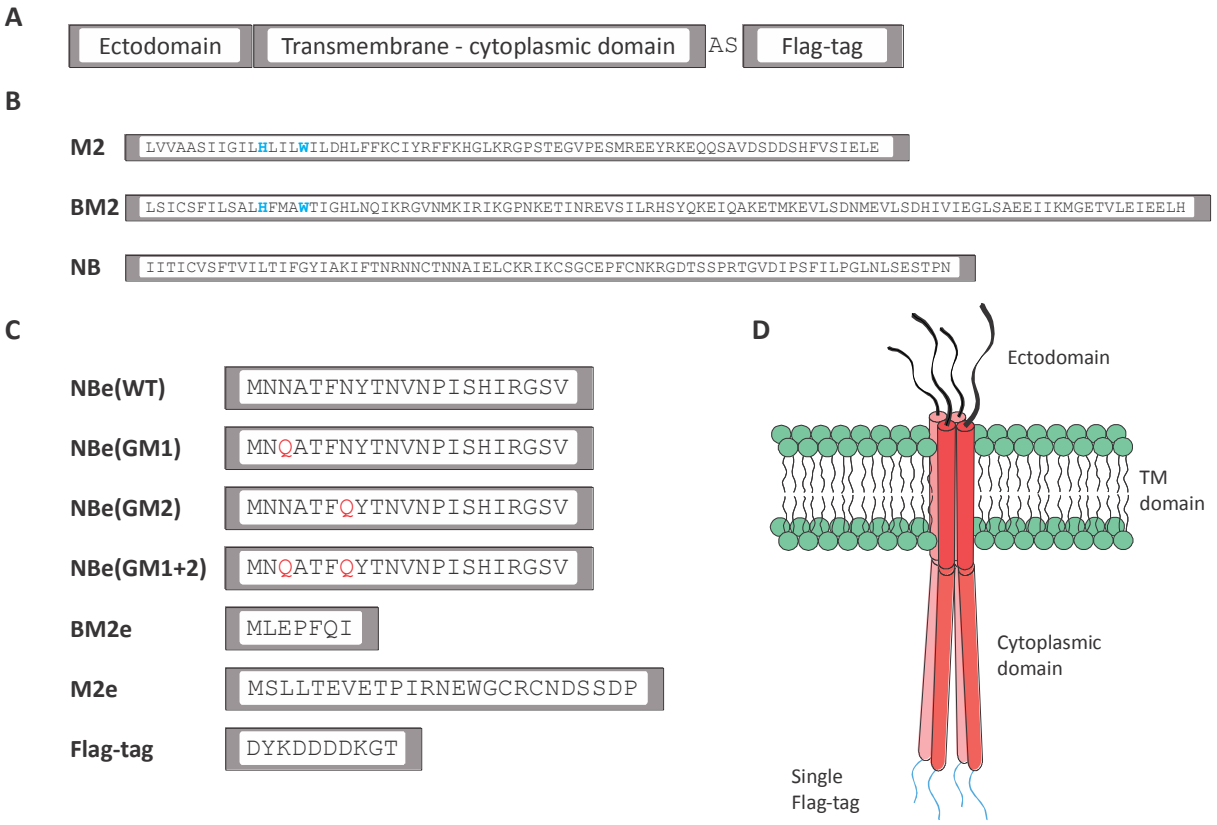


Figure 3.4. Construction and make-up of chimeric transmembrane proteins. A. General structure of transmembrane proteins, consisting of an ectodomain combined with a transmembrane and cytoplasmic domain. A C-terminal Flag-tag, following a Ala-Ser (AS) linker, allows easy detection of the proteins. **B.** Amino acid sequences of M2, BM2 and NB protein. For M2 and BM2, the amino acids responsible for proton-selective gating are shown bold and in blue. **C.** Sequences of the ectodomains used for domain-swapping in expression of chimeric transmembrane proteins and the single Flag-tag for detection. For the NBe-sequences, glycosylation mutants (GM), with removal of the first (GM1), the second (GM2) or both (GM1+2) N-glycosylation sites, are shown with altered residues in bold and red. **D.** Cartoon representation of membrane-associated chimeric proteins.

c) *Expression and purification of chimeric proteins*

For initial expression, purification and structural tests of M2e-, NBe- and BM2e-tGCN4 fusion proteins, tags were introduced C-terminally. These include both a hexahis-tag (His_6) and a triple Flag-tag. Expression of all these constructs was optimized using *E. coli* B strains (BL21 CodonPlus) and transcriptionally controlled by the *pL*-promotor. To avoid unwanted expression under non-optimal conditions, potentially leading to counter selection or mutation, the pICA2 repression plasmid was used (Fig. 3.5) [20]. In this system, a temperature sensitive variant of the cI-repressor [34] assures silencing of pL-promotor based transcription at temperatures below 30°C; repression can be lifted by

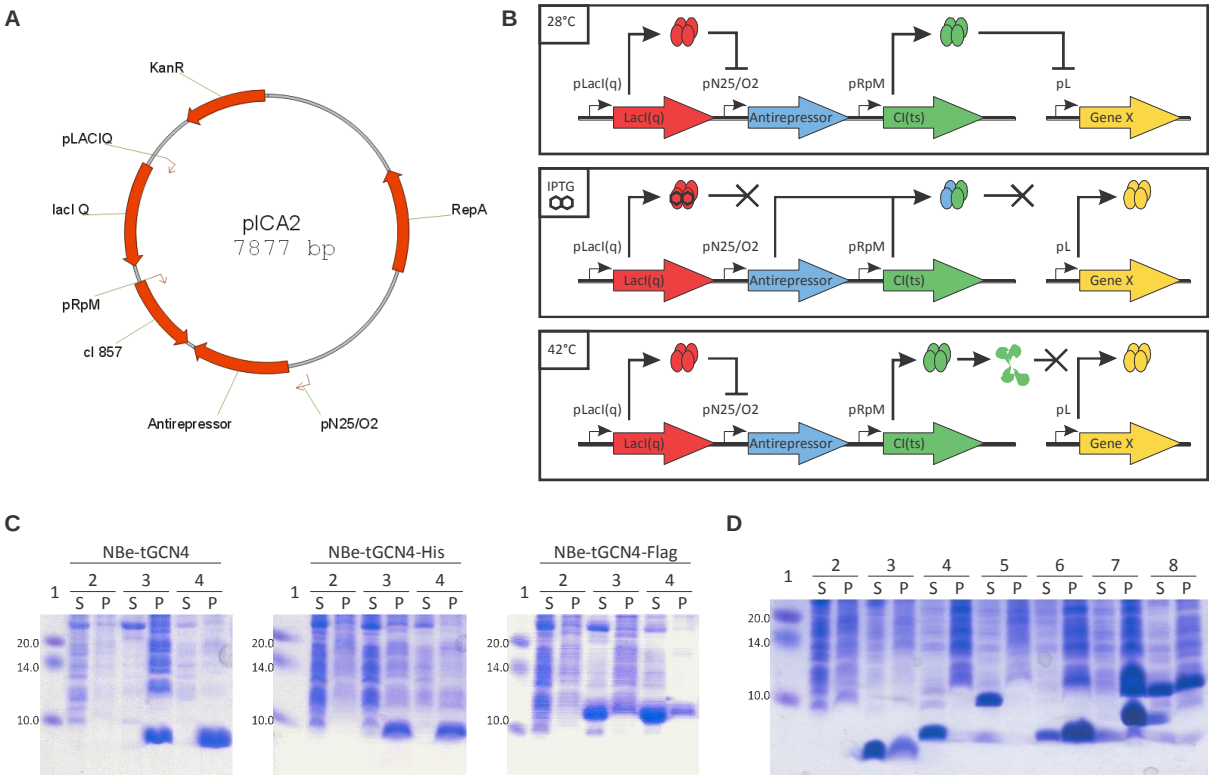


Figure 3.5. *E. coli* based expression of tGCN4-based proteins. A. Vector map of the pICA2 repression plasmid, used in the bacterial expression system for the production of tGCN4-based proteins. **B.** Schematic representation of the bacterial expression system used here. When incubating cultures below 30°C, *in trans* expression of the temperature-sensitive cI(ts) will block *pL*-promotor driven expression of the protein of interest (top). Increasing the cultivation temperature to 42°C will lead to inactivation of cI(ts) and expression of the protein of interest (bottom). The pICA2 repression plasmid also codes for the P22 anti-repressor, which can hetero-oligomerize with the cI-repressor and inactivate it as such. Expression of this anti-repressor is under control of a hybrid promotor containing *Lac*-repressor sites, which are bound by the LacI-repressor, also encoded by pICA2. Addition of lactose-analogues such as IPTG will alleviate LacI-based repression, lead to anti-repressor expression and block cI repression (middle). **C.** Expression tests of NBe-tGCN4 proteins without tag (left), with a His_6-tag (middle) or with a triple Flag-tag (right), analyzed by SDS-PAGE and Coomassie-staining after sonication of bacterial cultures (see Material and Methods). 1 = gel marker with mass in kDa, 2 = non-induced sample, 3 = IPTG-induced sample (30°C), 4 = temperature-induced sample (42°C); S = supernatant after sonication, P = pellet after sonication. **D.** Overview of all BM2e- and NBe-tGCN4 proteins expressed after temperature induction of bacterial cultures. 1 = gel marker with mass in kDa, 2 = non-induced sample, 3 = BM2e-tGCN4, 4 = BM2e-tGCN4-His, 5 = BM2e-tGCN4-Flag, 6 = NBe-tGCN4, 7 = NBe-tGCN4-His, 8 = NBe-tGCN4-Flag; S = supernatant after sonication, P = pellet after sonication.

raising cultivation temperature to 42°C. The system also allows induction at lower temperatures, for instances in which problems are anticipated with solubility of the expressed protein due to high induction temperature. The anti-repressor from the *Salmonella* specific bacteriophage P22 [35], able to hetero-oligomerize with the cI-repressor and inactivating it as such, is under control of a hybrid promotor containing *Lac*-repressor sites (pN25/02). *In cis* expression of the lac-repressor prevents expression of this cI-antirepressor. Addition of non-metabolizable lactose-analogues, such as isopropyl-β-D-1-thiogalactopyranosid (IPTG), blocks repression of the pN25/O2 promotor by the Lac-repressor, leading to expression of the cI-antirepressor and freeing of the pL-promotor (Fig. 3.5B).

The results of expression tests of NBe-tGCN4 are shown in Figure 3.5C. Untagged and His_6-tagged NBe-tGCN4 chimeric protein resulted in insoluble recombinant proteins upon induction, as does. The addition of the highly charged triple Flag-tag improves solubility significantly. Hence, this format was purified and used for further *in vitro* and *in vivo* analysis. For BMe-based proteins, the presence or absence of a tag did not alter solubility tremendously, since all combinations show high expression of soluble protein (Fig. 3.5D). Tagless BM2e-tGCN4 was purified and used for downstream analyses. As induction method for all tGCN4-proteins, temperature induction was chosen. Higher incubation temperatures did not affect solubility greatly here, but did lead to slightly lower culture densities and hence lower amounts of endogenous proteins in the lysates after sonication.

Purification of tGCN4-based proteins exploits their physicochemical properties as much as possible. The heat-stability of the tGCN4-tetramer [19] and the lack of effect of temperature-induction on solubility of the proteins, tempted us to assess the effect of heat-treatment on the protein samples for purification purposes. Incubating samples directly after sonication for 30 minutes at 65°C resulted in denaturation of most of the endogenous proteins, without affecting the solubility of the tGCN4-based proteins (Fig. 3.6A). This highly effective first step in the purification process was therefore applied before further chromatographical steps.

The purification process of NBe-tGCN4-Flag is illustrated in Figure 3.6. Following induction, sonication and heat precipitation, the mixture was centrifuged and filtered to remove insoluble particles. In the next step, the protein sample was applied to a SourceQ anion exchange column, with the main purpose being the removal of nucleic acids in the lysate. NBe-tGCN4-Flag eluted in two different peaks (Fig. 3.6C), most likely differing in overall charge. Applying the first peak on a gel filtration column after concentration (Fig. 3.6D) resolved two species that differ in size. Analyzing the second peak from the anion exchange chromatography on the gel filtration column showed presence of solely the first protein species with high molecular weight (MW; data not shown). Although not so outspoken for other chimers, tGCN4-proteins seem to behave as a mixture of different species with distinct size and charge. A potential explanation is association of some of the tGCN4 chimeric proteins with bacterial endogenous proteins, as previously seen for M2e-tGCN4 [20], although other explanations such as multimerization of tetramers cannot be ruled out. For further analyses, only protein corresponding to peak 2 of the gel filtration, with an estimated molecular mass corresponding to that of the tetramer, was retained for further analysis.

The yield for each protein after expression and purification illustrates the power of the bacterial expression system used. On average, M2e-tGCN4 and NBe-tGCN4-Flag purification resulted in 10-20 mg per liter of bacterial culture (shake flasks), while for BM2e-tGCN4 proteins this reached 30-100 mg per liter of culture. tGCN4-Flag purification yielded 300-600mg per liter of bacterial culture.

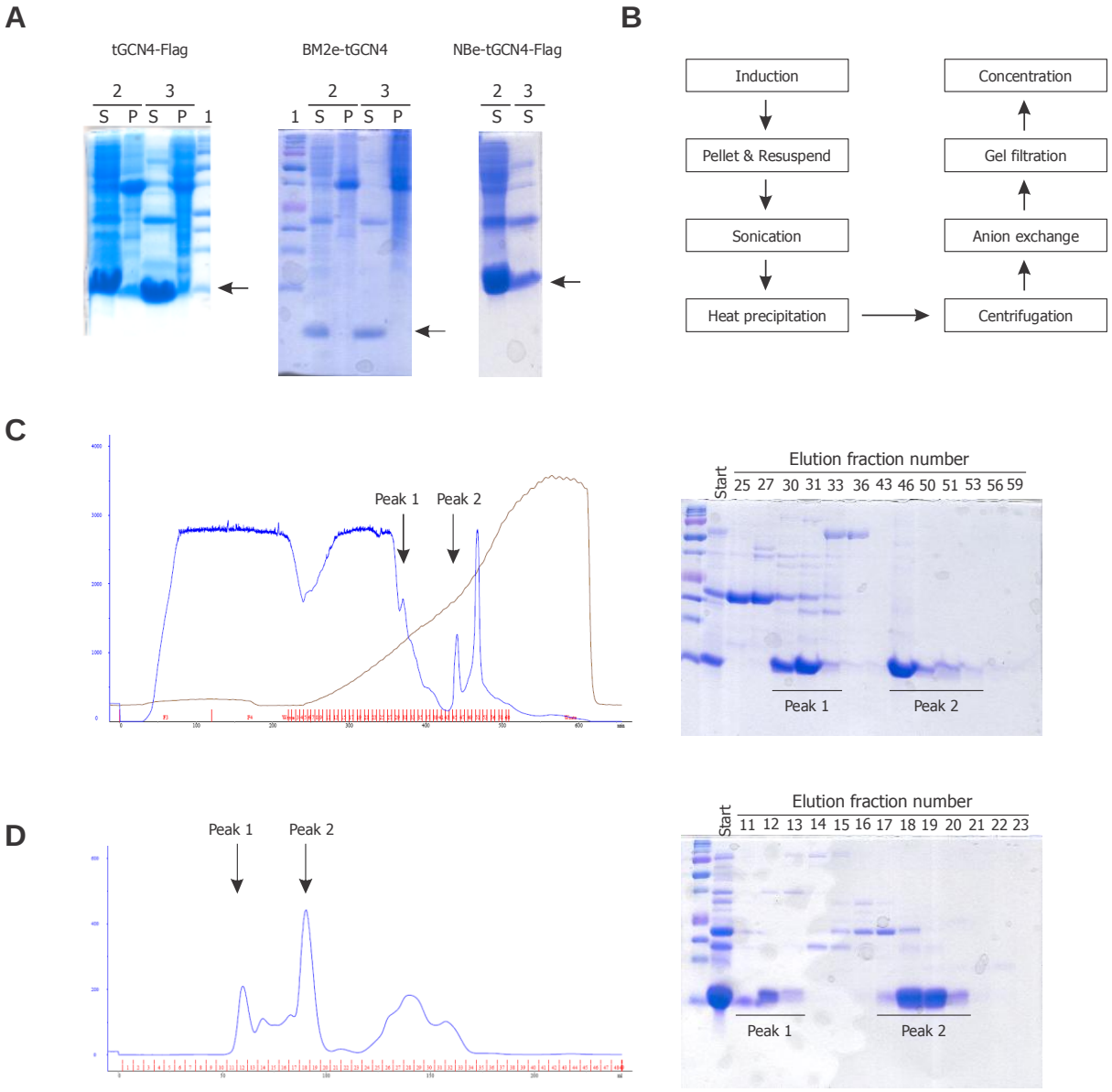


Figure 3.6. Purification of tGCN4-based proteins. A. Effect of heat-treatment (30min, 65C) of sonicates of tGCN4-Flag (left), BM2e-tGCN4 (middle) and NBe-tGCN4-Flag (right). Directly after sonication, lysates were centrifuged or heat-treated before centrifugation, followed by analysis of soluble and insoluble fractions through SDS-PAGE and Coomassie staining. Arrows indicate bands corresponding to tGCN4-proteins. 1 = gel marker, 2 = samples after sonication without heat-treatment, 3 = samples after sonication and heat-treatment; S = supernatant, P = pellet. **B.** Flow chart showing the different steps used for purification of tGCN4-proteins. **C.** Anion exchange profile and SDS-PAGE analysis of relevant samples of NBe-tGCN4-Flag purification. Elution peaks and corresponding fractions containing NBe-tGCN4-Flag are indicated. **D.** Gel filtration profile and SDS-PAGE analysis of relevant samples of NBe-tGCN4-Flag purification. Elution peaks and corresponding fractions containing NBe-tGCN4-Flag are indicated.

HBc-particles were expressed and purified using the same bacterial system relying on pL-promotor driven transcription and pICA2-encoded repression as was used for the tGCN4 fusions. In this instance, we opted for IPTG-based induction at lower cultivation temperatures, since induction at elevated temperatures led to higher amount of insoluble material (data not shown). After sonication and centrifugation, the cleared lysates were treated with $(NH_4)_2SO_4$ (30% saturation) to specifically

precipitate large proteins such as HBc-particles. Pellets were resuspended and dialyzed extensively, followed by fractionation by anion exchange chromatography. In a final gel filtration step on a Sephacryl S400 column, particles were selected on size and the solution buffer was changed to PBS for *in vitro* use. This protocol was applied for purification of 1646, 1651 and NM_2H. A number of particles, namely 3004, 1817 and unsubstituted HBc, were produced and purified by Acambis Inc. (Cambridge, MA).

For expression of N-glycosylated NBe-proteins, eukaryotic expression systems have to be used. The recombinant transmembrane proteins were expressed by transfection of mammalian cells (HEK293T, CHO, NIH3T3). Plasmids used for expression are pCAGGS based, relying on transcription from a constitutive chicken β-actin/rabbit β-globin hybrid promoter. For vaccination studies, transfected cells as such were used as immunogen. Glycosylated NBe-tGCN4 (gNBe-tGCN4) was produced by stable transformation of *Pichia pastoris* strains with methanol inducible expression plasmids. Two different strains of *Pichia* were used: the wild-type strain GS115 and a glyco-engineered strain (KE3) producing only proteins bearing a $Mannose_5$-glycostructure [35,36]. As analyzed on SDS-PAGE, the majority of yeast-produced gNBe-tGCN4 however did not show glycosylation (Fig. 3.7). Remarkably, in contrast to bacterially expressed NBe-tGCN4, solubility does not seem to be a problem in the *Pichia pastoris* expression system. Most likely reasons for this are the lower expression levels combined with secretion of the protein in the yeast expression system. Additionally, low-grade glycosylation of only a single monomer in the tetrameric structure might increase solubility as such.

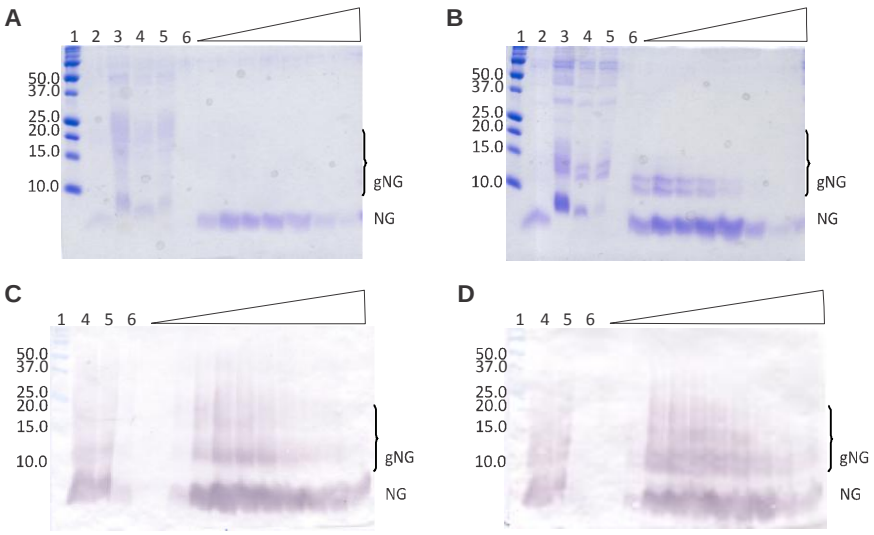


Figure 3.7. Cation exchange purification of gNBe-tGCN4 produced by wild type GS115 and glycomutant KE3 *P. pastoris* strains. SDS-PAGE analysis of samples from purification of gNBe-tGCN4 produced by wild type GS115 (A & C) and glyco-engineered KE3 (B & D) *P. pastoris*. Culture supernatants containing secreted gNBe-tGCN4 were concentrated and applied to a cation exchange column. The triangle depicts the elution phase with increasing salt concentrations. Presence of proteins was analyzed by Coomassie staining (A & B) or Western blot using tGCN4-specific rabbit serum (C & D). 1 = gel marker with mass in kDa, 2 = culture supernatant, 3 = concentrated culture supernatant, 4 = concentrate diluted in sample buffer, as applied on the column, 5 = flow-through fraction, 6 = wash fraction, triangle = elution with increasing ionic strength; NG = unglycosylated NBe-tGCN4, gNG = glycosylated gNBe-tGCN4.

Purification of gNBe-tGCN4, secreted by methanol-induced yeast cultures, started with concentration of the cell-free culture media by filtration or fractional $(NH_4)_2SO_4$-precipitation (specific precipitation between 40% and 80% $(NH_4)_2SO_4$). Because of the predicted overall positive charge of the protein at neutral pH (pI = 7,69), the concentrate was applied to a cation exchange column (Fig. 3.7). Analysis of the elution fractions on SDS-PAGE clearly shows gNBe-tGCN4 produced by wild type yeast shows a more dispersed glycosylation pattern compared to the $Mannose_5$-strain produced protein. Although not resembling the natural glycosylation state, the more uniform character of the $Mannose_5$-bearing gNBe-tGCN4 was selected for further analysis. Final purifications steps, gel filtration and concentration, were similar to *E.coli* produced tGCN4-protein purification.

3.2.2 *In vitro* characterization of tGCN4-based cross-reactive influenza B vaccine candidates

The quintessential property of the tGCN4-scaffold is its ability to tetramerize spontaneously, and thus present linked epitopes in a tetrameric fashion. To evaluate the quaternary structure of purified proteins, a number of *in vitro* tests were performed to assess the MW of these. N-terminal sequencing by Edmann-degradation showed that the primary sequence of the monomers was as expected (data not shown). For NBe-tGCN4-Flag, on average 80% of the protein retained the initiating methionine. Likewise, the majority of BM2e-proteins did not show cleavage of this residue. This last result was confirmed by mass-spectrometric determination of the MW of tagged and untagged BM2e-tGCN4 proteins, as described before [20]. Ionization led to disruption of the tetrameric structure, allowing detection of the MW of monomers only. These, however, did confirm the presence of the initiating methionine and showed that the monomers are intact. For BM2e-tGCN4 (theoretical mass of 4438.22Da), the detected mass was 4438.2Da; for BM2e-tGCN4-His_6 (theoretical mass of 5375.17Da), this was 5375.3Da; for BM2e-tGCN4-Flag (theoretical mass of 7339.15Da), a mass of 7326.6Da (with somewhat lower resolution) was detected.

a) Size-exclusion and native PAGE yield molecular weights corresponding to that of tetramers

The final step of purification of tGCN4-based proteins is gel filtration, achieving both size exclusion and productive buffer exchange. Additionally, calibration of the specific column used can generate information about the size and oligomeric state of the proteins. For gel filtration of the tGCN4-proteins, a Superdex75pg column was used, calibrated using a combination of bovine serum albumin (66 kDa), ovalbumin (43 kDa), chymotrypsinogen A (29 kDa) and RNase A (13.7 kDa). Figure 3.8A shows the calibration profile. Overlaying this profile with those of the tGCN4-proteins (Fig 3.8B-F) indicates that these are in an oligomeric state with molecular sizes agreeing with a tetramic structure.

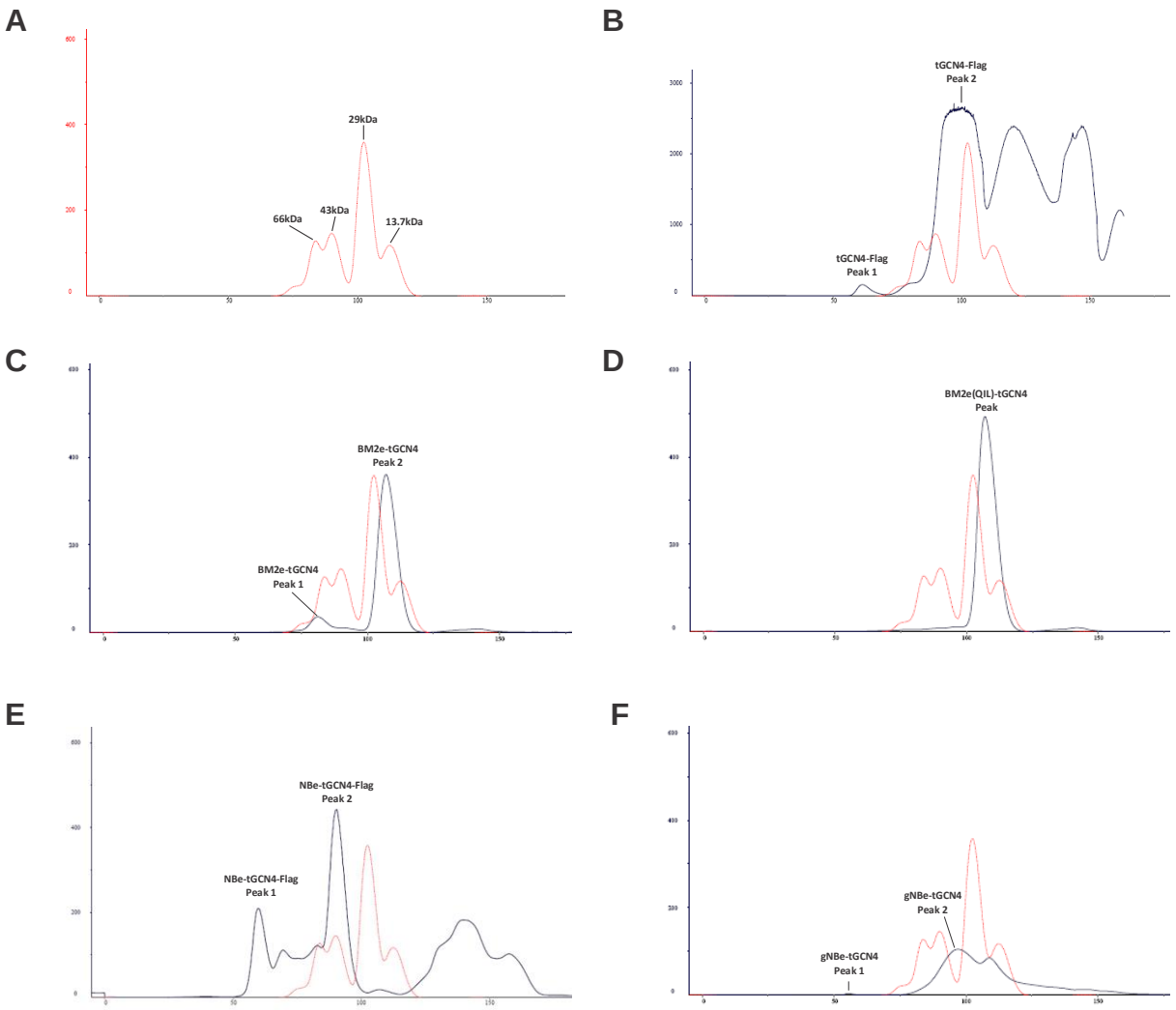
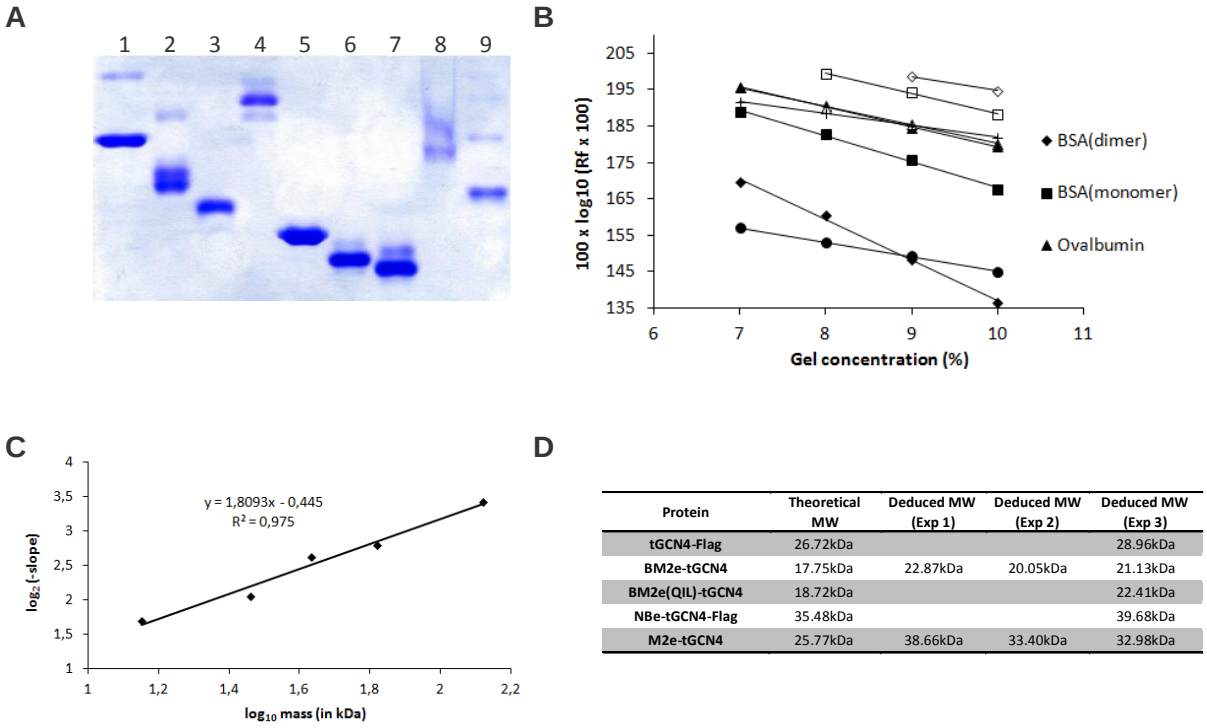


Figure 3.8. Gel filtration profiles of tGCN4-bases proteins using a calibrated column reveal that they adopt an oligomeric state. A. Gel filtration profile of a mixture of proteins with known sizes applied to the Superdex75pg column. This mixture includes BSA (66kDa), ovalbumin (43kDa), chymotrypsinogen A (29kDa) and RNase A (13.7kDa). **B-F.** Overlay of profiles from the calibration (A; in red) with that of (in black) tGCN4-Flag (B), BM2e-tGCN4 (C), BM2e(QIL)-tGCN4 (D), NBe-tGCN4-Flag (E) and gNBe-tGCN4 (F). Peaks containing the protein of interest are indicated; other peaks correspond to contaminating proteins or UV-absorbing molecules in general (*cf.* Fig.3.6D).

A second method to determine the oligomeric state of native proteins and their approximate molecular weight is native gel electrophoresis, based on the methods of Bryan and Davis [37,38] (Fig. 3.9). In this approach, proteins of interest together with a number of reference proteins are loaded on a series of native polyacrylamide gels with increasing acrylamide concentration. Based on the mobility (measured as ratio-to-front or Rf values) of a set of reference agents, a standard curve can be drafted showing the relationship between MW and the effect of gel density on the Rf value. From this, the molecular size of the proteins of interest can be deduced. Three different tGCN4-based proteins were evaluated in this way (Fig. 3.9D). For all of these, deduced MW are in agreement with theoretical numbers for tetrameric forms of the protein.



Protein	Theoretical MW	Deduced MW (Exp 1)	Deduced MW (Exp 2)	Deduced MW (Exp 3)
tGCN4-Flag	26.72kDa			28.96kDa
BM2e-tGCN4	17.75kDa	22.87kDa	20.05kDa	21.13kDa
BM2e(QIL)-tGCN4	18.72kDa			22.41kDa
NBe-tGCN4-Flag	35.48kDa			39.68kDa
M2e-tGCN4	25.77kDa	38.66kDa	33.40kDa	32.98kDa

Figure 3.9. Native PAGE approach to determine the molecular size of selected tGCN4-based proteins. Reference proteins and selected tGCN4-proteins were analyzed on native gels with different gel percentages. **A.** Coomassie stain of a 9% native gel. 1 = BSA (monomer, bottom; dimer, top), 2 = ovalbumin, 3 = carbonic anhydrase, 4 = α-lactalbumin, 5 = tGCN4-Flag, 6 = BM2e-tGCN4, 7 = BM2e(QIL)-tGCN4, 8 = NBe-tGCN4-Flag, 9 = M2e-tGCN4. **B.** Graph showing the relationship between gel concentration and mobility of the proteins (measured as ratio-to-front or Rf values), showing a linear relationship with negative slope. **C.** Standard curve showing the relationship between the size (MW) and slope of the mobility graph depicted in B. **D.** Deduced MW of native tGCN4-proteins based on the standard curve depicted in C and slopes of the mobility graphs depicted in B.

b) Cross-linking experiments reveal tetrameric and intermediate structures

To visualize tetramers or oligomeric structures in general, tGCN4-protein solutions were treated with two different homobifunctional cross-linkers, bis(sulfosuccinimidyl)suberate (BS^3) and dithiobis-(succinimidyl)propionate (DSP). Both react with primary amino-groups, but the spacer arm of DSP contains a reducible disulfide-bridge. Cross-linked samples were analyzed by SDS-PAGE in the presence or absence of β-mercapto-ethanol in the loading buffer, to assure effects seen on gel are a consequence of the cross-linking. Results of two independent cross-linking experiments are shown in Figure 3.10. In a first set-up, BM2e-tGCN4 was treated with both cross-linkers at two different concentration (Fig. 3.10A and B). In the absence of reducing agents, all treated samples show a main species running at just under 14kDa (Fig. 3.10A). With the theoretical MW of tetrameric BM2e-tGCN4 being 17.75kDa, this result is in line with the expected, since cross-linking might compact the structure to a certain extent and render it somewhat more mobile than expected. DSP-treated samples seem to have higher mobility than BS^3-treated samples; this can be explained by the difference in MW of both cross-linkers, with the larger BS^3 (572.43Da) increasing the size of the

structure more than the smaller DSP (404.42Da). Also note the presence of other bands in treated samples, corresponding to sizes of intermediate monomer, dimer and tetramer structures. With the addition of reducing agents (Fig. 3.10B), BS^3-treated samples behave the same, while DSP cross-linked species all fall back to the size of the monomer.

In a second experiment (Fig. 3.10C and D), BM2e(QIL)-tGCN4, M2e-tGCN4 and NBe-tGCN4-Flag were treated with BS^3 or DSP cross-linkers under the same conditions and analyzed in a similar manner. In the absence of reducing agents (Fig. 3.10C), the majority of cross-linked samples show mobility corresponding to that of tetramers. Note the uncross-linked M2e-tGCN4 sample (lane 3a) behaves as a dimer, indicating the influence of M2e-encoded cysteine-residues on the organization of the structure. Under reducing conditions (Fig. 3.10D) , BS^3-treated samples behave similarly, while DSP-treated proteins show more species of lower complexity (monomer, dimer and trimer). A third experiment, with equivalent results, is described further (Fig. 3.14). Overall, these cross-linking experiments show that tGCN4-proteins can be fixed in a complex with a size corresponding to that of the expected tetramer.

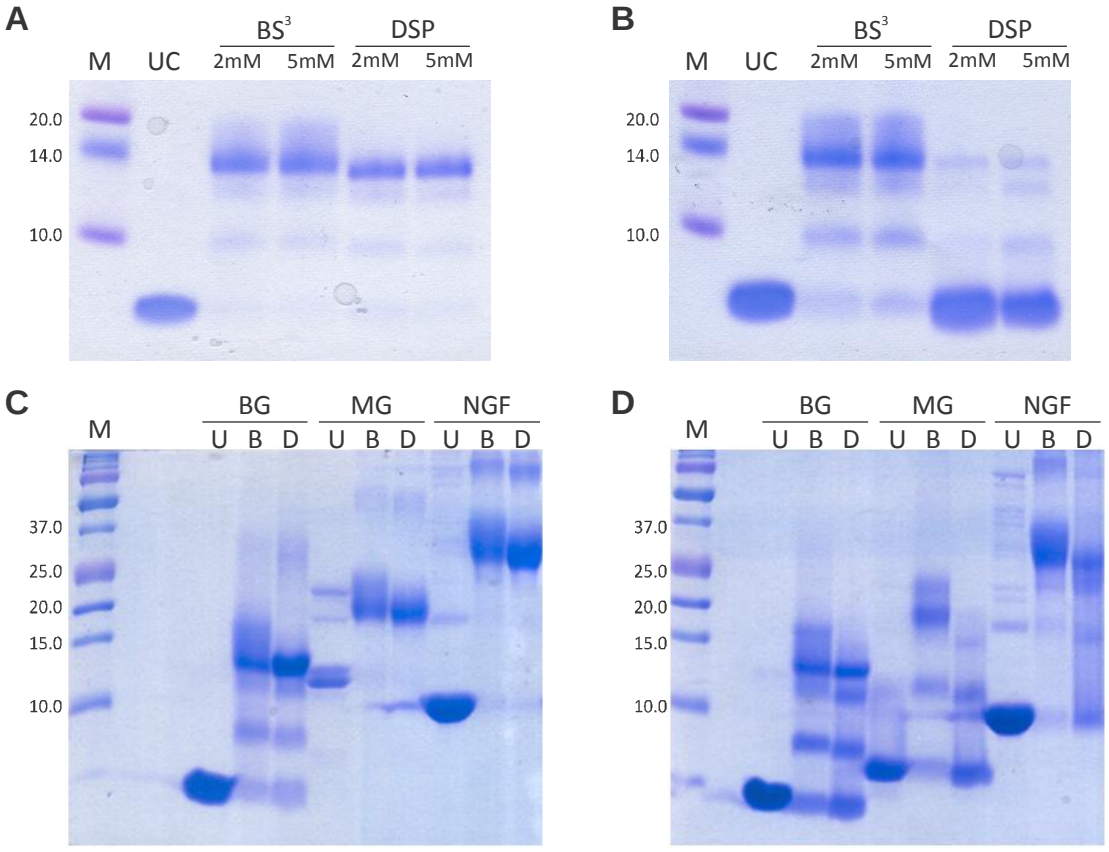
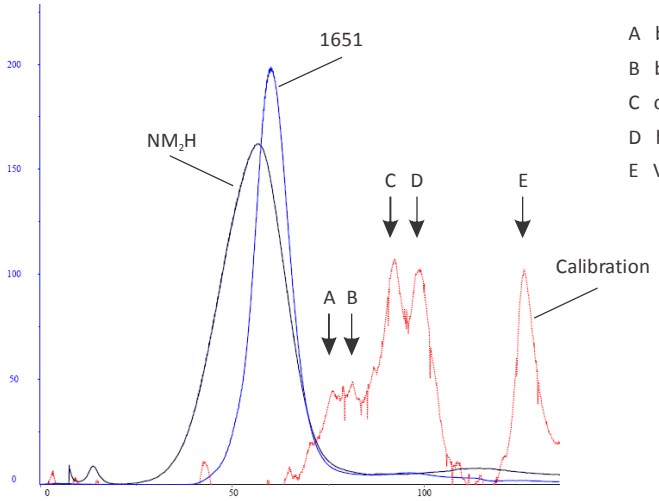


Figure 3.10. Cross-linking experiments show tetrameric and intermediate structures of tGCN4-proteins. Selected tGCN4-proteins were treated with two homobifuncional cross-linkers, BS^3 and DSP, of which DSP has a reducable disulfide bridge in its spacer arm. Analysis of cross-linking was done on SDS-PAGE with or without the addition of β-mercapto-ethanol as reducing agent. **A-B.** Cross-linking of BM2e-tGCN4 with two concentrations of cross-linker and analysis under non-reducing (A) or reducing (B) conditions. M = gel marker with mass in kDa, UC = uncross-linked sample, BS^3 = BS^3-treated sample, DSP = DSP-treated sample. **C-D.** Cross-linking of BM2e(QIL)-tGCN4, M2e-tGCN4 and NBe-tGCN4-Flag and analysis under non-reducing (C) or reducing (D) conditions. M = gel marker with mass in kDa, BG = BM2e(QIL)-tGCN4, MG = M2e-tGCN4, NGF = NBe-tGCN4-Flag; U = uncross-linked sample, B = BS^3-treated sample, D = DSP-treated sample.

3.2.3 Characterization of HBc-based proteins

To assess the assembly of HBc-based vaccine candidates, *i.e.* VLP formation, a number of assays were performed. Initial proof for multimerisation of HBc-proteins comes from the gel filtration profiles of the final purification step. Comparison of the profiles of the gel filtration run of NM_2H and 1651 with a calibration run is shown in Figure 3.11. The size exclusion column used was a Sephacryl S400 column, with a range of separation between 10kDa and 5MDa. The expected particle size of unsubstituted HBc149 VLP is appr. 3-5MDa, entailing these and any antigen-bearing particles will elute shortly after the void volume of the column. For calibration, a mixture of bovine thyroglobin (670kDa), bovine γ-globulin (158kDa), chicken ovalbumin (44kDa), horse myoglobin (17kDa) and Vitamin B12 (1.35kDa) was used (Biorad Gelfiltration Standard). As can be deduced from the profile overlay, both 1651 and NM_2H show a single peak larger than 670kDa, close to the void volume. This suggests that both proteins behave as particulate antigens.



A bovine thyroblobin (670kDa)
B bovine g-globulin (158kDa)
C chicken ovalbumin (44kDa)
D horse myoglobin (17kDa)
E Vitamin B12 (1.35kDa)

Figure 3.11. Gelfiltration profiles illustrate the particulate nature of HBc proteins. The profiles of a calibration run (dashed red) and gel filtration of 1651 (solid blue) and NM_2H (solid block) are shown, overlayed with each other. For the calibration run, proteins and their MW corresponding to each peak are indicated.

To assure the particulate proteins form VLPs and are not just aggregates, two additional tests were done. A first is based on separation of large (negatively charged) proteins using agarose gels. Full-length HBc-proteins (183 amino acids; HBc183) will spontaneously form particles with the ability to retain nucleic acids by binding of these through a C-terminal protamine-like domains. Truncated forms of HBc (149 amino acids; HBc149) loose this property. A third form of VLPs is constituted of HBc-monomers with an intermediate protein length (163 amino acids; HBc163), retaining part of the nucleic acid binding domain. These different particles can easily be visualized by agarose gel electrophoresis, followed by Ethidium Bromide (EtBr) in case nucleic acids are retained by the particles or Coomassie staining. If VLP formation is complete, HBc proteins will stain as a discrete band on Coomassie gel, and stain with EtBr if they contain nucleic acid binding motifs C-terminally. Additionally, because of the stabilizing role of disulfide bridges in particle formation [32], reduction of these VLPs by agents such as β-mercapto-ethanol (BME) is expected to abolish particle formation. Figure 3.12 depicts an agarose gel analysis of different HBc proteins in the absence or presence of BME. Both unsubstituted HBc163 and $M2e_3$-HBc163 run as a defined species visualized by Coomassie

staining; additionally, both contain nucleic acids since particles in the gel can be stained with EtBr. With the addition of BME, only M2e$_3$-HBc163 particles can be disrupted, indicating that substitution of HBc has an effect on general stability of the formed VLP. HBc149 and HBc183 proteins, provided as certified VLPs by Acambis Inc. (Cambridge, MA), lost their particulate integrity upon extended storage at -20°C, as apparent by staining as a smear on gel with Coomassie. Addition of BME however does focus these proteins into a single band, most likely corresponding to reduced monomers derived from dissociated particles. The NM$_2$H hybrid also shows a profile corresponding to a broken-down particle, with no defined staining in the absence of BME and appearance as a single band with higher mobility in the presence of BME. 1646, 1651 and 3004 are not visualized on gel because of low protein concentration; in other instances, staining of single bands has been possible (data not shown).

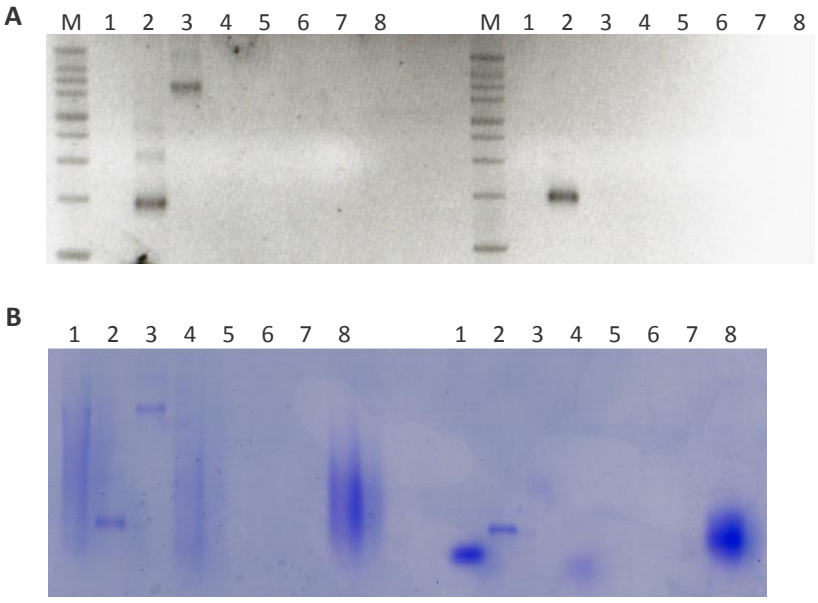


Figure 3.12. Agarose gel electrophoresis distinguishes particles formed by HBc proteins. Different HBc proteins were loaded on a 2% agarose gel in the absence or presence of the reducing agent β-mercapto-ethanol (BME) as indicated. After electrophoresis, gels were stained with EtBr (A) to reveal particles containing nucleic acids, or with Coomassie (B) to stain proteins. M = DNA marker, 1 = HBc149, 2 = HBc163, 3 = M2e$_3$-HBc163, 4 = HBc183, 5 = 1646, 6 = 1651, 7 = 3004, 8 = NBe-M2e$_2$-HBc.

A second method to evaluate VLP formation is based on light scattering induced by particles. The technique, called dynamic light scattering (DLS), essentially measures the Brownian movement of particles in solution and relates this to their size. By measuring the fluctuation in light scatter that particles in solution induce over time, the so-called translational diffusion coefficient of those particles, representing the velocity of the Brownian movement, can be determined. Based on this measurement, and taking into account the temperature and viscosity of the solution, the hydrodynamic diameter of the particles in solution can be calculated. Because DLS assumes particles measured are spherical in size, the diameter of the particles obtained by this technique will be equivalent to that of a sphere with the same diffusion coefficient. In initial measurements, the intensity of scattering is determined in relation to the size of the particles in solutions, giving an intensity-size distribution. However, because larger particles scatter light more than smaller particles,

the relative presence of larger particles will be strongly favored this way (intensity correlates with d^6, with d the diameter of the particle). By converting intensity signals in volume occupied by the particles (volume-size distribution, correlating with d^3), a closer approximation of the relative prevalence of particles of different sizes is obtained.

As a control, a validated HBc VLP was taken along, namely 1604 [26], consisting of a single M2e-copy N-terminally fused to the short (149 amino acids) HBc protein. The particulate nature of three HBc-chimeras was assessed here: an M2e-HBc fusion with full length HBc (183 amino acids; M2e-HBc183), the 3004 B/HA0-HBc protein and the NBe-M2e$_2$-HBc fusion. The expected diameter for an HBc-particle is appr. 30nm, which is detected by dynamic light scattering for the 1604 particle (Fig. 3.13). Note that in both the intensity-size and volume-size distributions a single peak is observed, indicating that the protein behaves as a single-size complex. For the three test-particles, other peaks in the intensity-size distribution can be seen. For M2e-HBc183 and the B/HA0-HBc proteins these resemble high-order complex, most likely aggregates of particles (Fig. 3.13A, C). These however have a relatively low prevalence in the mixture, as illustrated by the disappearance of the peaks through

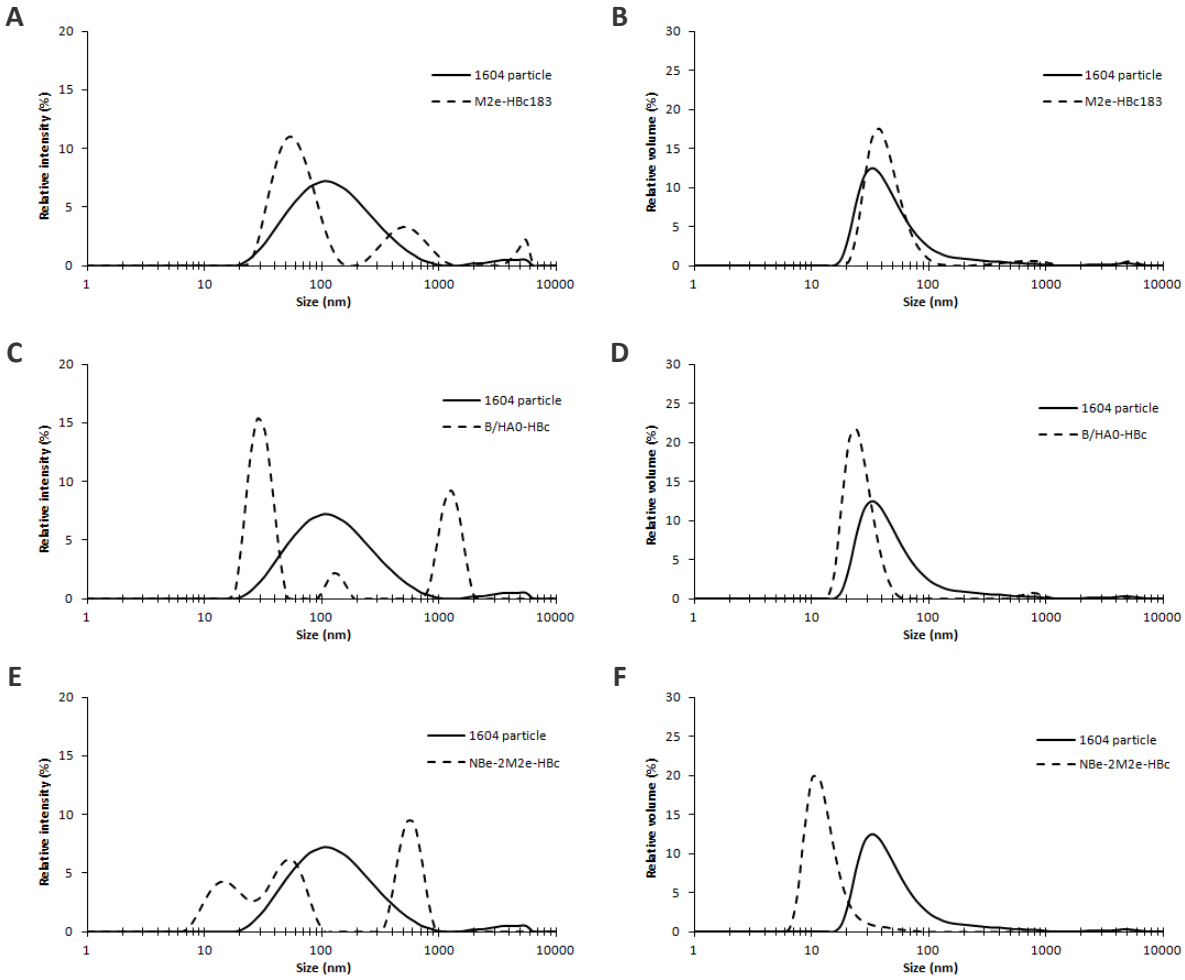


Figure 3.13. Dynamic light scattering (DLS) shows NBe-M2e$_2$-HBc does not behave as a VLP. DLS measurements were done for different HBc-particles. Shown is the hydrodynamic diameter or size of illuminated proteins in function of relative intensity of scattering (A, C, E) or relative volume (B, D, F). Profiles for M2e-HBc183 (A, B), B/HA0-HBc (C, D) and NBe-M2e$_2$-HBc (E, F) are represented by dashed lines and overlaid with the profile of 1604 particle (full line).

conversion to volume-size distribution (Fig. 3.13B, D). For NBe-M2e$_2$-HBc protein, a small peak corresponding to a diameter of 50nm can be seen (Fig. 3.13E); however, conversion into a volume-size distribution reveals the presence of a single major species with a size under that of a particle (Fig. 3.13F). Together with the above data, this shows the NBe-M2e$_2$-HBc protein does not behave as a particle, but as a dimer or aggregates/complexes thereof. Other HBc-based proteins used in this research, including the B/HA0-HBc (3004) chimera, do form particles.

3.2.4 Characterization of glycosylated proteins

a) P. pastoris *produced gNBe-tGCN4*

As described above (see 1.3), SDS-PAGE analysis of yeast-produced gNBe-tGCN4 indicates that the majority of monomers are non-glycosylated (Fig. 3.7). Evidence on the glycosylation state of tetramers comes from the profile and size exclusion analysis by gel filtration. As shown in Figure 3.14, no dissociation of glycosylated and non-glycosylated tetramers can be seen. The profile shows only one main peak containing gNBe-tGCN4 (peak 2), with analysis of fractions confirming co-elution of glycosylated and non-glycosylated species.

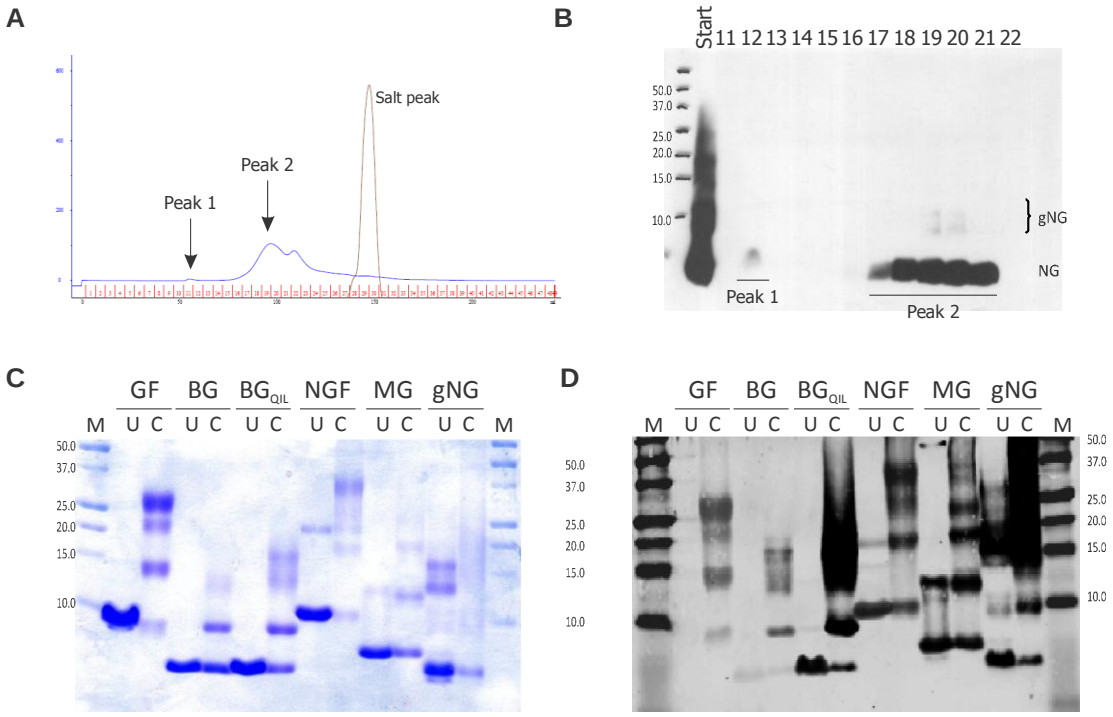


Figure 3.14. Gel filtration profile and molecular characterization of gNBe-tGCN4 reveal the presence of glycan structures. A-B. Gel filtration profile of gNBe-tGCN4 purification (A) and concomitant analysis of fractions on Western blot probed with anti-tGCN4 rabbit serum (B). NG = NBe-tGCN4 running at the MW of non-glycosylated protein, gNG = NBe-tGCN4 with indication of glycosylation. Elution peaks and corresponding fractions containing gNBe-tGCN4 are indicated. **C-D.** Cross-linking of tGCN4-proteins with BS^3, analyzed by SDS-PAGE and Coomassie-staining (C) or Western blot (D) with detection

using rabbit anti-tGCN4 serum, reveals structures corresponding to dimers, trimers and tetramers. For gNBe-tGCN4, higher structures run as a smear on gel, not discerning discrete multimeric species. M = gel marker (mass in kDa), GF = tGCN4-Flag, BG = BM2e-tGCN4, BG_{QIL} = BM2e(QIL)-tGCN4, NGF = NBe-tGCN4-Flag, MG = M2e-tGCN4, gNG = gNBe-tGCN4; U = uncross-linked, C = BS^3 cross-linked.

Because of the predicted positive charge of gNBe-tGCN4 at pH below 7.4, native gel electrophoresis to determine the MW of the native protein is not straightforward. As an alternative, fixation of the native multimeric structure with the homobifunctional BS^3 cross-linker was performed. Analysis on SDS-PAGE through Coomassie staining (Fig. 3.14C) and Western blot detected with rabbit anti-tGCN4 serum (Fig. 3.14D) revealed clear mono-, di-, tri- and tetrameric structures for all bacterial produced tGCN4-proteins. For gNBe-tGCN4 however, monomers are clearly distinguishable, but higher structures form a smear on gel, compatible with oligomers with mixed glycosylation state and mixed size of glycosyl-structure.

To ascertain the identity of the glycosyl-structure on gNBe-tGCN4, DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) analysis was performed [39]. gNBe-tGCN4 produced by both wild type GS115 and glyco-engineered KE3 *P. pastoris* were analyzed. Little glycosyl-structures were detected on wild type produced protein, and structures detected mainly existed of phosphorylated structures (data not shown). KE3 produced gNBe-tGCN4 is expected to contain structures not more complex as $GlcNAc_2$-Man_5 ($(N\text{-Acetyl-Glycosamine})_2$-$Mannose_5$). However, both $GlcNac_2$-Man_5 glycans and a higher order structure can be discerned. The precise identity of the latter structure is as yet undetermined.

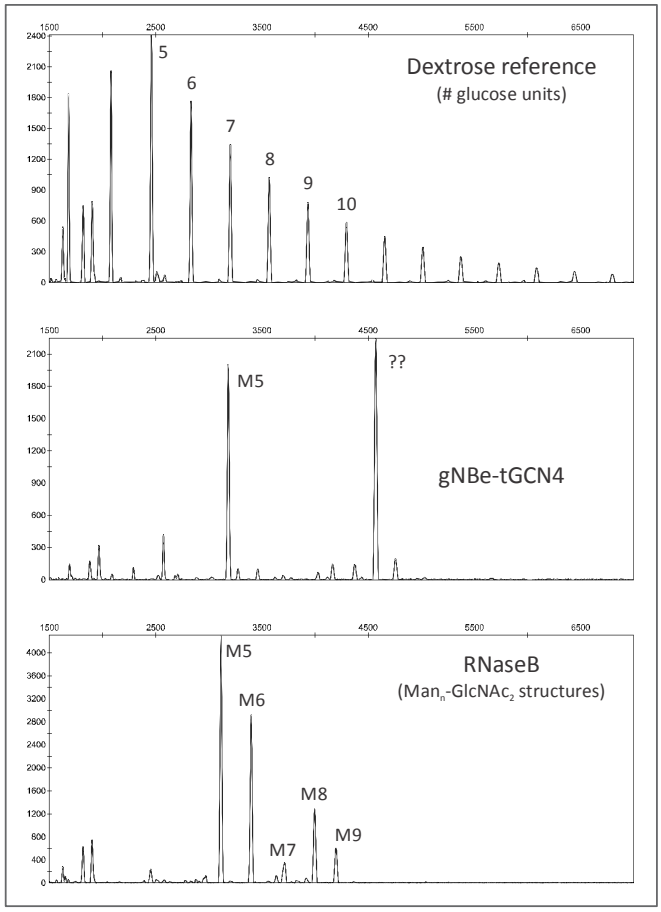


Fig. 3.15. DSA-FACE analysis ofglycan structures on gNBe-tGCN4. The top panel shows the profile of a maltodextrose reference; the numbers indicate the number of glucose units present in oligomers corresponding to the indicated peaks. The middle panel shows the analysis of KE3 *P. pastoris* produced gNBe-tGCN4, showing two glycan-peaks. Of these, the first corresponds to Man_5-$GlcNAc_2$, the second is as yet unidentified. The bottom panel shows reference glycans ($Man_{5\text{-}9}$-$GlcNAc_2$) derived from bovine RNaseB.

b) Expression of membrane-bound NBe-M2 chimers

pCAGGS-based plasmids were created for cellular expression of transmembrane Flag-tagged full-length NB protein. However, in our hands, expression levels of NB-Flag protein were near to undetectable by Western blot analysis (data not shown). For this reason, chimeras were created replacing the ectodomains of well-expressed BM2 and M2 proteins by that of NB (see 3.2.1b, Fig. 3.4). As additional testing ground, mutations were introduced deleting the glycosylation sites (here termed G1 and G2) in the NBe-sequence (N3Q and N7Q), alone or combined (Fig. 3.4). SDS-PAGE analysis of lysates from transiently transfected HEK293T cells clearly shows the influence of introduction of these mutation, both for NBe-M2 and NBe-BM2 combinations (Fig. 3.16). Proteins bearing the wild-type NBe-sequence clearly show three bands on immunoblot, corresponding to unglycosylated (UG), single glycosylated (G1/2) and double glycosylated (G1+2) monomers. Although natural glycosylation has been shown to be of the complex type [6], no great amounts of high MW NBe-based proteins were observed in gel analyses. As for gNBe-tGCN4, unglycosylated monomers are readily detectable on Western blot. Also striking, the chimeric proteins show similar properties as the protein providing the transmembrane and cytoplasmic domains. For example, NP40-based lysis of transfected cells will solubilize M2-based proteins completely, while BM2-based proteins will remain associated with cellular structures, regardless of the ectodomain.

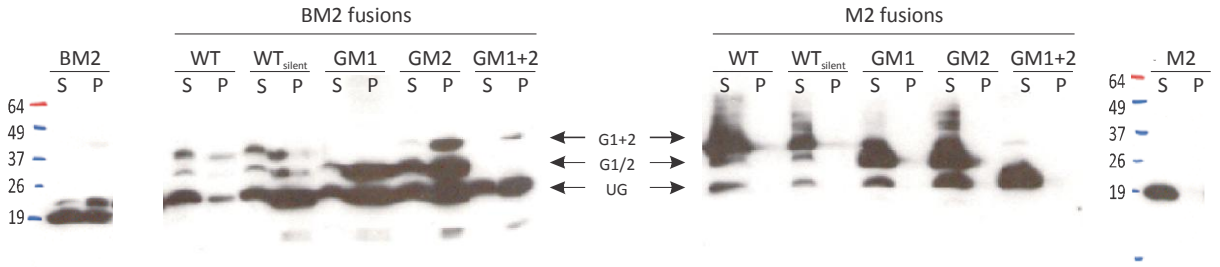


Figure 3.16. SDS-PAGE and Western blot analysis of eukaryotic cell expressed chimeric transmembrane NBe-proteins. HEK293T cells were transfected with pCAGGS-based vectors coding for different Flag-tagged M2- and BM2-based NBe-bearing proteins. NP40-detergent lysed cells were analyzed by SDS-PAGE and Western blot. These were detected using rabbit anti-Flag and anti-rabbit-HRP antibodies. Different NBe-ectodomains expressed are: WT = wild type sequence, WT_{silent} = wild type sequence with silent mutation deleting neuraminidase initiating codon (N3: AATGCT ➔ AACGCT), GM1 = glyco-mutant 1 (N3Q), GM2 = glyco-mutant 2 (N7Q), GM 1 + 2 = glyco-mutant 1+2 (N3Q + N7Q). Control proteins are full-length Flag-tagged BM2-protein (BM2) and M2-protein (M2). S = supernatant after lysis, P = pellet after lysis.

A more detailed expression profile of chimeric transmembrane proteins was obtained by immunofluorescence staining. For this, HEK293T cells were transfected with pCAGGS-vectors encoding the different chimeras. After fixation and permeabilization, stainings were done with a rabbit anti-Flag antibody and a mouse serum raised against the NBe-, BM2e- or M2e-ectodomain. These were obtained by immunizing mice with the different prokaryotic expressed and purified tGCN4-proteins. Figure 3.17 shows the results for NB-based proteins. Although full-length NB is hardly detectable when analyzing transfected cell lysates (obtained under similar conditions as for these stainings) on SDS-PAGE, clear Flag-staining and ectodomain detection is visible here. Serum

raised against NBe-tGCN4-Flag can clearly stain both wild type (NBe(WT)) and mutated NBe (lacking both glycosylation sites; NBe(GM1+2)). Is should be noted that using triple Flag-tagged NBe-tGCN4-Flag for raising the immune serum did not evoke antibodies cross-reactive with the single Flag-tagged transmembrane proteins, since control settings did not show cross-reactivity of this serum with other non-NBe bearing Flag-tagged proteins (data not shown).

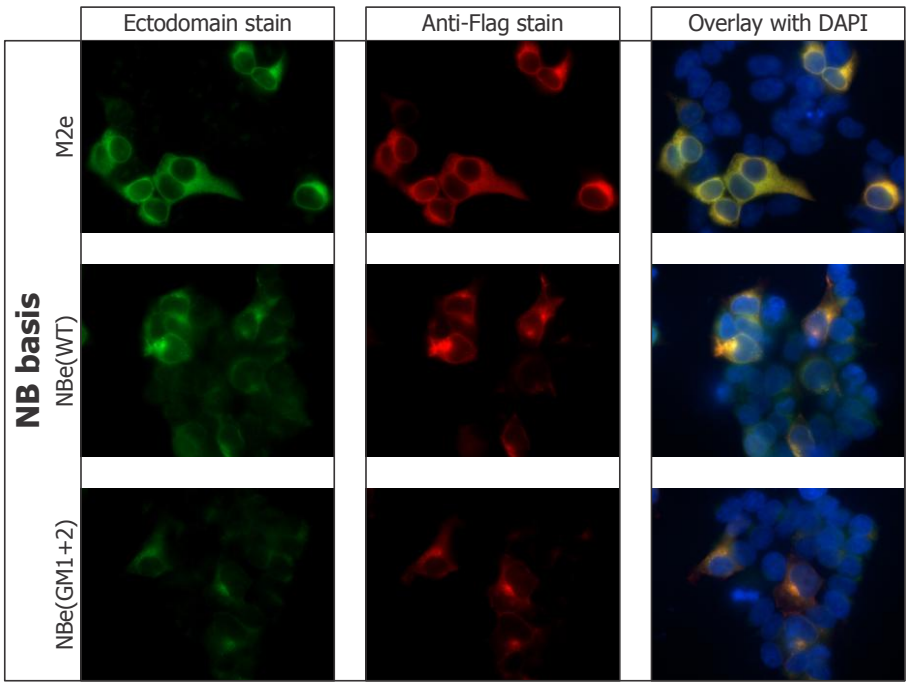


Figure 3.17. Immunofluorescence staining of HEK293T cells transfected with different NB-based proteins. Cells were transfected with pCAGGS-based vectors encoding the indicated ectodomains (M2e, NBe(WT), NBe(GM1+2)) fused to the NB transmembrane and cytoplasmic domain. Transfected cells were fixed, permeabilized and stained with rabbit anti-Flag and mouse antisera raised against the corresponding ectodomains using tGCN4-based proteins (see text). Antibody binding was visualized by secondary staining with anti-mouse-AlexaFluor488 and anti-rabbit-AlexaFluor568 antibodies. Cells were mounted using Vectashield containing DAPI for nuclear counterstain. Pictures shown are representative for each setting. Control settings using mock-transfected cells or secondary stain only were negative (data not shown).

Stainings of cells expressing proteins with BM2- or M2-basis (*i.e.* transmembrane and cytoplasmic domain) are shown in Figure 3.18. Corresponding with the Western blot analysis (Fig. 3.16), M2- and BM2-based proteins show a distinct localization pattern, in that BM2-proteins locate to perinuclear membranes (most likely Golgi-membranes), while M2 is more surface-expressed. Like for NB-fusions, antiserum raised with M2e-tGCN4 or NBe-tGCN4-Flag can clearly stain M2e- or NBe-fusions, both wild type and glycomutant for the latter. BM2e-tGCN4 antiserum however does not seem to contain anti-BM2e antibodies. Under certain conditions however, staining of IBV proteins with BM2e-tGCN4 antiserum can be seen (see Chapter 4).

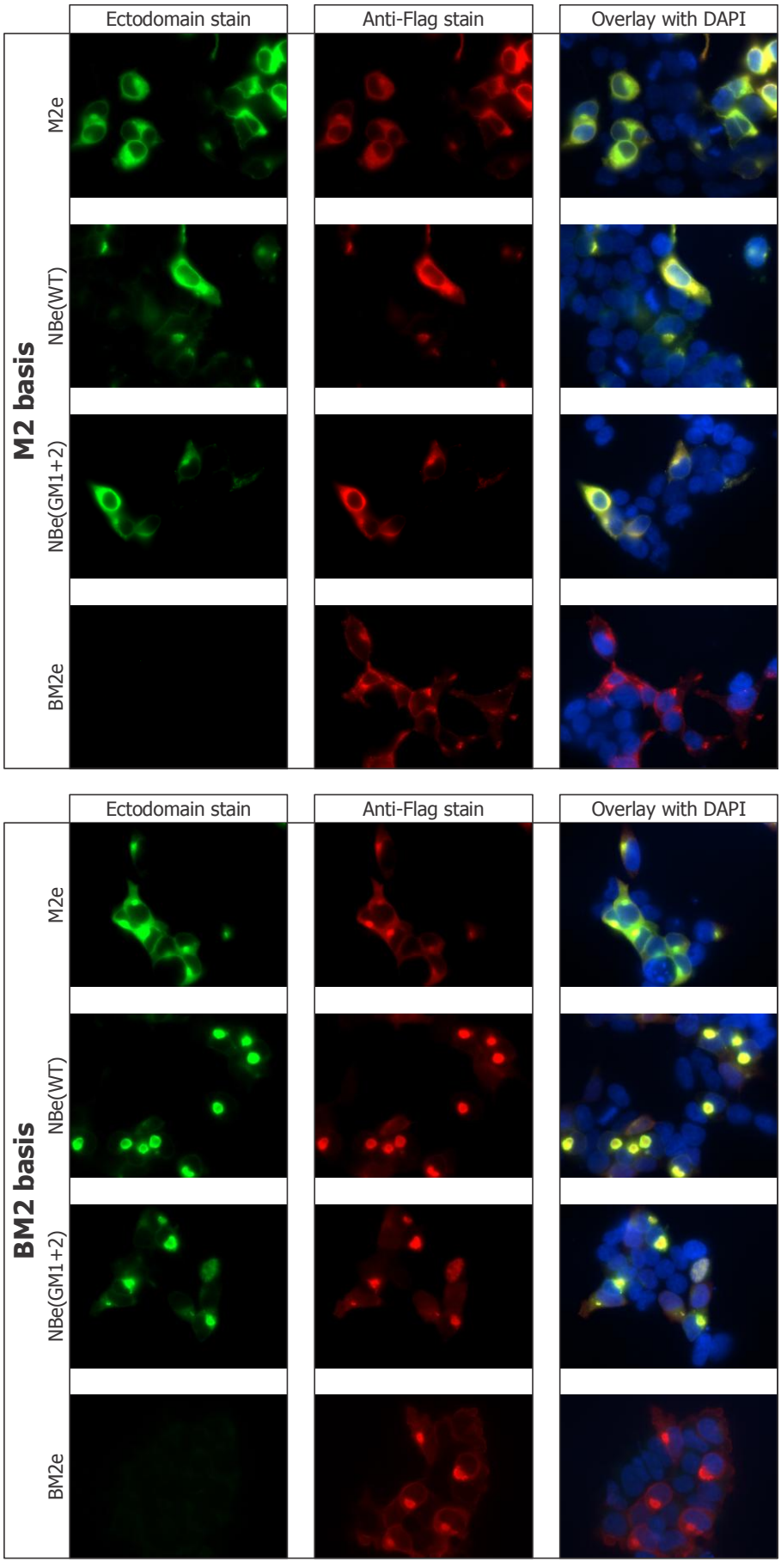

Ectodomain stain
Anti-Flag stain
Overlay with DAPI
M2 basis
M2e
NBe(WT)
NBe(GM1+2)
BM2e
Ectodomain stain
Anti-Flag stain
Overlay with DAPI
BM2 basis
M2e
NBe(WT)
NBe(GM1+2)
BM2e

Figure 3.18. Immunofluorescence staining of HEK293T cells transfected with different M2- and BM2-based proteins. Cells were transfected with pCAGGS-based vectors encoding the indicated ectodomains (M2e, NBe(WT), NBe(GM1+2), BM2e) fused to the BM2 (left) or M2 (right) transmembrane and cytoplasmic domain. Transfected cells were fixed, permeabilized and stained with rabbit anti-Flag and mouse antisera raised against the corresponding ectodomains using tGCN4-based proteins (see text). Antibody binding was visualized by secondary staining with anti-mouse-AlexaFluor488 and anti-rabbit-AlexaFluor568 antibodies. Cells were mounted using Vectashield containing DAPI for nuclear counterstain. Pictures shown are representative for each setting. Control settings using mock-transfected cells or secondary stain only were negative (data not shown).

To attest cellular expressed transmembrane proteins are in their native tetrameric organization, cell lysates were treated with the BS^3 cross-linker. Western blot analysis of treated samples, detected with rabbit anti-Flag serum, is shown in Figure 3.19. This confirms that monomers can be fixed together in structures with mobility on gel corresponding to dimers and multimers. For multimeric species, no discrete forms can be found for NBe-based proteins, as compared to *e.g.* M2-Flag, pointing to tetramers with mixed glycosylation state, as seen for gNBe-tGCN4 (Fig. 3.14). However, the complex nature of the cell lysates could influence the formation of homologously cross-linked oligomeric proteins.

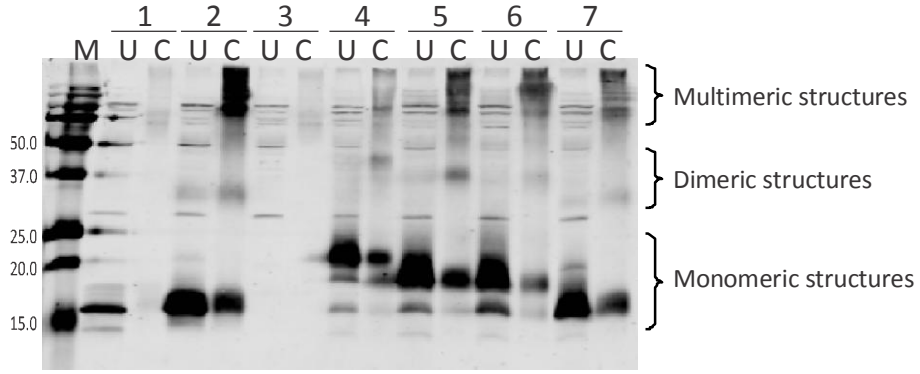


Figure 3.19. Cross-linking of cell-expressed viral and chimeric proteins shows the presence of higher oligomeric structures. Lysates of HEK293T cells transfected with pCAGGS vectors encoding different Flag-tagged transmembrane IAV and IBV proteins, or chimeric derivatives thereof, were treated with the BS^3 cross-linker. Samples were analyzed on Western blot through detection with rabbit anti-Flag antiserum. M = gel marker (mass in kDa), 2 = BM2-Flag, 3 = M2-Flag, 4 = NB-Flag, 4 = NBe(WT)-M2-Flag, 5 = NBe(GM1)-M2-Flag, 6 = NBe(GM2)-M2-Flag, 7 = NBe(GM1+2)-M2-Flag; U = uncross-linked sample, C = cross-linked sample.

Overall, these experiments show that NBe-proteins can be expressed successfully in cellular systems and assume their natural confirmation. Glycosylation of the NB ectodomain is evident from the Western blot analysis, clearly showing use of both sites individually or combined. Glycosylation of one site happens independently of the other, indicated by presence of monoglycosylated protein by mutation of either site. Removal of both sites also does not influence expression or transport to the cell surface, as described before [6]. Likewise, adopting a tetrameric structure is not dependent on glycosylation status for NBe (Fig. 3.19). The presence of both non-, mono- and double glycosylated monomers in tetramers, comparable to gNBe-tGCN4 expression (Fig. 3.7), makes it probable that tetramers consist of monomers with mixed glycosylation state, as confirmed by cross-linking (Fig.

3.19). In contrast to described data for naturally expressed NB [6], no evidence is seen for the addition of complex type carbohydrates, such as poly-N-lactosaminoglycans to the NBe-fusion constructs described here. However, the native conformation of glycosylated NBe-based proteins, as achieved by transfection of mammalian cells, formed the basis for assessing the *in vivo* protective effect of transfected cells as such. These cellular vaccines could lead to a proof-of-principle regarding protection against influenza B infection by using glyco-NBe proteins (see Chapter 4).

3.2.5 Generation and validation of NBe-specific monoclonal antibodies (mAbs)

a) Generation of NBe-directed mAbs

A good way of taxing the accessibility of antigens in their native (cell-associated) conformation, is by means of monoclonal antibody (mAb) binding assays. To develop NBe-specific mAbs, mice were repeatedly immunized with the NM_2H protein. After three consecutive immunizations, splenocytes of mice were used for fusion with myeloma cells to obtain hybridomas. On a regular basis, growing clones were tested for production of specific antibodies; an example of screening is shown in Figure 3.20A. After scaling up and further screening, five clones were retained for further use: the NBe-specific 11B1, 11H5, 12A7 and 15G5 and (as control) the HBc-specific 20C3. After 1-2 rounds of subcloning (through single-cell titration), monoclonal hybridomas were obtained which were used for large-scale production of mAbs. Titration of the purified mAbs showed that only 15G5 mAb had a significant affinity for NBe; the three other NBe-directed mAbs only reacted moderately with NBe-peptide coated on ELISA-plates (Fig. 3.20B). The concentration giving appr. 50% maximal binding in NBe-peptide ELISA was 197.28ng/mL for 15G5. The HBc-specific 20C3 mAb showed a higher affinity with its ligand (Fig. 3.20C), with a 50% maximum binding dose in HBc-ELISA of 20.01ng/mL. ELISAs using different substituted HBc-particles for coating revealed that 20C3 mAb is directed towards the immunodominant loop of the protein (data not shown). Reactivity of 15G5 mAb and 20C3 mAb towards different HBc- and NBe-HBc proteins in Western blot is shown in Figure 3.20D. Because of incomplete denaturation and reduction, both monomeric and dimeric HBc-species are visible in this figure. 20C3 mAb reacts with all HBc-proteins, except with 1646, the NBe-HBc chimeric protein bearing NBe in the immunodominant loop of HBc, confirming that this loop contains the 20C3 mAb epitope. Concomitantly, 15G5 mAb shows clear reaction with NBe-HBc fusions (1646, 1651, NM_2H). Because of the low affinity of other NBe-directed mAbs, focus remained on 15G5 mAb. Similar set-ups to develop mAbs directed to BM2e, B/HA0 or glycosylated NBe were performed numerous times, without any success of isolation of such specific mAbs or surviving hybridoma clones.

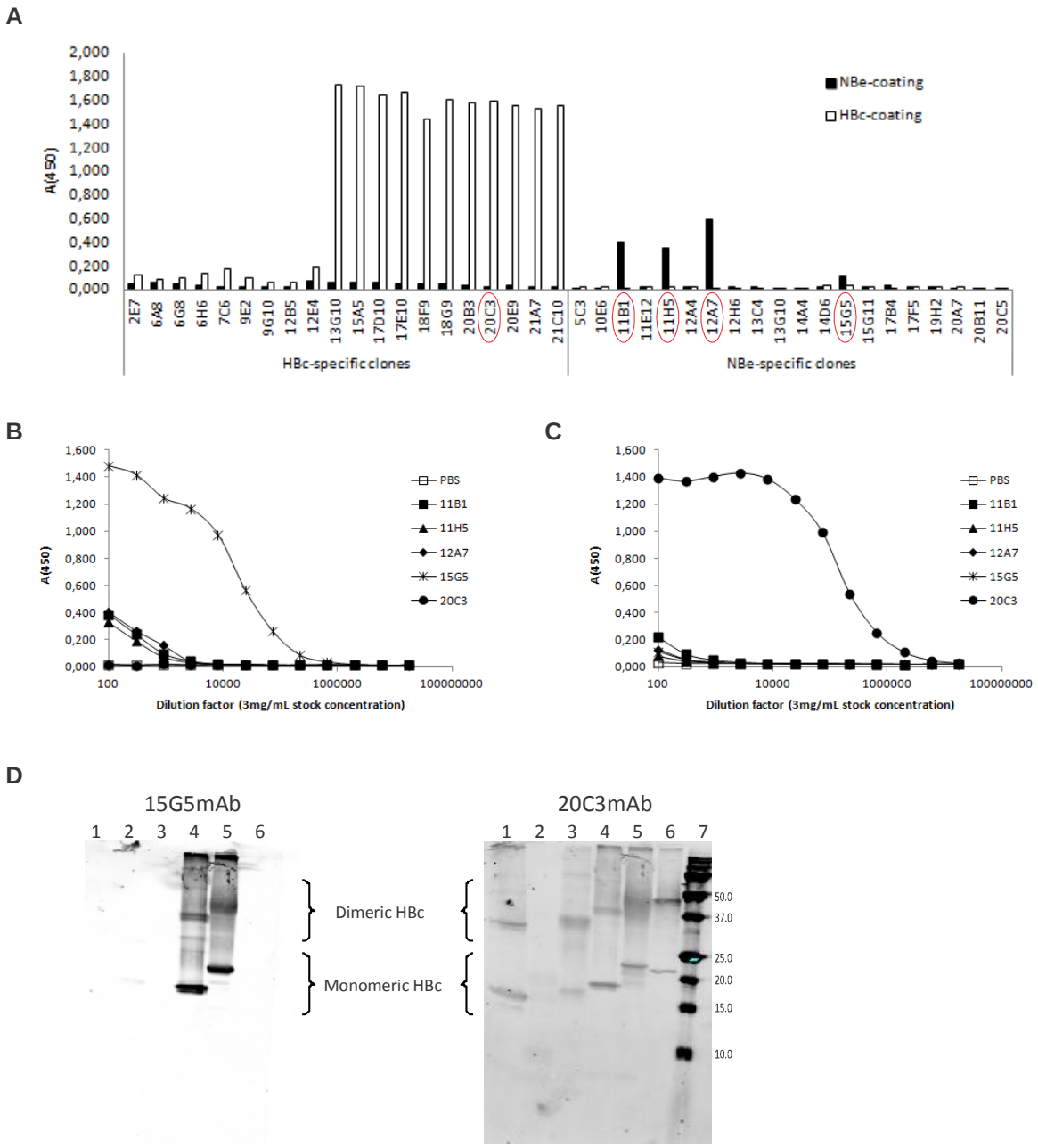


Figure 3.20. Development and validation of NBe-specific monoclonal antibodies (mAbs). Splenocytes of mice immunized with the chimeric NM_2H protein were used for the production of hybridomas secreting antibodies specific for HBc and NBe. **A.** Example of screening of hybridomas using HBc- and NBe-specific ELISAs. Clones which were selected based on their specificity and growth are circled in red. **B-C.** NBe- (B) and HBc-specific (C) ELISAs using purified mAbs at a starting concentration of 3mg/mL to detect specificity and affinity of the selected mAbs. **D.** Different HBc- and NBe-HBc-proteins (1µg each) were analyzed on Western blot with detection using the NBe-specific 15G5mAb (10µg/mL; left) or the HBc-specific 20C3mAb (1µg/mL; right). 1 = HBc (149 amino acids), 2 = 1646 (NBe-HBc, NBe in the loop), 3 = HBc (163 amino acids), 4 = 1651 (NBe-HBc, NBe N-terminal), 5 = NBe-M2e2-HBc , 6 = HBc (183 amino acids), 7 = gel marker with mass in kDa.

b) In vitro validation of native NB-binding by NBe-mAbs

Binding to native structures by 15G5 mAb was evaluated in a number of *in vitro* assays. In a first approach, cellular expressed Flag-tagged NBe-chimeric transmembrane proteins were immuno-precipitated. Lysates of transfected HEK293T cells were incubated with 15G5 mAb, 20C3 mAb (negative control) or mouse anti-Flag mAb (mαFlag, positive control). After pull-down of antibody-antigen complexes with Protein G beads, bound (eluted: EL) and unbound (UB) fractions were analyzed on Western blot by means of rabbit anti-Flag detection. As antigen, the NBe-M2 fusion protein was used, both with wild type NBe-sequence and the mutant sequence lacking both glycosylation sites. Figure 3.21A shows the result of the transfection and lysis, with clear presence of three distinct NBe-M2 bands in the wild type (WT) setting (corresponding to non-, mono- and double glycosylated NBe-M2) and a single band in the mutant setting. To assure that the immuno-precipitation was technically sound, the antibody bands of precipitating mAbs were also detected in Western blot (Fig 3.21B). The results of the pull-down are shown in Figure 3.21C-D. The control settings behave as expected: 20C3 mAb (and beads alone) do not bind any antigen, while mouse anti-Flag is able to precipitate the majority of NBe-M2-Flag protein. The 15G5 mAb can pull down a large part of the NBe-based proteins, irrespective of their glycosylation state. This might entail the binding site of 15G5mAb does not include the most N-terminal sequence containing the glycosylation sites, or that NBe-M2 tetramers are mixed in nature, with a part being non-glycosylated and accessible for 15G5 mAb. In the latter case, the relatively conservative nature of the N3Q and N7Q mutations might not influence the binding of 15G5 mAb dramatically, allowing association with this variant practically as efficient as wild type NBe.

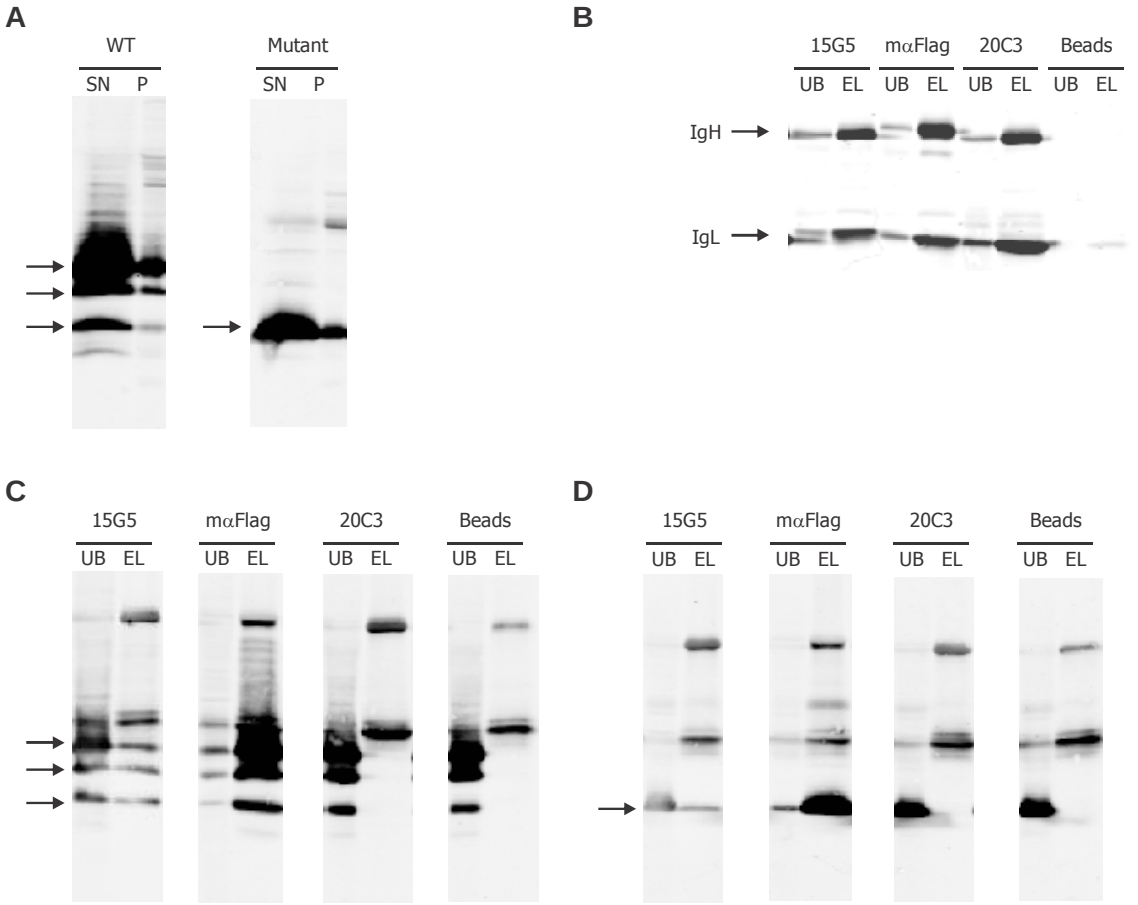


Figure 3.21. Immuno-precipitation (IP) of NBe-M2 proteins with 15G5 mAb. Lysates of transfected cells, expressing wild type (WT) or glyco-mutant (Mutant; lacking both glycosylation sites) NBe-M2-Flag, were incubated with the NBe-specific 15G5mAb, the HBc-specific 20C3 or mouse anti-Flag (all 10µg). After overnight incubation, antigen-antibody complexes were pulled down using Protein G beads. After centrifugation, unbound species and beads were separated and processed for analysis on Western blot. A control setting without the addition of antibody, to assess sticking of antigen to the beads, was also included (Beads). **A.** Western blot of the lysates of transfected cells, detected with rabbit anti-Flag. The arrows indicate bands corresponding to (non-, mono- and double glycosylated) NBe-M2-Flag proteins. SN = soluble fraction (supernatant) after lysis (used for the IP assay), P = insoluble fraction (pellet) after lysis. **B.** Western blot analysis after pull-down, with detection of antibody bands of the mAbs used for the IP. UB = unbound species, EL = eluted species from beads; IgH = antibody heavy chain, IgL = antibody light chain. **C-D.** Detection of precipitated wild type NBe-M2-Flag (C) or glyco-mutant NBe-M2-Flag (D) antigen by detection with rabbit anti-Flag. The arrows indicate bands corresponding to (non-, mono- and double glycosylated) NBe-M2-Flag proteins. UB = unbound species, EL = eluted species from beads.

A second approach to detect native NBe-binding by 15G5 mAb is immunofluorescence staining of influenza B (B/Memphis/12/97 and B/Harbin/7/1994) infected MDCK cells. Stainings were done before fixation on live cells at 37°C, to retain native structures for optimal access of 15G5 mAb as much as possible. As controls, influenza A/California/4/2009 (*i.e.* pandemic H1N1 2009) infected cells were taken along, as well as an M2e-specific mAb. After incubation with mAbs, cells were washed, fixed and permeabilized, followed by staining of the influenza expressed M1 protein using a polyclonal goat antiserum and secondary staining. Results of the live staining are shown in Figure 3.22. Even with some background staining of influenza A infected cells with 15G5 mAb (third panel row), clear staining of influenza B infected cells is evident (top two panel rows). Using an M2e-

specific mAb, M2-protein expressed during influenza A infection can nicely be visualized (last panel row), with almost no reaction towards influenza B infected cells (second to last panel row). Since viral expressed NB or M2 protein was stained pre-fixation, the ectodomain stainings reveal proteins expressed on the cell surface and line the entire surface of the infected cell. In the case of M2e-specific staining, it is very clear antibody-antigen complexes are internalized during incubation, giving a speckled staining pattern. For the anti-NBe stain, this is less clear, although surface staining of infected cells was clear for IBV-infected cells.

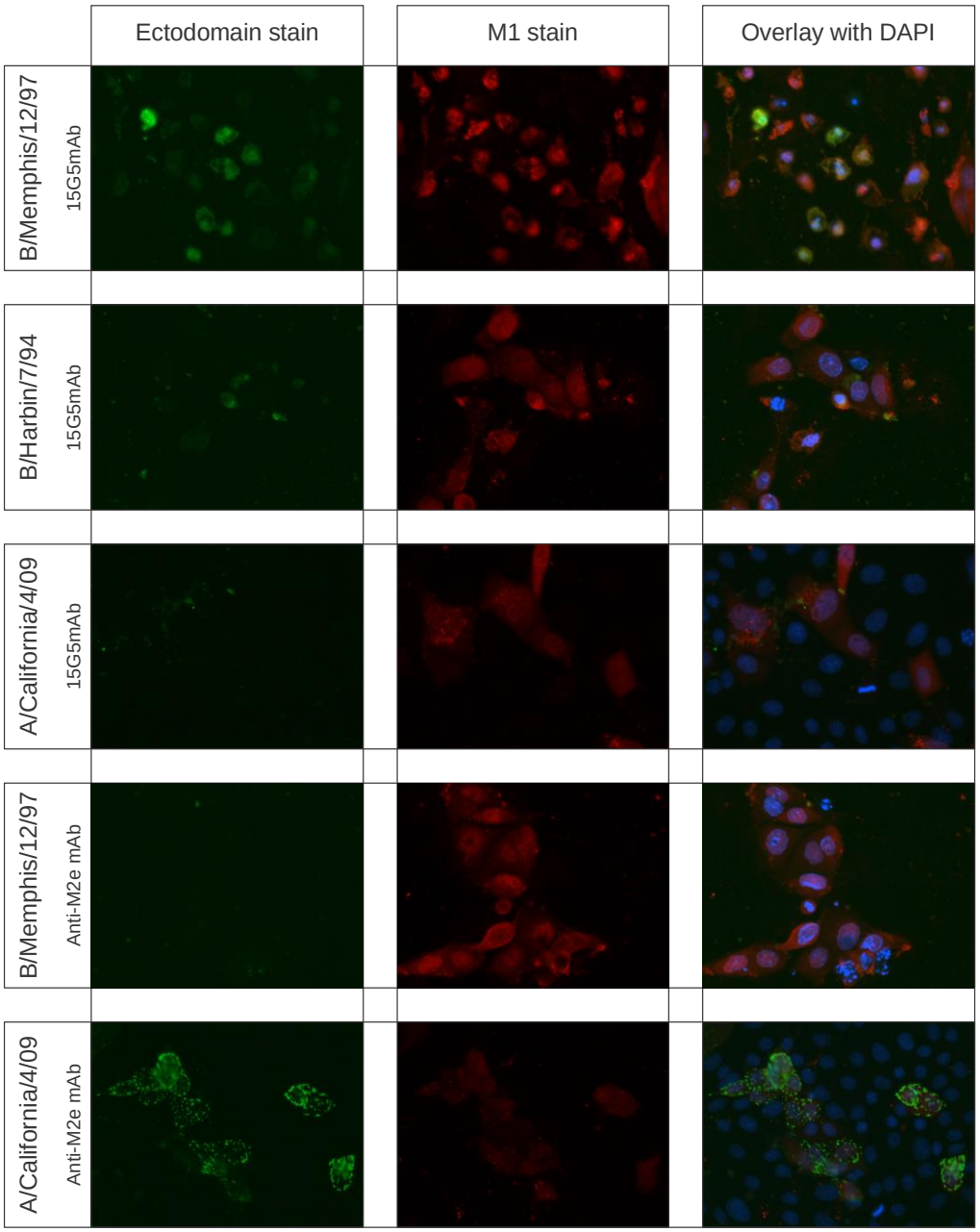


Figure 3.22. Immunofluorescence staining of infected MDCK cells using NBe- and M2e-directed mAbs. MDCK cells were infected with B/Memphis/12/97, B/Harbin/07/94 or A/California/04/09 as indicated. 16h after infection, cells were washed and incubated with NBe-specific 15G5mAb (10µg/mL) or an M2e-specific (1µg/mL) for 2h at 37°C. After extensive washing, cells were fixed, permeabilized and stained with goat anti-M1 antiserum. Shown are representative pictures for each condition.

Taken together, these stainings and the ones discussed earlier (see 3.2.4b, Fig. 3.17), along with the pull-down results, show that NBe-directed antibodies can react with (native) membrane-associated NBe-proteins. Because of clear presence of non-glycosylated monomeric NBe-proteins in our *in vitro* models of transfection and no clear indication about the glycosylation state of the NB protein during *in vitro* infection, the exact influence of NBe-glycosylation on antibody recognition is still largely undetermined. Nonetheless, these results do indicate that a certain level of NB protein remains non-glycosylated and shuttles to the plasmamembrane of infected (or transfected) cells in, presumably, tetramers with a mixed glycosylation state. It thus remains warranted to investigate the *in vivo* protective effect of (glycosylated) NBe-based vaccine candidates, next to BM2e- and B/HA0 chimeric proteins.

3.3. Material and methods

Construction of expression plasmids

Construction of 1646, 1651 and 3004 HBc particles was performed by Acambis Inc. (Cambridge, MA) using the *Lac*-promoter driven pKK223-3 expression vector (Amersham Pharmacia) [26]. For construction of NBe-M2e$_2$-HBc, the NBe-sequence was fused to the coding sequence of 1817 [26] by PCR methods. The entire sequence was recloned in the pLT32H vector using the *Nde*I and *Hin*dIII restriction sites [40], leading to *pL*-promotor driven expression in bacterial systems. Construction of tGCN4-Flag and NBe-tGCN4-Flag expressing vectors was done as previously described [20]. Briefly, overlapping oligonucleotides (Invitrogen) were used to create the coding sequences for the tetramerizing tGCN4 leucine zipper variant [41], the NB ectodomain and the triple Flag-tag. Using classical cloning techniques based on restriction enzyme digestion and PCR, the three domains were fused in frame and inserted in the pLT32H expression vector using the *Nde*I and *Hin*dIII restriction sites.

For secreted expression by *P. pastoris*, the pPIC9 expression plasmid was used. The NBe-tGCN4 coding sequence was picked up by PCR from the bacterial pLT32H expression plasmid and cloned in frame with the alpha mating factor secretion signal. For this, the forward primer included the *Xho*I restriction site (underscored) before the Kex2 and Dipeptidase cleavage sites and the NBe-sequence (italic) starting at amino acid 2 (5'-CCCCTGCAGAAAAGAGAGGCTGAAGCT*AACAATGCTACCTTCAACTAT*-3'), while the reverse primer introduced a stop codon after the tGCN4-sequence (italic) and allowed insertion at the *Eco*RI site (underscored; 5'-CCCGAATTCCTA*TTCGCCCAGCAGTT*-3').

Expression and purification of chimeric tGCN4-proteins

For expression of tGCN4-proteins, BL21 CodonPlus (Stratagene) bacteria, bearing the pICA2 repression plasmid [20], were transformed with the tGCN4-protein expressing pLT32H vectors. Inductions were done by diluting a 40mL bacterial culture, grown overnight at 28°C in Luria Broth (LB), 1/50 in 2L of LB. Cultures were grown further at 28°C until they reached an optical density at 600nm of 0.5-0.8. At this point, the cultivation temperature was raised to 42°C, leading to denaturation of the temperature-sensitive cI^{ts}-repressor [34], expressed by pICA2, liberating the *pL*-promotor and leading to expression of the chimeric tGCN4-proteins. After 4h of induction, bacteria were collected by pelleting the culture through centrifugation (10 min, 4°C, 6000g). If needed, bacterial pellets were stored dry at -20°C until use.

To purify the chimeric tGCN4-proteins, bacterial pellets were resuspended in 1/10 of the original volume using 50mM Tris/HCl pH8.0 + 150mM NaCl (4°C). Bacteria were disrupted by sonication and immediately treated for 30min at 65°C to precipitate contaminating bacterial proteins, relying on the heat-resistant properties of the

tGCN4 leucin zipper [41]. Next, denatured proteins and bacterial debris were removed by centrifugation (1h, 4°C, 20.000g) and filtered through a 0.45µm filter. Using an Akta purification station, cleared lysates were applied on a SourceQ anion exchange column, followed by a linear gradient elution using 50mM Tris pH8.0 + 1M NaCl, going from 0% to 100% elution buffer. Elution fractions were analyzed through SDS-polyacrylamide gel electrophoresis (SDS-PAGE); fractions containing the protein of interest were pooled and concentrated using Vivaspin columns (GE Healthcare) with a 5 kDa cut-off. Samples were loaded on a calibrated Superdex 75 prep-grade gel filtration column (120mL) and eluted with PBS. Fractions containing tGCN4-proteins of the desired size and purity (*cf.* Fig. 3.6) were pooled and concentrated using Vivaspin columns (5 kDa cut-off) to a concentration of 1-30 mg/mL without losing solubility. Protein solutions were batched out and stored at -80°C.

Expression and purification of chimeric HBc-proteins

Expression of HBc-based proteins was done using the same expression system as for tGCN4-proteins, with the exception induction was done using 1mM IPTG at 28°C for 4h. Expression of 1646, 1651 and 3004 relied on *lac*-promoter driven expression; for the NBe-M2e$_2$-HBc protein this was *pL*-promoter driven. Both systems rely on repression by pICA2 and allow de-repression by the addition of IPTG.

HBc-proteins were purified as follows. Bacterial pellets were resuspended in 1/10 of the original volume using 50mM Tris/HCl pH8.0 + 150mM NaCl (4°C). Bacteria were disrupted by sonication, bacterial debris was removed by centrifugation (1h, 4°C, 20.000g) and the supernatant was filtered through a 0.45µm filter. Next, $NH_4(SO_4)_2$ was added to the protein mixture to a final concentration of 30% (w/v) and incubated overnight at 4°C. Next, the precipitated HBc-proteins were pelletted by centrifugation (1h, 4°C, 20.000g) and dialyzed extensively against 50mM Tris/HCl pH 8.0 + 150mM NaCl. Using an Akta purification station, resolubilized proteins were applied on a SourceQ anion exchange column, followed by a linear gradient elution using 50mM Tris pH8.0 + 1M NaCl, going from 0% to 100% elution buffer. Elution fractions were analyzed through SDS-polyacrylamide gel electrophoresis (SDS-PAGE); fractions containing the protein of interest were pooled and concentrated using Vivaspin columns (GE Healthcare) with a 100 kDa cut-off. Samples were loaded on a calibrated Sephacryl S400 gel filtration column (120mL) and eluted with PBS. Fractions containing HBc-proteins of were pooled, concentrated using Vivaspin columns (100 kDa cut-off) and stored in batches at -80°C.

***Expression and purification of gNBe-tGCN4 by* Pichia pastoris**

To achieve expression by *P. pastoris* strains (GS115 and KE3), yeast cells were made competent as follows. A saturated culture of cells grown at 30°C was diluted in 500mL YPD medium, based on density (OD_{600}) and a doubling time of 2h at 30°C, to achieve an OD_{600} of appr. 1.4 after overnight incubation at 30°C. The next day cells were pelleted by centrifugation for 10' at 1500g. Pellets were resuspended in 100mL YPD + 20mL HEPES (1M, pH 8) containing 2.5mL 1M DTT and incubated for a further 15' at 30°C. After addition of 250mL sterile water, cells were pelleted by centrifugation (10', 1500g, 4°C). Pellets were resuspended in 250mL ice-cold sterile water, pelleted again (10', 1500g, 4°C) and washed once more in 125mL ice-cold sterile water and next 20mL ice-cold 1M sorbitol. After a final pelleting step (10', 1500g, 4°C), cells were gently resuspended in 1mL 20mL ice-cold 1M sorbitol and kept on ice.

For transformation, 40µL competent cells was mixed with 4µg *Sal*I linearized pPIC9-NBe-tGCN4 in a sterile electroporation cuvette. Immediately after electroshock (1.5kV, 25µFad), 1mL YPD was added to the cells and incubated at 30°C for 2h. Next, cells were plated on RDB-plates lacking His for selection of transformants and incubated at 30°C. Twelve colonies were picked, replated on RDB minus His plates to obtain single-cell colonies, and used for expression analysis.

Expression by *P. pastoris*, based on the AOX1 promoter, was achieved by inoculating a single-cell colony in BMGY medium. After overnight incubation at 30°C, OD_{600} was measured, cells were harvested by centrifugation (5', 3000g) and resuspended in BMMY medium to achieve a density of OD_{600} of 1. Cultures were induced for 48h, with addition of 0.5% methanol appr. every 12h. Finally, cultures were centrifuged, supernatants were collected and filtered and protease inhibitors (Complete ultra protease inhibitor tablets, Roche) were added.

Purification of gNBe-tGCN4 of *P. pastoris* culture medium started with differential $(NH_4)_2SO_4$ precipitation at 4°C. First, the $(NH_4)_2SO_4$ concentration was elevated to 40% by adding solid $(NH_4)_2SO_4$, followed by pelleting of insoluble material (1h, 20000g, 4°C). Next, the $(NH_4)_2SO_4$ concentration was further elevated to 80%. Insoluble material was collected by centrifugation (1h, 20000g, 4°C), resolubilized and dialyzed extensively to 50mM malonate pH 6.2 + 50mM NaCl using Vivaspin columns (5kDa cut-off; GE Healthcare). Using an Akta purification system, the dialyzed material was applied to a SP cation exchange column, followed by a linear gradient elution using 50mM malonate pH 6.2 + 1M NaCl, going from 0% to 100% of elution buffer. Elution fractions were analyzed through SDS-polyacrylamide gel electrophoresis (SDS-PAGE); fractions containing the protein of interest were pooled and concentrated using Vivaspin columns (GE Healthcare) with a 5 kDa cut-off. Samples were loaded on a calibrated Superdex 75 prep-grade gel filtration column (120mL) and eluted with PBS. Fractions containing gNBe-tGCN4 of the desired size and purity were pooled and concentrated using Vivaspin columns (5 kDa cut-off) to a concentration of 1-2 mg/mL without losing solubility. Protein solutions were batched out and stored at -80°C.

Construction and expression of chimeric transmembrane proteins

The cDNA coding for M2, BM2 and NB was obtained by reverse transcription using RNA prepared from A/Puerto Rico/8/34 or B/Memphis/12/97 virus-infected MDCK cells. Exchange of the ectodomains was done by fusion PCR, using oligonucleotides spanning the ectodomain on the one hand and transmembrane regions on the other. All coding sequences were cloned into the pCAGGS vector, allowing constitutive expression driven by the chicken β-actin/rabbit β-globin hybrid promoter. A single Flag-tag was introduced C-terminally to allow detection.

For transfections tests, HEK293 T cells were seeded at 2×10^5 cells in a 6-well plate in complete DMEM medium (DMEM + 10% FCS + L-Gln + sodium pyruvate + penicillin/streptomycin). 16h later, cells were transfected with 1µg plasmid DNA using calcium phosphate precipitation. 24h later, cells were lysed and analyzed by SDS-PAGE followed by Western blotting. Flag-tagged proteins were visualized using rabbit anti-Flag antibody detection, combined with alkaline phosphatase labeled goat anti-rabbit-AP secondary antibody and NBT/BCIP substrate (Roche) for detection.

Mass spectrometry

Mass spectrometry analyses were performed at the Department for Medical Protein Research (VIB, Ghent). A 1µl sample containing 0.3µg of protein was mixed with 1µl of saturated α-cyano-4-hydroxycinnamic acid that had been diluted 5-fold, and 0.5µl of this mixture was dried on the MALDI target of an Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany). Before measurement, the target was briefly rinsed with 10 mm ammonium citrate. MALDI mass spectra were generated by accumulation of 200 laser shots on the best target spots.

Native PAGE

The molecular weights of the M2e- and BM2e-tGCN4 proteins were estimated using a modification of the methods of Bryan and Davis [37,38]. Briefly, the chimeric protein and molecular weight standards were resolved by gel electrophoresis using various polyacrylamide concentrations. Electrophoretic mobility (Rf) was determined in each gel relative to the tracking dye, and 100 log (Rf x 100) values were plotted against the percent gel concentration for the reference proteins as well as M2e- and BM2e-tGCN4. The slope for each protein was determined and the logarithm of the negative slope was plotted against the logarithm of the molecular weight of each standard protein. This produces a linear plot from which the molecular weight of an unknown protein can be deduced.

Cross-linking experiments

All cross-linking experiments were performed as previously described [20]. Briefly, stock solutions of 50mM BS^3 (bis(sulfosuccinimidyl)suberate; Pierce) in water and 25mM DSP (dithiobis(succinimidylpropionate); Pierce) in DMSO were prepared freshly prior to use. These were diluted to 5 mM final concentration in PBS containing 10µg of the different tGCN4-proteins or 20µL lysates of transfected cells (equivalent to appr. 10^5 transfected cells). Cross-linking reaction was allowed to continue for 1h at room temperature, after which the reactions were stopped by adding 50 mM Tris, pH 6.8. When DSP and BS^3 were both used, samples were divided in two and boiled in non-reducing (50 mM Tris pH 6.8, 10% Glycerol) or reducing (50 mM Tris pH 6.8, 10% Glycerol, 20mM β-mercapto-ethanol) sample buffer, followed by analysis on SDS-PAGE and Coomassie-staining or Western blot analysis using rabbit anti-Flag (Sigma) or hopuse-made rabbit anti-tGCN4 serum.

HBc structural analyses

The efficiency of particle formation and the presence of particle-bound nucleic acids were analyzed by agarose gel electrophoresis. VLPs (5µg) were diluted in sample buffer (5% glycerol, 0,05% bromophenol blue) with or without the addition of 250mM β-mercaptoethanol and incubated for 2h at room temperature. Samples were loaded on a 2% agarose gel (Ultrapure agarose, Invitrogen) and run for 2h at 80V in TAE buffer. The gel was stained with ethidium bromide for nucleic acids and with Coomassie brilliant blue for proteins.

The dynamic light scattering (DLS) analysis was performed at the Faculty of Pharmaceutical Sciences. Briefly, protein solutions (in PBS) were diluted to contain 10mM salt and measured and analyzed on a polystyrene standard calibrated Zetasizer Nano S instrument (Malvern Instruments, Worcestershire, UK).

Immunofluorescense stainings

For immunofluorescence assays, HEK293T or MDCK cells were seeded on aceton-treated coverslips in 24-well plates at a density of 2 x 10^4 cells/well in complete DMEM. 16h later, HEK293T cells were transfected with 250ng pCAGGS-based constructs; MDCK cells were washed once with serum-free DMEM and infected with a MOI 3 A/Puerto Rico/8/34 or B/Memphis/12/97. 24h later, cells were fixed with 4% paraformaldehyde and permeabilized for 5 min using 0.05% Triton X-100 in PBS + 20 mM Glycine. After blocking for 1 h using a 1% (w/v) PBS-based BSA solution, cells were stained with rabbit anti-Flag (HEK293T cells) or goat anti-M1 serum (MDCK cells) and the indicated murine antisera or mAbs at the indicated dilution or concentration. Secondary staining was done using goat anti-rabbit Alexa-fluor-647 conjugated antibody and goat anti-mouse Alexa-fluor-488 antibody (Invitrogen) for HEK293T-stainings or donkey anti-rabbit Alexa-fluor-647 conjugated antibody and

donkey anti-mouse Alexa-fluor-488 antibody (Invitrogen) for MDCK stainings. Cells were mounted with Vectashield containing DAPI for nuclear counterstain before microscopic analysis.

Development of NBe-specific monoclonal antibodies (mAbs)

To obtain NBe-specific mAbs, mice were immunized three times three weeks apart with the NM_2H-protein (10µg) emulsified in Montanide ISA-720. Three week after the third immunization, mice were boosted a final time. Five days after this boost, splenocytes were isolated from the mice by gentle homogenization of the spleen in PBS, followed by red blood cell lysis using hypotonic ammonium chloride buffer. Next, cells were resuspended in RPMI + 0.5% FSC + penicillin/streptomycin and mixed with SP2/0-Ag14 myeloma cells in a ratio of 5:1 (splenocytes : myeloma cells). The cells were pelleted together (10min, 300g) and 1mL PEG-1500 (Roche) was added slowly under gentle mixing. Next, 10mL RPMI + 0.5% FSC + penicillin/streptomycin was added gently drop-by-drop to dilute the toxic PEG-1500. Fused cells were washed three times and resuspended in complete RPMI (RPMI + 10% FCS + L-Gln + sodium pyruvate + penicillin/streptomycin) containing 10% BM-Condimed-H1 (Roche). Cells were rested for 5h at 37°C and 5% CO_2, after which HAT selection (hypoxanthine – aminopterine – thymidine; Roche) was added and cells were seeded in 96well plates (200µL of 10^5 cells/mL). For the next two weeks, medium was replaced every 4-5 days and well were visually inspected for growth.

After a selection period of two weeks, growing clones showing production of NBe- or HBc-specific antibodies were transferred to sequential larger culture vessels (96well – 24well – 12well – 6well – 75cm^2 flask – 175cm^2 flask). For subcloning, cells were seeded at a density of 0.3cells/100µL in 96well plates, after which growth of subclones was monitored daily and productive and growing clones were expanded.

For purification of mAbs, a HiTrap recombinant Protein A MabSelect Sure column (GE Healthcare) was used according to the manufacturers protocol using an Akta purification station.

Pull-down experiments

For pull-down experiments, 50µL of cell lysate (corresponding to appr. 2x10^5 transfected cells) was incubated with 10µg of the indicated antibodies overnight in 500µL immunoprecipication (IP) buffer (10mM Tris/HCl pH7.4 + 150mM NaCl + 0.5% NP40 + 0.5% Triton X-100). 20µL of washed Protein G beads (GE Healthcare) was added to the antigen-antibody mixture and incubated for an additional three hours. Next, beads were collected, washed three times with IP buffer and treated with 50µL Laemmli buffer (50mM Tris pH6.8, 10% Glycerol, 100mM β-mercapto-ethanol). To analyze the unbound fraction, the 500µL solutions after precipitation were TCA-precipitated and resuspended in 50µL Laemmli buffer. For gel analysis, equivalent fractions were loaded, *i.e.* 25µL cell lysate, 25µL unbound fraction and 25µL eluted fraction.

3.4. Acknowledgements

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3.5. References

1. Belshe, R.B. The need for quadrivalent vaccine against seasonal influenza. *Vaccine* **28**, D45-D53 (2010).
2. Bandell, A., Woo, J. & Coelingh, K. Protective efficacy of live-attenuated influenza vaccine (multivalent, Ann Arbor strain): a literature review addressing interference. *Expert Review of Vaccines* **10**, 1131-1141 (2011).
3. Shaw, M.W., Choppin, P.W. & Lamb, R.A. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proceedings of the National Academy of Sciences* **80**, 4879 (1983).
4. Hatta, M. & Kawaoka, Y. The NB protein of influenza B virus is not necessary for virus replication in vitro. *Journal of virology* **77**, 6050 (2003).
5. Williams, M.A. & Lamb, R.A. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Molecular and Cellular Biology* **6**, 4317 (1986).
6. Williams, M.A. & Lamb, R.A. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Molecular and Cellular Biology* **8**, 1186 (1988).
7. Paterson, R.G., Takeda, M., Ohigashi, Y., Pinto, L.H. & Lamb, R.A. Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology* **306**, 7-17 (2003).
8. Mould, J.A*., et al.* Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. *Developmental cell* **5**, 175-184 (2003).
9. Watanabe, T., Watanabe, S., Ito, H., Kida, H. & Kawaoka, Y. Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. *Journal of virology* **75**, 5656 (2001).
10. Hatta, M., Goto, H. & Kawaoka, Y. Influenza B virus requires BM2 protein for replication. *Journal of virology* **78**, 5576 (2004).
11. Wang, Y*., et al.* Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity. *New Microbiologica* **33**, 311-317 (2010).
12. Graves, P.N., Schulman, J.L., Young, J.F. & Palese, P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology* **126**, 106-116 (1983).
13. Sagawa, H., Ohshima, A., Kato, I., Okuno, Y. & Isegawa, Y. The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region. *Journal of General Virology* **77**, 1483 (1996).
14. Steel, J*., et al.* Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain. *mBio* **1**, e00018 (2010).
15. Bommakanti, G*., et al.* Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. *Proceedings of the National Academy of Sciences* **107**, 13701 (2010).
16. White, J., Kartenbeck, J. & Helenius, A. Membrane fusion activity of influenza virus. *The EMBO journal* **1**, 217 (1982).
17. Chen, J*., et al.* Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* **95**, 409-417 (1998).
18. Bianchi, E*., et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
19. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science (New York, NY)* **262**, 1401 (1993).
20. De Filette, M*., et al.* An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. *Journal of Biological Chemistry* **283**, 11382 (2008).
21. Wang, J., Pielak, R.M., McClintock, M.A. & Chou, J.J. Solution structure and functional analysis of the influenza B proton channel. *Nature Structural & Molecular Biology* **16**, 1267-1271 (2009).
22. Clarke, B.E*., et al.* Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* **330**, 381-384 (1987).
23. Pumpens, P., Borisova, G.P., Crowther, R.A. & Grens, E. Hepatitis B virus core particles as epitope carriers. *Intervirology* **38**, 63-74 (1995).
24. Pumpens, P. & Grens, E. HBV core particles as a carrier for B cell/T cell epitopes. *Intervirology* **44**, 98-114 (2000).

25. Neirynck, S*., et al.* A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nature medicine* **5**, 1157-1163 (1999).
26. De Filette, M*., et al.* Universal influenza A vaccine: optimization of M2-based constructs. *Virology* **337**, 149-161 (2005).
27. De Filette, M*., et al.* The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. *Vaccine* **24**, 544-551 (2006).
28. De Filette, M*., et al.* Universal influenza A M2e-HBc vaccine protects against disease even in the presence of pre-existing anti-HBc antibodies. *Vaccine* **26**, 6503-6507 (2008).
29. Wingfield, P.T., Stahl, S.J., Williams, R.W. & Steven, A.C. Hepatitis Core Antigen Produced in Escherichia coli: Subunit Composition, Conformation Analysis, and in Vitro Capsid Assembly. *Biochemistry* **34**, 4919-4932 (1995).
30. Riedl, P*., et al.* Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. *The Journal of Immunology* **168**, 4951 (2002).
31. Crowther, R.A*., et al.* Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell* **77**, 943-950 (1994).
32. Nassal, M., Rieger, A. & Steinau, O. Topological analysis of the hepatitis B virus core particle by cysteine-cysteine cross-linking. *Journal of Molecular Biology* **225**, 1013-1025 (1992).
33. Böttcher, B., Wynne, S.A. & Crowther, R.A. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* **386**, 88 (1997).
34. Remaut, E., Stanssens, P. & Fiers, W. Plasmid vectors for high-efficiency expression controlled by the PL promoter of coliphage lambda. *Gene* **15**, 81-93 (1981).
35. Vervecken, W*., et al.* In vivo synthesis of mammalian-like, hybrid-type N-glycans in Pichia pastoris. *Applied and environmental microbiology* **70**, 2639 (2004).
36. Jacobs, P.P*., et al.* Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate. *Microbial Cell Factories* **9**, 93 (2010).
37. Bryan, J.K. Molecular weights of protein multimers from polyacrylamide gel electrophoresis. *Analytical Biochemistry* **78**, 513-519 (1977).
38. Davis, B.J. Disc Electrophoresis–II Method and Application to Human Serum Proteins. *Annals of the New York Academy of Sciences* **121**, 404-427 (1964).
39. Callewaert, N., Geysens, S., Molemans, F. & Contreras, R. Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment. *Glycobiology* **11**, 275 (2001).
40. Mertens, N., Remaut, E. & Fiers, W. Versatile, multi-featured plasmids for high-level expression of heterologous genes in Escherichia coli: overproduction of human and murine cytokines. *Gene* **164**, 9-15 (1995).
41. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* **262**, 1401 (1993).

Chapter 4

In vivo validation of cross-reactive IBV vaccine candidates

4.1. Introduction

In the previous chapter, the design and molecular characterization of broadly reactive IBV virus (IBV) vaccine candidates was described. Based on the combination of selected conserved IBV epitopes and carrier proteins, the rational design of these proteins is presumed to be efficient in eliciting immune responses against the presented epitopes. To evaluate this, and to assess the protective efficacy of elicited responses, mouse experiments were performed. These are based on a mouse model of IBV infection, a direct translation of that for influenza A virus infection. The immunogenic and protective capacity of HBc- and tGCN4-based broadly reactive IBV vaccines was assessed, using combinations with different adjuvants and challenge with different influenza B viruses. The merit of the selected epitopes derived from IBV, as construed from our mouse model, is discussed.

4.2. Results

4.2.1 The IBV mouse model

Compared to the influenza A virus (IAV) mouse model, this small animal model for IBV is more constrained because of the humanized phenotype of IBV [1,2]. In general, to obtain an influenza virus strain that replicates productively in the mouse lung and induces morbidity and mortality, primary human isolates are passaged in mice. For this, mice are infected with a virus suspension, and three days later the lungs are isolated, homogenized and resuspended. The soluble fraction will contain viruses selected on their ability to replicate successfully in the mouse lung environment. This selection procedure is repeated numerous times (in general, 5-30 times), until morbidity and mortality are observed. For most selected strains, this process is biphasic. In initial passages, the viral titer recovered from the lung homogenates drops drastically, through elimination of most (>99%) of the original viruses which are unable to replicate in the mouse lung environment. After initial passages, lung virus titer will typically incline again, through productive infection and replication of selected 'adapted' viruses [3,4].

Mouse-adaptation of in-house available IBV strains (B/Harbin/07/94, B/Yamagata/16/88, B/Lee/40) was attempted several times in this research. However, after initial passages (1-10 rounds of infection), no virus was recovered from lung homogenates, as determined by titration on MDCK cells (data not shown). Even in settings with immunosuppression, exogenously induced by γ-irradiation of mice or use of inhibitors of sugar-binding proteins present in the lung, such as the polysaccharide mannan [5,6], no successful recovery of virus was achieved. Therefore, research groups with published use of mouse-adapted IBV strains were contacted to obtain validated strains for *in vivo* use. A first viral strain obtained this way was B/HongKong/5/72, described by the group of Dr. Robert Sidwell (Utah State University, Logan, UT, USA) [7]. A second strain was kindly provided by Dr. Jonathan McCullers (St. Jude Children's Research Hospital, Memphis, TN, USA), and was prepared specifically during research into mouse-adaptation of IBV [8]. In our Balb/c mouse model, the LD_{50}-dose for B/Memphis/12/97 is appr. $1.25x10^6$ pfu and $8x10^6$ pfu for B/Hong Kong/5/72. A third strain used for initial (failed) mouse-adaptation and vaccine strain as inactivated virus is B/Harbin/7/94.

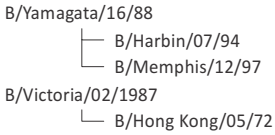
Figure 4.1 shows antigenic analysis of the IBV strains used here, compared to the reference strains of both lineages, *i.e.* B/Victoria/2/87 and B/Yamagata/16/88. Alignments were done using sequences available in the Influenza Sequence Database (www.fludb.org). Based on hemagglutinin (HA) sequences (nucleotides 481-501) [9], viruses can be attributed to one of both lineages. This way, the B/Harbin/7/94 and B/Memphis/12/97 can be identified as Yamagata-type viruses, while the B/Hong Kong/5/72 virus belongs to the Victoria-lineage (Fig. 4.1B).

Certain regions within the HA structure correspond to the antigenic sites responsible for neutralizing anti-HA responses and escape from homotypic immunity by mutation. Four major antigenic regions have been identified within the HA1-region, all in close proximity of each other, thus forming one continuous antigenic site [10]. These have been termed according to their location and structure: the 120 loop (residues 70-77 and 115-130), the 150 loop (residues 141-153), the 160 loop (residues 161-169) and the 190 helix (residues 192-202). Mutation of even a single residue in one of these sites, naturally or by reverse genetics, has been shown to abolish binding of monoclonal antibodies or antisera, and endow the naturally mutated strains with epidemic power [10-14]. The protruding 160 loop is specifically prone to insertion-deletion mutations, altering the antigenic site drastically [12,15]. The 190 helix contains a site (residues 194-196) allowing removal or introduction of an N-glycosylation site, influencing the epidemic and antigenic features of the virus [16,17]. As can be deduced from Figure 4.1C, a number of residues are specific for either lineage of IBV [10].

A

Strain	nucleotide sequence (HA)	amino acid sequence (HA)
B/Yamagata/16/88	GTTACCAGTAGAAACGGATTT	VTSRNGF
B/Harbin/07/94	GCTACCAGTAGAAGCGGATTT	ATSRSGF
B/Memphis/12/97	GCTACCAGTAGAAGCGGATTT	ATSRSGF
B/Victoria/02/1987	GTTACCAATGGAAACGGATTT	VTNGNGF
B/Hong Kong/05/72	GTTACCAATGGAAACGGATTT	VTNGNGF

B



C

Strain	120 loop (70-130)	150 loop (144-153)	160 loop (161-169)	190 helix (192-202)
B/Yamagata/16/88	PMCMGTIP...ENIRLSTHNVINAERA	PNVTSRNGFF	PR-D-NKTA	SDDKTQMKNLY
B/Harbin/07/94	PMCVGTTP...ENIRLSTQNVINAEKA	PNATSRSGFF	PR-DDNKTA	SDNKAQMKNLY
B/Memphis/12/97	PMCVGVTP...EKIRLSTQNVINAEKA	PNATSRSGFF	PR-DNNKTA	SDNKTQMKNLY
B/Victoria/02/87	PKCTGTIP...EHIRLSTHNVINAETA	PNVTNGNGFF	PKNDNNKTA	SDNEAQMVKLY
B/Hong Kong/05/72	PKCMGTIP...ENIRLSARNVINAETA	PNVTNGNGFF	PK---NKTA	SDNEIQMVKLY

D

Strain	B/HA0 sequence	NBe sequence	BM2e sequence
B/Yamagata/16/88	PAKLLKERGFFGAIAGFLE	MNNATFNYTNVNPISHIR	MLEPFQIL
B/Harbin/07/94	PAKLLKERGFFGAIAGFLE	MNNATFNYTNVNPISHIR	MLEPFQIL
B/Memphis/12/97	PAKLLKERGFFGAIAGFLE	MNNATFNYTNVNPIPHIR	MLEPFQIL
B/Victoria/02/87	PAKLLKEKGFFGAIAGFLE	MNNATFNYTNVNPISHIR	MLEPFQIL
B/Hong Kong/05/72	PAKLLKERGFFGAIAGFLE	MNNATFNCTNINPITHIR	MLEPFQIL

Figure 4.1. Classification and epitope sequences of the influenza B viruses used in this study. A-B. IBV used in this study (non-mouse-adapted B/Harbin/07/94 and mouse-adapted B/Memphis/12/97 and B/Hong Kong/05/72) can be attributed to one of the two lineages of IBV (Yamagata and Victoria lineage, with their prototypical strains B/Yamagata/16/88 and B/Victoria/02/72, respectively) according to specific antigenic sites. Shown in (A) are nucleotides 481-501 of the hemagglutinin (HA) gene with corresponding amino acid sequence. Based on these sequences, the B/Harbin/07/94 and B/Memphis/12/97 viruses can be assigned to the Yamagata-lineage and B/Hong Kong/05/72 to the Victoria-lineage (B). **C**. Amino acid sequences of antigenic determinants in HA1 for the viruses mentioned in (A). Residues distinctive for viruses of either lineage are shown bold in red. Boxed residues represent the presence of a N-glycosylation site altering the antigenicity of the virus. **D**. Amino acid sequences of selected conserved epitopes of IBV for the viruses mentioned in (A). Residues deviating from consensus sequences used in the construction of broad vaccine candidates are shown bold in red.

Alignment of the selected regions used for vaccine design shows complete conservation of the B/HA0- and BM2e-sequences (Fig. 4.1D). The NBe-sequence however deviates from the consensus-sequences for both mouse-adapted strains used. The B/Memphis/12/97 NBe-sequence differs at only one membrane-proximal residue (S15P). Therefore, it is unlikely that this variation will interfere greatly with potential antibody binding. For B/Hong Kong/5/72 however, three mutations can be observed, Y8C, V11I and S15T, spread over the sequence. These could potentially evade binding by NBe-specific antibodies. Sequencing of the NB open reading frame confirmed the presence of these mutations (data not shown).

4.2.2 Validation of broad HBc-based IBV vaccines

a) NBe-HBc fusions, 1646 and 1651

In a first experiment scrutinizing HBc-based vaccination to achieve immune protection against IBV infection, two recombinant NBe-HBc fusions, 1646 (with the NBe-sequence in the immunodominant loop of HBc) and 1651 (with NBe fused N-terminally to HBc), were used to immunize mice (see 3.2.1b) . As negative controls, unsubstituted HBc-particles were used alongside a buffer-only (PBS) treated group. As positive control, groups of mice were immunized with heat-inactivated IBV belonging to either lineage, *i.e*. B/Harbin/7/94 (Yamagata-lineage) and B/Hong Kong/5/72 (Victoria-lineage). Since the challenge virus was B/Hong Kong/5/72 in this set-up, the former inactivated virus-immunized group represents a heterologously vaccinated group, while the latter is a homologous (strain-matching) combination. Immunizations were given intraperitoneally (i.p.) using 10µg of protein (1µg for inactivated viruses) adjuvanted with LTR192G, a detoxified mutant of the heat-labile enterotoxin of *E. coli* as adjuvant [18]. Three immunizations were given, three weeks apart. The challenge with B/Hong Kong/05/72 virus was done twelve weeks after the third immunization.

Two weeks after each immunization, blood was drawn to evaluate the humoral responses in peptide- or protein-specific ELISA. Antibody titers specific for NBe-peptide, HBc-protein and the two inactivated viruses are shown in Figure 4.2. Mice clearly seroconvert towards NBe, with a slight Th2-skewing based on higher IgG1-specific titers. No anti-NBe serum IgG responses were observed following immunization with inactivated viruses. Immunization with 1646, carrying NBe in the immunodominant loop, elicited higher anti-NBe responses than 1651, with NBe N-terminal of HBc. Conversely, anti-HBc titers are higher in the 1651 immunized group compared to the 1646 group, indicating that a large part of HBc-specific antibodies are directed towards the aptly named immunodominant loop. Anti-virion ELISAs show that cross-reactive antibodies are induced, but homologous immune responses supersede heterologous responses by at least a factor ten.

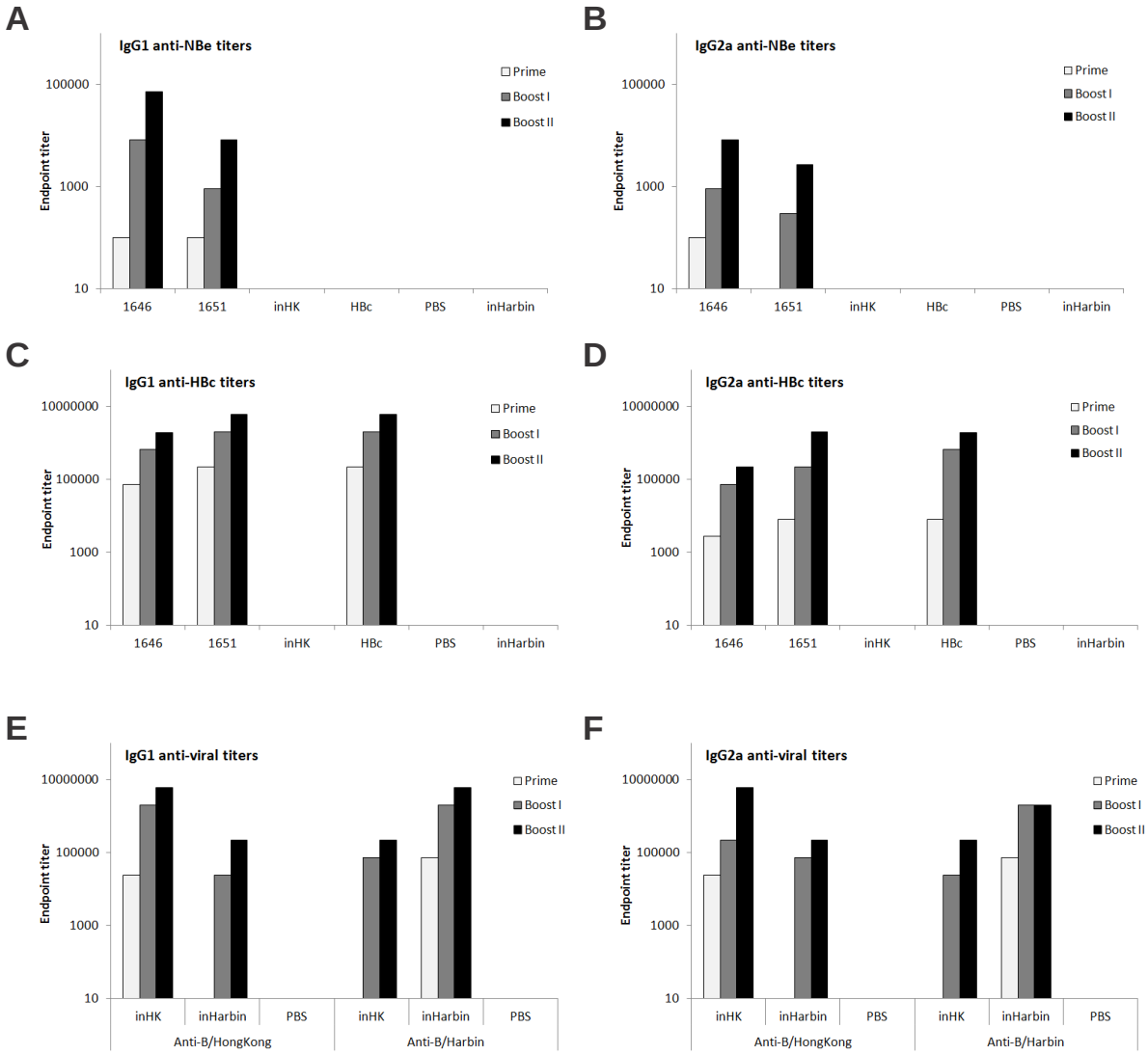


Figure 4.2. Serum antibody titers in vaccinated Balb/c mice . Groups of 9 (1646, 1651, HBc), 7 (PBS) or five (inHK, inHarbin) mice were immunized three times three weeks apart intraperitoneally with LTR192G-adjuvanted proteins (10µg) or inactivated B/Hong Kong/05/72 (inHK) or B/Harbin/07/94 (inHarbin) virus. Two weeks after each immunization, blood was drawn for serological analysis of antibody endpoint titers in pooled sera through ELISA. Shown are NBe-peptide specific (A, B), HBc-protein specific (C, D) and virus-specific (E, F) antibody titers. For the latter, ELISA plates were coated with inactivated B/Hong Kong/12/72 (B/HongKong) or inactivated B/Harbin/07/94 (B/Harbin) virus. Both IgG1-specific (A, C, E) and IgG2a-specific (B, D, F) titers were assessed.

Twelve weeks after the last immunization, mice were challenged with $4LD_{50}$ of the mouse-adapted IBV/Hong Kong/5/72 strain. The challenge results are shown in Figure 4.3. Clearly, immunization with inactivated viruses only provides protection in the homologous setting using inactivated B/Hong Kong/5/72 (inHK), leading to 100% survival and no signs of morbidity. Heterologous vaccination with inactivated B/Harbin/7/94 (inHarbin) offers no protection from mortality. Both negative control groups (HBc, PBS) also show no protection. Most interestingly, NBe-HBc vaccination can offer protection when the NBe-epitope is presented N-terminally on HBc (1651), saving 33% of mice from mortality. Statistically, inactivated B/Hong Kong/5/72 vaccination protects significantly compared to

all other groups (Kaplan-Meier analysis; p<0.05 compared to 1651, p<0.01 compared to inHarbin, p<0.001 compared to 1646, HBc, PBS). Protection by 1651 vaccination is statistically significant compared to 1646 immunization (p<0.05). Even with overall morbidity, measured by relative weight loss, being relatively high, it can be concluded that NBe-HBc based immunization using the 1651 particle can offer protection against a potential lethal IBV infection, even with a difference of three amino acids between the NBe-sequence of the vaccine and the challenge virus.

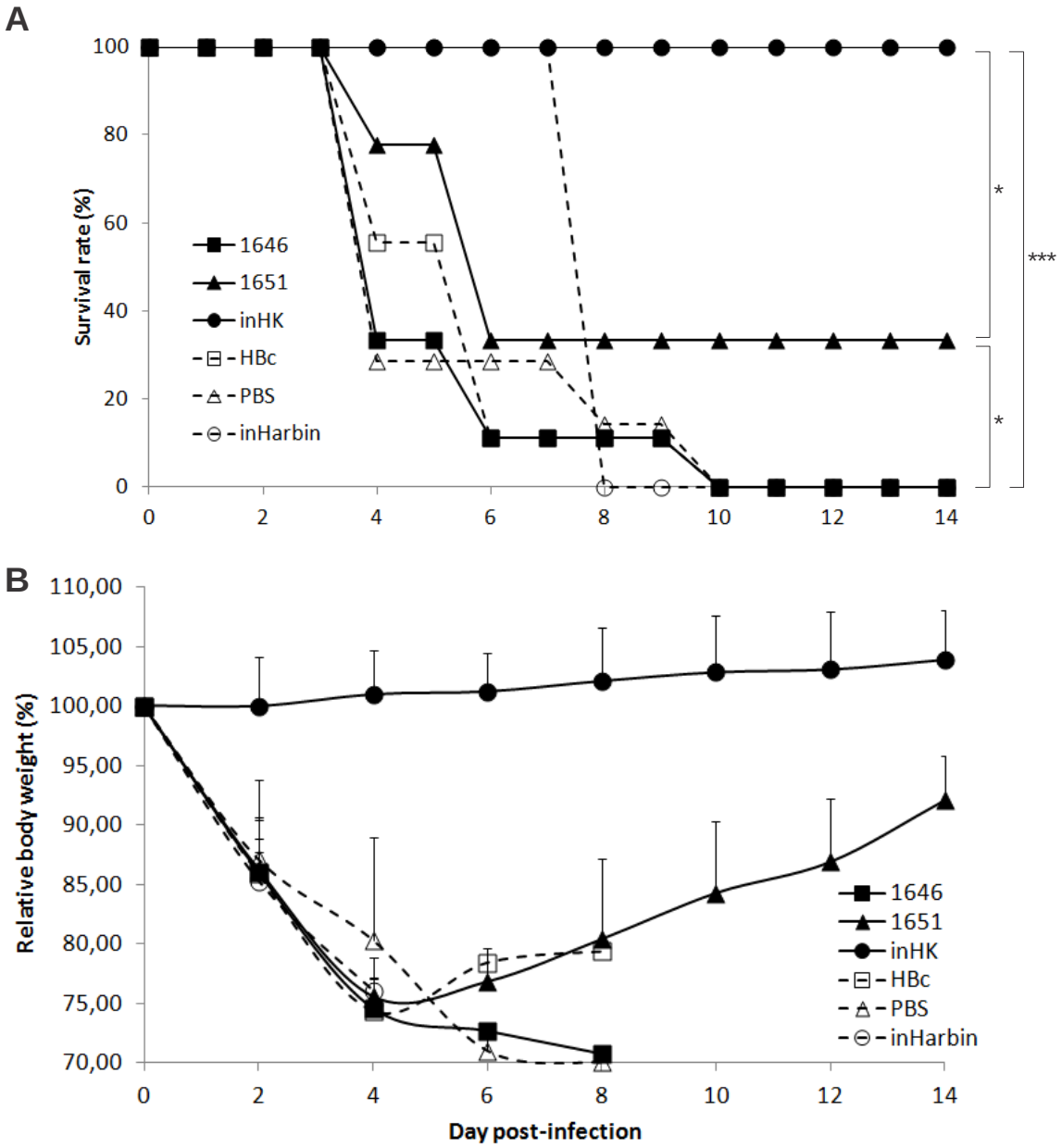


Figure 4.3. Protection in NBe-HBc (1646 and 1651) immunized mice against IBV/Hong Kong/05/72 challenge. Mice (n = 9, 1646, 1651, HBc; n = 7, PBS; n = 5, inHK, inHarbin) were vaccinated as described in Figure 4.2. Twelve weeks after the third immunization, mice were challenged with a potentially lethal dose ($4LD_{50}$) of IBV/Hong Kong/05/72. **A.** Relative survival after challenge. Kaplan-Meier analysis shows significant differences between inHK and all other immunized groups and between 1651 and 1646 immunized mice. *, p<0.05; ***, p<0.001. **B.** Relative body weight after challenge; group average +SD.

b) NBe-M2e$_2$-HBc (NM$_2$H) and B/HA0-HBc (3004) fusion

The third NBe-HBc fusion, based on coupling of the NBe-sequence N-terminally to a HBc-particle bearing two M2e-sequences (NBe-M2e$_2$-HBc, NM$_2$H), did not show particle formation in *in vitro* testing. However, because of its potential to provide heterotypic (*i.e.* against IAV and IBV) protection, the non-particulate protein was used in a challenge experiment using potentially lethal IAV and IBV infections. In a first set-up, groups of mice were immunized three times three weeks apart with the NM$_2$H hybrid, 1817 particle (M2e$_2$-HBc, the base on which NBe was grafted to construct NM$_2$H), unsubstituted HBc or buffer only (PBS). Immunizations were done with 10µg protein per dose, adjuvanted with Montanide ISA-720 [19], a metabolizable water-in-oil mixture used for subcutaneous immunizations. Three weeks after the last immunization, mice were challenged with mouse adapted X47 virus, a recombinant strain based on the internal genes of A/PuertoRico/8/34 and the hemagglutinin and neuraminidase of A/Victoria/3/75, or mouse-adapted IBV/Memphis/12/97. However, the latter challenge was unsuccessful because of low virulence of the IBV/Memphis/12/97 virus stock used at that time (data not shown); hence, only results for the influenza A part of the experiment are shown.

Two weeks after each immunization, and two weeks after the challenge, blood was drawn for preparation of sera and analysis of antibody responses in ELISA. Figure 4.4 shows IgG1- and IgG2a-specific antibody responses, as determined in NBe-peptide, M2e-peptide and HBc-protein ELISA. Clearly, despite lack of evidence for particle formation for NM$_2$H, mice immunized with this protein seroconverted for NBe, M2e and HBc. A minor difference compared to 1817 particle immunization however is the more Th2-skewed immune response the NM$_2$H protein evokes, with higher IgG1 titers and lower IgG2a titers raised against M2e and HBc. However, a role for the presence of the NBe-epitope on its own in eliciting this effect cannot be excluded.

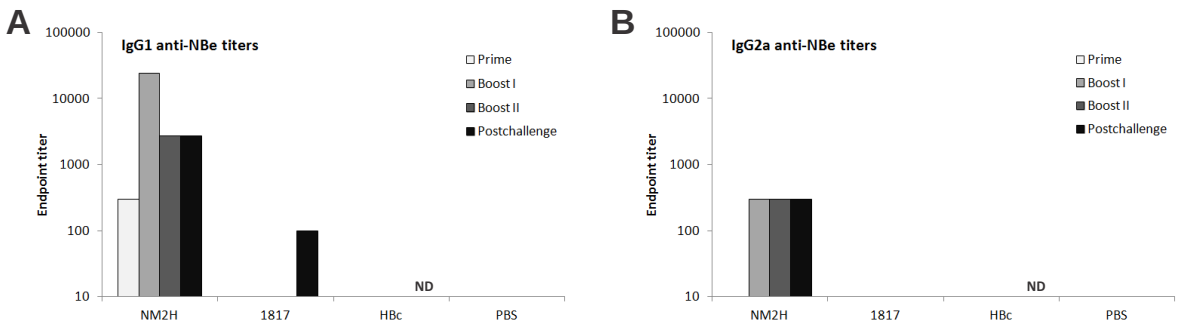


Figure 4.4. Antibody titers in mice vaccinated with different HBc-constructs and challenged with A/X47 virus. Mice (n = 9 for all groups) were immunized three times three weeks apart with 10µg NM$_2$H (NBe-M2e$_2$-HBc), 1817 (M2e$_2$-HBc), HBc or PBS emulsified in Montanide ISA-720 and injected subcutaneously. Three weeks after the last immunization, mice were challenged with a potentially lethal dose (4LD$_{50}$) of A/X47 virus. Two weeks after each immunization and after challenge blood was drawn from (surviving) mice for serological analysis of antibody endpoint titers in pooled sera by ELISA. Shown are NBe-peptide specific (A, B), M2e-peptide specific (C, D) and HBc-protein specific (E, F) antibody titers. Both IgG1-specific (A, C, E) and IgG2a-specific (B, D, F) titers were assessed. ND = not determined (no surviving mice).

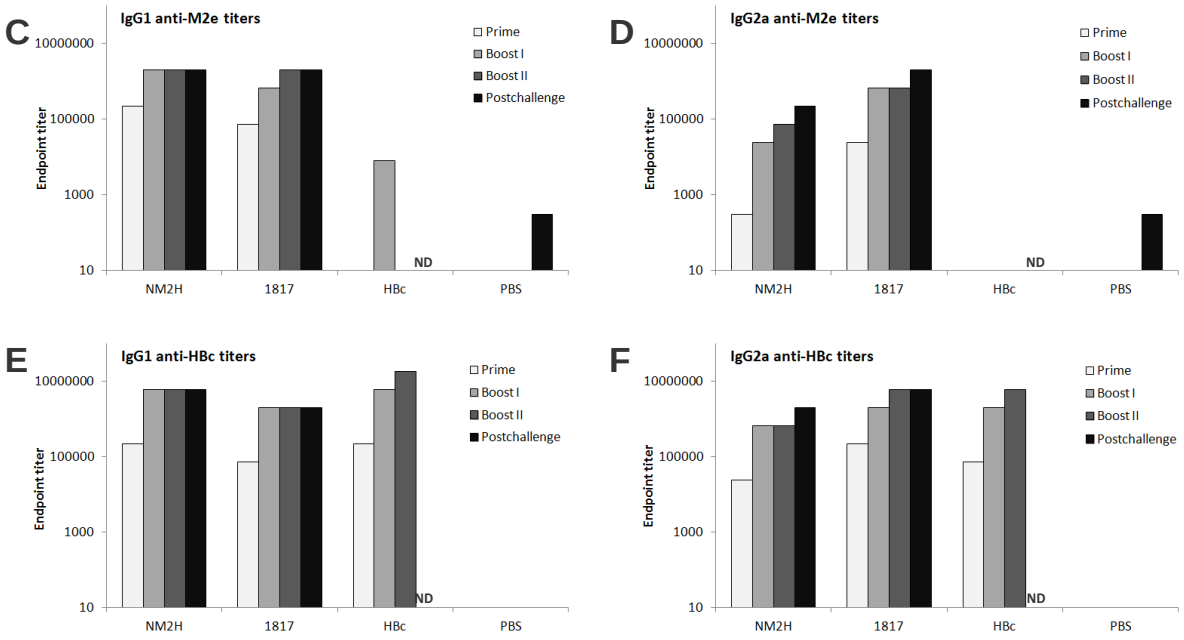


Figure 4.4. ***Continued.***

Challenge results after infection with X47 virus are shown in Figure 4.5. Using a potentially lethal viral dose of $4LD_{50}$, all HBc-immunized mice and almost 90% of buffer-treated mice succumbed to the infection. In contrast, immunization with NM_2H or 1817 fully protected mice from lethality and limit edmorbidity strongly, allowing recovery starting day 6 post-infection. Additionally, each group contained three immunized mice that were infected with a sublethal ($0.4LD_{50}$) influenza A/X47 dose. These mice were sacrificed day 6 post-challenge for preparation of lung homogenates and titration of lung virus titers. As depicted in Figure 4.5C, the two M2e-immune groups (NM_2H and 1817) contain significantly less virus in their lungs as do the negative control groups (HBc and PBS; one-way Anova, $p<0.001$). In conclusion, despite the lack of particle formation, the NM_2H protein is very immunogenic, with a slightly higher Th2-skewing than its particulate counterpart (1817), and is able to convey protection against a potentially lethal IAV infection to a similar extent as 1817.

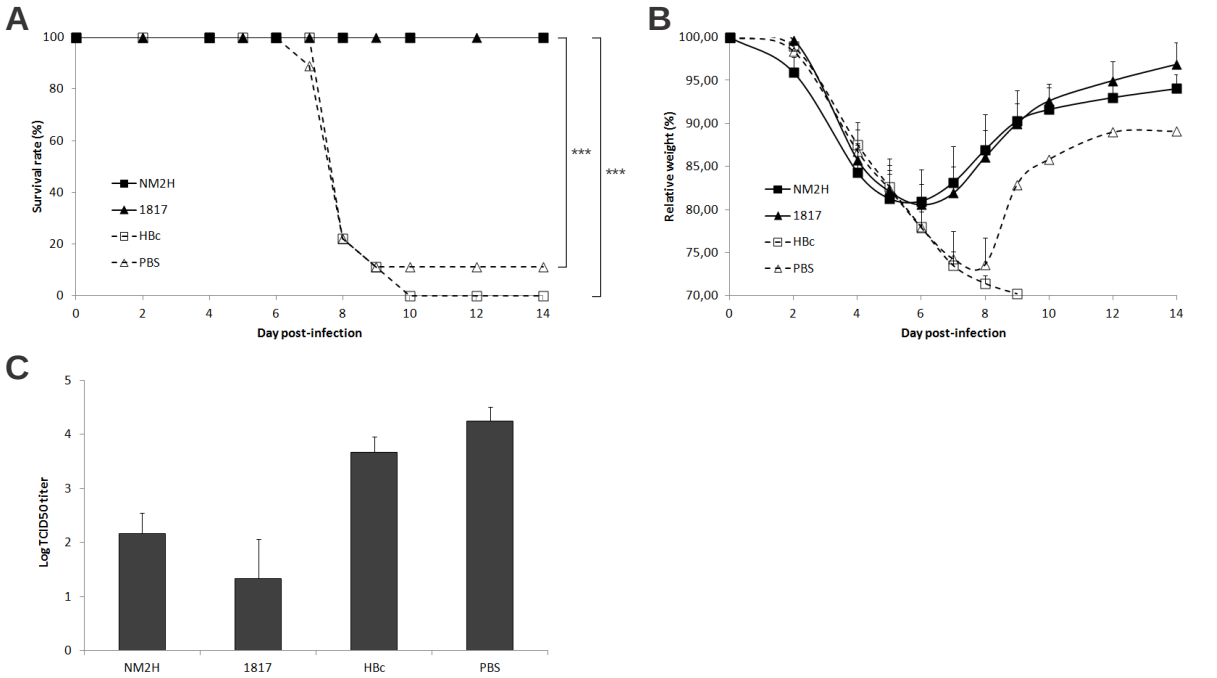


Figure 4.5. Protection of mice vaccinated with different HBc-constructs after a potential lethal influenza A/X47 virus challenge. Mice (n = 9 for all groups) were treated as described in Figure 4.4. Challenge results after a potential lethal infection ($4LD_{50}$) of A/X47 virus. **A.** Relative survival rate of challenged mice. Kaplan-Meier analysis: ***, $p<0.001$. **B.** Relative body weight of infected mice; group averages +SD. **C.** Three mice in each group were challenged with a sublethal influenza A/47 dose ($0.4LD_{50}$). Six days later, mice were sacrificed for preparation of lung homogenates and titration of the lung virus dose. Shown is the average $TCID_{50}$ titer (calculated log-value) ±SD.

To assess protection against IBV infection, the experiment was repeated. In this instance, mice were immunized with the NM_2H protein and the 3004 particle, displaying the B/HA0 cleavage sequence in the immunodominant loop of HBc. As negative controls, immunizations with unsubstituted HBc particles and buffer (PBS) were performed. As in the initial set-up, vaccinations were adjuvanted with Montanide ISA-720 and administered subcutaneously. Mice were immunized three times, three times apart, with 10µg of protein per dose (7.5µg for the 3004 particle). Using serum from blood samples taken two weeks after each immunization and after the challenge, IgG1 and IgG2a antibody titers directed against NBe, M2e, HBc and inactivated virions were determined in ELISA (Fig. 4.6). Antibody titers induced by the NM_2H protein against NBe, M2e and HBc appeared to be even higher in this experiment than in the formerly discussed set-up. Also here, Th2-skewing by this protein is evident when comparing anti-HBc titers induced by NM_2H and 3004. To detect B/HA0-specific responses induced by 3004, cellular ELISAs were carried out using fixed influenza A or B infected cell monolayers (Fig. 4.6G-H). NM_2H-specific antisera clearly react with IAV infected cells, corresponding with the presence of anti-M2e antibodies. However, no reaction is seen with IBV infected cells, despite the clear presence of anti-NBe antibodies in peptide ELISA. 3004-specific antisera on the other hand do react with IBV infected monolayers. With a lack of reaction with HBc-antisera, these data show the induction of B/HA0-specific antibodies which are able to bind infected cells. This finding was further confirmed by immunofluorescence staining of B/Memphis/12/97 infected cells with sera of mice taken after the second boost or the challenge (Fig. 4.7).

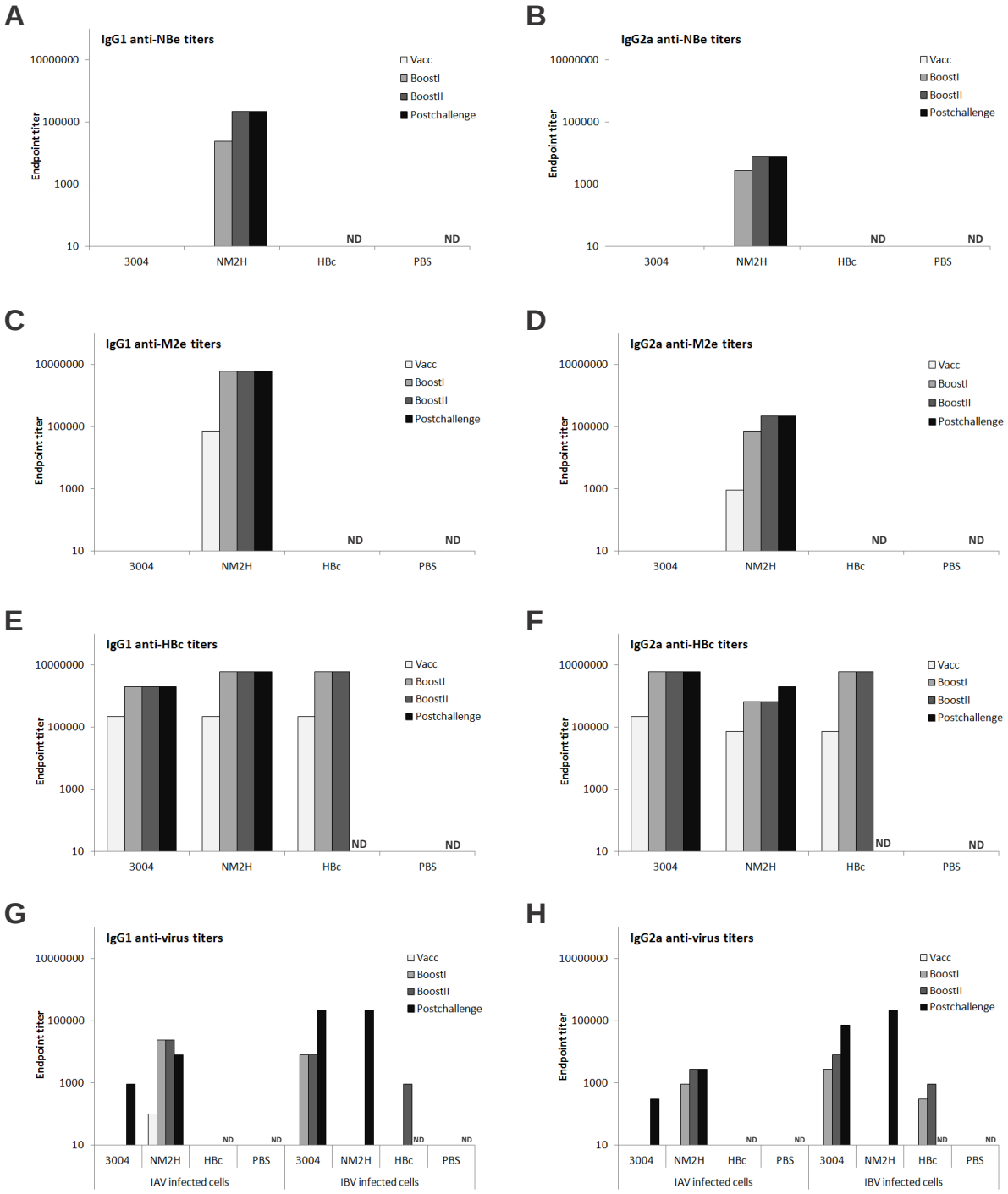


Figure 4.6. Antibody titers in mice vaccinated with different HBc-constructs and challenged with B/Memphis/12/97 virus. Mice (n = 12 for all groups) were immunized three times three weeks apart with 10µg NM_2H (NBe-M2e_2-HBc), 3004 (B/HA0-HBc), HBc or PBS emulsified in Montanide ISA-720 and injected subcutaneously. Three weeks after the last immunization, mice were challenged with a potential lethal dose ($4LD_{50}$) of B/Memphis/12/97 virus. Two weeks after each immunization and after challenge blood was drawn from (surviving) mice for serological analysis of antibody endpoint titers in pooled sera through ELISA. Shown are NBe-peptide specific (A, B), M2e-peptide specific (C, D), HBc-protein specific (E, F) and infected cell specific (G, H) antibody titers. For the latter, monolayers of MDCK cells were infected with influenza A/PuertoRico/8/34 or B/Memphis/12/97 virus (MOI 3), fixed after 24h of incubation and processed further using the standard ELISA protocol. Both IgG1-specific (A, C, E, G) and IgG2a-specific (B, D, F, H) titers were assessed. ND = not determined (no surviving mice).

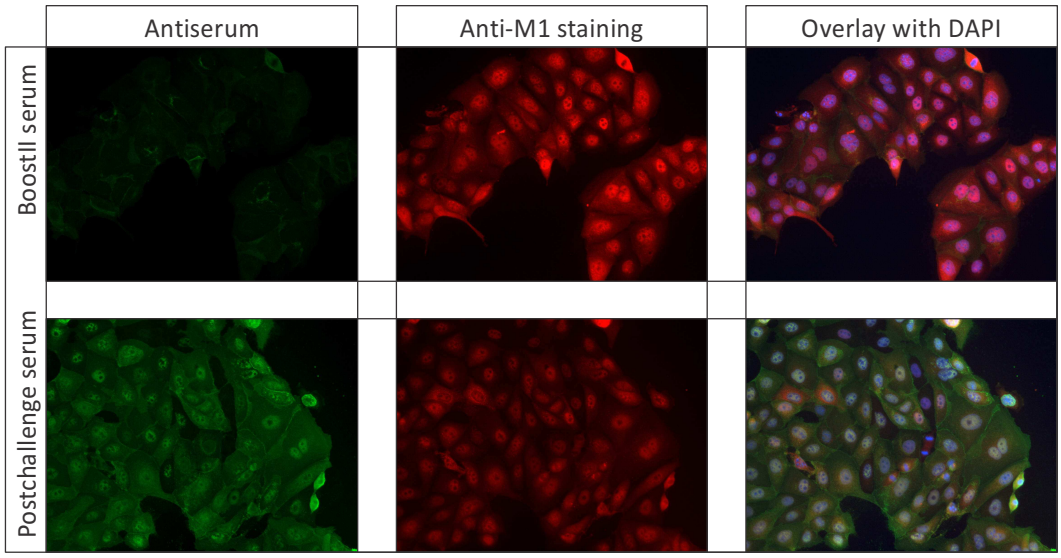


Figure 4.7. B/HA0 antiserum reacts with IBV infected MDCK cells in immunofluorescence staining. MDCK cells were infected with B/Memphis/12/97 virus (MOI 3) overnight, fixed and permeabilized. Cells were stained with mouse serum (1/300) taken after the second boost with 3004 particles or after the B/Memphis/12/97 challenge (green) and with goat anti-M1 antiserum (red). Representative pictures for each condition are shown. Control stainings using A/PuertoRico/8/34 or non-infected cells were negative.

Three weeks after the third immunization, mice were challenged with $4LD_{50}$ of mouse-adapted B/Memphis/12/97. All groups showed severe morbidity and high mortality rates (Fig. 4.8). With only 25% survival in the 3004 immunized group and 17% in the NM_2H group, no significant protection was seen (3004 vs HBc, p=0.064; NM2H vs HBc, p=0.060; Kaplan-Meier analysis). As a second read-out, viral replication in the lungs of infected mice was analyzed. To this end, two groups of three mice in each immunized group were challenged with $0.4LD_{50}$ B/Memphis/12/97 and sacrificed three and four days after challenge. This shorter infection period as compared to IAV infections was used to encompass the ideal timing to detect replication in the lungs of infected mice (data not shown). As depicted in Figure 4.8C, titers are significantly higher on day 3 in the PBS group ($p<0.05$; one-way Anova) but do not differ significantly on day 4 ($p=0.076$; one-way Anova). Looking at the differences between pulmonary virus levels on day 3 and day 4 post-challenge, the decline is only significant for the 3004 particle immunized and PBS-only treated mice ($p<0.01$, two-sided t-test). Overall, despite the induction of NBe-peptide reactive antibodies in the NM_2H-immunized group and B/HA0-specific antibodies reactive with infected cells in the 3004-vaccinated mouse group, no clear protective effect of either vaccination strategy against a potentially lethal IBV infection can be seen, although anti-B/HA0 antibodies inhibit viral replication slightly.

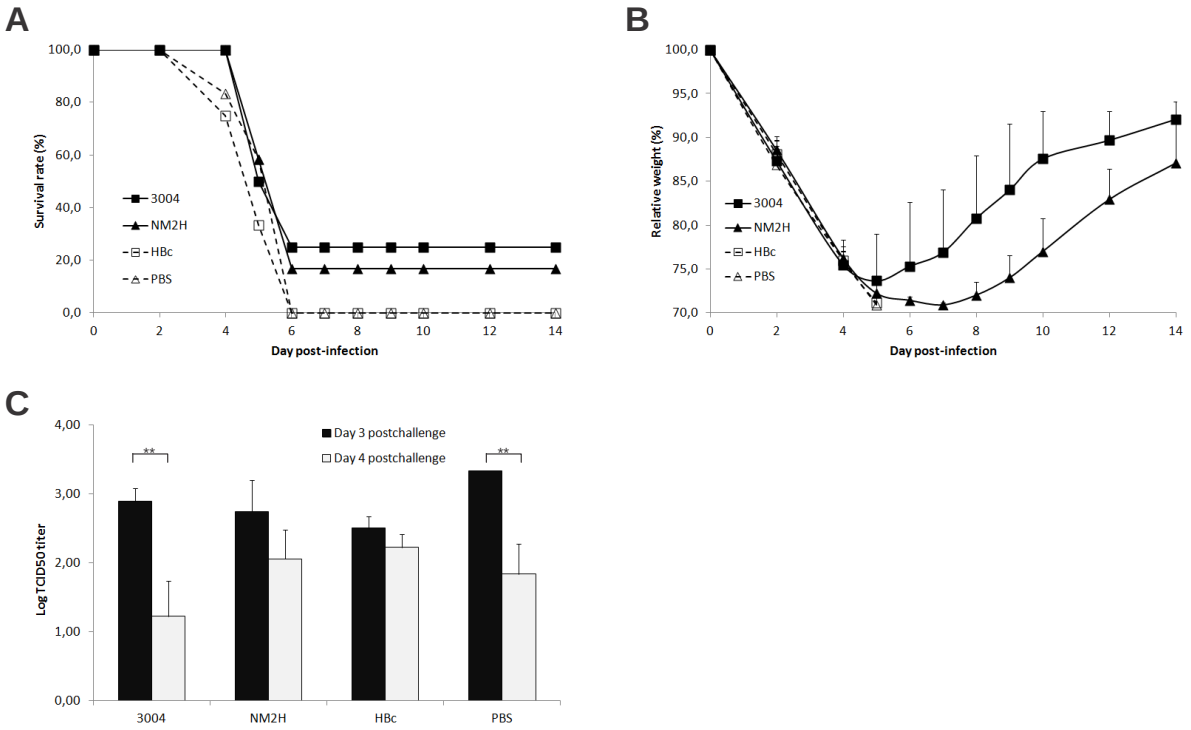


Figure 4.8. Protection of mice vaccinated with different HBc-constructs after a potentially lethal infection with B/Memphis/12/97 virus. Mice (n = 12 for all groups) were treated as described in Figure 4.6. Challenge results after a potentially lethal infection ($4LD_{50}$) with B/Memphis/12/97 virus. **A.** Relative survival rate of challenged mice. **B.** Relative body weight of infected mice; group averages +SD. **C.** Two groups of three mice in each group were challenged with a sublethal IBV/Memphis/12/97 dose ($0.4LD_{50}$). Three and four days later, mice were sacrificed for preparation of lung homogenates and titration of the lung virus dose. Shown is the average $TCID_{50}$ titer (calculated log-value) ±SD. **, $p<0.01$.

4.2.3 Protection provided by tGCN4-based broad IBV vaccine candidates

The second carrier protein used to display conserved IBV regions in a potentially immunogenic manner is tGCN4, the tetramerizing leucine zipper of the GCN4 transcription factor of yeast [20,21]. In a first *in vivo* experiment, immunogenicity and protective capacity against B/Hong Kong/5/72 infection of BM2e-tGCN4 and NBe-tGCN4-Flag was assessed. As negative control, adjuvanted buffer-only (PBS) immunizations were included. An additional group received inactivated B/Memphis/1/97 virus, to assess heterologous protection provided by this strain belonging to the Yamagata-lineage against the Victoria-like B/Hong Kong/5/72. Two different adjuvants were used for all treatments: Montanide ISA-720 and Alhydrogel, an aluminum salt based adjuvant. Antibody titers two weeks after each immunization and after the challenge are shown in Figure 4.9. In general, the tGCN4-carrier appears to be highly immunogenic in Balb/c mice (Fig. 4.9A, B), giving endpoint titers of over 10^7 after three immunizations. Immune responses are slightly Th1-skewed, with higher IgG1-specific titers than IgG2a-titers. Montanide ISA-720 adjuvanted proteins elicit higher antibody responses than Alhydrogel adjuvantation. Clear anti-NBe responses can be seen in peptide ELISA (Fig. 4.9C, D). Serum

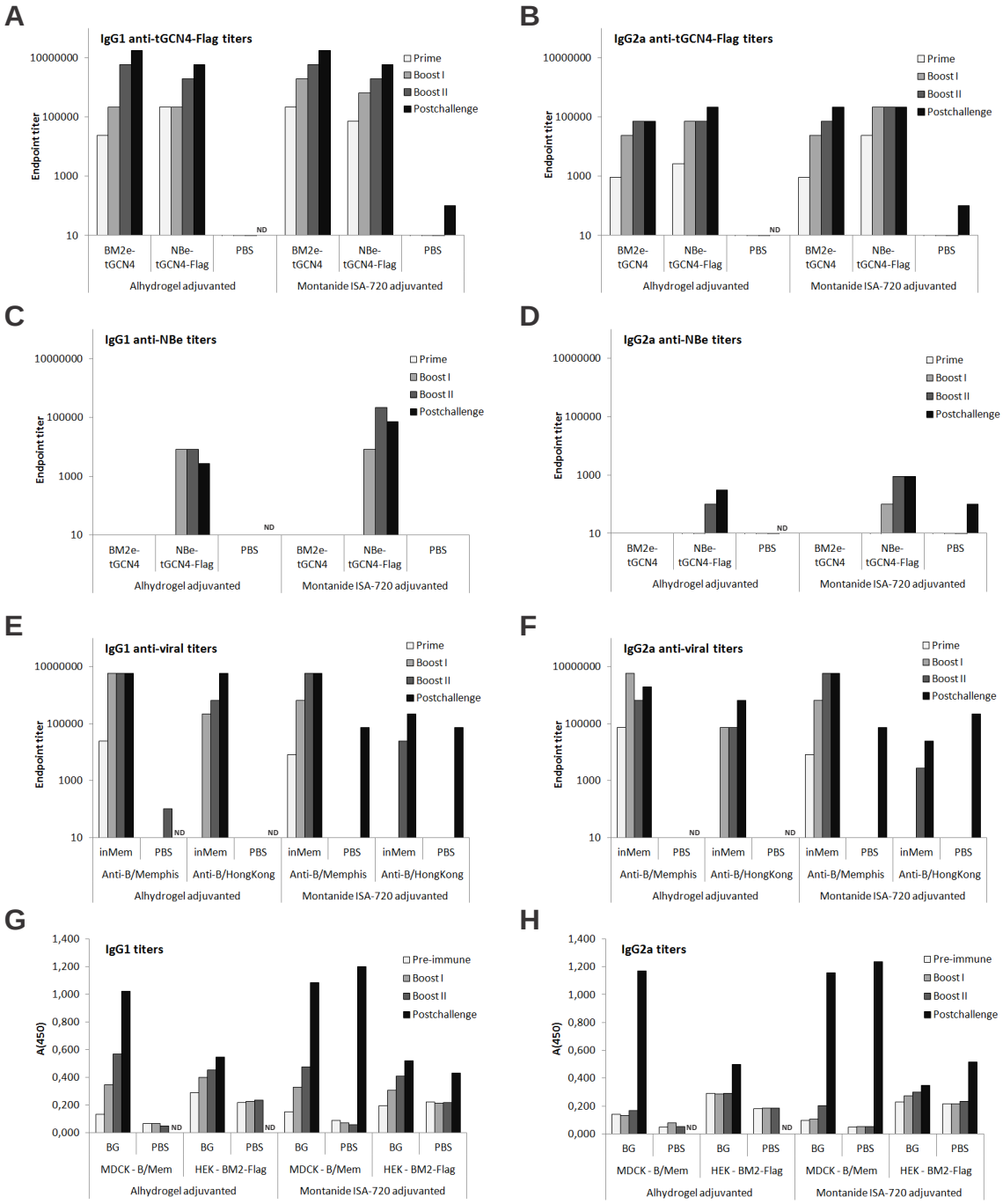


Figure 4.9. Antibody titers in mice vaccinated with different tGCN4-constructs and challenged with B/Hong Kong/5/72 virus. Groups of mice were immunized three times three weeks apart with 10µg BM2e-tGCN4 or NBe-tGCN4-Flag (n = 9), 1µg heat-inactivated B/Memphis/12/97 (n = 5) or PBS (n = 9), adsorbed to Alhydrogel and injected ip. or emulsified in Montanide ISA-720 and injected sc. Three weeks after the last immunization, mice were challenged with a potentially lethal dose ($4LD_{50}$) of B/Hong Kong/5/72. Two weeks after each immunization and after challenge blood was drawn from (surviving) mice for serological analysis of antibody endpoint titers in pooled sera by ELISA. Shown are tGCN4-Flag specific (A, B) and NBe-peptide specific (C, D) specific titers. For determination of virus-specific (E, F) antibody titers, ELISA plates were coated with 25ng/well inactivated B/Hong Kong/5/72 or B/Memphis/12/97 virus. To detect anti-BM2e reactivity, cellular ELISAs were performed using fixed monolayers of B/Memphis/12/97 infected MDCK cells (MOI 3; MDCK-B/Mem) or HEK cells transfected with a BM2-Flag expressing construct (HEK – BM2-Flag). Shown is the absorbance at 450nm for pooled sera of mice immunized with BM2e-tGCN4 (BG) or PBS. Both IgG1-specific (A, C, E, G) and IgG2a-specific (B, D, F, H) titers were assessed. ND = not determined (no surviving mice).

from mice immunized with inactivated B/Memphis/12/97 was tested in ELISA using plates coated with heat-inactivated B/Memphis/12/97 and B/Hong Kong/5/72 as antigens. Prechallenge sera clearly react stronger with B/Memphis/12/97 virus, but do show cross-reactivity with B/Hong Kong/5/72 virus (Fig. 4.9E, F). To detect anti-BM2e antibody responses, cellular ELISAs were performed using B/Memphis/12/97 infected MDCK cells or BM2-Flag transfected HEK cells (Fig. 4.9G, H). Relatively high background levels did not allow clear determination of anti-BM2e endpoint titers, but absorbance levels measured in ELISA do show an increasing reactivity with the BM2-expressing cells with increasing numbers of immunizations. Even more, after challenge, a boost-effect can be observed, as indicated by higher reactivity with BM2-Flag expressing HEK cells. Similar set-ups using serum from NBe-tGCN4-Flag immunized mice did not show any specific reactivity or boost-effect (data not shown).

Morbidity and mortality after a potentially lethal ($4LD_{50}$) IBV/Hong Kong/5/72 challenge is illustrated in Figure 4.10. Mice immunized with Alhydrogel adjuvanted BM2e-tGCN4 protein were significantly protected against mortality compared to the PBS group ($p<0.05$; Kaplan-Meier analysis). Immunization with inactivated B/Memphis/12/97 does not induce protective immunity against infection with the distinct B/Hong Kong/5/72 virus, despite the presence of cross-reactive antibodies as detected in the anti-virion ELISAs (Fig. 4.9E, F). Again, this indicates lack of cross-lineage protection, as observed before (Fig. 4.3). Morbidity, measured by relative body weight, was significantly reduced in BM2e-tGCN4 mice on days 8 and 9 post-infection compared to the three other groups (Fig. 4.10B).

Subcutaneous immunizations with Montanide ISA-720 as adjuvant gives a different result (Fig. 4.10C, D). In this set-up, NBe-tGCN4-Flag immunization can provide significant protection compared to PBS immunization ($p<0.05$; Kaplan-Meier analysis), whereas BM2e-tGCN4 gives the same survival rate as in combination with Alhydrogel (33%). Also here, no cross-lineage protection provided by inactivated B/Memphis/12/97 immunization is detected, and morbidity is similar in all groups (Fig. 4.10D).

In each group of mice, three additional mice were infected with a sublethal dose ($0.4LD_{50}$) of B/Hong Kong/5/72 for analysis of viral replication in the lung three days after the challenge (Fig. 4.10E, F). Irrespective of the adjuvant used, NBe- or BM2e-based tGCN4-protein immunization led to a significant drop in pulmonary virus titer compared to PBS-treated mice ($p<0.05$, one-way Anova). Noteworthy, immunization with inactivated B/Memphis/12/97 did not induce any inhibitory effect on viral replication, reflecting the morbidity and mortality results.

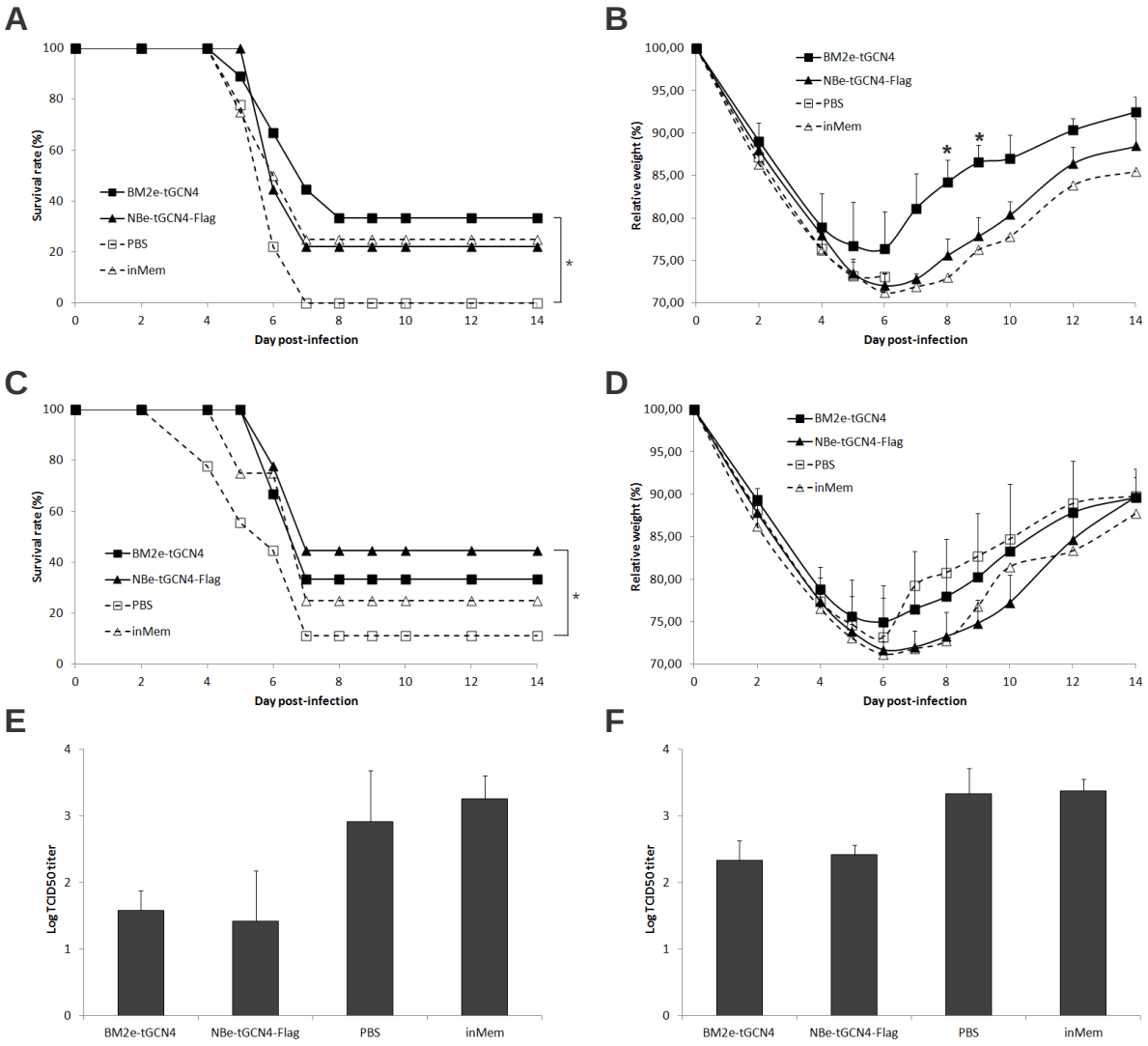


Figure 4.10. Protection of mice vaccinated with different tGCN4-constructs after a potentially lethal infection with B/Hong Kong/5/72 virus. Mice (n = 9 for all groups except inMem, n = 5) were treated as described in Figure 4.8. **A-D.** Challenge results after a potentially lethal infection ($4LD_{50}$) with B/Memphis/12/97 virus. Shown are the relative survival rate (A) and relative body weight of mice immunized with Alhydrogel-adjuvanted vaccines after challenge (B), and the relative survival rate (C) and relative body weight of mice immunized with Montanide ISA-720 adjuvanted vaccines after challenge (D). For survival, significant differences as determined by Kaplan-Maier analysis are indicated; *, $p<0.05$. For relative body weights, group averages are shown +SD; *, $p<0.05$ (one-way Anova). **E-F.** Two groups of three mice in each group were challenged with a sublethal IBV/Memphis/12/97 dose ($0.4LD_{50}$). Three days later, mice were sacrificed for preparation of lung homogenates and titration of the lung virus dose. Shown is the average $TCID_{50}$ titer (calculated log-value) ±SD.

In a follow-up experiment, BM2e(QIL)-tGCN4 and *P. pastoris* expressed gNBe-tGCN4 were used alongside BM2e-tGCN4, NBe-tGCN4-Flag, and M2e-tGCN4-Flag and PBS buffer as negative controls. Inactivated B/Memphis/12/97 virus was used again as immunogen, this time to assess induction of homologous protection against a potentially lethal B/Memphis/12/97 challenge. Combined with Montanide ISA-720, induction of antibody responses against the tGCN4-carrier, BM2-protein and NBe-peptide were similar as in the previous experiment (data not shown). Additionally,

immunofluorescence stainings of influenza infected MDCK cells were carried out to assess recognition of viral proteins by immune sera. Representative pictures are shown in Figure 4.11. Sera raised with BM2e- and NBe-based chimeric proteins react specifically with IBV infected cells, albeit with low intensity, as does M2e-tGCN4 serum with IAV infected cells. Strong staining is achieved with antiviral serum raised with inactivated B/Memphis/12/97, as would be expected.

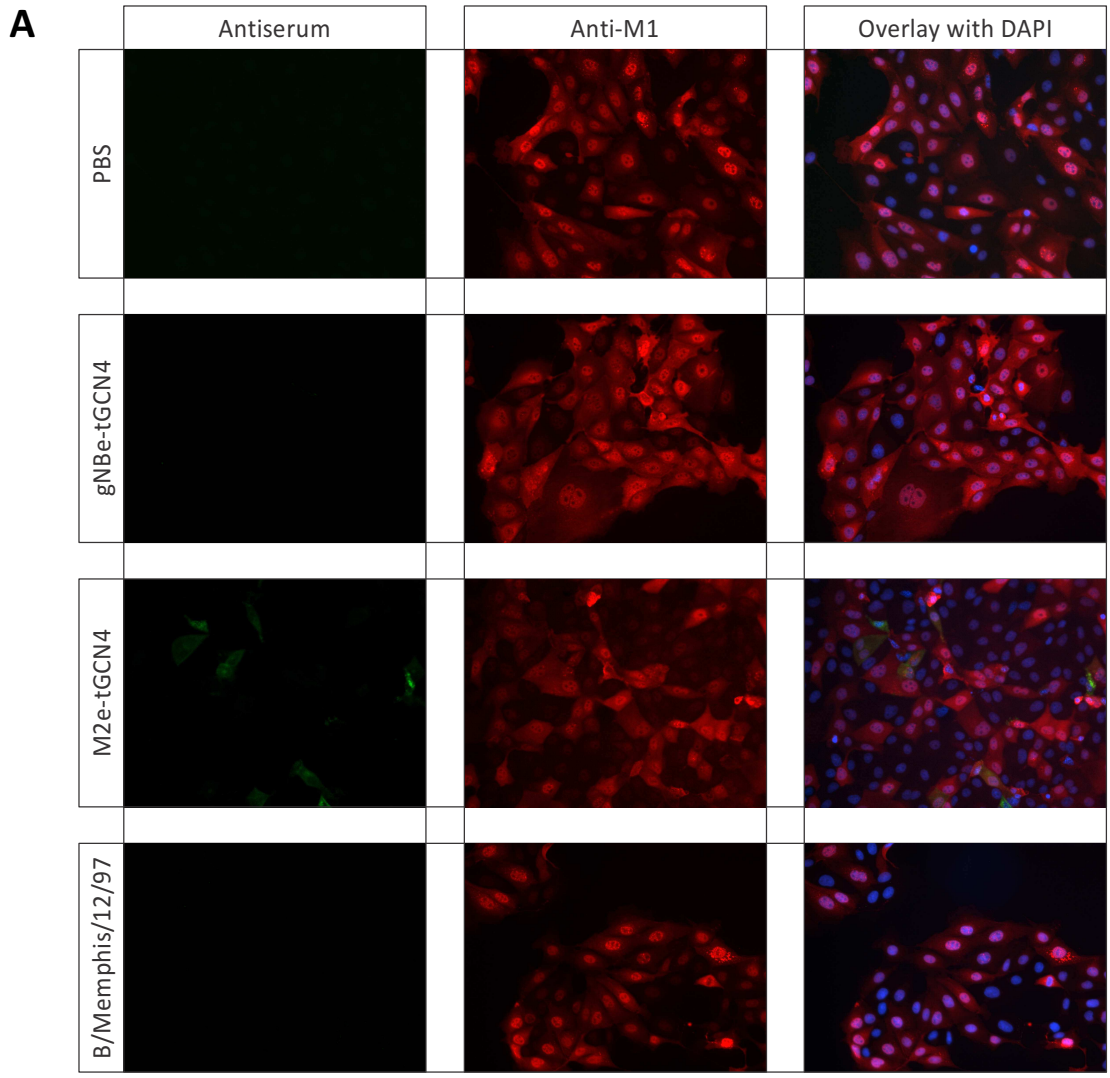


Figure 4.11. Immunofluorescence staining of IBV infected MDCK cells shows specific reactivity with tGCN4-protein raised mouse serum. MDCK cells were infected with A/PuertoRico/8/34 (MOI 3; A) or B/Memphis/12/97 (MOI 3; B) overnight, fixed and permeabilized. Staining was done with immune sera, taken after the third immunization, raised with the indicated immunogens (1/300 dilution) and with goat anti-IBV M1 antiserum. Representative pictures for each condition are shown. Control stainings using non-infected cells were negative.

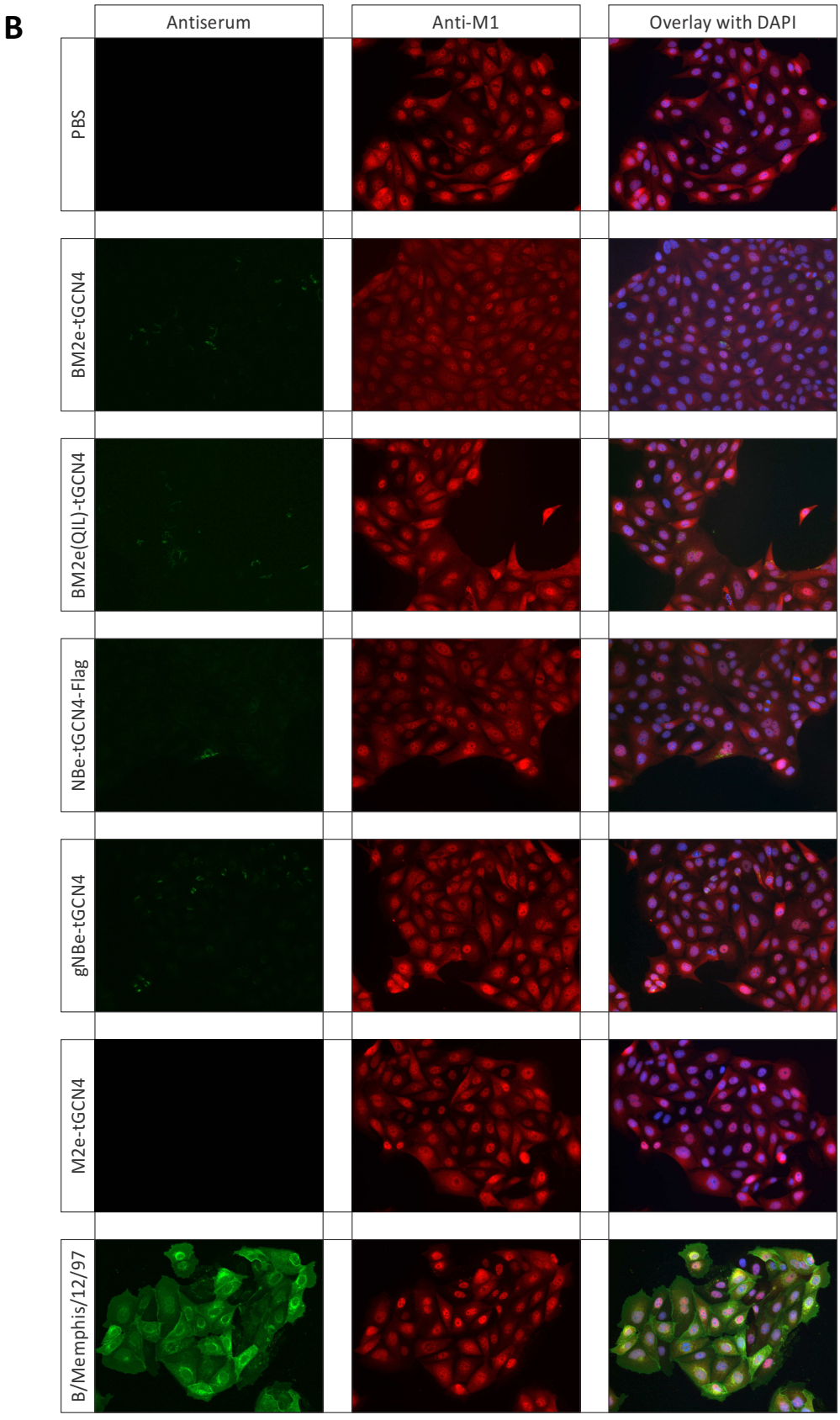


Figure 4.11. ***Continued.***

Challenge results are depicted in Figure 4.12. Immunization with inactivated B/Memphis/12/97 virus protects mice from a potentially lethal homologous challenge, leading to only minor morbidity shortly after infection. Both BM2e-based tGCN4-vaccines show significant protection from mortality compared to M2e-tGCN4 ($p<0.05$, Kaplan-Meier analysis). Compared to NBe-tGCN4-Flag, gNBe-tGCN4 and BM2e-tGCN4 provide significant protection ($p<0.05$, Kaplan-Meier analysis); BM2e(QIL)-tGCN4 does not reach significance ($p=0.051$). It should be clearly noted that in this instance, mortalitity results are highly influenced by the exclusion of mice losing over 30% of body weight. Additionally, on the level of morbidity, no differences are seen between the different tGCN4-immunized groups. Hence, for BM2e- and gNBe-based proteins showing significant protection from mortality, morbidity remains very high but simply does not exceed 30% weight loss for mice depicted in the mortality curve. This reflects the need for further improvement regarding the IBV mouse model in general and the induction of protective immunity by conserved antigens described here.

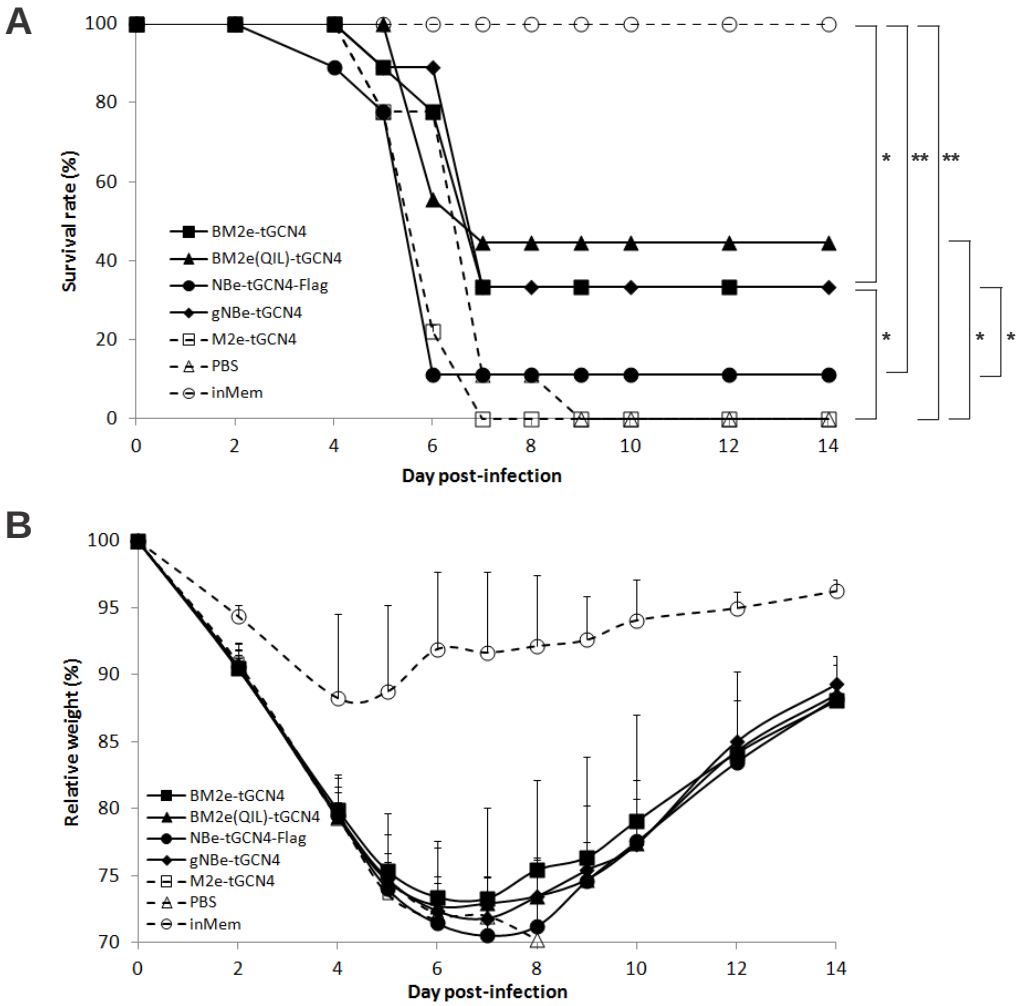


Figure 4.12. Protection of mice vaccinated with different tGCN4-constructs after a potentially lethal infection with B/Memphis/12/97 virus. Groups of mice were immunized three times, three weeks apart, with 10µg Montanide ISA-720 adjuvanted BM2e-tGCN4, BM2e(QIL)-tGCN4, NBe-tGCN4-Flag, gNBe-tGCN4-Flag or M2e-tGCN4-Flag (n = 9 for each group) protein, PBS buffer alone (n = 9) or 1µg inactivated B/Memphis/1/97 (n = 5). Three weeks after the last immunization, mice were challenged with a potential lethal ($4LD_{50}$) dose of B/Memphis/12/97 virus. **A.** Relative survival for each group, with indication of statistical significant differences, as determined by Kaplan-Meier analysis, *, $p<0.05$; **, $p<0.01$. **B.** Relative body weight after challenge. Shown are the group averages +SD.

4.2.4 Immunogenicity and protective efficacy of chimeric transmembrane NBe-protein expressing cells

The second tGCN4-based vaccination experiment indicated a superior effect of the glycosylated gNBe-tGCN4 immunogen compared to bacterial expressed NBe-tGCN4-Flag. Hence, we thought it appealing to further assess the protective capacity of glycosylated NBe-species. With a lack of good expression systems yielding appreciable amounts of soluble glycosylated NBe-proteins next to *P. pastoris*, we employed cellular-vaccine immunizations using mammalian cells transiently transfected with constructs expressing transmembrane chimeric NBe-proteins. In this set-up, three consecutive immunizations were given, three weeks apart, using HEK293T cells transfected with different pCAGGS-based plasmids through calcium phosphate precipitation. As a final heterologous boost (*i.e.* with a distinct cell line transfected with the same constructs), mice were immunized eight weeks after the third HEK-immunization with polyethylene-imide transfected NIH3T3 cells. The cell-expressed antigens used include NBe(WT)-M2-Flag, with the wild type NBe-sequence containing its two glycosylation sites, and NBe(Mut)-M2-Flag, with the mutant NBe-sequence lacking the two glycosylation sites (N3Q and N7Q mutations). An additional test-group was immunized with BM2-Flag expressing cells. As controls, M2-Flag expressing cells were included, alongside mock-transfected (GFP-expressing vector) cells. A representative Western blot analysis of expression levels in transfected cells used for immunization is shown in Figure 4.13A. All immunizations were done with appr. 2-4x10^6 cells per vaccination per mouse, suspended in PBS without an adjuvant. It should be noted that the high glycosylation level of NBe(WT)-M2-Flag (nearly 100% fully glycosylated species, as seen in Fig. 4.13A) was comparable for each immunization.

To analyze humoral responses, a number of assays were performed. In peptide ELISA, no anti-NBe responses were detected (data not shown). For the M2-Flag immunized group, low anti-M2e serum IgG responses were recorded, with endpoint titers just under 10^5 for both IgG1 and IgG2a (data not shown). To assess interaction of antisera with total Flag-tagged proteins or glycosylated NBe, cellular stainings were performed. In a first set-up, lysates of NBe(WT)-M2-Flag expressing HEK293T cells were analyzed on SDS-PAGE and transferred to Western blot. Using pooled sera of immunized mice (after the third HEK-immunization), the blot was detected to reveal anti-cellular and NBe(WT)-M2-Flag specific responses. As can be deduced from Figure 4.13B, antisera react with a number of cellular proteins. Immunization with cells expressing any of the M2-Flag based proteins elicits antibodies against the common M2-Flag transmembrane and cytoplasmic domain, indicating release and processing of the entire transmembrane protein. This might be due to disruption of transfected cells during vaccine preparation and injection, or might happen *in vivo* after immunization.

A second, similar analysis was based on detection of virion-associated antigens on Western blot (Fig. 4.13C). With the AM2, NB and BM2 content of viral particles being relatively low, loading a large amount of A/PuertoRico/8/34 and B/Memphis/12/97 pfu (app. 10^7-10^8 pfu per lane) on gel allowed for detection of minor reactivity. All antisera raised through M2-based immunization reacted with the M2-protein, while BM2-specific antisera revealed an influenza B specific protein with a MW corresponding to that of BM2. No NB-specific reaction was detected. Overall, using transfected cells as immunogens, viral protein specific antibodies can be elicited reactive with full-length proteins in Western blot.

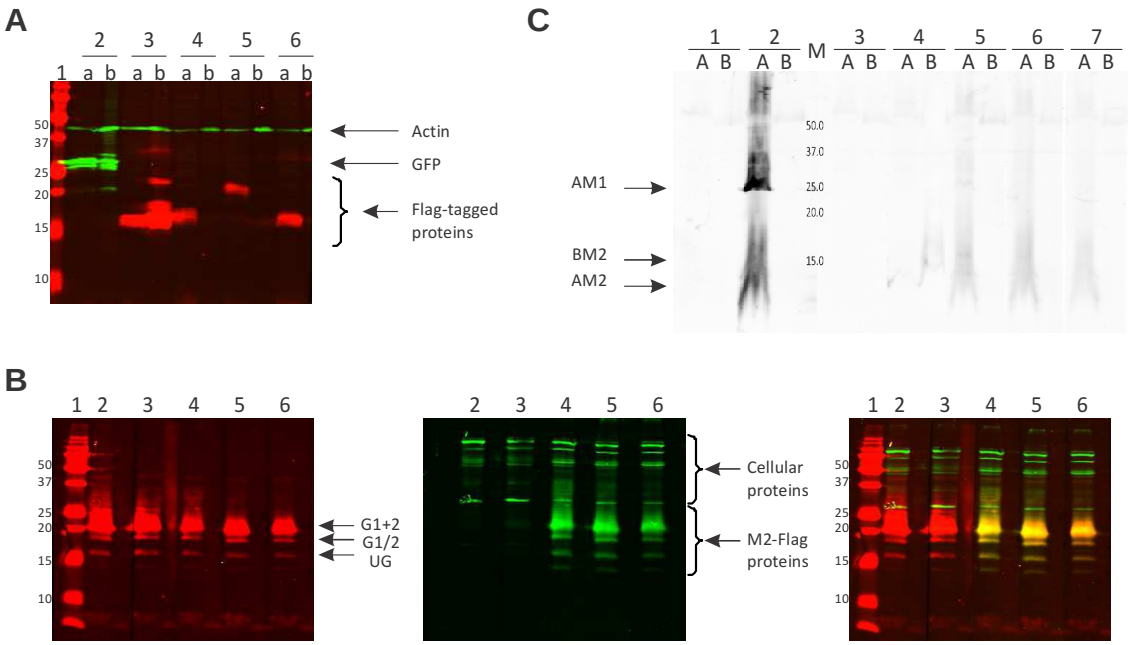


Figure 4.13. Expression levels in and immunogenicity of cells transfected with different constructs expressing influenza-derived transmembrane proteins. A. Analysis of cell lysates, showing the expression level of Flag-tagged proteins after transfection of HEK293T cells with pCAGGS-based vectors coding for the different proteins. Western blot analyzed with rabbit anti-Flag (red) and mouse anti-actine and anti-GFP (green), visualized with the Odyssey imaging system. 1 = gel marker (MW in kDa), 2 = mock transfected cells expressing GFP, 3 = pCAGGS-BM2-Flag, 4 = pCAGGS-M2-Flag, 5 = pCAGGS-NBe(WT)-M2-Flag, 6 = pCAGGS-NBe(Mut)-M2-Flag; a = supernatant after lysis, b = pellet after lysis. **B.** Reaction of pooled antisera of mice, immunized three times with transfected HEK cells expressing NBe(WT)-M2-Flag on Western blot. As internal control, the blot was detected with rabbit anti-Flag (red; left panel), showing expression of non-glycosylated (UG), single glycosylated (G1/2) and mainly double glycosylated (G1+2) NBe(WT)-M2-Flag. The middle panel shows reactivity of antisera with NBe(WT)-M2-Flag lysates, revealing reactivity with both cellular and M2-Flag based proteins. The right panel is an overlay of the left and middle panel. 1 = gel marker (MW in kDa), 2 = antiserum against mock-transfected cells, 3 = antiserum against BM2-Flag expressing cells, 4 = antiserum against M2-Flag expressing cells, 5 = antiserum against NBe(WT) M2-Flag expressing cells, 6 = antiserum against NBe(Mut)-M2-Flag expressing cells. **C.** Reaction of pooled antisera of mice after the fourth immunization with virion-associated proteins. Approximately 10^7-10^8 pfu of A/PuertoRico/8/34 (lanes A) or B/Memphis/12/97 (lanes B) were loaded on gel for Western blot analysis. Detection of viral proteins was done with immune sera (1/300 diluted). The expected location of bands corresponding to AM1, AM2 and BM2 protein are indicated by arrows. M = gel marker (MW in kDa), 1 = detection with NBe-specific 15G5mAb, 2 = M1- and M2e-specific 148mAb, 3 = antiserum against mock-transfected cells, 4 = antiserum against BM2-Flag expressing cells, 5 = antiserum against M2-Flag expressing cells, 6 = antiserum against NBe(WT) M2-Flag expressing cells, 7 = antiserum against NBe(Mut)-M2-Flag expressing cells.

Immunofluorescence staining of transfected NIH3T3-cells is a second approach used here to evaluate humoral responses elicited by cell-based immunizations. To be able to detect the potential presence of antibodies against glycosylated NBe, cells were transfected with BM2-Flag based constructs and used for staining after fixation and permeabilization (since the majority of BM2-based proteins is not surface-expressed). Background staining of endogenous cellular proteins was largely avoided by a pre-clearing step with incubation of immune sera with untransfected NIH3T3 cells before staining of transfected cells. Representative pictures of stainings with antiserum (from the final NIH3T3 cell-based boost) raised against NBe(WT)-M2-Flag are shown in Figure 4.14. In general, little background staining is visible because of the pre-clearing step, as evidenced by staining of mock (empty pCAGGS-vector) transfected cells (top panel). Staining of NBe(WT)- and NBe(Mut)-BM2-Flag expressing cells

shows the presence of cross-reactive antibodies, which might be specific for (glycosylated) NBe. Staining of BM2-Flag expressing cells however also shows some staining with antiserum, suggesting the presence of anti-Flag antibodies. Binding of M2-Flag expressing cells gives a very clear staining of the M2-protein, corresponding to the presence of anti-M2 antibodies as detected in Western blot stainings (Fig. 4.13). Corresponding stainings with NBe(Mut)-M2-Flag specific antiserum gave similar results (data not shown).

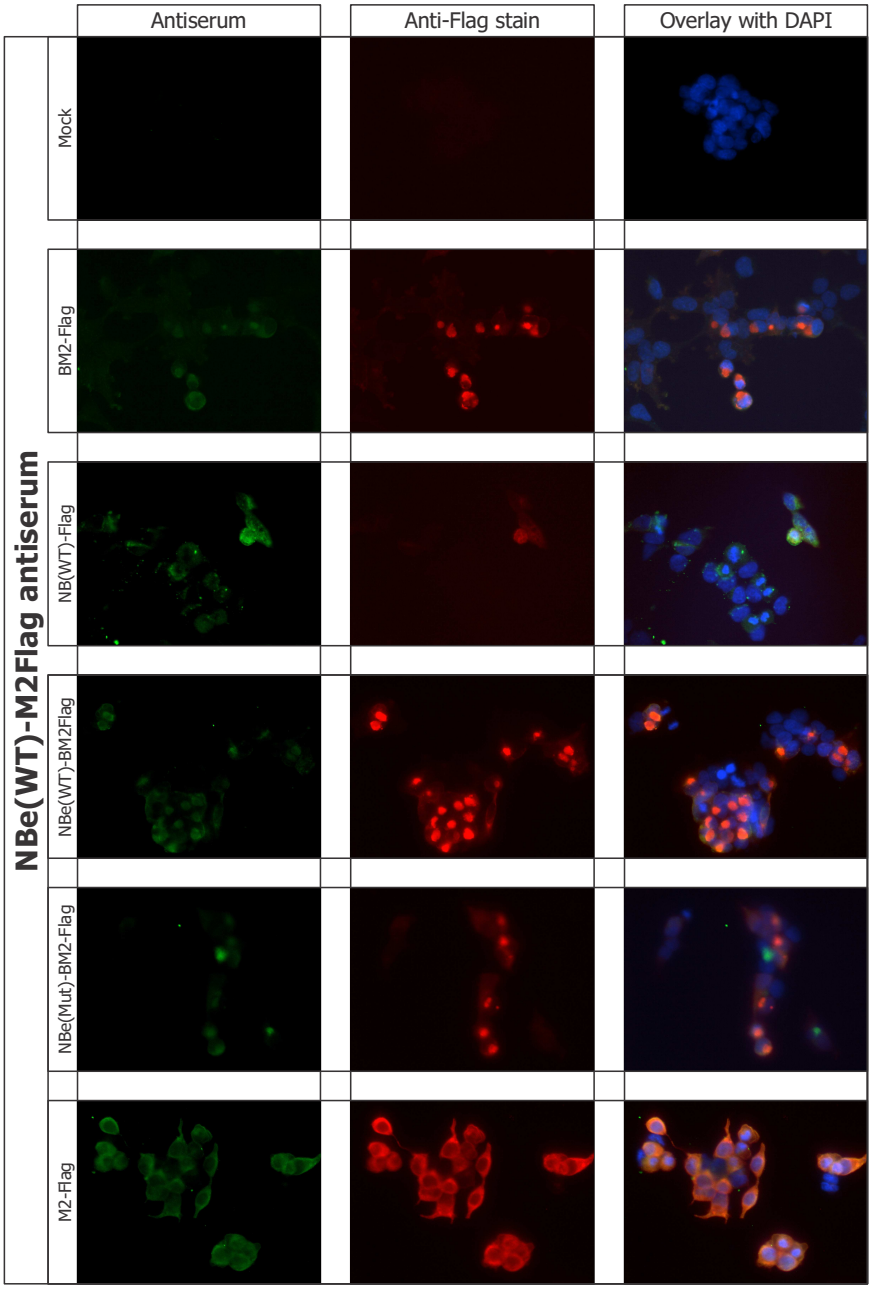


Figure 4.14. Immunofluorescence staining of transfected cells with pooled antisera raised against cells expressing influenza-derived transmembrane proteins. NIH3T3-cells were transfected with empty pCAGGS vector (top row) or pCAGGS-based vectors encoding BM2-Flag (second row), NB(WT)-Flag (third row), NBe(WT)-BM2-Flag (fourth row), NBe(Mut)-BM2-Flag (fifth row) or M2-Flag (bottom row). 16h after transfection, cells were fixed and permeabilized and stained with rabbit anti-Flag (red) and pooled antiserum (1/250 diluted; green) raised against NBe(WT)-M2-Flag by immunizing mice with cells transiently expressing this protein. Representative pictures for each setting are shown.

In control settings, transfected cells were stained with pooled antiserum raised against mock (GFP-expressing plasmid), BM2-Flag or M2-Flag transfected cells (Fig. 4.15). Staining of NBe(WT)-BM2-Flag expressing cells with M2-Flag specific antiserum revealed staining of Flag-tagged proteins, affirming the presence of anti-Flag antibodies in the serum providing cross-reactivity. The corresponding staining with BM2-Flag antiserum however shows a more intense binding of the antiserum, demonstrating the induction of BM2-protein reactive antibodies. Corresponding stainings of NB-Flag, NBe(Mut)-BM2-Flag and BM2-Flag expressing cells fall in line with discussed results (data not shown).

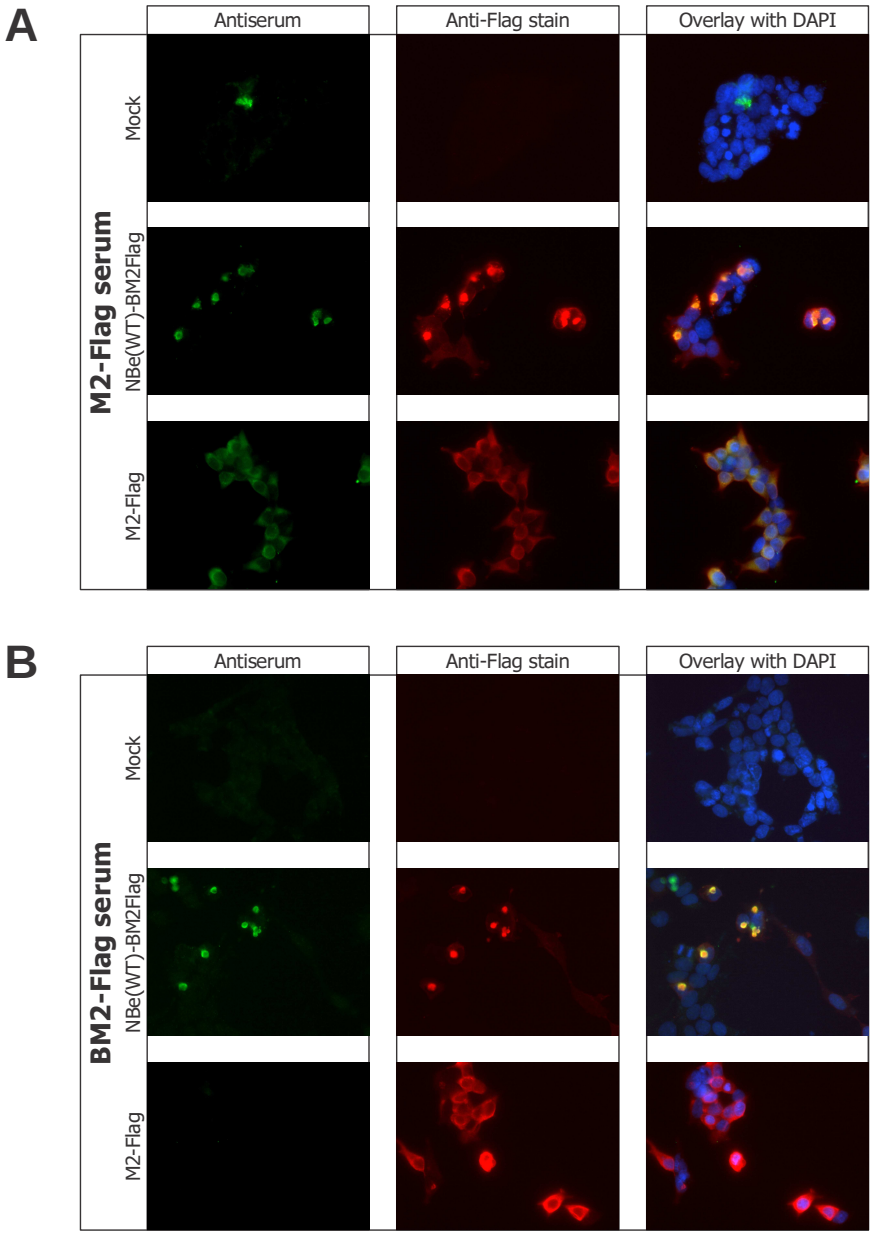


Figure 4.15. Immunofluorescence staining of transfected cells with pooled antisera raised against cells expressing influenza-derived transmembrane proteins. NIH3T3-ells were transfected with empty pCAGGS vector (top row of each panel) or pCAGGS-based vectors encoding NBe(WT)-BM2-Flag (middle row of each panel) or M2-Flag (bottom row of each panel). 16h after transfection, cells were fixed and permeabilized and stained with rabbit anti-Flag (red) and pooled antiserum (1/250 dilution; green) raised against M2-Flag (A), BM2-Flag (B) or mock transfected cells (C). Representative pictures for each setting are shown.

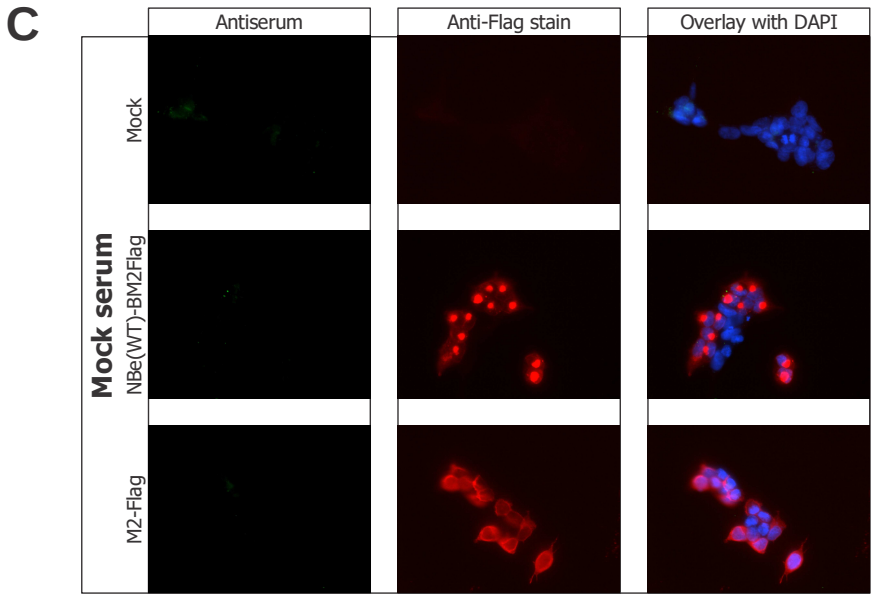


Figure 4.15. ***Continued.***

Four weeks after the fourth (heterologous) immunization, mice were challenged with a 2.5LD_{50} IBV/Memphis/12/97 dose. With this slightly lower dose compared to previous challenge experiments, it was anticipated that protective immunity would be more evident, while still retaining potential lethality. For the analysis of viral replication in the lungs of infected mice, a 0.025LD_{50} dose was administered (appr. $3x10^4$ pfu). Challenge results are shown in Figure 4.16. NBe(WT)-M2-Flag and BM2-Flag immunized mice show 43% and 29% survival, respectively, although morbidity, as determined by body weight measurement, is high. Compared to mock and M2-Flag transfected cells, NBe(WT)-M2-Flag can bestow significant protection ($p<0.05$, Kaplan-Meier analysis).

Day 3 and day 5 after the sublethal challenge (0.025LD_{50}), lung homogenates were prepared for virus titration. For unclear reasons, the titers on day 3 postchallenge for the mock-transfected cell immunized group are significantly lower compared to other groups ($p<0.05$; one-way Anova). However, only in this group do titers incline on day 5 compared to day 3 postchallenge. For all the M2-Flag based immunized groups, titers are lower on day 5 after infection. More interestingly, titers in NBe(Mut)-M2-Flag immunized mice are very low on day 5 postchallenge, and even undetectable in the NBe(WT)-M2-Flag immunized group. For the latter group, day 5 titers are statistically lower compared to mock- and M2-Flag transfected cell immunized mice ($p<0.01$ and $p<0.05$, resp.; two-sided t-test).

Combined with significant protection from mortality, absence of lung virus titers shows that immunization of mice with a glycosylated NBe-based vaccine has a beneficial effect on the induction of IBV antiviral immunity compared to control settings. The mutant counterpart, lacking glycosylation sites through mutation of the Asn-residues to Gln (N3Q and N7Q), can induce inhibitory effects on viral replication but fails to protect mice from mortality. These results indicate an additional advantage of glycosylation of the NBe-sequence in vaccine design in inducing protective immunity towards IBV infection.

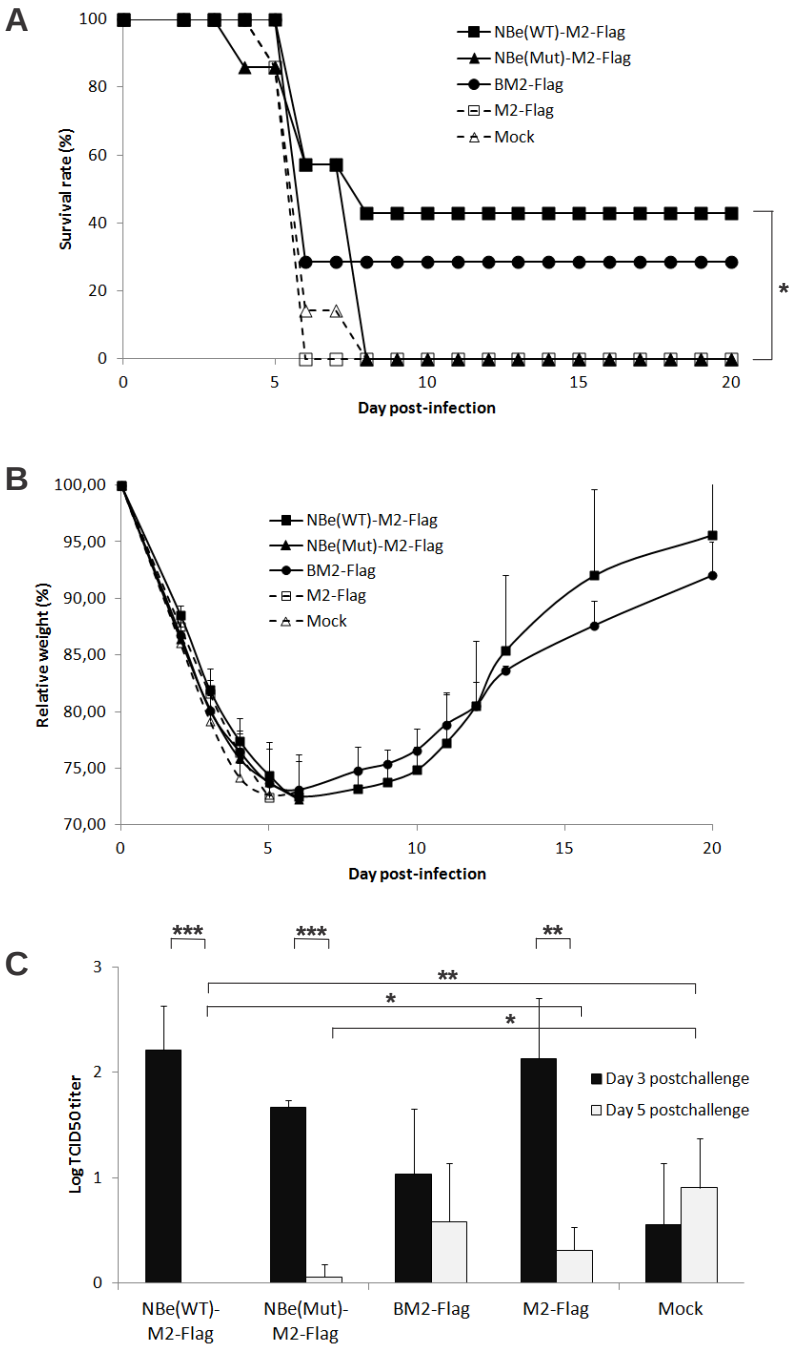


Figure 4.16. Protection of mice vaccinated with transfected cells, expressing transmembrane influenza-derived proteins, after a potential lethal infection with B/Memphis/12/97 virus. Groups of seven mice were immunized three times, three weeks apart, with transfected HEK293T cells and boosted eight weeks later with transfected NIH3T3 cells. For transfection, pCAGGS-based vectors were used encoding BM2-Flag, M2-Flag, NBe(WT)-M2-Flag or NBe(Mut)-M2-Flag. Mock transfections were performed with a GFP-expressing vector combined with empty pCAGGS. Three weeks after the last immunization, mice were challenged with a potential lethal ($2.5LD_{50}$) dose of B/Memphis/12/97 virus. **A.** Relative survival for each group, with indication of statistical significant differences, as determined by Kaplan-Meier analysis, *, $p<0.05$. **B.** Relative body weight after challenge. Shown are the group averages +SD. **C.** Two additional groups of three mice in each group were challenged with a sublethal IBV/Memphis/12/97 dose ($0.025LD_{50}$). Three and five days later, mice were sacrificed for preparation of lung homogenates and titration of the lung virus dose. Shown is the average $TCID_{50}$ titer (calculated log-value) ±SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.2.5 Passive immunization of mice using NBe-specific monoclonal antibodies

Experiments described above indicate that bacterial produced NBe-tGCN4-Flag can provide some protection, but that glycosylated NBe-based vaccine candidates (gNBe-tGCN4, NBe(WT)-M2-Flag) have superior effects compared to their non-glycosylated counterparts. To further assess the protective capacity of non-glycosylated NBe-bases vaccines in an indirect manner, passive immunization experiments were performed using the in-house developed NBe-specific monoclonal antibody (mAb) 15G5 (see section 3.2.5). Compared to other selected NBe-specific mAbs, 15G5 shows the highest affinity for its epitope. As controls, the simultaneously selected HBc-specific mAb 20C3 was used, alongside buffer only (PBS) and polyclonal post-challenge serum of B/Memphis/12/97 virus infected mice as positive controls. Passive immunizations was performed with 150µg mAbs or 50µL post-challenge serum. One day after transfer, blood was drawn to assess the presence of the transferred immune agents before mice were challenged with 2.5LD_{50} of B/Memphis/12/97.

Serology and challenge results are shown in Figure 4.17. All immune agents transferred are detected in sera collected the next day (Fig. 4.17A). Subsequent infection with a potentially lethal dose of B/Memphis/12/97 virus induced a high degree of morbidity and mortality in all mouse groups, including mice who received the homologous anti-viral serum. Only this latter group showed survival (42%), giving a significant difference with other groups ($p<0.05$, Kaplan-Meier analysis). Similar results are seen when analyzing lung viral titers, determined three and four days after a sublethal (0.1LD_{50}) challenge. Titers in mice receiving anti-viral serum are significantly lower on both days compared to other groups ($p<0.05$, one-way Anova). In conclusion, the NBe-specific mAb 15G5 is unable to convey protection in passive immunization, despite showing binding of infected cells in immune-fluorescence assays (see chapter 3, Figure 3.22).

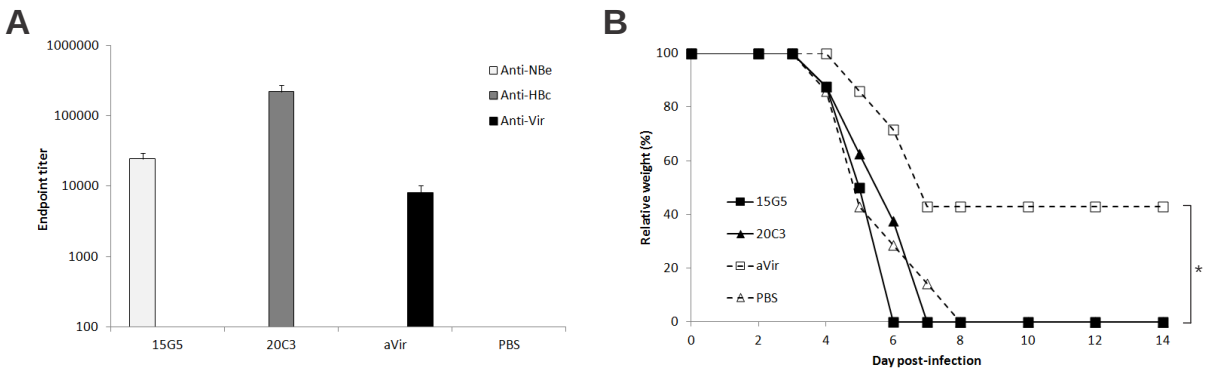


Figure 4.17. Passive immunization of mice with NBe-specific mAbs is unable to protect against a potential lethal IBV/Memphis/12/97 challenge. Groups of mice were passively immunized by i.p. injection with 150µg NBe- (15G5; n = 8) or HBc-specific (20C3; n = 8) monoclonal antibody, 50µL B/Memphis/12/97-specific postchallenge serum (aVir; n = 7) or PBS (n = 7). 16h after passive transfer, blood was drawn for serum analysis. 20h after transfer, mice were challenged with a potentially lethal (2.5LD_{50}) dose of B/Memphis/12/97 virus. **A.** Endpoint titers as determined in NBe-, HBc- or virus-specific (aVir) ELISA. For the latter, ELISA plates were coated with inactivated B/Memphis/12/97 virus. **B.** Relative survival for each group, with indication of statistical significant differences, as determined by Kaplan-Meier analysis, *, $p<0.05$.

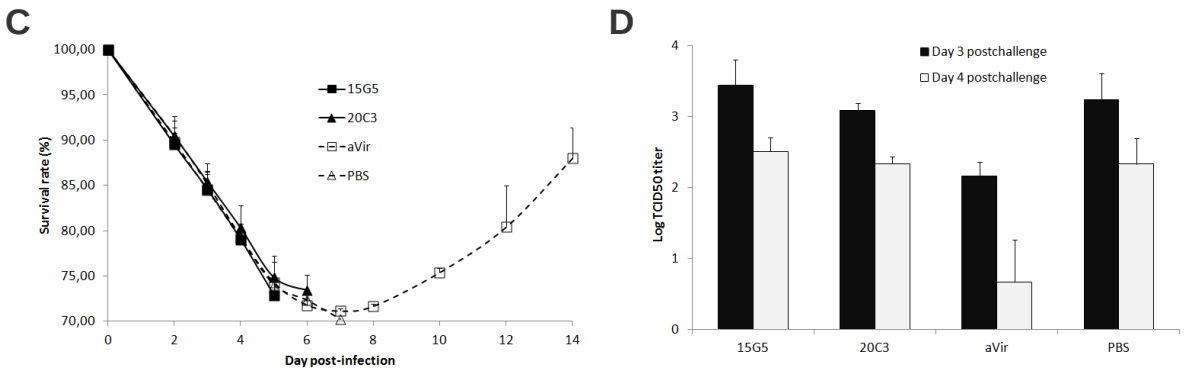


Figure 4.17. ***Continued.*** **C.** Relative body weight after challenge. Shown are the group averages +SD. **D.** Two additional groups of three mice in each group were challenged with a sublethal IBV/Memphis/12/97 dose ($0.1LD_{50}$). Three and four days later, mice were sacrificed for preparation of lung homogenates and titration of the lung virus dose. Shown is the average $TCID_{50}$ titer (calculated log-value) ±SD.

4.3. Discussion

The current epidemic situation of IBV is problematic. Co-circulation of two antigenically distinct lineages enables the virus to easily circumvent natural or vaccine-induced immunity by switching between strains of both lineages. Additionally, it provides a basis for recombination of genes or gene constellations, adding on to intragenic antigenic evolution based on mutation and immune selection[15,22-24]. Hence, new strategies are needed to combat future epidemics. Apart from implementation of quadrivalent vaccines, containing two IAV (H1N1 and H3N2) and two IBV (of both lineages) strains [25], recombinant vaccines can provide a basis for a broad, cross-lineage IBV vaccine. On these premises, the immunogenic and protective potential of a number of conserved IBV domains were scrutinized in this study.

The main obstruction in this and related work, is the lack of a good, small animal model for IBV. The mouse as model is an obvious choice, as for IAV this works well and mice, in general, are easy to handle and manipulate. However, compared to IAV, IBV is a 'humanized' form of the virus, with a very restricted host range. This entails results obtained in mouse models should not be taken for granted. The difficulties with this model were also apparent in our immunization-challenge experiments. Initial attempts to adapt a number of IBV to the murine host failed. To overcome this, mouse-adapted IBV strains were obtained from other research groups [7,8]. The dose needed to achieve lethality with these mouse-adapted strains however exemplifies the difficulties with the model. On average, the LD_{50} dose for IBV is over 4log values higher than for IAV. Such amounts of virus needed for lethality induce an enormous strain on the model as such. This is e.g. illustrated in the passive immunization experiment, where administration of homologous antiserum (with an endpoint anti-virion ELISA titer of appr. 10^5/50µL, equivalent to an HI titer of 160) can only protect 42% of treated mice, a set-up shown to protect mice from excessive IAV induced morbidity and mortality [26].

Despite the challenging IBV model, encouraging effects of our vaccine candidates were detected. A summary of the immunogenic and protective characteristics of proteins tested here is given in Table

4.1. Protection of mice against a potentially lethal IBV infection through immunization with HBc-based broad IBV vaccine candidates is only evident when using an NBe-HBc particle displaying the NBe-sequence N-terminally (1651). In contrast however, 1646 immunized mice show higher anti-NBe responses than 1651 immunized mice, as measured in peptide-ELISA (Fig. 4.2A, B), not indicating any correlation between protection and anti-NBe titers. A qualitative difference however might explain the results, in that the presentation of NBe at the N-terminal end of the chimeric protein might be superior to insertion of the NBe-sequence in the loop of HBc. Specific pools of antibody reactive with the free N-terminal end of (unglycosylated) NBe could have better access to virally expressed NB protein, providing protection through Fc-mediated downstream effects or direct activity on IBV infectivity. Also noteworthy in this regard, in this set-up mice were challenged with B/Hong Kong/05/72 virus, for which the NBe-sequence shows quite some disparity with that of the vaccine construct, but specifically towards the C-terminal end of the sequence (Fig. 4.1). Is should be stated however, that no clear specific staining of IBV infected cells was observed using sera raised with any of the NBe-HBc fusions reported here (data not shown).

NM_2H-protein immunization has a clear effect on influenza A/X47 infection, subsiding morbidity, mortality and pulmonary virus replication compared to HBc- and PBS-treated groups. This protein is able to elicit anti-M2e IgG responses to a similar extent as its non-NBe bearing particulate counterpart 1817, albeit with a slight skewing towards a higher Th2-biased response. Despite induction of high anti-NBe titers, encompassing those evoked by 1646 and 1651, no clear-cut result was seen when challenging immunized mice with B/Memphis/12/97. The 3004 particle, displaying the cleavage loop op B/HA0 in an immunogenic manner, also failed to induce protection, even though specific antibody levels were readily detected in cellular ELISA, and it has been reported that the conserved B/HA0 cleavage loop can be used as an immuno-protective antigen in mouse [27], although certain aspects in the experimental set-up do differ strongly. Here, $4LD_{50}$ was given in a volume of 50µL, penetrating deep into the lungs, while Bianchi et al. administered $1LD_{90}$ in a volume of 20µL. Nonetheless, the lack of a significant effect on IBV challenge in these instances further emphasizes the demanding aspects of the IBV mouse model, requiring high doses of virus to achieve mortality and measurable levels of morbidity.

The tGCN4-based proteins are expected to display the NBe- and BM2e-epitopes in a tetrameric fashion, mimicking their natural conformation [28-32]. Hence, immune serum raised with these is expected to recognize cell-expressed NB and BM2 proteins, as has been shown for M2e-tGCN4 and M2 protein recognition [21]. Cellular assays here show that anti-BM2e immune sera can react with BM2-expressing transfected or infected cells in ELISA, albeit at low intensity. Likewise, antisera directed towards glycosylated and non-glycosylated NBe can clearly stain transfected cells expressing NBe-chimeric transmembrane proteins. However, levels of reactive antibodies might not reach levels needed for full protection, resulting in high morbidity and mortality, as detected in described experiments.

The outcome of challenge with potentially lethal doses of IBV after immunizations seems to correlate with reactivity of antisera towards infected cells. BM2e-based immunogens can convey protection against IBV of both lineages, regardless of the length of the BM2e-sequence used. This is to some extent in line with recent data showing a direct *in vitro* antiviral effect of BM2e-specific antibodies [33]. *In vivo* data presented here enforce the value of the very short but highly conserved BM2 ectodomain in broad IBV vaccine development. For NBe, non-glycosylated immunogens might not

induce antibody pools able to react sufficiently with glycosylated viral expressed NB. In this regard, gNBe-tGCN4 vaccination seems superior to NBe-tGCN4-Flag immunization. Specific subpopulations of antibody, recognizing glycosylated NBe or epitopes outside the glycosylated domain, can theoretically provide antiviral immunity.

To supplement data generated using gNBe-tGCN4, whole-cell based vaccination using transfected cells expressing glycosylated NBe(WT)-M2-Flag was performed. This less elegant method of immunization was used to ascertain the influence of glycosylation of NBe, since in these immunizations nearly all of the protein was fully glycosylated (as compared to small-scale transfection tests, where non-, mono- and double glycosylated species are clearly distinguishable, *cf*. Fig. 3.16). Antisera raised this way react with the ectodomain, cytoplasmic domain and Flag-tag of the expressed proteins, indicating these are liberated during vaccine preparation or injection or through *in vivo* mechanisms. Quite strongly, the NBe(WT)-M2-Flag chimer can evoke protective immunity, lowering mortality rates in a significant manner and rendering lung of infected mice completely virus-free by day 5 postchallenge. Additionally, NBe(Mut)-M2-Flag, lacking glycosylation, also has a significant effect on pulmonary virus titers. With the only common denominator with IBV being the NB ectodomain, this set-up further endorsed the merit of glycosylated NBe-based vaccination in IBV vaccine development. The method of immunization could lead to the induction of specific T cells, conveying anti-IBV activity. However, no NBe-specific T cells are induced in the $H2^d$-background of Balb/c mice (see Chapter 5).

In conclusion, we have shown a beneficiary effect of BM2e- and glycosylated NBe-based (gNBe-) immunization on the outcome of IBV infection. Protection afforded by BM2e-tGCN4 is cross-reactive, thwarting infection with both a Yamagata-like IBV strain (B/Memphis/12/97) and a Victoria-like IBV strain (B/Hong Kong/5/72). Although no virus neutralizing effects were observed, BM2e- and gNBe-based immunogens can convey a restriction on pulmonary virus replication and infection-induced mortality. We feel these results contribute significantly to research on cross-protective IBV vaccine development.

Table 4.1. Overview of *in vitro* serological and *in vivo* antiviral characteristics of broad influenza B vaccine candidates. IF = immunofluorescence, WB = Western blot, PD = pull-down, ND = not determined, DNS = data not shown; B/HK = B/Hong Kong/5/72, B/Mem = B/Memphis/12/97.

Type	Immunogen	Reactivity of antiserum				*In vivo* protective effect	Challenge virus	Figure
		towards transfected cells	Figure	towards infected cells	Figure			
HBc proteins	NBe-HBc (1646, 1651)	ND	-	ND	-	+ (1651)	B/HK	4.3
	NBe-M2e$_2$-HBc	ND	-	++ (ELISA)	4.6	+++	A/X47	4.5
		ND	-	-	4.6	-	B/Mem	4.8
	B/HA0-HBc (3004)	ND	-	++ (ELISA, IF)	4.6, 4.7	-	B/Mem	4.8
tGCN4 proteins	BM2e-tGCN4 / BM2(QIL)-tGCN4	+ (ELISA)	4.9	+ (ELISA, IF)	4.9, 4.11	++	B/HK	4.10
						++	B/Mem	4.12
	NBe-tGCN4-Flag	++ (IF)	3.17, 3.18	+/- (IF)	4.11	+/-	B/HK	4.10
						-	B/Mem	4.12
	gNBe-tGCN4	+/-	DNS	+ (IF)	4.11	+	B/Mem	4.12
Full-length TM proteins	NBe(WT)-M2-Flag	++ (WB, IF)	4.13, 4.14	+ (WB)	4.13	++	B/Mem	4.16
	NBe(Mut)-M2-Flag	++ (IF)	DNS	+ (WB)	4.13	+/-	B/Mem	4.16
	BM2-Flag	++ (IF)	4.15	+ (WB)	4.13	+/-	B/Mem	4.16
mAb	15G5mAb	+ (PD)	3.21	+ (IF)	3.22	-	B/Mem	4.17

4.4. Material and methods

Mice, viruses and influenza challenges

For all *in vivo* studies, 6-8 week old female Balb/c (Charles River, Italy) mice were used. Mice were housed under specific pathogen free conditions and fed *ad libitum*. All infection experiments were conducted under BSL-2 containment and were approved by the local Institutional Ethics Committee on Experimental Animals of Ghent University.

IBV infections were performed using mouse-adapted B/Hong Kong/05/72, received from Dr. Robert Sidwell [7], or B/Memphis/12/97, received from Dr. Jonathan McCullers [8]. In our model, the LD_{50} dose of B/Hong Kong/05/72 corresponds to $8x10^6$ pfu, and 2.5×10^5 pfu for B/Memphis/12/97. For potential lethal infection, a $4LD_{50}$ or $2.5LD_{50}$ dose was administered, as indicated in the test; sublethal infections were done with $0.4LD_{50}$ or $0.025LD_{50}$ as mentioned in the text. For IAV infections, mouse-adapted X47 was used, with $1LD_{50}$ corresponding to appr. 50-100 pfu. For potential lethal infection, a $4LD_{50}$ dose was used. All infections were done by instilling virus diluted in 50µL phosphate-buffered saline (PBS) into the nostrils of ketamine (50mg/kg) + xylazin (7.5mg/kg) anaesthetized mice. Following infection, mice were monitored for weight loss as a measurement of morbidity. If mice lost more than 30% of their initial weight, they were regarded as not protected and euthanized. All experiments were approved by the Local Ethical Committee.

Immunizations and experimental set-ups

All protein immunizations were done using 10µg of protein. Intraperitoneal (i.p.) vaccinations using Alhydrogel (Brenntag Biosector A/S) as adjuvant were done as described previously [21]. For i.p. immunization adjuvanted with LTR192G [18], proteins were diluted in PBS and combined with 1µg LTR192G to a final volume of 100µL. Subcutaneous vaccinations were given in a volume of 100µL, consisting of 26µL PBS containing the tGCN4-protein and 74µL Montanide ISA-720 (Seppic). For immunization with transfected cells, cells were harvested in PBS and diluted to appr. $2\text{-}4x10^7$ cells/mL. 100µL of cell suspension was injected i.p. as such per mouse per vaccination. Passive immunizations were performed by injecting 150µg of antibody or 50µL polyclonal post-infection serum i.p. in a volume of 100µL PBS. For each experiment, the number of mice per group is indicated in the figure legends.

In active immunization experiments, one week before the first immunization and two weeks after every next immunization and infection, blood was drawn from the lateral tail veins for serological analysis. Subsequent immunizations had an interval of three weeks; the number of immunizations are mentioned with each experiment. Unless stated otherwise, challenge of mice was performed three weeks after the last immunization. In passive immunization, blood was drawn one day after transfer of antibodies to determine the serum titer of administered antibodies. The same day (*i.e.* one day after transfer), mice were challenged. In all follow-up studies, body weight of challenged mice was determined on a daily or two-daily basis. Lung homogenates were prepared on indicated days by tissue homogenization in 1mL PBS using a Heidolph RZR 2020 homogenizer. Viral titers were determined through $TCID_{50}$ titration on MDCK cells in quadruplicate according to the method of Reed and Muench [34].

ELISA procedure

To analyze antibody responses evoked by active vaccination or presence of antibodies after passive transfer, ELISAs were performed. For peptide-specific ELISA (NBe, M2e), Maxisorp immune-plates (Nunc) were coated overnight at 37°C with 50µL of a 2µg/mL peptide-solution in 100mM sodium carbonate buffer, pH 9.6. Anti-

tGCN4-Flag titers were determined using plates coated overnight at 4°C with 2µg/mL tGCN4-Flag in carbonate buffer. Anti-viral ELISAs using inactivated viruses as coating antigen were done using plates coated with 50µL 0.5µg/mL inactivated virus per well. For detection of anti-HBc titers, ELISA plates were coated with 50µL of 0.5µg/mL rabbit anti-HBc (Serotec) overnight at 4°C. After coating, plates were washed three times with PBS + 0.1% Tween20 and blocked with PBS + 3% Milk. For HBc-ELISAs, plates were incubated with 50µL 1µg/mL HBc-protein for 1.5h at room temperature before washing again. Sera from immunized mice were diluted (1/3 dilution series) in PBS + 0.05% Tween20 + 1.5% Milk and incubated on the ELISA plates for 1.5h at room temperature. After washing three times, subtype-specific HRP-linked goat anti-mouse secondary antibodies (Bio-Connect) were incubated on the plates for a further 1.5h at room temperature. Following a final wash step, titers were detected using TMB substrate (Pharmingen, BD). The reaction was stopped after 10min by adding an equal amount of 1M H_2SO_4. Plates were read at 450nm, with background measurement at 655nm. Endpoint titers are determined as the last dilution of immune serum giving an ELISA signal at least twice as high as pre-immune serum.

Cellular assays and vaccines

HEK293T cells were maintained in complete DMEM (DMEM + 10% FCS + L-Gln + sodium pyruvate + non-essential amino acids + penicillin/streptomycin); MDCK cells in complete DMEM without sodium pyruvate. NIH3T3 cells were cultivated in complete RPMI (RPMI + 10% FCS + L-Gln + sodium pyruvate + non-essential amino acids + penicillin/streptomycin).

For cellular ELISAs, MDCK cells were seeded in 96well tissue culture plates (BD) at $2x10^4$ cells per well. 24h later, cells were washed with serum-free DMEM and infected with indicated viruses at a multiplicity of infection (MOI) of 3. 16h later, cells were washed, fixed with 4% paraformaldehyde in PBS for 30min at room temperature and permeabilized for 5min with 0.05% Triton X100 + 20mM Glycine in PBS. After blocking with PBS + 3% milk, plates were further processed as described above.

For cellular immunizations, HEK293T or NIH3T3 cells were seeded in $175cm^2$ flasks (BD) at a density of $4x10^6$ cells/flask. 24h later, when cells reached 50-70% density, cells were washed once and transfected with 20µg pCAGGS-based vectors encoding the indicated proteins through calcium phosphate precipitation (for HEK293T cells) or using 120µg polyethylene imide (for NIH3T3 cells). 16h later, cells were washed once, collected using non-enzymatic dissociation buffer (Gibco) and washed three further times with PBS. Finally, cells were resuspenden in PBS at a concentration of $2\text{-}4x10^7$ cells/mL.

To analyze reaction of antisera with influenza-specific proteins, Western blots and immunofluorescence assays were used. For Western blots, the indicated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were cut into strips and each strip was incubated with antiserum 1/100 diluted in PBS + 1.5% Milk + 0.05% Tween-20. Binding of antisera was revealed using anti-mouse-HRP antibody or using the Odyssey imaging system (Li-Cor Biosciences). For immunofluorescence assays, NIH3T3 cells were seeded on aceton-treated coverslips in 24well plates at a density of 2×10^4 cells/well in complete RPMI. 16h later, cells were transfected with 250ng pCAGGS-based constructs using 1.5µg polyethylene imide. 24h later, cells were fixed with 4% paraformaldehyde and permeabilized for 5min using 0.05% Triton X-100 in PBS + 20mM Glycine. After blocking for 1h using a 1% PBS-based BSA solution, cells were stained with rabbit anti-Flag and murine antiserum (diluted 1/300). Secondary staining was done using goat anti-rabbit Alexa-fluor-647 conjugated antibody and goat anti-mouse Alexa-fluor-488 antibody (Invitrogen). Cells were mounted with Vectashield containing DAPI for nuclear counterstain before acquiring microscopic pictures.

4.5. Acknowledgements

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4.6. References

1. Sridharan, H., Zhao, C. & Krug, R.M. Species Specificity of the NS1 Protein of Influenza B Virus. *Journal of Biological Chemistry* **285**, 7852 (2010).
2. Versteeg, G.A*., et al.* Species-specific antagonism of host ISGylation by the influenza B virus NS1 protein. *Journal of virology* **84**, 5423 (2010).
3. Ledinko, N. & Perry, B. Studies with Influenza Virus B of Recent Human Origin. *The Journal of Immunology* **74**, 371 (1955).
4. Ledinko, N. An analysis of the process of adaptation of influenza virus B of recent human origin to the mouse lung. *Journal of General Microbiology* **15**, 47 (1956).
5. Reading, P.C., Morey, L.S., Crouch, E.C. & Anders, E.M. Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. *Journal of virology* **71**, 8204 (1997).
6. Smee, D.F., Wandersee, M.K., Wong, M.H., Bailey, K.W. & Sidwell, R.W. Treatment of mannan-enhanced influenza B virus infections in mice with oseltamivir, ribavirin and viramidine. *Antiviral Chemistry and Chemotherapy* **15**, 261-268 (2004).
7. Sidwell, R.W*., et al.* Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. *Antimicrobial agents and chemotherapy* **40**, 2626 (1996).
8. McCullers, J.A., Hoffmann, E., Huber, V.C. & Nickerson, A.D. A single amino acid change in the C-terminal domain of the matrix protein M1 of influenza B virus confers mouse adaptation and virulence. *Virology* **336**, 318-326 (2005).
9. Biere, B., Bauer, B. & Schweiger, B. Distinction of Influenza B virus lineages Yamagata and Victoria by real-time PCR. *Journal of clinical microbiology* **48**, 1425-1427 (2010).
10. Wang, Q., Cheng, F., Lu, M., Tian, X. & Ma, J. Crystal structure of unliganded influenza B virus hemagglutinin. *Journal of virology* **82**, 3011 (2008).
11. Rivera, K*., et al.* Probing the structure of influenza B hemagglutinin using site-directed mutagenesis. *Virology* **206**, 787-795 (1995).
12. Nakagawa, N*., et al.* Characterization of new epidemic strains of influenza B virus by using neutralizing monoclonal antibodies. *Journal of medical virology* **65**, 745-750 (2001).
13. Lugovtsev, V.Y., Vodeiko, G.M., Strupczewski, C.M., Ye, Z. & Levandowski, R.A. Generation of the influenza B viruses with improved growth phenotype by substitution of specific amino acids of hemagglutinin. *Virology* **365**, 315-323 (2007).
14. Nakagawa, N., Suzuoki, J., Kubota, R., Kobatake, S. & Okuno, Y. Discovery of the neutralizing epitope common to influenza B virus Victoria group isolates in Japan. *Journal of clinical microbiology* **44**, 1564 (2006).
15. McCullers, J.A., Wang, G.C., He, S. & Webster, R.G. Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *Journal of virology* **73**, 7343 (1999).
16. Nakagawa, N., Kubota, R., Maeda, A., Nakagawa, T. & Okuno, Y. Heterogeneity of influenza B virus strains in one epidemic season differentiated by monoclonal antibodies and nucleotide sequences. *Journal of clinical microbiology* **38**, 3467 (2000).
17. Nakagawa, N., Kubota, R., Maeda, A. & Okuno, Y. Influenza B virus Victoria group with a new glycosylation site was epidemic in Japan in the 2002-2003 season. *Journal of clinical microbiology* **42**, 3295 (2004).
18. Dickinson, B.L. & Clements, J.D. Dissociation of Escherichia coli heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infection and immunity* **63**, 1617 (1995).
19. Lawrence, G.W., Saul, A., Giddy, A.J., Kemp, R. & Pye, D. Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. *Vaccine* **15**, 176-178 (1997).

20. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* **262**, 1401 (1993).
21. De Filette, M.*, et al.* An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. *Journal of Biological Chemistry* **283**, 11382 (2008).
22. McCullers, J.A., Saito, T. & Iverson, A.R. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *Journal of virology* **78**, 12817 (2004).
23. Chi, X.S.*, et al.* Detection and characterization of new influenza B virus variants in 2002. *Journal of clinical microbiology* **43**, 2345 (2005).
24. Jian, J.W.*, et al.* Genetic analysis and evaluation of the reassortment of influenza B viruses isolated in Taiwan during the 2004-2005 and 2006-2007 epidemics. *Virus research* **131**, 243-249 (2008).
25. Belshe, R.B. The need for quadrivalent vaccine against seasonal influenza. *Vaccine* **28**, D45-D53 (2010).
26. Kistner, O.*, et al.* A whole virus pandemic influenza H1N1 vaccine is highly immunogenic and protective in active immunization and passive protection mouse models. *PLoS One* **5**, e9349 (2010).
27. Bianchi, E.*, et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
28. Williams, M.A. & Lamb, R.A. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Molecular and Cellular Biology* **6**, 4317 (1986).
29. Betakova, T., Nermut, M.V. & Hay, A.J. The NB protein is an integral component of the membrane of influenza B virus. *Journal of general virology* **77**, 2689 (1996).
30. Paterson, R.G., Takeda, M., Ohigashi, Y., Pinto, L.H. & Lamb, R.A. Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology* **306**, 7-17 (2003).
31. Wang, J., Pielak, R.M., McClintock, M.A. & Chou, J.J. Solution structure and functional analysis of the influenza B proton channel. *Nature Structural & Molecular Biology* **16**, 1267-1271 (2009).
32. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science (New York, NY)* **262**, 1401 (1993).
33. Wang, Y.*, et al.* Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity. *New Microbiologica* **33**, 311-317 (2010).
34. Reed, L.J. & Muench, H. A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* **27**, 493 (1938).

Chapter 5

T cell based immunity provided by the conserved NB ectodomain of influenza B virus

A potent cytotoxic T cell epitope in the extracellular domain of influenza B virus NB promotes virus clearance

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Running title

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The extracellular domain of influenza B virus NB harbors a potent cytotoxic T cell epitope

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Abstract

Influenza B viruses represent a considerable threat to human health as they are responsible for about 1 out of 3 influenza epidemics. Like influenza A, they drift easily. Furthermore, the co-circulation of two antigenically different lineages, necessitates frequent update of vaccines. Hence, we explored the potential broad protection provided by the conserved ectodomain of the NB transmembrane protein (NBe) of influenza B virus. To mimic its natural quaternary structure, we used a scaffold as fusion partner resulting in production of soluble tetrameric NBe. Surprisingly, immunization with this NBe-fusion protein induced strong NBe-specific cytotoxic T cell responses, which upon challenge enhanced influenza B virus clearance from the lung compartment. Such specific T cell responses were also observed following infection of naïve mice with influenza B virus. These cellular responses were specific for the $CD8^+$ T cell compartment and mapped to a highly conserved, membrane-distal T cell epitope in NBe. *In vivo* killing assays confirmed the induction of strong cytotoxic activity following immunization with NBe-fusion proteins, even when alum, a Th2-directing immune potentiator, was used as an adjuvant. This is the first identification of immune protection provided by the ectodomain of the NB-protein, and the first record of a protective $CD8^+$ T cell epitope for influenza B in the mouse.

Author summary

Influenza A and B viruses cause seasonal epidemics. Every three to four years, a drifted influenza B strain spreads globally in the human population leaving epidemics in its track. Most currently approved vaccines contain purified hemagglutinin subunits derived from virus strains predicted to circulate in the upcoming season. Hence, unexpected drift of the virus will invalidate predictions and derail influenza vaccination campaigns. Using conserved regions of viral proteins as vaccine components allows to avoid these and other uncertainties. Our aim was to develop an influenza B vaccine based on the conserved ectodomain of the NB protein (NBe). Remarkably, irrespective of the route or adjuvant used, immunizations with this vaccine led to induction of high numbers of NBe-specific $CD8^+$ cytotoxic T cells, specific for the $H2^b$-background of C57Bl/6 mice. Following challenge, this T cell immunity was responsible for strong anti-influenza B activity, as evidenced by rapid clearance of pulmonary influenza B virus. This cellular immune response is surprisingly strong and was observed following different NBe-based immunization strategies as well as upon infection by influenza B virus. We document here the opportunity of NBe-based immunization with a protein-only vaccine to establish cellular immunity protection in an appropriate MHC-mouse model.

Introduction

Influenza B virus is an important respiratory pathogen in humans. Influenza viruses belong to the family of *Orthomyxoviridae*, which are characterized by a segmented negative-stranded RNA genome. While influenza B and C infection occurs almost exclusively in humans, influenza A viruses also circulate in a much larger animal reservoir including aquatic and migratory birds, pigs, horses and some other mammals. This creates a genetic pool from which new influenza strains can emerge through processes of mutation, reassortment and recombination. This antigenic drift and shift is responsible for yearly epidemics and occasional pandemics in the human population [1-3]. The surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) are the major antigenic determinants of influenza A and B viruses. To date, 16 different HA subtypes and 9 NA subtypes have been classified for influenza A viruses, all present in the avian reservoir [4]. In contrast, influenza B viruses do not circulate in an animal reservoir. As a result influenza B viruses display antigenic drift, coerced mainly by antibody-based selection pressure induced by vaccination and previous infection [5,6]. Even though no subtypes of influenza B viruses are distinguished, two separate lineages are acknowledged, denoted by their prototypical parental strains B/Victoria/2/87 and B/Yamagata/16/88 [7,8], although lineage separation occurred already in the early 70s [8]. These two lineages co-circulate within the human population and cause alternate epidemics. Moreover, frequent genetic reassortment between Victoria and Yamagata lineage viruses occurs, creating new epidemic strains with altered antigenic and biological properties [5,9,10]. There is little or no natural cross-reactive immunity between the two influenza B virus lineages, supporting the claim to include both strains in seasonal influenza vaccines [11]. Indeed, in some instances a mismatch has been observed between the vaccine B-strain and circulating strains, resulting in failed vaccination campaigns and excessive morbidity in high risk target populations [12-15].

A number of research groups have attempted to identify cross-protective epitopes present in influenza viruses and develop vaccines that induce broader protection based on these [16,17]. For influenza A, one such an approach has focused on the conserved ectodomain (M2e) of the matrix protein 2 ion channel [18,19]. Alternatively, conserved regions within the HA2 domain of the HA molecule have been proposed as antigenic components of broad spectrum vaccines [20-22]. Also for influenza B, at least one reported broad-spectrum vaccination strategy was based on conserved regions of HA [23]. Immunity induced by these cross-protective vaccine relies on antibodies [17,24]. In addition to these approaches eliciting humoral immunity, broad protection can also be achieved by induction of cellular immunity. A number of T cell epitopes, mainly present in internal influenza proteins, have been shown to be responsible for broadly protective cellular immunity [16,25,26].

We previously reported that M2e of influenza A can be rendered immunogenic by using a tetramerizing scaffold protein. This is based on a mutant form of the leucine zipper of the yeast transcription factor GCN4 (tGCN4) [27,28]. We tried to extend these observations to a conserved influenza B virus antigen based on the ectodomain (NBe) of the NB protein [29].The NB protein is a homotetrameric, viroporin-like type III membrane protein and is encoded by segment 6 along with NA from overlapping reading frames [30,31]. NB is present on infected cells and in virions, and contains an N-terminal ectodomain of 18 amino acid residues in length with two N-glycosylation sites [31-33]. These are used for addition of poly-N-lactosaminoglycan structures, although low levels of unglycosylated NB protein can be found on infected cells [34]. The high conservation of NBe, irrespective of the influenza B lineage, encouraged us to explore the protective efficacy of a

tetramerized NBe-based vaccine. Surprisingly, high levels of NBe-specific $CD8^+$ cytotoxic T cells could be induced by protein vaccination with the NBe-tGCN4 construct, regardless of the route of vaccination or the adjuvant used. A similar strong NBe-specific $CD8^+$ T cell response was induced upon influenza B infection. The NBe- directed T cells were able to eliminate NBe-peptide pulsed cells *in vivo*, cleared influenza B virus from the lung of challenged mice and protected mice from influenza B virus induced morbidity. To our knowledge, this is the first report showing NBe-based protection against influenza B infection and the first identification of a murine-specific T cell epitope for influenza B.

Results

NB vaccine antigen design

To obtain a cross-reactive influenza B vaccine antigen, we focused on NBe, the ectodomain of the tetrameric NB protein. NBe comprises 18 amino acid residues, which are conserved across influenza B viruses (Figure 1A). We genetically fused NBe to the tetramerizing leucine zipper tGCN4, in order to obtain a soluble vaccine that mimics the natural conformation of NBe [27,28]. Increased solubility was obtained by addition of a Flag-tag at the C-terminus, resulting in NBe-tGCN4-Flag (Figure 1B).

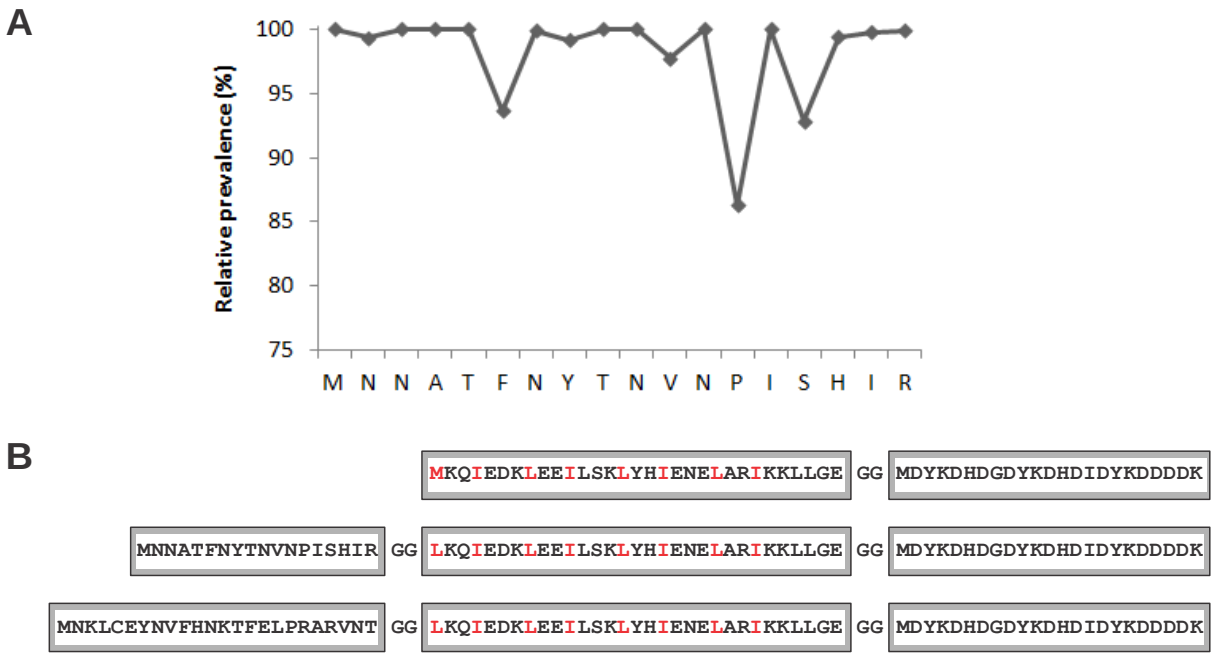

Figure 1. NBe-based vaccine design. A. Graphical representation of the conservation of the NBe-sequence (A) and vaccine design (B). A. For the sequence analysis, all available NBe-sequences (n = 854, from 1940 up until March 2011) were aligned and conservation of the consensus amino acids was calculated. Sequences were obtained from the Influenza Research Database at www.fludb.org. B. Overview of NBe-based vaccine design and control constructs. Shown are the tGCN4-Flag carrier only vaccine (top), the NBe-tGCN4-Flag vaccine (middle) and the irrelevant epitope-carrier fusion Irr-tGCN4-Flag (bottom). The different domains of the constructs are boxed and include the N-terminal NBe or irrelevant epitope, the central tetramerizing tGCN4 domain and the C-terminal triple Flag-tag. For the tGCN4-part, amino acids involved in tetramerization are shown in red.

The chimeric protein was purified by sonication of the resuspended bacterial pellet, heat-treatment, followed by anion exchange chromatography and size-exclusion by gel filtration. This last step yielded two distinct peaks containing NBe-tGCN4-Flag (Figure S1A). The first peak contains NBe-tGCN4-Flag in a higher molecular weight (MW) complex, possibly corresponding to NBe-tGCN4-Flag oligomers or a complex with bacterial contaminants [28]. The second peak corresponds to a protein with an estimated MW of approximately 40kDa, close to the expected MW of tetrameric NBe-tGCN4-Flag (35.2kDa) (Figure S1B). The latter fraction was used in all experiments, and these NBe-tGCN4-Flag preparations contained negligible amounts of LPS (data not shown).

The tetrameric assembly of NBe-tGCN4-Flag was confirmed by cross-linking experiments using different homobifunctional chemical cross-linkers: BS^3 and reducible DSP. M2e-tGCN4 and BM2e-tGCN4, displaying the M2-ectodomains of influenza A and influenza B in a tetrameric fashion [28], were included as controls. Under denaturing, non-reducing conditions BM2e-tGCN4 and NBe-tGCN4-Flag migrate with a relative MW corresponding to that of the monomeric proteins (4.68kDa and 8.81kDa, respectively), while M2e-tGCN4 (predicted MW of 6.45kDa) migrates predominantly as a dimer, with minor bands corresponding to trimers and tetramers (Fig S1C). Cross-linking results in a predominant fraction of proteins that migrates in SDS-PAGE with an electrophoretic mobility corresponding to that of the respective tetramers. Under reducing conditions uncrosslinked and BS^3-crosslinked proteins migrate as monomeric proteins (Figure S1D). Overall, these results demonstrate that the purified NBe-tGCN4-Flag vaccine is a tetramer.

Immunization with NBe-fusion protein promotes pulmonary virus clearance

To evaluate the protective efficacy of the NBe-tGCN4-Flag vaccine, we immunized mouse strains of different genetic backgrounds, namely BALB/c ($H2^d$), C57Bl/6 ($H2^b$) and C3H/HeN ($H2^k$). This allowed us to evaluate the influence of the haplotype on the NBe-specific immune response and compare strain-dependent protection against influenza B virus challenge. The importance of the genetic background of laboratory mice for susceptibility to influenza A virus infection and M2e-specific immune responses has already been reported [35,36].

Figure S2 depicts the serum antibody titers after immunization with NBe-tGCN4-Flag or carrier only. Immunized C57Bl/6 seroconverted weakly to NBe, whereas C3H/HeN mice displayed high NBe-specific titers and BALB/c mice responded intermediately. In contrast, anti-tGCN4-Flag (the carrier) serum IgG titers were high overall, but are completely lacking in C57Bl/6 and C3H/HeN mice that had been immunized with tGCN4-Flag (Figure S2 C,D). The latter observation suggests a lack of T helper epitope in tGCN4-Flag within the $H2^b$ and $H2^k$ background that is compensated for by the fusion to NBe. Challenge of immunized mice with a strain-matching potentially lethal dose ($2.5LD_{50}$) of mouse-adapted influenza B virus revealed no NBe-specific protection in any of the three inbred mouse strains (Figure 2A), indicating that NBe-specific serum IgG either does not contribute to immune-protection or is unable to protect against a high challenge inoculum in our model.

We therefore decided to determine the extent of lung virus clearance in NBe-tGCN4-Flag immunized mice as a more subtle readout for protection, using a sublethal ($0.075LD_{50}$; 7.5×10^4 pfu) challenge dose. NBe-immunized mice of all three mouse strains had a significantly lower lung virus titer on day 4 after challenge compared to control immunized mice (Figure 2B). From this, we concluded that

immunization with NBe-tGCN4-Flag enhances influenza B virus clearance from the lungs, but is overwhelmed by a high virus titer.

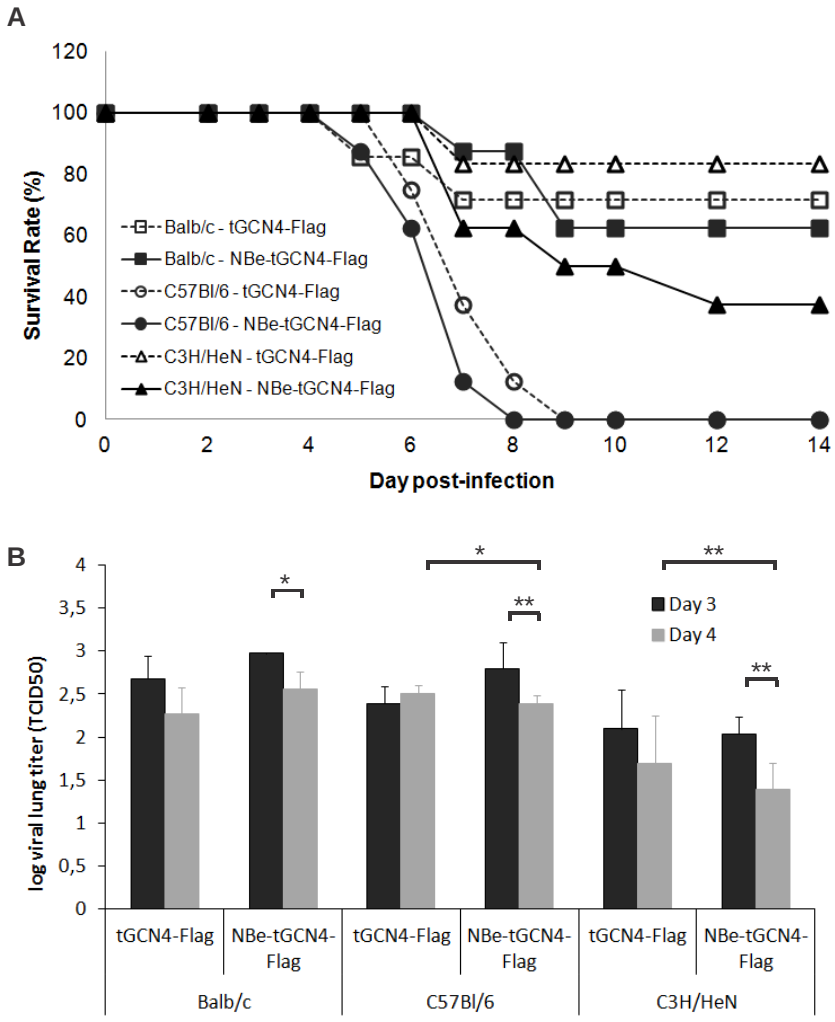

Figure 2. NBe-tGCN4-Flag vaccination fails to induce protective humoral immunity in mice. Mice from different genetic backgrounds, Balb/c ($H2^d$), C57Bl/6 ($H2^b$) and C3H/HeN ($H2^k$), were immunized three times with carrier only or NBe-tGCN4-Flag in combination with Alhydrogel. A. Three weeks after the second boost, mice were challenged with 2.5 LD_{50} of mouse-adapted IBV virus. Shown are the relative survival level during follow-up, taking a 30% weight loss limit in account. n=6-8 mice for all groups. B. Three and four day after a sublethal IBV challenge (0.1 LD_{50}) lung homogenates were made for titration of viral titers in a TCID50 assay. Asterisks above bars indicate significant differences with the titer for the carrier only group determined on the same day. n=3 for all groups. *, $p<0.05$; **, $p<0.01$.

Pre-existing NBe-specific CD8 T cell responses correlate with enhanced pulmonary virus clearance

The reduced influenza B virus replication in the lung of NBe-immunized mice encouraged us to look for adaptive T cell responses potentially elicited by NBe-based immunization. Because tGCN4-Flag carrier did not induce serum IgG responses in C57Bl/6 ($H2^b$ background) and C3H/HeN ($H2^k$ background) mice, whereas immunization with NBe-tGCN4-Flag induced both NBe-specific as well as carrier-specific antibodies (Figure S2), we included a tGCN4-Flag fusion construct displaying an irrelevant (Irr) human Respiratory Syncytial virus (RSV) derived peptide as an additional negative

control (Irr-tGCN4-Flag). Mice immunized with NBe-tGCN4-Flag or Irr-tGCN4-Flag in the presence of alum seroconverted, with clear induction of anti-tGCN4-Flag antibodies in both groups (Figure S3). As controls for the induction of cellular antiviral responses, mice that had recovered from a sublethal influenza A or B virus infection were used. In addition, a DNA-vaccine construct was generated by genetically grafting NBe onto the influenza A M2 transmembrane and cytoplasmic domain, in a mammalian expression plasmid. We found that this NBe-containing DNA vaccine construct allowed the expression of NBe-M2 chimeras with a higher expression level compared to full-length NB (Figure S4). Although it is controversial whether NB possesses ion-gating activity, it is possible that constitutive expression of NB is toxic to cells. In contrast, it has been shown that replacement of the M2e ectodomain by NBe is expected to abolish the ion channel activity (and associated cytotoxicity) of M2 [37].

IFNγ-specific ELISPOT assays and intracellular cytokine staining (ICS) were performed on splenocytes from immunized and convalescent mice. Full-length NBe-peptide (18 amino acids) and haplotype-matching influenza A virus nucleoprotein peptides (A/NP-peptide) were used for *in vitro* restimulation. ELISPOT analysis revealed clear NBe-specific cellular responses in splenocytes from NBe-immunized (NBe-tGCN4-Flag, NBe-M2 DNA and IBV infection) mice of $H2^b$-background (Figure 3A). Smaller, but still detectable spot counts were also observed in $H2^d$-background mice. Using ICS and differential staining for $CD4^+$ and $CD8^+$ cells, the NBe-specific cellular responses could be attributed to $CD8^+$ T cells (Figure 3B and C). In summary, NBe-based immunization in the $H2^b$-haplotype background elicited strong NBe-specific T cell responses that were mainly restricted to the $CD8^+$ T cell compartment. Furthermore, we also conclude that influenza B virus infection induces an $H2^b$-restricted NBe-specific $CD8^+$ T cell response.

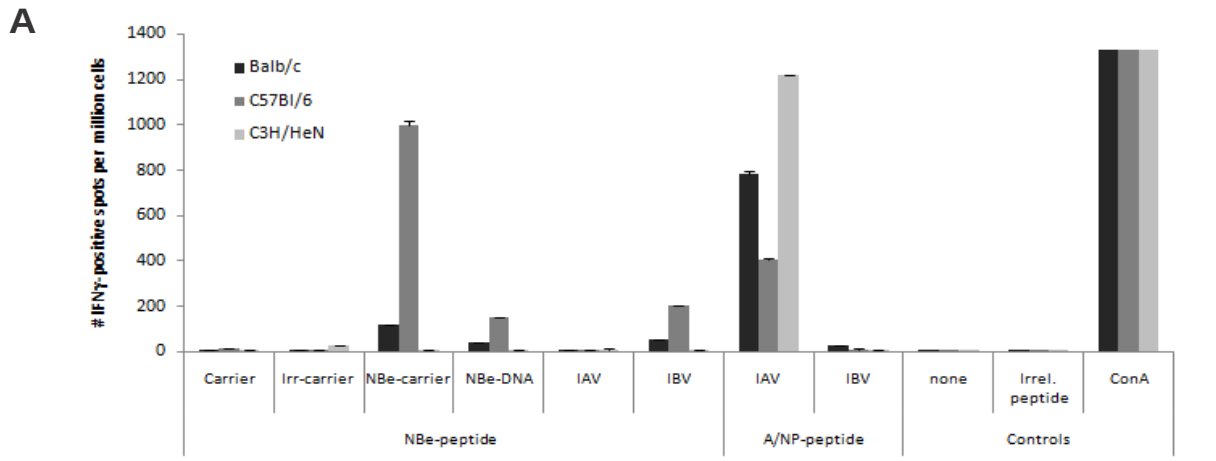

Figure 3. *Ex vivo* restimulation of splenocytes from immunized mice reveals the presence of NBe-restricted T-cell responses specific for the H2b-haplotype. Balb/c ($H2^d$), C57Bl/6 ($H2^b$) and C3H/HeN ($H2^k$) were immunized with the indicated immunogens twice, three weeks apart. Four weeks after the second immunization, induction of cellular responses was evaluated. A. IFNγ-specific elispot analysis detecting cellular responses. Splenocytes from immunized mice were restimulated in elispot plates using NBe-peptide or haplotype-specific A/NP peptides as positive controls. (Continues next page)

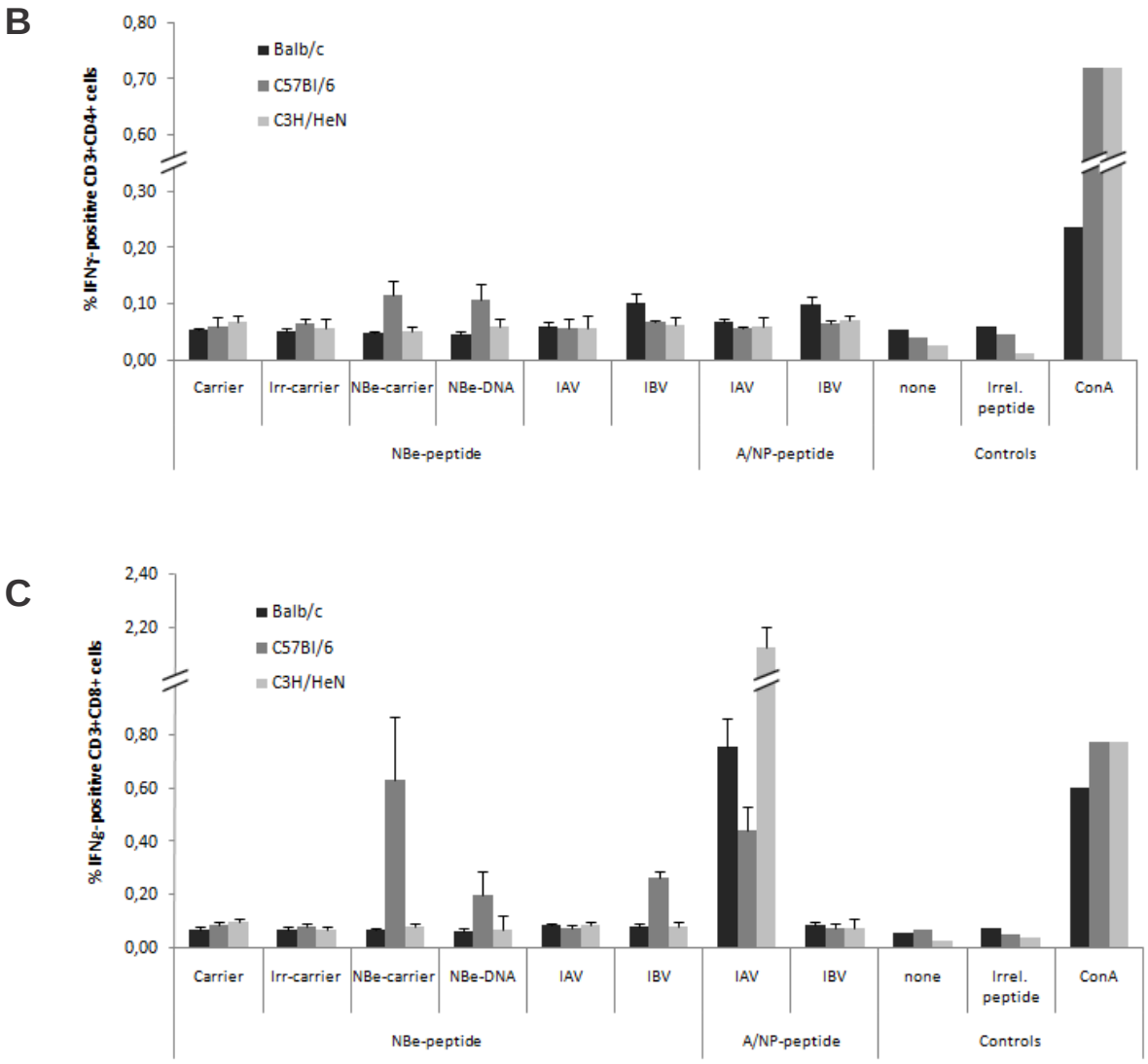

Figure 3. ***Continued.*** B and C. ICS analysis detecting cellular responses. Here, splenocytes were restimulated overnight with the same peptides as in the elispot assay and were stained for intracellular IFNγ-upregulation. The analysis was done for CD4+ T-cells (B) and CD8+ T-cells (C). For all peptide restimulations, n=3; control setting were only tested once.

To further evaluate the protective efficacy of the NBe- T cell response induced by immunization with NBe-tGCN4-Flag, C57Bl/6 mice were immunized three times with this NBe-fusion protein, Irr-tGCN4-Flag or tGCN4-Flag. Figure S5 shows pooled serum antibody titers against NBe-peptide, Irr-peptide and tGCN4-Flag carrier for all groups. As seen before, tGCN4-Flag immunized mice do not seroconvert, while NBe- or Irr-substituted tGCN4-Flag does evoke an anti-carrier response. Three weeks after the third immunization, mice were challenged with $0.075LD_{50}$ of influenza B/Memphis/12/97 virus. Lung homogenates were prepared on day three, four and five after infection, a window encompassing the likely timing for a specific T cell memory response, to evaluate antiviral activity [38-40]. As shown in Figure 4, immunization with NBe-tGCN4-Flag was associated with a significantly enhanced influenza B virus clearance from the lungs of challenged mice. In conclusion, the enhanced reduction of influenza B virus titers in the lungs of NBe-immune C57Bl/6 mice correlates with the presence of NBe-specific $CD8^+$ T cells.

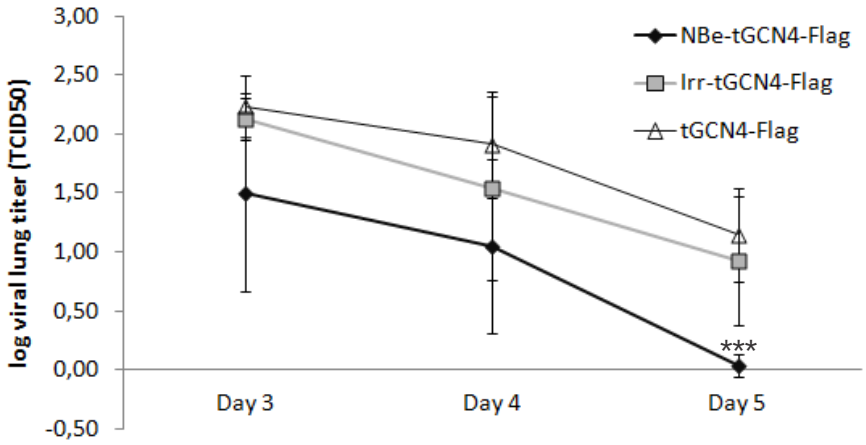

Figure 4. The NBe-tGCN4-Flag vaccine is able to induce anti-IBV immunity in C57Bl/6 mice. C57Bl/6 mice were immunized with tGCN4-Flag, NBe-tGCN4-Flag or the Irr-tGCN4-Flag vaccine. Viral lung titers were determined in TCID50 titration at the indicated time points after a sublethal (0.1 LD_{50}) IBV challenge three weeks after the third immunization. Asterisks above bars indicate significant differences with the titer of both control groups determined on the same day. For tGCN4-Flag and NBe-tGCN4-Flag groups, n=6 on all days; for Irr-tGCN4-Flag, n=5 on day 3 and n=4 on day 5 and 6. Shown are averages ± SD. ***, $p<0.001$.

Primed NBe-specific CD8 T cells are heterologously boosted by influenza B virus infection

Influenza B viruses circulate globally as epidemic strains. Therefore, we were interested in the effects of prior vaccination or infection on the magnitude of NBe-specific $CD8^+$ T cell responses upon subsequent vaccination or infection. Naïve C57Bl/6 mice were primed with NBe-tGCN4-Flag vaccination or exposed to a sublethal influenza B virus infection and 3 weeks later boosted homologously (*i.e.* with the same immunogen as for the priming) or heterologously (*i.e.* with the other immunogen as used for the priming). Cellular responses primed by NBe-vaccine were significantly boosted by a repeat vaccination (homologous boost). Strikingly, boosting with influenza B virus infection elicited a much higher NBe-specific cellular response as determined by ELISPOT (Figure 5A). This cellular booster effect was confirmed for NBe-tGCN4-Flag primed mice in an IFNγ-specific ICS assay. This experiment also confirmed that $CD8^+$ but not $CD4^+$ T cells are the main responders to NBe (Figure 5B). No NBe-peptide-specific boosting effect was observed by a double infection with influenza B virus, presumably due to the sterilizing immunity evoked by the primary infection. In contrast, NBe-tGCN4-Flag vaccination boosted infection-induced cellular responses of $CD8^+$ T cells. A polyclonal, influenza B virus-based restimulation analyzed by ICS, also showed clear $CD4^+$ T cell responses, mainly detectable when assayed on splenocytes from influenza B immunized mice (Figure 5B).

To further evaluate the breadth of the cellular anti-NBe response, the effect of different immunization routes and adjuvants on NBe-tGCN4-Flag vaccination was investigated. The NBe-immunogen was combined with the LTR192G, a detoxified mutant of the heat-labile *E. coli* enterotoxin [41], and administered intranasally or emulsified in Montanide ISA-720, a metabolisable, oil-based adjuvant [42], and injected subcutaneously. Alum-adjuvanted intraperitoneal and subcutaneous vaccination induced similar NBe-specific cellular responses, as measured in ELISPOT

(Figure 5C). Intranasal immunization with NBe-tGCN4-Flag in the presence of LTR192G however induced a higher level of splenocyte responses compared to either of the other immunization routes. Following heterologous boost with influenza B virus infection, both intraperitoneally and subcutaneously primed mice responded strongly (Figure 5C). Strikingly though, cellular immunity induced by intranasal priming was boosted more moderately by a subsequent influenza B virus infection. These results reveal that NBe-tGCN4-Flag immunization induces strong anti-NBe immune responses independent of the adjuvant or route of immunization used. In addition, heterologous boosting by influenza B virus infection strongly augments the vaccine-induced anti-NBe responses.

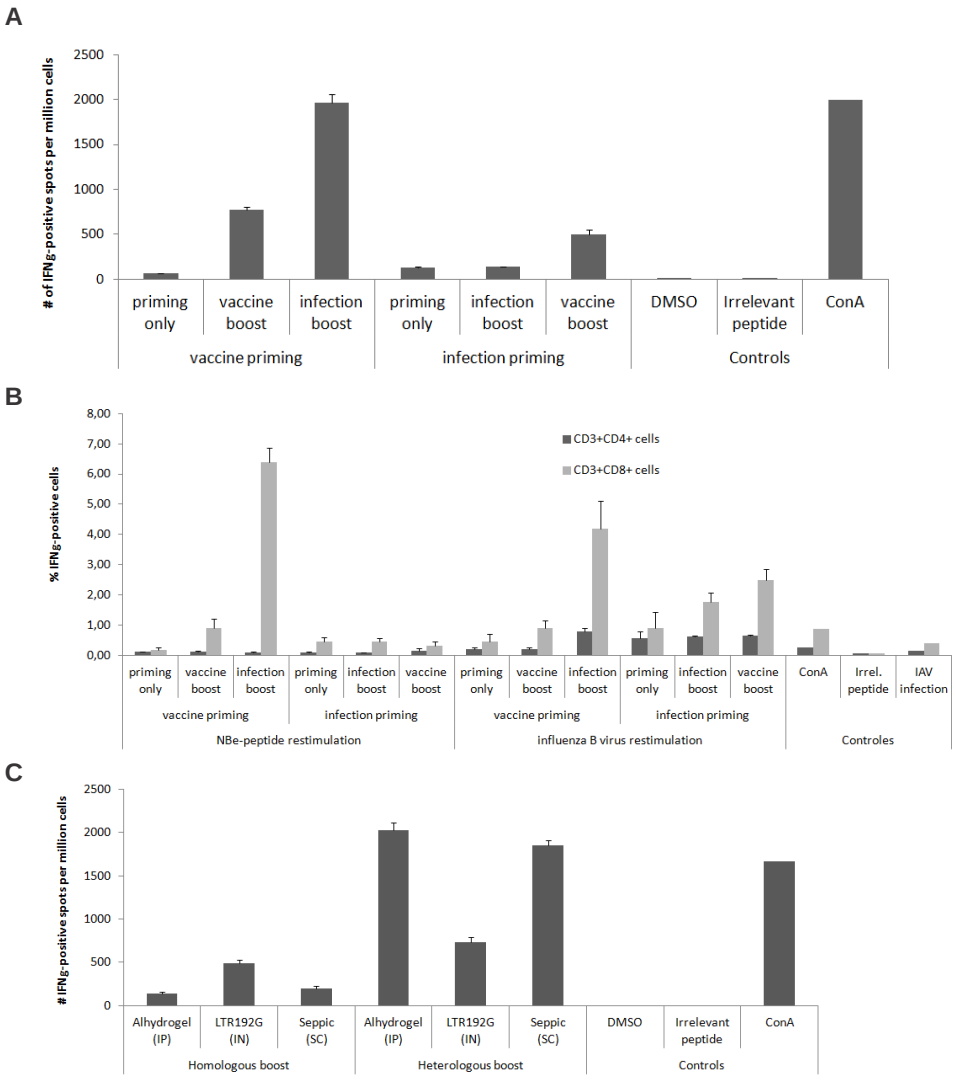

Figure 5. Homologous and heterologous prime-boost settings induce different levels of NBe-specific cellular responses. Mice were immunized once with NBe-tGCN4-Flag or IBV-infection or primed with NBe-tGCN4-Flag vaccination or IBV-infection and boosted homologously or heterologously with vaccination or infection. A. IFNγ-specific *ex vivo* elispot analysis through NBe-peptide restimulation of splenocytes. Vaccines were adjuvanted with alhydrogel, infections were done with a sublethal influenza B dose (0.1 LD_{50}). B. IFNγ-specific ICS of splenocytes from the same mice restimulated with NBe-peptide or IBV virus. C. IFNγ-specific *ex vivo* elispot analysis of splenocytes from homologously and heterologously immunized mice. For vaccine primings, NBe-tGCN4-Flag protein was mixed with different adjuvants as indicated and administered in appropriate ways. For the heterologous boost, 0.1 LD_{50} influenza B was used. IP = intraperitoneal, IN = intranasal, SC = subcutaneous. n=3 for all settings; controls were only measured once.

In vivo *killing assays reveal strong NBe-specific cytotoxic effects*

To functionally analyze the NBe-specific $CD8^+$ T cell responses, *in vivo* killing assays [43] were performed using differentially CFSE-labeled and peptide-pulsed splenocytes as targets in immunized recipient mice. In a first setup, mice were immunized twice with tGCN4-Flag carrier, NBe-tGCN4-Flag or were infected with a sublethal ($0.075LD_{50}$) dose of influenza B/Memphis/12/97 virus. NBe-directed cytotoxicity was assessed at four weeks and three months after the second immunization (Figure 6A and Figure S6A). In both instances, a clear shift in ratio between NBe-peptide and irrelevant peptide uploaded splenocytes was observed. This translates in specific NBe-directed killing when compared to the carrier only vaccinated groups (Figure 6A) and this specific cell killing activity is sustained over time. In a second setup, mice were infected twice with influenza A or influenza B virus or primed with NBe-tGCN4-Flag and heterologously boosted with influenza B virus, a protocol that results in very strong NBe-specific $CD8^+$ T cell levels (Figure 5B and 5C). Compared to naive mice, high NBe-directed *in vivo* killing was observed in the influenza B virus infected group (30% specific killing) and the heterologuosly boosted group (80% specific killing) (Figure 6B). In summary, these *in vivo* killing assays confirm the induction of NBe-directed cytotoxic responses in NBe-immune C57Bl/6 mice, either by vaccination or by sublethal influenza B virus infection, and these responses were sustained for at least three months post-immunization.

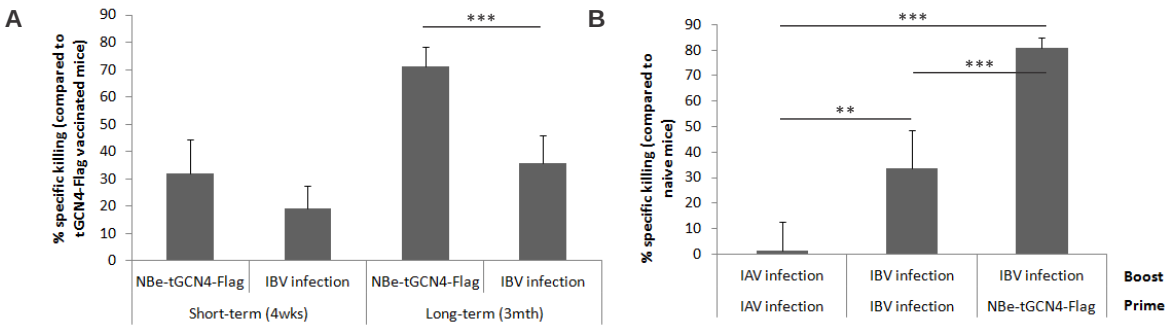

Figure 6. *In vivo* killing assays reveal strong anti-NBe cytotoxic effects of vaccine or infection induced T-cells. C57Bl/6 mice were prime-boost immunized as indicated. *In vivo* killing assays were performed by transfer of differentially CFSE-labelled peptide-pulsed splenocytes and FACS-analysis of the presence of CFSE-positive population in the spleen of recipient mice 24h post-transfer. Percent specific killing was calculated based on a control group. Shown are the averages for at least 4 mice per group ± SD. A. Mice were immunized twice with NBe-tGCN4-Flag or infected twice with a sublethal influenza B dose (0.1 LD_{50}). *In vivo* killing assays were performed 4 weeks or three months post-boost. The control group consisted of carrier only immunized mice (Figure S6). ***, $p<0.001$. B. Mice were infected twice with a sublethal dose (0.1 LD_{50}) of influenza A or B virus or NBe-tGCN4-Flag primed and boosted with influenza B infection. *In vivo* killing assays were performed 4 weeks post-boost using naïve mice as control group. n = 5 for all groups. **, $p<0.01$; ***, $p<0.001$.

$CD8^+$ T cell epitope mapping in NBe

Using the NetMHC 3.2 Server [44,45], NBe-derived peptides capable of binding $H2^b$, $H2^d$ and $H2^k$ alleles were predicted (Table 1). To assess if the predicted $H2^b$-restricted epitopes of NBe are generated *in vivo*, C57Bl/6 mice were immunized using different regimens. Homologously prime-boosted tGCN4-Flag, NBe-tGCN4-Flag, influenza A virus and influenza B virus infected mice were used. In an

additional group, NBe-tGCN4-Flag primed mice were subsequently boosted by a sublethal ($0.075LD_{50}$) influenza B virus infection. Controls for this set-up included carrier primed and influenza B boosted and NBe-tGCN4-Flag primed and influenza A virus boosted mice. This allowed us to ascertain the specificity of the heterologous boosting effect for NBe.

Table 1. Overview of predicted MHCI-restricted epitopes present in the NBe-sequence of influenza B. Predictions were made using the NetMHC 3.2 Server application.

Peptide length	H2b	H2d	H2k
8mer	3-ATFNYTNV-12	4-NYTNVNPI-13	
9mer	5-FNYTNVNPI-15		5-FNYTNVNPI-15
10mer			
11mer		4-NYTNVNPISHI-16	

As can be deduced from the ELISPOT results in Figure 7A, both the full-length NBe-peptide and the H2-K^b-restricted ATFNYTNV octameric peptide induce *in vitro* recall responses in splenocytes from NBe-immune mice. The outcome of the two control groups employing non-specific heterologous boosts (*i.e.* carrier only – influenza B virus infection and NBe-tGCN4-Flag – IAV infection) indicate that the boosting effect is NBe-specific, since the number of IFNγ-positive spots from this setting does not reach the level of the NBe-heterologous boost (NBe-tGCN4-Flag – IBV infection) group. From these data, we conclude that ATFNYTNV corresponds to the NBe-derived peptide that is presented by H2b MHC-I class molecules, following immunization with NBe-tGCN4-Flag or influenza B virus infection.

To identify the specific T cell players involved in NBe-based immunity, we performed a combination of in vivo killing assays with specific T cell depletions. For this, mice were primed with carrier only or NBe-tGCN4-Flag, and boosted homologously with the respective vaccine or heterologously by influenza B virus infection. Four weeks after the boost, mice were treated with PBS, anti-CD4 or anti-CD8 depleting antibody to achieve specific T cell depletion (Figure S7). Subsequently, an *in vivo* killing assay was performed to evaluate the effect of deletion of $CD4^+$ or $CD8^+$ T cell population on NBe-directed cytotoxicity. As depicted in Figure 7B, carrier only vaccinated mice did not show any baseline *in vivo* killing effect. In the homologously boosted NBe-tGCN4-Flag group, depletion of $CD8^+$ T cells strongly reduced the NBe-directed cytotoxicity compared to PBS-treated mice ($p<0.001$). Strikingly, CD4+ T cell depletion also had an effect, shifting specific killing from 61.8% to 32.7% ($p<0.01$). For the carrier-only primed group that was subsequently boosted with influenza B virus infection, the effect of depletion of both CD4+ and CD8+ T cell population completely abrogated *in vivo* NBe-specific killing ($p<0.001$). In contrast, CD4+ T cell depletion in NBe-tGCN4 primed mice that were boosted by an influenza B virus infection had no significant effect compared to PBS-treated mice ($p=0.082$), while CD8+ T cell deletion completely abolished specific cytotoxic effects ($p<0.001$).

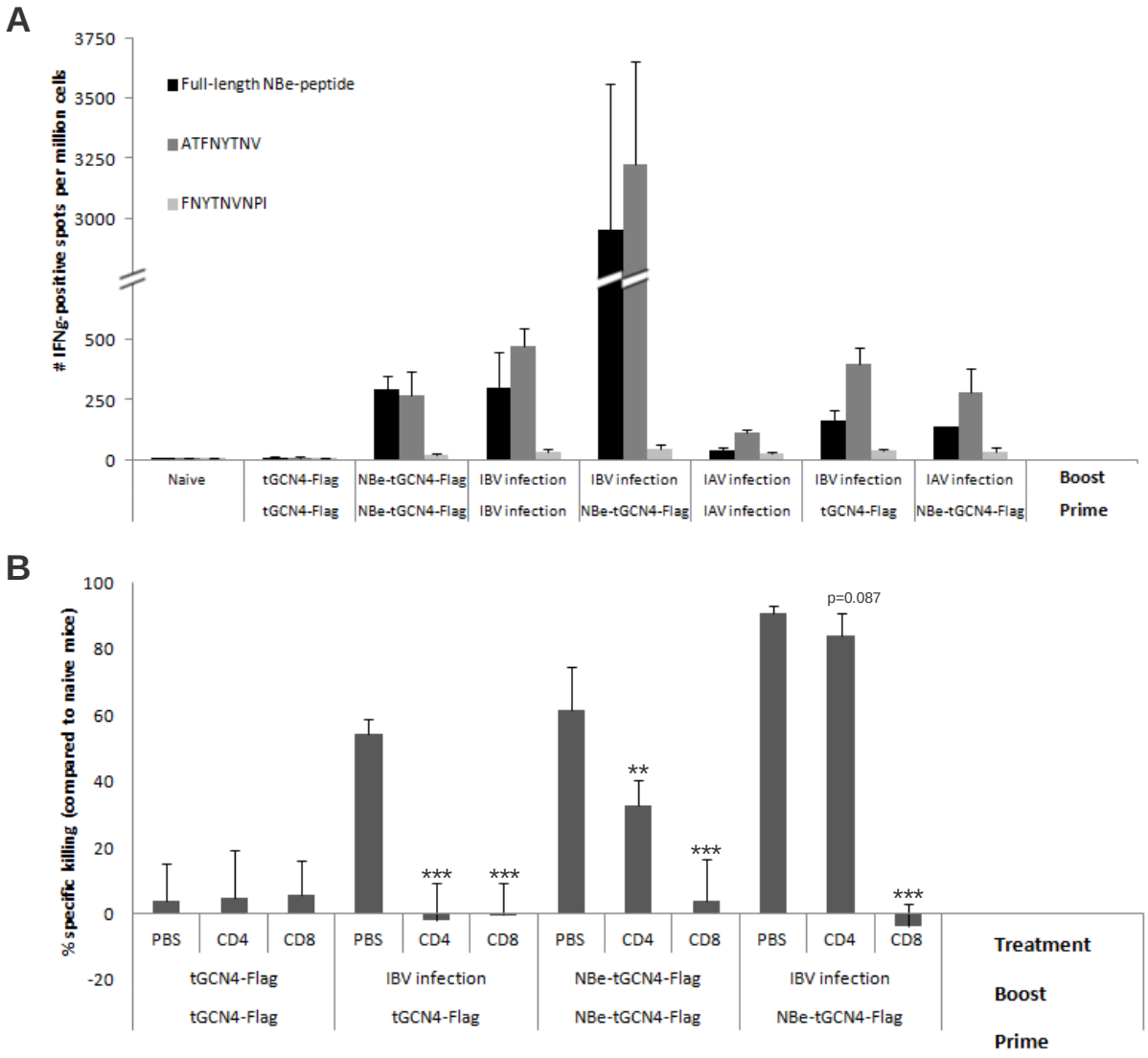

Figure 7. Identification of the NBe-specific H2^b-restricted epitope and the T-cell players involved. A. C57Bl/6 mice were immunized twice three weeks apart as indicated. Three weeks post-boost splenocytes were used for *ex vivo* elispot assays using the full-length NBe peptide, the octameric H2-K^b peptide or the nonameric H2-D^b peptide for restimulation. B. C57Bl/6 mice were immunized twice as indicated. Three weeks post-boost the mice were treated with PBS, 500µg anti-CD4 or 500µg anti-CD8 depleting antibody. 24 hours later, an *in vivo* killing assay was performed. Shown are the specific killing rates, analyzed 24 hours later by FACS, as compared to naïve recipient mice. Asterisks indicate significant differences compared to PBS treated mice. n=5 for all groups. **, p<0.01; ***, p<0.001.

In conclusion, the CD8$^+$ T cell restricted NBe-specific epitope maps to the more membrane distal ATFNYTNV peptide sequence in NB. In addition, depletion of CD8+ T cells completely abolished the killing of targe T cells that had been uploaded with this octameric NBe peptide, while an auxiliary role for CD4+ T cells was revealed in single and homologous immunization settings, possibly because these CD4$^+$ T cells provided help to CD8$^+$ T cells in killing NBe-uploaded targets.

Local immunity induced towards NBe is strongly protective

We noticed that heterologous immunization (by exposure to influenza B virus infection) after intranasal priming with LTR192G-adjuvanted NBe-tGCN4-Flag resulted in a more limited cellular NBe-specific boosting effect compared to intraperitoneal or subcutaneous priming (Figure 5C). Therefore, we immunized C57Bl/6 mice twice intranasally with NBe-tGCN4-Flag or tGCN4-Flag only, both in presence of LTR192G adjuvant. Mice that were mock-immunized with PBS only were included as additional controls to exclude an antiviral adjuvant effect, as has been reported for related molecules [46,47]. Four weeks after the boost immunization, cellular responses were analyzed in different lymph nodes and organs. In all lymph nodes tested, strong vaccine-induced NBe-specific responses were detected in ELISPOT. Strikingly, NBe-specific cellular responses in cells derived from the lung were remarkably high (Figure S8A), indicative of a powerful local adaptive cellular immune response.

The animals were challenged eight weeks after the booster immunization. A sublethal dose of $0.3LD_{50}$ influenza B virus was used to evaluate morbidity, while a $0.075LD_{50}$ dose was administered for analysis of pulmonary virus replication,. NBe-tGCN4-Flag vaccination significantly reduced morbidity following infection (Figure 8A). During the recovery phase, a small adjuvant effect was detectable, as PBS immunized mice recovered more slowly than the two adjuvanted groups. Quantification of the viral lung titers revealed that NBe-specific immunity inhibited viral replication in the lungs (Figure 8B). At all three time points evaluated, viral titers in the NBe-tGCN4-Flag group were significantly lower than in both control groups, and the challenge infection was cleared totally by day 6 post-challenge.

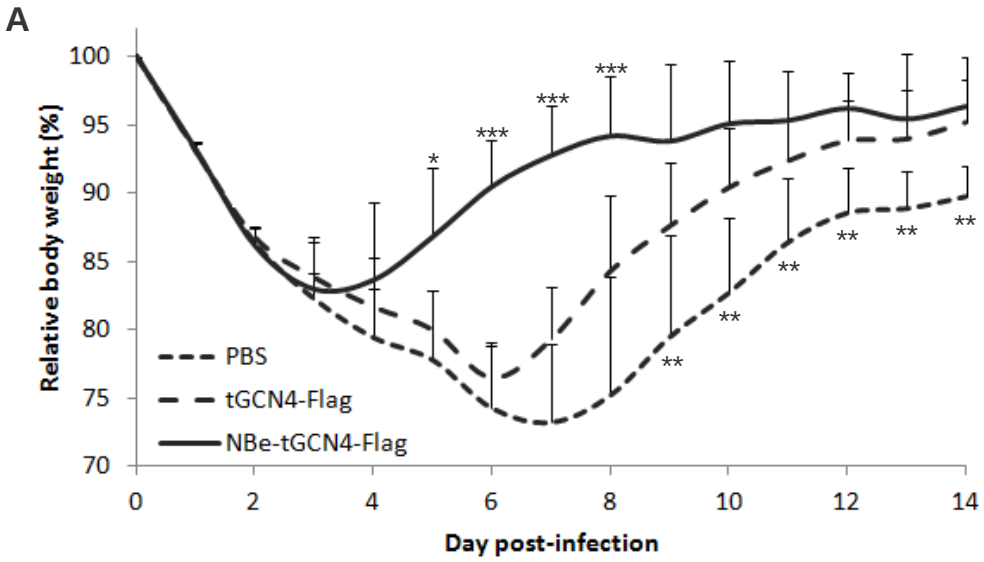

Figure 8. Mucosal immunization of mice with LTR192G adjuvanted NBe-tGCN4-Flag affords strong anti-influenza B immunity. C57Bl/6 mice were immunized with PBS, adjuvanted carrier only or adjuvanted NBe-tGCN4-Flag twice three weeks apart. Eight weeks post-boost, the mice were challenged with sublethal influenza B doses. A. Morbidity following sublethal challenge (0.3 LD_{50}), illustrated by relative weight. Asterisks indicate significant differences compared to both other groups. n=5 for PBS and carrier only groups, n=6 for NBe-tGCN4-Flag group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. B. Viral lung titers determined at the indicated timepoints following a sublethal challenge (0.067 LD_{50}). Asterisks indicate significant differences compared to both other groups. n=5 for all groups. **, $p<0.01$; ***, $p<0.001$.

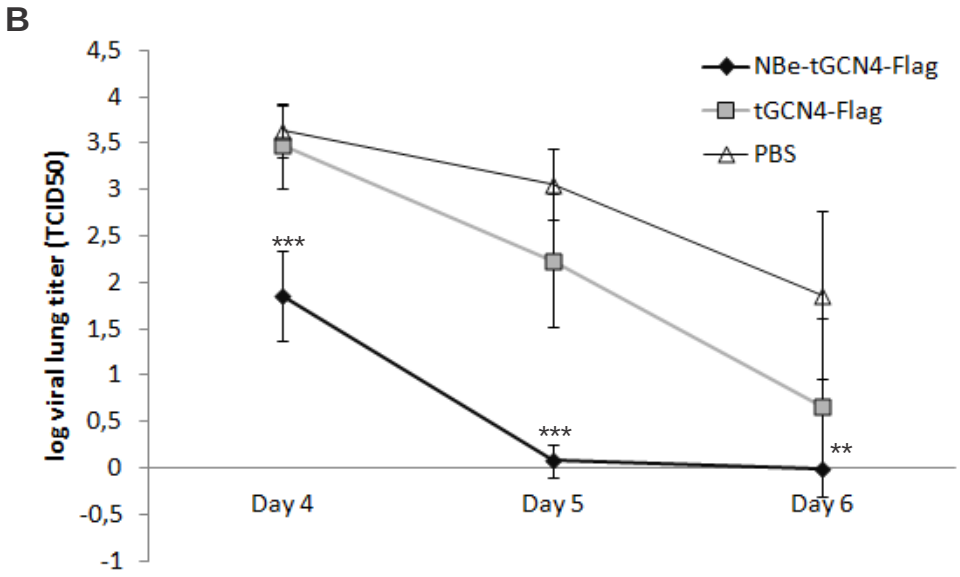

Figure 8. ***Continued.***

As a final control, the challenged mice were used for an *in vivo* killing assay two weeks after challenge. Adoptively transferred CFSE-labeled peptide-pulsed splenocytes were not only detected in the spleen of recipient mice, but also in the inguinal lymph nodes (ILN) and lungs. As shown in Figure S8B, specific killing was comparable in the three immune compartments analyzed, within each group. NBe-directed cytotoxicity was similar in both PBS and adjuvanted carrier only groups, reflecting anti-NBe immunity induced by the single infection. However, the NBe-tGCN4-Flag immunized mice showed a higher specific killing activity, reaching over 90%, reflecting the heterologous boosting effect observed above following intraperitoneal priming (Figure 6B).

Taken together, these data show that the adjuvanted NBe-tGCN4-Flag vaccine induces strong local NBe-directed cellular immunity, inhibiting viral replication in the lung and improving the clinical outcome of the infection.

Discussion

Even without an animal reservoir feeding novel genetic reassortments, the epidemic burden of influenza B viruses is quite substantial. The co-circulation of two antigenically distinct lineages in the human population offers a basis for gene shuffling, creating influenza B virus strains with improved fitness or a difficult to predict antigenic make-up [5,9,10]. Additionally, vaccination- or infection-induced immune pressure will cause the virus to drift and contributes to evasion of existing immunological memory [6]. Surveillance data scrutinizing a number of sequential influenza seasons has revealed that vaccine mismatches occur fairly frequently [48-51]. Co-circulation of viruses belonging to either of the influenza B lineages will lead to diminished vaccine efficacy and supports the drift of influenza B viruses [48]. Vaccine mismatch puts the (vaccinated) high-risk populations in a vulnerable position and increases the risk of potential hospitalization or death [14,15,48,49]. To avoid hurdles faced with classical vaccines, we approached influenza B vaccination from a different angle. We focused on the conserved ectodomain (NBe) of the NB protein in an attemt to induce cross-reactive immunity against influenza B virus infection in an experimental model. Within the $H2^b$-background of C57Bl/6 mice, NBe-based immunization induced strong NBe-specific CD8+ T cell responses that were associated with enhanced lung virus clearance and reduced morbidity. Furthermore, and although a number of human CTL epitopes of influenza B viruses have been identified [52,53], to our knowledge, the NBe-CTL epitope is the first murine-specific influenza B epitope identified.

Using a tetramerizing mutant of the leucine zipper of the GCN4 transcription factor [27,28], we initially aimed to develop an antibody-inducing vaccine. Surprisingly though, vaccination with NBe-tGCN4-Flag protein, even in the presence of a Th2-skewing adjuvant like Alhydrogel [54], induced strong cellular responses directed to NBe. In recent years, many studies have been performed to elucidate the mechanism by which aluminum salt, which have a track-record of over 80 years, exert their adjuvancy [55-60]. Aluminum adjuvantation is largely based on its crystalline structure, leading to lysosomal destabilization upon lipid membrane binding and uptake by dendritic cells (DCs), inducing the release of PGE_2, a Th2-mediator [57]. Aluminum salts also induce cell death, leading to the release of uric acid and host DNA, endogenous danger signals amplifying the crystalline aluminum signal [58,60]. Additionally, cytokines induced during immunization will stimulate a Th2-driven immune response. Nonetheless, NBe-tGCN4-Flag combined with Alhydrogel induces strong cellular responses, indicating effective cross-presentation upon vaccination. For this, soluble antigen has to be taken up by DCs through phagocytosis and processed for MHCI-presentation [61]. However, membrane binding of DCs by aluminum salts will usually not lead to productive endocytosis, disfavoring cross-presentation [56,61]. One way to overcome aluminum salt induced Th2-skewing, is the addition of pro-inflammatory proteins such as TLR agonists, as is the case with the ASO4 adjuvant system [62]. The specific mechanism by which NBe-tGCN4-Flag protein vaccination promotes a strong $CD8^+$ T cell response however, remains uncertain. Clearly though, the NBe-epitope on its own appears to be strong and naturally processed, irrespective of its way of presentation. The combination with the tGCN4-Flag motif may further strengthen the immunological properties of the NBe-epitope. The specific role of tGCN4 in potential immune modulation is under further scrutiny.

For respiratory diseases like influenza, mucosal immunization might serve as a preferred mode of vaccination. Not only local immune responses are induced, providing a first line of defense at the site of entry, but also systemic immunity is generated. Intranasal immunization with NBe-tGCN4-Flag induced enhanced protection compared to the intraperitoneal route. High local cellular responses

are induced, and two immunizations (with an eight week period between boost and infection) show stronger inhibitory effects on influenza B virus replication than three i.p. immunizations (Figure 4 and 8B). In the homologous and heterologous prime-boost setting we were initially surprised that intranasal priming is only boosted moderately by influenza B virus infection (Figure 5C). The higher (systemic) response that homologous intranasal boosting induced points to a stronger anti-NBe response during priming compared to other vaccination routes, which might be strong enough to neutralize the low-dose influenza B boost to a certain extent [63]. Although residual adjuvant effects of LTR192G limiting boosting cannot be excluded, clearly, intranasal NBe-based immunization significantly restrains pulmonary replication of influenza B virus.

Protective immunity against influenza A virus induced by M2e-fusion constructs is largely dependent on the induction of M2e-specific IgG [24,64]. However, the initial vaccination experiment with NBe-tGCN4-Flag using different mouse strains revealed no correlation between NBe-specific serum IgG and protection from morbidity and death following a potential lethal challenge with $2.5LD_{50}$ influenza B virus. It is important to note that in the mouse model for influenza B virus infection the challenge dose needed to obtain a lethal outcome is orders of magnitudes larger than the dose required for most mouse-adapted influenza A virus strains. We used mouse-adapted B/Memphis/12/97 [29] as challenge virus, for which, under our conditions, 1 LD_{50} corresponds to 10^6 pfu for C57Bl/6 mice, a dose up to 4-logs higher than required for influenza A viruses. The lack of protection seen in an initial challenge experiment (Figure 2), despite the induction of NBe-specific T cells with anti-influenza B activity (Figure 3 and 4), might be linked to the levels of T cell induction and viral dose used for infection. Using lower infectious doses of $0.075LD_{50}$, clear effects on viral replication can be seen (Figure 2B and 4). Moreover, two mucosal immunizations, inducing higher relative numbers of NBe-specific T cells (Figure 5C) and strong local immunity (Figure S8), reduced disease manifestation significantly after a $0.3LD_{50}$ sublethal influenza B infection in NBe-tGCN4-Flag immunized mice (Figure 8). A too low ratio between T cell level and viral dose might mask protective efficacy of induced T cells, an observation also made in adoptive transfer experiments using different levels of transferred T cells [65].

To identify the specific T cell players involved in NBe-directed immunity, we combined depletion of specific T cell populations with an *in vivo* killing assay. Based on intracellular cytokine staining assays, it was apparent that $CD8^+$ T cells are the prime NBe-reactive induced species (Figure 3). However, minor NBe-specific $CD4^+$ T cell contribution was consistently detected. The depletion assay did reveal a role for $CD4^+$ T cells in (non-infectious) NBe-directed killing. Single prime or homologous prime-boost immunizations clearly need $CD4^+$ T cell help to obtain maximal effect. This is in agreement with studies showing the influence of helper T cells on $CD8^+$ memory expansion and responsiveness [66,67]. For the heterologous boost setting, however, the cytotoxic NBe-directed killing response appears to operate without the help from CD4 T cells. The robust T cell induction that heterologous prime-boost immunizations evoke apparently circumvents the need for help for a recall response when encountering antigen. $CD4^+$ T cells specifically assist in the expansion and survival of memory $CD8^+$ T cells [68,69]; the high levels of $CD8^+$ T cells generated by the heterologous boosting scheme, and potentially their phenotype, might avoid the need for $CD4^+$ T cell induced expansion [70].

The finding that heterologous boosting schemes are so efficient in inducing cellular immune responses is encouraging. Evidently, many research groups have uncovered the power of heterologous prime-boost regimens in inducing T-cell and antibody responses to combat diseases

such as influenza, HIV and malaria [71,72]. Classically though, the prime immunization consists of a viral vector or a DNA vaccination, followed by a different viral vector or protein boost. There are only a limited number of studies involving a protein to achieve priming [73]. In our setting, boosting vaccine-induced responses by a sublethal influenza B virus dose is robust. NBe-tGCN4-Flag vaccination on its own elicits strong responses, inhibiting viral replication and reducing excessive morbidity. A following influenza B infection however boosts cellular immunity considerably. Translated to an epidemic setting, this implies that seasonal infection would boost immunity induced by the (infection-permissive) NBe-based vaccination. Hence, influenza B virus exposure would serve as a natural booster immunization, extending protection induced by the conserved NB ectodomain. This opens prospects for general influenza vaccination strategies, based on non-neutralizing, infection-permissive vaccines [74]. Additional candidates for this are the M2-ectodomain of influenza A, the LAH of influenza A/H3 viruses which induce non-neutralizing antibodies, and validated cytotoxic T cell epitopes. Immunity induced by these vaccine candidates would be boosted with every infection, which may not be completely blocked but might subside to subclinical levels.

Our results provide new insights into the potential of inducing effective cytotoxic T cell responses to combat infectious diseases. While exploring the value of a novel influenza B restricted $H2^b$-restricted epitope, we uncovered the strength of this epitope in depressing influenza B virus infection and the potential power of our carrier protein in inducing T cell responses. Moreover, the heterologous viral boosting effects add further evidence for the advantageous nature of infection-permissive vaccines.

Material and methods

Mice, viruses and influenza challenges

For all *in vivo* studies, 6-8 week old female mice were used. BALB/c mice were obtained from Charles River (Italy), C57Bl/6J mice from The Jackson Laboratory (France) and C3H/HeN mice from Harlan (The Netherlands). Mice were housed under specific pathogen-free conditions and fed *ad libitum*. All infection experiments were conducted under BSL-2 containment and were approved by the local Institutional Ethics Committee on Experimental Animals of Ghent University.

All influenza B infections were performed using the mouse-adapted M15-strain of B/Memphis/12/97 [29]. In our model, one LD_{50} dose corresponds to 10^6 pfu for C57Bl/6J mice, 1.25 x 10^6 pfu for Balb/c mice and 6 x 10^5 pfu for C3H/HeN mice. For potential lethal infection, a $2.5LD_{50}$ dose was administered; sublethal infections were done with 0.075 or 0.3 LD_{50} as indicated in the text. For influenza A virus infections, mouse-adapted A/Puerto Rico/8/34 was used, with $1LD_{50}$ corresponding to approximately 50-100 pfu. Sublethal infections as form of immunization were performed with 0.075 LD_{50}. All infections were done by instilling virus diluted in 50µL phosphate-buffered saline (PBS) into the nostrils of ketamine (50mg/kg) + xylazin (7.5mg/kg) anaesthetized mice. Following infection, mice were monitored for weight loss as a measurement of morbidity. Mice that had lost more than 30% of their initial weight, were euthanized, as demanded by the approved protocol, and regarded as not-protected .

Construction, purification and cross-linking of NBe-tGCN4-Flag

Construction of tGCN4-Flag and NBe-tGCN4-Flag expressing vectors was done as previously described [28]. Briefly, overlapping oligonucleotides (Invitrogen) were used to create the coding sequences for the tetramerizing tGCN4 leucine zipper variant [27], the NB ectodomain and the triple Flag-tag. Using classical cloning techniques based on restriction enzyme digestion and PCR, the three domains were fused in frame and inserted in the pLT32H expression vector using the *Nde*I and *Hin*dIII restriction sites [75], leading to *pL*-promotor driven expression in bacterial systems.

For expression, BL21 CodonPlus (Stratagene) bacteria, bearing the pICA2 repression plasmid [28], were transformed with the tGCN4-protein expressing pLT32H vectors. Inductions were done by diluting a 40mL bacterial culture, grown overnight at 28°C in Luria Broth (LB), 1/50 in 2L of LB. Cultures were grown further at 28°C until they reached an optical density at 600nm of 0.5-0.8. At this point, the cultivation temperature was raised to 42°C, leading to denaturation of the temperature-sensitive cI^{ts}-repressor [76], expressed by pICA2, liberating the *pL*-promotor and leading to expression of the chimeric tGCN4-proteins. After 4h of induction, bacteria were collected by pelleting the culture through centrifugation (10 min, 4°C, 6000g). If needed, bacterial pellets were stored dry at -20°C until use.

To purify the chimeric tGCN4-proteins, bacterial pellets were resuspended in 1/10 of the original volume using 50mM Tris/HCl pH8.0 + 150mM NaCl (4°C). Bacteria were disrupted by sonication and immediately treated for 30min at 65°C to precipitate contaminating bacterial proteins, relying on the heat-resistant properties of the tGCN4 leucin zipper [27]. Next, denatured proteins and bacterial debris were removed by centrifugation (1h, 4°C, 20.000g) and filtered through a 0.45µm filter. Using an Akta purification station, cleared lysates were applied on a SourceQ anion exchange column, followed by a linear gradient elution using 50mM Tris pH8.0 + 1M NaCl, going from 0% to 100% elution buffer. Elution fractions were analyzed through SDS-polyacrylamide gel electrophoresis (SDS-PAGE); fractions containing the protein of interest were pooled and concentrated using Vivaspin columns (GE Healthcare) with a 5 kDa cut-off. Samples were loaded on a calibrated Superdex 75 prep-grade gel filtration column and eluted with PBS. Fractions containing tGCN4-proteins of the desired size and

purity (Figure S1) were pooled and concentrated using Vivaspin columns (5 kDa cut-off) to concentration of 1-30 mg/mL without losing solubility. Protein solutions were batched out and stored at -80°C.

Purified NBe-tGCN4-Flag, M2e-tGCN4 and BM2e-tGCN4 were used for cross-linking experiments [28]. Briefly, stock solutions of 50mM BS^3 (bis(sulfosuccinimidyl)suberate; Pierce) in water and 25mM DSP (dithiobis(succinimidylpropionate); Pierce) in DMSO were prepared freshly prior to use. These were diluted to 5 mM final concentration in PBS containing 10µg of the different tGCN4-proteins. Cross-linking reaction was allowed to continue for 1h at room temperature, after which the reactions were stopped by adding 50 mM Tris, pH 6.8. Samples were divided in two and boiled in non-reducing (50 mM Tris pH 6.8, 10% Glycerol) or reducing (50 mM Tris pH 6.8, 10% Glycerol, 20mM β-mercapto-ethanol) sample buffer, followed by analysis on SDS-PAGE and Coomassie-staining.

Construction and expression of NBe-M2 chimeras

The cDNA coding for M2 and NB was obtained by reverse transcription using RNA prepared from A/Puerto Rico/8/34 or B/Memphis/12/97 virus-infected MDCK cells. Exchange of the two ectodomains was done by fusion PCR, using oligonucleotides spanning the NBe and the M2 transmembrane region. All coding sequences were cloned into the pCAGGS vector, allowing constitutive expression driven by the chicken β-actin/rabbit β-globin hybrid promoter. A single Flag-tag was introduced C-terminally to allow detection.

For transfections tests, HEK293 T cells were seeded at 2 x 10^5 cells in a 6-well plate in complete DMEM medium (DMEM + 10% FCS + L-Gln + sodium pyruvate + penicillin/streptomycin). 16h later, cells were transfected with 1µg plasmid DNA using calcium phosphate precipitation. 24h later, cells were lysed and analyzed by SDS-PAGE followed by Western blotting. Flag-tagged proteins were visualized using rabbit anti-Flag antibody detection, combined with alkaline phosphatase labeled goat anti-rabbit-AP secondary antibody and NBT/BCIP substrate (Roche) for detection.

For immunofluorescence assays, HEK293T cells were seeded on aceton-treated coverslips in 24-well plates at a density of 2 x 10^4 cells/well in complete DMEM. 16h later, cells were transfected with 250 ng pCAGGS-based constructs. 24h later, cells were fixed with 4% paraformaldehyde and permeabilized for 5 min using 0.05% Triton X-100 in PBS + 20 mM Glycine. After blocking for 1 h using a 1% PBS-based BSA solution, cells were stained with rabbit anti-Flag and murine antiserum raised against M2e-tGCN4 (diluted 1/500) and NBe-tGCN4-Flag (diluted 1/50). Secondary staining was done using goat anti-rabbit Alexa-fluor-647 conjugated antibody and goat anti-mouse Alexa-fluor-488 antibody (Invitrogen). Cells were mounted with Vectashield containing DAPI for nuclear counterstain before microscopic analysis.

Immunization methods and challenge experiments

All protein immunizations were with 5 µg of protein per dose. Intraperitoneal vaccinations using Alhydrogel (Brenntag Biosector A/S) as adjuvant were performed as described [28]. For intranasal immunization, proteins were diluted in 50 µL PBS and adjuvanted with 1 µg LTR192G. The vaccine preparation was instilled into the nostrils of ketamine/xylazine anaesthetized mice. Subcutaneous vaccinations were given in a volume of 100 µL, consisting of 26 µL PBS containing the tGCN4-protein and 74 µL Montanide ISA-720 (Seppic). DNA immunizations were administered intramuscularly, using 100 µg LPS-free plasmid DNA in a volume of 100 µL PBS.

One week before the first immunization and two weeks after every subsequent immunization, blood was drawn from the lateral tail veins for serological analysis. Subsequent immunizations had an interval of three

weeks; the number of immunizations is mentioned in the legends. Unless stated otherwise, challenge of mice was performed three weeks after the last immunization. For follow-up studies, body weight of challenged mice was determined on a daily basis. Lung homogenates were prepared on different days after challenge by tissue homogenization in 1 mL PBS using a Heidolph RZR 2020 homogenizer. Viral titers were determined by $TCID_{50}$ titration on MDCK cells in quadruplicate and calculated according to the method of Reed and Muench [77].

ELISA procedure

Antibody responses induced by vaccination were quantitated by ELISA. For peptide-specific ELISA (NBe, Irr), Maxisorp immune-plates (Nunc) were coated overnight at 37°C with 50 µL of a 2 µg/mL peptide-solution in 100 mM sodium carbonate buffer, pH 9.6. Anti-tGCN4-Flag titers were determined using plates coated overnight at 4°C with 2 µg/mL tGCN4-Flag in carbonate buffer. After coating, plates were washed three times with PBS + 0.1% Tween-20 and blocked with PBS + 3% milk. Sera from immunized mice were diluted (1/3 dilution series) in PBS + 0.05% Tween-20 + 1.5% milk and incubated on the ELISA plates for 1.5 h at room temperature. After washing three times, subtype-specific HRP-linked goat anti-mouse secondary antibodies (Bio-Connect) were incubated on the plates for a further 1.5 h at room temperature. Following a final wash step, titers were detected using TMB substrate (Pharmingen, BD). The reaction was stopped after 10 min by adding an equal amount of 1 M H_2SO_4. Plates were read at 450 nm, with background measurement at 655nm. Endpoint titers are determined as the last dilution of immune serum giving an ELISA signal at least twice as high as pre-immune serum.

ELISPOT and intracellular cytokine stainings (ICS)

Murine IFNγ-specific ELISPOT assays (U-Cytech Biosciences) were performed according to the manufacturer's instructions. Briefly, Maxisorp immune-plates were coated with monoclonal anti-IFNγ antibodies overnight at 4°C. Splenocytes were isolated from mice three weeks after the last immunization by gentle homogenization of the spleen in PBS, followed by red blood cell lysis using hypotonic ammonium chloride buffer. Cells were seeded at densities of 1-3 x 10^5 cells per well and restimulated overnight with 2 µg/mL of the described peptides (synthetized by Pepscan, The Netherlands). Cells were flicked out of the wells and plates were washed with PBS + 0.1% Tween-20. Next, plates were incubated for 1.5 h with biotinylated polyclonal anti-IFNγ antibodies, followed by a wash step and incubation with GABA anti-biotin antibodies. Spots were detected with specific reagent leading to silver staining. Spots were manually counted using an inverted light microscope. Control set-ups use restimulation with DMSO only, irrelevant Ova-peptide, and the polyclonal concanavalin A (ConA) stimulus.

For ICS, splenocytes were prepared and restimulated as for the ELISPOT assays. Restimulations however were performed in 12-well suspension plates using 1 mL cell-suspension at 3 x 10^6 cells per well. Further processing was done using the CytoFix/Perm kit (BD) according to the manufacturer's protocol. In short, after 4-6 h of incubation with peptides (2 µg/mL), 1 µg of Brefeldin A was added to stop secretion of cytokines. After a further incubation of 8-12 h, cells were collected, washed in PBS + 1 % BSA and incubated for 30 min at room temperature with anti-CD3ε-PE, anti-CD4-FITC and anti-CD8α-PerCP.Cy5 (all from BD) for staining of surface markers. After a further wash, cells were fixed, permeabilized, and stained for intracellular IFNγ. Cells were washed and subsequently analyzed on a FACS Calibur or LSRII cytometer using CelQuestPro (BD) software. Positive (ConA) and negative (irrelevant peptide) restimulations were included as controls as well as antibody-staining controls (i.e. single antigen stains, isotype controls). were included.

In vivo *killing assays*

The *in vivo* killing assays are based on the protocol of Barber *et al.* [43]. Briefly, splenocytes were prepared, as described above, from naive mice and resuspended in complete RPMI (RPMI + 10% FCS + L-Gln + sodium pyruvate + penicillin/streptomycin) to a final density of 3-5 x 10^6 cells per mL. One half of the cell population was pulsed with a specific NBe-related peptide (full-length NBe-peptide or octameric ATFNYTNV, as indicated) and the other half with an unrelated Ova-peptide (SIINFEKL). Peptides were added at a concentration of 10 µg/mL, followed by incubation for 1-2 h at 37°C in a CO_2-incubator. After this, peptide-pulsed cells were washed and stained differentially with 5 µM or 0.5 µM CFSE (carboxyfluorescein diacetate succinimidyl ester; *Invitrogen*) according to the manufacturer's protocol. Subsequently, cell populations were mixed in equicellular amounts and adoptively transferred to immunized mice at the indicated time points by intravenous injection of approximately 5 x 10^6 cells. Twenty four h later, spleens (or other immune organs, as indicated) were isolated and cells were prepared and analyzed on a FACS Calibur using CelQuestPro (BD) software. To calculate specific killing rates, ratios of CFSE (high) and CFSE (low) cell numbers were compared in test groups with an irrelevant (naive) mouse group or negative control, using the equation 100-100*[ratio (test setting) / ratio (control setting)].

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References

1. Scholtissek, C. Molecular evolution of influenza viruses. *Virus Genes* **11**, 209-215 (1995).
2. Hay, A.J., Gregory, V., Douglas, A.R. & Lin, Y.P. The evolution of human influenza viruses. *Philosophical Transactions of the Royal Society of London. Series B* **356**, 1861 (2001).
3. Hao, W. Evidence of intra-segmental homologous recombination in influenza A virus. *Gene*.
4. Fouchier, R.A.M*., et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of virology* **79**, 2814 (2005).
5. Chen, R. & Holmes, E.C. The evolutionary dynamics of human influenza B virus. *Journal of Molecular Evolution* **66**, 655-663 (2008).
6. Shen, J., Kirk, B.D., Ma, J. & Wang, Q. Diversifying selective pressure on influenza B virus hemagglutinin. *Journal of medical virology* **81**, 114-124 (2009).
7. Shaw, M.W*., et al.* Reappearance and global spread of variants of influenza B/victoria/2/87 lineage viruses in the 2000-2001 and 2001-2002 seasons. *Virology* **303**, 1-8 (2002).
8. Chen, J.M*., et al.* Exploration of the emergence of the Victoria lineage of influenza B virus. *Archives of virology* **152**, 415-422 (2007).
9. McCullers, J.A., Wang, G.C., He, S. & Webster, R.G. Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *Journal of virology* **73**, 7343 (1999).
10. McCullers, J.A., Saito, T. & Iverson, A.R. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *Journal of virology* **78**, 12817 (2004).
11. Belshe, R.B. The need for quadrivalent vaccine against seasonal influenza. *Vaccine* **28**, D45-D53 (2010).
12. Tsai, H.P*., et al.* Increasing appearance of reassortant influenza B virus in Taiwan from 2002 to 2005. *Journal of clinical microbiology* **44**, 2705 (2006).
13. Yang, C.F*., et al.* Genetic sequence analysis of influenza viruses and illness severity in ill children previously vaccinated with live attenuated or inactivated influenza vaccine. *Vaccine* **28**, 5128-5134 (2010).
14. Camilloni, B*., et al.* An influenza B outbreak during the 2007/2008 winter among appropriately immunized elderly people living in a nursing home. *Vaccine* **28**, 7536-7541 (2010).
15. Belshe, R.B., Coelingh, K., Ambrose, C.S., Woo, J.C. & Wu, X. Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. *Vaccine* **28**, 2149-2156 (2010).
16. Roose, K., Fiers, W. & Saelens, X. Pandemic preparedness: toward a universal influenza vaccine. *Drug News Perspect* **22**, 80-92 (2009).
17. Stanekova, Z. & Vareckova, E. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. *Virology Journal* **7**, 351 (2010).
18. Saelens, X. The influenza matrix protein 2 as a vaccine target. *Future Virology* **3**, 167-178 (2008).
19. Schotsaert, M., De Filette, M., Fiers, W. & Saelens, X. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. *Expert review of vaccines* **8**, 499 (2009).
20. Steel, J*., et al.* Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **1**, e00018-00010 (2010).
21. Bommakanti, G*., et al.* Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. *Proceedings of the National Academy of Sciences* **107**, 13701 (2010).
22. Wang, T.T*., et al.* Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 18979-18984 (2010).
23. Bianchi, E*., et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
24. El Bakkouri, K*., et al.* Universal vaccine based on ectodomain of matrix protein 2 of Influenza A: Fc receptors and alveolar macrophages mediate protection. *The Journal of Immunology* **186**, 1022 (2011).
25. Rimmelzwaan, G.F., Fouchier, R.A.M. & Osterhaus, A.D.M.E. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. *Current opinion in biotechnology* **18**, 529-536 (2007).
26. McMurry, J.A., Johansson, B.E. & De Groot, A.S. A call to cellular & humoral arms: enlisting cognate T cell help to develop broad-spectrum vaccines against influenza A. *Human vaccines* **4**, 148 (2008).
27. Harbury, P.B., Zhang, T., Kim, P.S. & Alber, T. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* **262**, 1401 (1993).

28. De Filette, M.*, et al.* An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. *Journal of Biological Chemistry* **283**, 11382 (2008).
29. McCullers, J.A., Hoffmann, E., Huber, V.C. & Nickerson, A.D. A single amino acid change in the C-terminal domain of the matrix protein M1 of influenza B virus confers mouse adaptation and virulence. *Virology* **336**, 318-326 (2005).
30. Shaw, M.W., Choppin, P.W. & Lamb, R.A. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proceedings of the National Academy of Sciences* **80**, 4879 (1983).
31. Williams, M.A. & Lamb, R.A. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Molecular and Cellular Biology* **6**, 4317 (1986).
32. Brassard, D.L., Leser, G.P. & Lamb, R.A. Influenza B virus NB glycoprotein is a component of the virion. *Virology* **220**, 350-360 (1996).
33. Betakova, T., Nermut, M.V. & Hay, A.J. The NB protein is an integral component of the membrane of influenza B virus. *Journal of general virology* **77**, 2689 (1996).
34. Williams, M.A. & Lamb, R.A. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Molecular and Cellular Biology* **8**, 1186 (1988).
35. Misplon, J.A., Lo, C.Y., Gabbard, J.D., Tompkins, S.M. & Epstein, S.L. Genetic control of immune responses to influenza A matrix 2 protein (M2). *Vaccine* **28**, 5817-5827 (2010).
36. Garigliany, M.M.*, et al.* Influenza A strain-dependent pathogenesis in fatal H1N1 and H5N1 subtype infections of mice. *Emerg Infect Dis* **16**, 595-603 (2011).
37. Betakova, T. & Hay, A.J. Stability and function of the influenza A virus M2 ion channel protein is determined by both extracellular and cytoplasmic domains. *Archives of virology* **154**, 147-151 (2009).
38. Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *The Journal of Immunology* **152**, 1653 (1994).
39. Flynn, K.J.*, et al.* Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* **8**, 683-691 (1998).
40. Deliyannis, G.*, et al.* Induction of long-term memory CD8+ T cells for recall of viral clearing responses against influenza virus. *Journal of virology* **76**, 4212 (2002).
41. Dickinson, B.L. & Clements, J.D. Dissociation of Escherichia coli heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infection and immunity* **63**, 1617 (1995).
42. Lawrence, G.W., Saul, A., Giddy, A.J., Kemp, R. & Pye, D. Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. *Vaccine* **15**, 176-178 (1997).
43. Barber, D.L., Wherry, E.J. & Ahmed, R. Cutting edge: rapid in vivo killing by memory CD8 T cells. *The Journal of Immunology* **171**, 27 (2003).
44. Lundegaard, C.*, et al.* NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. *Nucleic acids research* **36**, W509 (2008).
45. Lundegaard, C., Lund, O. & Nielsen, M. Accurate approximation method for prediction of class I MHC affinities for peptides of length 8, 10 and 11 using prediction tools trained on 9mers. *Bioinformatics* **24**, 1397 (2008).
46. De Filette, M.*, et al.* The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. *Vaccine* **24**, 544-551 (2006).
47. Tritto, E.*, et al.* The acquired immune response to the mucosal adjuvant LTK63 imprints the mouse lung with a protective signature. *The Journal of Immunology* **179**, 5346 (2007).
48. D'Agaro, P.*, et al.* The molecular epidemiology of influenza viruses: a lesson from a highly epidemic season. *Journal of clinical pathology* **61**, 355 (2008).
49. Goni, N.*, et al.* Influenza B viruses isolated in Uruguay during the 2002-2005 seasons: Genetic relations and vaccine strain match. *Virus research* **123**, 100-104 (2007).
50. Skowronski, D.M.*, et al.* Estimating vaccine effectiveness against laboratory-confirmed influenza using a sentinel physician network: results from the 2005-2006 season of dual A and B vaccine mismatch in Canada. *Vaccine* **25**, 2842-2851 (2007).
51. Belongia, E.A.*, et al.* Effectiveness of inactivated influenza vaccines varied substantially with antigenic match from the 2004–2005 season to the 2006–2007 season. *Journal of Infectious Diseases* **199**, 159 (2009).

52. Robbins, P.A., Garboczi, D.N. & Strominger, J.L. HLA-A* 0201 complexes with two 10-Mer peptides differing at the P2 anchor residue have distinct refolding kinetics. *The Journal of Immunology* **154**, 703 (1995).
53. Robbins, P.A., Rota, P.A. & Shapiro, S.Z. A broad cytotoxic T lymphocyte response to influenza type B virus presented by multiple HLA molecules. *International immunology* **9**, 815 (1997).
54. Aimanianda, V., Haensler, J., Lacroix-Desmazes, S., Kaveri, S.V. & Bayry, J. Novel cellular and molecular mechanisms of induction of immune responses by aluminum adjuvants. *Trends in pharmacological sciences* **30**, 287-295 (2009).
55. Kool, M.*, et al.* Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *The Journal of experimental medicine* **205**, 869 (2008).
56. Flach, T.L.*, et al.* Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nature Medicine* **17**, 479-487 (2011).
57. Kuroda, E.*, et al.* Silica Crystals and Aluminum Salts Regulate the Production of Prostaglandin in Macrophages via NALP3 Inflammasome-Independent Mechanisms. *Immunity* **34**, 514-526 (2011).
58. Kool, M. & Willart, M.A. An unexpected role for uric Acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity* **34**, 527-540 (2011).
59. Korsholm, K.S., Petersen, R.V., Agger, E.M. & Andersen, P. T helper 1 and T helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. *Immunology* **129**, 75-86 (2010).
60. Marichal, T.*, et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nature Medicine* **17**, 996-1002 (2011).
61. Rock, K.L. Exiting the outside world for cross-presentation. *Immunity* **25**, 523-525 (2006).
62. Didierlaurent, A.M.*, et al.* AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *The Journal of Immunology* **183**, 6186 (2009).
63. Wherry, E.J. & Ahmed, R. Memory CD8 T-cell differentiation during viral infection. *Journal of virology* **78**, 5535 (2004).
64. Neirynck, S.*, et al.* A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nature Medicine* **5**, 1157-1163 (1999).
65. Hillaire, M.L.B.*, et al.* Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. *Journal of General Virology* **92**, 2339-2349 (2011).
66. Ryu, S.J.*, et al.* Cognate CD4 help is essential for the reactivation and expansion of CD8 memory T cells directed against the hematopoietic cell–specific dominant minor histocompatibility antigen, H60. *Blood* **113**, 4273 (2009).
67. Xiao, Y., Peperzak, V., Keller, A.M. & Borst, J. CD27 instructs CD4+ T cells to provide help for the memory CD8+ T cell response after protein immunization. *The Journal of Immunology* **181**, 1071 (2008).
68. Matter, M.S., Claus, C. & Ochsenbein, A.F. CD4+ T cell help improves CD8+ T cell memory by retained CD27 expression. *European journal of immunology* **38**, 1847-1856 (2008).
69. Cui, W. & Kaech, S.M. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunological reviews* **236**, 151-166 (2010).
70. Hovav, A.H.*, et al.* The impact of a boosting immunogen on the differentiation of secondary memory CD8+ T cells. *Journal of virology* **81**, 12793 (2007).
71. Radosevic, K., Rodriguez, A., Lemckert, A. & Goudsmit, J. Heterologous primeboost vaccinations for poverty-related diseases: advantages and future prospects. *Expert review of vaccines* **8**, 577-592 (2009).
72. Lu, S. Heterologous prime-boost vaccination. *Current opinion in immunology* **21**, 346-351 (2009).
73. Bouillet, L.E.M.*, et al.* Long-term humoral and cellular immune responses elicited by a heterologous Plasmodium vivax Apical Membrane Antigen 1 protein-prime/adenovirus-boost immunization protocol. *Infection and immunity*, IAI. 05048-05011v05041 (2011).
74. Bodewes, R., Kreijtz, J.H.C.M. & Hillaire, M. Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8+ T-cell responses in mice. *Journal of general virology* **91**, 1743 (2010).
75. Mertens, N., Remaut, E. & Fiers, W. Versatile, multi-featured plasmids for high-level expression of heterologous genes in Escherichia coli: overproduction of human and murine cytokines. *Gene* **164**, 9-15 (1995).

76. Remaut, E., Stanssens, P. & Fiers, W. Plasmid vectors for high-efficiency expression controlled by the PL promoter of coliphage lambda. *Gene* **15**, 81-93 (1981).
77. Reed, L.J. & Muench, H. A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* **27**, 493 (1938).

Supplementary Figures

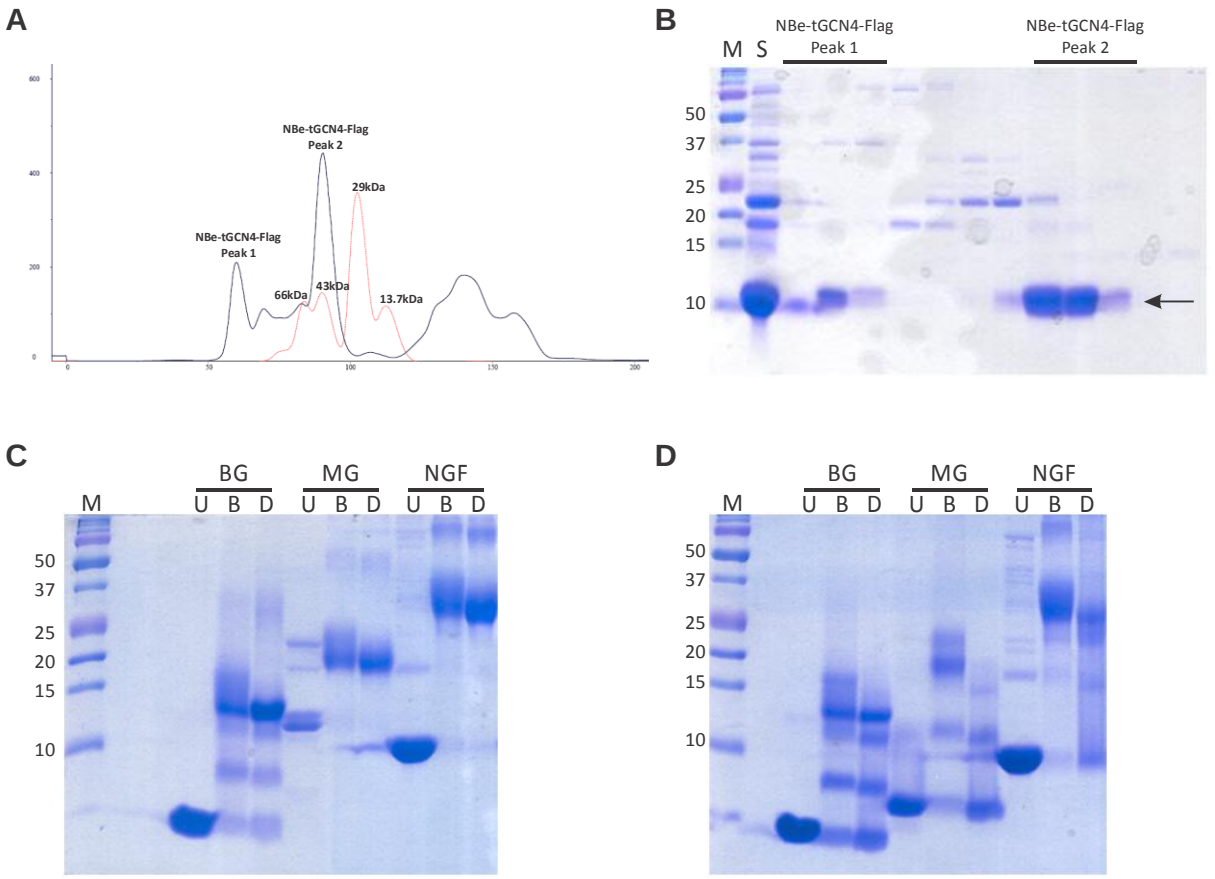


Figure S1. The NBe-tGCN4-Flag protein behaves as a tetrameric protein. A-B. Gel filtration profile of the final purification step of NBe-tGCN4-Flag (A), overlayed with a calibration run using proteins of different sizes. Analysis of fractions corresponding to the NBe-tGCN4-Flag gel filtration on polyacrylamide gel shows the protein of interest (indicated by the arrow) to be present in two distinct peaks (B). Peak 2 corresponds to NBe-tGCN4-Flag protein with the expected molecular weight (35,24kDa), while peak 1 contains aggregated chimeric NBe-protein, or protein associated with bacterial contaminants. The purest fractions of this peak were further used throughout this study. M = gel marker (mass in kDa), S = starting material for the gel filtration. **C-D.** Cross-linking experiment using two homobifunctional cross-linkers with similar reactivity, DSP and BS^3, of which DSP contains a reducable disulfide bridge in its spacer arm. Validated tGCN4-based tetrameric proteins were taken along as controls [28]. Analysis was done on polyacrylamide gel under non-reducing (C) and reducing (D) conditions. M = gel marker (mass in kDa), BG = BM2e-tGCN4, MG = M2e-tGCN4, NGF = NBe-tGCN4-Flag; U = uncross-linked, B = BS^3-cross-linked, D = DSP-cross-linked.

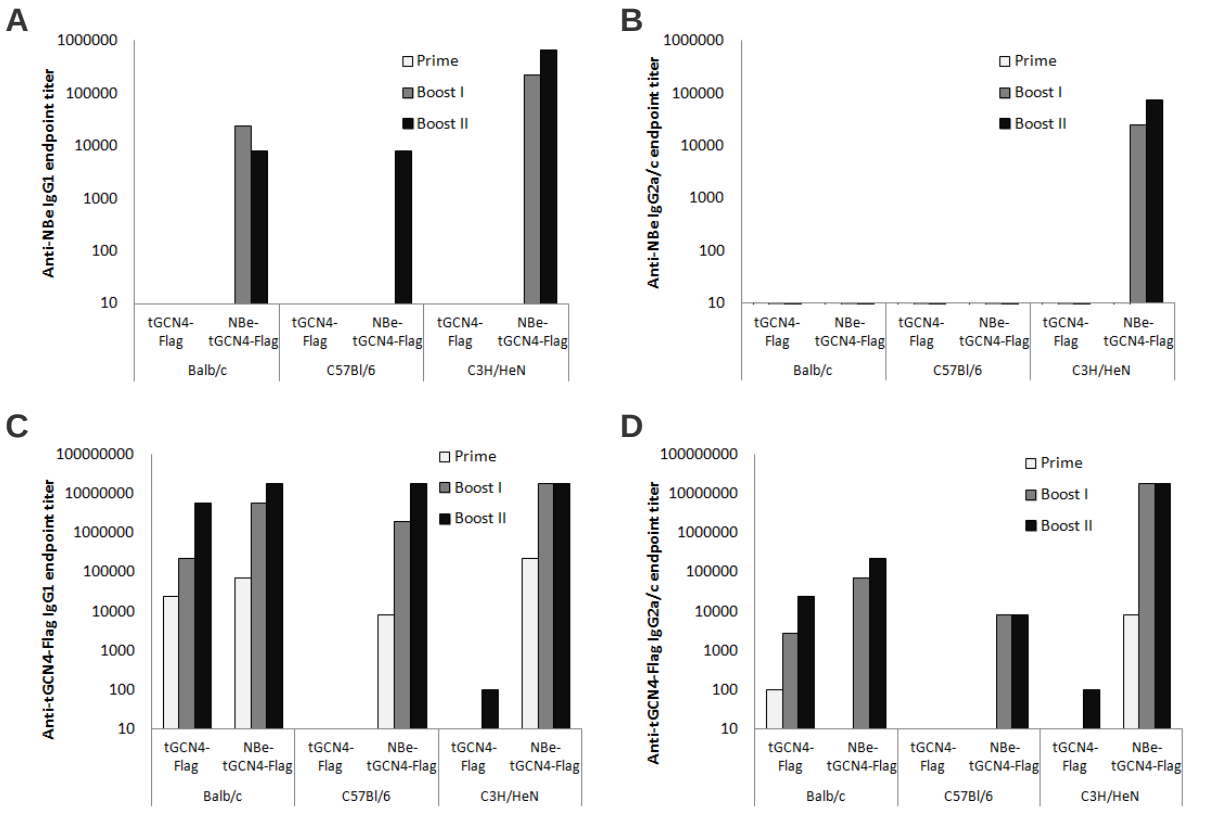


Figure S2. Mice of different genetic backgrounds react differently to NBe-tGCN4-Flag and carrier only vaccination. Mice from different genetic backgrounds, Balb/c ($H2^d$), C57Bl/6 ($H2^b$) and C3H/HeN ($H2^k$), were immunized three times with carrier only or NBe-tGCN4-Flag in combination with Alhydrogel. Serum endpoint titers of pooled sera determined two weeks after each immunization in NBe-peptide (A & B) or tGCN4-Flag-specific (C & D) ELISA. Both IgG1 (A & C) and IgG2a or IgG2c (B & D) specific titers were analyzed.

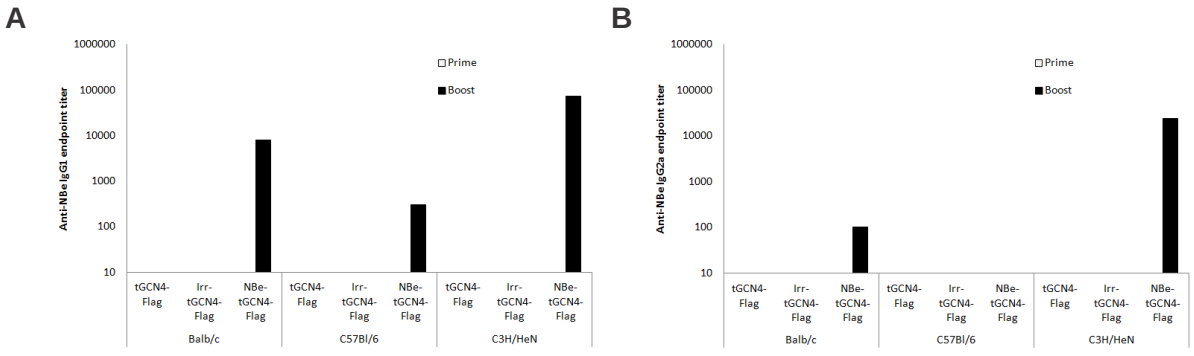


Figure S3. Irr-tGCN4-Flag immunization evokes anti-Irr and anti-tGCN4-Flag responses in mice of different genetic backgrounds. Mice from different genetic backgrounds, Balb/c ($H2^d$), C57Bl/6 ($H2^b$) and C3H/HeN ($H2^k$), were immunized twice with carrier only, Irr-tGCN4-Flag or NBe-tGCN4-Flag in combination with Alhydrogel. Serum endpoint titers of pooled sera determined two weeks after each immunization in NBe-peptide (A & B), tGCN4-Flag (C & D) or Irr-peptide specific ELISA. Both IgG1 (A, C, E) and IgG2a or IgG2c (B, D, E) specific titers were analyzed.

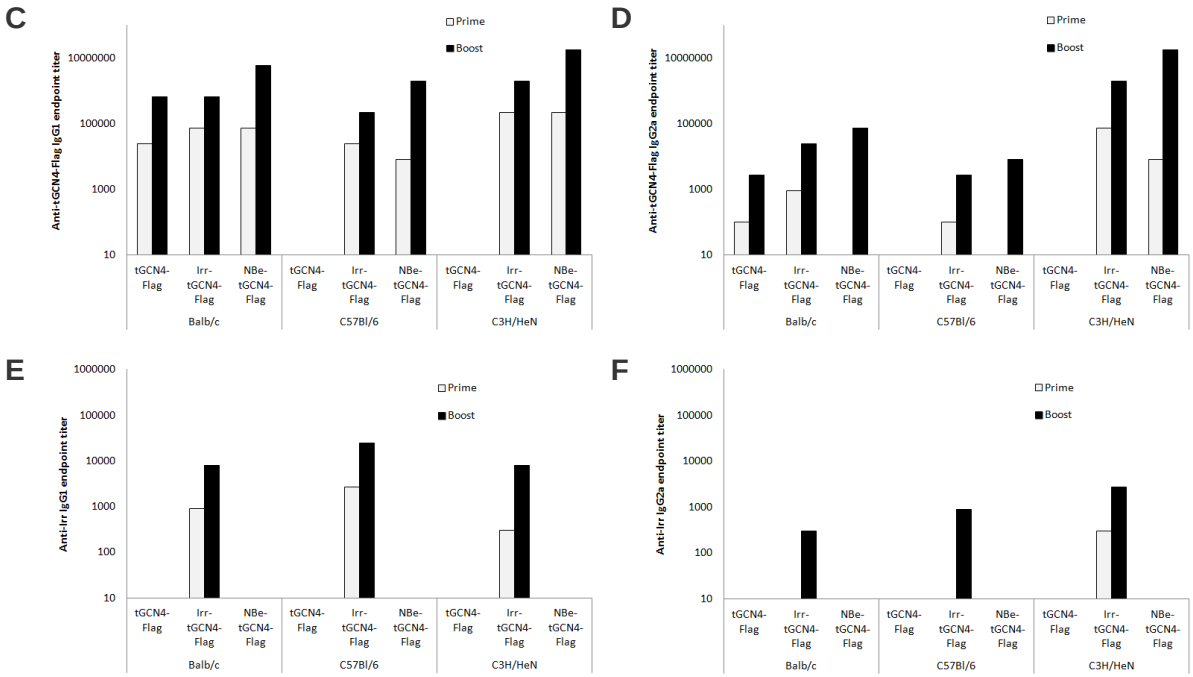


Figure S3. ***Continued.***

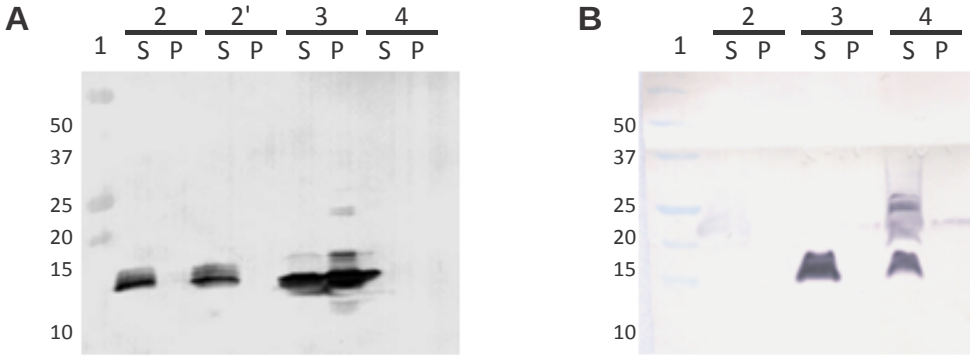


Figure S4. Eukaryotic expression of chimeric NBe-M2-Flag protein after transient transfection. A. Western blot analysis of lysates of HEK cells transiently transfected with pCAGGS-based constructs expressing Flag-tagged full-length influenza ion channels, showing nice expression of M2 and BM2, but no detectable levels of NB. 1 = protein gel marker (mass in kDa), 2, 2' = M2-Flag, 3 = BM2-Flag, 4 = NB-Flag; S = supernatant after cell lysis, P = pellet after cell lysis. **B.** Expression of chimeric Flag-tagged NBe-M2-Flag, in which the M2-ectodomain is replaced by NBe. 1 = protein gel marker (mass in kDa), 2 = mock transfection (empty pCAGGS), 3 = M2-Flag, 4 = NBe-M2-Flag; S = supernatant after cell lysis, P = pellet after cell lysis. **C.** Immunofluorescense staining of HEK cells transiently transfected with pCAGGS vectors expressing NB-Flag (top), M2-Flag (bottom) or NBe-M2-Flag (middle). Staining was done with immune serum raised using M2e-tGCN4 (Balb/c mice) and NBe-tGCN4-Flag (C3H/HeN mice). As control rabbit anti-Flag staining was used, showing clear expression of all three proteins in this set-up. Note immune serum from mice immunized with NBe-tGCN4-Flag, carrying a triple Flag-tag, does not cross-react with the single Flag-tag of M2-Flag, indicating staining of NBe with this serum is specific.

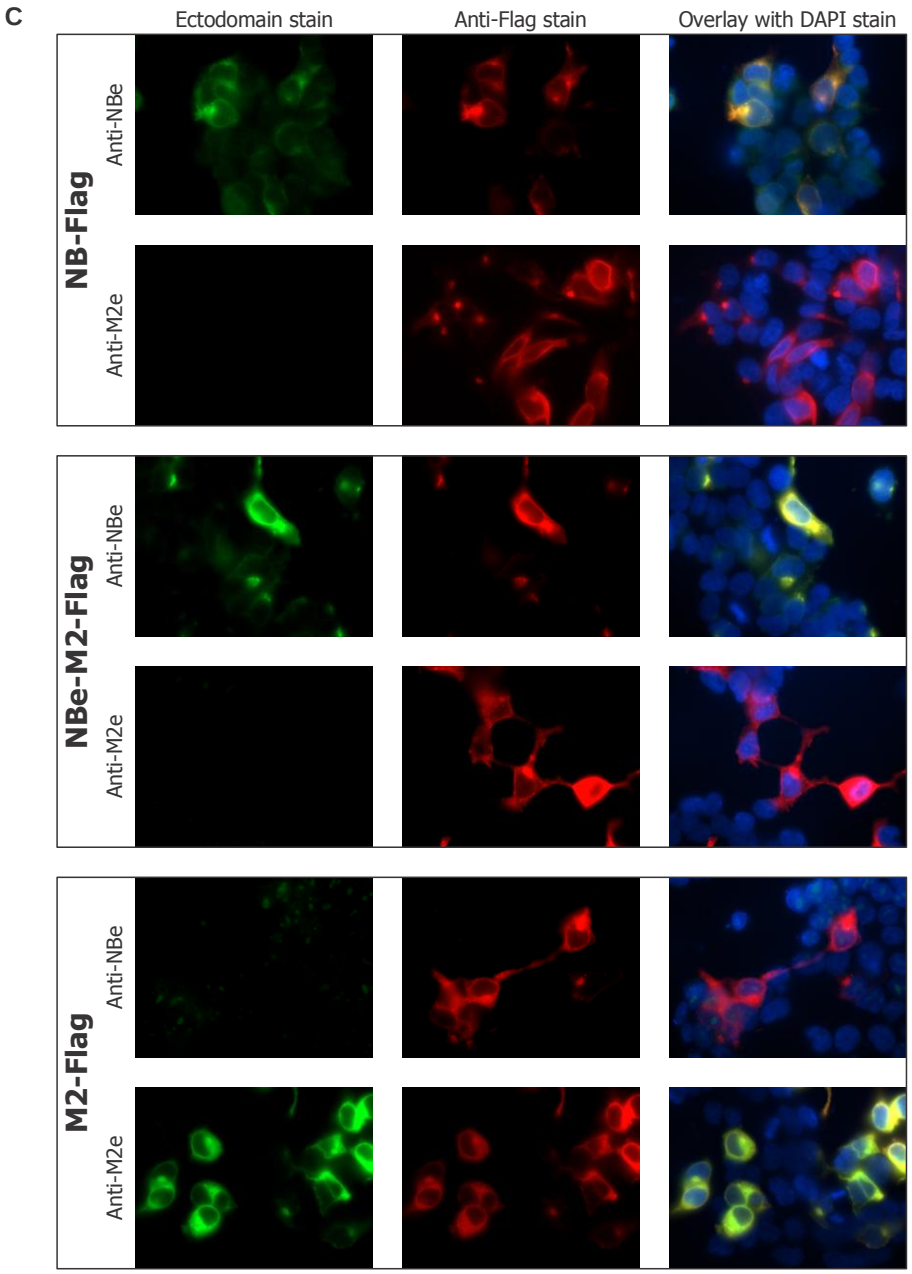


Figure S4. ***Continued.***

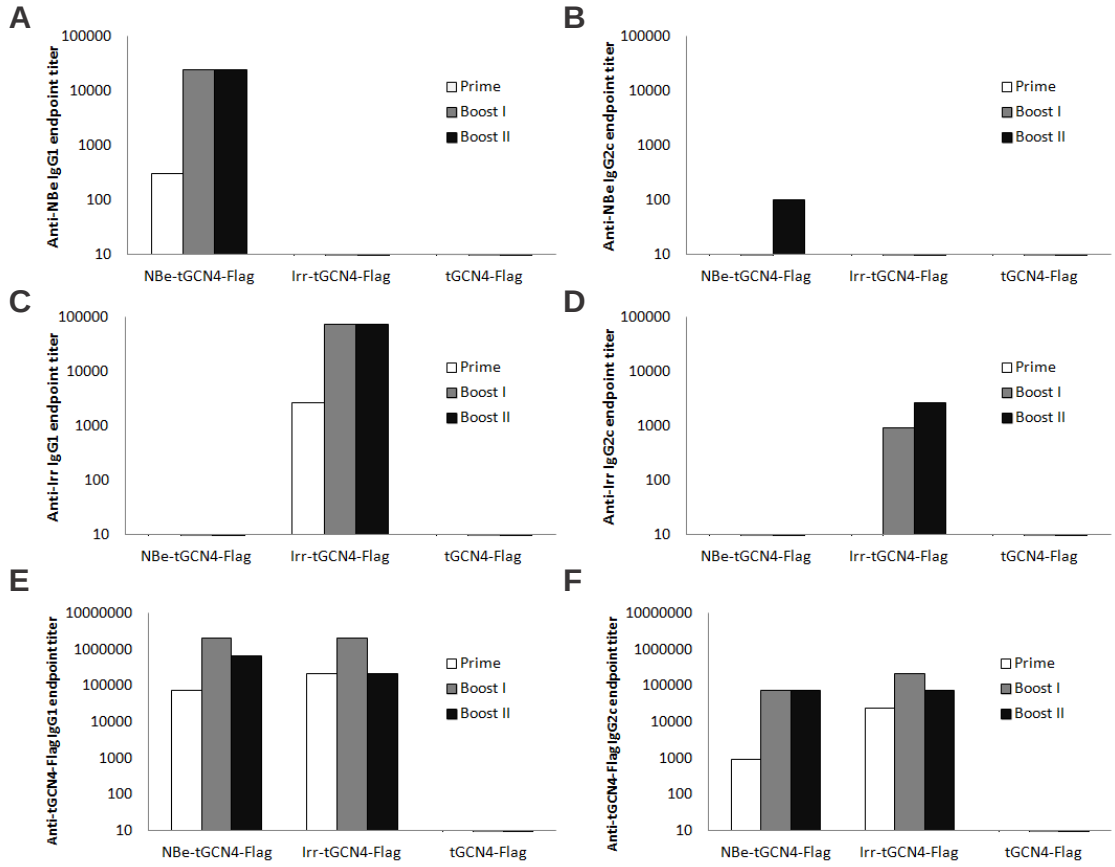


Figure S5. Induction of antibody titers using different carrier-based immunogens in C57Bl/6 mice. C57Bl/6 mice were immunized three times with tGCN4-Flag carrier only, NBe-tGCN4-Flag or Irr-tGCN4-Flag, as depicted in Figure 1, in the presence of alum. Serum endpoint titers of pooled sera were determined two weeks after each immunization in NBe-peptide (A & B), irrelevant peptide (C & D) or tGCN4-Flag-specific (E & F) ELISA. Both IgG1 (A, C, E) and IgG2c (B, D, F) specific titers were analyzed.

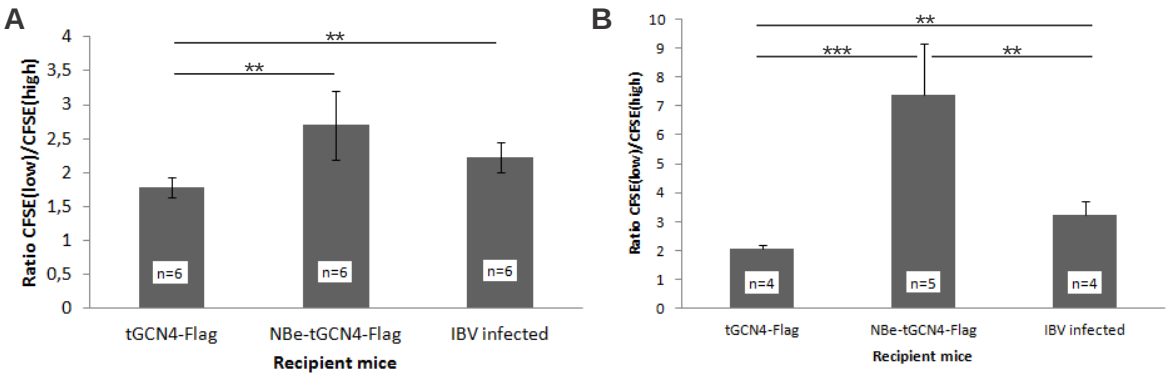


Figure S6. Short- and long-term *in vivo* killing assays reveal strong and long-lasting NBe-directed cytotoxicity. C57Bl/6 mice were immunized twice as indicated. *In vivo* killing assays were performed by transfer of differentially CFSE-labelled peptide-pulsed splenocytes 4 weeks (A) or three months (B) post-boost. The presence of CFSE-positive population in the spleen of recipient mice was determined through FACS-analysis 24h post-transfer. Shown are the ratios of Ova-peptide pulsed cells labeled with 0.5mM CFSE (low) and NBe-peptide pulsed cells labeled with 5mM CFSE (high). The number of mice in each group is indicated in the bars. **, $p<0.01$; ***, $p<0.001$.

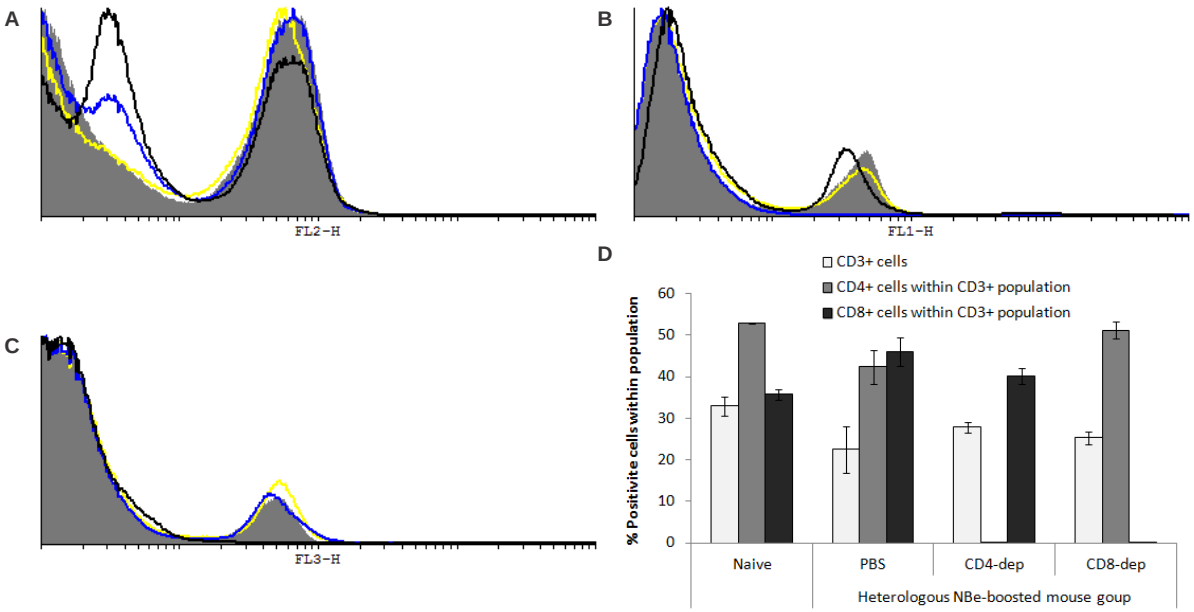


Figure S7. CD4- and CD8-specific T cell depletion. A-C. FACS profiles of splenocytes stained for CD3 (A), CD4 (B) or CD8 (C) after treatment with PBS (yellow graph), anti-CD4 (blue graph) or anti-CD8 (black graph). The gray filled profile corresponds to the staining of splenocytes from untreated naive mice. **D.** Relative positivity for CD3 within the total splenocyte population and for CD4 and CD8 within the $CD3^+$-population, determined by FACS for naive untreated mice and PBS-, anti-CD4- and anti-CD8-treated heterologous NBe-based prime-boosted mice.

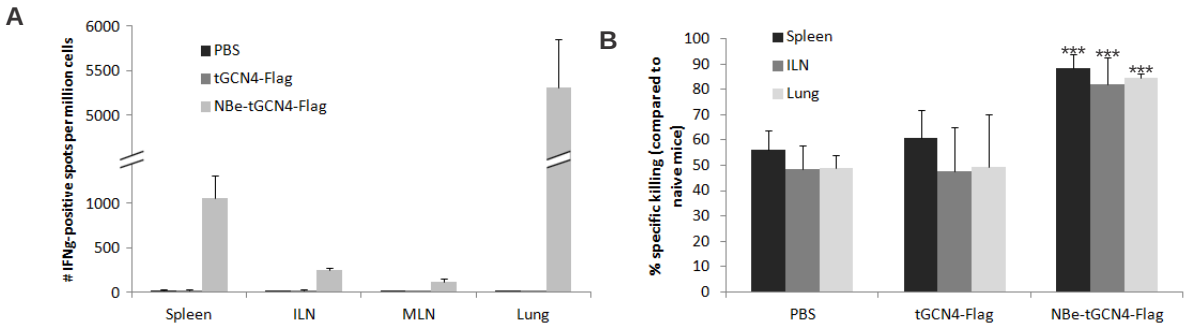


Figure S8. Mucosal immunization with LTR192G adjuvanted NBe-tGCN4-Flag vaccine induces strong local immunity. C57Bl/6 mice were immunized with PBS, adjuvanted carrier only or adjuvanted NBe-tGCN4-Flag twice three weeks apart. **A.** Four weeks post-boost, cellular responses were evaluated in an *ex vivo* elispot assay using the indicated lymph nodes and organs. For restimulation, the H2-K^b restricted octameric ATFNYTNV NBe-peptide was used. n=3 for all groups. **B.** Eight weeks post-boost, mice were challenged with a sublethal influenza B dose (0.3 LD_{50}) for follow-up of morbidity (Figure 7). Two weeks post-challenge, mice were subjected to an *in vivo* killing assay using irrelevant peptide and the H2-K^b restricted octameric ATFNYTNV NBe-peptide. Shown are the specific killing rates compared to a naïve mouse group. Asterisks indicate significant differences compared to both other groups. n=5 for PBS and carrier only groups, n=6 for NBe-tGCN4-Flag group. ***, $p<0.001$.

Additional data

Correlation between viral lung titers and post-challenge cellular responses

Next to intranasal NBe-tGCN4-Flag immunization, ip. vaccination with the tetrameric protein is able to inhibit IBV replication in the mouse lung (cf. Figure 4 and Figure 8). In an attempt to repeat the results of the initial experiment (Figure 4), C57Bl/6 mice were immunized ip. with tGCN4-Flag or NBe-tGCN4-Flag in combination with Alhydrogel. In this instance however, only two immunizations were given instead of three, to assess whether this also yields protective levels of NBe-directed cellular responses against IBV infection. As positive control, mice were sublethally pre-challenged twice with homologous IBV/Memphis/12/97 virus, further controlled by a sublethal influenza A infected mouse group. Three weeks after the second immunization or infection, mice were challenged with a 0.067LD_{50} dose of B/Memphis/12/97. On day 5 post-challenge, lung homogenates were made for determination of the lung virus titer. As can be deduced from Figure A1, NBe-tGCN4-Flag immunization had little effect on IBV replication in the lung compared to tGCN4-Flag immunization. As expected, IBV pre-challenged mice did not show any pulmonary viral load. Surprisingly though, also influenza A pre-infected mice were almost completely devoid of IBV as well. This is most likely caused by so-called non-specific temporary immunity induced by a previous unrelated influenza infection [1].

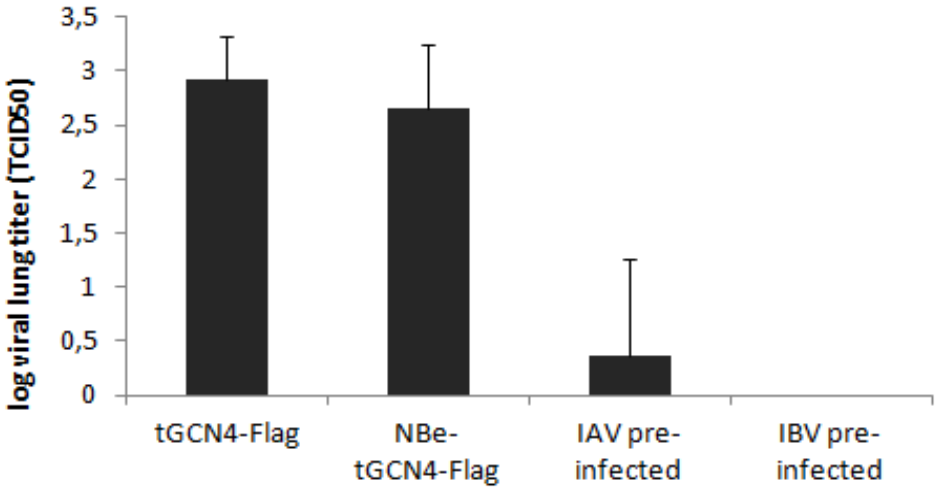


Figure A1. Intraperitoneal NBe-tGCN4-Flag immunization does not provide confiding protection against IBV infection. Mice were immunized twice three weeks apart with tGCN4-Flag or NBe- tGCN4-Flag, or infected twice with a sublethal influenza A or IBV dose. Three weeks after the second immunization, mice were challenged with a 0.067LD_{50} dose of IBV. On day 5 post-challenge, viral lung titers were determined in a $TCID_{50}$ titration. n = 6 for all groups.

Concomitantly with lung virus titer determination, NBe-specific cellular responses were ascertained using splenocytes of the infected mice. All groups reacted strongly towards NBe-peptide restimulation in ELISPOT analysis, including non-NBe immunized mice (Fig. A2A). Such strong immediate post-challenge induction of NBe-specific cellular responses was seen in other instances as well (data not shown). When comparing viral lung titers with NBe-specific cellular responses (Fig. A2B-D), striking correlations can be found. Both for tGCN4-Flag immuned and influenza A virus (IAV) pre-infected mice, higher pulmonary virus titers correspond to higher NBe-specific cellular responses detected in ELISPOT. For the NBe-tGCN4-Flag immunized mice however, higher virus titers correlate with a lower number of cellular responses. This indicates NBe-directed cellular responses do provide a certain degree of protection in NBe-tGCN4-Flag mice, limiting viral replication in an apparent dose-dependent manner. In non-NBe immune mice, higher viral loads simply induce higher levels of NBe-responsive T cells.

Although non-protective in this instance, NBe-directed cellular responses do correlate with anti-IBV activity. This is reminiscent of the study of McMichael *et al*. [2], which showed for the first time a correlation between virus-directed cytotoxicity and viral shedding in the absence of cross-reactive antibodies. With antisera containing undetectable amounts of anti-IBV titers and not showing any neutralization effect *in vitro* (data not shown), conditions here are similar.

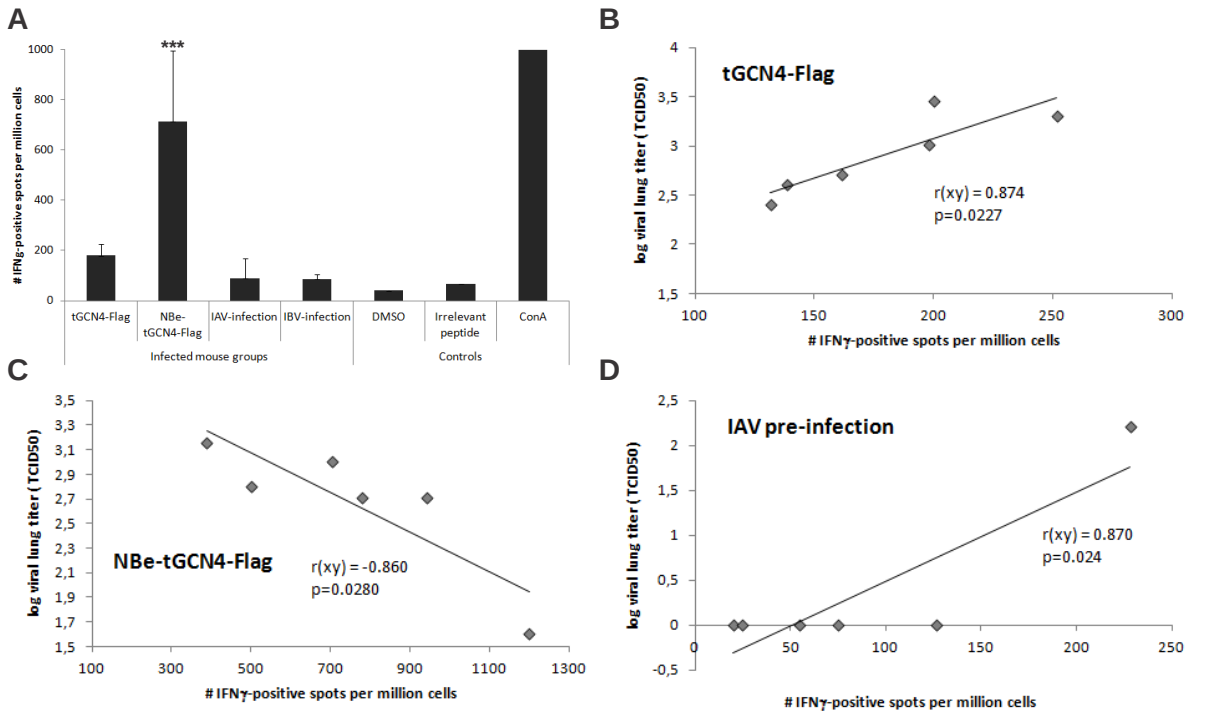


Figure A2. Correlation between pulmonary viral load and NBe-specific immune responses in NBe-tGCN4-Flag immunized mice. Splenocytes from mice used for the determination of IBV replication in the lungs day 5 post-challenge (Fig. A2), were restimulated with NBe-peptide to asses cellular responses in an IFNγ-specific ELISPOT. **A.** Number of spots for each immunized mouse group. n = 6 for all groups. ***, p<0.001 (Two-way ANOVA). **B-D.** Correlation between number of spots induced by NBe-peptide restimulation and viral lung titer, assessed on the same day (day 5) after a sublethal IBV challenge, for tGCN4-Flag (B), NBe-tGCN4-Flag (C) and influenza A virus (IAV) pre-infected (D) mice. Indicated are the Pearson's coefficients of correlation with their respective p-value.

NBe-vaccination based on different carriers can induce NBe-specific T cells

Using NBe-tGCN4-Flag, cytotoxic NBe-specific T cell responses can be induced using different adjuvants and immunization routes. Additionally, IBV infection and NBe-based DNA immunization also evokes such responses. To evaluate the value of other NBe-carrier fusion proteins, mice were immunized ip. with a number of proteins combined with Alhydrogel. Next to validated NBe-tGCN4-Flag and NBe-M2 DNA immunization, three different HBc-based vectors were used [3,4]. In 1646 and 1651, a single copy of the NB ectodomain is fused to the N-terminus (1651) or inserted into the immunodominant loop (1646) of the short version of HBc, lacking the nucleic acid binding protamine region. In NBe-2M2e-HBc, a single NBe-copy is fused N-terminally to two M2e-copies coupled a short-version HBc-particle. The 2M2e-HBc particle has been validated in influenza A based challenges [5], and provides a more soluble basis for carrying the NBe-sequence. To check the influence of the highly-charged Flag-tag on induction of cellular responses, tag-less *P. pastoris* NBe-tGCN4 was used, produced by both wild type (PpWT) and glyco-engineered yeast producing only glycoproteins bearing mannose$_5$-structures (PpM5) [6,7]. IFNg-specific ELISPOT revealed all NBe-based immunization methods induce specific cellular responses, albeit to a different extent, both in homologous (two protein immunizations, Fig. A3A) and heterologous (protein immunization boosted by a sublethal IBV infection, Fig. A3B) setting. In general, it can be concluded that, in addition to using different adjuvants and immunization routes in combination with NBe-tGCN4-Flag, different carriers can lead to the induction of cellular responses directed towards the coupled NB ectodomain.

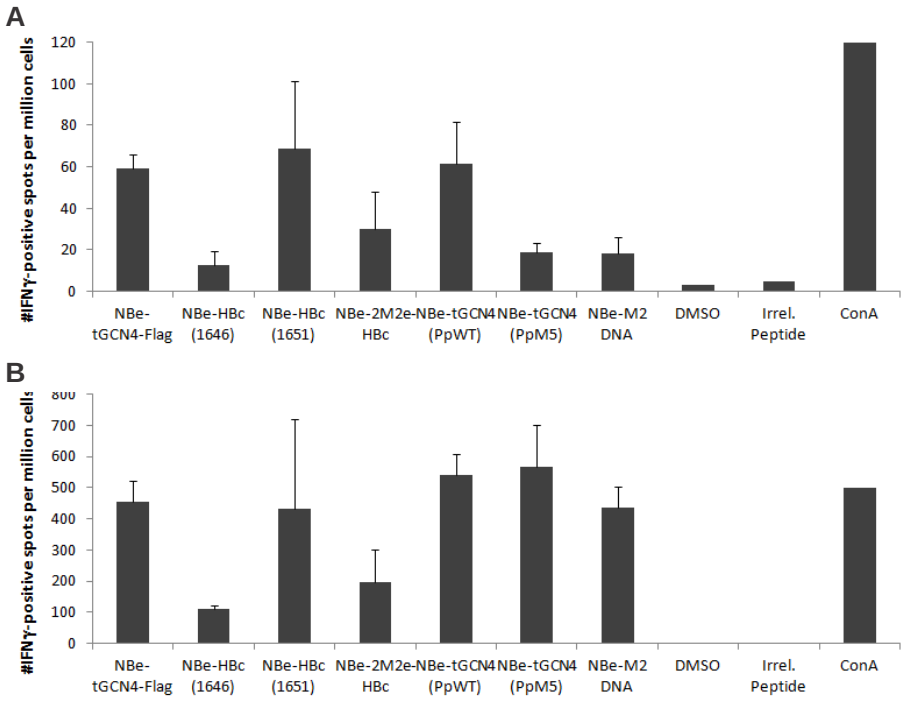


Figure A3. NBe-specific cellular responses can be induced by different NBe-carrier fusions. Mice were immunized intraperitoneally in combination with Alhydrogel using the indicated immunogens, except for NBe-M2 given intramuscularly as a DNA vaccine. Three weeks after the first immunization, mice were boosted homologously with the same immunogen (A) or heterologously with IBV infection (B). Cellular anti-NBe responses were assessed three weeks after the boost in IFNγ-specific ELISPOT.

The tGCN4-carrier can induce T cells against different epitopes

Even with the NBe-epitope being a strong natural antigen, and different carrier systems being able to induce NBe-directed cellular responses, we wondered what the applicability of the tGCN4-carrier is. The ease of construction and purification of epitope-tGCN4 fusions, combined with the high yield in the prokaryotic expression system is extremely inviting for a broad T cell inducing vaccine basis. To assess this, a number of validated cytotoxic T cell epitopes were genetically fused to the tGCN4-Flag carrier (Table A1). These include $H2^b$-, $H2^d$- and $H2^k$-restricted influenza A-derived NP epitopes [8-10], alone or fused together into one construct, and the well-known $H2^b$-restricted OVA epitope [11,12].

Table A1. Overview of epitopes and their sequences fused to tGCN4-Flag used for immunization. The sequences shown include the epitope (underlined) flanked by three amino acids on either side derived from the natural context. These sequences are fused together or the tGCN4-Flag carrier using a Gly-Gly (GG) linker.

Epitope	Sequence
$H2^b$-specific NP-epitope [NP(b)]	MVQIASNENMETMESS-GG-[tGCN4-Flag]
$H2^d$-specific NP-epitope [NP(d)]	MNDATYQRTRALVRTG-GG-[tGCN4-Flag]
$H2^k$-specific NP-epitope [NP(k)]	MLKLSDYEGRLIQNS-GG-[tGCN4-Flag]
Combined NP-epitopes [NPc]	NP(k)-GG-NP(d)-GG-NP(b)-GG-[tGCN4-Flag]
$H2^b$-specific OVA-epitope [Ova]	MQLESIINFEKLTEW-GG-[tGCN4-Flag]

For initial testing, small-scale purification were done, based on pull-down using the Flag-tag. Bacterial pellets from low-volume inductions (giving high relative yields) were sonicated, followed by immunoprecipitation of the Flag-tagged product using mouse anti-Flag. As internal control, both for purification and immunization, NBe-tGCN4-Flag (NGF) was used. Figure A4 shows the purification results. Most of the protein remains unbound to Protein G beads, mainly due to a low antibody-antigen ratio. However, for each protein, a small amount is detectable in the eluate. Also note, because of the non-covalent association of beads and antibody, the elution fractions contain both antigen and anti-Flag antibody.

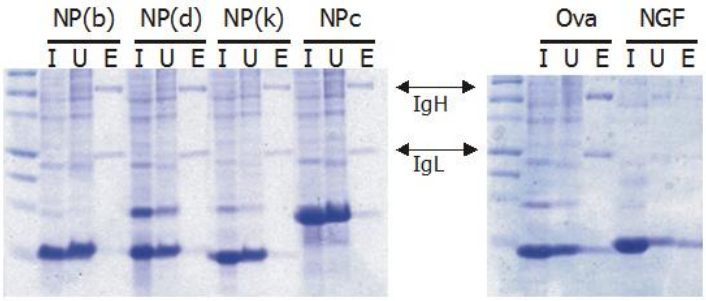


Figure A4. Small-scale purifications of chimeric tGCN4-Flag proteins. Analysis of fractions after anti-Flag pull-down using Protein G agarose beads of tGCN4-Flag based proteins on Coomassie-stained polyacrylamide gels. The highly intense stained bands correspond to the protein of interest. The different fractions are indicated as input (I), unbound (U) and eluted (E). The eluates also contain the anti-Flag antibody used for the pull-down of the tGCN4-Flag proteins, as indicated by the presence of heavy (IgH) and light (IgL) antibody chains.

To test the immunogenicity of the chimeric proteins, mice with genetic backgrounds responsive to the epitopes used were immunized twice three weeks apart ip. with the eluted proteins, mixed with Alhydrogel as adjuvant. This entails Balb/c mice were immunized with NP(d)-tGCN4-Flag and NPc-tGCN4-Flag, C3H/HeN mice with NP(k)-tGCN4-Flag and NPc-tGCN4-Flag and C57Bl/6 mice with NP(b)-tGCN4-Flag, NPc-tGCN4-Flag, OVA-tGCN4-Flag and NBe-tGCN4-Flag.

As a first analysis, tGCN4-Flag specific antibody responses after the second immunization were determined in ELISA (Fig. A5A). IgG2a/c titers (in comparison to IgG1 titers) are higher than would be expected from aluminum salt adjuvanted vaccination. The most likely cause for this is the presence of mouse anti-Flag antibodies together with the eluted protein provides a complexed vaccine preparation, leading to a more pronounced immune response [13]. This would also explain the reasonably high antibody response directed towards the small amount of protein.

Cellular responses were ascertained in IFNγ-specific ELISPOT. NP-based immunization resulted in epitope-specific and haplotype-restricted responses in each of the mouse strains (Fig. A5B). In the control set-up, using immunoprecipitated NBe-tGCN4-Flag, cellular responses were high and specific for the octameric peptide (Fig. A5C). The tGCN4-Flag protein bearing the $H2^b$-restricted Ova-peptide also induced epitope-specific cellular responses (Fig. A5C). In conclusion, the tGCN4-Flag carrier protein is able to induce epitope-specific cellular responses in different genetic murine backgrounds when immune-complexed with anti-Flag antibody. Future experimental settings using tGCN4-based proteins purified through conventional chromatographic methods should provide additional evidence for the broad immunogenicity of such chimeric proteins.

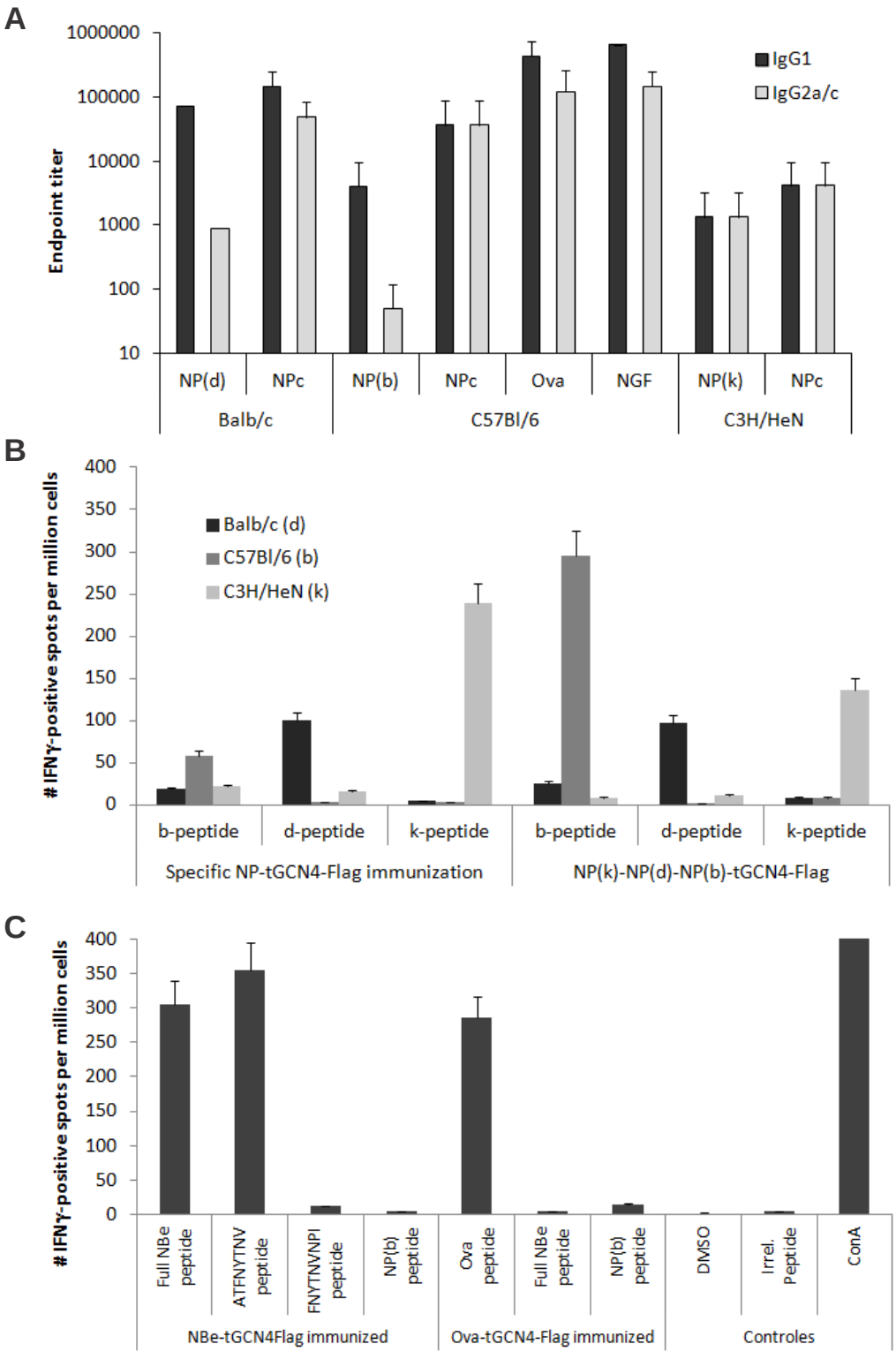


Figure A5. Immunogenicity of tGCN4-Flag proteins bearing validated T cell epitopes in different mouse strains. Mice were immunized twice ip. with Alhydrogel adjuvanted tGCN4-Flag proteins bearing different influenza A/NP-epitopes, the $H2^b$-restricted SIINFEKL Ova-peptide, or the NBe-ectodomein, purified through immunoprecipitation with mouse anti-Flag (Fig. 4). **A.** Antibody titers (IgG1 and IgG2a/c) two weeks after the second immunization, detected in tGCN4-Flag specific ELISA. **B-C.** Detection of cellular responses, through IFNγ-specific ELISPOT. NP-specific responses were assessed, showing haplotype-dependent reactivity (B). Ova- and NBe-restricted responses, as determined in C57Bl/6 immunized mice (C).

Viruses with a deviating NBe-sequence confirm the defined consensus epitope sequence

As shown in Figure 1, a number of NBe-sequences contain mutations specifically in the mapped ATFNYTNV T cell epitope. The H2-K^b MHC molecule, responsible for binding the peptide, imparts a number of restrictions on its ligand. The major anchor residues for octameric peptides are located at position 5, with a preference for Tyr or Phe, and position 8, with a preference for residues with a hydrophobic side chain, such as Leu, Met, Ile or Val [14]. Table A2 describes all alternative NBe-sequences found in the influenza database (www.fludb.org), with a sequence deviating from the consensus epitope sequence. A total of 66 sequences containing a mutation can be identified. Based on predictions using the NetMHC 3.2 server, only 6 of these will cause a drastic drop in affinity, with half of those responsible for complete abolishment of binding.

Table A2. NBe-sequences with deviating H2-K^b-restricted epitope sequence. All NBe-sequences available in the influenza sequence database (www.fludb.org; n = 854, from 1940 till March 2011) were aligned to identify all possible sequences corresponding to the identified H2-K^b restricted T cell epitope. In 788 instances (92.3%), the consensus sequence was found back. For all other situations, the H2-K^b binding was predicted using the NetMHC 3.2 server and ordered according to affinity (+++, very strong binding; ++, strong binding; +, relative strong binding; +/-, weak binding; -, no binding predicted). For sequences with weak or no predicted binding, the lineage to which the corresponding virus belongs is indicated (V = B/Victoria/02/87 lineages, Y = B/Yamagata/16/88 lineage, P = virus pre-dates lineages separation, ND = not determined).

Sequence	Occurrence	H2-K^b binding	Lineage
ATFNYTNV	788/854	+++	ND
ATFDYTNV	1/854	++	V
ATFNYTNI	10/854	++	V(5) / Y(5)
ATLNYTNV	48/854	++	V(22) / Y(26)
ATLNYTNI	1/854	+	V
ATFNHTNV	1/854	+/-	V
ATFNYTNA	2/854	+/-	V(1) / Y(1)
ATINHTNV	1/854	-	V
ATFNCTNI	2/854	-	V(1) / P(1)

To assess the influence of mutation of the NBe-restricted epitope, groups of C57Bl/6 mice were infected with a number of different IBVes (Fig. A6A). Of these, the B/Hong Kong/05/72 virus contains three residues deviating from the consensus NBe-sequence, with two of them residing in the T cell epitope. Specifically, the mutation of Tyr at position 8 (Y8C) is predicted to completely abolish MHCI-restricted binding [14]. Cellular responses were assessed through IFNγ-specific ELISPOT; results are shown in Figure A6B. Clearly, while B/Memphis/12/97, B/Harbin/07/94 and B/Yamagata/16/88 virus, all with consensus sequence, induce NBe-specific cellular responses, B/Hong Kong/05/72 is unable to do so.

A

Strain	Nucleotide sequence (HA)	amino acid sequence (NBe)
B/Yamagata/16/88	GTTACCAGTAGAAACGGATTT	MNNATFNYTNVNPISHIR
├ B/Harbin/07/94	GCTACCAGTAGAAGCGGATTT	MNNATFNYTNVNPISHIR
└ B/Memphis/12/97	GCTACCAGTAGAAGCGGATTT	MNNATFNYTNVNPI**P**HIR
B/Victoria/02/87	GTTACCAATGGAAACGGATTT	MNNATFNYTNVNPISHIR
└ B/Hong Kong/05/72	GTTACCAATGGAAACGGATTT	MNNATFN**C**TN**I**NPI**T**HIR

B

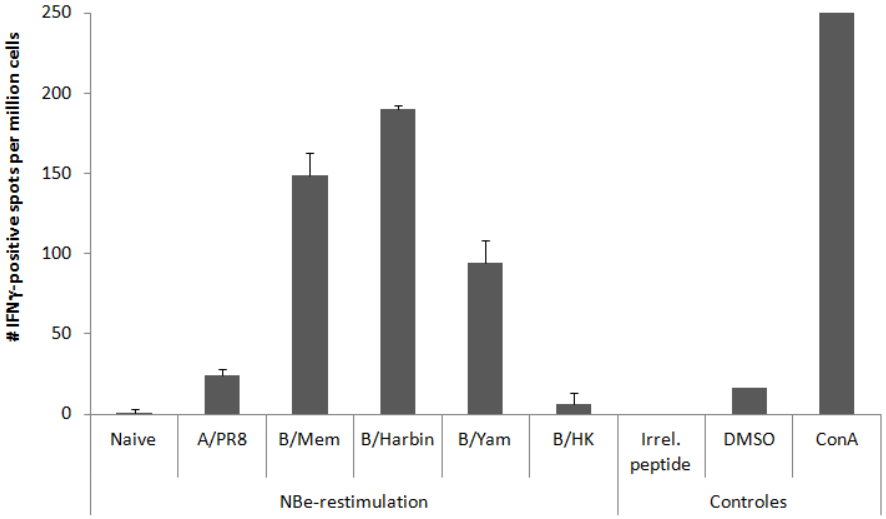


Figure A6. Mutation of the NBe-specific T cell epitope abolishes induction of cellular responses. Groups of three mice were infected twice, three weeks apart with buffer only (naive) or with $7.5x10^4$ pfu IBV/Memphis/12/97 ($0.075LD_{50}$), B/Harbin/04/94, B/Yamagata/12/88 or B/Hong Kong/05/72 or with $0.075LD_{50}$ A/Puerto Rico/08/34 virus. Three weeks after the second immunization, NBe-specific cellular responses were assessed in IFNγ-specific ELISPOT using the octameric ATFNYTNV peptide for restimulation. A. Classification of viruses can be done according to a specific stretch of nucleotides in the hemagglutinin (HA) sequence (nucleotides 481-501), assigning them to one of either IBV lineage (B/Yamagata/16/88 or B/Victoria/02/87 lineage) [15]. The corresponding amino acid sequence of the NB ectodomain of each virus is also shown. Residues deviating from the consensus sequence are shown bold in red. B. IFNγ-specific ELISPOT results after restimulation with the ATFNYTNV peptide. Shown is the average of the response of the three mice, each measured in triplicates, ±SD.

It should be noted that the occurrence of specific mutations of the Y8 anchor residue is very low. One of the viruses with this feature is the first isolated IBV (B/Lee/40), passaged numerous times in *in vitro* systems, and a single primary isolate (B/Mississippi/UR06_0345/2007). However, the two other viruses with deviating NBe-sequence established themselves as epidemic strains and were included in the trivalent vaccines for the 1974-1979 seasons (B/Hong Kong/05/72 virus) and the 1984-1986 seasons (B/USSR/100/83 virus).

The consequences of these observations cannot be ignored. Using predictive online tools (NetMHC 3.2 server; IEDB predictions), a substantial number of hits were identified showing high affinity of NBe-derived epitopes for a number of human HLA alleles (data not shown). Several of these hits correspond to the octameric ATFNYTNV murine H2-K^b specific epitope and are predicted to bind HLA-molecules of the quite common A2-supertype. The identification of mutations in the NBe-sequence in epidemic strains interfere with this exciting finding. The potential natural occurrence of human

NBe-specific T cell responses in the population and influence of (common) NBe-mutations is under further scrutiny. The mere fact epidemic IBV strains, establishing themselves for a long period of time in the human population, contain mutations abolishing theoretical T cell recognition of the NB ectodomain, could reflect natural NBe-specific immunity. After all, for IAV, a number of strains have been identified bearing mutations in validated human NP epitopes, which have been linked to immune escape [16-18].

Generally, any prophylactic or therapeutic strategy based on induction of cytotoxic T cells is restricted by the stringent characteristics imposed by the peptide-MHC-TCR interaction. In some instances, naturally presented epitopes have a low affinity for MHC binding, thus suboptimally inducing T cells. Using peptides with a high (predicted) affinity for their cognate MHC molecule as a replacement for epitopes naturally present in the target protein, so-called heteroclitic peptides, is a common approach used in *e.g.* cancer treatments [19]. The main alterations introduced in heteroclitic compared to natural peptides are replacing anchor residues with those predicted to have a higher affinity for MHC binding. It is anticipated that using such a strategy will induce high number of T cells able to react both the inducing (high affinity) and the natural (low affinity) epitope. However, recent studies have shown that both *in vitro* and *in vivo* T cell repertoires induced by heteroclitic epitope based immunizations can differentiate between natural and sequence-optimized peptides [18,20,21]. Hence, although predicted, the use of the consensus NBe-sequence might not induce T cells reactive to *e.g.* the alternative ATLNYTNV (5.6% prevalence, between 1999 and 2005) or ATFNYTNA sequences. Careful selection of cross-reactive or novel immune-dominant epitopes is warranted in such vaccination strategies[18].

Material and methods

Mice, viruses and influenza challenges

For all *in vivo* studies, 6-8 week old female mice were used. BALB/c mice were obtained from Charles River (Italy), C57Bl/6J mice from The Jackson Laboratory (France) and C3H/HeN mice from Harlan (The Netherlands). Mice were housed under specific pathogen-free conditions and fed *ad libitum*. All infection experiments were conducted under BSL-2 containment and were approved by the local Institutional Ethics Committee on Experimental Animals of Ghent University.

Influenza virus infections were performed as described in the main text. Next to A/PuertoRico/08/34 and B/Memphis/12/97, other non-mouse-adapted influenza viruses used here include B/HongKong/05/72, received from Dr. Robert Sidwell [22], B/Harbin/04/97 and B/Yamagata/16/88. For sublethal infections with these, $7.5x10^4$ pfu was inoculated, the equivalent of $0.075LD_{50}$ mouse-adapted B/Memphis/12/97.

Construction and small-scale purification of tGCN4-based proteins

Chimeric tGCN4-based proteins were constructed by PCR methods using primer sets containing the sequence of the epitope to be linked to tGCN4 and a sequence homologous to the N-terminal tGCN4 coding sequence. Upon amplification of epitope and tGCN4-Flag coding sequences, both were fused in frame through PCR. Fused sequences were cloned into pLT32H, as described in the main text. For protein expression, small-scale inductions (3mL) were performed based on the method described in the main text.

Purifications were done on a small-scale level. Briefly, 50µL of bacterial lysate after sonication (corresponding to 500µL bacterial culture) was incubated with 10µg mouse anti-Flag (Sigma) overnight in 500µL immunoprecipication (IP) buffer (10mM Tris/HCl pH7.4 + 150mM NaCl + 0.5% NP40 + 0.5% Triton X-100). 20µL of washed Protein G beads (GE Healthcare) was added to the antigen-antibody mixture and incubated for three hours. Next, beads were collected, washed three times with IP buffer and treated with 300µL 100mM sodium citrate pH3.0 for 30min at room temperature to elute the antibody-antigen complexes. Immediately after pelleting of the beads, the supernatant was collected and neutralized using 30µL 1M Tris pH8.8. The IP was analyzed on SDS-PAGE by loading 1/10 of each fraction (*i.e.* the input material, the unbound fraction and the eluted fraction) on gel.

Immunization procedures

Immunizations using NBe-HBc and *P. pastoris* produced NBe-tGCN4 (described in Chapter 3) were done with 5µg of protein per immunization given i.p. in combination with Alhydrogel, as described in the main text. NBe-M2 DNA was administered as a DNA vaccine by injecting 100µg LPS-free plasmid in 100µL PBS intramuscularly. For the heterologous boost, $0.075LD_{50}$ of IBV/Memphis/12/97 was given. Novel tGCN4-based proteins administered after IP purification were given ip. in combination with Alhydrogel in a volume of 300µL, using 30µL eluted sample (appr. 1/10 of the eluate) diluted with PBS to 150µL and combined with 150µL diluted Alhydrogel.

ELISA and IFNγ-specific ELISPOT procedure

As described in the main text.

References

1. Kelly, H., Barry, S., Laurie, K. & Mercer, G. Seasonal influenza vaccination and the risk of infection with pandemic influenza: a possible illustration of non-specific temporary immunity following infection. *Euro Surveill* **15**, 19722 (2010).
2. McMichael, A.J., Michie, C.A., Gotch, F.M., Smith, G.L. & Moss, B. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *Journal of general virology* **67**, 719 (1986).
3. Clarke, B.E*., et al.* Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* **330**, 381-384 (1987).
4. Pumpens, P., Borisova, G.P., Crowther, R.A. & Grens, E. Hepatitis B virus core particles as epitope carriers. *Intervirology* **38**, 63-74 (1995).
5. De Filette, M*., et al.* Universal influenza A vaccine: optimization of M2-based constructs. *Virology* **337**, 149-161 (2005).
6. Vervecken, W*., et al.* In vivo synthesis of mammalian-like, hybrid-type N-glycans in Pichia pastoris. *Applied and environmental microbiology* **70**, 2639 (2004).
7. Jacobs, P.P*., et al.* Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate. *Microbial Cell Factories* **9**, 93 (2010).
8. Townsend, A.R.M*., et al.* The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959-968 (1986).
9. Rötzschke, O*., et al.* Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* **348**, 252-254 (1990).
10. Gould, K.G., Scotney, H. & Brownlee, G.G. Characterization of two distinct major histocompatibility complex class I Kk-restricted T-cell epitopes within the influenza A/PR/8/34 virus hemagglutinin. *Journal of virology* **65**, 5401 (1991).

11. Carbone, F.R. & Bevan, M.J. Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization. *The Journal of experimental medicine* **169**, 603 (1989).
12. Stura, E.A.*, et al.* Crystallization of murine major histocompatibility complex class I H-2Kb with single peptides* 1,* 2. *Journal of molecular biology* **228**, 975-982 (1992).
13. Hioe, C.E.*, et al.* The use of immune complex vaccines to enhance antibody responses against neutralizing epitopes on HIV-1 envelope gp120. *Vaccine* **28**, 352-360 (2009).
14. Falk, K., Rötzschke, O., Stevanović, S., Jung, G. & Rammensee, H.G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290-296 (1991).
15. Biere, B., Bauer, B. & Schweiger, B. Distinction of Influenza B virus lineages Yamagata and Victoria by real-time PCR. *Journal of clinical microbiology* **48**, 1425-1427 (2010).
16. Rimmelzwaan, G.F.*, et al.* Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants. *Journal of general virology* **86**, 1801 (2005).
17. Wahl, A.*, et al.* T-cell tolerance for variability in an HLA class I-presented influenza A virus epitope. *Journal of virology* **83**, 9206 (2009).
18. Valkenburg, S.A.*, et al.* Protective efficacy of cross-reactive CD8+ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold. *PLoS Pathogens* **6**, e1001039 (2010).
19. Fikes, J.D. & Sette, A. Design of multi-epitope, analogue-based cancer vaccines. *Expert Opinion on Biological Therapy* **3**, 985-993 (2003).
20. Speiser, D.E.*, et al.* Unmodified self antigen triggers human CD8 T cells with stronger tumor reactivity than altered antigen. *Proceedings of the National Academy of Sciences* **105**, 3849 (2008).
21. Cole, D.K.*, et al.* Modification of MHC anchor residues generates heteroclitic peptides that alter TCR binding and T cell recognition. *The Journal of Immunology* **185**, 2600 (2010).
22. Sidwell, R.W.*, et al.* Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. *Antimicrobial agents and chemotherapy* **40**, 2626 (1996).

IV. General discussion and perspectives

General discussion and perspectives

Cross-protection induced by B/HA0-, BM2e- and NBe-based IBV vaccine candidates

The current situation of influenza vaccination needs to be addressed urgently. Licensed trivalent vaccines, consisting of inactivated or life-attenuated viruses of influenza B (IBV) and the influenza A (IAV) A/H1N1 and A/H3N2 subtype, do provide strong sterilizing immunity to homologous or closely related strains. However, with influenza viruses constantly diversifying under vaccine or infection induced immunity, selection of an inappropriate seed strain to prepare vaccines will fail to induce protective immunity in vaccinated people [1]. Specifically for IBV this is a contemporary problem. For IAV, two strains are included in vaccines, covering both H1N1 and H3N2 subtypes currently circulating in the population as seasonal viruses. IBV is not characterized by subtypes, but is subdivided in lineages (Victoria and Yamagata lineage) which are antigenically distinct [2-4]. Inclusion of a single IBV strain in the vaccine, belonging to one of both lineages, entails a failure to choose a strain of the circulating lineage will lead to a vaccine mismatch. Indeed, the past decade, nearly one in two seasons has been characterized by a lineage mismatch between circulating and vaccine IBV strain, resulting in vaccine failure. This is particularly troublesome for people more sensitive to IBV infection and related complications, such as children and the elderly [5-7]. Moreover, failure to protect vaccinees will diminish belief in the merit of influenza vaccination, and will have strong socio-economic consequences. In this thesis we wanted to address the problem of IBV vaccination by exploring the protective efficacy of properly presented conserved features of the virus in a mouse model. With this, we wanted to add on to the wealth of information related research with IAV has given (Chapter 1) [8] and supplement the few studies into cross-protective IBV vaccine development (Chapter 2) [9-13]. A general overview of observations made in this study, is given in Figure IV.1.

We selected three different extravirionic epitopes, presented in high numbers on infected cells, as target antigens. The rationale for this was to, in analogy with the M2 ectodomain of IAV [14], develop a vaccine capable of inducing humoral immunity. These included the ectodomains of the BM2 and NB transmembrane proteins (BM2e and NBe, respectively) and the maturational cleavage loop of HA (B/HA0). To mimick their natural conformation as much as possible, specific carrier proteins and expression systems were selected. Insertion of B/HA0 in the immunodominant loop of HBc clearly induced virus specific antibodies, although challenge of immunized mice did not reveal protection. This is in strong contrast with the results of Bianchi *et al*. [9], who could protect mice with a B/HA0-OMPC vaccine against challenge with IBV strains of different lineages. Minor differences in the antigen dose and challenge model might however explain this discrepancy. The B/HA0-HBc protein showed very low solubility, requiring dual expression of unsubstituted HBc and B/HA0-HBc to yield a soluble protein (termed 3004) with mixed substitution rate. Hence, while the OMPC vaccine induced very high B/HA0-specific titers, as assayed in B/HA0-peptide ELISA, 3004 induced only moderate virus specific titers (Fig. 4.6). Our challenge model is based on administering $4LD_{50}$ of IBV in a volume of 50µL, while Bianchi *et al*. used $1LD_{90}$ in a volume of 20µL. The smaller volume and lower infectious dose (although a direct comparison of this is not possible), combined with a better immune response, might tilt the balance towards stronger protection.

The N-terminal NB ectodomain was presented by HBc in three different manners: a direct N-terminal fusion (1651), coupled to $M2e_2$-HBc extending the accessibility of NBe (forming NM_2H) or inserted in the immunodominant loop to improve the immunogenicity and accessibility (1646). Surprisingly, only 1651 was able to provide protection against a potential lethal IBV infection. Even with the use of mouse-adapted B/Hong Kong/5/72 virus, containing a strongly deviating NBe-sequence as compared to the consensus sequence used for vaccine construction, NBe-HBc immunization can protect mice from excessive lethality. The presentation of NBe, providing a non-ligated free N-terminus, on a particulate HBc-protein appears to be superior to the more immunogenic 1646 particle. The NM_2H-protein, forming small aggregates but not particles, can thwart an IAV infection as efficiently as the $M2e_2$-HBc particle, but fails to induce protective NBe-responses acting against an IBV infection. From the NBe-peptide ELISA is can be deduced the non-particulate NM_2H protein elicits lower anti-NBe responses than 1651, most likely related to the T-cell independent induction of antibody responses by particulate HBc [15]. Hence, it seems an immunogenic presentation of N-terminally presented NBe is required to provide effective protection against IBV.

With BM2 and NB forming tetramers, we coupled the BM2e and NBe sequences to a tetramerizing variant of the leucine zipper of GCN4 (tGCN4). Four different formats were used: a short (5AA) and a long (8AA) BM2e-sequence coupled to tGCN4, NBe coupled to a triple Flag-tag bearing tGCN4 scaffold and NBe-tGCN4 without tag produced in *P. pastoris* to achieve glycosylation of NBe (gNBe-tGCN4), as naturally occurs [16,17]. Using size-exclusion chromatography, native PAGE MW determination and chemical cross-linking experiments, tetramerization of the proteins was confirmed. Immunization of mice with tGCN4-based proteins revealed an extremely immunogenic protein format, eliciting carrier-specific antibody titers exceeding 10^7 endpoint dilution. Both BM2e-based chimeras show induction of BM2- and IBV-reactive antibodies, protective against a potential lethal challenge with both B/Memphis/12/97 (Yamagata-like virus) and B/Hong Kong/5/72 (Victoria-like virus). NBe-tGCN4-Flag on the other hand induces clear anti-NBe peptide responses, reactive with NBe-expressing transfected cells but only slightly with IBV infected cells. The main reason for this is most likely the glycosylation state of the three species. Using non-glycosylated NBe-tGCN4-Flag, antibodies can be elicited reactive with transfected cells expressing NBe-proteins, since these display non-, mono- and di-glycosylated NBe (Fig. 3.16). For infected cells, the precise glycosylation state of NB within our *in vitro* system should be addressed, but could be more complex, disfavouring anti-NBe detection. Like for N-terminal NBe-HBc fusions, it simply might be a question of quantity. When comparing Alhydrogel and Montanide-ISA-720 adjuvanted NBe-tGCN4-Flag immunization, the latter induces higher anti-NBe titers and concomitantly provides better protection compared to the former. If anti-NBe responses could be boosted further, protection might be even more confiding. The observation the NBe-specific 15G5mAb can stain IBV infected cells, but is unable to protect mice, might also reflect a difference in accessibility of NBe *in vitro* and *in vivo*, something which should be investigated within our mouse model. Alternatively, it might reflect a qualitative problem, in that a polyclonal response evoked *in vivo* offers better IBV recognition and protection *in vivo*.

The gNBe-tGCN4 protein displays glycosyl-structures less complex as determined on native NB [17], but do provide a general glycosylated form of NBe. As vaccine, it offers significant protection against a potential lethal B/Memphis/12/97 infection compared to bacterial expressed NBe-tGCN4-Flag, highlighting the merit of glycosylated NBe as antigen, even if glycosylation is far for complete. These observations were extended with the use of transfected cells as immunogens. In this case, NBe(WT)-M2-Flag expressing cells induce significant protection compared to NBe(Mut)-M2-Flag expressing

cells. Obviously, the introduction of the mutations in the NBe sequence, removing the glycosylation sites, can affect the primary recognition of the wild type NBe sequence, although the inverse situation (recognition of NBe(Mut) by NBe(WT) antiserum) does not show problems of that sort. Nonetheless, NBe(WT)-M2-Flag con evoke significant protection, reinforcing the value of glycosylated NBe-vaccine constructs. Development of a cellular expressed and secreted NBe-based protein to further evaluate this is therefore warranted.

In a number of set-ups, heat-inactivated IBV viruses were used as immunogen to assess homologous or heterologous protection. In general, no heterologous protection was observed in any of the experiments. This is in accordance with other animal studies [18], although under specific vaccination conditions the immune response evoked by inactivated IBV can be broadened to be reactive with both lineages [19]. The method of inactivation used (30min incubation at 56°C) might destroy antigenic structures more than formalin or detergent inactivation. In humans, cross-reactive titers have been detected in primed individuals [18,20-22]. Hence, it deems worthwhile to look for epitopes residing in HA responsible for the cross-reactivity. This could be achieved by *e.g.* reverse vaccinology as has been done for the LAH structure of IAV HA [23,24].

As main conclusion it can be stated both BM2e- and glycosylated NBe-based vaccine candidates can offer significant protection against a potential lethal IBV infection. Anti-BM2e responses can afford protection against both Yamagata- and Victoria-like strains of IBV, making it a cross-protective vaccine basis. However, it should be stated protection seen is based purely on survival of challenged mice. When looking at morbidity, none of the cross-reactive vaccine candidates can avoid induction of morbidity when using $2.5LD_{50}$ or $4LD_{50}$ doses of challenge virus. Most likely, this is related to the mouse model, requiring high IBV doses (in pfu) for inducing lethality. Indeed, for assaying pulmonary virus replication we used appr. ten-fold lower doses, and in many instances this revealed stronger antiviral responses compared to controls. Hence, it would be interesting to retest the vaccine candidates described here in a low-dose challenge model, utilizing sublethal doses of *e.g.* $0.3LD_{50}$, and score protection based on prevention of morbidity.

The NB ectodomain harbours a T cell epitope affording strong IBV protection

In an attempt to improve NBe-tGCN4-Flag induced immunity, two other inbred mouse strains, C3H/HeN and C57Bl/6, were used next to Balb/c mice. Based on a small-level reduction in lung virus titers, further research uncovered a strong $CD8^+$ T cell epitope within the NBe-sequence specific for the $H2^b$-background of C57Bl/6 mice. We showed this epitope to induce cytotoxic effects towards peptide-pulsed cells and induce a strong impairment on virus replication in the lungs of immunized mice. Moreover, a sublethal infection with $0.3LD_{50}$ showed that NBe-based cellular immunity, although not neutralizing, can improve the clinical outcome of infected mice drastically.

It is quite remarkable parenteral immunization with a soluble antigen (NBe-tGCN4-Flag), adjuvanted by aluminum salt, induced such strong cellular responses. Indeed, it is a central paradigm that for efficient induction of T cell responses, the intracellular pathway of antigen presentation needs to be followed. Known exceptions include the use of cell-penetrating peptides (CPP) or protein transduction domains (PTD), allowing direct delivery of the protein in the cytoplasm [25], or use of specific adjuvants which exert similar actions or which specifically activate immune cells such as

monocytes or dendritic cells (DCs) [26-30]. DCs specifically possess the ability to cross-present exogenous antigens for the induction of cellular responses [31,32]. The specific mechanisms responsible for T cell induction here remain unclear. One possibility is the formation of immune complexes (ICs), consisting of protein combined with a large amount of antibodies. ICs generally have a negative effect on immune responses, leading to exacerbation of disease or induction of autoimmune diseases [33-35]. However, in some instances, they are used specifically in immunizations to induce cellular or humoral responses [36,37]. With the tGCN4-Flag moiety of the protein being so immunogenic, it is not inconceivable free antigen (be it during priming or with boosting) is coated with antibody. Such ICs can be taken up by DCs through FcγRI- (CD64-) mediated phagocytosis, leading to cross-presentation of the antigen [38,39]. Some evidence is in line with this theory. First of all, immunization of C57Bl/6 mice with NBe-peptide mixed with tGCN4-Flag protein, which is not immunogenic in the $H2^b$-background, did not induce T cell responses (data not shown). Second, other proteinaceous NBe-carrier protein fusions, based on the highly immunogenic HBc- or tagless tGCN4-scaffold (Chapter 5, Additional data), also induced NBe-specific T cell responses. And third, using a very low amount of chimeric proteins consisting of NP-epitopes fused to tGCN4-Flag in combination with mouse IgG1 anti-Flag antibodies, relative high T cell responses were evoked. Although circumstantial, it thus seems there is a link between high antibody responses and cellular responses. To address this, several models could be used. If for instance a chemical coupling of NBe-peptide and tGCN4-Flag is not immunogenic in the C57Bl/6, the influence of complexing the chemical conjugate with anti-tGCN4-Flag antibodies could be assessed. Similarly, immunization of B cell deficient mice (*e.g.* $Ig\mu^{MT/MT}$ mice [40]) or mice lacking FcγRI ($CD64^{-/-}$ mice) or the common FcRγ-chain could provide more insight. If so, the value of tGCN4-Flag or a highly immunogenic carrier in general in eliciting a broad (*i.e.* humoral and cellular) immune response would be enforced dramatically.

A second intriguing observation was the fact cellular immunity induced by a single immunization with NBe-based immunogens (NBe-tGCN4-Flag, NBe-HBc, NBe-DNA) was boosted drastically by a sublethal IBV infection. Such a heterologous boost effect is encouraging, if it can efficiently be translated to human immunology. Recent clinical trials with HIV- or malaria-derived immunogens did show heterologous boost effects in humans when using DNA vaccines or viral vectors [41-43]. The concept of 'original-antigenic-sin' is well-known, and anecdotic, epidemiological and immunological data has shown this concept can also be applied to influenza infections. Examples include data from past influenza pandemics [44,45], the IBV vaccination studies mentioned above [18-22] and any reverse vaccinology approach identifying cross-reactive antibodies [46]. The derived vaccination strategy seems extremely appealing: any infection occurring after immunization based on a conserved viral feature would boost immunity further. A drawback of such an approach is that the immunity induced by the initial vaccination would not or cannot be neutralizing, since it has to allow a boosting effect by infection. However, it has become increasingly clear over the past few years that heterologous anti-influenza immunity is naturally induced by repetitive heterologous infections [47,48]. Hence, any vaccination strategy aiming at neutralizing infection will completely abrogate such an effect. The identification of the first murine $CD8^+$ T cell restricted epitope of IBV in this thesis will allow future research to monitor the induction of heterosubtypic immunity against IBV by vaccine-induced non-sterilizing immunity. Additionally, the cross-protective nature of this epitope, and other NBe- or IBV-restricted epitopes specific for mouse and man, deserves future attention.

Influenza vaccination: The Good, The Bad and The Ugly

In a sense, influenza vaccination tactics can be coined as 'The Good, The Bad and The Ugly'. Conventional vaccination with inactivated viruses will induce a strong anti-HA response able of neutralizing homologous infection completely; The Good. However, a vaccine mismatch will not allow protection induced by such a vaccination, leading to a lack of protection of vaccinees: The Bad. Using vaccines based on CTL epitopes, certain HA epitopes, M2e of IAV and BM2e or NBe or IBV, infection would not be neutralized, inducing some clinical symptoms: The Ugly. However, as indicated by animal studies [49-53], these symptoms could be subdued to subclinical levels, and immunity towards (other) cross-reactive epitopes would be favored and built up over time. Like many other research groups [54], we feel this is the way forward for inducing effective influenza immunity, exploiting natural mechanisms for heightening heterologous immunity.

One main consideration to take into account when using an infection-permissive vaccination approach, either antibody or CTL inducing, is the effect this could have on viral antigenic selection and evolution. Immunity directed against a single or a select set of epitopes might drive the selection of viruses containing specific mutations within these epitopes. This is clear from conventional influenza vaccination using TIV vaccines or sequential infection, which drives antigenic mutation of HA and NA and selects for CTL epitope variants [55-58]. Additionally, sequential infection of mice in the presence of M2e-directed antibodies has been shown to induce mutations within the M2e-sequence [59]. However, in many instances, such escape is limited because of ramifications on viral fitness. For instance, the M2-escape mutants detected in the study by Zharikova *et al*. were limited to a single residue (P10) [59], a mutation which does not influence its recognition by polyclonal anti-M2e sera (unpublished data). CTL escape mutations on the other hand often have detrimental effects on viral fitness and require compensatory co-mutations [60-63]. This might be more challenging to overcome from a vaccination point-of-view, although a certain flexibility in epitope sequence is allowed for recognition by CTLs [64,65].

A more difficult question to address in preclinical settings is the effect infection-permissive vaccines could have on general evolution of influenza viruses. Under neutralizing pressure, only viruses able to escape immunity before initial infection will be able to generate progeny; when host immunity matches circulating seasonal strains, the level of viruses circumventing antiviral immunity will be rather low. Under infection-permissive immunity, selection of escape variants will occur both before (for antibody-inducing vaccines) and after initial infection. This entails selection of escape mutants could occur more rapidly. It is also not inconceivable this will drive evolution of influenza viruses as a whole faster than neutralizing immunity will do. Moreover, infection-permissiveness will also allow a higher level of progeny virus to be produced, which in turn could lead to higher transmissibility of (selected) viruses, fueling selection of escape variants and evolution even further. Theoretically, this means infection-permissive vaccines could have more detrimental than beneficial effects on the level of viral ecology. It remains unclear if broad immunity induced by infection-permissive vaccines can keep up with vaccine-induced viral evolution.

To date, the use of live attenuated influenza vaccines (LAIV) has not been reported to speed up antigenic evolution of seasonal influenza virus. Since such vaccines stimulate both the humoral and cellular arm of the immune system, they induce combined strain-specific neutralizing immunity and heterosubtypic broad infection-permissive immunity [66,67]. For single-epitope based infection-

permissive vaccines it might also be worthwhile to combine them with a vaccine inducing neutralizing immunity, to cover all bases. This has been shown advantageous for M2e-supplementation of conventional inactivated vaccines [68,69] and hence might be extended to other antigenic targets. Future will tell if such strategies are feasible and achievable for human influenza vaccination.

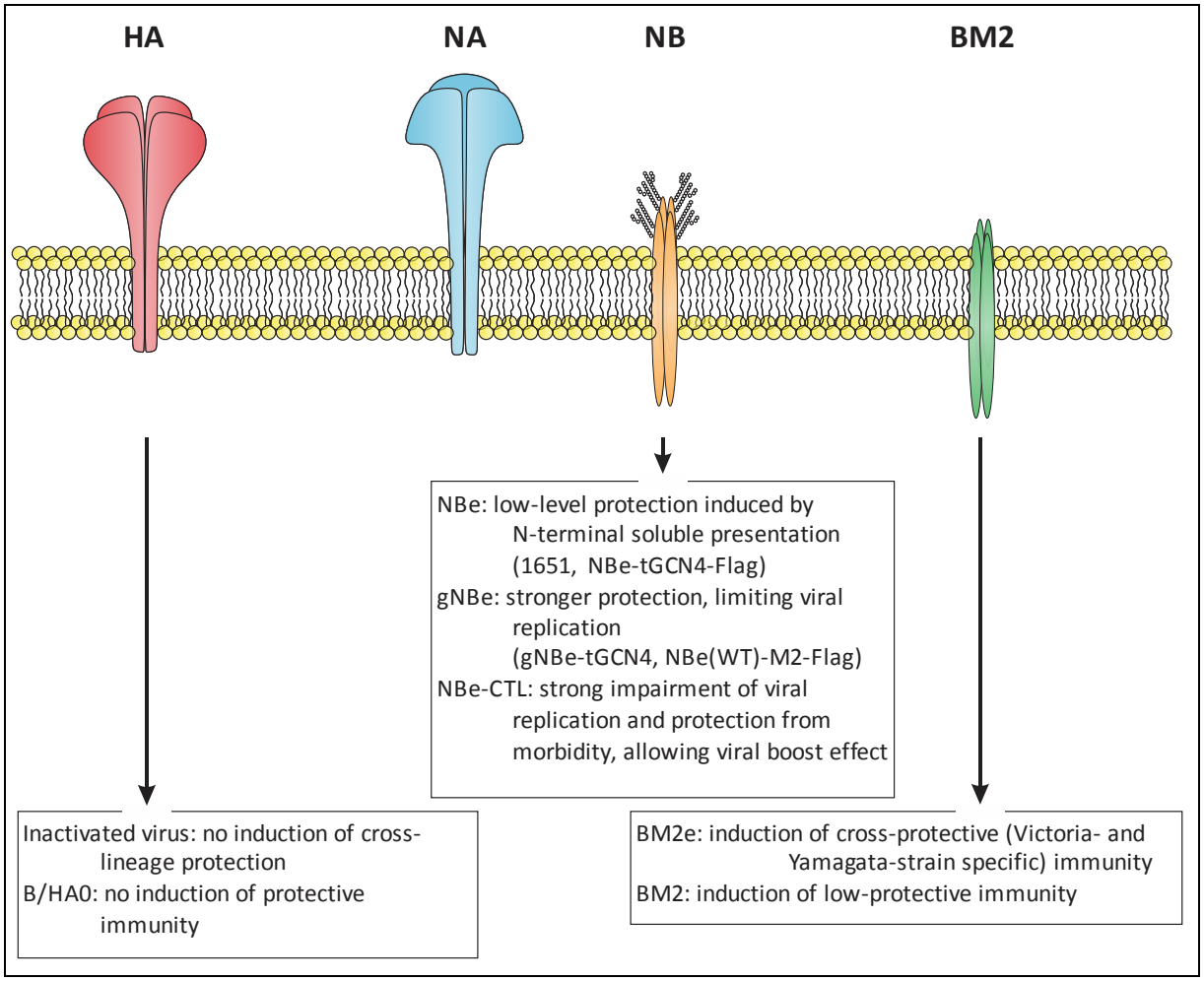


Figure IV.1. Overview of (non-)protective immune responses observed in this thesis.

Algemene discussie en perspectieven

Kruisbescherming geïnduceerd door B/HA0-, BM2e- en NBe-gebaseerde IBV kandidaat-vaccins

De huidige situatie van influenza vaccinatie moet dringen herbekeken worden. Goedgekeurde trivalente vaccins, bestaande uit geïnactiveerde of levend verzwakte virussen van influenza B (IBV) en de influenza A (IAV) A/H1N1 en A/H3N2 subtypen, geven sterke steriliserende bescherming tegen homologe of sterk gerelateerde stammen. Echter, gezien influenza virussen continue diversifiëren onder druk van vaccinatie of infectie geïnduceerde immuniteit, zal een verkeerde keuze van stam om vaccins aan te maken niet leiden tot inductie van beschermende immuniteit in gevaccineerde personen [1]. Specifiek voor IBV is dit een hedendaags probleem. Voor IAV worden twee stammen ingesloten in vaccins, behorende tot zowel het H1N1 als H3N2 subtype die momenteel circuleren in de menselijke populatie als seizoensgebonden stammen. IBV wordt weliswaar niet gekenmerkt door virus subtypes, maar er worden wel twee antigenisch verschillende lijnen erkend (de Victoria en de Yamagata lijn) [2-4]. Inclusie van slechts één IBV stam in het vaccin, behorende tot één van de twee lijnen, houdt in dat de keuze van een stam die tot de tegenovergestelde lijn behoort dan deze die circuleert zal leiden tot een vaccin mismatch. Het is inderdaad zo dat in het afgelopen decennium bijna de helft van de griepseizoenen gekenmerkt werden door een lijn mismatch tussen IBV vaccinstam en circulerende stam. Dit is vooral problematische voor personen die extra gevoelig zijn voor IBV infectie en afgeleide complicaties, zoals kinderen en ouderen [5-7]. Bovendien zal het niet beschermen van gevaccineerde personen het geloof in de voordelen van influenza vaccinatie teniet doen, wat zware socio-economische gevolgen kan hebben. In deze thesis wilden we probleem omtrent IBV vaccinatie aankaarten door het beschermend effect van op gepaste wijze gepresenteerde geconserveerde delen van IBV na te gaan in een muismodel. Zodoende wilden we bijdragen tot de weelde aan informatie dat gerelateerd onderzoek met IAV heeft opgeleverd (Hoofdstuk 1) [8] en de schaarse studies rond kruisbeschermende IBV vaccins te ondersteunen (Hoofdstuk 2) [9-13]. Een algemeen overzicht van vaststellingen gemaakt in deze studie is gegeven in Figuur IV.1.

We hebben als doelwit antigenen drie verschillende extravirionische epitopen gekozen, in aanzienlijke aantallen gepresenteerde door geïnfecteerde cellen. De idee achter dit was om in analogie met het M2 ectodomain van IAV [14] een vaccin te ontwikkelen dat humorale immuniteit kan opwekken. De gekozen antigenen omvatten het ectodomain van het BM2 en NB transmembraaneiwit (respectievelijk BM2e en NBe) en de klievingssequentie van HA (B/HA0). Om hun natuurlijke conformatie zo goed mogelijk na te bootsen werden specifieke dragereiwitten en expressiesystemen gekozen. Insertie van B/HA0 in de immunodominante lus van HBc kon duidelijk virusspecifieke antilichamen induceren, alhoewel infectie van geïmmuniseerde muizen geen bescherming kon aantonen. Dit is in schril contrast met de studie van Bianchi *et al.* [9] die met behulp van een B/HA0-OMPC vaccine muizen konden beschermen tegen infectie met IBV stammen van de verschillende lijnen. Kleine verschillen in de antigendosis en het infectiemodel kunnen deze discrepantie echter verklaren. Het B/HA0-HBc eiwit toonde een lage oplosbaarheid en vroeg om dubbelexpressie van zowel vrij HBc als B/HA0-HBc om een oplosbaar product te geven (3004 genaamd) met gemengde substitutiegraad. Terwijl het OMPC vaccin duidelijke hoge B/HA0-

specifieke antilichaamtiters kon opwekken, bepaald in B/HA0-peptide specifieke ELISA, kon 3004 slecht gemiddelde virusspecifieke titers opwekken (Fig. 4.6). Ons infectiemodel is gebaseerd op toediening van $4LD_{50}$ IBV in een volume van 50µL, terwijl Bianchi *et al.* een dosis van $1LD_{50}$ gebruikten in een volume van 20µL. Het kleiner volume en lagere virusdosis (alhoewel een exacte vergelijking van dit niet mogelijk is), gecombineerde met een betere immuunrespons de balans kan doen overhellen naar robuuste bescherming.

Het N-terminale NB ectodomain werd met behulp van HBc gepresenteerd op drie manieren: een directe N-terminale fusie (1651), gekoppeld aan $M2e_2$-HBc (NM_2H) om de bereikbaarheid van NBe te verhogen of ingevoegd in de immunodominante lus van HBc om zowel immunogeniciteit als bereikbaarheid te verhogen (1646). Verassend genoeg had enkel 1651 de mogelijkheid om beschermende immuniteit op te wekken tegen een potentieel letale dosis IBV. Zelfs gebruik makende van muisgeadapteerd B/Hong Kong/05/72 virus, met een sterk afwijkende NBe-sequentie in vergelijking met de consensus sequentie gebruikt voor vaccin constructie, kon NBe-HBc muizen beschermen tegen excessieve letaliteit. De presentatie van NBe, met een vrije N-terminus, op een partikelvormend HBc-eiwit blijkt superieur te zijn in vergelijking met het meer immunogene 1646 partikel. Het NM_2H eiwit, dat kleine aggregaten vormt in plaats van partikels, kan weliswaar even efficiënt als $M2e_2$-HBc een IAV infectie afzweren, maar kan geen beschermende NBe-responsen opwekken effectief tegen een IBV infectie. Uit de NBe-peptide specifieke ELISA kan afgeleid worden dat het niet-partikelvormig NM_2H eiwit lagere anti-NBe responsen opwekt dan 1651, waarschijnlijk gelinkt aan de T cel onafhankelijke inductie van antilichaamresponsen door partikelvormig HBc [15]. Het blijkt dus dat een immunogene presentatie van N-terminaal gepresenteerd NBe nodig is om afdoende bescherming op te wekken tegen IBV.

Gezien BM2 en NB tetrameren vormen, hebben we BM2e en NBe gekoppeld aan een tetrameriserende variant van de leucine zipper van GCN4 (tGCN4). Vier verschillende fusies werden gebruikt: een korte (5AA) en een langere (8AA) BM2e-sequentie gekoppeld aan tGCN4, NBe gekoppeld aan tGCN4 met een C-terminale driedubbele Flag-sequentie en NBe-tGCN4 geproduceerd door *P. pastoris* om glycosylatie van NBe te bekomen (gNBe-tGCN4), wat tijdens infectie ook gebeurt [16,17]. Door middel van chromatografische analyse, natieve PAGE en chemische cross-linking experimenten konden we tetramerisatie van de eiwitten bevestigen. Immunisatie van muizen met de tGCN4-gebaseerde eiwitten toonde aan dat het een extreem immunogene eiwitvorm is, met inductie van antilichaamtiters die 10^7 eindpuntsverdunning overschrijden. Beide BM2e-gebaseerde chimeren tonen inductie van BM2- en IBV-specifieke antilichamen, die bescherming kunnen bieden tegen een potentieel letale infectie met zowel B/Memphis/12/97 (een Yamagata-lijn virus) als B/HongKong/5/72 (een Victoria-lijn virus). NBe-tGCN4-Flag op zijn beurt induceert duidelijke anti-NBe responsen die reageren met NBe-expresserende getranfecteerde cellen, maar slechts lichtjes IBV geïnfecteerde cellen. De hoofdreden voor dit verschil is waarschijnlijk de glycosylatiestatus van de drie species. Door niet-geglycosyleerd NBe-tGCN4-Flag te gebruiken worden antilichamen opgewekt die reageren met getransfecteerde cellen die NBe-eiwitten expresseren, aangezien deze zowel niet-, mono- als di-geglycosyleerd NBe dragen (Fig. 3.16). Voor geïnfecteerde cellen is de exacte glycosylatiestatus van NB binnen ons *in vitro* systeem niet gekend maar kan complexer zijn en kan zo anti-NBe detectie benadelen. Zoals bij de N-terminale NBe-HBc fusies kan het silpelweg een kwestie van hoeveelheid zijn. Wanneer we immunisaties met NBe-tGCN4-Flag geadjuvanteerd met Alhydrogel en Montanide-ISA-720 vergelijken kan de laatste hogere anti-NBe titers induceren en biedt bijgevolg betere bescherming. Als anti-NBe responsen verder verhoogd zouden kunnen

worden, kan de bescherming nog meer sluitend zijn. De vaststelling dat het NBe-specifieke 15G5mAb IBV geïnfecteerde cellen kan kleuren, maar muizen niet kan besschermen, kan ook een verschil in beschikbaarheid van NBe *in vitro* en *in vivo* reflecteren, iets dat onderzocht zou moeten worden binnen ons muismodel. Alternatief kan het ook een kwalitatief probleem voorstellen, waarbij een *in vivo* opgewekte polyklonale respons betere IBV herkenning en bescherming geeft *in vivo*.

Het gNBe-tGCN4 eiwit draagt glycosylstructuren die minder complex zijn als op natief NB [17], maar geven wel een algemeen geglycosyleerde vorm van NBe. Als vaccin kan het significant meer bescherming bieden tegen een mogelijks letale infectie met B/Memphis/12/97 in vergelijking met NBe-tGCN4-Flag, wat het voordeel van een geglycosyleerd NBe als antigen verder onderstreept. Deze vaststelling werd verder uitgebreid door het gebruik van getransfecteerde cellen als immunogen. In deze opstelling konden NBe(WT)-M2-Flag expresserende cellen significante bescherming opwekken in vergelijking met NBe(Mut)-M2-Flag expresserende cellen. De introductie van mutaties in de NBe-sequentie om glycosylatie te vermijden kan uiteraard herkenning van de wild type NBe-sequentie verhinderen, alhoewel de inverse situatie (herkenning van NBe(Mut) door immuunserum opgewekt met NBe(WT)) geen gelijkaardige problemen toont. Desalniettemin, NBe(WT)-M2-Flag kan significante bescherming opwekken, wat verder het voordeel van een geglycosyleerd NBe-vaccin onderstreept. De ontwikkeling van een cellulair geproduceerd en gesecreteerd NBe-eiwit om dit verder te evalueren is daarom zeer aangewezen.

In een aantal opstellingen werden hitte-geïnactiveerde IBV virussen aangewend als immunogen om homologe en heterologe bescherming na te gaan. Algemeen gezien kon geen heterologe bescherming worden vastgesteld. Dit is in overeenkomst met andere dierstudies [18], alhoewel onder specifieke condities van vaccinatie de immuunrespons opgewerkt door geïnactiveerd IBV verbreed kan worden om reactief te zijn met beide lijnen [19]. De methode voor inactivatie (30min incubatie bij 56°C) kan antigenische structuren meer vernietigen als formaline of detergens inactivatie. Bij mensen kunnen kruisreactieve titers gedetecteerd worden in voorheen geïmmuniseerde personen [18,20-22]. Het lijkt dus de moeite waard om epitopen te identificeren aanwezig in HA die verantwoordelijk zijn voor kruisreactiviteit. Dit kan gerealiseerd worden door bv. *reverse vaccinology*, zoals uitgevoerd voor identificatie van de LAH structuur in IAV HA [23,24].

Als algemeen besluit kan gesteld worden dat zowel BM2e- als geglycosyleerd NBe-gebaseerde kandidaatvaccins aanzienlijke bescherming kunnen bieden tegen een mogelijk letale IBV infectie. Anti-BM2e responsen kunnen bovendien bescherming bieden tegen zowel Yamagata- als Victoria-lijn IBV stammen, wat het een kruisbeschermende vaccin basis maakt. Het moet echter vermeld worden dat de detectie van bescherming gebaseerd is op overleving van geïnfecteerde muizen. Wanneer naar morbiditeit wordt gekeken, kan geen enkel kruisbeschermend kandidaatvaccin morbiditeit vermijden wanneer dosissen van $2.5LD_{50}$ of $4LD_{50}$ gebruikt worden. Dit is waarschijnlijk gerelateerd aan het muismodel, waarbij hoge dosissen (uitgedrukt in pfu) nodig zijn om letaliteit te induceren. Om pulmonaire replicatie van virus na te gaan word een ongeveer 10voudig lagere dosis gebruikt voor infectie, en dit toonde in veel gevallen inderdaad sterkere antivirale responsen in vergelijking met controles. Het is dus interessant om de vaccinkandidaten hier beschreven te hertesten in een lage-dosis infectiemodel, gebruik maken van bv. dosissen van $0.3LD_{50}$, en bescherming na te gaan op basis van het voorkomen van morbiditeit.

Het NB ectodomein bevat een T cel epitoop dat sterke bescherming tegen IBV biedt

In een poging om immuniteit opgewekt door NBe-tGCN4-Flag te verhogen, werden naast Balb/c muizen twee andere inteeltstammen gebruikt, C3H/HeN en C57Bl/6. Vertrekkende van een kleine reductie in long virustiters kon verder onderzoek een sterk $CD8^+$ T cel epitoop identificeren binnen de NBe-sequentie, specifiek voor de genetische $H2^b$-achtergrond van C57Bl/6 muizen. We konden aantonen dat dit epitoop cytotoxische effecten kan opwekken gericht tegen peptide-opgeladen cellen en een sterke reductie in virusreplicatie in de longen van geïmmuniseerde muizen kan opwekken. Bovendien kon NBe-gebaseerde cellulaire immuniteit, ook al is ze niet neutraliserend, de klinische afloop van muizen geïnfecteerd met een subletale dosis van $0.3LD_{50}$ drastisch verbeteren.

Het is opmerkelijk dat parenterale immunisatie met een oplosbaar antigen (NBe-tGCN4-Flag), geadjuvanteerd door een aluminiumzout, dergelijke sterke cellulaire responsen kan induceren. Een van de centrale paradigma's voor de efficiënte inductie van T cel responsen stelt immers dat in dit geval de intracellulaire weg voor antigenpresentatie gevolgd moet worden. Gekende uitzonderingen omvatten het gebruik van celpenetrerende peptiden (CPP) of eiwittransductie domainen (PTD) die directe aflevering van het eiwit in het cytoplasma toelaten [25], of het gebruik van specifieke adjuvantia met gelijkaardige effecten of die immuuncellen zoals monocyten of dendritische cellen (DCs) kunnen activeren [26-30]. DCs bezitten specifiek de eigenschap om exogene antigenen te kruispresenteren, leidend tot de inductie van cellulaire responsen [31,32]. Het specifieke mechanisme verantwoordelijk hier voor de inductie van T cellen blijft echter onduidelijk. Eén mogelijkheid is de vorming van zgn. immuuncomplexen (ICs), bestaande uit eiwit gebonden door een grote hoeveelheid antilichamen. Algemeen gezien hebben ICs een negatief effect op immuunresponsen, leiden tot een ontsporing van ziekte of inductie van auto-immuun afwijkingen [33-35]. In sommige gevallen echter kunnen ze specifiek aangewend worden bij immunisaties om cellulaire en humorale responsen op te wekken [36,37]. Aangezien het tGCN4-Flag deel van het vaccin zo immunogeen is, is het aannemelijk dat vrij antigen (tijdens de initiële immunisatie of bij verdere immunisaties) bedekt wordt door antilichamen. Zo gevormde ICs kunnen vervolgens opgenomen worden door DCs via FcγRI- (CD64-) gemedieerde fagocytose, resulterend in kruispresentatie van het antigen [38,39]. Een aantal vaststellingen steunen deze theorie. Ten eerste, immunisatie van C57Bl/6 muizen met een mengsel van NBe-peptide en tGCN4-Flag, wat niet immunogeen is in deze muizen, kon geen T cel responsen opwekken (data niet getoond). Ten tweede, andere NBe-dragereiwit fusies die als proteïne toegediend werden, zijn gebaseerd op het sterk immunogene HBc en tGCN4 (Hoofdstuk 5, Additionele data); ook deze konden NBe-specifieke T cel responsen opwekken. En ten derde, door gebruik te maken van een lage hoeveelheid chimeer eiwit bestaande uit een NP-epitoop gefusioneerd aan tGCN4-Flag in combinatie met muis IgG1 anti-Flag antilichamen, konden relatief hoge T cel responsen opgewekt worden. Alhoewel indirect duiden deze vaststellingen aan dat er een link is tussen hoge antilichaamresponsen en cellulaire responsen. Om dit te bevestigen kunnen verschillende modellen gebruikt worden. Als bv. een chemische koppeling van NBe-peptide en tGCN4-Flag niet immunogeen blijkt in C57Bl/6 muizen, kan het effect van anti-tGCN4-Flag antilichamen op de cellulaire immuunrespons bekeken worden. Analoog kan immunisatie van B cel deficiënte muizen (bv. $Ig\mu^{MT/MT}$ muizen [40]) of muizen zonder FcγRI ($CD64^{-/-}$ muizen) of de gemeenschappelijke FcRγ-keten meer inzicht geven. Indien aantoonbaar wordt de waarde van tGCN4-Flag, of een gelijkaardige immunogene drager in het algemeen, in het induceren van een breed (t.t.z. humoraal en cellulair) immuun antwoord aanzienlijk versterkt worden.

Een tweede intrigerende vaststelling was het feit dat cellulaire immuniteit opgewekt met een enkele vaccinatie met een NBe-gebaseerd immunogen (NBe-tGCN4-Flag, NBe-HBc, NBe-DNA) aanzienlijk kon verhoogd worden door een subletale IBV infectie. Een dergelijk heterologe boosteffect is stimulerend, als het efficiënt vertaald kan worden naar humane immunologie. Recente klinische testen met HIV of malaria afgeleide antigenen konden dergelijke effecten aantonen in mensen, wanneer gebruik gemaakt werd van DNA vaccins of virale vectoren [41-43]. Het begrip van de 'originele antigenische zonde' is gekend, en anekdotische, epidemiologische en immunologische data hebben aangetoond dat dit begrip ook toepasbaar is op influenza infecties. Voorbeelden omvatten data van voorbije influenza pandemieën [44,45], de IBV vaccinatie studies hierboven vermeld [18-22] en elke *reverse vaccinology* benadering die kruisreactieve antilichamen heeft kunnen identificeren [46]. De afgeleide vaccinatie strategie is aantrekkelijk: elke infectie die plaatsvindt na immunisatie met een geconserveerd viraal kenmerk zal immuniteit versterken. Een nadeel van deze aanpak is echter dat de immuniteit opgewekt door de initiële vaccinatie niet kan of niet mag neutraliserend zijn, aangezien het een boost effect door infectie moet toelaten. De laatste jaren is het echter duidelijk geworden dat heterologe anti-influenza immuniteit natuurlijk wordt opgewekt door herhaaldelijke heterologe infecties [47,48]. Zodoende zal elke vaccinatie strategie die neutraliserende immuniteit opwekt een dergelijk effect blokkeren. De identificatie van het eerste muriene $CD8^+$ T cell epitoop voor IBV in deze thesis zal verder onderzoek toelaten de inductie van heterologe immuniteit tegen IBV door vaccin geïnduceerde niet-steriliserende immuniteit te volgen. Daarnaast verdienen het kruisbeschermend potentieel van dit epitoop, en andere NBe- of algemeen IBV-afgeleide epitopen specifiek voor de muis en de mans, meer aandacht.

Influenza vaccinatie: *The Good, The Bad and The Ugly*

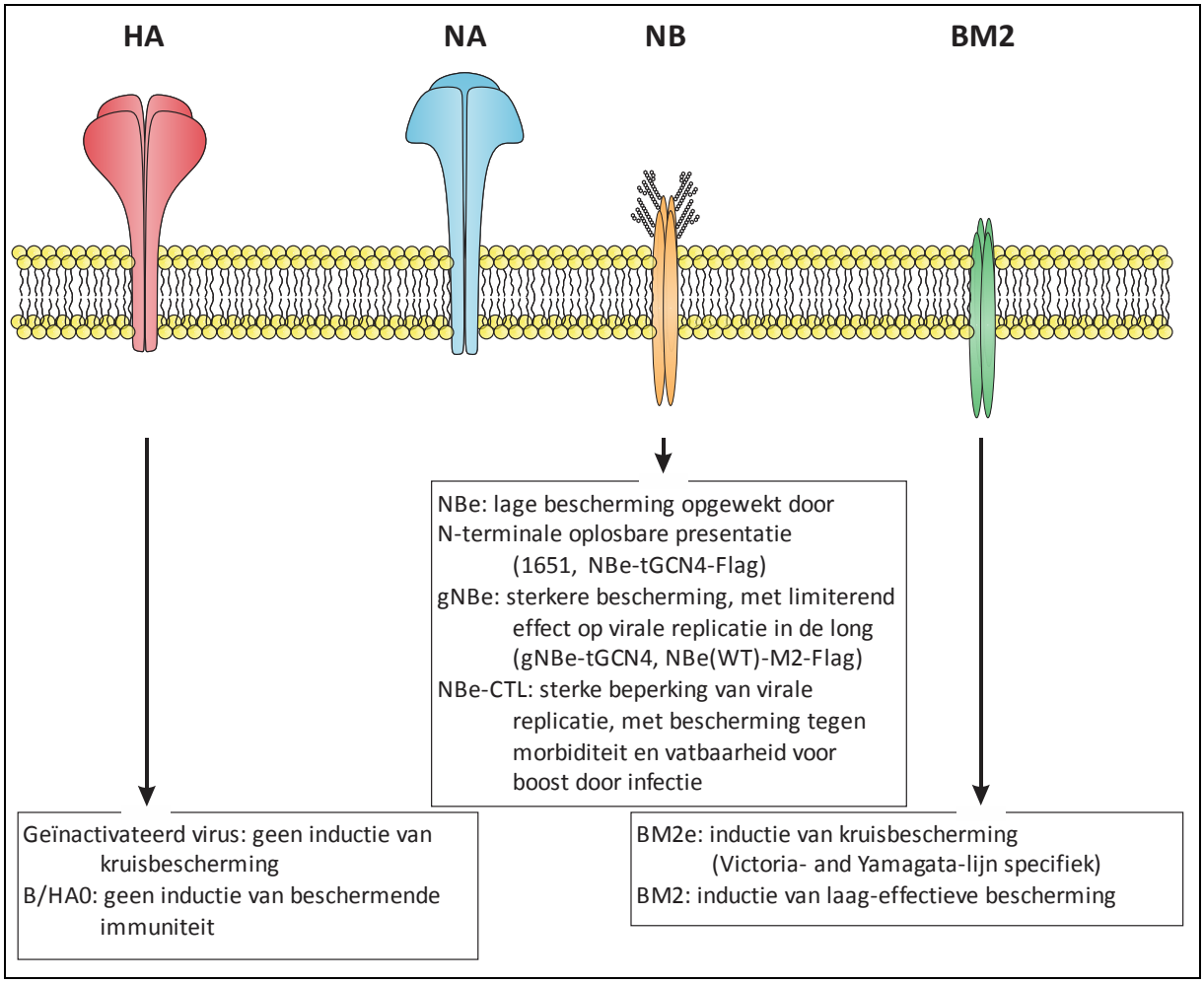
In zekere zin kan influenza vaccinatie omschreven worden met het adagium: "*The Good, The Bad and The Ugly*". Conventionele vaccinatie met geïnactiveerde virussen zal sterke anti-HA respons induceren, die infectie met een homologe virusstam kan neutraliseren: *The Good*. Een mismatch tussen vaccin en circulerende virusstam zal echter geen bescherming kunnen bieden bij gevaccineerde personen: *The Bad*. Wanneer gebruik gemaakt wordt van CTL epitopen, bepaalde HA epitopen, M2e van IAV en BM2e of NBe van IAV voor vaccinconstructie, zal infectie niet geneutraliseerd worden, waarbij lichte symptomen geïnduceerd zullen worden: *The Ugly*. Echter, deze symptomen kunnen tot subklinisch niveau herleid worden, zoals aangetoond door dierstudies [49-53], en immuniteit tegenover (andere) kruisbeschermende epitopen zou bevoordeeld worden en opgebouwd worden met de tijd. Zoals andere onderzoeksgroepen [54] is het onze mening dat dit de juiste aanpak is om effectieve immuniteit op te wekken tegen influenza, gebruik makend van natuurlijke mechanismen om heterologe immuniteit op te bouwen.

Een belangrijke bedenking die gemaakt kan worden betreffende het gebruik van infectiepermissieve vaccins, zowel antilichaam als CTL inducerend, is het effect deze kunnen hebben op antigenische selectie and evolutie van influenza virus. Immuniteit gericht tegen een enkel of een beperkte groep van epitopen zou de selectie van virussen met specifieke mutaties binnen deze epitopen kunnen drijven. Dit is zo voor conventionele vaccinatie met geïnactiveerde virussen of sequentiële infectie, wat de antigenische variatie van HA en NA beïnvloedt en selecteert voor CTL epitoop mutanten [55-58]. Bovendien werd aangetoond dat na opeenvolgende rondes van influenza A infectie in muizen in de

aanwezigheid van anti-M2e antilichamen, mutaties worden geïnduceerd in de M2e-sequentie [59]. Echter, meestal is dergelijke omzeiling van immuniteit door mutatie beperkt door weerslag op de virale fitness. Zo waren bvb. de M2e-mutanten gedetecteerd in de studie van Zharikova *et al*. beperkt tot een enkel residu [59], een mutatie die geen invloed heeft op de herkenning van M2e door polyklonale sera (ongepubliceerde data). CTL-epitoop mutaties op hun beurt hebben vaak een zware terugslag op viabiliteit van het virus en vragen om compenserende co-mutaties [60-63]. Vanuit een vaccinatie standpunt kan dit een uitdaging zijn om te omzeilen en verhelpen, alhoewel een zekere flexibiliteit in epitoopsequentie toegelaten is voor herkenning door CTLs [64,65].

Een moeilijkere vraag om te beantwoorden via preklinische testen is het effect infectiepermissieve vaccins zouden kunnen hebben op de algemene evolutie van influenzavirus. Onder neutraliserende druk zullen enkel virussen die vóór de initiële infectie immuniteit kunnen omzeilen nageslacht geven; wanneer de immuniteit van de gastheer overeenstemt met circulerende virusstammen, zal zo het niveau van virussen dat antivirale immuniteit kan ontwijken relatief laag liggen. Onder infectiepermissieve immuniteitsdruk zal de selectie van mutante virussen zowel vóór (voor antilichaaminducerende vaccins) als na de initiële infectie gebeuren. Dit houdt in dat de selectie van virussen die immuniteit omzeilen sterker zal zijn en vlugger kan gebeuren. Het is ook niet ondenkbaar dat dit de evolutie van influenzavirussen in het algemeen meer kan leiden dan neutraliserende immuniteit dit kan. Bovendien zal infectiepermissiviteit ook toelaten dat er een hoger aantal virussen geproduceerd wordt bij infectie, wat kan leiden tot hogere transmissie van (geselecteerde) virussen en zo verder selectie en evolutie van virussen kan drijven. Theoretisch kan dit inhouden dat infectiepermissieve vaccins meer nadelen dan voordelen kunnen hebben op het niveau van virale ecologie. Het blijft onduidelijk of de brede immuniteit geïnduceerd door infectiepermissieve vaccins de vaccin-geïnduceerde virale evolutie kan bijbenen.

Tot nog toe werd niet bericht over een eventuele invloed die levend geattenueerde influenza virussen (LAIV) als vaccin hebben op de evolutie van seizoensgriep. Aangezien dergelijke vaccins zowel de humorale als de cellulaire arm van het immuunsysteem activeren, induceren ze zowel stamspecifieke neutraliserende immuniteit als brede heterosubtypische infectiepermissieve immuniteit [66,67]. Voor infectiepermissieve vaccins gebaseerd op een enkel epitoop kan het de moeite zijn deze te combineren met een vaccin dat neutraliserende immuniteit opwekt, om zo op beide paarden te wedden. Dit werd al voordelig aangetoond voor M2e-supplementatie van conventionele geïnactiveerde vaccins [68,69] en kan misschien ook toegepast worden op andere antigenen. De toekomst zal uitwijzen of een dergelijke aanpak doenbaar en haalbaar is voor influenza vaccinatie van de menselijke populatie.



Figuur IV.1. Overzicht van (niet-)beschermende immuunresponsen vastgesteld in deze thesis.

References

1. Carrat, F. & Flahault, A. Influenza vaccine: the challenge of antigenic drift. *Vaccine* **25**, 6852-6862 (2007).
2. Rota, P.A*., et al.* Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59-68 (1990).
3. Chen, J.M*., et al.* Exploration of the emergence of the Victoria lineage of influenza B virus. *Archives of virology* **152**, 415-422 (2007).
4. McCullers, J.A., Saito, T. & Iverson, A.R. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *Journal of virology* **78**, 12817 (2004).
5. Win, M.K*., et al.* Influenza B Outbreak among Influenza-vaccinated Welfare Home Residents in Singapore. *Annals of the Academy of Medicine, Singapore* **39**, 448-452 (2010).
6. Camilloni, B*., et al.* An influenza B outbreak during the 2007/2008 winter among appropriately immunized elderly people living in a nursing home. *Vaccine* **28**, 7536-7541 (2010).
7. Goni, N*., et al.* Influenza B viruses isolated in Uruguay during the 2002-2005 seasons: Genetic relations and vaccine strain match. *Virus research* **123**, 100-104 (2007).
8. Roose, K., Fiers, W. & Saelens, X. Pandemic preparedness: toward a universal influenza vaccine. *Drug News Perspect* **22**, 80-92 (2009).
9. Bianchi, E*., et al.* Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology* **79**, 7380 (2005).
10. Camilloni, B., Neri, M., Lepri, E. & Iorio, A.M. Cross-reactive antibodies in middle-aged and elderly volunteers after MF59-adjuvanted subunit trivalent influenza vaccine against B viruses of the B/Victoria or B/Yamagata lineages. *Vaccine* **27**, 4099-4103 (2009).
11. Wang, Y*., et al.* Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity. *New Microbiologica* **33**, 311-317 (2010).
12. Stech, J*., et al.* Influenza B Virus With Modified Hemagglutinin Cleavage Site as a Novel Attenuated Live Vaccine. *Journal of Infectious Diseases* **204**, 1483-1490 (2011).
13. Horimoto, T., Iwatsuki-Horimoto, K., Hatta, M. & Kawaoka, Y. Influenza A viruses possessing type B hemagglutinin and neuraminidase: potential as vaccine components. *Microbes and infection* **6**, 579-583 (2004).
14. El Bakkouri, K*., et al.* Universal Vaccine Based on Ectodomain of Matrix Protein 2 of Influenza A: Fc Receptors and Alveolar Macrophages Mediate Protection. *The Journal of Immunology* **186**, 1022-1031 (2011).
15. Milich, D.R*., et al.* Comparative immunogenicity of hepatitis B virus core and E antigens. *The Journal of Immunology* **141**, 3617 (1988).
16. Williams, M.A. & Lamb, R.A. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Molecular and Cellular Biology* **6**, 4317 (1986).
17. Williams, M.A. & Lamb, R.A. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Molecular and Cellular Biology* **8**, 1186 (1988).
18. Levandowski, R.A*., et al.* Cross-reactive antibodies induced by a monovalent influenza B virus vaccine. *Journal of clinical microbiology* **29**, 1530 (1991).
19. Asahi-Ozaki, Y*., et al.* Secretory IgA antibodies provide crossâ€protection against infection with different strains of influenza B virus. *Journal of medical virology* **74**, 328-335 (2004).
20. Levandowski, R.A*., et al.* Antibody responses to influenza B viruses in immunologically unprimed children. *Pediatrics* **88**, 1031 (1991).
21. Zuckerman, M.A*., et al.* Serological responses in volunteers to inactivated trivalent subunit influenza vaccine: antibody reactivity with epidemic influenza A and B strains and evidence of a rapid immune response. *Journal of medical virology* **33**, 133-137 (1991).
22. Pyhälä, R*., et al.* Immune response to inactivated influenza virus vaccine: antibody reactivity with epidemic influenza B viruses of two highly distinct evolutionary lineages. *Vaccine* **10**, 631-636 (1992).
23. Wang, T.T*., et al.* Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proceedings of the National Academy of Sciences* **107**, 18979-18984 (2010).
24. Steel, J*., et al.* Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain. *mBio* **1**, e00018 (2010).

25. Brooks, N.A., Pouniotis, D.S., Tang, C.K., Apostolopoulos, V. & Pietersz, G.A. Cell-penetrating peptides: application in vaccine delivery. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* **1805**, 25-34 (2010).
26. Chikh, G. & Schutze-Redelmeir, M.P. Liposomal delivery of CTL epitopes to dendritic cells. *Bioscience reports* **22**, 339-353 (2002).
27. Aranda, F.*, et al.* Adjuvant combination and antigen targeting as a strategy to induce polyfunctional and high-avidity T-cell responses against poorly immunogenic tumors. *Cancer research* **71**, 3214 (2011).
28. Klinman, D.M., Klaschik, S., Sato, T. & Tross, D. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. *Advanced drug delivery reviews* **61**, 248-255 (2009).
29. Bungener, L., Huckriede, A., Wilschut, J. & Daemen, T. Delivery of protein antigens to the immune system by fusion-active virosomes: a comparison with liposomes and ISCOMs. *Bioscience reports* **22**, 323-338 (2002).
30. Kronenberg, K.*, et al.* Vaccination with TAT-Antigen Fusion Protein Induces Protective, CD8+ T Cell-Mediated Immunity Against Leishmania Major. *Journal of Investigative Dermatology* **130**, 2602-2610 (2010).
31. Jondal, M., Schirmbeck, R. & Reimann, J. MHC class I-restricted CTL responses to exogenous antigens. *Immunity* **5**, 295 (1996).
32. Brossart, P. & Bevan, M.J. Presentation of exogenous protein antigens on major histocompatability complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* **90**, 1594-1599 (1997).
33. Monsalvo, A.C.*, et al.* Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nature medicine* **17**, 195-199 (2010).
34. Aurora, A.B.*, et al.* Immune complex-dependent remodeling of the airway vasculature in response to a chronic bacterial infection. *The Journal of Immunology* **175**, 6319 (2005).
35. Tanaka M, K.S., Sieling PA, Lee DJ, Rea TH, Modlin RL. Activation of Fc gamma RI on monocytes triggers differentiation into immature dendritic cells that induce autoreactive T cell responses. *J Immunol* **183**, 2349-2355 (2009).
36. Hioe, C.E.*, et al.* The use of immune complex vaccines to enhance antibody responses against neutralizing epitopes on HIV-1 envelope gp120. *Vaccine* **28**, 352-360 (2009).
37. Schuurhuis, D.H.*, et al.* Immune complex-loaded dendritic cells are superior to soluble immune complexes as antitumor vaccine. *The Journal of Immunology* **176**, 4573 (2006).
38. Regnault, A.*, et al.* FcÎ³ receptorâ€“mediated induction of dendritic cell maturation and major histocompatibility complex class Iâ€“restricted antigen presentation after immune complex internalization. *The Journal of experimental medicine* **189**, 371 (1999).
39. Schuurhuis, D.H.*, et al.* Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *The Journal of Immunology* **168**, 2240 (2002).
40. Kitamura, D., Roes, J., KÃ¼hn, R. & Rajewsky, K. AB cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin Î¼ chain gene. (1991).
41. Aboud, S.*, et al.* Strong HIV-specific CD4+ and CD8+ T-lymphocyte proliferative responses in healthy individuals immunized with an HIV-1 DNA vaccine and boosted with recombinant modified vaccinia virus ankara expressing HIV-1 genes. *Clinical and Vaccine Immunology* **17**, 1124 (2010).
42. Keefer, M.C.*, et al.* A phase I trial of preventive HIV vaccination with heterologous poxviral-vectors containing matching HIV-1 inserts in healthy HIV-uninfected subjects. *Vaccine* **29**(2011).
43. Sheehy, S.H.*, et al.* Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. *Molecular Therapy* (2011).
44. Epstein, S.L. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. *Journal of Infectious Diseases* **193**, 49 (2006).
45. Adalja, A.A. & Henderson, D.A. Original antigenic sin and pandemic (H1N1) 2009. *Emerging infectious diseases* **16**, 1028.
46. Sui, J.*, et al.* Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature structural & molecular biology* **16**, 265-273 (2009).
47. Bodewes, R.*, et al.* Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus. *PLoS One* **4**, e5538 (2009).

48. Bodewes, R., Kreijtz, J.H.C.M. & Hillaire, M. Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8+ T-cell responses in mice. *Journal of general virology* **91**, 1743 (2010).
49. McLain, L. & Dimmock, N.J. Protection of mice from lethal influenza by adoptive transfer of non-neutralizing haemagglutination-inhibiting IgG obtained from the lungs of infected animals treated with defective interfering virus. *Journal of General Virology* **70**, 2615 (1989).
50. Neirynck, S*., et al.* A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nature medicine* **5**, 1157-1163 (1999).
51. De Filette, M*., et al.* Universal influenza A vaccine: optimization of M2-based constructs. *Virology* **337**, 149-161 (2005).
52. Carragher, D.M., Kaminski, D.A., Moquin, A., Hartson, L. & Randall, T.D. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *The Journal of Immunology* **181**, 4168 (2008).
53. Santiago, F.W., Fitzgerald, T., Treanor, J.J. & Topham, D.J. Vaccination with drifted variants of avian H5 hemagglutinin protein elicits a broadened antibody response that is protective against challenge with homologous or drifted live H5 influenza virus. *Vaccine* **29**(2011).
54. Bodewes, R., Kreijtz, J.H.C.M. & Rimmelzwaan, G.F. Yearly influenza vaccinations: a double-edged sword? *The Lancet infectious diseases* **9**, 784-788 (2009).
55. Price, G.E., Ou, R., Jiang, H., Huang, L. & Moskophidis, D. Viral Escape by Selection of Cytotoxic T Cell-Resistant Variants in Influenza a Virus Pneumonia. *The Journal of experimental medicine* **191**, 1853 (2000).
56. Voeten, J.T.M*., et al.* Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *Journal of virology* **74**, 6800 (2000).
57. Boon, A.C.M*., et al.* Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Journal of virology* **76**, 2567 (2002).
58. Rimmelzwaan, G.F*., et al.* Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus research* **103**, 97-100 (2004).
59. Zharikova, D., Mozdzanowska, K., Feng, J., Zhang, M. & Gerhard, W. Influenza Type A Virus Escape Mutants Emerge In Vivo in the Presence of Antibodies to the Ectodomain of Matrix Protein 2. *Journal of virology* **79**, 6644 (2005).
60. Rimmelzwaan, G.F., Berkhoff, E.G.M., Nieuwkoop, N.J., Fouchier, R.A.M. & Osterhaus, A. Functional compensation of a detrimental amino acid substitution in a cytotoxic-T-lymphocyte epitope of influenza a viruses by comutations. *Journal of virology* **78**, 8946 (2004).
61. Berkhoff, E.G.M*., et al.* Functional constraints of influenza A virus epitopes limit escape from cytotoxic T lymphocytes. *Journal of virology* **79**, 11239 (2005).
62. Rimmelzwaan, G.F*., et al.* Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants. *Journal of general virology* **86**, 1801 (2005).
63. Berkhoff, E.G.M*., et al.* Fitness costs limit escape from cytotoxic T lymphocytes by influenza A viruses. *Vaccine* **24**, 6594-6596 (2006).
64. Boon, A*., et al.* Recognition of homo-and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. *The Journal of Immunology* **172**, 2453 (2004).
65. Valkenburg, S.A*., et al.* Protective efficacy of cross-reactive CD8+ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold. *PLoS Pathogens* **6**, e1001039 (2010).
66. Chen, G.L., Lau, Y.F., Lamirande, E.W., McCall, A.W. & Subbarao, K. Seasonal influenza infection and live vaccine prime for a response to the 2009 pandemic H1N1 vaccine. *Proceedings of the National Academy of Sciences* **108**, 1140 (2011).
67. Hoft, D.F*., et al.* Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. *Journal of Infectious Diseases* **204**, 845-853 (2011).
68. Wu, F., Yuan, X.Y., Huang, W.S. & Chen, Y.H. Heterosubtypic protection conferred by combined vaccination with M2e peptide and split influenza vaccine. *Vaccine* **27**, 6095 (2009).
69. Song, J.M., Van Rooijen, N., Bozja, J., Compans, R.W. & Kang, S.M. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. *Proceedings of the National Academy of Sciences* **108**, 757-761 (2011).

Part 2.

Confidential

Part 3.

Addenda

Additional publications

Supplemental Material can be found at:
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An Influenza A Vaccine Based on Tetrameric Ectodomain of Matrix Protein 2*[S]

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Matrix protein 2 (M2) of influenza A is a tetrameric type III membrane protein that functions as a proton-selective channel. The extracellular domain (M2e) has remained nearly invariable since the first human influenza strain was isolated in 1933. By linking a modified form of the leucine zipper of the yeast transcription factor *GCN4* to M2e, we obtained a recombinant tetrameric protein, M2e-tGCN4. This protein mimics the quaternary structure of the ectodomain of the natural M2 protein. M2e-tGCN4 was purified, biochemically characterized, and used to immunize BALB/c mice. High M2e-specific serum IgG antibody titers were obtained following either intraperitoneal or intranasal administration. Immunized mice were protected fully against a potentially lethal influenza A virus challenge. Antibodies raised by M2e-tGCN4 immunization specifically bound to the surface of influenza-infected cells and to an M2-expressing cell line. Using a M2e peptide competition enzyme-linked immunosorbent assay with M2-expressing cells as target, we obtained evidence that M2e-tGCN4 induces antibodies that are specific for the native tetrameric M2 ectodomain. Therefore, fusion of an oligomerization domain to the extracellular part of a transmembrane protein allows it to mimic the natural quaternary structure and can promote the induction of oligomer-specific antibodies.

Influenza has one of the highest infection rates of all human viruses and can kill healthy persons of all ages (1). It is estimated that influenza infection during seasonal epidemics kills 1 in 1000 infected individuals, whereas an unpredictable pandemic is likely to kill millions. In addition, increased hospitalization and absenteeism from school and work are direct consequences of the flu. At present, the best way to protect against influenza is to vaccinate against the ever-mutating strains (2). However, antigenic drift and occasional shift of the two major membrane glycoproteins, hemagglutinin and neuraminidase, make vaccine production cumbersome and necessitate yearly revision of the vaccine seed strains by the World Health Organization.

Influenza A also encodes a third integral membrane protein, M2,[2] a homotetramer, the subunit of which has a small external domain (M2e) of 23 amino acid residues (3). Natural M2 protein is present in a few copies in the virus particle but in abundance on virus-infected cells (4). In contrast to hemagglutinin and neuraminidase, M2e is almost nonimmunogenic (5), and its sequence is highly conserved. Capitalizing on these properties, we developed a universal influenza A vaccine by linking the M2e peptide to a virus-like particle based on the hepatitis B virus core (HBc) (6). In this context, M2e is highly immunogenic, and the M2e-HBc vaccine induces antibodies that protect mice against influenza-induced death and morbidity.

Oligomeric proteins found in vaccines derived by inactivating or attenuating a pathogen often function as their major antigenic determinant. Conformational epitopes embedded in the quaternary structures may critically contribute to immunogenicity, but the oligomeric status of the antigenic structures may change during vaccine preparation, leading to aggregation or disassembly into monomers. For example, when producing influenza split vaccines, the hemagglutinin and neuraminidase oligomeric antigens may lose their oligomeric structure during the virus disruption step, or they may form aggregates. Specific protein oligomerization is critical for the function of many proteins. For example, influenza virus hemagglutinin is a homotrimer (7), and neuraminidase is a homotetramer composed of two disulfide-linked dimers (8, 9, 10, 11). Remarkably, the enzymatic activity of neuraminidase is associated only with the tetrameric form (12). Furthermore, tetrameric neuraminidase molecules are considerably more immunogenic than the monomers and dimers. The quaternary structure of an oligomeric protein is often determined by a subdomain with strong oligomerization properties. In many instances, an oligomerization subdomain can be substituted by a heterologous motif with similar conformation-inducing properties. For example, the p53 tetramerization domain can be replaced with a tetrameric coiled-coil motif, in this case an engineered leucine zipper that

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: M2, matrix protein 2; M2e, extracellular domain of M2; BM2e, influenza B M2 protein ectodomain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BS3, bis(sulfosuccinimidyl) suberate; HBc, hepatitis B virus core; DSP, dithiobis(succinimidyl propionate); MALDI, matrix-assisted laser desorption ionization; MDCK, Madin-Darby canine kidney; HEK, human embryonic kidney; Bistris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

Drug News Perspect 22(2), March 2009

LOOKING AHEAD

PANDEMIC PREPAREDNESS: TOWARD A UNIVERSAL INFLUENZA VACCINE

The idea of a broad or even universal vaccine capable of protection against any virus strain, epidemic or pandemic, is a goal that has been pursued for decades.

by Kenny Roose, Walter Fiers and Xavier Saelens

The biology of influenza viruses is a challenge for classical vaccination. The short generation time, the relatively low replication fidelity typical for RNA polymerases, and the segmented RNA genome allow rapid selection of mutant viruses. The membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA) are prime targets of host immune responses following influenza virus infection. Furthermore, hemagglutination inhibition (HI) titers of 1:40 or greater in serum, as determined by an in vitro assay based on the ability of influenza virions to agglutinate red blood cells, correlate with protection against seasonal influenza, but not necessarily against a new, potentially highly pathogenic, pandemic strain.[1] Under immunological selection pressure, virus strains with antigenic escape mutations in HA and NA are rapidly selected. This phenomenon, termed antigenic drift, necessitates yearly revision of the licensed trivalent vaccines, which are currently directed against influenza strains A/H1N1, A/H3N2 and B. Moreover, the segmented genome of the virus and the presence of an animal reservoir in the case of influenza A virus contribute to antigenic diversity through reassortment of RNA segments between different viral strains. Reassortment also frequently occurs between seasonal influenza virus strains and can rapidly lead to the formation of fit variant viruses that become dominant in a particular population.[2–5]

Since 1977, H1N1 and H3N2 influenza A virus strains, as well as influenza B virus strains, have been responsible for annual influenza outbreaks in humans. No influenza B virus subtypes have been identified and B viruses do not cause pandemics. A pandemic could start when an influenza A virus carrying an HA subtype other than H1 or H3 infects humans and starts spreading rapidly in the population. To date, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified for influenza A virus, all of which have been isolated from aquatic birds.[6] However, only influenza viruses encoding H1, H2, H3, N1 and N2 have been found to circulate within the human population. Recently, however, several zoonotic transmissions of H5, H7 and H9 viruses from poultry to man have occurred, highlighting the potential for emergence of a new pandemic strain.[7]

Two natural processes are known to cause an influenza pandemic outbreak. Firstly, on very rare occasions, reassortment of HA- and NA-coding genome segments may occur between human and avian influenza A viruses of different subtypes, resulting in a new progeny virus strain. When an appropriate constellation of human internal gene segments has been retained, the new subtype virus may be well-adapted to the human host and spread rapidly.[8,9] Molecular analysis of the 1957 H2N2 and 1968 H3N2 pandemic viruses provided proof for this scenario.[10–12] Secondly, an avian influenza virus of a non-H1, non-H3 subtype can occasionally infect humans and subsequently accumulate mutations that contribute to adaptation, including transmission, to the new host. This mechanism might have been at the root of the 1918 Spanish flu pandemic and may occur in more recent zoonotic H5N1 infections. In this scenario, reassortment between a

SUMMARY

The possible emergence of a new influenza pandemic is considered a major threat for human health worldwide. Pandemics start by the introduction or reintroduction and spread in the human population of an influenza virus subtype against which almost nobody has protective immunity. Currently used influenza vaccines provide good protection only against antigenically matching influenza strains. However, neither the timing nor the subtype of the next pandemic virus is known. Therefore, different approaches are being pursued in anticipation of this pandemic threat. In the present article, we review approaches that aim to induce heterosubtypic immunity, that is, protection against challenge with influenza A viruses belonging to two or more subtypes. Experimental and epidemiological studies indicate that natural infection can provide some heterosubtypic immunity, possibly involving cellular immune responses directed against matrix and/or nucleoprotein as well as humoral responses against neuraminidase. Other approaches have focused on the use of conserved epitopes of the viral proteins, including matrix protein 2 ectodomain (M2e) and nucleoprotein (NP). Proof-of-concept of protection by these novel vaccines has been obtained in animal models, and promising results from several clinical trials have recently been reported. Demonstrating the efficacy of these new vaccines against a potential pandemic influenza endowed with human transmissibility remains a major challenge.

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M2e-based universal influenza A vaccine

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ABSTRACT

Human influenza causes substantial morbidity and mortality. Currently, licensed influenza vaccines offer satisfactory protection if they match the infecting strain, but they come with significant drawbacks. These vaccines are derived from prototype viruses, containing the hemagglutinin of influenza viruses that are likely to cause the next epidemic. Their usefulness against a future pandemic, however, remains problematic. A vaccine based on the ectodomain of influenza matrix protein 2 (M2e) could overcome these drawbacks. M2e is highly conserved in both human and avian influenza A viruses. The low immunogenicity against natural M2e can be overcome by fusing M2e to an appropriate carrier such as Hepatitis B virus-derived virus-like particles. Such chimeric particles can be produced in a simple and safe bacterial expression system, requiring minimal biocontainment, and can be obtained in a pure form. Experiments in animal models have demonstrated that M2e-based vaccines induce protection against a lethal challenge with various influenza A virus subtypes. Furthermore, the production and use of an effective M2e-vaccine could be implemented at any time regardless of seasonality, both in an epidemic as well as in a pandemic preparedness program. In animal models, M2e-vaccines administered parenterally or intranasally protect against disease and mortality following challenge with various influenza A strains. Adjuvants suitable for human use improve protection, which correlates with higher anti-M2e antibody responses of defined subtypes. Recently, Phase I clinical studies with M2e-vaccines have been completed, indicating their safety and immunogenicity. Further clinical development of this universal influenza A vaccine candidate is being pursued in order to validate its protective efficacy in humans.

1. Introduction

Currently licensed influenza vaccines are tripartite, aiming to protect against two influenza A strains and one influenza B, as guided by WHO. They exist in various forms, inactivated whole virus, split virus, subunit, or cold-adapted, live virus vaccine. These seasonal vaccines have a protective efficacy in the range of 60–90% [1]. They have had a major impact on human health by reducing viral infections, and influenza-related morbidity and mortality. All currently licensed influenza vaccines are derived from viruses and are based on hemagglutinin (HA) as dominant antigen. Large-scale growth of virus for vaccine production, either on embryonated chicken eggs or in tissue culture, implies the availability of a suitable seed vaccine strain (usually made by gene reassortment between a new field strain and a trusted production strain such as PR8). There is a 6–8 months delay between identification of a new field strain with pandemic potential and the start of delivery of the matched conventional vaccine. The major drawback of relying on HA as an antigen is the strain specificity of the vaccine. The peculiarity of influenza virus really is that most of the external amino acids of the two major glycoproteins, HA and neuraminidase (NA), can change in several directions without loss of functionality of the active sites located in crevasses, the sialic acid binding site and the sialidase active site, respectively. And change these amino acids do, a process known as *drift*, resulting almost each year in the emergence of new epidemic influenza strains. This drift phenomenon is presumably due to selection pressure as a result of herd immunity in the susceptible human population.

An even greater concern is the expected emergence of a new influenza pandemic. Most influenza virologists believe that this is only a question of *when* not *if*. Our understanding of the genesis of influenza pandemics is limited, as only three occurred in the previous century. Especially the 1918 "Spanish flu" pandemic was a major world-wide catastrophe with a global human mortality toll in the order of 50 million. It would be naive to believe that such an event cannot re-occur. A pandemic strain arises by the introduction

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Short Communication

Antiserum against the conserved nine amino acid N-terminal peptide of influenza A virus matrix protein 2 is not immunoprotective

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The recent emergence and rapid spread of the pandemic H1N1 swine influenza virus reminded us once again of the need for a universal influenza vaccine that can elicit heterosubtypic protection. Here, we show the superior immunogenicity and immunoprotective capacity of the full-length matrix protein 2 ectodomain (M2e) peptide coupled to keyhole limpet haemocyanin (KLH) compared with the N-terminal 9 aa residues of M2e (SP1). Immunization with M2e–KLH protected mice against a lethal challenge with influenza A virus and significantly reduced weight loss and lung virus titres. In addition, passive transfer of serum raised in rabbits against M2e–KLH protected mice against a lethal influenza virus challenge, whereas serum from rabbits immunized with SP1–KLH did not. Nevertheless, immunofluorescence staining revealed that rabbit serum raised against SP1–KLH bound specifically to infected Madin–Darby canine kidney cells. We conclude that the peptide SP1 contains an immunogenic epitope that is not sufficient for immunoprotection.

Influenza is possibly the most important respiratory infectious disease in humans worldwide. Yearly epidemics, and more importantly occasional pandemics, often cause considerable morbidity and mortality. Vaccination is the most important protective measure against seasonal influenza epidemics (Fiore *et al.*, 2009). However, effective vaccination against influenza is severely hampered by the structural variability of haemagglutinin (HA) and neuraminidase, the two major antigens of the virus. This frequent change in antigenic appearance is referred to as 'drift', while 'shift' refers to the emergence of a new HA gene in the human influenza gene pool and is usually the starting point of a new pandemic. Avian species are the major reservoir of different HA genes. Occasionally, avian influenza A subtypes cross the species barrier and might initiate a pandemic, such as the highly pathogenic Spanish flu during 1918–1919 and the recent, fairly mild, H1N1 swine influenza virus (H1N1v) pandemic (Dawood *et al.*, 2009). Different approaches have been followed to obtain intra- or even heterosubtypic protective immunity, that is, immunity to all influenza A strains regardless of subtype, mainly by exploiting more-conserved influenza targets for the development of novel vaccine candidates (Grebe *et al.*, 2008; Mozdzanowska *et al.*, 2003; Neirynck *et al.*, 1999).

We previously described an influenza vaccine based on the highly conserved external domain of influenza matrix protein 2 (M2e) linked to an appropriate carrier, such as hepatitis B virus core particle (Neirynck *et al.*, 1999). The protection provided by M2e-based vaccination presumably targets infected cells by a mechanism that is antibody- and Fc receptor-dependent (Jegerlehner *et al.*, 2004; Wang *et al.*, 2008).

Lamb *et al.* (1985) were the first to describe the influenza A M2 protein as an 'infected-cell surface antigen' with a minimum of 18 aa exposed on the cell surface. M2 is an essential integral membrane protein of influenza virus that forms a highly selective and pH-regulated proton channel (Pinto *et al.*, 1992). Although M2 protein is scarce on virus particles, it is expressed abundantly on the surface of infected cells (Lamb *et al.*, 1985). These authors also described an immune serum raised in rabbits by injecting a 9 aa peptide (SP1) that was chemically fused to the carrier keyhole limpet haemocyanin (KLH). SP1 corresponds to the N terminus of the mature influenza M2 protein. The antiserum specifically bound to influenza-infected cells.

In this study, we compared the immunogenic and immunoprotective properties of the oligopeptide, SLLTEVETP

Curriculum vitae

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Name	Kenny Roose
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Education	
2006	Basic Course in Laboratory Animal Science, Category C, Ghent University
2002-2004	Master in Biotechnology, Ghent University
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2007-2011	Practical Course Molecular Biology I (Bachelor Biotechnology)
2010	Dag van de Biotechnologie, 26th June 2010, Virology module.
2009-2010	Sanne D'hondt, Master I Project, "Engineering of recombinant influenza monoclonal antibodies".
2008-2009	Kevin Titeca, Master I Project, "Development of a fast and reliable detection method for influenza viruses in clinical and field samples: a comparison of techniques".
2007-2008	Muriel Smet, Master I Project, "Comparison of methods for the detection of Influenza A in mouse lung samples".
2006-2007	Sarah De Baets, Master II Project, "Letaliteit van influenza B bij muizen: rol van het matrix eiwit en bescherming door CTA1-DD geïnduceerde aangeboren immuniteit".

Conference proceedings and oral presentations

Oral presentations

VIB seminar, Blankenberge, 10th March 2006, "Development of recombinant Influenza B vaccines with cross-strain protective potential"

Poster presentations

Keystone Symposium, Pathogenisis of Influenza: Virus-Host Interactions, 23th – 28th May 2011, Sheraton Hotel, Hong Kong, "Cellular immunity induced by the influenza B extracellular domain of NB in the H2b-background can inhibit viral replication in the mouse lung"

IVW 2009, Influenza Vaccines for the World, 27th – 30th April 2009, Palais des Festivals et des Congres, Cannes, "An Influenza A Vaccine Based on the Tetrameric Ectodomain of Matrix Protein 2"

Other

Symposium & Master Classes on Frontiers of Mucosal Immunity, WTC Rotterdam, 26th – 27th January 2006

Vaccine symposia on Infection and Immunity, UMC University, Utrecht, 22nd November 2007 / 24th April 2008 / 19th November 2009 / 26th November 2010

Allergy & Asthma symposium, Bruges, May 29th – 30th 2009

Scientific publications

De Filette, M, Martens, W, **Roose, K**, Deroo, T, Vervalle, F, Bentahir, M, Vandekerckhove, J, Fiers, W, Saelens, X. (2008). "An Influenza A vaccine based on tetrameric ectodomain of Matrix Protein 2". J Biol Chem 283, 11382-11387.

Roose, K, Fiers, W, Saelens, X. (2009). "Pandemic preparedness: Toward a universal influenza vaccine". Drug News Perspect 22, 80-92.

Fiers, W, De Filette, M, El Bakkouri, K, Schepens, B, **Roose, K**, Schotsaert, M, Birkett, A, Saelens, X. (2009). "M2e-based universal influenza A vaccine". Vaccine 27, 6280-6283.

De Filette, M, Ysenbaert, T, **Roose, K**, Schotsaert, M, Roels, S, Goossens, E, Schepens, B, Fiers, W, Saelens, X. (2011). "Antiserum against the conserved nine amino acid N-terminal peptide of influenza A virus matrix protein 2 is not immunoprotective". J Gen Virol 92, 301-306.

Maelfait, J, **Roose, K,** Bogaert, P, Sze, M, Saelens, X, Pasparakis, M, Carpentier, I, van Loo, G, Beyaert, R. "A20 (*Tnfaip3*) deficiency in myeloid cells protects against influenza A virus infection". Plos Pathogens. *Accepted for publication*.

Roose, K, Schotsaert, M, Schepens, B, Smet, A, Ysenbaert, T, Vandekerckhove, J, Fiers, W, Saelens, X. "A potent cytotoxic T cell epitope in the extracellular domain of influenza B virus NB promotes virus clearance". *Ready for submission.*

Ibañez, LI, **Roose, K**, De Filette, M, Schotsaert, M, De Sloovere, J, Roels, S, Fiers, W, Saelens, X. "Vaccination with virus-like particles displaying the matrix protein 2 ectodomain and containing nucleic acids improves protection against influenza A virus infection". *In preparation.*

Roose, K, Fiers, W, Saelens, X. "Influenza B virus: etiology, biology and epidemiology". *In preparation.*

Roose, K, Smet, A, Ysenbaert, T, Vervalle, F, Schepens, B, Martens, W, Fiers, W, Saelens, X. "NB and BM2 Ectodomains can offer cross-protection against Influenza B infection". *In preparation.*

Book chapters

Bredan, A, **Roose, K**, Schotsaert, M, Saelens, X. (2008). "Chapter 18. Vaccine development by recombinant DNA technology. Part I: DNA Vaccines". In The Oea Review of Medicine (Hadeli, K, Benamer, H, eds.), Tripoli, Oea Medical Publisher , 419-434.

Bredan, A, **Roose, K**, Schotsaert, M, Saelens, X. (2008). "Chapter 19. Vaccine development by recombinant DNA technology. Part II: Virus-like Particles". In The Oea Review of Medicine (Hadeli, K, Benamer, H, eds.), Tripoli, Oea Medical Publisher , 435-453.

Roose, K, Schotsaert, M, Schepens, B, Fiers, W, Saelens, X. "Cutting edge approaches towards novel and cross-protective influenza vaccines". In Skills, knowledge and translational technologies leading to novel vaccines (von Gabain, A, Nagy, E, eds.). *In press.*

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Willy en Marina, jullie waren eigenlijk degene die mij in het labo ingelijfd hebben. Dankzij jullie en Elke ben ik (rustig weg) begonnen met het influenza B werk wat mij toch wel enkele jaren heeft zoet gehouden…! Jullie lagen ook aan de basis van mijn beginstapjes in de wereld van kloneren en muizen hanteren, waarvoor dank!

Prof. Fiers, ik denk dat iedereen het over eens is dat uw blijvende inzet en interesse in de wetenschap uitermate bewonderenswaardig zijn en blijven. Zelfs bij herhaaldelijke tegenslag of doodlopende weg blijft u aan alternatieven of oplossingen denken. Uw ongebreideld geloof in projecten die u actief opvolgt is inspirerend te noemen.

Na zeven jaar in het labo heb ik uiteraard heel wat mensen zien komen en gaan. Karim, immer stil en rustig, maar met een inzet die tegen alle frustraties en tegenslagen in kan gaan. Jou doorzettingsvermogen kan alleen maar stimulerend werken! Els, ook jij was een doorbijter, maar vooral kon je goed van jezelf afbijten… Mostafa en Jonas, jullie hebben de hybridoma techniek in ons labo binnengebracht; maar misschien interessanter waren de gesprekken die we daarnaast gevoerd hebben. En ik zal zeker nooit die ene editie van het VIB seminarie vergeten (ook al herinner ik me blijkbaar niet meer alles…); wie erbij was, weet wel waarover het gaat. Wouter, je was erbij van in het begin van mijn tijd in het labo (en misschien wel een extra drijfveer om er te blijven ;-)) en hebt mij dan ook veel basistechnieken aangebracht en véél geholpen bij mijn eerste eiwitzuiveringen. Maar daarbuiten was je vooral een plezanten om mee op stap te gaan en es goe te zeveren! Francis, jij hebt er mij toe aangezet zonder veel poespas op de Akta te leren werken; een man die van aanpakken weet! Maar ook wel megaschattig als hij ietwat beneveld nog 'guanidinium hydrochloride' probeert uit te spreken… Kimberly, ook al was je niet direct mijn type, ik vond het een supertrieste dag toen je besliste om ons te verlaten. Ook al betreurde ik die keuze, ik bewonder wel dat je ze kon maken. Het was even aanpassen aan de stilte die volgde, maar das wel gelukt! Wanneer is je trouw nu eigenlijk ook alweer gepland? Veronique, jouw beslissing om het ergens anders te proberen kwam nog onverwachter, maar ik vond ze even moedig om te maken. Ik ben er zeker van dat wat je ook onderneemt, je het tot een goed einde zal brengen. Itati, till lately the 'mother' of the

lab, I miss your drive and dedication, very inspiring to say the least! And I definitely miss your cooking; you can always send me a bag of palitto's as well! I hope we'll see each other again in the near future. En tenslotte, onze voetballer, Frederik. Om het met een adagium te stellen: *I hate to see you leave, but I love to see you walking away…* Het labo is niet meer hetzelfde sinds je vertrek, en dat op zoveel vlakken. Het ga je goed bij de buren; af en toe door het raam zwaaien é!

En dan zijn we bij de vaste waarden – het meubilair als het ware – beland. En dan beginnen we met de oudste van de hoop, ons Anouk. Ik denk dat je niet direct beseft, en dat het je ook niet genoeg gemeld wordt, hoe belangrijk je voor het labo bent. In zekere zin ben jij de draaischijf (en soms ook schietschijf als het nodig is!) van ons groepje. Zowel praktisch (ik kan nog altijd geen deftig cellulair experiment doen zonder je visuele hulp te vragen…) als ondersteunend (koffie, koekskes en de gazet!) ben je onmisbaar geworden, maar vooral daarbuiten kon ik altijd op je steun rekenen. Onze dagelijkse wandelingen naar Ally's zijn nog steeds essentieel om ons gezond verstand te behouden en onze frustraties te luchten, ook al is het vaak éénrichtingsverkeer… ;-p. Merci voor de voorbije zeven jaar, en alvast voor de jaren die nog komen! Michel, zonder introductie van alle cellulaire immuniteit gerelateerde know-how door jou ging het labo, en zeker ook mijn doctoraat, op zijn gat gezeten hebben. Daarbuiten hebben we héél wat fijne momenten beleefd in het labo en daarbuiten, culminerend in een avondje *sex on the beach* in Hong Kong. Trouwens, nog niet al mijn troeven zijn uitgespeeld om je de franse beginselen af te leren…! Nu we toch in de juiste sfeer zitten: Bert, nooit gedacht dat ik het zou zeggen van een bioloog, maar jouw praktisch inzicht is soms fenomenaal. Gebeten door alle vormen van wetenschap, en geobsedeerd door nog zo veel meer dingen. Samen met je buurman ontspruiten er soms vreemde gedachtenkronkels uit jullie hoekje, ik hou mijn hart al vast voor een eventueel filmpje… En dan de meisjes! Toen Veronique en Kimberly er nog waren, zaten we effectief met een kiekenkot, maar ondertussen is het kliekje uitgedund en is iedereen wat volwassener geworden. Sarah (of is het toch Sara?), ooit nog mijn thesisstudentje, toen ik nòg minder wist en kon als nu, ik ben ervan overtuigd dat je van je project een prachtig verhaal zal kunnen maken, zonder probleem! En van Judith verwacht ik ook niet minder; tussen het loodgieteren en dergelijke door supergestructureerd deftige experimenten voeren, chapeau! Ik wens jullie allebei nog veel succes de komende tijd bij het afwerken van alles en kijk al uit naar het eindresultaat. En dan Tine, het manusje van alles dat zonder problemen verbouwingen, een eigen project, werk van meerdere andere en het begeleiden van studenten combineert, en er toch nog zo schitterend blijft uitzien, nie normaal! Samen met Anouk ben je het bewijs dat een labo staat of valt met de laboranten die erin rondlopen. Merci voor alle hulp die je mij en ons labo al hebt gegeven! And then, Mister Mexico: working at times when most people are at home, attending every social function there is, showering all the ladies with attention… Now that Itati has gone, I wonder who's going to check up on you? ;-) En tenslotte de 'nieuwe garde': Silvie, ik ben er vrij zeker van dat we nog niet alles weten en gezien hebben van je, maar dat zal zeker nog gebeuren! Koen, ik wens je vooral succes met het in toom houden van de vrouwen daar in je buurt… ;-p Lei, all the best for the coming years, I'm sure your dedication will pay off!

Nu zal ik mijn blik verruimen tot buiten ons labo… Francis, ons dubbelspel vraagt nog een beetje sleutelwerk, maar ooit worden we Europees bedrijfsportkampioen, ik voel het! Als je trouwens binnenkort es ene wil gaan drinken in den Duvel, let me know; ik ben de perfecte chaperon om naar daar te gaan! Kristof (Pluisje voor de vrienden), we moeten meer een kamer in Duitsland delen; de volgende keer kruip ik wel in bed met jou in plaats van Francis, beloofd. En geen nood, *what happens in Hamburg, stays in Hamburg*… Katrien, onze stuntdubbel, laten we geregeld toch es gaan eten om

'bij te praten', belangrijk om op de hoogte te blijven é! Charlotje, immer dartelend door de gang aan het bewegen, als je nog es gaat shoppen in Parijs, mag ik dan mee? Thx! ;-p. Bram, Dieter, Annelies, Evelyn, Leander, Petra, Mouna, Sylviane, Isabelle en Pieter, merci voor alle technische hulp, maar vooral om zulke leuke buren te zijn, nu en in het verleden!

Tante Ria, nu je een verdiep omhoog bent gekropen zal ons contact vluchtiger en minder frequent zijn jammer genoeg… Of misschien beter zo, anders zou er van werken niet veel meer in huis komen. Tot 21h staan tetteren (lees: roddelen) met Anouk erbij is ook niet bevorderlijk voor mijn huisvrede, dus laten we het vooral bij geplande dinertjes en dergelijke houden, een verse traditie om te koesteren! Muriel, nog zo'n bron van waarheidsgetrouwe, pittige informatie, ooit nog studentje onder mijn vleugels (meest memorabele practicum tot nu toe!). Blijven komen met alle verhalen! Iedereen van Unit 7 die mij getroost heeft als Vanessa weer eens zoek was, thx!

De mensen van alle core-facilities en technische diensten mag ik zeker niet over het hoofd zien; steeds zijn ze actief, vaak op de achtergrond, maar wel onmisbaar! Marita, Veerle, Chantal, Myriam en Rik; Didier, Steven en Wim; Nancy, Charly en Geert; Ann en Wilma; Daisy, Thomas, Korneel, Tijl, Tim, Natalie, Laetitia en alle andere dierverzorgers; Ann, Carine en Christiane; Eef, Wies, Sam, Amanda en Evelien; Chantal en Jannick; Pieter (eerste prijs ook voor de meest herkenbare aftershave!); en alle andere die ik hier vergeten ben (sorry!).

Laten we het DMBR even verlaten nu… De voorbije jaren hebben een groot aantal mensen mij gesteund als het nodig was, of op zijn minst mij veel plezier en liefde bezorgt. Een kleine waarschuwing wel: het kan melig worden met momenten… Schete, wie had ooit gedacht dat de GB zo'n invloed op een mens zijn leven kon hebben! Ik mag er niet aan denken waar ik zou staan moest ik je niet leren kennen hebben in Middelkerke en terug tegen het lijf was gelopen in Gent; ik ben je oprecht dankbaar voor zóveel zaken. *Love ya, hunnybunny*, en Seba en Julien ook! Mollie, ik zie je nog altijd zwaaien naar me de eerste keer dat we elkaar ontmoet hebben in de Irish Pub en met trots declareerde dat je goed was in chemie enzo… De band die we door de jaren heen hebben opgebouwd is onbreekbaar, me dunkt; als we al samen naar de 'CD-winkel' kunnen gaan, zit het wel snor. Prinses Wendy, telkens ik je zie, beeld ik me in dat we terug in Juan-les-pins, Antibes of Monaco zitten, *you stylisch lady*! Katy B, jonglerend tussen verbouwer, gids, dierenoppasser en ambtenaar, zelden iemand gekend die een tent op Werchter zo proper kan houden als jij! Wel nog effe oefenen met die plastuit zou ik zeggen… Misschien moet je Maarten es een aantal tips vragen. Oh ja, als Spencer Tunik nog es in de buurt es, *hold the presses, frontpage new on the way*! Iiris and Don, good luck with the new house but even more with the family addition! I wonder how a Finnish-Irish baby will look like… As long as he/she is better behaved than Casper! Marlien, altijd rustig en gereserveerd, maar in de R&B hebben we je pas echt leren kennen! And last but least (hèhè), Dididel de opperklet, veel noten op je zang maar blaffende hondjes bijten niet. Laten we Londen volgende zomer maar es goe onveilig maken!

Sara, Gert, Jeroen, Ryfka en Pjotr, weekendjes en reisjes met jullie zijn steeds educatief (begraafplaatsen, hoezee! Eben-Emael, nice!) maar ook zeker ontspannend (Jungle speed met bijbehorende kwetsuren, uren plezier in doolhoven verspreid over gans Europa, …). Vooral de bonte avonden zijn memorabel… Tis trouwens nog es dringend tijd dat we een quiz winnen, ik wil toch op zijn minst éénmaal in mijn leven in De Serrist terecht komen. De Messalina's *rule*! Samen kunnen we

ook zonder moeite Anderlecht kunnen verheffen tot de beste ploeg die er is (alsof onze hulp daarvoor nodig is…). Ik kijk ook uit naar ons eerste bezoekje aan ‘de Hof van Creve’!

En dan komen we bij de belangrijkste vermeldingen aan. Om te beginnen, mijn ouders en grootmoeder. Mama, papa en mémé, ik wil jullie bedanken om mij ten eerste de kans te geven te kunnen studeren in Gent. Het blijft een financiële aderlating, zeker als zoonlief net dat iets duurder kot (studio dus) wou huren. Bedankt voor de onvoorwaardelijke steun tijdens mijn studies, daarvoor en daarbuiten. Ook bedankt aan Tante Lea voor alle goeie zorgen in het verleden en aan de rest van de familie (maar die zijn net te talrijk om allemaal te vernoemen!)

Schete, hoe zou het labo-leven eruit zien zonder jou? Je bent gewoonweg een deel van mijn dag geworden. Hoe het zo ver is kunnen komen weet ik zelfs niet meer, maar eigenlijk maakt het niet zoveel uit. Ik denk niet dat ik zonder jou in de buurt het tot een goed einde had kunnen brengen; merci voor alle steun en zorg van de voorbije jaren! We gaan er sebiet een spetterend feestje van maken! Oh ja, en zeg maar tegen Koen dat ik hem ooit wel zal krijgen… ;-)

Misschien een klein postuum woordje van dank aan Alan Alexander Milne. Wiedadde, denk je nu? Wel, het fantastisch brein waaruit Teigetje en Co zijn ontsproten, toch wel een vaste waarde in mijn leven geworden ondertussen. Niets kan mij zo opbeuren als de dubbeldikke-dwarsgestreepte-superstrakke-stuit (gewoon youtube checken, *you’ll see*!).

En dan gaan we eindigen in schoonheid. Zoetje, ik weet, je hebt afgezien de afgelopen maanden (of meer?). Ik ga het moeilijk hebben om alle maaltijden, afwasbeurten, winkelsessies, … terug te betalen. Zonder de liefde, steun en vooral geduld die je me hebt kunnen geven de voorbije jaren was dit alles niet mogelijk geweest. Ook al zijn we nu al effen samen, ik ben er zeker van dat onze reis samen nog maar net begonnen is. Niets liever zou ik willen dan nog meer mooie en innige momenten met je te beleven, zoals we er al veel hebben beleefd; ik hou van je, mijn lieveling!


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