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William C. Weldon Date

STABILIZATION OF AN OLIGOMERIC PROTEIN ANTIGEN ENHANCES IMMUNOGENICITY BY ALTERNATIVE ROUTES OF IMMUNIZATION

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Doctor of Philosophy

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William C. Weldon
B.S., Georgia Institute of Technology, 2003

Advisor: Richard W. Compans, PhD

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ABSTRACT

STABILIZATION OF AN OLIGOMERIC PROTEIN ANTIGEN ENHANCES IMMUNOGENICITY BY ALTERNATIVE ROUTES OF IMMUNIZATION

By William C. Weldon

Structural studies of recombinant sHA indicate that the oligomeric status varies with HA subtype and immunogenicity is associated with trimeric proteins. To stabilize the oligomeric structure, we modified the C-terminus of the sHA derived from the H3N2 influenza virus A/Aichi/2/68 was modified with the GCN4pII trimerization repeat. This modification was found to stabilize the H3 sHA trimers while unmodified H3 sHA was a mixture of trimers, dimers and monomers. Conformation-specific monoclonal antibodies indicate that the trimeric sHA presented native epitopes while the unmodified sHA presented low-pH conformation epitopes. In vaccination studies, we observed enhanced immune responses with the stabilized trimeric sHA resulting in 100% protection against lethal challenge. Our results suggest that stabilization of trimeric H3 sHA enhances immunogenicity and confers protective immune responses by preserving epitopes present in the native trimeric structure of HA.

We also investigated the efficacy of skin-based vaccination using microneedles coated with recombinant H3 sHA. Our data indicate that the modified trimeric sHA induces improved immune responses resulting in 100% protection against lethal challenge, and improved clearance of influenza virus from lungs of vaccinated mice after lethal challenge compared to subcutaneous vaccination. Coated-microneedle vaccination with the trimeric sHA induced increased serum and mucosal IgA compared to the unmodified sHA. In addition, we observed an increased Th1 helper T cell phenotype following microneedle vaccination with trimeric sHA. Our results demonstrate the efficacy of skin-based vaccination with a recombinant HA subunit vaccine.

We extended our studies of stabilized H3 sHA trimers to investigate the effect of trimer stabilization of the H1 sHA derived from the swine-origin influenza virus A/California/04/2009. Modification of the H1 sHA with GCN4pII generated stabilized trimers while unmodified sHA formed monomers. However, vaccination studies indicate that the trimeric sHA was less immunogenic than the monomeric sHA, with neither recombinant H1 sHA conferring complete protection from lethal challenge. These data indicate that a different strategy for antigen design is required for the H1 sHA.

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1.1. Influenza Virus

1.1.1. Overview of virus structure

Influenza virus belongs to the *Orthomyxovirus* family which consists of single-stranded segmented RNA viruses. There are 3 distinct serotypes designated A, B and C, with types A and B being the most relevant in human disease. The genome of influenza A and B viruses consists of 8 segments encoding 10 proteins: PB1, PB2, and PA (RNA-dependent RNA polymerase), nucleoprotein (NP), M2, M1, hemagglutinin (HA), neuraminidase (NA), NS1, and NEP. However, in some strains a +1 frameshift mutation generates an additional open reading frame on segment 2 in the PB1 ORF resulting in synthesis of PB1-F2. The viral RNA genome segments range in size from 890 to 2341 nucleotides and include 3' and 5' untranslated regions (UTR) 20 to 45 nucleotides and 23 to 61 nucleotides long respectively[1]. The UTR posseses viral promoter activity, however there is no polyadenylation signal. Evidence suggests that the UTR may contribute to the segment packaging signal during viral assembly by associating with M1 (Table 1).

Electron microsocope imaging of mature virus particles reveals the envelope structure of influenza viruses. Influenza viruses have a spherical or filamentous shape[2]. The lipid bilayer is derived from the host cell and contains transmembrane viral proteins including HA, NA, and M2. The interior surface of the lipid bilayer is coated with M1 protein forming the matrix of the virus. Within the matrix, the viral genome is packaged with the viral RNA dependent polymerase and nucleoprotein forming the RNP complex.

Table 1. Influenza virus proteins

Viral Protein	**Abbreviation**	**RNA segment**
Basic Polymerase subunit 2	PB2	1
Basic Polymerase subunit 1	PB1	2
Acidic Polymerase subunit	PA	3
Hemagglutinin	HA	4
Nucleoprotein	NP	5
Neuraminidase	NA	6
Matrix protein 1 and 2	M1+M2	7
Non-structural protein, Nuclear export protein	NS1+NEP	8

1.1.2. Hemagglutinin

HA is a trimeric type I transmembrane protein consisting of two subunits, HA1 and HA2 linked by disulfide bonds and is responsible for receptor binding and membrane fusion (Figure 1). The HA protein is translated as the HA precursor, HA_0, weighing approximately 75 KDa (monomer). X-ray crystallography of the H3 HA has shown that the trimer is an elongated cylinder approximately 135 angstrom long and a radius of 15 to 40 angstrom. A peptide of 27 amino acids spans the lipid bilayer followed by a small hydrophilic domain on the internal side of the membrane [3].

The receptor binding site is located at a highly conserved "pocket" located on the membrane distal HA_1 subunit. For the H3 subtype, amino acids involved in binding α(2-6) siayllactose receptor analogs were determined by x-ray crystallography data of receptor bound HA: S136, H183, E190, Y98, W153, and L194 [4,5]. Mutations at Y98F, H183F, and L194A completely abrogate receptor binding supporting their role in receptor binding. Substitutions S136T/A, Y195F, G225D, and L226P reduce receptor binding suggesting that these residues are directly or indirectly involved in receptor binding. Conversely, mutations at residues E190 and S228 were reported to increase receptor binding [6].

Each HA monomer is modified by host cell translation machinery with N-linked carbohydrates at the mammalian N-glycosylation sequon N-X-S/T. Five glycosylation sites on the stem region of the HA_2 chain are highly conserved. For the H3 subtype, the number of N-glycosylation sites on the HA_1 has increased over time from 2 sites (1968-

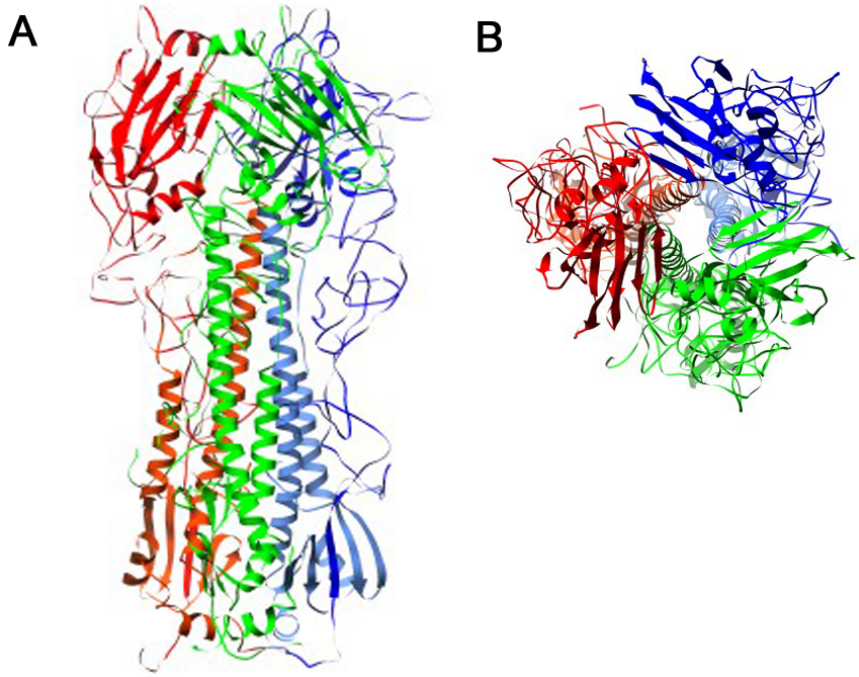

Figure 1. Influenza A hemagglutinin trimer derived from the H3N2 virus A/Aichi/2/68. A. Side view. B. Top view. PDB: 1HGI

1974) to 6 or 7 sites (1999)[7]. N-glycosylation sites on the HA monomer have been shown to play important roles in protein folding and transport [8,9], receptor affinity [10], immune evasion [11], and HA cleavage [12].

In order for influenza to efficiently infect cells, HA must be processed into the HA1 and HA2 chains. This activation of the HA is accomplished by proteolytic cleavage of the HA at the glycine-rich fusion peptide. The cleavage site for human and low pathogenic avian influenza viruses is at a single arginine followed by a glycine in the loop structure of residues 323-328 (HA1) and 1-12 (HA2). In cell culture, the addition of trypsin or plasmin to the growth media is capable of cleaving HA proteins with a single arginine cleavage site for activation of infection [13]. For highly pathogenic avian influenza viruses, the HA cleavage site is polybasic with multiple arginine residues at the C-terminus of the HA1 chain which is cleaved by intracellular furin proteases [14]. In cell culture, viruses with HAs which have a polybasic cleavage site do not need to have media supplemented with a protease as cleavage takes place intracellularly. In eggs, cleavage of influenza HA has been attributed to a factor Xa-like protease which is localized to the allantoic and amniotic fluids where virus is shown to replicate [15]. In mammals, the trypsin-like protease tryptase Clara which is associated with bronchial epithelial cells of the lung has been shown to cleave human influenza HA [16].

1.1.3. Neuraminidase

NA is a tetrameric type II transmembrane glycoprotein that consists of a stalk region at the N-terminus followed by an enzymatic head at the C-terminus. At the C-terminus of

NA in influenza A viruses, there are 6 conserved hydrophilic amino acids followed by a series of hydrophobic residues representing the transmembrane domain. At the N-terminus of NA, the enzymatic "head" of the protein consists of approximately 425 amino acids and is preceded by a 40 amino acid "stalk" [17].

The enzymatic activity for influenza NA was first described by Hirst et al [18]. The enzyme active site is part of a "pocket" of conserved amino acid residues amongst all A and B strains of influenza. The catalytic mechanism is proposed to involve H274 and E276 and as such mutation at these sites results in loss of enzymatic activity at neutral pH [17,19]. Other residues making up the enzymatic active site are R118, E119, D151, R152, D198, R224, E227, E243, E277, R292, D330, L350, and E425 [20].

The influenza NA is responsible for cleaving the sialic acid receptor from both viral and host glycoproteins. This role is critical in the dissemination of budding virus from infected cells evidenced by the aggregation of NA-deficient virus at the surface of MDCK cells [21]. The stalk region of viral NA is able to tolerate deletions of up to 28 amino acids and insertion of up to 41 amino acids with no effect on viral assembly and enzymatic activity. Mutation of the C76, eliminating a disulfide bridge site, results in the lack of infectious virus [22]. Other mutations in the NA protein result in the generation of cold sensitive and temperature sensitive mutants which lack NA activity at 40°C [23].

The biological role of NA as a viral enzyme has led to the development of enzyme inhibitors for anti-viral therapy. Zanamivir (4-guanidino-Neu5Ac2en) binds to the NA

active site by interacting with E119, which is conserved in influenza viruses, and is an effective treatment for influenza when administered intranasally [24,25]. However, due to delivery of the zanamivir through the inhalational route, focus was shifted to the design of an oral influenza NA anti-viral. Oseltamivir (ethyl-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate), the oral influenza anti-viral currently in use, must be modified by host enzymes in order to generate the active ingredient. In addition, unlike zanamivir, oseltamivir is able to inhibit influenza B NA [25,26]. Surveillance data now suggests that mutations in NA allow resistance to both oseltamivir and zanamivir [27].

1.1.4. Internal proteins and non-structural proteins

The internal viral proteins are more highly conserved than the surface glycoproteins and include PB1, PB2, and PA which form the viral RNA polymerase, NP, M1 and M2. The influenza nucleoprotein is a 498 amino acid long protein rich in ariginine, glycine and serine residues. In influenza A strains, the NP is modified by phosphorylation and is strain-specific [28]. Experimental evidence indicates that influenza NP is capable of binding to PB1 and PB2 subunits of the viral polymerase, the M1 protein, other NP forming large oligomers, and most notably single-stranded RNA [29,30,31]. Binding to ssRNA is estimated at a ratio of 1 NP per 24 nucleotides of RNA, and once fully assembled forms the ribonucleoprotein (RNP) complex [32]. The NP protein plays an important role in RNA synthesis, viral genome replication, RNP trafficking from the nucleus, and viral assembly and packaging [30,33,34,35].

The influenza RNA-dependent polymerase consists of 3 subunits: PB1, PB2, and PA. Biochemical studies have suggested that direct contact is made between NP and the polymerase in the virus particle [36]. The polymerase catalyzes replication of the viral RNA genome through a positive-sense cRNA intermediate which is then copied back into the negative sense viral RNA [37]. The PA subunit is approximately 80 KDa and contains endonuclease activity which is required for RNA synthesis [38]. The PB1 protein catalyzes RNA chain elongation and contains amino acid motifs common to all viral RNA-dependent RNA polymerases. In addition, the C-terminus of PB1 interacts with PA and the N-terminus interacts with PB2 allowing formation of a functional trimeric polymerase [39,40]. In some strains of influenza, a +1 frameshift mutation generates an alternative open reading frame encoding the PB1-F2 protein which has been shown to play a role in inducing apoptosis in infected cells by forming pores in the outer membrane of mitochondria [41,42]. The PB2 protein is responsible for cap snatching of cellular mRNA required for viral RNA transcription [43,44].

The M1 protein is a peripheral membrane protein approximately 252 amino acids long and forms dimers. According to the x-ray crystal structure, a positively charged region of the dimer can bind viral RNA while a hydrophobic surface interacts with the viral lipid membrane [45]. M1 is the most abundant protein in an influenza virus particle and plays roles in regulation of RNP transport between cytoplasm and the nucleus, regulation of transcription and replication of RNP, interaction with envelope proteins, and viral budding. The role in virus budding was demonstrated by generating virus-like particles using only influenza M1 in expression systems [46,47]. At the inner layer of the

membrane, M1 interacts via hydrophobic interactions with the lipids causing an asymmetry in the membrane initiating budding and can influence the morphology of influenza virus [48]. The M2 protein is a homotetrameric, type III integral membrane, 96 amino acid protein and functions as proton channel acidifying the interior of the virus during cell entry initiating release of the viral genome into the cytoplasm [49,50]. The antiviral drugs amantadine (1-adamantanamine) and rimantadine target the M2 protein and selectively inhibit influenza infection by blocking acidification of the viral core [51]. However, mutations in the M2 protein at residues 27, 30 31 and 34 confer resistance to amantadine and rimantadine [52,53,54].

The non-structural proteins, NS1 and NS2, are encoded on the segment 8. NS1 is a 26 KDa dimeric protein that functions as an antagonist to the host innate immune system [55]. The NS1 protein consists of two domains: an RNA binding domain at the N-terminus and an effector domain which binds cellular proteins involved in 3'-end processing of cellular mRNA [56]. The cellular localization of NS1 varies with cell types infected, strains, and expression levels but it is predominantly localized to the nucleus of host cells. NS1 interferes with the innate immune response by limiting IFN-β production [57,58] and protein kinase R (PKR) [59] and 2'-5' oligoadenylate synthase (OAS) activity [60]. The role of NS1 in modulation of the innate immune response to influenza infection was further demonstrated in NS1 mutants lacking NS1 or with non-functional NS1 [61,62].

The influenza nuclear export protein (NEP, previously known as NS2) is a 121 amino acid protein derived from splicing of the NS1 mRNA [63]. Influenza A NEP was originally described as a non-structural protein, however evidence suggested that the N-terminus of NEP associated with the cellular export protein Crm1 and the C-terminus of the M1 protein, which was bound to viral RNPs [64]. The role of NEP in nuclear transport has been proposed to involve a bridge interaction with NEP, the viral RNP and cellular nucleoporins [65].

1.2. Influenza Virus Infection Cycle

1.2.1. Receptor binding and entry

The cellular receptor for influenza virus is terminal sialic acid on host cell surface glycoproteins and glycolipids which is bound by the viral HA. In nature, there are two major sialic acid linkages to another sugar residue of carbohydrate chains: Neu5Acα(2,3)-Gal and Neu5Acα(2,6)-Gal. In the H3 subtype a mutation at residue 226 will result in receptor switching from a 2-6 sialic acid to a 2-3 sialic acid [66]. However, in the H1 subtype mutations at residues 190 and 225 will result in a switch of receptor usage[67]. Biologically, the receptor usage by a human versus avian influenza virus is relevant during establishing infections in a new host. In general, avian influenza HA has a higher affinity to α(2-3) sialic acid while human influenza HA has a higher affinity to α(2-6) sialic acid. This receptor usage is reflected by the presence of α(2-3) sialic acid receptors on duck intestine epithelial cells and the α(2-6) sialic acid receptors on human bronchial epithelial cells[68]. Using fluorescently labeled virus and lectins, the human airway epithelium was shown to expresses both 2-6 and 2-3 sialic acids suggesting that

both avian and human influenza viruses can infect human airway epithelium [69,70]. However, avian influenza virus replication in human cells also requires mutations in the PB2 and NS1 proteins [71].

Attachment induces clathrin-mediated endocytosis and entry through the endosomal compartment[72]. As the endosome trafficks through the cytoplasm, it becomes acidified inducing a conformational change in the HA structure exposing the fusion peptide. The fusion peptide inserts itself into the host membrane resulting in membrane fusion and release of the core into the cytoplasm[73]. The M2 proton pump acidifies the core of the virus resulting in the release of the genetic material from the core[74].

1.2.2. Viral transcription and genome replication

Once released into the cytoplasm, the viral RNA is transported into the nucleus where the RNA-dependent polymerase (PB1,PB2,PA) begins to synthesize positive sense mRNA using the negative sense genomic RNA as a template. In order to initiate the synthesis of viral mRNA, the PB2 subunit of the viral polymerase "steals" the caps from the 5' end of cellular mRNA which serve as primers for the viral mRNA synthesis [75,76,77]. During transcription of mRNA the gene segment is fed through the RNA-dependent polymerase until it reaches a point in the gene segment close to the binding site of PB1. This results in reiterative transcription of a tandem repeat of uridine nucleotides results in the addition of a poly-A tail to the 3' end of viral mRNA[78]. The switch to genomic replication is controlled by the amount of free NP and the ability of the polymerase to

initiate synthesis without a primer [79,80]. The PA protein is required for initiation of both the positive and negative strands without a primer [81].

1.3. Antigenic variation of influenza HA and NA

1.3.1. Antigenic drift and shift

Influenza viruses evade the host immune system by two molecular mechanisms: antigenic drift and antigenic shift. In antigenic drift, mutations are acquired in the glycoproteins HA and NA over time due to the high error rate of the RNA-dependent RNA polymerase. These mutations occur in regions of each protein which are recognized by host antibodies and therefore allow evasion of the host humoral immune response. For the HA protein, the rate of mutation is approximately 4×10^{-3} substitutions per nucleotide per year with most of these changes occurring in the HA1. In addition, these changes have been mapped to antigenic regions recognized by monoclonal antibodies [5,11]. In the H1 subtype, the rate of mutation in HA is similar to the H3 subtype [82]. The N2 subtype also undergoes antigenic drift at a similar rate to H3 with most changes occurring in the head region surrounding the catalytic site [20].

Antigenic shift occurs in influenza A viruses because they infect various animal reservoirs which serve as sources for new antigenic subtypes. The result of antigenic shift is the generation of a novel influenza virus subtype, known as a reassortant virus, containing a new combination of viral RNA segments. Reassortant viruses can be generated when two different viruses co-infect the same host. It has been established that the 1968 H3N2 pandemic was a result of antigenic shift in which the NA and the internal

proteins except for PB1 were derived from an H2N2 virus circulating in humans while the HA and PB1 are postulated to be from an avian virus[83]. This mechanism of antigenic shift is possible due to the segmented nature of the influenza genome; segments from two viruses can be packaged together generating new combinations of viral proteins resulting in a reassortant virus. Another possible mechanism would be the ability of a newer subtype to be transmitted from an avian reservoir and infect a human host such as the 1997 and 2005 H5N1 outbreaks and the H9N2 outbreaks in humans [84,85,86]. A third mechanism has been proposed in which a reservoir maintains an influenza subtype until it is established in the human population again, such as the reemergence of the H1N1 subtype in 1977 following its replacement by H2N2 influenza in 1957.

1.3.2. Influenza epidemics and pandemics

Seasonal influenza occurs in both the northern and southern hemisphere during the fall and winter months. For the United States, the average influenza season begins in October and ends in March. The reason for this cycling of influenza infection is thought to be due in part to the stability of the virus in moist, cold environments which are common during cold weather [87,88].

Antigenic shift has occurred 4 times since 1918 resulting in pandemic influenza: 1) H1N1 “Spanish flu” – 1918, 2) H2N2 “Asian flu” – 1957, 3) H3N2 “Hong Kong flu” – 1968, and 4) H1N1 “swine-origin H1N1” – 2009. A pandemic of H3N8 in 1898 preceding the 1918 H1N1 pandemic has been suggested based on historical documents and serology [89,90]. In 1977, the H1N1 subtype reemerged as the Russian flu. In 1997

the first human cases of H5N1 avian influenza was detected followed by a reemergence in 2005 in Hong Kong and mainland China and has caused sporadic cases totaling more than 500 cases un until the present time [91,92]. Each pandemic resulted in increased mortalities over the average seasonal influenza with the worst influenza pandemic, the 1918 "Spanish flu", resulting in the death of approximately 675,000 people in the United States and approximately 50 million worldwide.

2.1. Immune responses to influenza

2.1.1. Innate immunity

In humans, influenza infects and replicates in the polarized epithelial cells of the respiratory tract. In the human lungs, virus is shed to the apical side of the airway epithelium and into the respiratory cavity where the virus is able to spread by aerosol and contact transmission. Upon infection the airway epithelial cells produce pro-inflammatory cytokines including IL-6 and IFN-α, while IL-8 and TNF-α are produced later in infection [93].

In response to infection, human cells begin to express MxA, an anti-viral protein produced in response to infection by orthomyxoviruses and bunyaviruses and induced by interferon [94]. Mx belongs to the class of dynamin-like large guanosine tripphosphatases (GTPase) which are known to be involved in intracellular vesicular trafficking and organelle homeostasis [95]. MxA accumulates in the cytoplasm and partially associates with the endoplasmic reticulum where it interferes with viral protein synthesis and genome replication [95,96].

In the human innate immune system, pattern recognition receptors (PRR) recognize pathogen associated molecular patterns (PAMP) and signal the detection of a pathogen. The toll-like receptors (TLR) are PRRs evolutionary related to the Drosophila toll receptors. In humans, 11 TLRs have been identified which recognize diverse PAMPs including peptidoglycan (TLR2), bacterial flagellin (TLR5), lipopolysaccharide (TLR4), and unmethylated CpG DNA (TLR5) [97]. For influenza, TLR7 and TLR8 recognize the single stranded RNA genome as a heterodimer and TLR3 recogizes the double stranded RNA intermediate produced during transcription and genome replication. In addition to the TLRs, the cellular PRR, RIG-I and NLRs also recognize the single stranded RNA genome of influenza in the cytoplasm of the infected cell [98].

Natural killer (NK) cells form an important branch of the cellular innate immune system. Following infection with influenza, a transient increase in the activity of NK cells in the mouse lungs is observed [99]. In mice, influenza A HA has been shown to signal through the NK cell activating receptor NKp46 resulting in activation of cytolytic activity and IFN-γ production by NK cells[100,101].

2.1.2. Humoral immune response to influenza

The two influenza glycoproteins HA and NA are the primary targets for the humoral immune response. In mice, antibody secreting cells (ASC) are detectable in the respiratory tract tissues 5 to 7 days following infection and peaking on day 10 to 20[102]. During the primary antibody response, the predominant antibody in sera is IgM, IgG, and

IgA [103]. Upon re-exposure to virus, IgG and IgA isotypes dominate the serum antibody response. Because HA is the dominant glycoprotein on the virus particle the antibody response is skewed to recognizing the HA protein, although some antibody is directed towards the NA and M2 proteins. Some antibody responses can be detected against the NP and M1 proteins although only antibodies against NP have been demonstrated to provide protection against challenge in mice [104].

The mucosal antibody response in the lung airway is vital to protection against infection[105]. Influenza specific dimeric IgA dominates the lung mucosal response and a role for tissue localized IgG has been demonstrated via transcytosis into the lung airway by FcRn[106,107]. Protection against infection with influenza virus correlates with respiratory sIgA and IgG[108].

Antigenic maps of the H3 and H1 HAs have been generated using monoclonal antibodies and point mutations. For the H3 HA, 5 antigenic regions have been determined on the HA1 unit. Sites A, B, C and E all map to regions of the HA protein in close proximity to the receptor binding domain. Site D is located at the interface between monomers in the native trimeric structure[11]. For the H1 HA, 5 antigenic regions have also been identified which are homologous to the H3 antigenic sites[109]. For the H1 HA, 5 antigenic regions have also been identified which overlap with those defined for the H3 subtype.

For the N2 NA, a total of 4 antigenic sites have been identified by monoclonal antibody selection of antigenic variants [110]. Three of these sites overlap and are located on the enzymatic head surrounding the sialic acid binding site [111]. One of the sites was further characterized with the N9 subtype using a monoclonal Fab fragment which was mapped to the external amino acids of the enzyme active site[112]. A second site was characterized on N8 NA and was mapped to the interface between two adjacent monomers in the tetrameric NA [113]. Although the N1 NA has not been mapped as extensively, sequence alignments with other NA sequences suggests that the N1 subtype shares the same antigenic regions as the N2 subtype [114].

Antibodies directed to the extracellular domain of M2 (M2e) have a role in anti-influenza immunity, although the antibody response is weak to non-existent in natural infections [115,116]. The mechanism of protection for these anti-M2e antibodies has been proposed to involve FcγRIII on NK cells which recognize the antibody binding to M2e expressed on the cell surface of infected cells resulting in antibody dependent cell-mediated cytotoxicity [117].

2.1.3. Cellular immune response to influenza

In humans during a natural influenza infection, CD8+ cytotoxic T cells (CTL) are detected in the blood from day 6 to 14 and disappear by day 21 [118]. These MHC class I restricted CTLs recognize HA, M, NP, and PB2 allowing for subtype specific and heterosubtypic responses [119]. In mice these CTLs are characterized by the expression of perforin, granzyme B, and IFN-γ [120]. In mice, A population of CD4+ cytotoxic T

cells has been identified with a similar viral protein recognition pattern to their CD8+ counterparts, although this recognition is MHC class II restricted [121,122,123]. The induction of these MHC class II restricted CTLs depends on stimulation with non-infectious virus [122].

Influenza specific helper T cells are also induced by infection with influenza. In mice, these class II restricted CD4+ T cells recognize HA, M or NP antigens and provide help to antigen specific B cells [124,125]. T cell help is provided to B cells during the germinal center reaction in lymphatic tissues by signaling through CD40-CD40L interactions and cytokines. The type of cytokines being produced, along with CD40 signaling, can augment and skew the antibody response to different isotypes [126]. In mice, the Th1 cytokine IFN-γ induces production of the IgG2 isotype while the Th2 cytokine IL-4 induces production of the IgG1 isotype [127,128]. The production of IgA is induced by IL-5 and TGF-β and a proliferating inducing ligand (APRIL) has been implicated in playing a role in indcution of IgA in lungs following infection[129].

2.2. Influenza vaccines

Influenza is a vaccine preventable disease. In the United States, influenza virus is responsible for 36,000 deaths and over 200,000 hospitalizations in an average influenza season[130]. Worldwide it is estimated that influenza is responsible for up to 1.5 million deaths annually[131]. Accordingly, the Centers for Disease for Control and Prevention and the World Health Organization currently recommend vaccination for all populations.

To account for antigenic shift and drift, vaccine strains are selected based on worldwide surveillance data of the major circulating viruses.

2.2.1. Vaccine production

In 1945, the first commercial influenza vaccines were approved for use in the United States using whole inactivated virus as the vaccine antigen. Influenza vaccines consist of 3 viruses: two influenza type A strains (H1N1 and H3N2) and 1 influenza B strain. In the United States, there are two currently used vaccine types: an inactivated, split vaccine (TIV) and a live-attenuated vaccine (LAIV). For the TIV, the influenza A vaccine strains is a reassortant virus selected for rapid and high yield growth in eggs. These strains contain the internal genes from the A/Puerto Rico/8/34 (H1N1) influenza A virus with the HA and NA genes derived from the predominant circulating virus in a given influenza season. The inactivated split vaccine is grown in embryonated hen eggs, purified, treated with detergent and inactivated using formalin or β-propiolactone.

For the LAIV, the vaccine strain contains the internal genes derived from the A/Ann Arbor/6/60 (H2N2) influenza A virus that has been cold-adapted by passaging preventing growth at 37°C. The mutations that are acquired to confer the cold-adapted phenotype have been determined to be in the PB1 and PB2 subunit of the viral RNA polymerase and the NP [132]. Like the TIV vaccine strain, each cold-adapted reassortant also contains the HA and NA of the predominant circulating influenza virus.

2.2.2. Next generation influenza vaccines

Influenza vaccine production in eggs requires the generation of high-yield egg growth reassortant viruses and a large supply of embryonated chicken eggs, and the vaccine has potential safety concerns in individuals with egg allergies [133,134,135,136]. For these reasons, alternative vaccine production methods are being pursued which could be used to rapidly produce influenza vaccine in response to pandemics. Influenza vaccines derived from mammalian, insect, bacterial and plant cell lines have been investigated for efficacy. Due to antigenic shift and drift, next generation influenza vaccines should induce cross reactive and long-lasting immune responses, eliminating the need for annual vaccinations. Therefore, in addition to the source of the influenza vaccine, alternative vaccine antigens have been investigated including recombinant protein (HA, NA, M2e), virus like-particles (M1, HA/NA), and recombinant viral vectors.

3.1. Skin Immunology

3.1.1. Skin anatomy

The skin acts as a mechanical barrier against the environment and provides the first line of defense against pathogens. Mammalian skin is composed of three layers: the epidermis, dermis and hypodermis. The epidermis is the outermost layer of skin consisting of stratified squamous epithelial cells. Because there are no blood vesssels in the epidermis, nutrients are obtained by diffisuion from blood capillaries in the upper layers of the dermis. Cell types found in the epidermis are keratinocytes, melanocytes, Merkel cells, and Langerhans cells. The epidermis is further divided into 5 strata: corneum, lucidum, granulosum, spinosum, and germinativum. The dermis lies beneath

the epidermis and consists of connective tissue and contains hair follicles, sweat glands, lymphatic vessels, and blood vessels.

3.1.2. Immune cell populations localized to skin

The skin-associated lymphoid tissue (SALT), which was first described by Streilein et al [137], represents an ideal target for skin-based vaccinations because it contains by keratinocytes, professional APCs, and lymphocytes. The skin-associated APCs and keratinocytes function as immune sentinels in the skin and have been shown to express several pattern recognition receptors (PRRs) including TLR9, TLR2, TLR3, and TLR7 [138,139]. Within the epidermis, the most prominent professional APC in the skin is the Langerhan cell distinguished by the Birbeck granules and named for their discoverer Paul Langerhan in 1868. The function of LCs as APCs in the skin wasn't described until 1980 by Ralph Steinman[140]. The LC is identified by the prototypic marker Langerin (CD207), although other populations of skin-associated DCs have been shown to express Langerin. In the dermis, the population of immune cells is more diverse. In addition to dermal dendritic cells, macrophages and plasmacytoid dendritic cells also reside in the dermis. The skin is also home to lymphocytes including CD4+ Th17, Th1, and Th2 helper T cells, NKT cells, CD8+ T cells, mast cells, and γδ T cells.

The LC and DDC have been shown to take up antigen, migrate to draining lymph nodes and induce an antigen-specific adaptive immune response[141]. Migration is initiated by expression of TNF-α and IL-1β by keratinocytes and Langerhan cells. This induces the production of prostaglandin E_2 which signals the down regulation of E-cadherin and up

regulation of CCR7, resulting in migration of the LC from the skin to the draining lymph node. In addition to presenting antigen on MHC class II, human LCs have been shown to cross-present exogenous antigens on MHC class I allowing priming of both CD4+ and CD8+ T cells.

The role of LC in adaptive immunity is not absolute. Evidence suggests that LCs might also play an important role in the induction of tolerance. In the absence of infection and inflammation, disruption of E-cadherin will induce incomplete maturation of LCs leading to upregulated MHC class II, CCR7, and costimulatory molecules. Instead of T cell stimulating cytokines such as IL-12, these immature LCs have been shown to induce IL-10 producing regulatory T cells.

3.1.2. Skin-based vaccination

Cutaneous vaccinations have been shown to be highly efficacious, inducing strong humoral, cellular, and mucosal responses. Their effectiveness is highlighted by the global eradication of smallpox which was accomplished by administering vaccine using a bifurcated needle that pierced the skin and deposited a liquid vaccine. Recent investigations support a cutaneous based influenza vaccine by demonstrating that the intradermal administration of trivalent inactivated influenza vaccine produced equivalent responses to vaccine administered intramuscularly at a higher dose[142]. Alternatives to intradermal injections of vaccines have been investigated including liquid jet inection[143], powder jet,[144,145] electroporation[146], or topical application[147,148]. A transdermal immunization approach using microneedles has been studied using viral

and bacterial antigens in animal models [149,150,151,152]. Microneedle immunization produced a strong immune response that was equivalent to intramuscular vaccinations using 10-100 fold higher doses [152]. Thus, influenza immunizations administered via the skin by a microneedle would have the advantages of having improved efficacy and requiring a lower dose. However, hypodermic needle-based vaccination through the skin lacks accuracy and can cause pain[153,154]. In order to deliver vaccine into the skin, the stratum corneum must be disrupted by mechanical disruption, chemical treatment, or electroporation [148,155,156,157].

3.1.3. Microneedle vaccination

Microneedles have become an important vaccine delivery format; they have the advantage of relatively pain-free insertion, cost-effective production, little to no biohazardous waste generated, and the potential for self-administration. Numerous microneedle formats are being investigated including solid metal needles with a vaccine coating, dissolvable needles with encapsulated vaccine, and hollow metal needles containing a small volume of vaccine.

4.1. Research objectives of the dissertation

This dissertation aims to investigate the efficacy of an improved vaccine design based on stabilizing the structure of viral proteins and to determine how the route of delivery can affect the outcome of vaccination. In the first study, the objective is to test the hypothesis that stabilization of the native trimeric structure of a soluble form of the influenza hemagglutinin will improve the immune response to this recombinant vaccine. We

truncated the HA from the H3N2 virus A/Aichi/2/68, eliminating the transmembrane and cytosolic domains, and fused the trimerization repeat GCN4pII derived from the yeast transcription factor GCN4 to the C-terminus. SDS-PAGE analysis of crosslinked recombinant HA and monoclonal antibody binding were performed to determine the quarternary structure of the modified soluble HA compared to the unmodified protein. Vaccination studies were performed in the mouse model and vaccine efficacy was evaluated using ELISA, hemagglutination inhibition assay, and lethal viral challenge.

In the second study, the objective is to test the hypothesis that delivery of a recombinant influenza subunit vaccine to the skin via solid coated microneedles will induce a protective immune response. Using the previously characterized proteins, we determined the quarternary structure of the recombinant proteins after microneedle coating. Vaccination studies were performed using the mouse model and vaccine efficacy evaluated using ELISA, HAI assay, microneutralization assay, intracellular cytokine staining, and lethal viral challenge. In addition, we determined the mucosal antibody response following vaccination.

In the third study, we expand the use of the GCN4pII trimerization domain to the H1 subtype of influenza viruses and test the hypothesis that modification of the soluble H1 HA from the swine-origin A/California/04/2009 enhances immunogenicity. We evaluated the quarternary structure of these recombinant proteins using the same techniques used with the H3 recombinant proteins. Vaccination studies were performed

using the mouse model and vaccine efficacy evaluated using ELISA, HAI, and lethal viral challenge.

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Chapter 1. Enhanced Immunogenicity of Stabilized Trimeric Soluble Influenza Hemagglutinin

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Abstract

Background: The recent swine-origin H1N1 pandemic illustrates the need to develop improved procedures for rapid production of influenza vaccines. One alternative to the current egg-based manufacture of influenza vaccine is to produce a hemagglutinin (HA) subunit vaccine using a recombinant expression system with the potential for high protein yields, ease of cloning new antigenic variants, and an established safety record in humans. **Methodology/Principal Findings:** We generated a soluble HA (sHA), derived from the H3N2 virus A/Aichi/2/68, modified at the C-terminus with a GCN4pII trimerization repeat to stabilize the native trimeric structure of HA. When expressed in the baculovirus system, the modified sHA formed native trimers. In contrast, the unmodified sHA was found to present epitopes recognized by a low-pH conformation specific monoclonal antibody. We found that mice primed and boosted with 3 µg of trimeric sHA in the absence of adjuvants had significantly higher IgG and HAI titers than mice that received the unmodified sHA. This correlated with an increased survival and reduced body weight loss following lethal challenge with mouse-adapted A/Aichi/2/68 virus. In addition, mice receiving a single vaccination of the trimeric sHA in the absence of adjuvants had improved survival and body weight loss compared to mice vaccinated with the unmodified sHA. **Conclusions/Significance:** Our data indicate that the recombinant trimeric sHA presents native trimeric epitopes while the unmodified sHA presents epitopes not exposed in the native HA molecule. The epitopes presented in the unmodified sHA constitute a “silent face” which may skew the antibody response to epitopes not accessible in live virus at neutral pH. The results demonstrate that the

trimeric sHA is a more effective influenza vaccine candidate and emphasize the importance of structure-based antigen design in improving recombinant HA vaccines.

INTRODUCTION

Influenza virus is one of the most common causes of serious respiratory illness. Since the beginning of the 2009-2010 influenza season, the CDC has reported that all subtyped influenza A viruses isolated from hospitalizations were the novel 2009 H1N1 virus [1]. Monovalent H1N1 vaccines have been distributed in the United States; however, due to lower than expected production levels a vaccine shortage occurred. Influenza vaccine production in eggs requires the generation of high-yield egg growth reassortant viruses and a large supply of embryonated chicken eggs, and the vaccine has potential safety concerns in individuals with egg allergies [2,3,4,5]. For these reasons, efforts have been made to develop alternative vaccine production methods. Production of influenza proteins in recombinant expression systems is one such approach; recombinant HA vaccine has been produced in bacterial, plant, mammalian, and recombinant baculovirus (rBV) expression systems. The rBV expression system has several advantages over traditional egg-based vaccine production techniques including the high levels of expression of recombinant protein[6], rapid scaling of production to meet high demands[2], an established safety record[2,5,7], and more rapid production of new vaccines in response to antigenic shift or drift[2,8]. One disadvantage to recombinant HA vaccines is that other viral proteins are not present such as neuraminidase or M2.

Proteins produced in the rBV system use the same glycosylation sites as mammalian proteins. However, in the insect cell system the major carbohydrate moiety is high-mannose carbohydrate while mammalian systems have elongated carbohydrates with the common high-mannose intermediate[6]. Previous studies have demonstrated that the recombinant HA produced in insect and mammalian expression systems did not have significantly altered receptor binding[9]. Despite the difference in glycosylation, the immunogenicity of insect derived HA is comparable to mammalian expressed HA[10].

The seasonal influenza vaccine is trivalent, containing two type A influenza strains (H1N1 and H3N2) and one type B influenza strain. The major component of influenza vaccine is the viral hemagglutinin (HA), a trimeric type I transmembrane protein consisting of two chains, HA1 and HA2, linked by inter-chain disulfide bonds[11]. During the influenza virus life cycle, HA functions to bind terminal sialic acid residues on host cell glycoproteins, initiating receptor mediated endocytosis and allowing the virus to enter the cell in an endosome. Once internalized, HA mediates fusion of viral and host membranes via a low pH- induced structural conformation change [11,12,13].

Currently, there are 16 distinct antigenic subtypes of HA in influenza A viruses[14]. There are two major mechanisms enabling influenza A viruses to evade the immune response and spread more rapidly amongst a population, antigenic drift and antigenic shift. Antigenic drift is a result of point mutations in the HA and NA genes which are under selective pressure from the host antibody response. Antigenic shift is the term used

to describe the emergence of novel viruses by the genetic reassortmant of viral RNA segments.[11,15]

Because the HA is the most immunologically important component of influenza vaccines, we generated a system for producing a protein subunit vaccine using a rBV-derived soluble influenza HA modified with a trimerization heptad repeat derived from the transcription factor GCN4 [16,17,18], to stabilize the native trimeric structure of the HA protein. We determined the effect of this stabilization on the immunogenicity of the protein vaccine and its ability to induce a protective immune response to challenge with homologous virus in mice primed and boosted with a low dose of vaccine without adjuvants.

RESULTS

Recombinant baculovirus derived Aichi/2/68 soluble HA is expressed as the HA0 precursor. Diagrams of recombinant baculovirus expression constructs for the soluble HA derived from the H3N2 virus A/Aichi/2/68 with the GCN4pII modification are shown in Figure 1. Sf9 insect cells expressed and secreted ~ 8 μg of purified recombinant protein per mL of Sf9 cell culture. The sHA and sHA.GCN4pII proteins migrated on SDS-PAGE as single polypeptides of approximately 70 and 75 kDa proteins, respectively, indicating that the recombinant proteins were expressed as HA precursors (HA0). The larger molecular weight of the sHA.GCN4pII protein indicates that the GCN4pII modification was added to the C-terminus of the H3N2 HA protein. Western

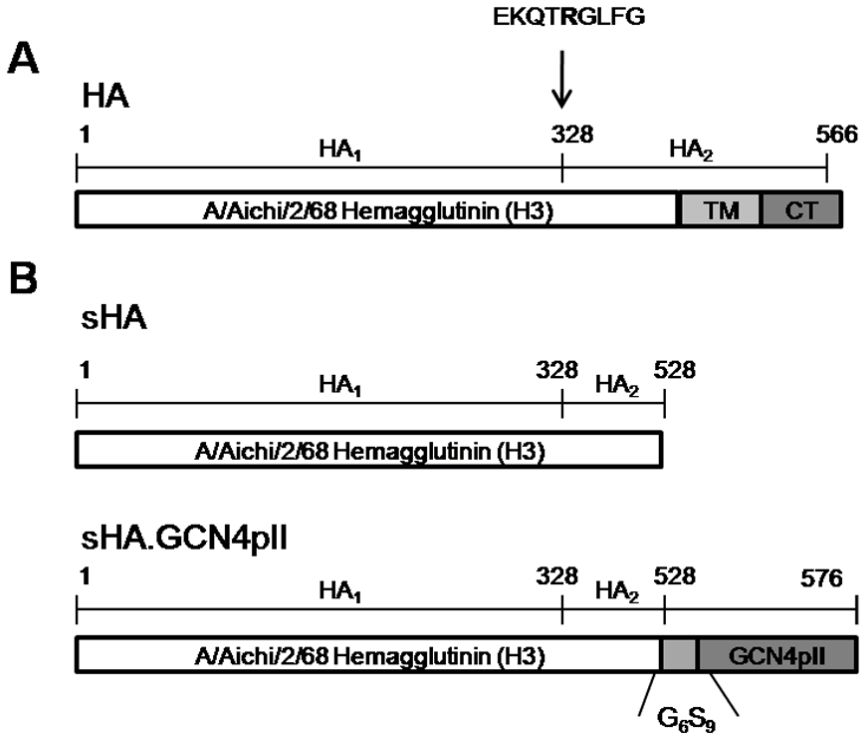

Figure 1. Recombinant soluble HA constructs. A. Full length H3N2 A/Aichi/2/68 hemagglutinin precursor (HA0). TM = transmembrane domain, CT = cytosolic domain. Cleavage site at R328 is indicated by arrow. **B.** Soluble H3N2 A/Aichi/2/68 HA constructs. Soluble H3N2 HA was generated by eliminating the transmembrane (TM) and cytosolic (CT) domain. The modified trimeric soluble H3N2 HA was generated by fusing the GCN4pII trimerization repeat to the C-terminus of HA with a short peptide linker (G6S9).

blot analysis of the recombinant HA proteins using reference sera from A/Aichi/2/68 infected mice indicates that the recombinant proteins are antigenically similar to the viral HA (Figure 2A) and the anti-His monoclonal antibody binding demonstrates that the His-tag was included in the protein (Figure 2B)(Lane 1 = sHA, Lane 2 = sHA.GCN4pII). Coomassie blue staining after SDS-PAGE separation of the sHA and sHA.GCN4pII following nickel-bead chromatography showed a single band corresponding to the recombinant proteins indicating a relatively high level of purity (Figure 2C). Furthermore, both recombinant proteins are produced as the HA0 precursor at a high yield in the baculovirus expression system.

To determine the oligomeric structure of the recombinant proteins, cross-linking was performed using BS3, a water soluble cross-linker approximately 11 angstrom long which reacts with primary amines thus covalently linking proteins together. Multimeric proteins exposed to this crosslinker will have each subunit crosslinked together, allowing them to be analyzed on denaturing gels for Western blot analysis [19,20]. Using this approach, we determined the oligomeric structure of the recombinant soluble HA with and without the GCN4pII modification. Separation of reaction products on gradient SDS-PAGE and Western blot analysis indicates that the sHA is observed as a mixture of trimeric (~210 kDa), dimeric (~140 kDa), and monomeric proteins (~70 kDa)(Figure 3A – Lane 1,2). However, the modification of the sHA protein at the C-terminus with the GCN4pII trimerization repeat stabilized the trimeric structure of the secreted recombinant protein (~220 KDa)(Figure 3A – Lane 3,4).

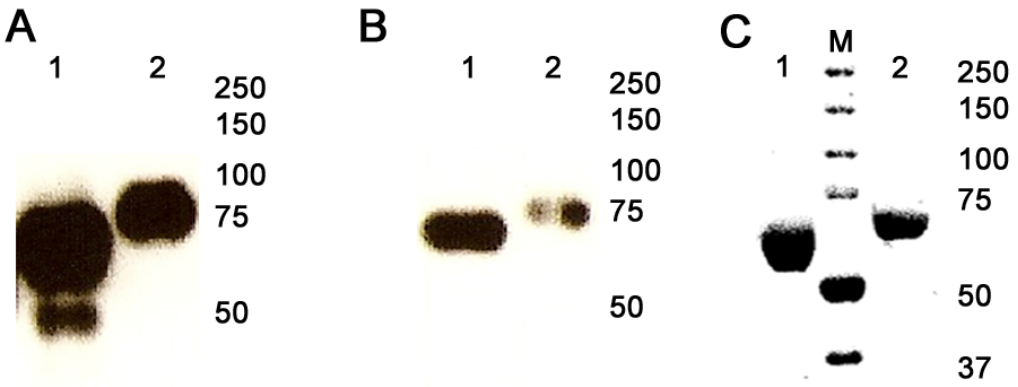

Figure 2. Recombinant H3N2 A/Aichi/2/68 is expressed as the hemagglutinin precursor (HA0). A,B. Western blot analysis of SDS-PAGE separated sHA (Lane 1) and sHA.GCN4pII (Lane 2) using polyclonal sera or anti-histidine tag monoclonal antibody (primary antibody). Protein bands were developed using goat anti-mouse HRP (secondary antibody) and ECL Plus (GE Healthcare). **C**. Coomassie blue stain of sHA (Lane 1) and sHA.GCN4pII (Lane 3) after nickel-bead column purification. Lane 2 - molecular weight marker. Each lane was loaded with 1 µg of recombinant protein.

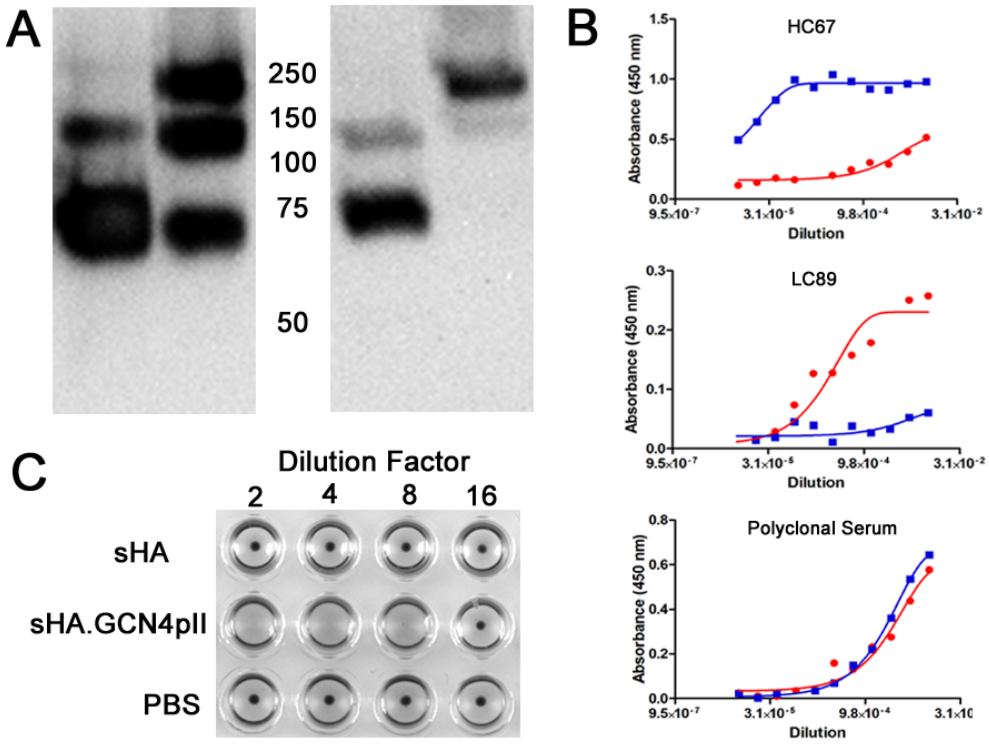

Figure 3. GCN4pII modification stabilizes the trimeric structure of A/Aichi/2/68 soluble HA. A. BS_3 crosslinking was performed as described in Materials and Methods. Lanes 1 and 2 – sHA, Lanes 3 and 4 – sHA.GCN4pII. Lane 1 and 3, no BS3, Lanes 2 and 4, 3 mM BS3. Trimeric HA corresponded to a band of ~220 kDa, dimeric HA corresponds to a band of ~140 kDa, and monomeric HA corresponded to a band of ~75 kDA Western blot primary antibody, anti-histidine monoclonal antibody. **B**. Sandwich ELISA. 20 µg of sHA (red circle) or sHA.GCN4pII (blue square) were captured using guinea pig anti-A/Aichi/2/68 then binding affinity of HC67, LC89 or polyclonal sera was detected by absorbance at 450 nm. **C**. Hemagglutination test using 1 µg of recombinant protein (sHA or sHA.GCN4pII) or PBS. Proteins were diluted in PBS and incubated at room temperature for 30 minutes with 0.05% chicken red blood cells (washed).

In addition to analysis by chemical crosslinking of the recombinant proteins, monoclonal antibody binding was used to determine the conformation of the recombinant soluble HA. Antibody binding by sandwich ELISA indicates that the trimeric A/Aichi/2/68 HA specific monoclonal antibody HC67 bound to sHA.GCN4pII with a higher affinity compared to the sHA, indicating that trimeric HA was the predominant form in the modified soluble HA (Figure 3B). This supports our findings using crosslinking of the recombinant proteins. The low-pH A/Aichi/2/68 HA specific monoclonal antibody LC89 bound to the unmodified sHA with a higher affinity than the trimeric sHA indicating that the unmodified but not the modified trimeric sHA exhibits epitopes which are exposed in the low-pH conformation.

Low-pH treated bromelain-treated HA (BHA) forms rosettes in solution via hydrophobic interactions between the fusion peptide of adjacent BHA, allowing the protein to agglutinate red blood cells [20,21]. We compared recombinant sHA and sHA.GCN4pII in a hemagglutination assay, and found that the sHA.GCN4pII protein was able to agglutinate chicken RBC while the sHA was not (Figure 3C). This demonstrates that the modified protein folds into the native structure of the HA and has retained the ability to bind sialic acid receptors on red blood cells, causing agglutination. Therefore, modification of the H3N2 Aichi HA at the C-terminus with the GCN4pII repeat does not prevent the receptor binding activity of the trimeric protein.

sHA.GCN4pII induces a robust humoral immune response. To compare the immunogenicity of the trimeric sHA.GCN4pII and the unmodified sHA, female Balb/c

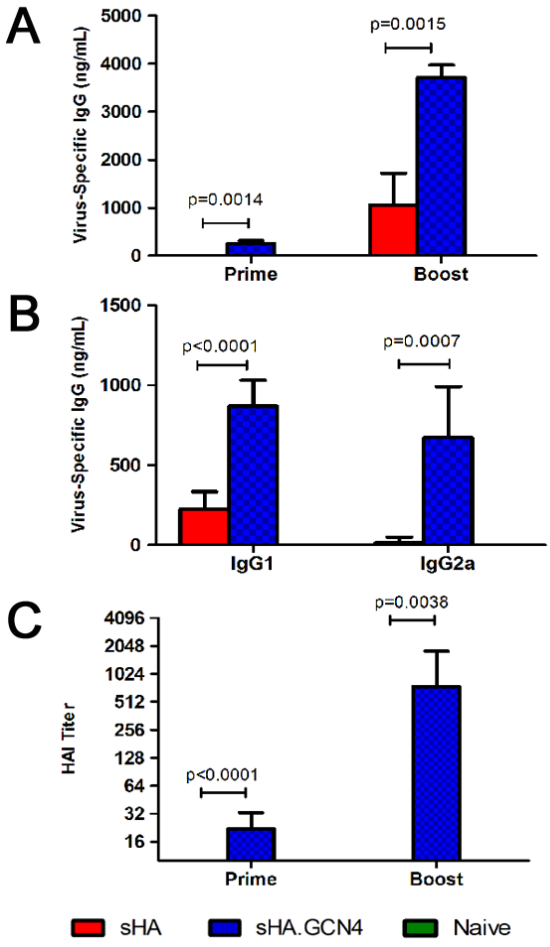

Figure 4. sHA.GCN4pII induces more robust humoral responses compared to sHA. A. Mice were primed and boosted 3 weeks later with 3 µg of sHA (red), sHA.GCN4pII (blue) or PBS (naïve – green). Blood was collected day 21 after priming (prime) and 21 days after boosting (boost). ELISA plates were coated with 4 µg/mL of A/Aichi/2/68 virus and antigen specific serum IgG ELISA was measured for prime and boost. HRP-conjugated goat anti-mouse IgG was used for detection. **B.** IgG subtype ELISA. Sera collected 21 days after boosting was tested antigen-specific IgG subclass by ELISA as described in Materials and Methods. ELISA plates were coated with 4 µg/mL of A/Aichi/2/68 and antigen specific IgG subclasses were detected using HRP-conjugated goat anti-mouse IgG1 or IgG2a. **C**. Hemagglutination inhibition test of prime and boost sera was performed as described in Materials and Methods using live MDCK grown A/Aichi/2/68. (n=6 per group)

mice (6-8 weeks) were vaccinated subcutaneously (s.c.) with 3 µg of sHA or sHA.GCN4pII at day 0 and boosted at day 21. Mice primed with the sHA.GCN4pII had serum IgG responses to homologous inactivated A/Aichi/2/68 virus while mice primed with sHA had no detectable antigen-specific IgG by ELISA (Figure 4A). Following boosting, the sHA.GCN4pII vaccinated group had approximately 15-fold higher serum IgG titers than the sHA vaccinated mice (Figure 4A).

The HAI test is used to measure antibodies which bind to the HA receptor binding domain, blocking binding to sialic acid receptors. In general, an HAI titer of ≥40 is correlated with vaccine-induced protection in humans[22]. Therefore, serum collected from mice primed and boosted with the recombinant proteins were tested for HAI titers. At 21 days after priming, the sera from sHA.GCN4pII vaccinated mice had a geometric mean HAI titer of 16 while the sera from the sHA vaccinated group had no detectable HAI activity. Following boosting, sera from mice vaccinated with sHA.GCN4pII had a geometric mean HAI titer of approximately 700 while the sHA group had no detectable HAI titer (Figure 4C). Thus, the modified trimeric H3N2 sHA vaccine has a significantly enhanced ability to induce functional anti-influenza antibodies compared to the unmodified H3N2 sHA antigen.

Mice vaccinated with sHA.GCN4pII express similar levels of serum IgG1 and IgG2a. The differences in biological functions of the IgG subclasses are well known [23] and the helper T cell cytokines responsible for class switching have been studied extensively [24]. Therefore, we compared the antigen specific IgG1 and IgG2a levels in

mice vaccinated with sHA or sHA.GCN4pII by ELISA. On day 21 after boosting, mice vaccinated with sHA.GCN4pII had higher serum levels of antigen specific IgG1 than mice vaccinated with sHA. In addition, mice vaccinated with sHA.GCN4pII had antigen specific serum IgG2a while the mice vaccinated with sHA had no detectable antigen specific IgG2a (Figure 4B). For the sHA.GCN4pII vaccinated mice, similar relative amounts of IgG1 and IgG2a were observed suggesting a balanced Th1/Th2 cytokine environment.

sHA.GCN4pII induces a protective immune response to lethal challenge with mouse adapted A/Aichi/2/68 virus. To compare the efficacy of the trimeric sHA and the monomeric sHA as vaccine antigens, mice vaccinated with 3 µg of sHA or sHA.GCN4pII were challenged with 5 x LD50 of homologous mouse adapted A/Aichi/2/68 virus by intranasal instillation. Body weights and survival were monitored for 14 days post challenge. Following challenge, the sHA group had an average body weight loss of approximately 20% at a rate comparable to the PBS treated (naïve) mice (Figure 5A). However, the sHA.GCN4pII vaccinated group maintained their initial body weight for the duration of the experiment. Notably, mice that received the sHA.GCN4pII vaccine had a survival rate of 100% (5/5) compared to mice vaccinated with sHA which had a survival rate of 33% (2/6) (Figure 5B)($p=0.0269$). This difference in weight loss and survival demonstrates the enhanced efficacy of the modified trimeric Aichi GCN4pII HA vaccine compared to the unmodified H3N2 HA vaccine.

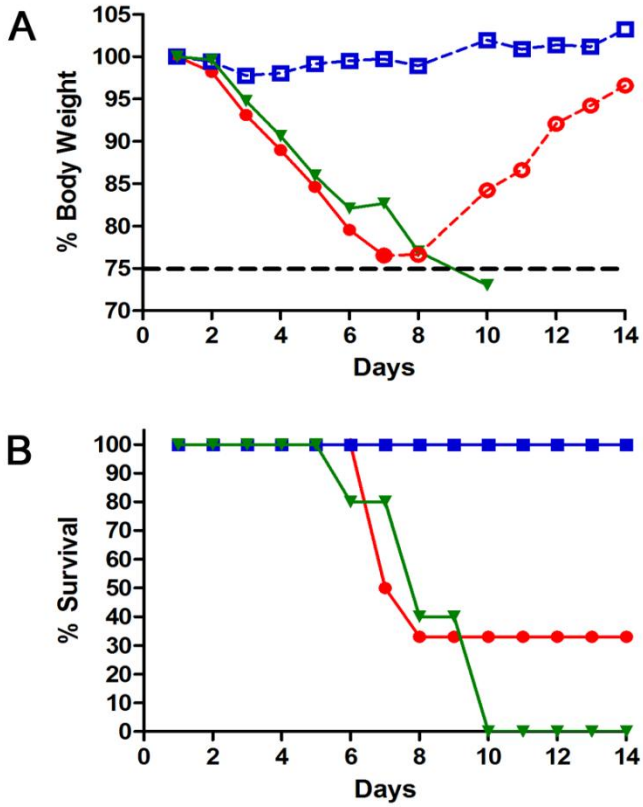

Figure 5. sHA.GCN4pII provides complete protection after two vaccinations unlike sHA. Mice primed and boosted were challenged with 5 xLD_{50} of mouse adapted H3N2 A/Aichi/2/68. **A, B**. Body weights and survival were followed for 14 days post challenge. sHA (circle), sHA.GCN4 (square), PBS (inverted triangle). Open symbols with dashed line indicate % mean initial body weight of surviving mice for each group. Closed symbols with solid line indicate % mean initial body weight of mice showing signs of morbidity. (n=6 per group)

A single immunization with sHA.GCN4pII increases survival and decreases signs of morbidity. To determine the efficacy of a single vaccination, female Balb/c mice (6-8 weeks) were vaccinated s.c. with 3 µg of sHA or sHA.GCN4pII. Serum was collected on days 14 and day 28 post-vaccination and serum IgG and HAI titers were measured as above. On day 14, sHA.GCN4pII induced a virus-specific serum IgG response of approximately 52 ng/mL while sHA induced no detectable virus specific IgG. On day 28 following vaccination, sHA.GCN4pII induced a 144-fold higher virus-specific serum IgG response compared to animals vaccinated with sHA (Figure 6A). Two of six animals from the sHA.GCN4pII group were positive for HAI titer while sHA vaccinated mice had no detectable HAI titer (data not shown).

Mice were challenged with 5 x LD_{50} of mouse adapted A/Aichi/2/68 by intranasal instillation. sHA.GCN4pII vaccinated mice had limited body weight loss compared to sHA vaccinated mice or control mice (Figure 6B). Despite modest IgG and HAI titers, 5/6 of the sHA.GCN4pII vaccinated mice survived the challenge compared to sHA vaccinated mice in which only 1/6 survived (Figure 6C). These results demonstrate that stabilization of the trimeric structure of the H3N2 soluble HA enhances protective immune responses following a single vaccination in the absence of adjuvants ($p=0.0195$).

DISCUSSION

Influenza virus remains an important respiratory pathogen with the potential to cause worldwide pandemics, such as the “Spanish” influenza virus in 1918 and the novel

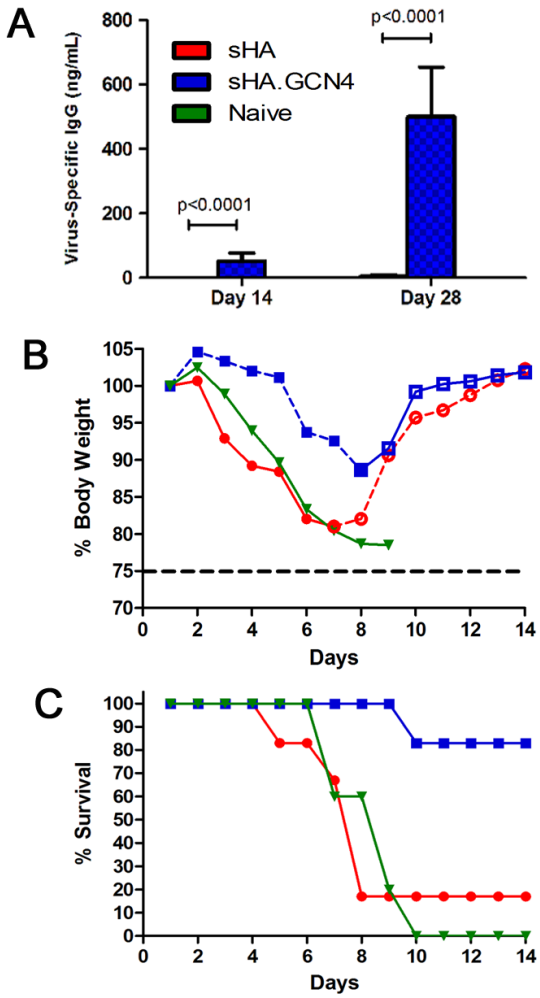

Figure 6. sHA.GCN4pII induces stronger protective immune response after a single vaccination compared to sHA. A. Serum IgG ELISA. Plates were coated with 4 μg/mL of A/Aichi/2/68 and antigen specific IgG was detected in sera collected on day 14 and day 28 post-vaccination using HRP-conjugated goat anti-mouse IgG. Mice vaccinated with recombinant Aichi HA proteins were challenged on day 31 after vaccination with 5 x LD50 of mouse adapted A/Aichi/2/68. **B, C**. Body weights and survival were followed for 14 days post challenge. sHA (circle), sHA.GCN4 (square), PBS (inverted triangle). Open symbols with dashed lines indicate % mean initial body weight of surviving mice for each group. Closed symbols with solid lines indicate % mean initial body weight of mice showing signs of morbidity. (n=6 per group)

swine-origin H1N1 pandemic in 2009 [25]. Furthermore, the recent delays in the production of the monovalent H1N1 vaccine illustrate the need for a rapidly produced and safe alternative to egg-based influenza vaccines. Here we investigated the use of recombinant baculovirus expression to produce soluble forms of the HA protein, which is the primary target of neutralizing antibody. This system has several advantages including the high levels of protein expression [6] and an established safety record in humans[2,5,7]. Current influenza vaccine production requires the generation of a high-yield egg growth reassortant to obtain a sufficient yield for vaccine production. Using molecular cloning, the HA gene from circulating viruses can be cloned directly from clinical isolates and a recombinant protein vaccine can be rapidly produced to prevent new seasonal outbreaks or pandemics as a result of antigenic drift or shift [2,8].

In this study, we generated a rBV-derived soluble hemagglutinin (sHA) modified at its C-terminus with the GCN4pII trimerization heptad repeat to stabilize the trimeric structure of the secreted protein. The heptad repeat is derived from the yeast transcription factor GCN4, which plays a role in the biosynthesis of amino acids during starvation. The wildtype heptad repeat forms stable dimers; however by mutating the ‘a’ and ‘d’ positions in the heptad repeat, trimeric or tetrameric coiled coils can form [16,17]. This strategy has been used recently to study the structure and function of the HIV-1 envelope glycoprotein and parainfluenza virus 5 F protein [26,27].

The sHA selected for this study was derived from the H3N2 virus A/Aichi/2/68 due to the extensive data on its structure and function previously published [12,28,29,30]. Our

data indicated that the GCN4pII modification stabilized the trimeric sHA in solution as determined by BS3 crosslinking and analysis by Western blot. Mice primed and boosted with a low dose of recombinant sHA.GCN4pII without an adjuvant had an improved humoral response to the H3N2 HA compared to mice receiving the unmodified sHA protein. Our results are also consistent with results using the H3N2 virus A/Victoria/3/75 soluble HA in which the monomeric HA induced HA specific antibodies; however there was no detectable binding to the trimeric viral HA[31]. In addition, mice vaccinated with the A/Victoria/3/75 sHA were not protected against challenge with homologous mouse adapted virus.

Other studies using recombinant influenza sHA have used HA from H1 [4,5,8], H3 [3,5,8,32,33], H5 [10,21,34], and H7 [21] subtypes in addition to influenza B hemagglutinin [5,8] as a vaccine antigen. The properties of these sHA have been analyzed using hemagglutination assay, trypsin susceptibility, and/or electron microscopy [4,7,21]. Overall, the ability of these recombinant proteins to form stable trimers varies among subtypes and viruses. In the current study, we have employed a water-soluble crosslinker, BS3, which links monomers within an oligomeric structure to characterize the trimeric structure of the recombinant H3 sHA [19,20]. Crosslinking provides the advantage of being able to determine the approximate ratio of oligomers in solution without the need to use complex size-exclusion chromatography techniques. Modification of the H5 HA with the foldon trimerization domain from the T4 fibritin protein also was reported to generate soluble trimeric HA in the recombinant baculovirus system which was more immunogenic than the unmodified protein when mice were

vaccinated with recombinant protein with adjuvant [10]. In addition, the GCN4 trimerization repeat has been used to study the immunogenicity of H5 HA in poultry[35]. Our data clearly demonstrate that the enhanced immunogenicity of the trimeric H3 HA is due to the presentation of native trimeric epitopes in the GCN4pII stabilized protein, while the unmodified protein presents epitopes associated with the altered low-pH conformation of the HA protein.

Our data indicate that sHA.GCN4pII generates higher levels of both IgG2a and IgG1 than the soluble Aichi HA. The ratio of IgG1:IgG2a that we observe following boosting suggests that the cytokine environment is a balanced Th1/Th2 phenotype in the sHA.GCN4pII group while the mice immunized with sHA exhibit a Th2 phenotype. In the influenza mouse model, virus specific class 1 helper T cells (Th1) have been suggested to play a more important role in viral lung clearance compared to class 2 helper T cells (Th2) which are associated with increased airway inflammation and enhanced morbidity[36]. Furthermore, the higher serum levels of IgG2a, which is regulated by Th1-associated cytokines[37], in sHA.GCN4pII vaccinated mice might suggest that the finding of more efficient clearance of virus could be related to the effector functions of IgG2a such as complement activation and Fc receptor binding[23,38,39]. Therefore, both the quantity and quality of the humoral immune response in addition to the helper T cell phenotype should be considered in evaluating influenza vaccine efficacy.

It is noteworthy that the protection observed did not require the use of any adjuvants in the vaccination, although we cannot exclude any undocumented adjuvant properties of

the GCN4pII repeat. Using the modified recombinant HA, we were able to induce complete protection in mice following a lethal challenge with mouse adapted A/Aichi/2/68 after two vaccinations. Furthermore, we were able to provide protection to 5/6 of mice following a single vaccination with 3 μg of sHA.GCN4pII in the absence of adjuvants. Thus, the trimeric antigen has a high potential to induce protective immune responses with just a single vaccination, which may be further enhanced by including approved adjuvants.

These results demonstrate that using the expression construct employed in this study, the instability of HA is eliminated by stabilizing the trimeric structure through the GCN4pII trimerization heptad repeat. Furthermore, the stabilized trimeric HA has been demonstrated to exhibit enhanced immunogenicity over unmodified HA with just one vaccination in the absence of adjuvants. These differences could be explained by the presence of the low-pH conformation in the unmodified sHA detected by monoclonal antibody binding. Previous studies on the structure of the X31 HA (A/Aichi2/68) monomer indicate that the trimeric protein is dissociated into monomeric subunits[40]. Antibodies that recognize this low pH structure might interact with epitopes at the monomer-monomer interface, or the "silent face", of the hemagglutinin molecule. By presenting the "silent face" in the unmodified sHA, the antibody repertoire maybe skewed to epitopes not accessible in live virus at neutral pH. Therefore, by conserving the native trimeric structure of the HA, the sHA.GCN4pII represents a better influenza subunit vaccine candidate.

MATERIALS AND METHODS

Ethics Statement. Mice were sterile housed and treated according to Emory University School of Medicine (Atlanta, GA) guidelines and all animal studies were approved by the Emory University Institutional Animal Care and Use Committee.

Generation of a soluble influenza hemagglutinin.

The HA gene from A/Aichi/2/68 was obtained from David Steinhauer. The soluble HA (sHA) gene was generated by introducing a stop codon upstream of the transmembrane domain by PCR (K528). The sHA gene was cloned (Quickligase, New England Biolabs – Ipswich, MA) into the pET22b(+) vector (Novagen/ Merck KGaA, Darmstadt, Germany) using BamHI and SalI to introduce a 6X His tag at the C-terminus and then subcloned into the recombinant baculovirus vector pFastBac1 (Invitrogen - Carlsbad, CA - Carlsbad, CA) using BamHI and XbaI. The DNA segment encoding the trimeric GCN4pII heptad repeat (RMKQIEDKIEEILSKIYHIENEIARIKKLVGER) derived from the wildtype dimeric GCN4 repeat found in *Saccharomyces cerevisiae* was constructed using two rounds of PCR then cloned into pET22b(+)[16,17]. The GCN4pII sequence was then subcloned into pFastBac1containing the sHA gene using EagI and SalI as described above (sHA.GCN4pII). All plasmids were sequenced to confirm deduced amino acid sequences (Agencourt/Beckman Coulter Genomics – Danver, MA).

sHA and sHA.GCN4pII recombinant baculovirus production. pFastBac1 vectors containing the sHA and sHA.GCN4pII genes were transformed into DH10Bac cells and recombinant baculoviruses were generated according to the manufacturer's protocol

(Invitrogen - Carlsbad, CA). Sf9 cells were transfected with bacmids as previously described [41].

Recombinant sHA protein expression and His-tag affinity purification. Plaque assays were performed according to the Bac-to-Bac Kit protocol (Invitrogen - Carlsbad, CA). Sf9 cells were infected at a MOI of 1 and incubated for 48 hours to express recombinant protein. Supernatants were collected, clarified by centrifugation then affinity purification was performed by incubating recombinant proteins overnight at 4^{o}C with nickel-agarose beads (Qiagen – Valencia, CA) and washing in empty columns and to remove rBV and cellular proteins. Non-specific nickel-bead binding was eliminated by washing with 10 mM imidazole (Sigma – St. Louis, MO) and recombinant protein eluted with a 250 mM imidazole solution. Protein elution was monitored using a UV detector at 280 nm. Imidazole was removed by dialysis against PBS (Gibco - Carlsbad, CA)

Purified proteins were separated on a 10% SDS-PAGE under reducing conditions (1% mercaptoethanol) then blotted and analyzed by Western blot using anti-A/Aichi/2/68 serum, anti-6xHis monoclonal antibody (Qiagen – Valencia, CA) and Coomassie blue stain (Biorad – Hercules, CA). Western blot was developed using the substrate ECL-Plus (BioRad – Hercules, CA).

Bis[sulfosuccinimidyl] suberate (BS3) crosslinking. The oligomeric status of purified recombinant proteins was determined using the water soluble BS3 crosslinker (Pierce -

Rockford, IL). Crosslinking was performed as described by De Fillette et al [42], with the following modifications. Briefly, 1 µg recombinant protein was incubated at room temperature in the presence of BS3 (final concentration – 3 mM) for 30 minutes. Crosslinking was stopped by the addition of 1M Tris-HCl pH 8.0 to a final concentration of 50 mM. After crosslinking, proteins were separated on a 5-15% SDS-PAGE under reducing conditions (1% mercaptoethanol) then blotted and analyzed by Western blot using anti-6xHis antibody and developed using ECL-Plus.

Sandwich ELISA. Sandwich ELISAs were performed as described by Vanlandschoot et al [31,33], with modifications. Briefly, 96-well Immunoplates (Nunc) were coated with guinea pig anti-A/Aichi/2/68 polyclonal sera (0.02 µg/mL)(BEI Resources). After three washes (0.05% Tween-20 in PBS), wells were blocked with 3% BSA in wash buffer for 2 hours at 37°C, followed by three washes. Recombinant proteins were added to each well (20 ug/mL in PBS) and incubated for 1.5 hours at 37°C, followed by three washes. To determine antibody binding, 2-fold serial dilutions (PBS) of anti-influenza A/Aichi/2/68 sera (mouse), LC89, or HC67 (a kind gift of David Steinhauer) were added and incubated for 1.5 hours at 37°C, followed by three washes. Wells were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:500 in PBS) for 1.5 hours at 37°C, followed by three washes. OPD substrate was added to each well and color allowed to develop. Color development was stopped using 1 M phosphoric acid. Absorbances were read at 450 nm on a Biorad Model 680 microplate reader.

Vaccination and Serological Immune Responses. Female Balb/c mice (6-9 weeks old)(Harlan Laboratories) were lightly anesthetized with a mixture of xylazine and ketamine and vaccinated subcutaneously with 3 µg of sHA, sHA.GCN4pII, or PBS (naïve) on day 0 and day 21. Mice were then retro-orbitally bled on d21 and d42 and serum collected for hemagglutination inhibition tests (HAI) and Ig ELISA.

HAI tests were performed on vaccinated animal sera based on the WHO protocol [43]. Briefly, sera was treated with receptor destroying enzyme (Denka Seiken Co. Ltd, Tokyo, Japan) for 16 hours at 37°C then heat inactivated for 30 minutes at 56°C. Treated sera was diluted to a final concentration of 1:10 in PBS and incubated with pack chicken RBC for 1 hour at 4°C to remove cryoglobulins. Treated sera were serially diluted and incubated with 4 HA units of A/Aichi/2/68 virus for 30 minutes at room temperature. An equal volume of 0.5% chicken RBC was added to each well and incubated for 30 minutes at room temperature. The HAI titer was read as the reciprocal of the highest dilution of serum that inhibited hemagglutination. Values were expressed as the geometric mean with a 95% confidence interval.

Virus and challenge. To determine vaccine efficacy, vaccinated mice were lightly anesthetized with isofluorane and challenged by intranasal inoculation of 50 µL with 5 x LD50 of live mouse adapted A/Aichi/2/68. Body weight loss and survival rates were monitored daily for 14 days post challenge. Weight loss ≥25% was used as end-point and mice were euthanized according to IACUC guidelines.

Statistical analysis. Statistical analysis was done using the one-way ANOVA analysis of grouped data with GraphPad Prism5 software. Survival curves were analyzed using the log-rank test. All data followed normal Gaussian distributions unless otherwise noted. A P value less than 0.05 was defined as a significant difference.

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Chapter 2. Microneedle vaccination with stabilized recombinant influenza hemagglutinin induces improved protective immunity

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Abstract

The emergence of the swine-origin 2009 pandemic illustrates the need for improved vaccine production and delivery strategies. Skin-based immunization represents an attractive alternative to traditional hypodermic needle vaccination routes. Microneedles can deliver vaccine to the epidermis and dermis, which are rich in antigen presenting cells (APC) such as Langerhans cells and dermal dendritic cells. Previous studies using coated or dissolvable using coated or dissolving microneedles emphasized the use of inactivated influenza virus or virus-like particles as skin-based vaccines. However, most currently available influenza vaccines consist of solublized viral protein antigens. Here we test the hypothesis that a recombinant subunit influenza vaccine can be delivered to the skin by coated microneedles and can induce protective immunity. We found that mice vaccinated via microneedle delivery with a stabilized recombinant trimeric hemagglutinin (HA) derived from A/Aichi/2/68 (H3) virus had significantly higher immune responses than mice vaccinated with unmodified sHA. These mice were fully protected against a lethal challenge with influenza virus. Analysis of post-challenge lung titers showed that MN-immunized mice had completely cleared the virus from their lungs in contrast to mice given the same vaccine by a standard subcutaneous route. In addition, we observed a higher frequency of IFN-γ+CD4+ T cells in trimeric sHA vaccinated mice and a greater mucosal antibody response. Our data therefore demonstrate the improved efficacy of a transdermal recombinant subunit influenza vaccine and emphasize the advantage of this route of vaccination for a protein subunit vaccine.

Introduction

The skin acts as a mechanical barrier against the environment and provides the first line of defense against pathogens. The skin-associated lymphoid tissue (SALT), which was first described by Streilein et al [1], represents an ideal target for skin-based vaccinations because it contains keratinocytes, Langerhans cells (LC), dermal dendritic cells (DDC), and T cells. Skin-associated APC and keratinocytes have been shown to express several pattern recognition receptors (PRRs) including TLR9, TLR2, and TLR3[2, 3], which are important enhancers of the immune response. The LC and DDC, present in the epidermis and dermis respectively, have been shown to take up antigen, migrate to draining lymph nodes and induce an antigen-specific adaptive immune response[4]. Therefore, targeting vaccine to the skin has been shown to enhance immunogenicity[5-8].

The 2009 swine-origin influenza pandemic illustrates the need for rapid and effective vaccination. Transdermal influenza vaccines have utilized approaches including tape stripping [9], epidermal powder immunization [10], and microneedles[11]. These strategies have used diverse antigens including virus-like particles[12], inactivated influenza virus[13], and HA DNA vaccines[14]. However, a recombinant HA subunit vaccine, which has the advantage of rapid, high yield production in an expression system with a high level of purity, has not been developed.

Microneedle arrays are designed to penetrate the stratum corneum, the outer layer of the skin, and deposit vaccine or drug into the epidermis and dermis. Using this approach,

vaccine is coated on the surface of metal microneedles or encapsulated in a polymer making up the microneedle [15]. Delivery of soluble protein via coated microneedles suggests that antigen can be was delivered quickly into the skin. Furthermore, this immunization method generated an antigen specific antibody response that was superior to subcutaneous (s.c.) and intramuscular (i.m.) routes [16-19].

We previously demonstrated that modified form of soluble HA derived from the H3N2 influenza virus A/Aichi/2/68 containing the GCN4pII trimerization repeat stabilized the trimeric structure of the HA protein. In the current study, we tested the hypothesis that MN delivery of the recombinant vaccine would induce superior or at least equivalent levels of protective immunized as compared with subcutaneous immunization. Specifically we investigated the efficacy of transdermal delivery of stabilized trimeric influenza HA from the H3 virus A/Aichi/2/68 via coated microneedles. In addition we determined whether the stabilized trimeric sHA microneedle vaccination induces an improved humoral and cellular responses compared to s.c. immunization. To compare the effect of immunization on post-challenge virus clearance we determined virus lung titers after challenge infection. The work presented here illustrates the first analysis of transdermal delivery of a recombinant influenza subunit HA vaccine using microneedle technology.

Materials and Methods

Recombinant trimeric soluble influenza hemagglutinin (sHA). The HA gene derived from the H3N2 influenza virus A/Aichi/2/68 was truncated and the trimeric GCN4pII sequence from *Saccharomyces cerevisiae*, encoding the trimerization motif, fused to the C-terminus and cloned into the recombinant baculovirus (rBV), pFastBac1 expression vector as previously described[20]. rBV carrying genes for the sHA and sHA.GCN4pII proteins were generated and recombinant proteins expressed and purified as previously described[20]. For purification, a His-tag was added to the C-terminus of each protein construct.

Microneedle fabrication and coating. Microneedles were fabricated from stainless steel sheets (Trinity Brand Industries, SS 304, 50 μm thick, GA) by wet etching. Individual microneedles had a length of 750 micrometers and width of 200 micrometers.

The coating solution was composed of 1% (w/v) carboxymethylcellulose sodium salt (low viscosity, USP grade, Carbo-Mer - San Diego, CA), 0.5% (w/v) Lutrol F-68 NF (BASF - Mt. Olive, NJ) and soluble HA protein at 5 mg/ml. In order to reach a high vaccine concentration in coating solution we used evaporation for 5-10 minutes at room temperature (+23C) at the final step of preparation (Vacufuge - Eppendorf, NY). The coating step was performed by a dip coating process [21]. The apparatus had a chamber with coating solution and microneedle holder which was attached to a linear stage that allowed the microneedle array to move in two dimensions with 0.4 micrometer accuracy. The coating was performed automatically and was monitored by a video camera (Prosilica - MA) attached to a computer.

To measure the amount of vaccine coated per row of microneedles, three rows out of each batch of coated microneedles were each submerged into 200 µL of PBS buffer for 5 minutes. The concentration of protein in the solution was measured by BCA protein assay and was consistent within each batch (Pierce – Rockford, IL).

Bis[sulfosuccinimidyl] suberate (BS3) crosslinking. The oligomeric status of purified recombinant proteins was determined using the water soluble BS3 crosslinker (Pierce - Rockford, IL). Crosslinking was performed as described by De Fillette et al [22], with the following modifications. Briefly, 1 µg of recombinant protein was incubated at room temperature in the presence of BS3 (final concentration – 3 mM) for 30 minutes. Crosslinking was stopped by the addition of 1M Tris-HCl pH 8.0 to a final concentration of 50 mM. After crosslinking, proteins were separated on a 5-15% SDS-PAGE under reducing conditions (1% mercaptoethanol) then blotted and analyzed by Western blot using anti-6xHis antibody and developed using ECL-Plus.

Vaccinations. Female Balb/c mice (6-8 weeks) were anesthetized with a xylazine/ketamine cocktail i.p. and hair on the lower back removed with a hair-removal cream (Nair, Chrurch & Dwight Co. -) two days prior to microneedle vaccination. Mice were anesthetized again and microneedle arrays were inserted into the skin on days 0 (Prime) and 28 (Boost) and left in place for 5 minutes to allow the vaccine coating to dissolve.

Tissue and mucosal secretion collection. Vaginal washes were collected as previously described [23]. For lung homogenates, mice were anesthetized with a xylazine/ketamine cocktail and perfused with sterile PBS to remove circulating blood. Lung tissue was removed and stored in PBS with protease cocktail (Thermo Scientific – Rockford, IL) and homogenized.

Virus and challenge. To determine vaccine efficacy, vaccinated mice were lightly anesthetized with isofluorane and challenged by slow intranasal inoculation of 50 µL containing 5 x LD50 of live mouse adapted A/Aichi/2/68 (H3N2). Body weight loss and survival rates were monitored daily for 14 days post-challenge. Weight loss ≥25% was used as the end-point at which mice were euthanized according to IACUC guidelines.

Viral lung titers. Mouse lungs were collected on day 4 post-challenge as previously described [18]. MDCK cells were maintained at a low passage number in DMEM supplemented with 10% FBS (Hyclone, ThermoFisher - Rockford, IL). Plaque assays were performed on lung homogenates from challenged mice as previously described [24].

ELISA. IgG ELISA was performed on serum and lung homogenates as previously described [18]. All horseradish-peroxidase (HRP) conjugated secondary antibodies to mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 were purchased from Southern Biotechnology Associates (Birmingham, AL). Briefly, 96-well immunoplates (Nunc, Co - Rochester, NY) were coated overnight at 4°C with 4 µg of inactivated A/Aichi/2/68 virus per well. Plates were washed with PBS-Tween (0.05%) and blocked with PBS-Tween

supplemented with 3% BSA. Sera were diluted 1:100 and incubated for 1.5 hours at 37°C. Plates were washed 3 times with PBS-Tween (0.05%) and incubated for 1.5 hours at 37°C in a 1:1000 dilution of goat, anti-mouse HRP-conjugated secondary antibody (Southern Biotechnology – Birmingham, AL). Plates were washed 3 times with PBS-Tween (0.05%) and OPD substrate (Invitrogen – Carlsbad, CA), added to each well and color allowed to develop. Color development was stopped using 1 M phosphoric acid. Absorbances were read at 450 nm on a Biorad Model 680 microplate reader. Immunoglobulin concentrations were determined by linear regression of a standard curve of known concentrations.

The IgA ELISA procedure was modified from A. Rodriguez et al [25]. Briefly, 96-well immunoplates (Nunc, Co – Rochester, NY) were coated as above and blocked with 1% BSA. Vaginal washes were diluted 1:5 and sera were diluted 1:100 then incubated overnight at 4°C. A 1:500 dilution of biotin-conjugated rat anti-mouse IgA was used and streptavidin-HRP followed by OPD substrate as a detection method (obtained from BD Pharminigen – San Jose, CA).

HAI and microneutralization. HAI tests were performed on vaccinated animal sera based on the WHO protocol [26]. Briefly, sera was treated with receptor destroying enzyme (Denka Seiken Co. Ltd, Tokyo, Japan) for 16 hours at 37°C then heat inactivated for 30 minutes at 56°C. Treated sera was diluted to a final concentration of 1:10 in PBS and incubated with packed chicken RBC for 1 hour at 4°C to remove cryoglobulins. Treated sera were serially diluted and incubated with 4 HA units of A/Aichi/2/68 virus

for 30 minutes at room temperature. An equal volume of 0.5% chicken RBC was added to each well and incubated for 30 minutes at room temperature. The HAI titer was read as the reciprocal of the highest dilution of serum that inhibited hemagglutination. Values were expressed as the geometric mean with a 95% confidence interval.

The microneutralization assay was performed as described by Rowe et al [27] with modifications. Briefly, mouse sera were heat inactivated for 30 minutes at 56°C and serially diluted in virus diluent (DMEM +1% BSA) in a 96-well tissue culture plate. Virus (200 $TCID_{50}$) was added to diluted serum in virus diluent supplemented with 2 µg/mL of TPCK-treated trypsin and incubated for 2 hours at 37°C. Freshly trypsinized MDCK cells were added to all wells and incubated overnight at 37°C. Cells were then fixed in 80% acetone/PBS and washed 3 times with PBS-Tween (0.05% Tween-20). A 1:2000 dilution of biotin-conjugated anti-influenza A nucleoprotein (clone A3)(Milipore - Billerica, MA) and streptavidin-HRP was added for the detection of infected cells. For each plate, the 50% of specific signal value was calculated as follows: ((average of virus only wells)-(average of cells only well))/2 + average of cells only well. The 50% endpoint neutralization titer is reported as the last dilution to score below the 50% specific signal value.

Cellular immune responses. Single cell suspensions were prepared from spleens of mice 14 days post vaccination or naïve mice, by mincing the tissue through a 70 µm cell strainer (BD Falcon), followed by incubation in red blood cell lysing buffer (Sigma) to

remove red blood cells. CD4+ T cells were purified by negative selection using BD iMag magnetic cell separation (BD Biosciences – San Jose, CA). Naïve splenocytes were treated with Mitomycin C for 30 minutes and used as accessory cells after incubation with 20, 5 or 0 µg of vaccine in complete RPMI overnight at 37°C in a 5% CO2 incubator. Purified CD4+ T cells were added to accessory cells at a ratio of 2:1 (responder:accessory) in complete RPMI medium supplemented with 30 U of recombinant human IL-2 (BD Biosciences – San Jose, CA). In vitro antigen-specific stimulation was measured after incubation for 5 days by intracellular cytokine staining.

Antibodies and flow cytometry. Cells were washed with PBS, 1% BSA buffer and surface stained with fluorochrome-conjugated antibodies to CD4 and CD3, followed by intracellular staining of IFN-γ and IL-4. Antibodies were purchased from eBiosciences and BD Biosciences. For intracellular cytokine staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm manufacturer's protocol and reagents (BD Bioscience – San Jose, CA). The data were acquired on a BD LSR-II and analyzed with FloJo Software (Tree Star, Inc. v7.6.1).

Statistics. Statistical analysis was done using the one-way ANOVA analysis of grouped data with GraphPad Prism5 software. Survival curves were analyzed using the log-rank test. All data followed normal Gaussian distributions unless otherwise noted. Viral lung titers were analyzed using one-way ANOVA. A P value less than 0.05 was defined as a significant difference.

Results

Trehalose stabilizes the trimeric structure of sHA.GCN4pII

The GCN4pII trimerization repeat has been used to investigate the immunogenicity and protein structure for several viral proteins including HIV gp120/41, parainfluenza virus 5 F protein, and influenza HA[28-30]. We have previously demonstrated that modification of the sHA at the C-terminus with the GCN4pII trimerization repeat generates a stabilized trimeric sHA[20]. Because the trimeric structure was important for enhanced immunogenicity, we determined if this structure of sHA.GCN4pII was disrupted by the microneedle coating process. We coated solid metal microneedle arrays with 3 µg of recombinant protein in the presence or absence of 15% trehalose, allowed the coating to dry, and then redissolved the vaccine coating. As demonstrated by SDS gel electrophoresis when redissolved samples were analyzed after chemical crosslinking, the sHA.GCN4pII protein dissociates into dimeric and monomeric species (Figure 1 – Lane 3. However, the trimeric structure is maintained when 15% trehalose is added to the coating buffer (Figure 1 – Lane 4). In contrasts, the unmodified sHA remains a mixture of trimers, dimers, and monomers independent of the presence of trehalose (Figure 1 – Lane 1 and 2). These data suggest that the trimeric sHA protein structure disrupted during the microneedle coating and drying process, however the structure is preserved by the addition of trehalose to the coating solution.

Microneedle delivery of sHA.GCN4pII induces antigen specific humoral response

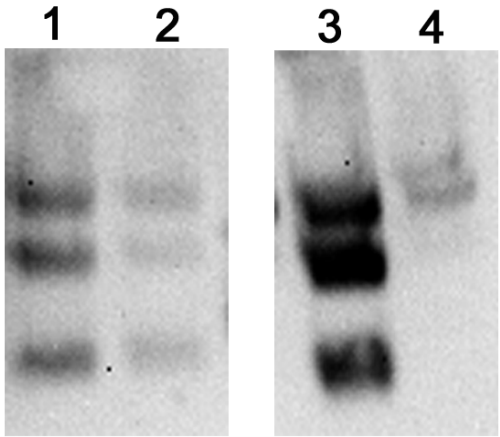

Figure 1. Trehalose supplemented microneedle coating solution preserves the sHA.GCN4pII trimeric structure. BS^3 crosslinking of recombinant baculovirus produced sHA and sHA.GCN4pII after dissolving the coating from the microneedles coataed in the presence or absence of 15% trehalose. Lane 1 – sHA without 15% trehalose. Lane 2 – sHA with 15% trehalose. Lane 3 – sHA.GCN4pII without 15% trehalose. Lane 4 – sHA.GCN4pII with 15% trehalose. Crosslinked proteins were separated on 5-15% SDS-PAGE and western blot performed using mouse anti-His primary antibody.

To test the immunogenicity of recombinant soluble HA delivered to the skin by coated microneedles, female Balb/c mice (6-8 weeks old) were vaccinated with 3 μg of sHA or sHA.GCN4pII on day 0 and boosted on day 28. After priming, mice vaccinated with sHA.GCN4pII had approximately 2.6-fold higher A/Aichi/2/68 specific serum IgG titers than mice boosted with sHA ($p=0.0032$), indicating the enhanced immunogenicity of the HA when in its trimeric native form. In addition, 28 days after boosting with sHA.GCN4pII induced approximately 3.7-fold higher A/Aichi/2/68 specific serum IgG titers than mice boosted with sHA ($p<0.0001$) (Figure 2).

The influenza-specific IgG subtype profile in the serum of MN vaccinated mice indicates that sHA.GCN4pII induced higher IgG1 (1.4-fold, $p<0.0001$), IgG2a (2.2-fold, $p=0.0053$), and IgG2b (2-fold, $p=0.0148$) levels than mice receiving sHA or uncoated microneedles (Figure 3). No significant differences were found in the proportion of IgG1 to IgG2a/b between the groups, which indicate that the nature of the antigen did not influenced the expression of the IgG subtype. There was no detectable antigen specific IgG3 in either vaccinated group (data not shown). The serum immunoglobulin levels indicate that the transdermal delivery of recombinant trimeric sHA induces a robust IgG response and enhances the levels of IgG2a, IgG2b and IgG1 subtypes when compared with sHA-vaccinated mice.

sHA.GCN4pII induces improved HAI and neutralizing antibody responses

The HAI test is used to measure antibodies which bind to the HA receptor binding domain, blocking binding of HA to sialic acid receptors. In general, an HAI titer of ≥40

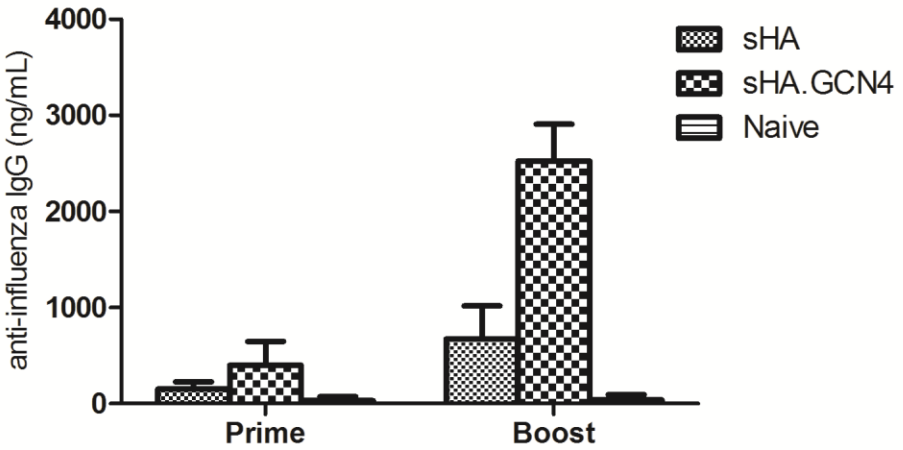

Figure 2. Microneedle vaccination with sHA.GCN4pII induces greater serum levels of A/Aichi/2/68 specific IgG. Mice were primed and boosted 3 weeks later with 3 µg of sHA, sHA.GCN4pII, or blank microneedles (naïve). Blood was collected day 21 after priming (prime) and 21 days after boosting (boost). ELISA plates were coated with 4 µg/mL of A/Aichi/2/68 virus and antigen specific serum IgG ELISA was measured for prime and boost. HRP-conjugated goat anti-mouse IgG was used for detection. (n=12)

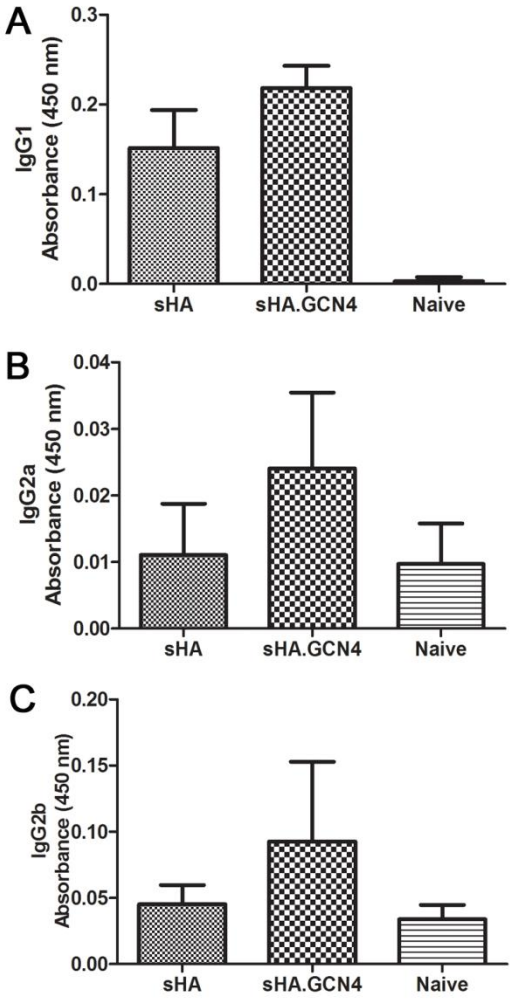

Figure 3. IgG subtype ELISA. Sera collected 21 days after boosting with antigen coated microneedles were tested for antigen-specific IgG subclasses by ELISA as described in Materials and Methods. ELISA plates were coated with 4 μg/mL of A/Aichi/2/68 and antigen specific IgG subclasses were detected using HRP-conjugated goat anti-mouse IgG1 (**A**), IgG2a (**B**), or IgG2b (**C**).

is correlated with vaccine-induced protection in humans[31]. Therefore, serum from microneedle vaccinated mice was tested for HAI titers. At 21 days after priming no detectable HAI titer was observed in the vaccinated mice. However, after boosting the sHA.GCN4pII vaccinated mice had an HAI titer of 190, while sHA vaccinated mice had no detectable HAI titer ($p<0.0001$)(Figure 4A). We previously observed similar enhancement of HAI titers when the stabilized trimeric HA was administered by subcutaneous immunization [20].

In order to measure the ability of serum antibodies to neutralize virus infectivity we performed a microneutralization assay. sHA.GCN4pII vaccinated mice had an approximately 70-fold higher neutralization endpoint titer than mice vaccinated with sHA ($p<0.0001$) (Figure 4B). Therefore, MN vaccination with the sHA.GCN4pII protein induced high levels of functional antibodies but similar immunization with the sHA protein did not induce such responses.

Microneedle vaccination with sHA.GCN4pII induces a robust antibody response at mucosal sites

Influenza virus is a respiratory pathogen that infects the airway epithelia. A strong mucosal antibody response has been associated with prevention of viral pathology in the upper respiratory tract of mice[32]. Previous studies using intradermal or transdermal influenza vaccinations have investigated the induction of secretory IgA (sIgA) at mucosal sites.[9]. Therefore, to determine the induction of secretory IgA by microneedle vaccination with recombinant soluble HA we measured sIgA in vaginal washes by

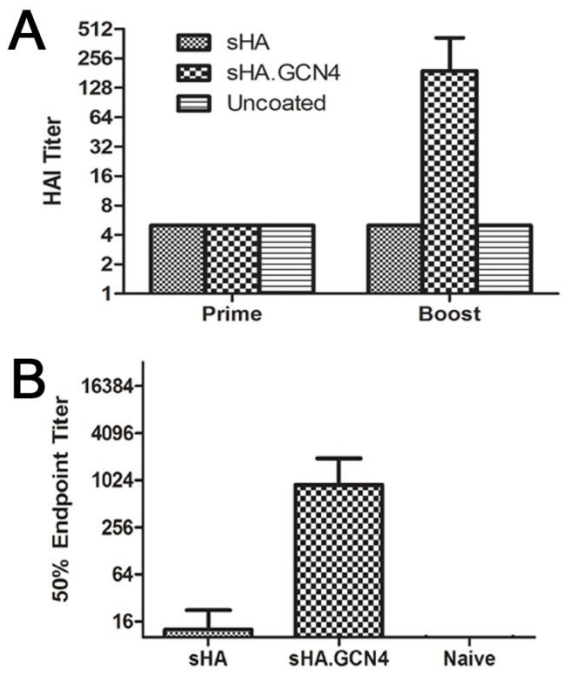

Figure 4. Transdermal vaccination with sHA.GCN4pII induces higher HAI and microneutralization titers. **A.** Hemagglutination inhibition test of prime and boost sera was performed as described in Materials and Methods using MDCK grown A/Aichi/2/68 virus. HAI titers were reported as the reciprocal of the last dilution of serum to inhibit agglutination of RBCs **B.** Microneutralization assay using boost serum was performed as described in Materials and Methods with 200 $TCID_{50}$ of MDCK grown A/Aichi/2/68. Virus infection was detected using biotin conjugated anti-NP monoclonal antibody and streptavidin-HRP. 50% endpoint titers were reported as the dilution of serum to inhibit 50% of signal observed in virus infected MDCK cells.

ELISA. On day 28 after boosting, sHA.GCN4pII vaccinated mice had approximately 6.7-fold higher sIgA than mice vaccinated with sHA ($p=0.0105$)(Figure 5A). When serum IgA levels were measured we found that in the sHA.GCN4pII microneedle-vaccinated mice were approximately 3.3-fold higher than sHA vaccinated mice ($p<0.0001$), correlating to the higher mucosal IgA levels we observed (Figure 5B).

It has been demonstrated that IgG antibodies have an important role in neutralizing viruses after infection has been established in addition to preventing lung pathology [32]. Therefore, we measured the total virus-specific IgG in the lung homogenates of microneedle-vaccinated mice. Lung homogenates from mice vaccinated with sHA.GCN4pII had 1.4-fold higher virus specific IgG compared to mice vaccinated with sHA ($p=0.0002$)(Figure 5C). These data suggests that skin delivery by microneedle vaccination with the trimeric HA, sHA.GCN4pII, induces improved humoral mucosal immune responses when compared to the sHA.

Microneedle vaccination with sHA.GCN4pII induces complete protection against challenge infection.

To determine the efficacy of microneedle vaccination with recombinant soluble HA, we challenged vaccinated mice intranasally with 5 x LD_{50} (lethal) or 0.1 x LD_{50} (sub-lethal) of mouse adapted A/Aichi/2/68. Body weights and survival were monitored for 14 days post-challenge. Following lethal challenge, mice vaccinated with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated

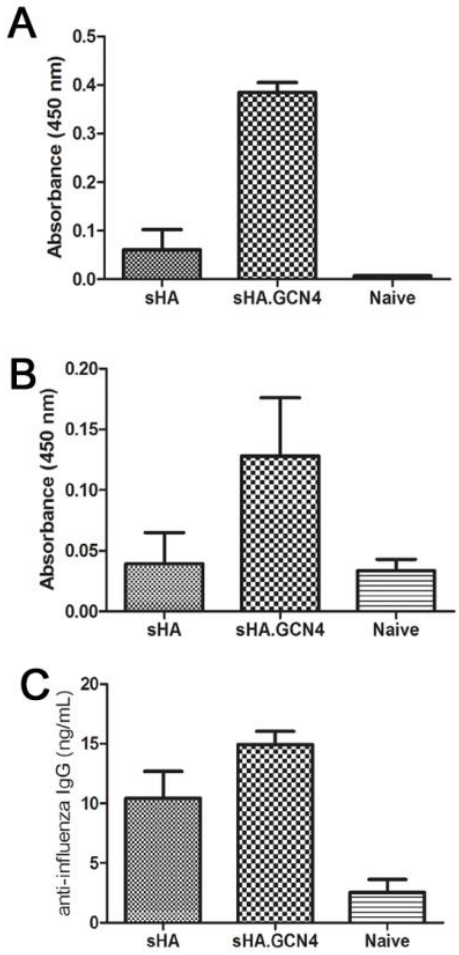

Figure 5. sHA.GCN4pII induces higher serum IgA and mucosal antibody responses. IgA ELISA was performed as described in Materials and Methods. ELISA plates were coated with 4 μg/mL of A/Aichi/2/68 and IgA detected using biotin-conjugated rat anti-mouse and streptavidin-HRP. IgG ELISA was performed as described previously. IgA was assayed 21 days after vaccination in vaginal washes (**A**) and serum (**B**). IgG was assayed 21 days after vaccination in lung homogenate (**C).**

with sHA lost approximately 20% of their body weight (Figure 6A). Microneedle vaccination with sHA.GCN4pII confers 100% protection (6/6) while sHA confers 50% protection (3/6), although this difference was not significantly different ($p=0.0559$)(Figure 6B). This data was further supported in an independent study following sub-lethal infection with the mouse adapted virus in which both vaccinated groups maintained their body weight over the course of the infection (Figure 6C). Thus, transdermal vaccination with microneedles coated with soluble trimeric HA coated microneedles is an effective route of inducing protective immunity against influenza virus. We previously observed that s.c. immunization with this antigen was also effective in conferring complete protection against live virus challenge [20].

Microneedle vaccination induces improved viral clearance after lethal challenge. As a sensitive approach to determine the ability of vaccinated mice to compare viral replication following lethal challenge we examined the viral lung titers 4 days post-challenge. Mice vaccinated with sHA.GCN4pII coated microneedles had no detectable viral lung titers at 4 days post challenge, representing a reduction of over 10^6-fold compared with the unimmunized group ($p<0.001$). In contrast, mice vaccinated subcutaneously with sHA.GCN4pII had a residual virus titer of about 10^2 pfu/g demonstrating less efficient clearance. Independent of route of immunization, the mice immunized with sHA coated microneedles had less than 1-log reduction in viral lung titers compared to unimmunized control mice (Figure 7). These data suggest that the microneedle route of vaccination induces improved clearance of replicating virus for both sHA and sHA.GCN4pII compared to the s.c. route.

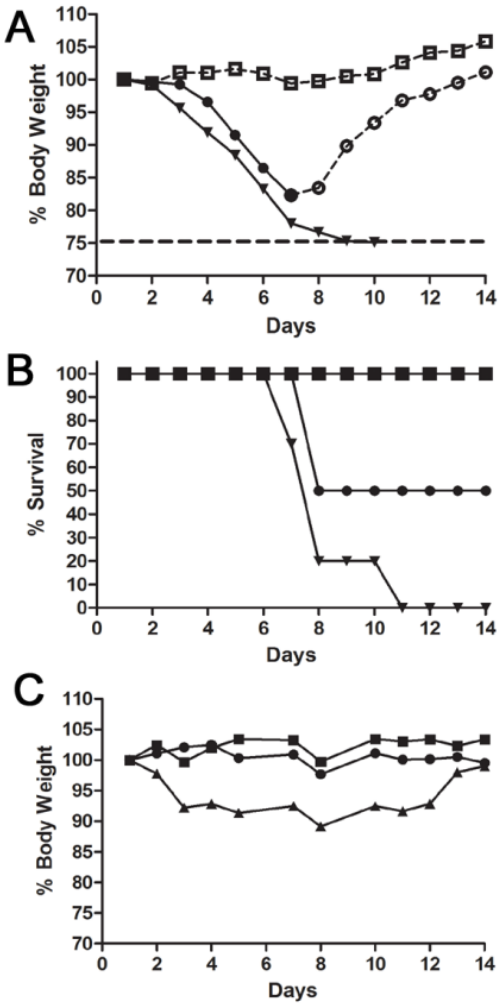

Figure 6. MN immunization with trimeric sHA induces improved protective immune responses against lethal challenge with mouse adapted A/Aichi/2/68. **A, B**. Mice primed and boosted were challenged with 5 xLD_{50} of mouse adapted H3N2 A/Aichi/2/68. Body weights and survival were followed for 14 days post challenge. sHA (circle), sHA.GCN4 (square), PBS (inverted triangle). Open symbols with dashed line indicate % mean initial body weight of surviving mice for each group. Closed symbols with solid line indicate % mean initial body weight of mice showing signs of morbidity. (n=6) **C.** Sub-lethal infection of microneedle vaccinated mice. Body weights were followed for 14 days post infection. (circle), sHA.GCN4 (square), PBS (inverted triangle). (n=6 per group)

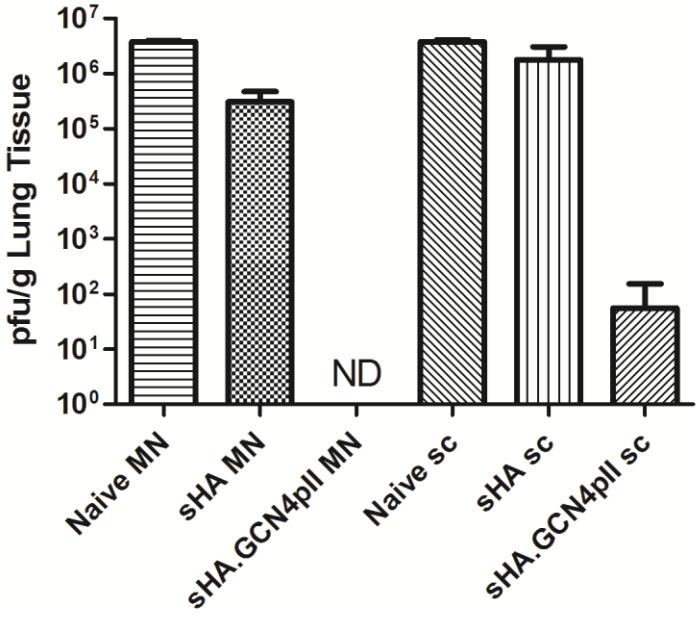

Figure 7. MN vaccination with trimeric sHA induces improved clearance of virus from the lung. Mice primed and boosted with 3 µg of recombinant soluble HA by the microneedle or subcutaneous routes were challenged with 5 x LD_{50} of mouse adapted A/Aichi/2/68. Lungs were removed on day 4 post-challenge and processed for virus plaque assays. Lung viral titers are reported as the arithmetic mean ± standard deviation (n=3). ND = not detectable

sHA.GCN4pII induces a type 1 helper T cell population after microneedle vaccination

The effector phenotype of the helper T cell compartment can influence the B cell response and the CD8+ T cell response [33, 34]. Therefore, to determine the helper T cell phenotype, we purified CD4+ T cells from vaccinated mice and restimulated the cells with vaccine being presented by accessory cells. The cytokines IFN-γ and IL-4 are known to be produced by helper T cell type 1 and helper T cell type 2, respectively. The frequency of IFN-γ+ CD4+ T cells was assayed by intracellular cytokine staining after in vitro re-stimulation. We observed that mice vaccinated with sHA.GCN4pII had approximately 2-fold higher frequency of IFN-γ+ CD4+ T cells than mice vaccinated with sHA at both antigen concentrations (20 μg, $p=0.117$ and 5 μg, $p=0.0262$)(Figure 8A). Conversely, mice vaccinated with sHA had approximately 1.3-fold higher frequency of IL-4+CD4+ T cells than mice vaccinated with sHA.GCN4pII at both antigen concentrations, although this difference was not significant (Figure 8B). These results indicated that microneedle vaccination with sHA.GCN4pII induced a more robust Th1 response in mice. The ratio of IFN-γ+CD4+ T cells to IL-4+CD4+ T cells suggests that the helper T cell phenotype is dominated by Th1 cells in mice vaccinated with sHA and sHA.GCN4pII (Figure 8C). These results indicated that microneedle vaccination with sHA.GCN4pII induced a more robust Th1 response in mice.

Discussion

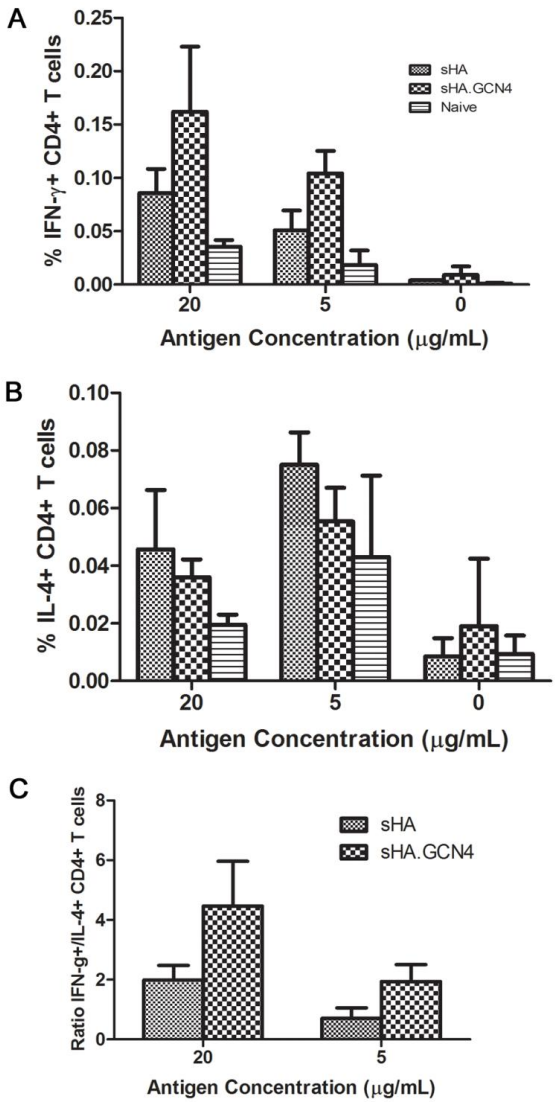

Figure 8. Microneedle vaccination with trimeric recombinant sHA induces greater Th1 responses. Purified CD4+ T cells were restimulated with 20, 5, or 0 ug/mL of vaccine for 5 days supplemented with 30 U/mL of recombinant human IL-2. **A,B.** On day 5, restimulated cells were fixed stained for surface markers and the indicated cytokines. **C.** Ratio of frequencies of IFN-γ+:IL-4+ helper T cells in microneedle vaccinated mice.

The recent H1N1 influenza pandemic has illustrated the need for convenient alternatives to traditional influenza vaccine. Previous studies from our lab and others have demonstrated the successful use of both coated and dissolving microneedles transdermal delivery of influenza vaccines using virus-like particles or inactivated virus[18, 19, 35]. However, little information is available on using this approach to deliver a protein subunit vaccine. Microneedles have the advantage of delivering vaccine to an area rich in APCs such as the epidermis and dermis without causing pain [36]. These vaccinations induced robust antibody and cellular responses resulting in protection against lethal challenge with several subtypes of influenza virus.

We have previously demonstrated that the influenza HA protein modified at the C-terminus with the trimerization repeat, GCN4pII, generates stable trimeric soluble HA. Following subcutaneous vaccination, the modified trimeric sHA was able to induce higher virus-specific serum IgG and HAI titers [20]. These enhanced humoral responses translated to a greater vaccine-induced protection following challenge with homologous virus. In the present study, we have tested the hypothesis that MN-mediated delivery via the transdermal route would be effective in enhancing the immune response to an influenza subunit vaccine. We demonstrated that the structure of the recombinant trimeric sHA was preserved when 15% w/v trehalose was included in the coating formulation. However, the unmodified sHA remained as a mixture of trimers, dimers and monomers in the presence or absence of 15% trehalose. A stabilizing effect of trehalose on the HA activity of influenza virus was previously observed when coating microneedles with the whole inactivated virus [37].

Microneedle vaccination with the modified trimeric sHA induced an improved humoral systemic response when compared with unmodified sHA as determined by ELISA. This improved response to trimeric vs. unmodified sHA was particularly evident in HAI and microneutralization titers. These results strongly support the view that the trimeric sHA induces greater levels of functional antibodies than the unmodified sHA, while both recombinant proteins induce binding antibodies as measured by ELISA. This difference in induction of functional antibodies could be attributed to the better presentation of native epitopes present in the stabilized trimeric structure of the modified sHA corresponding to those in the live virus. The unmodified sHA, in ctonrast, probably presents additional epitopes found at the interfaces between monomers, which are not exposed in the native virus. Importantly, sHA.GCN4pII immunization also induced 100% protection while sHA vaccination provided only partial protection.

The post-challenge viral lung titers observed in mice vaccinated with recombinant soluble HA coated microneedles demonstrates that the soluble trimeric HA induced immune responses that were efficient at clearing replicating virus. In addition, comparing the post-challenge viral lung titers of mice vaccinated subcutaneously or with coated microneedles indicated that the skin-based immunization resulted in greater clearance of replicating virus independent of the recombinant protein used. These data support the conclusion that skin-based delivery of a recombinant protein antigen using microneedles increases the efficacy of such vaccines.

Further investigation of the vaccine induced responses indicated that sHA.GCN4pII induced a balanced IgG subtype profile contrasting to the sHA which induces a predominantly IgG1 profile. The cytokines expressed by CD4+ helper T cells influence the B cell isotype switch; therefore we assessed the CD4 T cell phenotype in terms of a Th1 cytokine (IFN-γ) and a Th2 cytokine (IL-4) by intracellular cytokine staining. We observed that both recombinant sHA vaccines induced both IFN-γ+ producing T cells as well as IL-4+ producing T cells. Notably, sHA.GCN4pII induced a higher frequency of IFN-γ+CD4+ T cells. Helper T cells which express IFN-γ have been shown to play an important role in inducing isotype switching to IgG2 in Balb/c mice [38]. In addition, IFN-γ induces an anti-viral state, upregulation of MHC I and II, and costimulatory molecules on APCs [39]. The sHA induced approximately 1.3-fold higher IL-4+CD4+ T cells than sHA.GCN4pII in vaccinated mice ($p=0.1191$) which correlated to the presence of IgG1 and low concentration of IgG2a in serum. Here, we showed that both Th1 and Th2 effector cells are generated after antigen delivery to the skin and that Th1 effector CD4 T cells are predominant in the sHA.GCN4pII group correlating with the highest vaccine efficacy. The microneedle route for vaccination has the potential to enhance the CD4+ T cell response over traditional routes of immunization [40]. Thus, the role of CD4+ T cells should be further examined to determine their significance in determining the efficacy of transdermal vaccination.

For influenza virus infection, secretory IgA (sIgA) has been shown to bind and neutralize virus in the upper respiratory tract and nasal cavity [32]. In addition, transcytosis of influenza-specific IgG across the lung epithelial cell layer via FcRn plays an important

role in preventing lung pathology [32, 41]. We observed that mice receiving sHA.GCN4pII induced higher levels of tissue localized virus-specific IgG and mucosal sIgA suggesting that microneedle vaccination induced the critical antibody responses at the site of infection. The differences observed at the mucosal site could be the result of lower immunogenicity of sHA or perhaps because sHA results in a different homing pattern.

These results demonstrate the efficacy of transdermal vaccinations using recombinant sHA derived from the A/Aichi/2/68 (H3N2) virus. Microneedle vaccination with the stabilized HA trimers induced protective immune responses in mice. It is noteworthy that in comparison to our previous work with subcutaneous vaccination, the MN immunization with the trimeric HA induced similar serum IgG and HAI titers and induced the same level of protection against lethal challenge compared to the s.c. vaccinated mice. Comparisons between intramucscular, intranasal, subcutaneous, and microneedle vaccinations using VLP and inactivated virus antigens have all demonstrated the enhanced immunogenicity of the transdermal route[18, 35, 42]. Taken together the results emphasize the conclusion that in the delivery route as well as the nature of the vaccine antigen is important in determining the efficacy of an influenza vaccine.

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Chapter 3. Effect of trimer stabilization on immunogenicity of soluble H1 hemagglutinin from A/California/04/2009 virus

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Introduction

Influenza virus is one of the most common causes of serious respiratory illness. The novel swine-origin H1N1 2009 virus is antigenically distant from recent seasonal H1N1 influenza strains. Since the beginning of the 2009-2010 influenza season, the CDC has reported that all subtyped influenza A viruses isolated from hospitalizations were the novel 2009 H1N1 virus [1]. In June 2009, the World Health Organization declared the start of the 2009 H1N1 pandemic [2]. In response, monovalent H1N1 vaccines were distributed in the United States; however, due to lower than expected production levels distribution was delayed approximately 4 months after declaration of the pandemic. This delay illustrates the need to find alternatives to traditional influenza vaccine production.

Currently, influenza vaccine is produced in eggs which require the generation of high-yield egg growth reassortant viruses and a supply of embryonated chicken eggs, and has potential safety concerns in individuals with egg allergies [3,4,5,6]. For these reasons, effort has been taken to find alternative vaccine production methods. One such method uses recombinant baculovirus to produce a vaccine in insect cells. The rBV expression system has several advantages over traditional egg-based vaccine production techniques including the high levels of expression of recombinant protein[7], rapid scaling of production to meet high demands[3], an established safety record[3,6,8], and more rapid production of new vaccines in response to antigenic shift or drift[3,9].

Previously, we have demonstrated the effectiveness of using a trimerization repeat to stabilize the trimeric structure of soluble HA derived from the H3 virus A/Aichi/2/68. This trimeric sHA induced improved humoral immune responses and protective immune responses[10]. In the current study, we extend this strategy of structure-based antigen design to the H1 subtype of influenza viruses. We determined the effect of the stabilization of the trimeric structure of the H1 HA derived from A/California/04/2009 on the immunogenicity of the protein vaccine and its ability to induce a protective immune response to challenge with homologous virus.

Materials and Methods

Recombinant trimeric soluble influenza hemagglutinin (sHA). The HA gene derived from the H1N1 influenza virus A/California/04/2009 was truncated and the trimeric GCN4pII sequence from *Saccharomyces cerevisiae*, encoding the trimerization motif, fused to the C-terminus and cloned into the recombinant baculovirus (rBV), pFastBac1 expression vector as previously described[11]. rBV carrying genes for the sHA and sHA.GCN4pII proteins were generated and recombinant proteins expressed and purified as previously described[11]. For purification, a His-tag was added to the C-terminus of each protein construct.

Bis[sulfosuccinimidyl] suberate (BS3) crosslinking. The oligomeric status of purified recombinant proteins was determined using the water soluble BS3 crosslinker (Pierce - Rockford, IL). Crosslinking was performed as described by De Fillette et al [12], with

the following modifications. Briefly, 1 µg of recombinant protein was incubated at room temperature in the presence of BS3 (final concentration – 3 mM) for 30 minutes. Crosslinking was stopped by the addition of 1M Tris-HCl pH 8.0 to a final concentration of 50 mM. After crosslinking, proteins were separated on a 5-15% SDS-PAGE under reducing conditions (1% mercaptoethanol) then blotted and analyzed by Western blot using anti-6xHis antibody and developed using ECL-Plus.

Glycan Microarray. HA–antibody pre-complexes were prepared and assayed for receptor binding on glycan microarray as previously described [13]. Briefly, pre-complexes are prepared by mixing 15 microgram HA, 7 microgram Alexa Fluor488 labeled mouse anti-penta His (Qiagen - Germantown, MD) and 4.6 microgram Alexa Fluor488 labeled goat anti-mouse IgG (Invitrogen - Carlsbad, CA) at a molar ratio of 4:2:1 respectively in a total volume of 52.3 µL. Pre-complexed HA-antibody mixtures are incubated for 15 minutes on ice and diluted with 50 µl PBS buffer supplemented with 3% (w/v) BSA and 0.05% Tween-20.

Before adding pre-complex mixture 1 µl of cyanine-5 (1 mg/ml) is added to CFG Glycan array slide to indicate Grid positioning spots then rehydrated with PBS buffer (pH 7.4) with 0.05% Tween-20 for 5 min. Pre-complex mixture is added to the CFG Glycan array slide and incubated under a microscope cover-glass in a humidified chamber for 60 minutes at room temperature and protected from light. The slide is subsequently washed by successive rinses in PBS with 0.05% Tween-20, PBS, and deionized water then centrifuged for 30 seconds. The relative fluorescent units is read using a PerkinElmer

ProScan fluorescence scanner (Waltham, MA) and image analysis performed using Imagene (V.6) from BioDiscovery (El Segundo, CA).

Vaccinations. Female Balb/c mice (6-9 weeks old)(Harlan Laboratories) were lightly anesthetized with a mixture of xylazine and ketamine and vaccinated subcutaneously with 3 µg of sHA, sHA.GCN4pII, or PBS (naïve) on day 0 and day 21. Mice were then retro-orbitally bled on d21 and d42 and serum collected for hemagglutination inhibition tests (HAI) and Ig ELISA.

ELISA. IgG ELISA was performed on serum and lung homogenates as previously described [14]. All horseradish-peroxidase (HRP) conjugated secondary antibodies to mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 were purchased from Southern Biotechnology Associates (Birmingham, AL). Briefly, 96-well immunoplates (Nunc, Co - Rochester, NY) were coated overnight at 4°C with 4 µg of inactivated A/Aichi/2/68 virus per well. Plates were washed with PBS-Tween (0.05%) and blocked with PBS-Tween supplemented with 3% BSA. Sera were diluted 1:100 and incubated for 1.5 hours at 37°C. Plates were washed 3 times with PBS-Tween (0.05%) and incubated for 1.5 hours at 37°C in a 1:1000 dilution of goat, anti-mouse HRP-conjugated secondary antibody (Southern Biotechnology – Birmingham, AL). Plates were washed 3 times with PBS-Tween (0.05%) and OPD substrate (Invitrogen – Carlsbad, CA), added to each well and color allowed to develop. Color development was stopped using 1 M phosphoric acid. Absorbances were read at 450 nm on a Biorad Model 680 microplate reader.

Immunoglobulin concentrations were determined by linear regression of a standard curve of known concentrations.

Hemagglutination inhibition (HAI). HAI tests were performed on vaccinated animal sera based on the WHO protocol [15]. Briefly, sera were treated with receptor destroying enzyme (Denka Seiken Co. Ltd, Tokyo, Japan) for 16 hours at 37°C then heat inactivated for 30 minutes at 56°C. Treated sera were diluted to a final concentration of 1:10 in PBS and incubated with packed chicken RBC for 1 hour at 4°C to remove cryoglobulins. Treated sera were serially diluted and incubated with 4 HA units of A/California/04/2009 virus for 30 minutes at room temperature. An equal volume of 0.5% chicken RBC was added to each well and incubated for 30 minutes at room temperature. The HAI titer was read as the reciprocal of the highest dilution of serum that inhibited hemagglutination. Values were expressed as the geometric mean with a 95% confidence interval.

Virus and challenge. To determine vaccine efficacy, vaccinated mice were lightly anesthetized with isofluorane and challenged by slow intranasal inoculation of 50 µL containing 5 x LD50 of live mouse adapted A/California/04/2009 (H1N1). Body weight loss and survival rates were monitored daily for 14 days post-challenge. Weight loss ≥25% was used as the end-point at which mice were euthanized according to IACUC guidelines.

Results

Modification of A/California/04/2009 soluble HA with GCN4pII stabilizes the native trimeric structure

The Cal/04 soluble HA (sHA) and sHA.GCN4pII proteins migrated on SDS-PAGE as single polypeptides of approximately 70 and 75 kDa proteins, respectively, indicating that the recombinant proteins were expressed as HA precursors (HA0). The higher molecular weight of the sHA.GCN4pII protein indicates that the GCN4pII modification was added to the C-terminus of the H1N1 HA protein. Western blot analysis of the recombinant HA proteins using sera from A/California/04/2009 infected mice indicates that the recombinant proteins are antigenically similar to the viral HA (Figure 1 – Lane 1 = sHA, Lane 2 = sHA.GCN4pII) and the anti-His monoclonal antibody binding demonstrates that the His-tag was included in the protein (Figure 1 - Lane 3 = sHA, Lane 4 = sHA.GCN4pII). Furthermore, both recombinant proteins are produced as the HA0 precursor at a high yield in the baculovirus expression system.

Other studies have utilized the GCN4pII trimerization repeat to investigate the structure of viral proteins such as HIV gp120 and parainfluenza 5 F protein. We have previously demonstrated the trimer stabilization of H3 soluble HA derived from A/Aichi/2/68 using the trimerization repeat. Therefore, to determine the oligomeric structure of the Cal/04 sHA and sHA.GCN4pII proteins, cross-linking was performed using BS3 followed by SDS-PAGE separation and Western blot analysis. The crosslinked A/California/04/2009 sHA.GCN4pII suggests that the native trimeric structure of the viral protein is stabilized by the addition of the GCN4pII trimerization repeat (Figure 2A).

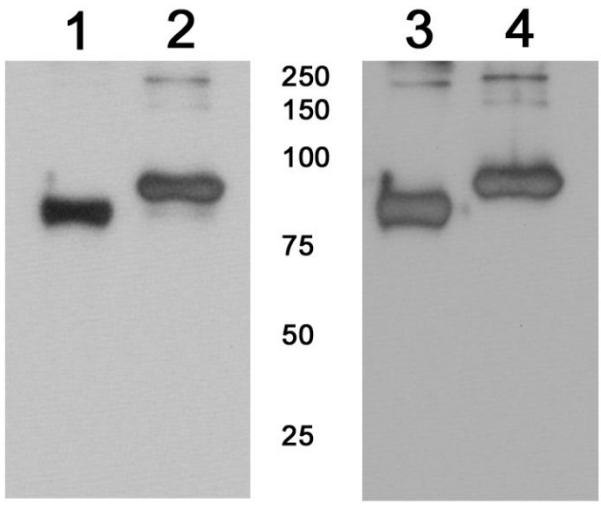

Figure 1. Recombinant HA protein from H1N1 A/California/04/2009 virus is expressed as the hemagglutinin precursor (HA0). Western blot analysis of SDS-PAGE separated sHA (Lane 1 and 3) and sHA.GCN4pII (Lane 2 and 4) using polyclonal sera (Lane 1 and 2) or anti-histidine (Lane 3 and 4) tag monoclonal antibody (primary antibody). Protein bands were developed using goat anti-mouse HRP (secondary antibody) and ECL Plus (GE Healthcare).

Glycan arrays represent a new approach to measuring receptor binding by glycan binding proteins, such as influenza hemagglutinin. These arrays contain printed glycans with known compositions and sialic acid linkages and have been used extensively to investigate influenza virus receptor usage. Therefore, we used glycan arrays to determine the receptor binding specificity of the Cal/04 recombinant trimeric sHA. The recombinant trimeric HA bound to α(2-6) sialic acid containing-glycans with a high affinity over background (Figure 2B). These results indicate that the Cal/04 sHA.GCN4pII forms stable trimers and fold appropriately.

A/California/04/2009 sHA.GCN4 induces increased titers of functional antibodies following sHA priming

To test the immunogenicity of modified Cal/04 soluble HA, female Balb/c mice were primed and boosted at 3 week intervals with 3 μg of recombinant protein (Table 1). On day 21 (Prime) and day 42 (Boost) after priming, mice were bled and sera tested for anti-Cal/04 antibodies by ELISA and HAI. On day 21 after boosting, Group 1 mice had 8-fold higher serum anti-Cal/04 IgG than Group 2 mice. Group 3 mice had comparable serum IgG compared to Group 1 mice while Group 4 mice had approximately 1.2 fold higher serum IgG than Group 1 mice (Figure 3A). Therefore, the preservation of the trimeric structure of the A/California/04/2009 soluble HA did not enhance immunogenicity.

The HAI test is used to measure antibodies which bind to the HA receptor binding domain, blocking binding of HA to sialic acid receptors. In general, an HAI titer of ≥40

Table 1. Vaccination groups

Group #	Prime Antigen	Boost Antigen
1	sHA	sHA
2	sHA.GCN4pII	sHA.GCN4pII
3	sHA	sHA.GCN4pII
4	sHA.GCN4pII	sHA
5	----	----

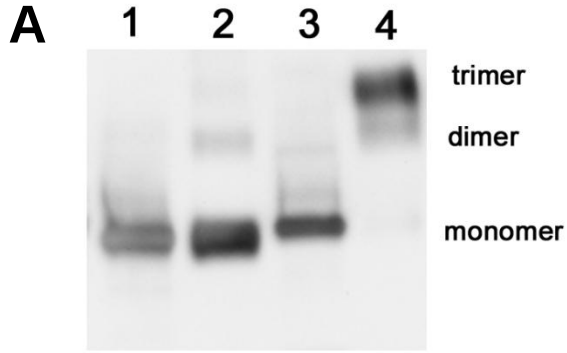

Figure 2. GCN4pII modification stabilizes the trimeric structure of A/California/04/2009 soluble HA. A. BS_3 crosslinking was performed as described in Materials and Methods. Lanes 1 and 2 – sHA, Lanes 3 and 4 – sHA.GCN4pII. Lane 1 and 3, no BS3, Lanes 2 and 4, 3 mM BS3. Western blot primary antibody, anti-histidine monoclonal antibody.

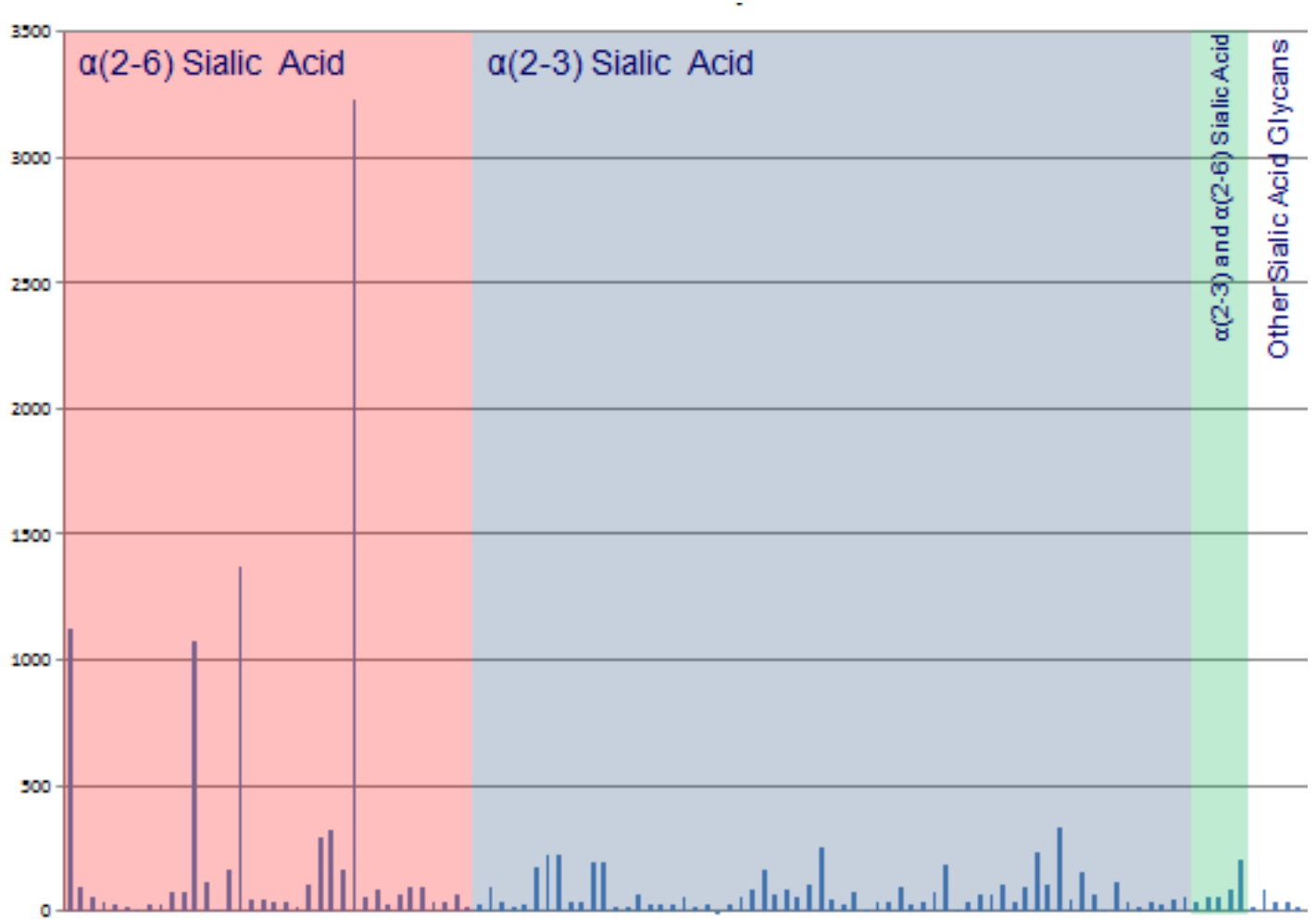

Figure 2. B. Receptor binding of Cal/04 sHA.GCN4pII by glycan array analysis. Recombinant protein (15 µg) was complexed with anti-His (rabbit) and anti-rabbit-Alexa488 antibodies at a ratio of 4:2:1 (HA protein:anti-His antibody:anti-rabbit-Alexa488).

is correlated with vaccine-induced protection in humans. Therefore, serum from vaccinated mice was tested for HAI titers. On day 21 after boosting, Group 3 mice had approximately 5.5-fold higher HAI titer than Group 4 and approximately 5-fold higher HAI titer than Group 1 mice. Group 2 mice had no detectable HAI titer (Figure 3B). These data suggest that the trimeric Cal/04 soluble HA is less immunogenic than the unmodified monomeric soluble HA. Boosting with the trimeric soluble HA following monomeric soluble HA priming induced greater HAI titers suggesting that trimeric protein presented epitopes present in the native trimeric protein.

Cal/04 sHA.GCN4pII induces protective immune responses following priming with sHA

To determine the efficacy of the recombinant H1N1 Cal/04 soluble HA proteins, mice were challenged intranasally with 5 x LD_{50} of mouse adapted A/California/04/2009. Body weights and survival were monitored for 14 days post-challenge. Following lethal challenge, all groups of mice lost body weight, though Group 3 mice were the most improved vaccinated group with approximately 10% body weight loss (Figure 4A). Priming with sHA and boosting with sHA.GCN4pII or sHA resulted in approximately 83% survival (5/6) while priming with sHA.GCN4pII and boosting with sHA resulted in 50% survival (3/6). Priming and boosting with sHA.GCN4pII induced only 17% protection (1/6) following lethal challenge (Figure 4B). These results suggest that priming with sHA then boosting with sHA.GCN4pII did not significantly enhance protection against lethal challenge. Interestingly, boosting with sHA did enhance induction of protective immune responses.

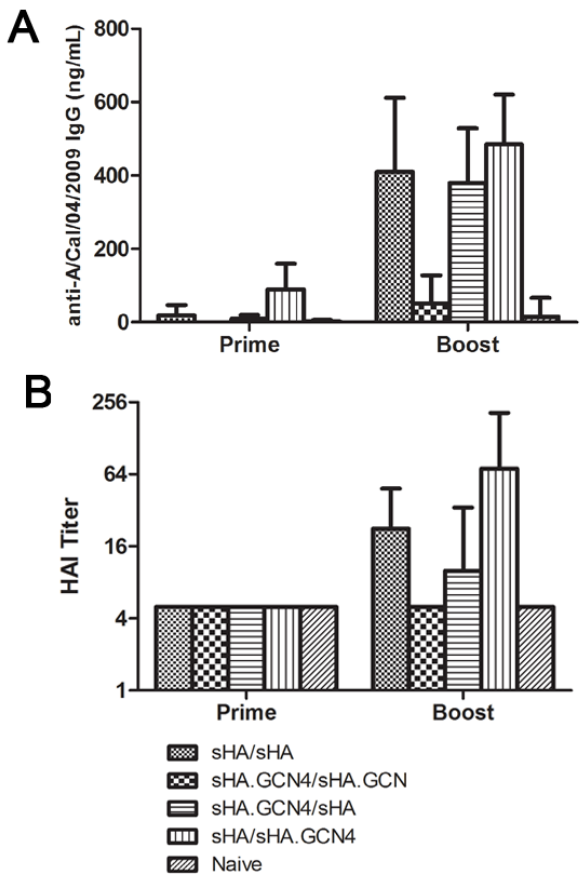

Figure 3. sHA induces more robust humoral responses compared to sHA.GCN4pII. A. Mice were primed and boosted 3 weeks later with 3 μg of sHA , sHA.GCN4pII or PBS. Blood was collected day 21 after priming (prime) and 21 days after boosting (boost). ELISA plates were coated with 4 μg/mL of A/California/04/2009 virus and antigen specific serum IgG ELISA was measured for prime and boost. HRP-conjugated goat anti-mouse IgG was used for detection. **B.** Hemagglutination inhibition test of prime and boost sera was performed as described in Materials and Methods using live MDCK grown A/California/04/2009. (n=6 per group)

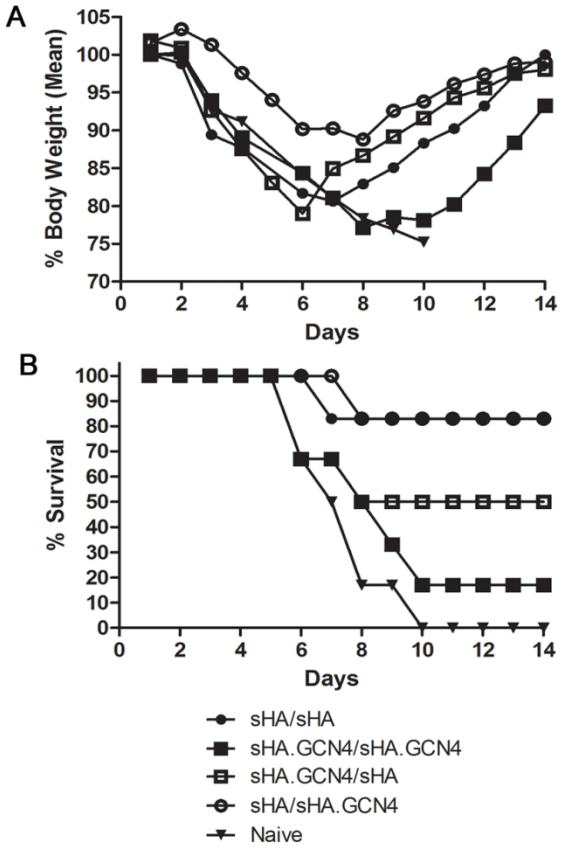

Figure 4.Vaccine-induced protective immunity. Mice primed and boosted were challenged with 5 xLD_{50} of mouse adapted H3N2 A/Aichi/2/68. **A, B**. Body weights and survival were followed for 14 days post challenge. (n=6 per group)

Discussion

One year from the start of the swine-origin H1N1 influenza virus in 2009, an estimated 61 million cases of H1N1 influenza and approximately 12,470 H1N1-related deaths in the United States[16]. The disadvantages of using traditional egg-based influenza vaccine production were illustrated by the delay in production of the monovalent H1N1 vaccine during the pandemic in 2009. We have previously demonstrated the increased immunogenicity of recombinant trimeric soluble HA derived from the H3N2 A/Aichi/2/68 influenza virus.

In the current study we sought to apply this strategy to the A/California/04/2009 SOIV by modifying the soluble HA at the C-terminus with the GCN4pII trimerization repeat. Our data suggest that the modified Cal/04 soluble HA formed stabilized trimers as determined by BS3 crosslinking and Western blot analysis. Furthermore, this recombinant protein could bind to sialic acid receptors suggesting that the receptor binding domain was folded properly. However in contrast to what we observed with the H3 subtype, mice primed and boosted with the monomeric sHA had protective immune responses while the trimeric sHA induced undetectable binding and functional antibodies or responses just above detection levels. When mice were primed with monomeric sHA and boosted with trimeric sHA we observed higher antibody and HAI titers with improved protection against lethal challenge.

The protection data we observed with the recombinant Cal/04 trimeric sHA are consistent with results obtained using a similar trimeric Cal/04 sHA construct in ferrets [17]. Compared to the trimeric H3 sHA that we have previously characterized, the H1 sHA is less immunogenic even in the unmodified monomeric form. This observation supports data obtained in human safety trials using trivalent recombinant influenza HA vaccines produced in the baculovirus system which have also indicated that the H1 HA is less immunogenic than the H3 and influenza B components [18]. In addition, codon-optimized DNA vaccines encoding H1 and H3 hemagglutinin genes demonstrated that the H3 HA is more immunogenic than the H1 HA when the transmembrane domain has been removed [19]. The reduced immunogenicity we observed could also be demonstrated by the reduced stability of the Cal/04 HA compared to seasonal H1N1 viruses (Skoutzou I, et al., unpublished data). Thus, a different strategy for design of a soluble H1 HA antigen is required for an effective recombinant subunit vaccine.

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DISCUSSION AND CONCLUSIONS

In this dissertation study, we investigated influenza vaccine antigen design and skin-based vaccination with recombinant subunit influenza vaccines. The results of our studies allow a better understanding of how the oligomeric structure of recombinant influenza hemagglutinin affects the immunogenicity of subunit vaccines and points to a strategy applicable to all subtypes of influenza virus and other pathogens. In addition, our results demonstrate an effective method of skin-based immunization with recombinant influenza subunit vaccine. More specifically, our results show that 1) modification of influenza HA with the trimerization repeat GCN4pII generates a stabilized soluble HA trimer, 2) stabilization of the H3 HA with GCN4pII significantly enhances immunogenicity and vaccine efficacy, 3) the skin route of immunization with recombinant influenza HA subunit vaccine is effective at producing protective immune responses, and 4) in contrast to H3, the GCN4pII stabilized H1 trimer does not exhibit enhanced immunogenicity. This dissertation shows the feasibility of structure-based antigen design for improving influenza vaccines and the efficacy of skin-based recombinant subunit influenza vaccines.

GCN4pII repeat forms stable trimers of H3 influenza sHA

Influenza A HA is a type I transmembrane glycoprotein which forms trimers in the membrane of virus particles. Because the HA is the most immunologically important component of influenza vaccines, recombinant influenza subunit vaccines have focused on production of HA in bacterial, mammalian, plant, and baculovirus expression systems.

Studies investigating the oligomeric structure of soluble HA using recombinant baculovirus have demonstrated that the H3 A/Victoria/3/75 is expressed as a mixture of trimers, dimers, and monomers [1,2].

To investigate the effect of stabilizing soluble trimeric influenza HA on immunogenicity, we fused GCN4pII, a trimerization repeat derived from the *Saccharomyces cerevisiae* transcription factor GCN4. The H3 HA derived from A/Aichi/2/68 was selected for this study due to structural data and availability of conformation dependent monoclonal antibodies. Our results clearly demonstrated that modification of the soluble HA with GCN4pII maintained the native trimeric structure, consistent with the oligomeric structure of the GCN4pII coiled-coil. This modified soluble HA was able to agglutinate chicken red blood cells by binding to sialic acid receptors. Other studies have shown that modification of H5 soluble HA with GCN4pII or a foldon sequence, a trimerization repeat derived from T4 fribitin, also results in generation of trimers [3,4]. However, our data provides the first evidence for the presence of trimer specific epitopes in the stabilized trimeric soluble H3 HA. On the other hand, the unmodified soluble H3 HA presented low-pH associated epitopes. Thus, stabilization of the HA trimer preserves conformational epitopes and masks the epitopes associated with the low-pH conformation.

Stabilization of trimeric H3 sHA enhances immunogenicity and vaccine efficacy

Other studies using recombinant influenza soluble protein vaccines have used HA from H1 [5,6,7], H3 [2,5,6,8,9], H5 [3,10,11], and H7 [11] subtypes in addition to influenza B

hemagglutinin [5,6] as a vaccine antigen. Vanlandschoot et al demonstrated that the monomeric fraction of A/Victoria/3/75 (H3N2) soluble HA was not immunogenic in mice resulting in low binding antibody titers and undetectable functional antibody titers[2]. More recent studies have recapitulated these results using stabilized trimeric recombinant H5 soluble HA vaccines, although these studies used adjuvants to study the immunogenicity in poultry or mice [3,4].

Our structural data led us to hypothesize that the trimeric protein would present native conformational epitopes, while the unmodified HA would skew the immune response to the "silent face" of the low-pH conformation. To investigate the immunogenicity of the unmodified and trimeric recombinant A/Aichi/2/68 soluble HA, we vaccinated mice with a low dose of antigen and determined binding and functional antibodies and protective immune responses. Our results demonstrate that the stabilization of the trimeric structure of the H3 soluble HA significantly enhances the immunogenicity even after a single low-dose vaccination, although we cannot rule out any adjuvant effect caused by fusion of GCN4pII with the H3 soluble HA. These results support the conclusion that for recombinant H3 HA vaccines the native trimeric structure must be maintained in order to present immunologically relevant epitopes for protection. The ineffectiveness of the unmodified soluble H3 HA vaccine could relate to the presentation of the low-pH epitopes which is masked by the native HA structure and is only presented during virus entry.

Skin-based immunization with recombinant trimeric influenza HA subunit vaccine induces protective immune responses

Recent investigations support a skin-based influenza vaccine by demonstrating that the intradermal administration of trivalent inactivated influenza vaccine produced equivalent responses to vaccine administered intramuscularly at a higher dose [12]. However, needle-based intradermal immunization is problematic and more often results in inefficient vaccine delivery and pain for patients receiving the vaccine [13]. Others groups have investigated alternative needle-free skin-based influenza vaccinations [14,15,16,17,18,19]. Our lab has demonstrated the successful use of both coated and dissolving microneedles delivering influenza vaccines using virus-like particles or inactivated virus antigens into the skin [20,21,22]. However, little information is available on using this approach to deliver an influenza protein subunit vaccine.

To investigate the efficacy of a recombinant influenza subunit vaccine delivered to the skin by coated microneedles, we immunized mice with a low dose of antigen and determined the antibody and cellular response and whether the vaccine induced a protective immune response. This work represents the first use of a skin-based recombinant influenza subunit vaccine. We clearly demonstrate that the trimeric soluble H3 HA was able to induce improved antibody and cellular immune responses, which resulted in complete protection against lethal challenge, compared to the unmodified soluble HA. Also, our results suggest that microneedle immunization with a recombinant influenza subunit vaccine induces improved immune responses compared to the

traditional needle-based vaccination route. Thus, our data show that the skin represents a potent vaccination site for influenza subunit vaccines.

Monomeric H1 sHA is more immunogenic than trimeric sHA

In June 2009, the World Health Organization declared the start of the 2009 H1N1 pandemic [23]. Currently, influenza vaccine is produced in eggs which require the generation of high-yield egg growth reassortant viruses and a supply of embryonated chicken eggs, and has potential safety concerns in individuals with egg allergies [6,7,9,24]. In response to the pandemic, monovalent H1N1 vaccines were distributed in the United States; however, due to lower than expected production levels distribution was delayed approximately 4 months after declaration of the pandemic. Studies investigating the efficacy of the monovalent H1N1 vaccine indicate that the vaccine is immunogenic and induced protective HAI titers in both young and elderly humans [25].

Our results clearly demonstrate that the GCN4pII trimerization repeat also stabilizes the trimeric structure of HA derived from the swine-origin H1N1 influenza virus. Unlike the unmodified H3 soluble HA, the unmodified H1 soluble HA formed monomers with no higher order oligomers. This oligomerization pattern for the H1 HA is also true for modified soluble HA derived from the H1N1 virus A/Puerto Rico/8/38 (data not shown). Using a receptor binding assay, we demonstrated that the recombinant trimeric H1 soluble HA was able to bind influenza virus receptors suggesting that the HA was properly folded.

The structural results we observed led us to hypothesize that the trimeric soluble H1 HA would present the native trimeric epitopes and shield the "silent face" presented by the monomeric soluble HA. Surprisingly, our data indicated that the monomeric soluble H1 HA induced increased antibody titers compared to the trimeric soluble H1 HA. Thus, despite presenting native trimeric epitopes the trimeric soluble H1 HA was less immunogenic than the monomeric soluble H1 HA. This data agrees with other studies that the recombinant H1 HA is less immunogenic in comparison to the H3 and influenza B components of trivalent recombinant vaccine [26]. Other recent studies have also investigated the effect of GCN4pII modified soluble HA from the A/California/04/2009 virus and found that an immune response can only be induced with the inclusion of an adjuvant with the recombinant HA protein [27]. Therefore, other factors must play a role in structure and immunogenicity of soluble H1 HA that do not affect the immunogenicity of soluble H3 HA.

Implications for structure based vaccine design

Stabilization of oligomeric proteins with immunologic importance derived from viral pathogens represents an important strategy in the development of structure-based antigen design. The work presented here demonstrates the feasibility of this strategy with influenza HA derived from the H3 and H1 subtypes. Using this method of antigen design and vaccine production, an immunogenic recombinant protein could be produced with relative ease in response to future pandemic influenza viruses resulting from antigenic shift or reduced vaccine coverage for circulating seasonal influenza viruses resulting from antigenic drift. Furthermore, our data strongly suggests that the structure of

recombinant HA proteins be determined before implementing as reagents for diagnostics or influenza subunit vaccines.

Stabilizing the oligomeric structure of other influenza proteins is also possible using the system we have employed in our studies. Influenza neuraminidase (NA), the other major glycoprotein present in the virus, exists in its native form as a tetramer. The antigenic structure of influenza NA has already been determined and numerous studies have suggested that only the tetrameric NA is immunogenic. By mutating the GCN4 repeat we can generate the tetramerization repeat GCN4pLI which could be used to stabilize tetramers of recombinant soluble influenza NA. This strategy was recently used with the swine origin pandemic influenza virus A/California/04/2009 [27]. The extracellular domain of the M2 protein (M2e) has been proposed as a target for a universal influenza vaccine. The M2 protein exists as a tetramer in the viral membrane, thus the same stabilization strategy is used to present the native structure of this protein in a soluble form. Studies using this approach have demonstrated the induction of oligomer-specific antbodies and protective responses in lethally challenged mice [28].

The strategy we have employed here is not limited to influenza alone but could be applied to other pathogens with immunologically relevant oligomeric proteins such as Ebola GP, HIV-1 Env, or rabies G protein [29,30,31]. These stabilized viral glycoproteins could potentially function as recombinant subunit vaccines, reagents for conformational epitope analysis, or targets for development of monoclonal antibodies for diagnostic or therapeutic uses.

According to our results, the stabilized trimeric soluble H1 HA is less immunogenic than the monomeric H1 HA. Other studies have indicated that removal of the transmembrane domain from H1 HA adversely affects the immunogenicity which we could not overcome by fusion of the GCN4pII sequence to the membrane-proximal region. This observation suggests that a different approach should be taken in designing the trimeric soluble H1 HA. Due to the shared function of the HA2 chain between the H3 and H1 influenza HAs, swapping the membrane proximal region of the HA2 chain of the H3 HA with that of the H1 HA could stabilize this membrane-proximal region restoring the immunogenicity of the recombinant protein.

Implications for the delivery of vaccines to the skin

The skin represents an important target for vaccination and we have demonstrated the efficacy of the first skin-based influenza subunit vaccine. This work suggests that delivering protein antigens derived from diverse human pathogens such as *Bacillus anthracis*, HIV-1, and hepatitis B would be an efficient vaccination route for the induction of protective immune responses [32,33]. The microneedles utilized in this study have the advantage of being relatively painless, efficient at delivering vaccine into the skin, and have the potential for self-administration.

The prototypical APC for the skin is the Langerhans cell which is present in the epidermis. This population of cells expresses the C-type lectin DEC-205 (CD205) which has been proposed to recognize apoptotic and necrotic self-antigens [34]. Using antibodies or small chain variable fragments (scFv), antigen has been targeted to this

receptor resulting in antigen presentation on both MHC class I and class II [35,36]. When antigen is presented using this targeting strategy with DC maturation signals, antigen-specific immune responses were generated [37,38]. However, when antigen is presented using CD205-targeting without DC maturation signals, antigen-specific induction of regulatory T cell subsets, anergy, and clonal deletion occurs [39,40].

Our ability to deliver antigen consistently to skin-associated APC using the microneedles allows for targeting antigen to the CD205+ cells present in the skin. Adding DC maturation signals to the CD205-targeted antigen and delivery by microneedles to the skin would be expected to increase the activation of antigen-specific B and T cells resulting in a higher quality immune response. The antigen used for CD205-targeting could be any protein antigen from pathogenic models such as influenza or HIV or tumor models. Conversely, the induction of tolerance to self-antigens delivered by microneedles into the skin could be used for treatment of type I diabetes.

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