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William Arthur Langley, III Date

Studies on the Influenza A Virus Hemagglutinin and Immune Responses to Influenza Vaccine Vectors and RNA Viral Infections in Mice

By

William Arthur Langley, III
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

_____	_____
Rafi Ahmed Advisor	David Steinhauer Advisor
_____	_____
Richard Compans Committee Member	Joshy Jacob Committee Member
_____	_____
Jacqueline Katz Committee Member	Samuel Speck Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____
Date

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By

William Arthur Langley, III
B.S., University of North Carolina-Chapel Hill, 2001

Advisors: Rafi Ahmed, Ph.D.
and David Steinhauer, Ph.D.

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Abstract

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Influenza is a major health concern for humans and despite intensive efforts, strategies to prevent and/or cure it have not proven to be very successful. Viral mutants resistant to available drugs are common and antigenic changes in the glycoproteins make it necessary to frequently reformulate vaccine components. The work presented herein examines numerous aspects of influenza structure and immune responses to viruses in hopes of providing insights that may aid in the development of improved anti-viral strategies and vaccines. Drugs and vaccines that are able to effectively target conserved components of influenza are likely to prove effective at targeting multiple influenza subtypes. We have extended studies on one of these potential targets, the fusion peptide of the influenza HA, and demonstrated that single residue deletions in this domain prevent it from mediating fusion. These studies demonstrate length constraints for the fusion peptide and may help lead to the design of drugs that can target this conserved region of the virus. Further work focuses on the ability of candidate influenza vaccines containing disrupted NS1 proteins to elicit CTL responses. We show that, although these viruses are severely attenuated, they are able to generate potent memory CTLs and these are able to mediate viral clearance. Several properties make influenza attractive as a vaccine vector. We demonstrate that recombinant influenza vectors containing inserts of *Bacillus anthracis* are able to elicit antibody responses against these inserted domains and that these responses can be boosted by heterologous vectors to levels that are able to neutralize the anthrax toxin. However, CTLs recognizing multiple strains of influenza inhibit previously infected mice from developing antibody or CTL responses after immunization with influenza vectors, although, these cross-reactive cells are able to protect mice from challenges with heterosubtypic viruses. Finally, we look at the longevity of humoral responses to influenza, LCMV, and VSV in mice. We show that depletion of naïve and memory B cells by rituximab results in a decrease of virus-specific plasma cells. These data indicate that plasma cells are intrinsically long-lived, but that some re-seeding by memory B cells may be necessary to maintain their numbers long-term.

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Chapter 1: Introduction

Influenza background

Classification

Influenza A virus is a major health concern for humans, a fact highlighted by the current H1N1 pandemic. It is a member of the *Orthomyxoviridae* family of viruses, which consists of five genera, all of which contain segmented single-stranded negative sense RNA genomes. The five genera are the *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, and *Thogotovirus* [1]. A sixth genus, consisting of the previously unclassified Quaranfil virus, Johnston Atoll virus, and the Lake Chad virus has recently been proposed [2].

The *Thogotovirus* genus consists of three viruses, the Thogoto virus, the Dhori virus, and the Araguari virus. Members of this genus can consist of six or seven segments [3]. Thogoto virus was first isolated from ticks in Kenya and livestock have been shown to have high levels of antibodies against it [4]. Thogoto virus infections have been known to cause spontaneous abortions in sheep [5] and Dhori virus was reported to have caused encephalitis and viral illness after the accidental infection of five laboratory workers [6]. Araguari virus was isolated from an opossum and has not been reported in humans [7]. The only member of the genus *Isavirus*, the infectious salmon anemia virus, consists of eight segments [8]. It is known to infect fish [9] and is a major concern for salmon farmers, but has not been reported in humans [10].

In contrast to the *Thogotovirus* and *Isavirus* genera, the *Influenzavirus* genera are all known to circulate in humans and a high degree of homology exists between the 5' and 3' ends of these viruses indicating a probable common evolutionary ancestor [11]. Influenza C virus was first isolated in 1949 [12] and shown to consist of only seven segments [12-13]. It has been isolated from humans and pigs [14], but these viruses are of little clinical significance [15-16]. Influenza B virus consists of eight segments and can be widespread in some years. First isolated in 1940 [17], it is normally thought of as a pathogen of humans, but has also been isolated from seals [18]. Its hemagglutinin (HA) protein shares stretches of homology with the HA protein of influenza A viruses [19].

Of the *Orthomyxoviridae*, influenza A virus generally causes the most severe infections in humans and will be the focus of this dissertation. It consists of eight segments [20-21] encoding ten or eleven proteins, depending on the strain [22]. In addition to humans, influenza A virus has also been detected in domestic and wild fowl [23-27], dogs [28], horses [29], whales [30], pigs [31-33], seals [34], minks [35], and cats [36], among others. The pathogenesis and sustained transmission of the virus in these animals can vary greatly. Influenza was first demonstrated to be caused by a virus when a filterable agent isolated from pigs suffering from influenza was used to infect other pigs and recreate the illness. Although, it was also necessary to co-infect the pigs with *Haemophilus influenzae suis* bacteria in order to reproduce the clinical symptoms [37]. Smith, Andrewes, and Laidlaw at Mill Hill outside of London were the first to isolate

human influenza virus in 1933. They demonstrated that the virus could be infectious in ferrets and used them to maintain their stocks in the laboratory [38]. The virus was first plaque purified and maintained in MDCK cells in 1975, following the discovery that the addition of trypsin to culture media was necessary [39].

Genome description

The eight segments of the influenza genome can encode ten or eleven different proteins, depending on the strain. The polymerase shows RNA-dependent RNA polymerase activity and consists of one acidic (PA) and two basic subunits (PB1 and PB2) based on isoelectric focusing and electrophoresis [40]. These subunits are encoded by the three largest segments of the viral genome. PB1 and PB2 consist of 2341 nucleotides, while PA is 2233 nucleotides [41-43]. The PB1 subunit has been shown to have polymerase activity and is responsible for transcribing the anti-genomic copies of the genome (cRNA) and then transcribing these back into multiple copies of the genome [44-45]. It also contains nuclear localization signals [46] and mutations within it have been shown to inhibit transcriptional activity [44]. PB1-F2 is the most recently discovered influenza protein and is encoded by an alternate reading frame of the PB1 segment. It is a virulence factor, causing apoptosis of infected host cells, and is not found in all strains of influenza viruses [22].

The PB2 subunit is a cap-binding protein that plays a role in initiation of viral mRNA synthesis by recruiting capped RNA primers [47-48]. It had been

shown to have protease activity which would allow it to cleave the 7-methyl guanosine triphosphate caps from host mRNAs [49], however, more recently, it has been demonstrated that while PB2 is able to bind to the host mRNA caps, it is actually the PB1 subunit that cleaves them [50]. The PB2 subunit has also been shown to contain nuclear localization signals [51] and has been demonstrated to play a role in restricting the host range of influenza virus [52-53].

The PA function is not entirely clear, but it has been shown to play a role in both transcription and replication, where it may initiate strand synthesis without a primer being present [54-56]. It also plays a crucial role in bringing together the three polymerase subunits by binding to PB1 [57] and has been reported to have protease activity, although the significance of this is still unknown [58].

The nucleocapsid protein (NP) consists of 1565 nucleotides [59]. It encapsulates cRNAs and vRNAs and interacts with the polymerase proteins allowing transcription to occur by bringing them together with the template RNA [60-61]. Together with the polymerase proteins, it forms the ribonucleoprotein complex (RNP) and one RNP is associated with each segment of the viral genome [62].

The second smallest segment of the genome is 1027 nucleotides long and encodes two proteins [63]. One of which, the matrix protein, M1, is the most abundant viral protein [64]. It has a structural role in the virion and it also plays a fundamental role in virus assembly [65]. The other protein encoded by this segment, M2, is a small transmembrane protein derived from spliced mRNA. It is a homotetramer and has proton channel activity allowing virus disassembly to

occur during the initial stages of infection [66-68]. It is the least abundant surface protein with only 20-60 molecules per virion [69].

The smallest segment in the influenza genome is only 890 nucleotides long, but also encodes two proteins, non-structural protein 1 (NS1) and non-structural protein 2 (NS2) [70-72]. NS2 functions to mediate the export of newly synthesized RNPs from the nucleus and therefore, is also referred to as the nuclear export protein (NEP) [73]. It has also been shown to interact with the M1 protein and may be important in defining viral structure [73].

The NS1 protein of has multiple functions in infected cells. It has been demonstrated to control both viral RNA replication [74] and viral protein synthesis [75]. It is also a major virulence factor of the virus, as it is known to inhibit host interferon responses [76]. One way in which it is able to accomplish this is by inhibiting the IRF-3, NF-κB and c-Jun/ATF-2 transcription factors [77-78]. It probably does this by binding to RIG-I, preventing intracellular sensing of viral single-stranded RNA [79-80]. It can also inhibit 2'-5'-oligoadenylate synthetase (OAS) and serine/threonine protein kinase R (PKR) [81-82] and activate the PI3K pathway [83]. It is thus able to influence multiple aspects of innate and, therefore, adaptive immune responses. These functions make viruses containing deletion of or truncations within the NS1 protein viable options for constructing attenuated influenza vaccines. This will be discussed in further detail below and is the focus of Chapter 3.

The NA is encoded by its own segment which is 1413 nucleotides long [84]. It forms a mushroom-like tetramer on the viral surface, where it is the

second most abundant glycoprotein, behind HA [85]. There are nine known, antigenically distinct, subtypes of this protein that, along with the HA protein, form the basis for classifying influenza subtypes [86]. It functions to mediate the release of mature virions from infected cells towards the end of the viral life cycle by cleaving the sialic acid residues that are interacting with the viral HA proteins [87-88]. Inhibition of this protein leads to virus clumping on the surface of host cells and is the target of the NA inhibitor drugs which will be discussed below [89].

The HA protein plays a major role in the viral life cycle and has historically been the most studied segment of the influenza genome. The HA of the A/Aichi/2/68 H3N2 subtype influenza virus is one of the most well-studied HA proteins and all subsequent discussions will refer to the HA from this influenza strain, unless noted otherwise. It is synthesized as a 550 amino acid monomer that folds into non-covalently linked homotrimers in the endoplasmic reticulum before being transported to cellular surfaces through the Golgi complex [90-91]. During this process, it is N-glycosylated at seven asparagines residues [91]. The precursor peptide, HA0, is proteolytically cleaved into the disulfide-linked units HA1 and HA2 by proteases found in the respiratory tract of hosts [92-94]. This cleavage is necessary for viral replication and will be discussed in more detail in the section below describing the viral life cycle.

There are sixteen antigenically distinct HA subtypes [27] and it is the major surface antigen to which neutralizing antibodies are produced [95]. It binds to sialic acid residues on the ends of glycans on host cell membranes enabling

the virus to enter host cells by clathrin-dependent receptor-mediated endocytosis [96-98]. Human influenza isolates show a preference for α2,6 linked sialic acids, while avian virus isolates typically show more affinity to glycans with α2,3 linkage [99-100].

This protein has also been demonstrated to be a major determinant of influenza virus pathogenicity. As mentioned above, in order for viral particles to be infective, the precursor molecule, HA0, must be cleaved into the HA1 and HA2 subunits [101-102]. This cleavage occurs at a site referred to as the cleavage loop and uncleaved HA proteins are still able to bind to host cells, but they are unable to mediate membrane fusion which is what makes the viral particles noninfectious [92-93]. The protease recognition motif (R-X-R/K-R) in the HA cleavage loop allows for cleavage by extracellular trypsin-like proteases and some highly pathogenic avian strains have been found to contain multiple basic amino acid insertions at this site allowing proteases that are ubiquitously expressed in many organs and tissues to access and cleave this loop. This can result in more virulent viruses that may spread systemically [103-107]. The absence of a nearby carbohydrate has also been associated with increased virulence in influenza viruses, presumably due to the increased accessibility of the cleavage loop for proteases [108-109]. In fact, the tropism of the neurovirulent A/WSN/33 influenza strain is increased to include neurons because of the loss of this glycosylation site [110].

The HA structure and function will be discussed in more detail as it relates to specific studies in this dissertation in subsequent sections.

Replication cycle

The RNA polymerase lacks proofreading resulting in replication errors at a rate of about one in 10^4 bases [111-112]. These errors result in many variants that are not viable, however, some of these mutations can prove to be advantageous to the virus. Amino acid substitutions in the HA and NA proteins are commonly observed and can allow the viruses to escape neutralization in previously immunized hosts [113]. This phenomenon is referred to as genetic drift and is responsible for yearly epidemics of influenza.

The segmented nature of the influenza virus genome allows reassortment of gene segments between different strains of the virus to take place in multiply infected hosts, referred to as genetic shift. Pigs have been shown to be susceptible to both avian and human influenza viruses and can serve as mixing vessels where this reassortment can occur [24, 114-115]. The reassortment of genetic segments can lead to the introduction of novel influenza subtypes to which a population does not have prior immunity and result in pandemic strains of the virus. This has most recently been seen with the triple reassortant 2009 H1N1 pandemic strain [116]. While pandemic viruses are usually associated with the introduction of novel HA and NA proteins, the PB1 protein, the PB2 protein [52-53], the NP protein [117-119] and the NS proteins [120] have also been demonstrated to play a role in restricting the host range of influenza virus [52-53]. These pandemic strains will be discussed in more detail in a subsequent section.

As mentioned above, the influenza HA plays an integral role in the replication of the virus. Upon cleavage of HA0 into HA1 and HA2, the N-terminus

of HA2 is released. This highly conserved hydrophobic domain is generally considered to consist of the first 23 amino acids of the newly formed chain and is referred to as the fusion peptide [121]. Following cleavage, the fusion peptides of each monomer insert into a cavity in the trimer interior, and the HA molecule is transformed into a metastable protein that can be subsequently triggered to induce membrane fusion upon acidification [122-123]. This metastable state is formed despite the physical rearrangement of only six residues at the C-terminus of the newly formed HA1 subunit and twelve residues of the fusion peptide [103]. Following receptor binding and internalization of the virus by receptor-mediated endocytosis (described above), the acidification of endosomes triggers the HA to undergo extensive structural rearrangements, transforming from the metastable conformation into a highly thermostable form of the protein. As mentioned above, uncleaved HA proteins are still able to bind to host cells, but they are unable to mediate membrane fusion making the viral particles noninfectious [92-93]. It is also known that the cleaved HA must be exposed to low pH environments before fusion can occur [122, 124]. This drop in pH is targeted by some classes of anti-influenza drugs and will be discussed in detail in the section below covering antivirals.

The neutral pH and the low pH conformation of HA following acidification have been well characterized and crystal structures of both conformations have been resolved [125-127]. The known conformational changes leading to membrane fusion are summarized below and depicted in Chapter 2, Figure 2. It is not known whether these changes occur in a specific order.

1) The membrane distal domains of the HA1 subunit detrimerize.

2) The fusion peptide is extruded out of the trimer interior.

3) The extended chain region that links the long and short helices of HA forms a helix and extends the central coiled coil in the N-terminal direction, directing the fusion peptide toward the target membrane.

4) A helix-to-loop transition within the long helix of the neutral pH HA near the fusion peptide reorients residues C-terminal to it by 180 degrees converting the molecule into a rod-like structure that brings the fusion peptide and the membrane anchor domain into close proximity with one another.

The mechanism by which acidification triggers conformational changes is not known, but mutations leading to increases in the pH at which fusion takes place have been reported at various regions throughout the trimer. One study found that mutations leading to increases in the pH at which fusion occur fell into two groups, those that resulted in the destabilization of the fusion peptide position within the neutral pH conformation and those that disturbed intersubunit contacts [128]. Mutations that destabilize the neutral pH conformation have also been reported by others [129-130] and give insight into how the conformational changes may be triggered. Other studies have found that the spacing of glycines within the fusion peptide may be important [131] and that shortened fusion peptides or those in which the N-terminal glycines have been substituted for by other amino acids are unable to mediate fusion [130]. Studies with double

mutants suggest that upon acidification, changes in this region may precede or dictate the events elsewhere in the molecule [132].

Based in part on structural features within the fusion peptide region, HA subtypes can be segregated into two clades. The kinetics of membrane fusion differ between these clades, and can be linked to specific residues. In all HAs, HA2 residues K51, D109, and D112 are completely conserved, whereas the residues at positions HA1 17, and HA2 106, and 111 are group-specific [27, 133]. These differences could play a role in the ecology of influenza viruses. Chapter 2 explores some of the structural constraints of the fusion peptide and offers further insight into its role in the fusion process.

After fusion of the viral and cellular membranes takes place, the viral contents are released into the host cell cytoplasm. The RNA segments, coated in NP, are imported into the nucleus by the importin α/importin β pathway [134-135]. Subsequently, replication of the viral genome and mRNA synthesis both occur in the nucleus of host cells [136-137]. Because the genomes of influenza viruses are negative sense RNA, they cannot be directly translated into protein, so mRNA must be transcribed from vRNA before proteins can be made. The synthesis of mRNA from vRNA involves the snatching of capped primers from nascent host cell mRNA by the polymerase complex, as described above. This “cap-snatching” also serves the purpose of blocking the translation of host cell mRNAs, thereby increasing viral output [138]. The viral mRNA is then exported to the cytoplasm of the cells where it can be translated. Newly synthesized NP and NS1 proteins migrate back to the nucleus where it is believed that the increased

concentration of free NP may trigger the shift from mRNA synthesis to replication of the viral genome [139-140].

The replication process involves synthesis of cRNA from a vRNA template, followed by synthesis of vRNA from the cRNA (vRNA→cRNA→vRNA). Newly synthesized vRNAs are encapsulated in NP within the nucleus (forming vRNPs) and exported to the cell surface for inclusion in new viral particles via interactions with the NS2 (NEP) and M1 proteins [73, 141]. The HA, NA, and M proteins are transported to the cell surface through the endoplasmic reticulum and the Golgi where they are integrated into the cell membrane [64, 69, 127, 142].

The vRNPs interact with the M1 protein [143], which buds outward at lipid rafts in the cell membrane forming new virions [144-146]. Interactions between M1 and the cytoplasmic domains of HA, NA, or M2 have been proposed as signals for budding [147], although M1 alone has been reported to be sufficient for virus-like particle formation [148]. It had been hypothesized that incorporation of vRNPs into new viruses was random [149], but recent studies support the model that the individual segments contain packaging signals that enable them to be packaged as a complete set [150-152]. Finally, the NA proteins cleave sialic acids from the HA receptors, releasing them from the host cell, starting the viral replication cycle again.

Epidemiology and pathogenesis

All 16 HA subtypes have been shown to circulate in waterfowl and these are considered to be the natural reservoir for the virus [23-27]. Most strains replicate in the respiratory tract and intestines of these birds without causing any overt symptoms [153-154]. In the United States, it has been estimated that somewhere between 5-20% of the population becomes infected with influenza each year leading to more than 200,000 hospitalizations and around 41,000 deaths [155-156]. Worldwide, influenza is responsible for three to five million cases of severe illness, and 250,000-500,000 deaths each year [157].

Influenza viruses are transmitted directly via aerosolized droplets or indirectly by contact with contaminated surfaces [158-159]. Symptoms in humans can include fever, coughing, sneezing, sore throat, runny or stuffy nose, achy body, headache, chills, or nausea and vomiting [156]. People 65 years and older, people with chronic medical conditions, pregnant women, and young children are the most likely to suffer life-threatening complications from influenza infections.

Influenza infections in healthy humans result in peak viremia around forty-eight hours post-infection and are usually cleared by around the eighth day [160-162]. Viral clearance is mediated by both the cellular and humoral branches of the immune system and influenza-specific T and B lymphocytes are detectable as early as two days after viral exposure [163-166]. Studies have found that mice having only $CD8^+$ T cells [167-168] or only B cells are still capable of clearing influenza infections. However, $CD4^+$ T cells alone were unable to clear influenza virus in the absence of B and $CD8^+$ T cells, despite proliferating to similar levels

compared to non-depleted mice. The study demonstrating this concludes that the main role of $CD4^+$ T cells in controlling influenza infections is the promotion of the T-dependent antibody response [169]. Even though CTL responses are capable of clearing influenza virus in the absence of B cells, B cell knockout mice have been shown to have a 50-100-fold greater susceptibility to influenza virus infection than do WT mice [168]. A majority of antibody in natural infections has been demonstrated to recognize the HA and NA proteins and these are the main immunogens in current influenza vaccines [170-171]. B cell responses against influenza and their role in influenza vaccines are both discussed in further detail in sections below.

Influenza-like illnesses have been recorded as far back as ancient Greece, and a survey of historical literature concluded that at least thirteen pandemics have probably occurred since the year 1500 [156], although, 1889 is the earliest pandemic for which concrete evidence exists. Retroactive studies on human serum suggest that this pandemic was caused by an H2N2 subtype virus and that an H3N8 subtype virus circulated shortly thereafter [172-174]. The 1918 “Spanish flu” was caused by an H1N1 virus and was the worst pandemic in recorded history, estimated to have killed around fifty million people worldwide, and is believed to have been a wholly avian virus that did not arise from reassortment [175-177]. The 1957 “Asian flu” was caused by an H2N2 subtype virus and was less severe [178]. It contained PB1, HA and NA segments from avian viruses and the other five segments were retained from the 1918 H1N1 strain [108, 179]. Likewise, the “Hong Kong flu” of 1968 was caused by an H3N2

subtype virus that acquired the HA and PB1 segments from an avian virus and the six other segments from the 1957 H2N2 strain [179-180]. The currently circulating 2009 strain is caused by an H1N1 subtype virus and is a triple reassortant, containing segments from avian, swine, and human influenza strains [181-182]. H1N1 and H3N2 subtype viruses are still circulating in humans today, while the H2N2 strain appears to have been displaced by the H3N2 strain's emergence in 1968 [183]. Whether the current pandemic strain will eventually displace the H3N2 strain remains to be seen.

As mentioned above, pigs have been postulated to be the mixing vessels where avian and human influenza viruses can undergo reassortment leading to the generation of progeny that can cause pandemics in man. It has further been postulated that pigs could act as hosts for avian viruses allowing them to gradually adapt to humans without any reassortment at all [184].

Although direct transmission from birds to humans is rare, a number of cases of human infection by avian influenza viruses have been documented. Prior to the 2009 H1N1 pandemic, it had been widely hypothesized that the next pandemic strain would be the H5N1 avian influenza. This strain was first recognized as a severe threat in 1997, when an outbreak was reported in humans that resulting in acute respiratory distress and a high fatality rate among infected individuals [185-187]. These were the first documented cases of avian influenza viruses being directly transmitted to humans. The WHO reports that as of December 30, 2009, there have been 467 confirmed cases worldwide resulting in 282 deaths [188]. This is a shocking 60% fatality rate, indicating that this virus

could be catastrophic if it mutates into a form that is able to more efficiently infect humans.

Other influenza subtypes have also been documented to have infected humans. H7N7 subtype viruses have been associated with conjunctivitis in humans as far back as 1977, although one death in a 2003 outbreak has been linked to it as well [189-193]. H9N2 has also been detected in humans, but only seems to cause relatively mild respiratory disease with no reported fatalities [194-195].

Influenza vaccines and antivirals:

Despite intensive efforts, current strategies aimed at preventing and/or curing influenza infections have not proven to be very successful. Viral mutants resistant to currently available drugs are common and antigenic changes in the glycoproteins make it necessary to reformulate the current vaccines almost annually. The history of influenza pandemics, the ongoing pandemic, and the potential for future pandemics clearly warrants further research into influenza vaccine and drug development.

Current vaccines

Vaccines are the most effective means of preventing influenza infections, and influenza vaccines have been developed and tested since the late 1930s

[196-197]. Antibodies have been demonstrated to be the major mediator of protection in immunocompetent hosts and current influenza vaccines are designed mainly to elicit these humoral responses [95, 198].

There are currently two types of influenza vaccines licensed for human use in the U.S., the inactivated influenza vaccine and the live attenuated, cold-adapted influenza vaccine (LAIV). Both of these vaccines are grown in eggs and are trivalent, containing H1N1, H3N2, and influenza B strains without any adjuvant. Influenza strains circulating throughout the world are continuously being monitored by scientists and each year the WHO meets and recommends the specific strains that should be included in that season's vaccine. In the U.S., the Vaccines and Related Biologicals Advisory Committee reviews the WHO recommendations and manufacturers working seeds are approved by Center for Biologics Evaluation & Research [199].

The inactivated vaccine is administered intramuscularly and is 70-90% effective in healthy individuals [170, 200]. During this year's pandemic, a monovalent H1N1 vaccine targeting the new strain was licensed by the FDA and is manufactured in an identical manner to the trivalent vaccine [201].

The first inactivated influenza vaccines used in humans consisted of whole viruses inactivated either by formalin or β-propiolactone [202-203]. Two other types of inactivated vaccines that have been used in humans are the split virion and the subunit. Both are derived by disrupting whole viruses with detergents, but subunit vaccines are then further purified, enriching for HA and NA proteins, getting rid of many of the byproducts of the production process that have been

associated with adverse reactions [204]. Whole virus and split virion vaccines were shown in the late 1960s to be correlated with elevated reactogenicity among the vaccinated, leading to the increased use of subunit vaccines [204-206].

While inactivated vaccines do elicit strong humoral responses against the HA and NA proteins, they have been shown to be poor inducers of cytotoxic T lymphocytes (CTL) [95, 170]. It is believed by many that this lack of CTL response makes this vaccine ineffective at protecting against heterologous strains of influenza, especially when compared to the LAIV [207-209].

The LAIV has been available since 2003 [210] and contains the same three vaccine strains as the inactivated vaccine [203]. A version protecting against the pandemic H1N1 strain has also been made available and is manufactured and administered in the same fashion as the seasonal vaccine [201]. The vaccine is both cold adaptated (ca), able to grow at 25° C, and temperature sensitive (ts), unable to grow at 37° C, which correlates with reduced virulence in vaccinated individuals because of limited replication in the lower respiratory tract [211-212]. The viruses are manufactured by combining the six internal segments, containing the mutations responsible for both the ca and the ts phenotypes, from the A/Ann Arbor/6/60 influenza A strain or the B/Ann Arbor/1/66 influenza B strain with the HA and NA surface proteins from the strain against which vaccination is desired [213-214]. These are referred to as 6/2 cold reassortant vaccines and, although immunized individuals have been shown to

excrete virus, they are considered safe because the mutations have been shown to be genetically stable [211, 215-216].

A major advantage of this vaccine over the inactivated vaccine is that it is administered intranasally, so no needles are involved. It has also been shown to be better at stimulating CTL responses, including IFN-γ and TNF-α responses, better at generating mucosal IgA responses, and to have a better capacity to interact with and be presented by antigen presenting cells (APC) [170, 217-218]. The responses to this vaccine have been shown to confer at least some protection against heterologous influenza strains [207-209].

Novel vaccine approaches

The currently licensed vaccines are ineffective when it comes to protecting against newly introduced influenza strains and currently circulating strains that have undergone genetic drift. Vaccines that are able to elicit heterologous immunity to many influenza subtypes are desirable. Although the HA and NA proteins are highly variable, the internal proteins of influenza viruses, including the NP and polymerase genes, are much more conserved [219] and immune responses directed against these may provide immunity to heterologous strains of influenza [208]. Another problem with current vaccines is that highly pathogenic avian strains that are likely to cause pandemics do not grow to high titers in eggs, making vaccine production difficult using currently licensed methods. For these reasons, novel approaches to influenza vaccination have

been undertaken by numerous laboratories in order to improve both their efficacy and their production.

One experimental approach to producing improved influenza vaccines involves the generation of viruses with truncated or deleted NS1 proteins. The NS1 protein and its many functions were described above, but, one of its functions that is of particular interest when it comes to vaccine design is its ability to inhibit type I interferon responses in infected animals. Type I interferons (IFN-I) are produced by infected or activated cells during viral infections and are required for optimal priming of both $CD8^+$ and $CD4^+$ T cell responses [220-222]. This production of interferons has been shown to play a role in clearing influenza virus from infected hosts and T cells lacking the IFN-I receptor show reduced expansion and memory formation after infections [223-227].

Influenza viruses lacking NS1 function are highly attenuated and induce robust IFN-I responses, yet they are still able to be grown to high titers in cells and eggs [76-77]. These viruses have also been shown to be potent activators of dendritic cells [228]. In fact, robust humoral and cellular immune responses resulting in protection against WT homologous strains has been seen after vaccination with these viruses in mice [77, 229-230], pigs [231], horses [232], and macaques [233]. In the macaque study, it was also demonstrated that the immune responses generated by these live-attenuated strains were more protective than those elicited by inactivated vaccines.

Importantly, the immune responses generated by these viruses have been shown to reduce replication of heterologous influenza strains in vaccinated

animals, indicating the induction of broad cross-protective responses [230-231]. To date, no human data have been generated for these viruses. Chapter 3 further explores the immune responses that are generated during infection with influenza viruses containing either full or truncated versions of the NS1 protein, focusing in depth on the quality of effector and memory $CD8^+$ T cell responses that these attenuated viruses are able to elicit.

One drawback of these vaccines is the potential for them to undergo reassortment with WT viruses. This has been addressed by incorporating further attenuating mutations into both the HA and the PB2 segments in these viruses, thereby making reassortment to a virulent subtype more unlikely [234].

DNA vaccines are also an enticing alternative to the currently influenza licensed vaccines. They are noninfectious, nonreplicating, stable, and easily produced, relatively, in mass quantities [235]. When injected into animals they are transcribed and translated, producing proteins that can be presented by the major histocompatibility complex [236-237]. They are also easily manipulated and have been demonstrated to elicit cellular and humoral immune responses against a wide variety of pathogens [238-242].

Currently, DNA vaccines have only been licensed for use in fish and horses [243], but human trials analyzing their safety and efficacy as components of HIV vaccines are underway [244-245]. Like the LAIV, DNA vaccines are able to elicit CTL responses and could potentially protect against heterolosubtypic influenza strains [246]. Influenza DNA vaccines have been demonstrated to

confer protection against drifted influenza strains in ferrets [247-248] and both drifted and heterosubtypic strains in murine models [236, 249].

However, several disadvantages exist with DNA vaccines. They must be administered by either intramuscular injection or a gene gun and there are questions about whether or not the DNA is able to incorporate into human genomes and the potential consequences this might have. DNA immunizations have also been shown to induce relatively low immune responses compared to some other vaccination methods [250].

Recombinant proteins have been proven effective in protecting against human papillomavirus (HPV) [251] and this approach also holds promise for future influenza vaccines. Recombinant proteins can be produced by the baculovirus expression system in insect cells and, because these are eukaryotic cells, the recombinant proteins have similar glycosylation patterns to those produced by natural influenza infections [252]. This process provides a safe, efficient way to produce highly purified proteins without the use of eggs and these proteins are non-replicating and non-infectious.

Immunization with recombinant HA proteins was shown to protect against homosubtypic influenza challenges in mice [253] and chickens [254] and a recombinant M2 protein immunization was shown to protect against both homolosubtypic and heterolosubtypic challenges in mice [255]. Human trials with recombinant influenza proteins have found them to be safe and immunogenic [256-257], able to generate antibodies at titers that are considered to be protective in humans [258-259].

A similar approach to recombinant protein immunizations is vaccination with virus-like particles (VLPs). These are produced in a similar fashion to the recombinant proteins in insect cells, but they also include the influenza matrix protein which results in budding particles containing the other recombinant proteins on their surface [260]. VLPs have the advantage of expressing recombinant proteins in their native conformations on a viral surface and of being able to be administered IN. They have been demonstrated to activate CTLs [261], elicit mucosal antibodies [262], and to be protective against homologous [263] and heterologous [262, 264] influenza strains in mice. VLPs are licensed in the U.S. for vaccines against hepatitis B and HPV and human trials of VLPs as influenza vaccines are currently underway.

In addition to novel types of influenza vaccines, increasing the immunogenicity of current and future vaccines is desirable and a number of methods are being tested to accomplish this goal, including the addition of adjuvants and the administration of the vaccines by more immunogenic routes. Clinical trials in humans have demonstrated that influenza vaccines for subtypes to which humans have not been previously exposed are often poorly immunogenic [259]. For this reason, adjuvants that can safely increase the response to these vaccines are desired. Aluminum salts are the only adjuvant that is currently licensed for use in humans in the U.S., however, studies with this adjuvant in influenza vaccines administered to humans have yielded mixed results as to its effectiveness [265-266]. MF59 is an oil in water emulsion that is licensed for use in influenza vaccines in Europe [267] and has not been

correlated with adverse events in humans that have received vaccines containing it [268]. Clinical trials in the U.S. have shown it to increase antibody responses among those vaccinated with it in trials involving both H5N3 [269] and H9N2 subtype viruses [270]. Although, the latter study also reported more discomfort at the injection site in people that received the adjuvant than in those that received non-adjuvanted vaccine. Other adjuvants that have been tried in humans, including the saponin QS21, have not been proven effective at boosting immune responses to vaccines [271]. On the other hand, the addition of granulocyte macrophage colony stimulating factor to VLPs was able to increase immune responses to influenza vaccines in mice [272]. However, the concerns with using adjuvants in human vaccines are great and, even if proven safe and effective, the perception of them may deter some people from getting vaccinated.

In addition to increasing immunogenicity of influenza vaccines by the use of adjuvants, other routes and means of administering the vaccines may prove to be more effective. One example of this is transcutaneous administration. This may prove to be a better route of administration because of the numerous APCs that reside in the skin [273]. One way to immunize by this route is through the use of microneedle vaccines. These consist of patches containing microscopic structures coated in antigen that are designed to pierce the skin and release the antigen to the APCs that are present [274-275]. This route of vaccination has been shown to require less influenza antigen to elicit the same level of response compared to IM vaccinations [276] and H1N1 coated microneedles have been demonstrated to be more protective than traditional IM vaccinations in mice

[277]. In human trials, these patches were also found to be more immunogenic than IM injections [278]. A few of the advantages of this type of vaccine include ease of administration, it could be applied in the absence of trained healthcare workers, and the fact that it has been reported to be painless [275, 279-280].

Current antivirals

Antivirals can offer an alternative to vaccination or, more likely, be utilized as treatment after an infection occurs. Currently, there are two classes of drugs available for the prevention and treatment of influenza, the adamantanes and the NA inhibitors.

The adamantane derivatives amantadine and rimantadine have been shown to inhibit influenza replication in infected cells [281-282]. They work by targeting the M2 proton channel and inhibiting the acidification of the virus interior in endosomes and thus, viral uncoating [283]. Resistance to amantadine is common among influenza viruses, including H5N1 and H1N1 subtypes, and can usually be traced to mutations in the M2 protein, although mutations in the HA allowing the pH-specific conformational change to occur at elevated pH levels have also been reported [128, 284-286]. In fact, resistance to amantadine and rimantadine has become so common that the U.S. Advisory Committee on Immunization Practices has recommended that neither be used for the treatment or chemoprophylaxis of influenza A in the United States [287].

The second class of influenza antivirals is the NA inhibitors, which include oseltamivir (Tamiflu) and zanamivir (Relenza). As mentioned above, these drugs

act on the NA protein of the virus and inhibit the budding viruses from being released from cellular surfaces [288-289]. They are able to reduce the severity of infections, but need to be administered early on to be most effective [89, 290-291]. Drug resistant mutants are less common than those seen with the M2 inhibitors, but have been reported [292-297]. Viral strains resistant to both M2 inhibitors and NA inhibitors were reported in immunocompromised patients that had received dual therapy [298].

Novel antivirals

Due to the emergence of influenza viruses resistant to the above classes of drugs, new drugs, new routes of administration of existing drugs, and new drug targets within the virus are being explored. Among these novel targets are the polymerase complex and the HA protein.

Polymerase inhibitors include the nucleoside T-705, which has broad activity with influenza A, B and C, as well as other RNA viruses [299-301]. The PB1 subunit of the polymerase has proven to be a popular target and drugs inhibiting its polymerase activity [302] as well as its cap-snatching endonuclease activity [303] have been developed.

The HA protein has historically been the most well-studied protein of the influenza virus. The first x-ray structure of the HA was reported in 1981 from the H3 subtype A/Hong Kong/1968 virus [127] and structures of many of the other HA subtypes, including the cleaved, uncleaved, and low pH conformations, have subsequently been solved [103, 125-126, 304-307]. The structural data, along

with other known properties of this protein should make it ideal for the development novel anti-influenza drugs and, in fact, several drugs are being, or have been, developed to act on this protein. These include the receptor analogues targeting the receptor binding site [308-310] and inhibitors of serine proteases [311-312].

Other drugs have been found to inhibit the conformational change that occurs during membrane fusion, although several only act on specific HA subtypes. One group of these is the benzoquinones and hydroquinones [313]. The most potent example of this class of drugs is *tert*-butyl hydroquinone (TBHQ). Although it was shown by viral infectivity assays to be a relatively weak inhibitor, it does lend credence to the belief that the HA can be stabilized in its neutral-conformation state [314]. A drug screen by the pharmaceutical company Wyeth-Ayerst Research found several drugs that were shown to bind near the fusion peptide and inhibit fusion [315]. Analysis of viral mutants resistant to another experimental drug, 180299, that showed inhibiting activity against influenza were analyzed and was similarly shown to bind the HA near the fusion peptide [316]. Finally, a compound isolated from broth containing fungi of the genus *Stachybotrys* was also shown to interfere with the low pH conformational change and a derivative of this drug demonstrated anti-influenza activity *in vivo* in mice [317-319].

The above studies suggest that a greater understanding of the roles that individual fusion peptide residues play in the initiation of fusion may aid in the development of new generations of anti-influenza drugs designed to inhibit

membrane fusion function. Chapter 2 focuses on the composition of the fusion peptide in influenza viruses and may add some insight into the rigidity and conservation seen in this particular stretch of amino acids.

Influenza virus vaccine vectors and anthrax

Influenza vectors

Viral vectors have been shown to make effective vaccines for a number of pathogens, including some for which vaccine development has otherwise proven to be problematic. Replicating viral vectors have been demonstrated to elicit both humoral and cellular immune responses that are greater in magnitude and longer lasting when compared to many non-replicating vaccines [320-323]. One likely reason for increased B cell responses is the ability of viral vectors to display the antigens in their native state on viral surfaces where they can interact with B cell receptors. The ability of these live vectors to replicate in the cytoplasm of host antigen-presenting cells (APCs) allows them to be presented via major histocompatibility complex (MHC) class I molecules and probably leads to the increases seen with $CD8^+$ T cell responses [324].

Numerous viruses are currently being developed for use as vaccine vectors. These include adenovirus [325], canarypox virus [326], rhabdovirus [327], measles virus [328], and modified vaccinia virus Ankara [329], to name just a few. A number of factors must be taken into account when choosing a virus

vector for vaccine purposes. Viral vectors must be safe, immunogenic, and genetically stable. They should also be efficiently targeted to the appropriate tissue to generate responses at the correct locations, whether directed by vector tropism or the route of inoculation by which it is administered. Another major factor that must be considered is preexisting immunity to the vector itself. Preexisting immunity is likely to render these vectors ineffective or at least less effective than those to which no prior immunity exists.

Influenza virus has many properties that make it a viable candidate for use as a viral vector. Influenza vaccines have an extensive record of safety and effectiveness in the human population and are manufactured in both live and inactivated forms annually [165-166, 330]. Influenza virus does not form a DNA intermediate during its replication cycle and is not able to integrate into the host's chromosomes. Exposure to live influenza viruses, either through infection or vaccination with the LAIV, elicits robust humoral and cellular immune responses in hosts in as little as 2-6 days [165, 170-171]. In addition, extensive knowledge of both the structure and function of the hemagglutinin (HA) glycoprotein exists allowing for manipulation of this protein [121, 127, 331-332]. Furthermore, a reverse genetics system has been developed for influenza which facilitates the manipulation of individual viral segments and allows for the generation of recombinant influenza strains [333]. The reverse genetics systems consist of DNA plasmids containing cDNA from influenza flanked by RNA I and II promoters. The original method for generating recombinant influenza viruses, developed in 1999, required transfecting cells with 17 plasmids [333]. More

recently, however, methods using only eight plasmids [334] or even a single plasmid have been developed [335]. These are more feasible for use in cell lines that may be used in manufacturing vaccines for humans. Finally, the HA protein has been reported to have an adjuvant effect when administered as a component of virus-like particles containing epitopes derived from simian immunodeficiency viruses [336].

Although influenza infections are common in humans, infecting 5-20% of Americans each year [337], immunity to influenza viruses is largely mediated by antibodies against the HA and neuraminidase (NA) glycoproteins [171], both of which continually undergo genotypic and phenotypic changes in response to host selective pressures, making them highly divergent from year to year. There are sixteen antigenically distinct HA subtypes and nine distinct NA subtypes and immunity against one subtype does not confer immunity to the others [27]. Furthermore, as mentioned in a previous section, only three of the HA (H1, H2, and H3) and two of the NA (N1 and N2) subtypes have circulated extensively in humans during the past century, making it possible to develop influenza vectors expressing HA and NA subtypes to which preexisting immunity would be unlikely [27, 183]. The effects of preexisting immunity to homosubtypic and heterosubtypic influenza viruses on responses generated by influenza virus vectors is the focus of Chapter 5.

Li et al. were the first to demonstrate that influenza viruses could be effective vaccine vectors [338]. They inserted a 12-amino-acid peptide derived from the V3 loop of gp120 of HIV type 1 into the HA protein of recombinant

influenza viruses and were able to elicit T cells against this insert and generate neutralizing antibody responses in a cell culture assay as well. Since then, a number of papers have been published demonstrating that influenza vectors containing HIV epitopes are able to elicit T and/or B cell responses against these inserts in mice and that, importantly, these responses can be both mucosal and systemic when the virus is given IN [339-342]. Influenza vectors containing foreign epitopes have also been used in mice to generate protective $CD8^+$ T cell responses against LCMV [343] and protective B and T cell responses against malaria [344]. An entire protein from *Mycobacterium tuberculosis* was inserted into the NS gene segment of influenza and these recombinant viruses were able to protect both mice and guinea pigs against a mycobacterial challenge [345]. Chapters 4 and 5 explore the ability of influenza vectors to elicit immune responses against anthrax in mouse models.

Anthrax

Bacillus anthracis is a Gram-positive bacterium that is the causative agent of anthrax, primarily a pathogen of herbivores. It can cause cutaneous, gastrointestinal, or pulmonary anthrax (the most deadly form), depending on the site where infections occur [346]. It can be fatal in humans and was utilized in an act of bioterrorism in 2001 [347]. The potential for future attacks of a similar nature make research into effective vaccines against it an important undertaking.

The major virulence factor of anthrax has been shown to be the bacterial toxin [348], which is composed of three polypeptide subunits, the PA, the lethal

factor (LF), and the edema factor (EF) [349]. The LF and EF subunits derive their names after the effects they have in infected hosts. The PA alone is unable to cause pathology when injected into hosts, while PA and EF alone cause skin edema, but not death, and PA and LF alone is lethal, but does not cause edema [350].

The PA subunit is composed of 735 amino acids that fold into four structurally distinct domains (Chapter 4, Figure 1a) serving unique functions [351]. Domain 4 is 140 amino acids in length and is responsible for binding to the host cell receptors, tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) [351-354]. It is, therefore, referred to as the receptor binding domain, or RBD. After binding to host cells, the protein is endocytosed and domain 1 is cleaved by the protease furin leaving domain 1' [351]. Domain 1' is responsible for binding the LF and EF subunits, and is therefore referred to as the lethal factor and edema factor binding domain, or LEF [355]. Upon entering host cells, the PA forms ring-shaped heptamers via interactions between domain 3 on different PA proteins [356] and domain 2 forms pores in the endosomes through which the LF and EF can be excreted [357].

Protection against anthrax is mediated primarily by antibodies to the bacterial toxin. Ivins and Welkos demonstrated for the first time that the PA alone could confer protective immunity in a guinea pig model [358], although, the presence of the EF or LF was suggested to augment this protection [359]. Two of the subunits, domain 1' (LEF) and domain 4 (RBD), have been shown to be specifically targeted by neutralizing antibodies [349, 360].

Currently, only one FDA approved vaccine against anthrax is available in the U.S., the anthrax vaccine absorbed (AVA). The AVA is prepared by adsorbing filtered culture supernatant fluids from the *B. anthracis* V770-NP1-R strain onto aluminum hydroxide gel and then adding benzethonium chloride and formaldehyde as preservatives [359]. It requires five or six immunizations over an 18 month period, as well as yearly boosts, to retain its effectiveness [361]. The intramuscular (IM) route of inoculation does not induce strong mucosal antibody responses and mucosal surfaces are the sites where infection with BA is the most deadly and where initial contact would likely occur in the event of a terrorist attack [362-363]. Furthermore, lot-to-lot variability in composition and adverse reactions to the vaccine in humans have also been observed [364]. Clearly, the present vaccine is less than ideal and would be impractical should anthrax be released into the public.

Since the 2001 attacks, a number of strategies have been explored in efforts to improve the costs, logistics, and efficacy of anthrax vaccines. These experimental vaccines include purified PA with aluminum adjuvants [365], purified PA with novel adjuvants [366], live-attenuated BA strains [367], viral vectors [327], and plasmid based vaccines [368]. In addition to these novel vaccines, administration via IN [369] or transcutaneous [370] routes have also been tested.

Our laboratory demonstrated that chimeric HA proteins containing either the LEF domain (domain 1') or the RBD domain (domain 4) inserted at the amino terminus (Chapter 4, Figure 1B) are able to be expressed on viral surfaces, are

able to undergo the low pH conformational change, and are able to mediate fusion. Furthermore, recombinant influenza viruses containing these chimeric proteins are able to grow to titers comparable to WT viruses, and to elicit antibody responses specific for the inserted epitopes. The inserts are also stable, remaining intact for at least six passages on MDCK cells. It is also noteworthy that plasmids containing the chimeric HA proteins elicited larger responses against the anthrax domains than plasmids containing the domains alone [371].

In the previous study, the serum from immunized mice was not tested for neutralizing ability against the anthrax toxin. The work described in Chapter 4 further characterizes the responses generated by these chimeric influenza vectors and explores the ability of various regimens utilizing heterologous virus vectors for priming or boosting to generate toxin neutralizing antibodies in mice.

One of the suggested advantages of using influenza virus vectors is the fact that there are many antigenically distinct subtypes of the virus and that preexisting immunity to these vectors in humans could be overcome by constructing vectors of different subtypes. None of the published studies describing influenza vectors, however, have explored the effects of preexisting immunity to heterologous influenza virus subtypes on the responses that are able to be generated against them. This is a very important question to answer if influenza vectors should ever be seriously developed and is the focus of Chapter 5.

Maintenance of humoral immunity against viruses

Humoral immunity following viral infections is can be measured by secreted antibodies. These are a major component of the immune response against a large variety of pathogens and the ability to generate long-lasting protective humoral responses is a requisite for most vaccines, including those against influenza. Humoral immunity is mediated by B lymphocytes, which develop in the fetal liver prior to birth and in the bone marrow after birth. In the bone marrow, B cells undergo rearrangement of their immunoglobulin genes, and non-self-reactive cells expressing IgM migrate to the spleen where they continue maturing into naïve B cells expressing varying levels of IgM and IgD on their surfaces [372-374]. Approximately 1-2 x 10^7 immature B cells are produced daily in humans, but only about three percent of these successfully mature into naïve B cells [375-376]

Some antigens are able to elicit B cell responses that do not require T cell help and are referred to as T-independent antigens, however, the generation of effective B cell responses against most antigens requires T cell help, and it is these T-dependent responses that are the focus of the work presented here [377-378]. The T-dependent responses are initiated when antigen is encountered by dendritic cells near the site of entry which then present the antigen to cognate T and B cells in the secondary lymphoid organs [324, 379]. The size of the B cell response, as well as the longevity of B cell survival following encounter with antigen, can be greatly increased by co-stimulatory signals generated by activated $CD4^+$ T cells. Mice deficient in CD40 or CD28 have been demonstrated

to have impaired B cell formation and mice with $CD4^+$ T cells that do not express Slam-associated protein (SAP) have been shown to be impaired in the development of long-term humoral immunity [380-382]. Similarly, antigen complexed with complement has also been demonstrated to increase signaling by receptors on the B cell surfaces and enhance long-term humoral responses [383].

After being exposed to antigen and T cell help, B cells can either become short-lived plasma cells, with half-lives of 3-5 days, or migrate into B-cell follicles where they participate in germinal center (GC) reactions [384]. Chemokines, such as CXCL13, secreted by follicular dendritic cells (FDC) and follicular stromal cells are known to control this migration [385-386]. Once in GCs, B cells interact with FDCs and $CD4^+$ T cells and undergo affinity maturation and somatic hypermutation. After these GC reactions, the B cells go on to become either memory B cells or long-lived plasma cells [387-388]. The factors determining the fate of individual B cells is not completely understood, but is thought to be determined by the signals that B cells receive during this time. Cells receiving CD40L stimulation or coming into contact with B cell-specific activator protein have been shown to preferentially become memory B cells [389-390], while those exposed to OX40, CD23, or B-lymphocyte-induced maturation protein-1 (Blimp-1) are more likely to become plasma cells [391-393]. Other data suggests that B cells with higher antigen affinity may preferentially become plasma cells [394].

Most of the cells emerging from GCs have switched immunoglobulin isotypes from IgM and IgD primarily to IgG, and the plasma cells that emerge

generally secrete higher affinity antibodies compared to the short-lived plasma cells that do not migrate to GCs [395]. Although both memory B cells and plasma cells can be found in many sites, the major reservoir of memory B cells has been shown to be spleen and a majority of plasma cells have been demonstrated to reside in the bone marrow following LCMV infection in mice [396-397].

The humoral responses generated by most acute infections is known to be long-lived, even in the absence of re-exposure to the original antigen by natural occurrences or vaccination. This was first reported in 1846 by Peter Panum when he published the observation that adults who had survived a measles outbreak in the Faroe Islands were protected from the virus when a second outbreak occurred 65 years later, despite no documented cases of measles on these islands during the intervening years [398]. In 2008, antibodies recognizing the 1918 pandemic influenza strain were able to be derived from the memory B cells of people who survived the pandemic 90 years earlier [399]. Long-lived humoral immunity has also been reported in humans immunized against smallpox [400-402] and one study that followed antibody levels in humans for up to 26 years estimated the half-life of the humoral response to some viral antigens in humans to be more than 200 years [320]. Our laboratory has reported antibody levels following LCMV infection in mice to persist at relatively constant levels for the lifetime of the mice [397], however, the half-life of antibodies has been demonstrated to be less than three weeks [403-405], leading to the conclusion that plasma cells must somehow be maintained for long periods of time in hosts in the absence of re-exposure to antigen.

The mechanism behind long-term plasma cell maintenance is a debated topic, with several, non-exclusive, models having been proposed. Some evidence exists that memory B cells are needed to maintain plasma cell numbers over time, while other data suggest that plasma cells are long-lived and do not require replenishment by memory B cells. In 1997, Manz, Thiel, and Radbruch demonstrated through BrdU labeling that plasma cells can persist for at least 90 days with no significant turnover [406] and two, more recent studies, have reported no decline in plasma cell numbers following memory B cell depletion in NP immunized mice [407-408]

Evidence supporting the model that memory B cells are needed to maintain plasma cells long-term includes the fact that memory B cells, themselves, have been shown to be long-lived and to undergo homeostatic renewal [409-411]. However, there are two opposing models of how this may occur *in vivo.* One model postulates that antigen or antigen-antibody complexes are retained for long periods of time on FDCs and that memory B cell interaction with these complexes is a requirement for the memory B cells to be transformed into plasma cells [411-412]. This has been partially debunked by the finding that mice with B cells that were not able to secrete antibody and only able to express membrane bound IgM were still able to mount immune responses and secondary B cell responses comparable to WT mice [409], although, this does not rule out that non-complexed antigen could still persist long-term. Another model postulates that non-specific stimuli, including bystander T cell help and toll-like receptor signaling, can stimulate the memory B cells to become plasma cells in

the absence of persisting antigen. The fact that memory B cells can be non-specifically stimulated *in vitro* and transformed into plasma cells supports this model [408, 413]. Other data supporting this include a study reporting that anti-tetanus and anti-measles antibody levels correlated with memory B cell numbers in the peripheral blood of humans [413]. Our laboratory reported a decline in plasma cell numbers over time in LCMV immune mice that had been sub-lethally irradiated in order to deplete memory B cells and estimated the half-life of these plasma cells to be around 138 days [396]. However, the radiation may have also damaged the plasma cells and the bone marrow stromal cells.

Work presented in Chapter 6 takes a different approach to this question. We look at the longevity of plasma cells in mice infected with influenza, LCMV, or VSV following memory B cell depletion by antibody therapy. We utilize transgenic mice expressing the human CD20 protein [414]. While the exact function of this glycoprotein is unknown, it has been variously proposed to play a role in B-cell activation, proliferation, and calcium transport. A homologue of human CD20 has been described in mice, which also shows a similar pattern of expression [415]. It is known to be expressed on naïve and memory B cells, but is down-regulated on plasma cells, allowing for the specific depletion of naïve and memory B cells, but not plasma cells by rituximab treatment [414, 416-417]. Rituximab is a chimeric monoclonal anti-CD20 antibody that is used in the treatment of many lymphomas, leukemias, and some autoimmune disorders in humans. Rituximab destroys B cells via complement-dependent cytotoxicity, antibody-dependent effector cell-mediated cytotoxicity, and apoptosis when cross-linked by Fc-γ

receptor-expressing cells [418]. Our results not only offer insight into the longevity of plasma cells following viral infections in the absence of memory B cells, but may also have implications for human rituximab treatment.

The work presented in the following chapters explores both the biology of influenza viruses, as well as the immune response generated following immunization with influenza vaccine vectors and infections with RNA viruses in mice. The overall aim of these studies is to gain a better understanding of influenza virus structure and immune responses against influenza and other RNA viruses with the hope that these insights could potentially lead to improvement of anti-viral drugs, vaccines, and vaccine regimens. The findings presented here could prove beneficial in the development of new drugs and vaccines to combat influenza, the development of novel vectors that could be used for vaccinating against a wide array of pathogens, and the understanding of the mechanisms involved in long-term humoral immunity against viruses in general.

Chapter 2

Single residue deletions along the length of the influenza HA fusion peptide lead to inhibition of membrane fusion function

The work in this chapter was published in *Virology*.

Single residue deletions along the length of the influenza HA fusion peptide lead to inhibition of membrane fusion function

William A. Langley[a], Sudha Thoennes[a], Konrad C. Bradley[a], Summer E. Galloway[a], Ganesh R. Talekar[a], Sandra F. Cummings[a], Eva Varecková[b], Rupert J. Russell[c] and David A. Steinhauer[a,]

[a]Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322, USA

[b]Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic

[c]School of Biology, University of St. Andrews, Fife KY16 9ST, UK

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Abstract

A panel of eight single amino acid deletion mutants was generated within the first 24 residues of the fusion peptide domain of the of the hemagglutinin (HA) of A/Aichi/2/68 influenza A virus (H3N2 subtype). The mutant HAs were analyzed for folding, cell surface transport, cleavage activation, capacity to undergo acid-induced conformational changes, and membrane fusion activity. We found that the mutant ΔF24, at the C-terminal end of the fusion peptide, was expressed in a non-native conformation, whereas all other deletion mutants were transported to the cell surface and could be cleaved into HA1 and HA2 to activate membrane fusion potential. Furthermore, upon acidification these cleaved HAs were able to undergo the characteristic structural rearrangements that are required for fusion. Despite this, all mutants were inhibited for fusion activity based on two separate assays. The results indicate that the mutant fusion peptide domains associate with target membranes in a non-functional fashion, and suggest that structural features along the length of the fusion peptide are likely to be relevant for optimal membrane fusion activity.

Keywords: Influenza; Hemagglutinin; Membrane fusion; Fusion peptide

Introduction

All enveloped viruses encode glycoproteins that function to mediate fusion of their membranes with those of host cells during the initial stages of infection. Such viral fusion proteins (VFPs) are designed to respond to external stimuli at the appropriate time and place, to rearrange their molecular structures and initiate the fusion process. Membrane fusion can occur at the plasma membrane of host cells, often following the engagement of receptor molecules, or within subcellular compartments such as endosomes following internalization and acidification of the local environment ([Harrison, 2008], [Kielian and Rey, 2006], [Lamb and Jardetzky, 2007], [Weissenhorn et al., 2007] and [White et al., 2008]). Often, more than one viral protein is involved, and for some viruses multiple cellular components take part in the fusion process. However, for most enveloped viruses a single membrane anchored VFP serves as the principal protagonist for drawing the membranes into proximity with one another to initiate the fusion event. For these VFPs, the structural rearrangements that trigger fusion are generally coincident with the exposure of relatively hydrophobic domains to allow for their interaction with cellular target membranes. These “fusion peptide” domains are known to occur in one of three basic forms; (i) they can be present at the N-terminus of the fusion subunit of the protein, (ii) they can reside within the polypeptide chain as single loop internal fusion peptide domains, and (iii) they can exist as internal bipartite loops.

Most of the VFPs that have been characterized appear to share some of the mechanistic features for bringing viral and cellular membranes into proximity with

one another in the initial stages of the fusion process. However, VFPs are often segregated into three classes, based primarily on common structural considerations. Among these, representatives of Class I VFPs from members of the orthomyxoviridae, paramyxoviridae, and retroviridae families have been particularly well characterized. For these VFPs, polypeptide precursors associate to form trimers that are cleaved to generate N-terminal fusion peptides on their membrane-anchored subunit. During the fusion process, these membrane-anchored subunits undergo conformational changes to generate highly stable helical rod structures. As a consequence, the N-terminal fusion peptides are relocated to the same end of the molecule as the C-proximal viral membrane anchor domain. The molecular rearrangements and end-state structures for Class I VFPs are consistent with a model for membrane fusion, in which N-terminal fusion peptides are directed to interact with cellular target membranes, and formation of helical rod structures bring the viral and cellular membranes into proximity to one another as a prelude to the fusion process.

The influenza A virus hemagglutinin glycoprotein (HA) serves as the prototype for the Class I VFPs. It is synthesized as polypeptide chains of approximately 550 amino acids that associate non-covalently as homotrimers. The precursor form of the trimer (HA0) requires proteolytic cleavage of each monomer into the disulfide-linked subunits HA1 and HA2 in order to activate membrane fusion potential and virus infectivity ([Appleyard and Maber, 1974], [Klenk et al., 1975] and [Lazarowitz and Choppin, 1975]). Cleavage of HA0 not only liberates the HA2 N-terminal fusion peptide domain, but allows the HA to assume a neutral pH

conformation that can subsequently be triggered by acidification to undergo the conformational changes required for membrane fusion ([Bizebard et al., 1995], [Bullough et al., 1994] and [Chen et al., 1999]).

Although the fusion peptides of all Class I VFPs feature several large hydrophobic amino acids and include a number of glycine residues interspersed along their length, there is no direct sequence homology outside of the individual virus families. By contrast, the HA fusion peptide domains of all16 HA subtypes of influenza A viruses, as well as those of influenza B viruses, are highly homologous. This is particularly evident within the N-terminal 11 residues, and at positions with large hydrophobic or glycine residues (Fig. 1A). The reason for such conservation is likely due to constraints on the folding of the precursor HA0 structure, the capacity of this structure to be cleaved into a neutral pH structure that is responsive to acidification, and the requirement that fusion peptides be capable of adopting functional structures while interacting with target membranes to initiate the process of membrane fusion.

A number of studies on expressed proteins and mutant influenza viruses have focused on the membrane fusion properties of mutant HAs with changes in the fusion peptide domain ([Cross et al., 2001], [Daniels et al., 1985], [Gething et al., 1986], [Korte et al., 2001], [Lai and Tamm, 2007], [Lin et al., 1997], [Nobusawa et al., 1995], [Qiao et al., 1999], [Steinhauer et al., 1995] and [Yewdell et al., 1993]). Among the implications derived from these studies, the N-terminal glycine has been demonstrated as particularly important for fusion activity, and large

hydrophobic residues at positions 2, 3, 6, 9, and 10 are desirable for optimal function. The conserved tryptophan at HA2 position 14 also appears to play a fundamental role for fusion. The glycine at position 4 has been shown to tolerate changes in functional HAs, but the glycine at position 8 may be more critical, and numerous observations suggest that the spacing of glycine residues in fusion peptides may be important for fusion peptides to adopt functional structures.

The actual length of fusion peptide domains has not been addressed in great detail, particularly as components of intact HA molecules. Experiments on mutants with a deletion of either the N-terminal glycine or the leucine at HA2 position 2 were shown to be non-functional, using assays with synthetic peptide analogs or full length expressed HAs ([Steinhauer et al., 1995] and [Wharton et al., 1988]). Further evidence for length constraints in the terminal region of the influenza fusion peptide derive from studies on cleavage activation mutants selected for growth in the presence of the protease thermolysin (Orlich and Rott, 1994). This protease cleaves HA0 between the residues that normally constitute HA2 Gly1 and Leu2, which generates an HA2 subunit with leucine at the N-terminus and a fusion peptide that is truncated by one residue. However, mutants selected for growth in the presence of thermolysin were found to contain single residue insertions just downstream of the N-terminal leucine, which functioned to restore authentic fusion peptide length.

For the present study, we extended previous analyses on the length requirements for functional influenza HA fusion peptide sequences using

expressed HAs with single residue deletions that span this domain. We found that with one exception, the mutants fold into native HAs that were expressed on cell surfaces, cleaved into HA1 and HA2, and were able to undergo the acid-induced conformational changes requisite for fusion. However, all mutants were debilitated for membrane fusion function, suggesting either an overall length requirement for these fusion peptide sequences, a requirement for particular structural elements along the length of this domain, or constraints on the relative spacing of such elements within fusion peptide sequences.

Results and discussion

In previous studies, single residue deletion mutants of glycine at the HA2 N-terminus and leucine at position 2 have been characterized using fusion peptide analogs, expressed HAs, and laboratory-selected mutants ([Orlich and Rott, 1994], [Steinhauer et al., 1995] and [Wharton et al., 1988]). The results of these studies suggest that single residue deletions at Gly1 or Leu2 lead to inhibition of fusion activity. Here we assess the structural properties and membrane fusion activity of seven additional single amino acid deletions along the length of the fusion peptide using expressed HAs derived from the parental influenza virus A/Aichi/2/68, an H3 subtype. The nomenclature and positions of deletion mutants examined in this study are shown in Fig. 1B. Fig. 1 also includes the fusion peptide sequences of representatives of the 16 HA subtypes as a reference. The ΔL2 mutant has been characterized previously, but was included in the current

study as an additional control. The previous study showed that ΔL2 HA to fold properly and undergo conformational changes in response to acidic pH, but was negative for fusion activity (Steinhauer et al., 1995).

Fig. 2 shows three conformations that the HA assumes during the influenza life cycle, with fusion peptide residues highlighted in yellow. In the HA0 precursor structure, the residues that constitute the N-terminal portion of the fusion peptide form the membrane proximal half of the surface loop that is recognized by activating proteases (Chen et al., 1998). In the cleaved neutral pH HA, these residues are relocated into a cavity in the interior of the trimer, where they make contacts with ionizable residues that are thought to be significant for priming the HA for subsequent conformational changes in response to acidification ([Chen et al., 1998], [Thoennes et al., 2008] and [Wilson et al., 1981]). In the low pH structure that results following the conformational changes required for fusion, the fusion peptide is relocated to the end of the helical rod structure to facilitate interaction with the target membrane ([Bullough et al., 1994] and [Chen et al., 1999]). Therefore, residues that constitute the fusion peptide provide critical roles in the context of each of the major HA conformations. The rationale behind the choice of HA2 positions for the current deletion studies was to cover the length of the HA2 N-terminal region from the N-terminus through the conserved aromatic residue (Y or F) at position 24, as residue 25 is not conserved and is often a charged or polar amino acid (Fig. 1). Individual positions for deletion were selected on the basis of previous observations on natural isolates or laboratory mutants, which showed that residues with alternative side chains at these

positions were operative in fusion-positive HAs ([Cross et al., 2001], [Daniels et al., 1985], [Gething et al., 1986], [Korte et al., 2001], [Lai and Tamm, 2007], [Lin et al., 1997], [Nobusawa et al., 1995], [Qiao et al., 1999], [Steinhauer et al., 1995] and [Yewdell et al., 1993]). As such, it is unlikely that the side chains present in the WT fusion peptide at these positions are obligately required for folding of the native HA, or for fusion activity following the acid-induced conformational changes. Therefore, any consequences of their deletion are likely to result from either a decrease in the overall length of the fusion peptide, the disruption of critical motifs or structural elements within this domain, or the relative length or spacing of such features.

A. Fusion peptide sequences of representatives for each HA subtype

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
H1	G	L	F	G	A	I	A	G	F	I	E	G	G	W	T	G	M	I	D	G	W	Y	G	Y	H
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	V	-	-	-	-	-	Y	H
H3	-	-	-	-	-	-	-	-	-	-	-	N	-	-	E	-	-	-	-	-	-	-	-	F	R
H4	-	-	-	-	-	-	-	-	-	-	-	N	-	-	Q	-	L	-	-	-	-	-	-	F	R
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	V	-	-	-	-	-	Y	H
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	H
H7	-	-	-	-	-	-	-	-	-	-	-	N	-	-	E	-	L	V	-	-	-	-	-	F	R
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	F	H
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	L	V	A	-	-	-	-	F	Q
H10	-	-	-	-	-	-	-	-	-	-	-	N	-	-	E	-	-	V	-	-	-	-	-	F	R
H11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	L	-	N	-	-	-	-	F	Q
H12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	L	V	A	-	-	-	-	F	Q
H13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	L	-	N	-	-	-	-	F	Q
H14	-	-	-	-	-	-	-	-	-	-	-	N	-	-	Q	-	L	-	-	-	-	-	-	F	R
H15	-	-	-	-	-	-	-	-	-	-	-	N	-	-	E	-	L	-	-	-	-	-	-	F	R
H16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	L	-	N	-	-	-	-	F	Q

B. H3 subtype fusion peptide deletion mutants for this study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
WT	G	L	F	G	A	I	A	G	F	I	E	N	G	W	E	G	M	I	D	G	W	Y	G	F	R
ΔL2	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ΔG4	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ΔI6	-	-	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ΔF9	-	-	-	-	-	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ΔN12	-	-	-	-	-	-	-	-	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-
ΔE15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-
ΔD19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Δ	-	-	-	-	-	-
ΔF24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Δ	-

Fig. 1. A) HA Fusion peptide sequences from representatives of each of the 16 HA subtypes. H1: A/PR/8/34, H2: A/Japan/305/57, H3: A/Aichi/2/68, H4: A/Duck-/Czechoslovakia/56, H5: A/Chick/Pennsylvania/1370/83, H6: A/Shearwater-/Australia/1/72, H7: A/FPV/Rostock/34, H8: A/Turkey/Ontario/6118/68, H9: A/Turkey/Wisconsin/66, H10: A/Chicken/Germany/N/49, H11: A/Duck/England-/56, H12: A/Duck/Alberta/60/76, H13: A/Gull/Maryland/704/77, H14: A/Mallard-/Astrakhan/263/1982, H15: A/Duck/Australia-/341/1983, H16: A/Black-Headed Gull/Sweden/2/99. B) Nomenclature and fusion peptide sequences of the HA deletion mutants addressed in this study. The symbol Δ denotes the deletion of the amino acid residue at this position.

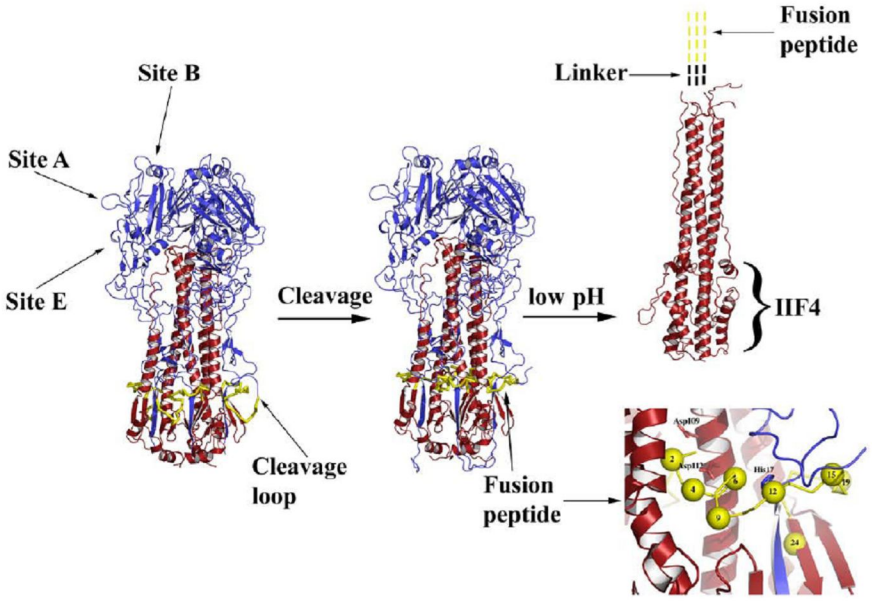

Fig. 2. Ribbon diagrams of the three conformations of the HA trimer. The HA0 precursor structure is shown on the left. Residues that will constitute the HA1 subunit following cleavage are depicted in blue, and the HA2 subunits in red. In all three structures, the residues that ultimately form the fusion peptide at the N-terminus of HA2 are shown in yellow. In the left panel, the cleavage loop of one of the HA0 monomers can be seen extending from the trimer surface and the cleavage site is indicated. The center panel shows that most of the HA structure remains unchanged following cleavage, the major structural consequence being the relocation of N-terminal HA2 “fusion peptide” residues from the bottom of the cleavage loop to the interior of the trimer. The right panel shows the structure of the HA2 trimer following the acid-induced conformational changes required for membrane fusion. In this thermostable rod-like structure, the fusion peptide and the viral transmembrane domain are located at the same end. Dashed lines indicate that the structure is unknown for both the fusion peptide and the 10-residue peptide that links it to HA2 residues of known structure. The antigenic sites A, B, and E are indicated for HA0 in the left panel, but these sites remain unchanged following proteolytic activation into the neutral pH cleaved structure shown in the center panel. The region bound by low pH HA-specific antibody IIF4 is shown in the right panel. The panel in the lower right corner depicts a magnified view of the fusion peptide region of cleaved neutral pH HA. The structural locations of HA2 residues that were deleted in this study are shown as yellow spheres. The positions of HA1 residue 17 and HA2 residues 109 and 112 are also identified.

Cell surface expression by ELISA

To analyze the effects of fusion peptide deletions on the structure and function of mutant HAs, recombinant vaccinia viruses were generated for their expression (Blasco and Moss, 1995). To assess HA folding and cell surface transport, ELISA assays were employed to determine antibody reactivity to recombinant vaccinia virus-infected HA-expressing HeLa cell monolayers. The panel of antibodies utilized included a rabbit polyclonal serum (α-X31) raised against purified trimeric HA ectodomains (BHA), and several monoclonal antibodies known to bind in conformation-specific fashion to the distinct antigenic sites A, B, or E of the HA membrane distal head domains ([Daniels et al., 1983] and [Wiley and Skehel, 1987]). The antibodies HC100 (site E), and HC159 (site A) bind to locations within the HA that remain structurally intact after the low pH conformational change occurs (Fig. 2). By contrast, the antibodies HC31 and HC68 bind to regions within site B at the membrane distal trimer interface, which are altered by de-trimerization of monomer head domains following acidification. The antibody reactivity to the mutant HA proteins is summarized in Table 1 and is represented as the percent binding compared to the WT HA as determined by OD_{450} ELISA readings. All of the mutants displayed significant reactivity with the rabbit polyclonal serum, indicating that they are capable of being transported to the cell surface. The results with the HC100 and HC159 monoclonal antibodies were consistent with this, as antibody reactivities for all mutant HAs were comparable to WT HA. However, the results with the neutral pH-specific antibodies HC31 and

HC68 demonstrated a significantly reduced reactivity for the ΔF24 mutant, suggesting an altered conformation for this mutant HA.

Table 1
Antibody reactivity of cell-surface HAs by ELISA (% of WT).

	α-X31	HC100	HC159	HC31	HC68
WT	100	100	100	100	100
ΔL2	94	95	99	99	104
ΔG4	108	105	114	97	78
ΔI6	100	94	103	94	104
ΔF9	101	104	103	86	91
ΔN12	102	104	122	96	105
ΔE15	108	110	120	109	102
ΔD19	96	103	112	100	100
ΔF24	86	85	108	40	43

Values represent averages derived from at least three separate experiments.

Analysis of HA0 cleavage of cell-surface HAs

The HA0 precursor protein must be cleaved into the HA1 and HA2 subunits to activate membrane fusion potential. Using the recombinant vaccinia virus system, the WT Aichi HA that we utilized in this study is expressed on the surfaces of infected cells in its uncleaved HA0 precursor form. Therefore, the capacity for cleavage activation of HA0 following exposure of HA-expressing cell monolayers to exogenous trypsin serves as an alternative assay for transport of mutant HAs to the plasma membrane. Furthermore, western blot analysis of the

migration patterns of cleavage products can be used to verify that mutant proteins are processed into HA1 and HA2 subunits of characteristic size, which is a requirement for fusion function. Fig. 3 shows a western blot analysis of trypsin cleavage of WT and mutant HAs expressed on the surface of recombinant vaccinia virus-infected cells. The results demonstrate that, with the exception of the ΔF24 HA, all mutant proteins were transported to the surface of infected cells and display HA1 and HA2 cleavage products that resemble WT HA. These results, in conjunction with the ELISA data described above, suggest that the ΔF24 HA is misfolded on the cell surface relative to WT HA and the other mutants. We have observed similar results with several mutant HAs in previous studies ([Li et al., 2008] and [Thoennes et al., 2008]), and our interpretation is that such mutants are expressed on the cell surface in a conformation that is distinct from that of WT HA, and that some features of this conformation are similar to those of the low pH HA structure. HA2 residue 24 is located in the membrane distal portion of an antiparallel β-sheet structure. In a previous study designed to examine the length of peptide segment linked to the fusion peptide, many of the pairwise deletions N-terminal to HA2 residue 24 and located in the antiparallel β-sheet structure of HA0 and neutral pH cleaved HA displayed folding and expression properties similar to ΔF24 HA (Li et al., 2008).

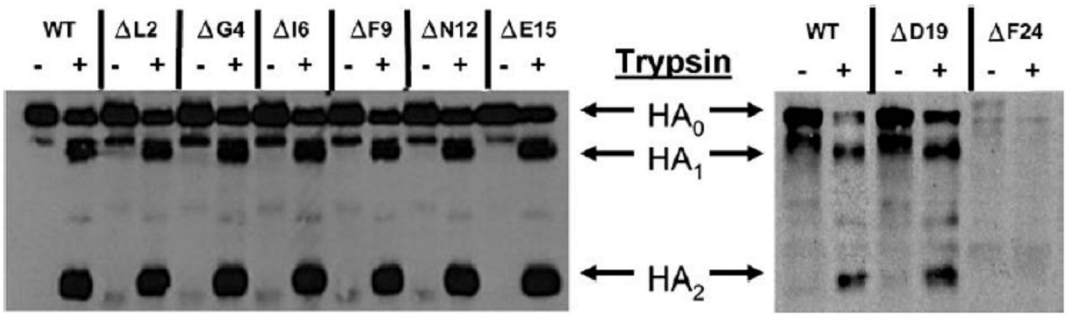

Fig. 3. Cell surface expression of HAs as assayed by trypsin cleavage of HA0 into HA1 and HA2. Recombinant vaccinia virus-infected HA-expressing cell monolayers were incubated with or without trypsin and cell lysates were analyzed by western blot following SDS-PAGE under reducing conditions.

Analysis of the pH of conformational change

Having verified that all mutant proteins except the ΔF24 HA express in native form on the cell surface and are capable of cleavage activation, we next examined the ability of the expressed HAs to undergo the acid-induced conformational changes required for fusion activity. First, we used an ELISA-based assay to assess the relative reactivities of the mutant HAs with two conformation-specific antibodies: HC3, which recognizes site A of both the neutral and low-pH HA conformations, and HC68, which preferentially binds to the neutral pH HA at site B (Fig. 2). Recombinant vaccinia virus-infected HeLa cells that were expressing HA0 on the cell surface were treated with trypsin to cleave HA0 into HA1 and HA2, washed with PBS, and incubated with PBS/citrate buffers in increments ranging in pH from 5.6 down to 4.5. Monolayers were then

neutralized, fixed, and assessed for reactivity to HC68 and HC3 by ELISA. The relative ratios of HC68 to HC3 reactivity as determined by ELISA OD_{450} were plotted as a function of pH and are shown in Fig. 4. A reduction in these ratios is indicative that the HA can undergo the acid-induced conformational changes required for membrane fusion. The results demonstrate that the Δ24F HA binds poorly to HC68 at all pH values examined, confirming that this mutant is expressed in an altered conformation on the cell surface. Most other mutants exhibited a decrease in HC68:HC3 reactivity over the range of pH reductions that were tested, indicating that they are capable of undergoing the characteristic conformational changes associated with membrane fusion. The reactivity profile for the ΔE15 and ΔD19 HA mutants are not significantly different from that of WT HA, indicating that they undergo conformational changes at a similar pH. The ΔL2 HA clearly displayed an elevated pH of conformational change relative to WT HA, confirming results that were obtained in a previous study (Steinhauer et al., 1995). Similarly, the ΔG4 and ΔI6 also appear to undergo structural rearrangements at elevated pH compared to WT. This suggests that these mutations might alter interactions that occur in native WT HA between fusion peptide residues and those located in the trimer interior, resulting in a less stable neutral pH structure. Previous reports demonstrate that a majority of fusion peptide substitution mutants with changes within the first 10 N-terminal residues of HA2 display an elevated pH of conformational change ([Cross et al., 2001], [Daniels et al., 1985], [Gething et al., 1986], [Lin et al., 1997], [Qiao et al., 1999] and [Steinhauer et al., 1995]). This is likely due to the fact that fusion peptide

residues in this region make extensive contact with ionizable residues such as HA1 H17, HA2 D109, and HA2 D112 in this region of neutral pH HA following HA0 cleavage. The aspartic acid side chains of HA2 residues 109 and 112 form multiple hydrogen bonds with residues 1 through 5 of HA2, and HA1 H17 forms hydrogen bonds via a water molecule with HA2 residues 6 and 10. Therefore, the deletion of residues L2, G4, I6, or F9 could result in the disruption of one or more of the interactions between fusion peptide residues and ionizable residues in the neutral pH structure, thus destabilizing the neutral pH structure.

The pH profile observed for the ΔF9 HA was somewhat aberrant compared to the others, and was not consistent with a loss of HC68 reactivity at reduced pH, even though this mutant appeared to be expressed in the correct conformation on the surfaces of infected cells and was efficiently cleaved into HA1 and HA2. In addition, the ΔI6 and ΔN12 mutant HAs displayed a gradual loss of HC68 reactivity over a broader range of pH than we generally observe for WT and most other mutant HAs. The disruption of interactions with HA1 H17 could offer a possible explanation for this, as HA1 H17 has been implicated previously for its potential role in the initial triggering of the acid-induced conformational changes (Thoennes et al., 2008). A further examination of the capacity for these mutants to undergo structural rearrangements was carried out using an alternative conformation-specific monoclonal antibody, IIF4. This anti-peptide antibody was generated against residues 125-175 of the HA2 subunit and binds preferentially to the low-pH form of the protein at the end of the helical rod structure opposite to the fusion peptide ([Vareckova et al., 2002] and [Wharton et al., 1995]). As

shown in Fig. 5, an increase in IIF4 reactivity upon reduction in pH can be observed for ΔI6, ΔF9, and ΔN12, as well as other HAs, indicating that structural rearrangements have occurred.

To confirm the antibody reactivity data regarding the capacity of mutant HAs to undergo acid-induced conformational changes, we carried out trypsin digestion experiments on cleaved HAs at neutral pH, or following incubation at pH 5.0. Once the HA0 precursor has been cleaved into HA1 and HA2, the WT protein is resistant to further protease digestion at neutral pH. However, upon acidification, the structural changes render the molecule susceptible to digestion by a variety of proteases, including trypsin (Skehel et al., 1982). For this experiment, HA-expressing cell monolayers were initially treated with trypsin to cleave HA0 into HA1 and HA2, as described for Fig. 3. The monolayers were then washed, and either subjected to an additional round of trypsin digestion at pH 7.2, or incubated at pH 4.6 prior to further trypsin treatment. Cell lysates were prepared and analyzed by SDS-PAGE under reducing conditions. Fig. 6 shows that the HA1 subunit of all mutants was digested by trypsin if the cleaved HA had been exposed to acidic pH, demonstrating that the mutants had gone through the characteristic conformational changes required for fusion activity. For the neutral pH samples, the results of HA1 digestion for ΔF24 HA confirm our interpretation that this HA is expressed in an altered conformation. However, the results with the ΔF9 HA indicate that neutral pH HA1 was partially degraded by the second trypsin digestion, as an additional band above HA2 was observed, which is not present in the other samples. This product is not observed during initial trypsin

cleavage of HA0 into HA1 and HA2 at neutral pH (Fig. 3). This suggests that the ΔF9 HA may be unstable relative to WT and most other mutant HAs, and this could explain the aberrant results for HC68:HC3 antibody reactivities observed for the experiments shown in Fig. 4. The conformational changes required for fusion occur within seconds for WT Aichi HA, and the digestion of ΔF9 HA1 was still incomplete after two rounds of trypsin treatment over several minutes. Therefore, it is quite possible that cell surface expressed ΔF9 HA, or at least a significant percentage of it, would be capable of going through the structural rearrangements required for fusion despite inherent instability. Cumulatively, the data suggest that all deletion mutants other than ΔF24, and possibly ΔF9 HA, are expressed and folded properly, and are capable of undergoing the acid-induced structural rearrangements requisite for membrane fusion.

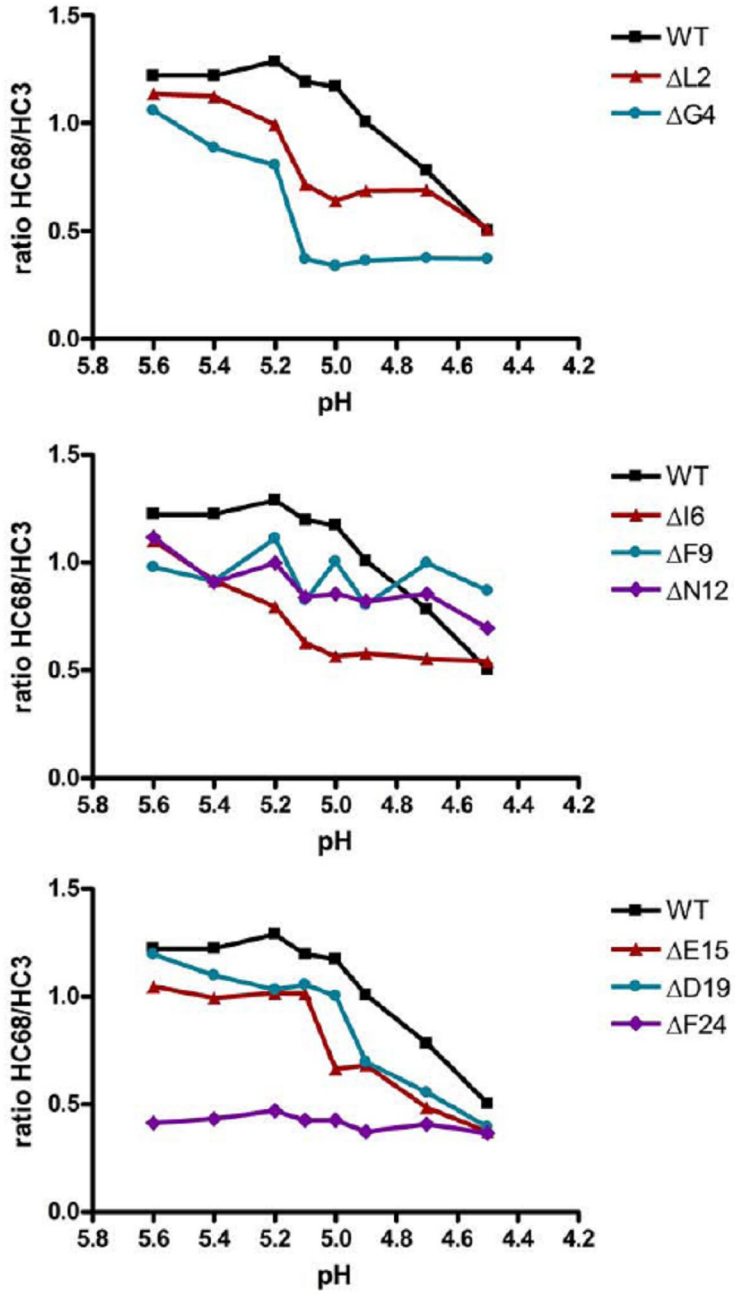

Fig. 4. Graphs of ELISA experiments to demonstrate HA conformational changes resulting from incubation at reduced pH. Graphs plot the ratios of HC68/HC3 reactivity as a function of pH. HC68 binds well with neutral pH HA, but poorly to the low pH structure. HC3 binds equally well with both HA conformations.

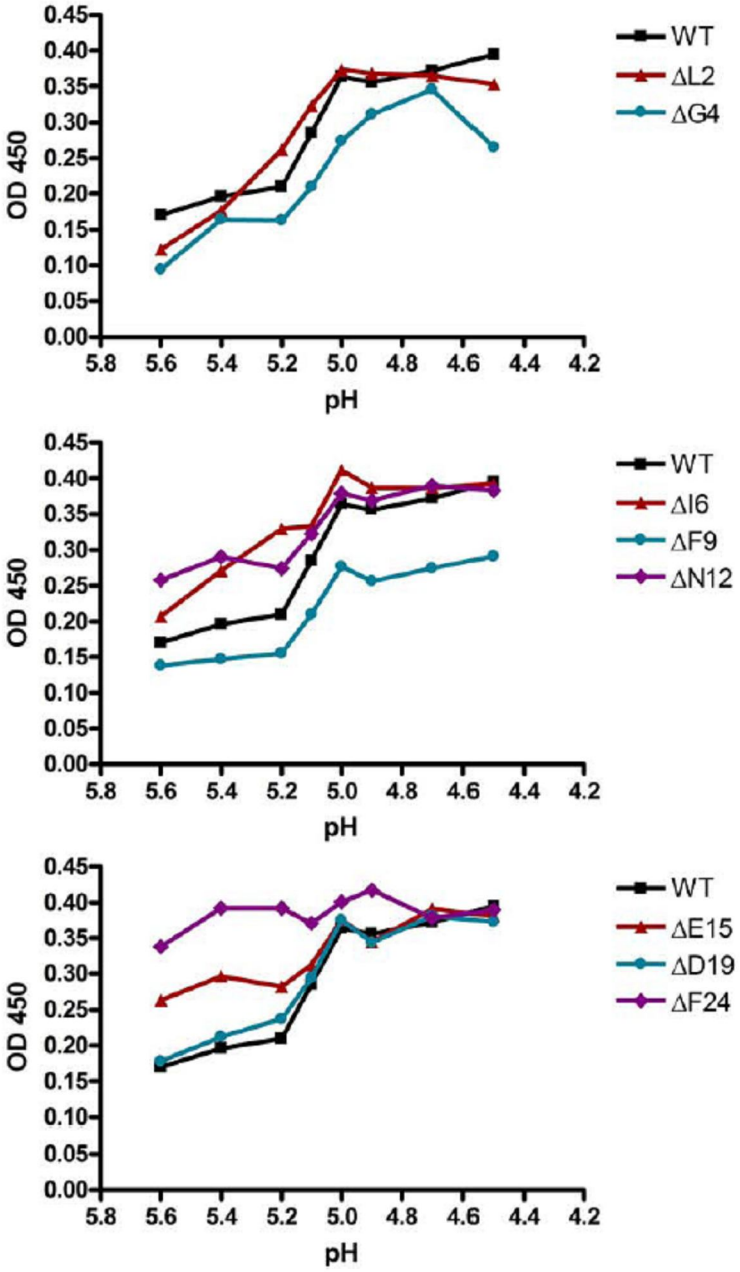

Fig. 5. Graphs of ELISA experiments showing reactivity with the low pH-specific monoclonal antibody IIF4 as a function of pH.

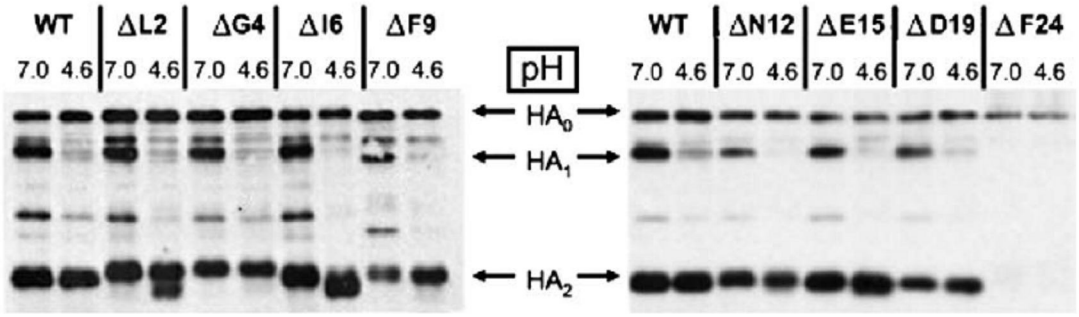

Fig. 6. Western blot analysis for the determination of the pH of conformational change by trypsin susceptibility. HA expressing cells were treated with trypsin to cleave HA0, washed, pH was adjusted to pH 4.6, and monolayers were again treated with trypsin. Lysates were then analyzed by western blot following SDS-PAGE under reducing conditions. The disappearance of HA1 bands indicates that the HA conformational changes have been triggered.

Analysis of membrane fusion by polykaryon formation and dye transfer assays

The ability of the mutant proteins to mediate fusion was assessed using an assay for polykaryon formation by recombinant vaccinia virus-infected HA-expressing BHK21 cell monolayers. HA-expressing cells were treated with trypsin to cleave HA0, the pH was adjusted by incubation in PBS/citrate buffer, monolayers were then incubated in complete medium at neutral pH, and monolayers were monitored for polykaryon formation by light microscopy. Over multiple independent experiments, no significant polykaryon formation was detected for any of the mutant HAs, even when tested in pH increments down to 4.2. Fig. 7 shows representative fields of cells for one such assay carried out at pH 4.8,

which provides evidence that all mutants were inhibited for membrane fusion activity.

To confirm these results, a dye transfer assay was also performed using the fusion peptide deletion mutants. For this assay, human erythrocytes were labeled with two dyes, the lipophilic probe R18 and the soluble dye calcein ([Ellens et al., 1990] and [Morris et al., 1989]). These loaded erythrocytes were allowed to adhere to HA-expressing cell monolayers, washed, and the pH of the monolayers were adjusted. The dyes are transferred from the smaller erythrocytes to the larger HA-expressing HeLa cells if the HA is capable of mediating fusion. The transfer of R18 is indicative of lipid mixing, and the transfer of calcein demonstrates content mixing and therefore, biologically relevant fusion activity. The transfer of R18, but not calcein is indicative of a “hemifusion” phenotype, in which the lipid mixing stage of membrane fusion does not proceed to full fusion with content mixing (Kemble et al., 1994). The dye transfer assays were carried out multiple times with the deletion mutants, and results for one such experiment are shown in Fig. 8. None of the mutants were found capable of mediating WT levels of membrane fusion using this assay. No evidence for calcein transfer and content mixing was observed for any mutant at all pH examined. However, for the Δ15 and Δ19 mutants, R18 transfer was detectable at levels above background in some examples, suggesting that these mutants might be capable of hemifusion activity, as has been observed for particular HA mutants in previous studies ([Cross et al., 2001], [Kemble et al., 1994] and [Qiao et al., 1999]). Dye transfer experiments were repeated in the presence of

chlorpromazine (CPZ) in attempts to induce the transition from a hemifusion to full fusion state (Melikyan et al., 1997); however, these did not allow us to further define the mutant phenotypes. Concentrations of CPZ ranging from 0.1 mM to 0.5 mM were tested, and at the lower concentrations no changes in fusion properties were detected, whereas at higher concentrations the compound appeared to cause cytopathology of the HA-expressing cells (data not shown).

One of the most well characterized of the “hemifusion” HA mutants is the fusion peptide substitution mutant G1S at the HA2 N-terminus. This serine mutant was found to display hemifusion activity by White and colleagues (Qiao et al., 1999) and in our laboratory (Cross et al., 2001), and the biophysical and structural properties have also been analyzed for G1S fusion peptide analogs ([Li et al., 2003] and [Li et al., 2005]). Despite the well-documented hemifusion phenotype, we were able to rescue the HA2 G1S mutant as an infectious virus (Cross et al., 2001). However, this mutant was severely attenuated for replication, and it rapidly reverted to WT despite the requirement for two nucleotide substitutions to do so. This suggests that, while such mutants provide useful tools for dissecting the mechanistic stages of fusion at the biophysical level, they are likely to be irrelevant in any natural environment.

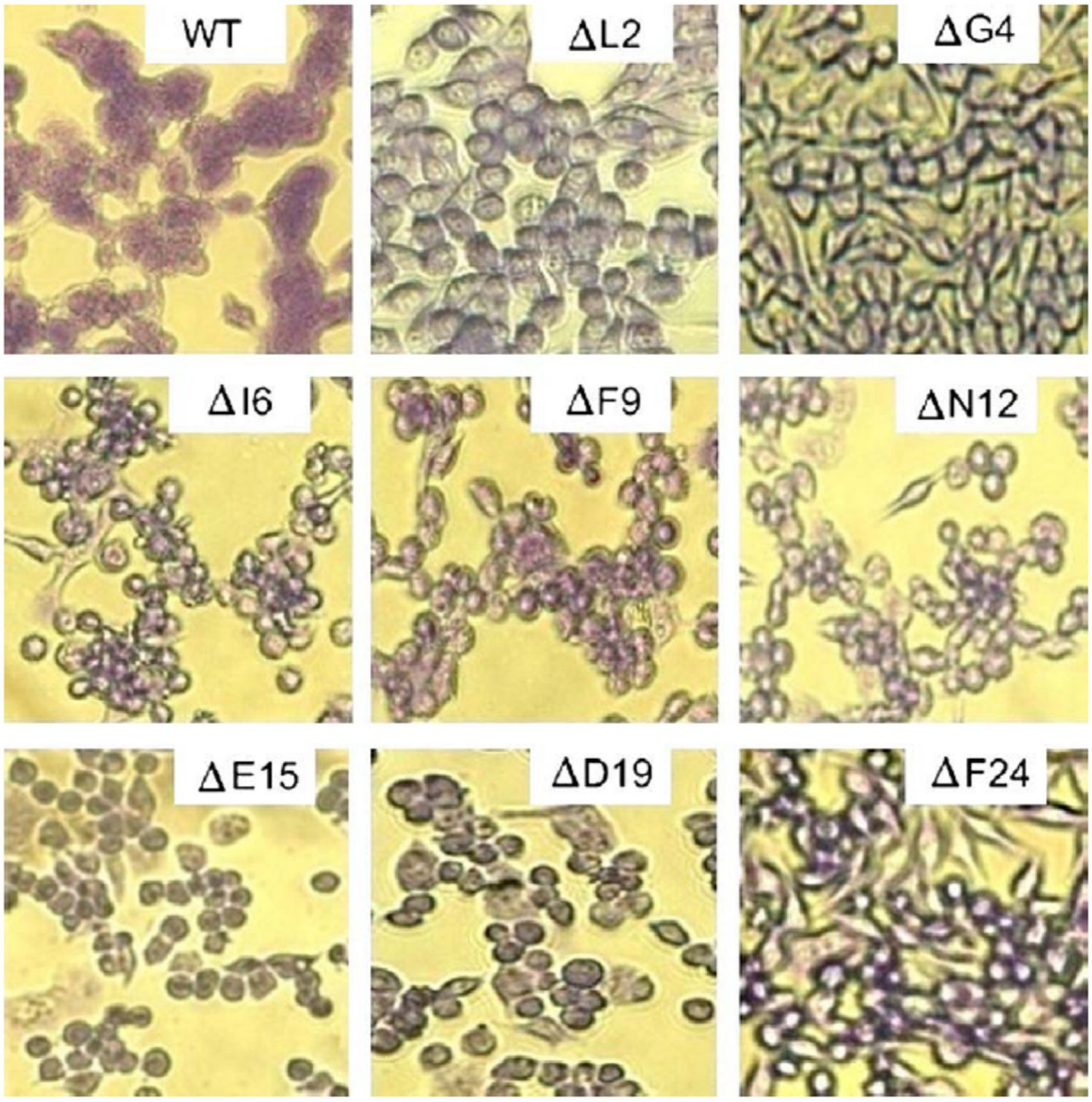

Fig. 7. Polykaryon formation by HA-expressing BHK cells following incubation at pH 4.8.

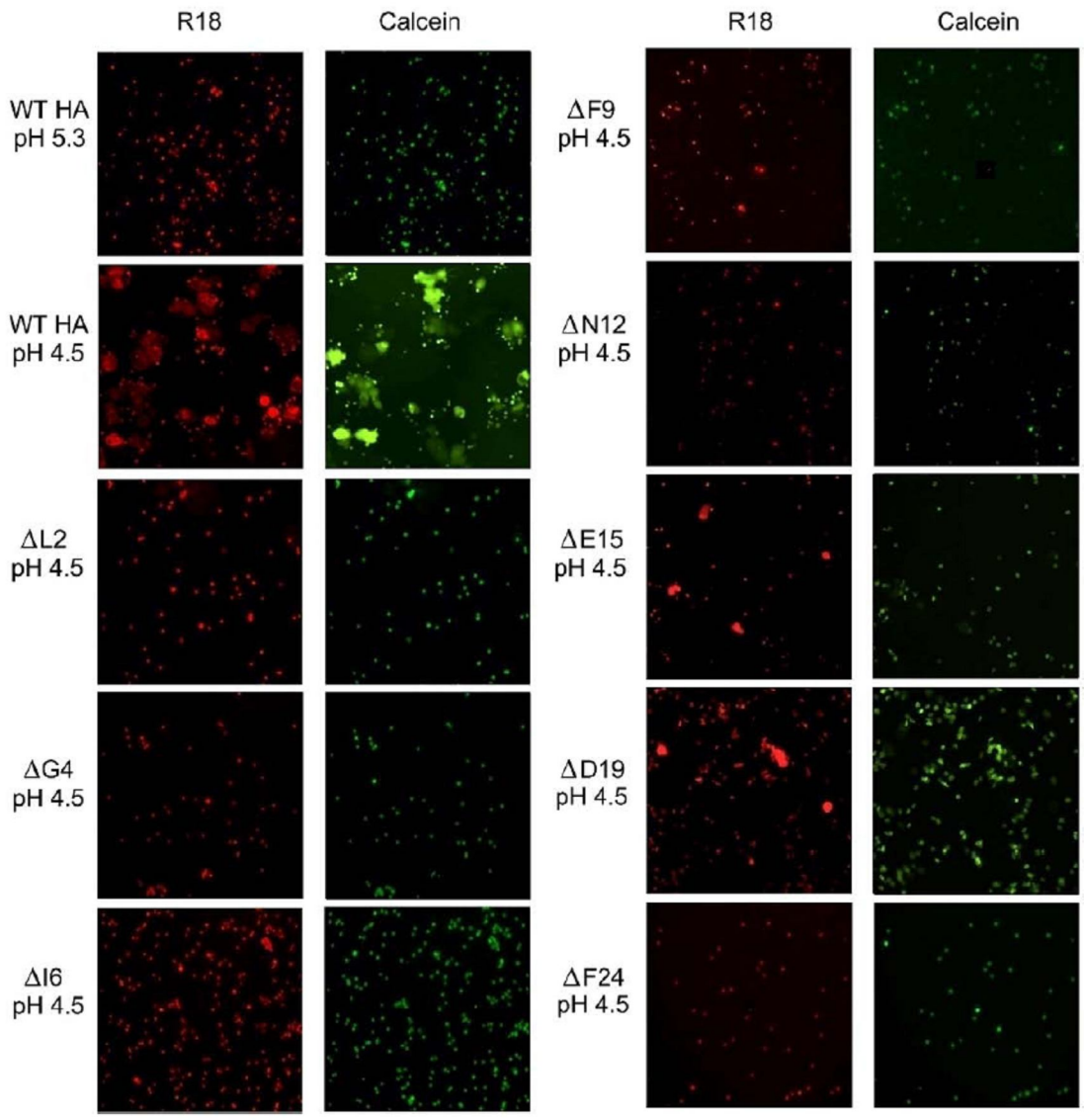

Fig. 8. Dye transfer assay for hemifusion and full fusion activity of HA mutants. Human erythrocytes loaded with R18 and calcein were adsorbed to HA-expressing cells and exposed to acidic pH to monitor HA mediated transfer of the lipid-soluble red dye R18 (lipid mixing) or the water-soluble green dye calcein (content mixing).

Deletion mutants and models for fusion peptide structure

Overall, our studies suggest that the length of the fusion peptide domain of influenza HA can influence the functional properties of the molecule at different levels, but appears to be most critical for the latter stages of fusion when this domain interacts with target membranes. All mutants except the ΔF24 HA were expressed in native conformation on the cell surface, can be cleaved into HA1 and HA2, and could be triggered by acidification to undergo the structural changes required for membrane fusion. Therefore, the most profound effects of the deletions are likely to involve the structure that these domains adopt in association with target membranes. The structure of the fusion peptide domain associated with membranes in the context of full length HA is unknown. The structure of the HA transmembrane anchor domain near the C-terminus of the molecule is also unknown, and there is evidence that this domain may also have a role in fusion ([Armstrong et al., 2000], [Chang et al., 2008] and [Melikyan et al., 2000]).

Hydrophobic photolabeling analyses have indicated that the 22 N-terminal residues of HA2 interact with target membranes during fusion, and the periodicity of labeling suggests that this segment adopts an amphipathic α-helical structure ([Durrer et al., 1996] and [Harter et al., 1989]). Circular dichroism and FTIR spectroscopy studies with membrane-bound fusion peptide analogs indicated that they may contain from 40% to 60% α-helical structure, with some studies also suggesting the presence of β-structure ([Gray et al., 1996], [Han et al.,

1999], [Lear and DeGrado, 1987], [Murata et al., 1987], [Rafalski et al., 1991] and [Wharton et al., 1988]). In some of these studies, a loose correlation between helical content and fusogenicity was observed ([Burger et al., 1991], [Lear and DeGrado, 1987] and [Wharton et al., 1988]).

The oligomeric state adopted by fusion peptide domains in association with target membranes remains to be determined, and models involving monomeric peptides, trimeric peptides, and fusion peptides complexed with HA transmembrane domains have been proposed. Experiments using spin-labeling electron paramagnetic resonance (EPR) techniques with cysteine mutants of the fusion peptide region suggest that they associate with membranes in monomeric form (Macosko et al., 1997). Monomeric fusion peptide structures have also been proposed by Tamm and colleagues based on work with synthetic fusion peptide analogs containing a seven residue solubilization domain (GCGKKKK) attached to the C-terminal end of the first 20 residues of HA2 ([Han et al., 2001], [Han and Tamm, 2000], [Lai et al., 2006], [Lai and Tamm, 2007] and [Li et al., 2005]). These synthetic peptides have been shown to possess pH-dependent membrane fusion activity, and their NMR structures in DPC micelles showed that the WT peptide adopted an inverted V-shaped structure at both neutral pH and pH 5.0, with the apex of the V located at the aqueous interface (Han et al., 2001). In the neutral pH structure, an N-terminal helix comprised of residues L2 to F9 is followed by a turn, which is stabilized by hydrogen bonds formed by residues G8 and F9 with residues E11 and N12. In the C-terminal portion of the peptide, residues W14-G20 form an extended structure with a bend between residues

G16 and M17. By comparison, in the pH 5 structure, residue I10 extends the N-terminal helix and the turn region C-terminal to this helix contains a slightly more acute bend. This kink region may be stabilized by hydrophobic interactions between F9 and W14. Additionally, the C-terminal half of the fusion segment folds into a short 3_{10} helix, and residues E15 and D19 are reoriented such that apolar residues become aligned at the bottom face of the kinked peptide. This generates a hydrophobic pocket, and allows the peptide to assume a more closed and deeply inserted structure within the membrane at acidic pH. NMR structural studies on mutant fusion peptide analogues that are inhibited for fusion activity suggested a structural significance for the hinge region that lies in the middle of the fusion peptide sequence ([Hsu et al., 2002], [Lai et al., 2006], [Lai and Tamm, 2007] and [Li et al., 2005]). In the context of these structures, the deletion mutants analyzed in our current study would clearly disrupt structural features that may be critical for function, including the N-terminal alpha-helical domain, the bend region, or the orientation or presence of acidic residues at positions 15 and 19.

Other studies and models suggest that fusion peptides associate with membranes in oligomeric form. Experiments with HA fusion peptide analogs linked to coiled coil domains designed to promote their trimerization, indicate that the trimeric peptides promote a greater degree of liposomal content leakage and membrane mixing than the monomeric form (Lau et al., 2004). A series of studies by Chang and colleagues indicated that influenza HA fusion peptides can loosely assemble in lipid bilayers ([Chang et al., 2000], [Chang et al., 2008] and [Cheng

et al., 2003]), but further suggested that they form oligomeric complexes with the HA transmembrane domains when both species are present. These results are consistent with a role for the HA transmembrane anchor domain in the fusion process, as suggested by mutagenesis studies ([Armstrong et al., 2000], [Chang et al., 2008] and [Melikyan et al., 2000]).

Our results with recombinant influenza virus mutants within the first 10 residues of the fusion peptide (Cross et al., 2001) are compatible with a model in which helical fusion peptides orient such that the relatively polar glycine residues form a trimeric interface, and the large hydrophobic side chains of residues 2, 6, and 10 reside on the surface to interact with membrane lipids (Skehel et al., 2001). In fact, many of the studies and models cited above suggest that the N-terminal region of the fusion peptide domain is predominantly α-helical. However, in the absence of structural information on fusion peptide and transmembrane domains associated with membranes in the context of full-length HA molecules, these models will continue to be speculative. Regardless, our results with the deletion mutants reported here suggest that structural elements along the length of the fusion peptide, or the spacing or orientation of such elements relative to one another, are critical for a functional association of fusion peptide sequences with target membranes during the fusion process.

Materials and methods

Mutagenesis and expression of HAs

The HA cDNA from the H3N2 subtype virus A/Aichi/2/68 was mutated by site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene). The presence of the desired mutations and the absence of extraneous mutations were confirmed by nucleotide sequencing of entire HA coding regions. The mutant cDNAs were expressed as recombinant vaccinia viruses using the plaque-selection system (Blasco and Moss, 1995). The generated viruses were propagated on CV1 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Cell surface expression trypsin cleavability and ELISA

HA cell surface expression was analyzed using a trypsin cleavage assay with recombinant vaccinia-infected CV1 cells as described previously (Steinhauer et al., 1995), except that blots were developed using chemiluminescence rather than radiolabeled secondary antibody. Following electrophoresis, blots were incubated with anti-HA rabbit polyclonal rabbit serum, washed, and incubated with a Protein A-HRP conjugate (Sigma cat# P8651). Blots were developed with the Enhanced Chemiluminescence Reagent (Amersham Pharmacia) according to the manufacturer's instructions. Quantitative cell surface expression by ELISA was carried out on recombinant vaccinia virus-infected HA-expressing HeLa cells

using a panel of monoclonal antibodies that recognize distinct antigenic regions of wild-type HA as described (Steinhauer et al., 1991).

Conformational change analysis by ELISA and trypsin digestion

Analysis of conformation changes was performed on recombinant vaccinia-infected HeLa cell monolayers by ELISA using monoclonal antibodies HC3 and HC68 as described previously (Steinhauer et al., 1991). HC3 recognizes both native and low pH HA, while HC68 recognizes only the native conformation. Trypsin susceptibility of low-pH HAs were determined as described previously (Thoennes et al., 2008).

Polykaryon formation and dye transfer assays for membrane fusion activity

The pH of membrane fusion was assayed by polykaryon formation as described previously (Steinhauer et al., 1991, cited by in Scopus (39)). For dye transfer assays, fresh heparinized human erythrocytes (HRBCs) were co-labeled with the membrane probe octadecyl rhodamine B chloride (R18) and the aqueous dye calcein-AM (Sigma) as described previously (Ujike et al., 2005). Ten milliliters of freshly prepared HRBCs (1% in Dulbecco's phosphate-buffered saline [DPBS]) was mixed with 10 µl of R18 (2 mM in ethanol) with vigorous shaking. The mixture was incubated in the dark for 30 min at room temperature, followed by the addition of 30 ml of 7.5% FBS- DMEM for 20 min at room temperature to

remove unbound R18. The R18-labeled HRBCs were washed three times and resuspended in DPBS (4% R18-labeled HRBCs). A 10-µl aliquot of 4 mM calcein-AM in dimethyl sulfoxide was added to 1 ml of 4% R18-labeled HRBCs in the dark and incubated at 37°C for 1 h, followed by the addition of 30 ml of 7.5% FBS-DMEM for 20 min at room temperature, three washes with DPBS to remove unbound calcein, and resuspension in DMEM (0.02% HRBCs). To analyze hemifusion and fusion pore formation by wildtype HA and HA mutants, an R18 and calcein transfer assay was performed. Recombinant vaccinia virus-infected HeLa cells expressing HA were pretreated with neuraminidase (NA) (30 mU/ml; Sigma) at 37 °C for 60 min, washed once with DPBS, and treated with TPCK-trypsin (5 µg/ml) at 37 °C for 5 min. Cells were washed with soybean trypsin inhibitor (5 µg/ml), washed, and incubated with R18- and calcein-labeled HRBCs at room temperature for 30 min for hemadsorption. After unbound HRBCs were removed by three washes, the cells were washed and incubated for 1 min at 37 °C in low-pH buffer (10 mM HEPES, 20 mM sodium citrate [pH 5.0], 150 mM NaCl, 2 mM $CaCl_2$, and 20 mM raffinose to prevent colloidal-osmotic swelling of the erythrocytes that could be induced by HA-mediated leakage) (Melikyan et al., 1999). The medium was replaced with DMEM supplemented with 7.5% FBS. After incubation for 15 min at 37 °C, hemadsorption and the transfer of fluorescence were observed with a phase-contrast microscope and a fluorescence microscope, respectively.

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Chapter 3

Immunization with Live Attenuated Influenza Viruses That Express Altered NS1 Proteins Results in Potent and Protective Memory $CD8^+$ T-Cell Responses

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Immunization with Live Attenuated Influenza Viruses That Express Altered NS1 Proteins Results in Potent and Protective Memory CD8$^+$ T-Cell Responses

Scott N. Mueller,[1,†,‡] William A. Langley,[1,†] Elena Carnero,[2,§] Adolfo García-Sastre,[2,3,4] and Rafi Ahmed[1*]

[†]S.N.M. and W.A.L. contributed equally to this study.

Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322,[1] Department of Microbiology,[2] Department of Medicine, Division of Infectious Diseases,[3] Global Health and Emerging Pathogens Institute, Mount Sinai School of Medicine, New York 10029[4]

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ABSTRACT

The generation of vaccines that induce long-lived protective immunity against influenza virus infections remains a challenging goal. Ideally, vaccines should elicit effective humoral and cellular immunity to protect an individual from infection or disease. Cross-reactive T- and B-cell responses that are elicited by live virus infections may provide such broad protection. Optimal induction of T-cell responses involves the action of type I interferons (IFN-I). Influenza virus expressed nonstructural protein 1 (NS1) functions as an inhibitor of IFN-I and promotes viral growth. We wanted to examine the priming of $CD8^+$ T-cell responses to influenza virus in the absence of this inhibition of IFN-I production. We generated recombinant mouse-adapted influenza A/PR/8/34 viruses with NS1 truncations and/or deletions that also express the gp33-41 epitope from lymphocytic choriomeningitis virus. Intranasal infection of mice with the attenuated viruses primed long-lived T- and B-cell responses despite significantly reduced viral replication in the lungs compared to wild-type virus. Antigen-specific $CD8^+$ T cells expanded upon rechallenge and generated increased protective memory T-cell populations after boosting. These results show that live attenuated influenza viruses expressing truncated NS1 proteins can prime protective immunity and may have implications for the design of novel modified live influenza virus vaccines.

INTRODUCTION

Influenza virus infections remain an important global health issue, particularly among the young and elderly. The natural host of influenza viruses is water birds, however, influenza viruses can also infect a wide variety of other hosts, including other birds, humans and pigs (36). The ability of influenza viruses to survive and adapt in different hosts has precipitated three human pandemics in the last century alone (in 1918 [H1N1], 1957 [H2N2], and 1968 [H3N2]), as well as numerous epidemics, including the recent H1N1 swine influenza outbreak (12). Despite our increasing understanding of influenza viruses, their life cycle, pathogenicity, and immunogenicity, the production of vaccines that generate long-lived cross-protective immunity against seasonal strains or pandemic strains of the virus remains a challenging goal (5).

An effective vaccine against influenza should ideally elicit both humoral and cellular immunity. Current inactivated influenza virus vaccines induce largely antibody-mediated responses that are effective in providing protection against homologous influenza virus infections and yet are inadequate against heterologous infections, where many of the viral proteins are distinct (3, 31). Influenza virus causes repeated infections by undergoing antigenic drift and occasionally antigenic shift to evade the host immune response. Neutralizing antibodies against the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) are required for resistance against respiratory infection, potentially by slowing the rate of viral replication and spread to allow time for the cellular

immune response to mediate viral clearance (7). Indeed, protective cell-mediated immunity to virulent influenza virus infection requires $CD8^+$ T cells (4), and these may need to reside in the respiratory tract to control initial viral replication until secondary effectors arrive (38).

Interestingly, influenza viruses can still cause disease in immune individuals despite the high conservation of T-cell epitopes, suggesting that the virus may also use mechanisms to subvert the immune system. The nonstructural (NS1) protein of influenza virus is a virulence factor with multiple functions in infected cells. In addition to potentially controlling viral RNA replication (9) and viral protein synthesis (18), one of the major functions of the NS1 protein is the inhibition of host interferon (IFN) responses (14). This can occur via inhibition of the IRF-3, NF-κB, and c-Jun/ATF-2 transcription factors (16, 32), possibly by preventing intracellular sensing of viral single-stranded RNA by preventing RIG-I activation (13, 27). The NS1 protein can also block the function of 2'-5'-oligoadenylate synthetase and serine/threonine protein kinase R (25, 26), as well as inhibit host mRNA processing and activate the phosphatidylinositol 3-kinase pathway (15), thus potentially influencing multiple aspects of innate immune activation and apoptosis in infected host cells.

Type I IFNs (IFN-I) are produced by infected or activated cells during viral infection. Some specialized cell types, such as plasmacytoid DC, are capable of producing very large amounts of IFN-I. The optimal priming of both $CD8^+$ and $CD4^+$ T-cell responses involves direct signaling through the IFN-I receptor (IFN-

IR) (8, 19, 24). T cells lacking the IFN-IR show reduced expansion and memory formation after infection. It has been demonstrated that expression of the NS1 protein by influenza viruses can significantly reduce the production of inflammatory cytokines after infection (21), as well as increase pathogenicity in a manner independent of its IFN-I blocking action (22). Viruses lacking NS1 function are highly attenuated and may be useful for the design of new generation influenza virus vaccines (32). In the absence of NS1, or in virus mutants with truncated NS1 proteins, influenza viruses can induce adaptive immune responses in different animal models, such as mice, pigs, and macaques (2, 10, 29, 33) and stimulate more effective dendritic cell maturation and migration (11).

To better understand the capacity of mutant influenza viruses with compromised NS1 function to elicit protective cell-mediated immune responses, in particular $CD8^+$ T-cell responses, we inserted the lymphocytic choriomeningitis virus (LCMV) gp33-41 epitope into the influenza virus A/PR/8/34 NA stalk. Using reverse genetics, this segment was incorporated into recombinant influenza viruses that expressed truncated NS1 proteins or lacked expression of NS1 via a complete deletion. These mutant viruses displayed reduced viral growth and pathology in mice after intranasal infection and yet generated long-lived antigen-specific T- and B-cell responses. Responses were readily detectable both systemically and in the lungs after infection. Mice containing effector or memory $CD8^+$ T cells primed by the live attenuated viruses cleared virus more rapidly after rechallenge, whereas prime-boost vaccination could further expand the size

of the memory pool generated. Together, our data suggest that NS1 mutant viruses might provide a safe and effective means of generating potent cellular and humoral immune responses against influenza viruses.

MATERIALS AND METHODS

Generation of recombinant influenza viruses. Recombinant influenza viruses were produced by using an established eight-plasmid influenza virus reverse genetics system (20, 28). The plasmids used in the construction of the recombinant influenza viruses have been previously described (35). The LCMV gp33-41 epitope (KAVYNFATM) was inserted into the NA of A/PR/8/34 (H1N1) at residue 42 by PCR mutagenesis using the primers 5'-CAAACTGGA-AGT*AAAGCCGTTTATAATTTTGCCACCATG*AACATCATTACC-3' and 5'-GGT-AATGATGTT*CATGGTGGCAAAATTATAAACGGCTTT*ACTTCCAGTTTG-3' (ital-iccs represent the inserted KAVYNFATM epitope). A corresponding number of amino acids (nine, QNHTGICNQ) were deleted from the recombinant viruses to maintain the protein length equal to the wild-type NA protein. In order to generate NS1 truncations, we pursued a previous strategy described by Solorzano et al. (30). We generated every NS1 truncation by amplification of two PCR fragments and subsequent ligation in the pDZ vector (28): the 5'-end portion of the segment, common for all truncations, was obtained with two primers, 5'-GCGCTTAATTAAGAGGGAGCAATTGTTGGCG-3' (NS1-153) and 5'-CATCGC-

TCTTCTATTAGTAGAAACAAGGGTGTTTTTTATTATTAAATAAG-3'. The 3'-end portion containing either the first 73, 113, or 126 amino acids of NS1 was obtained by using the primers 5'-GCGCTTAATTAATCAAGATCTAGGATTCTTC-TTTCAGAATCC-3' (NS1-73), 5'-GCGCTTAATTAATCAAGATCTAGCCTGCCA-CTTTCTGCTTGGG-3' (NS1-113), and 5'-GCGCTTAATTAATCAAGATCTAC-TTATCCATGATCGCCTGG-3' (NS1-126), respectively, together with 5'-GAT-CGCTCTTCTGGGAGCAAAAGCAGGGTGACAAAGAC-3'. NS1 truncations did not affect the sequence of NEP. To generate the NS segment containing a complete deletion of NS1, we amplified NEP by two PCRs using two pairs of primers: (i) NS1-BspMI,3 (GCGCACCTGCTTTTTCAGGACATACTGCTGAGG-ATG and 5'-GATCGCTCTTCTGGGA-GCAAAAGCAGGGTGACAAAGAC-3') and (ii) NS1-BspMI,5 (GCGCACCTG-CTTTTCTGAAAGCTTGACACAGTG and 5'-CATCGCTCTTCTATTAGTAGAAA-CAAGGGTGTTTTTTATTATTAAATAAG-3').

Mice, virus, and infections. Thy1.1$^+$ (B6.PL-Thy1^a/CyJ) mice were bred to P14 transgenic mice and maintained in our colony. Splenocytes from naive Thy1.1$^+$ P14 transgenic mice containing 10^5 antigen-specific CD8$^+$ T cells were transferred into 6-week-old female C57BL/6J (B6) mice (the Jackson Laboratory). Given a "take" of ca. 10%, this transfer reflected ~10^4 P14 CD8$^+$ T cells per recipient mouse. The following day, the mice were infected with 200 PFU of recombinant influenza virus PR8-33 (referred to as PR8 or wild-type [WT] virus) or either of the NS1 mutant viruses (NS1-73, NS1-113, NS1-126, or ΔNS1) intranasally. For challenge experiments, mice were infected with 5 x 10^6 PFU of a recombinant vaccinia virus expressing gp33-41 (VVgp33) (17) intranasally. Titers

of VVgp33 in lung homogenates were determined by plaque assay on Vero cells as previously described (17). Influenza virus titers were determined by using monolayers of Madin-Darby canine kidney (MDCK) cells. MDCK cells were infected with dilutions of lung tissue homogenates in Dulbecco modified Eagle medium (DMEM) for 1 h at 37°C before they were overlaid with 1% agarose in DMEM supplemented with 5% fetal bovine serum and 1 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin/ml. Cells were incubated for 3 days and stained with crystal violet (0.1% [wt/vol] in 20% methanol) to count plaques.

Hemagglutinin inhibition assay. One part serum was added to three parts receptor destroying enzyme (RDE; Accurate Chemical & Scientific) and incubated at 37°C overnight. The RDE was inactivated the following morning by incubating the samples at 56°C for 1 h. Samples were then serially diluted with phosphate-buffered saline (PBS) in 96-well V-bottom plates, and eight hemagglutination units (as determined by incubation with 0.5% turkey red blood cells [RBCs] in the absence of serum) of influenza virus was added to each well. After 30 min at room temperature, 50 μl of 0.5% turkey RBCs (Lampire Biological Laboratories) suspended in PBS-0.5% bovine serum albumin was added to each well, and the plates were shaken manually. After an additional 30 min at room temperature, the serum titers were read as the reciprocal of the final dilution for which no hemagglutination was observed.

Lymphocyte isolation. Lymphocytes were isolated from lungs by treatment with 1.3 mM EDTA in Hanks balanced salt solution for 30 min at 37°C with shaking at

200 rpm, followed by incubation with 100 U of collagenase (Invitrogen Life Technologies)/ml in 5% RPMI 1640 supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (60 min at 37°C, shaking at 200 rpm). Single-cell suspensions were obtained by pushing spleens, lymph nodes, or digested lungs through 70-μm-pore-size nylon mesh filters (Becton Dickinson). Lymphocytes from lungs were purified by centrifugation on a 44/67% Percoll gradient (800 x *g* for 20 min at 20°C).

Antibodies and flow cytometry. Single-cell suspensions were stained with anti-CD8α-APC (53-6.7), Thy1.1-PerCP (OX-7), CD62L-FITC (MEL-14), CD43-FITC (1B11), gamma interferon (IFN-γ)-FITC (XMG1.2), tumor necrosis factor alpha (TNF-α)-APC (MP6-XT22), and interleukin-2 (IL-2)-APC (JES6-5H4) (BD Pharmingen); CD127-PE (A7R34) (eBioscience); or anti-human granzyme B-PE (Caltag Laboratories). Intracellular staining for granzyme B directly ex vivo or for IFN-γ, TNF-α, or IL-2 after 5 h *in vitro* stimulation with 0.1 μg of LCMV gp33-41 peptide or influenza np366-374 peptide/ml was performed by using a Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Samples were analyzed by using a Becton Dickinson FACSCalibur apparatus.

Memory cell transfers. Thy1.1$^+$ P14 transgenic CD8$^+$ T cells were isolated from the spleens or lungs of mice infected with the indicated viruses. Naive B6 recipient mice (Thy1.2$^+$) received 1 x 10^4 Thy1.1$^+$ CD8$^+$ memory cells to track

expansion in the blood or 5 x 10^4 cells to measure protection upon recall with VVgp33.

Statistical analysis. Data are expressed as the means ± the standard deviation. Statistical analysis was performed by two-tailed Student *t* test with 95% confidence intervals, using Prism software (GraphPad).

RESULTS

Generation of recombinant influenza viruses expressing mutant NS1 proteins. The influenza A virus NS1 protein can be divided into three domains: the N-terminal RNA-binding domain (amino acids [aa] 1 to 73), the effector domain (aa 74 to 207), and a short C-terminal region (~20 aa). Numerous functions have been attributed to the NS1 protein, including binding to double-stranded RNA via the N-terminal region. In addition, the C-terminal effector domain may stabilize and support the function of the NS1 RNA-binding domain (34). We previously demonstrated that progressive truncation of the carboxy-terminal region of the NS1 protein results in recombinant influenza viruses with different degrees of inhibition of IFN-I induction that correlate with their attenuation *in vivo* (28, 30, 32). Infection with NS1 mutant viruses primed immune responses and protection against lethal infection by heterologous influenza virus (Fig. 1A). We wanted to more carefully examine $CD8^+$ T-cell responses primed by influenza viruses with truncated or deleted NS1 proteins. To do this, we

generated a recombinant influenza A/PR/8/34 virus (H1N1) with a $CD8^+$ T-cell epitope from the LCMV glycoprotein (gp33-41) inserted into the NA stalk (Fig. 1B). Using reverse genetics, PR8-33 viruses (referred to as wild-type PR8 from hereon) were then produced that lacked expression of the NS1 protein (ΔNS1) or expressed truncations of the NS1 protein representing the first 73, 113, or 126 aa of the protein from the N-terminal end (Fig. 1B) instead of the 230-aa wild-type NS1 protein.

We first examined the ability of the mutant NS1 viruses to infect mice after intranasal administration. Administration of a low titer of recombinant wild-type PR8 virus (10^2 PFU) resulted in rapid viral growth in the lungs within 2 days, peaking at around 4 days after infection (Fig. 1C). Since NS1-deficient viruses are highly attenuated in IFN-competent systems, we infected mice with a higher dose of the mutant viruses (10^5 PFU). In mice infected with the NS1 mutant viruses, no significant changes in body weight or signs of morbidity were observed after infection. Infection of mice with the mutant viruses expressing truncated NS1 proteins demonstrated different degrees of viral growth in the lungs. Virus titers were highest in mice infected with the PR8/NS1-73 (73) virus, followed by the 113 and 126 viruses, respectively (Fig. 1C). Similar to that observed with truncated NS1 proteins from swine or equine influenza viruses, the length of the protein correlated inversely with viral growth and IFN inhibition (28, 30). This likely reflects differential stability of the truncated NS1 proteins in infected cells (30). All viruses with truncated NS1 proteins stimulated long-lived humoral immunity in the mice, albeit at lower titers than that after infection with wild-type

virus (Fig. 1D). No virus was detected in the lungs of mice after infection with the ΔNS1 virus (Fig. 1C). However, low yet detectable hemagglutination inhibition (HI) titers were observed in these mice for at least 90 days (Fig. 1D). Thus, the NS1 mutant viruses were capable of infecting mice via the lungs and stimulating long-lived host immunity.

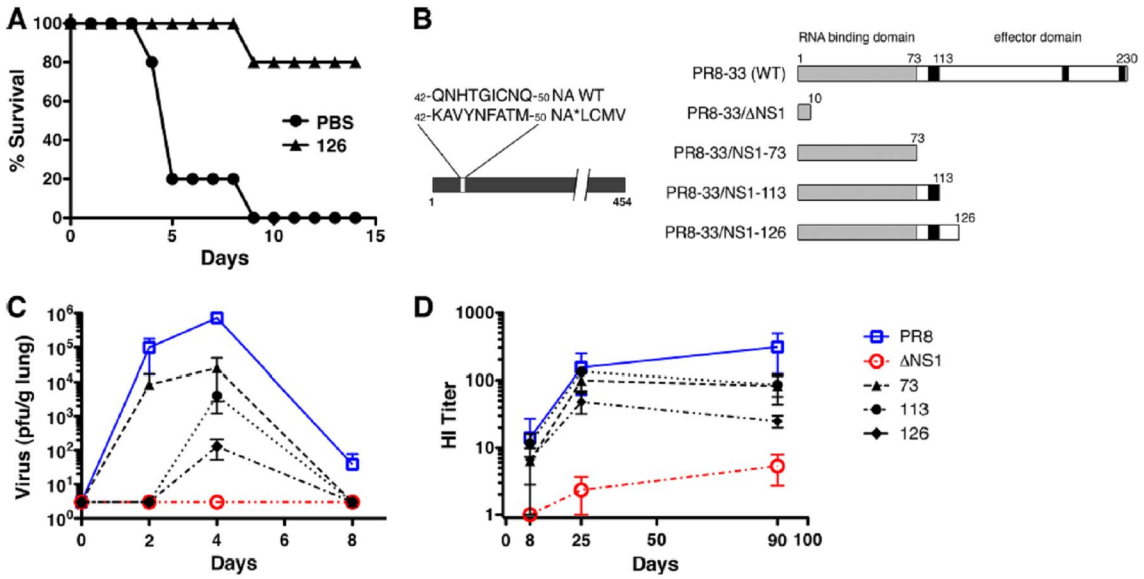

FIG. 1. (A) Mice were infected intranasally with 10^5 PFU of a recombinant influenza A/PR/8 virus (H1N1) containing the first 126 aa at the N-terminal end of NS1. Control mice were given PBS. Three weeks later the mice were challenged with 10^6 PFU of WT influenza A/HK-x31 virus (H3N2) and survival monitored over 14 days. (B) Mouse-adapted influenza virus A/PR/8/34 was modified to express the LCMV gp33-41 peptide (KAVYNFATM) in the NA stalk (PR8-33). This recombinant virus was rescued with plasmids encoding truncations of the influenza virus PR/8 NS1 containing just the first 73, 113, or 126 aa at the N-terminal end or complete deletion of the NS1 protein (ΔNS1). Viruses were designated PR8 (WT), ΔNS1, 73, 113, or 126. Virus titers were measured in the lungs (C) and hemagglutination inhibition (HI) titers were measured in the blood (D) of C57BL/6 mice at various times after intranasal infection with 10^2 PFU of WT PR8 or 10^5 PFU of the NS1 mutant viruses. Virus was undetectable in mice infected with the ΔNS1 virus (*n* = 3 mice per group).

Priming of effector CD8$^+$ T-cell responses by NS1 mutant influenza viruses. To determine CD8$^+$ T-cell responses after infection with the attenuated influenza viruses, mice were given TCR transgenic P14 T cells specific for the LCMV gp33-41 epitope prior to infection. These transferred T cells were undetectable in the spleens of uninfected mice. Eight days after infection, responding Thy1.1$^+$ CD8$^+$ T cells were examined in the spleen and lungs of the mice. Substantial responses were detected in the tissues of mice infected with each of the NS1 mutant viruses (Fig. 2A). Quantitation of the responding effector T-cell responses in the spleen and lungs (Fig. 2B) reflected the degree of viral infection in the lungs (see Fig. 1C). Although an order of magnitude less than that after infection with wild-type PR8 virus, infection of mice with the ΔNS1 virus primed considerable T-cell responses in the spleen and lungs, despite a lack of any detectable virus in the lungs after infection. Furthermore, P14 CD8$^+$ T cells primed by the NS1 mutant viruses upregulated CD44 after infection, demonstrating effective activation of the cells (data not shown).

Effector CD8$^+$ T cells primed after NS1 mutant influenza virus infection were functional and produced the cytokines IFN-γ and TNF-α after restimulation with peptide (Fig. 2C). Interestingly, a higher proportion of cells produced IL-2 8 days after infection with the 126 or ΔNS1 viruses. Downregulation of CD62L and upregulation of the activated isoform of CD43 recognized by the antibody 1B11 also correlated with the size of the expanded CTL populations and viral growth in the lungs (Fig. 2D). Moreover, expression of the effector molecule granzyme B was lowest after ΔNS1 virus infection, whereas higher expression was detected

in effector $CD8^+$ T cells primed by the 73 and 113 viruses (Fig. 2E). Together, this suggested that the $CD8^+$ T cells primed by the NS1 mutant viruses were less highly activated 8 days after infection, possibly reflecting incomplete activation of the antigen-specific $CD8^+$ T-cell population or a more rapid transition to memory.

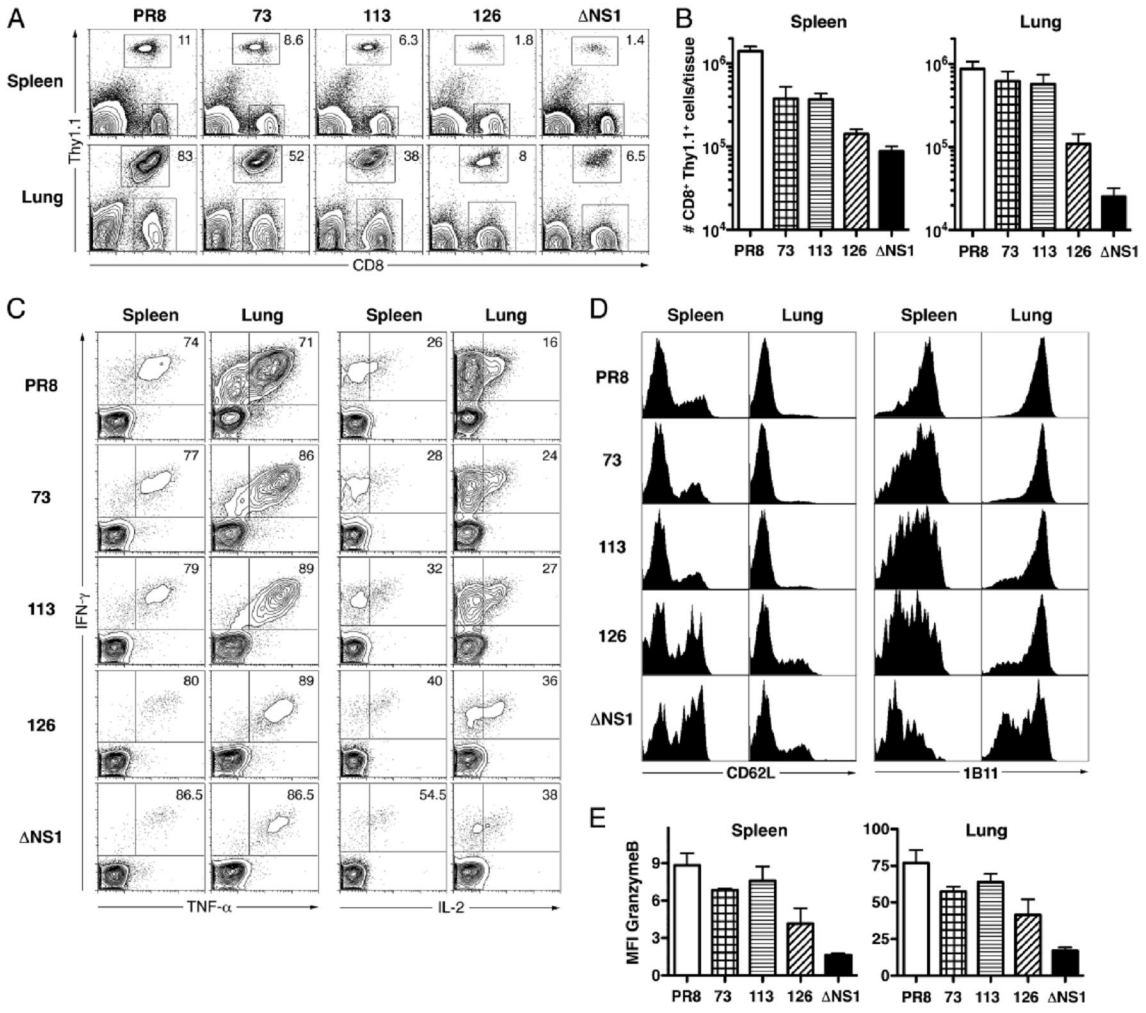

FIG. 2. T-cell responses in the spleen and lungs of mice 8 days after infection with the NS1 mutant viruses. (A) Mice were given LCMV gp33-41-specific P14 Thy1.1$^+$ CD8$^+$ T cells prior to infection, and responding P14 cells were examined in the indicated tissues. The numbers in the upper right corners of each subpanel indicate the proportions of Thy1.1$^+$ of CD8$^+$ T cells. (B) Responding antigen-specific P14 T cells were quantitated in the spleen and lungs. (C) Functional expression of cytokines after *in vitro* peptide stimulation of P14 cells taken from the indicated tissues 8 days after infection. The numbers in the upper right corners of each subpanel indicate the proportions of CD8$^+$ T-cells expressing both indicated cytokines. The expression of CD62L and CD43 (1B11) (D) and granzyme B (E) on effector P14 CD8$^+$ T cells in the spleen and lungs after infection were evaluated. MFI, mean fluorescence intensity (*n* = three to four mice per group). The results of one representative experiment of three are shown.

Long-lived memory CD8$^+$ T-cell responses after NS1 mutant influenza virus infection. Memory CD8$^+$ T-cell populations were analyzed in mice 3 months after infection with the different NS1 mutant influenza viruses (Fig. 3). All recombinant viruses primed populations of memory cells that were readily detectable in the spleen and lungs (Fig. 3A). The size of the memory populations detected in the tissues largely reflected the size of the effector pools observed 8 days after infection (Fig. 3B). However, contraction of the CD8$^+$ T-cell pool in the spleen after ΔNS1 infection was reduced, such that the size of the memory population remained similar to that detected at day 8. This may have reflected early or reduced contraction of the response after infection with the highly attenuated virus.

Memory cells primed by the mutant NS1 viruses were highly functional and produced IFN-γ and TNF-α after restimulation (Fig. 3C). A considerable proportion of the memory cells produced IL-2 in both the spleen and the lungs, a finding indicative of long-lived resting memory T cells. The majority of the memory cells had also regained expression of CD62L and expressed low levels of 1B11 (Fig. 3D). Similarly, endogenous influenza virus np366-specific CD8$^+$ T cells were primed after infection with the mutant NS1 viruses, and significant numbers of cells remained detectable in these mice for at least 3 months (Fig. 3E). Together, this demonstrates that infection of mice with the mutant NS1 viruses primed CD8$^+$ T cells with the phenotypic and functional properties of long-lived memory cells.

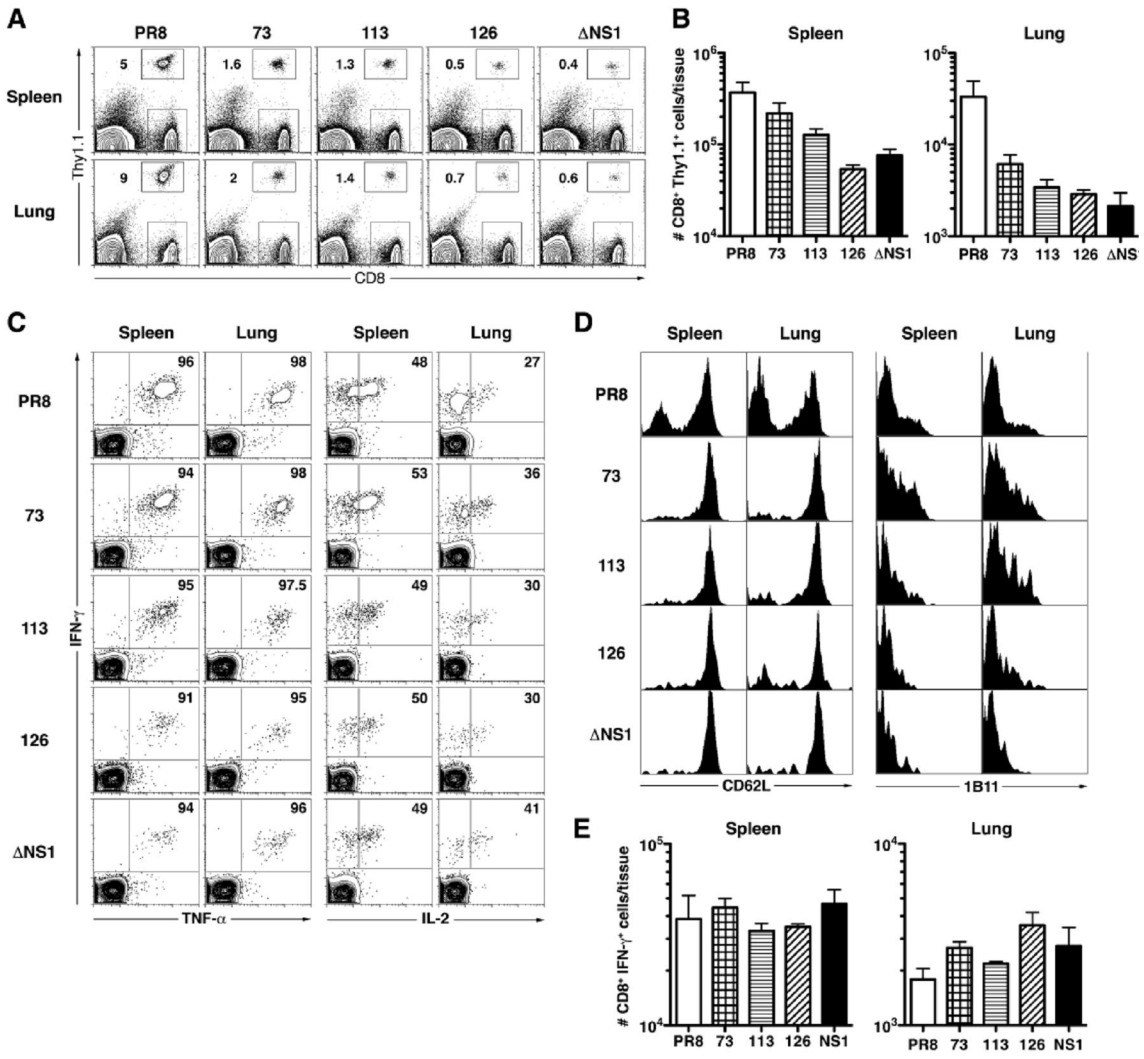

FIG. 3. Long-lived memory CD8 T-cell populations are primed by infection with the NS1-mutant viruses. (A) Memory Thy1.1$^+$ CD8$^+$ T cells were examined in the indicated tissues 3 months after infection with the indicated viruses. The numbers in upper right corners of each subpanel indicate the proportions of Thy1.1$^+$ of CD8$^+$ T cells. (B) Responding antigen-specific P14 T cells were quantitated in the spleen and lungs. (C) Functional expression of cytokines after *in vitro* peptide stimulation of P14 cells taken from the indicated tissues. The numbers in upper right corners of each subpanel indicate the proportions of CD8$^+$ T cells expressing both indicated cytokines. (D) Expression of CD62L and CD43 (1B11) on memory P14 CD8$^+$ T cells in the spleen and lungs after infection. (E) Endogenous np366-specific CD8$^+$ T cells from mice infected 3 months earlier were detected by intracellular cytokine staining after *in vitro* peptide stimulation (*n* = three to four mice per group). The results of one representative experiment of three are shown.

Recall responses by memory cells after NS1 mutant influenza virus infection. We next wanted to evaluate the recall capacity of memory $CD8^+$ T cells generated after infection of mice with the NS1 mutant influenza viruses. Normalized numbers of P14 $Thy1.1^+$ $CD8^+$ memory T cells (5×10^4), isolated from the spleens of immune mice, were transferred into naive mice prior to intranasal challenge with a recombinant vaccinia virus expressing the gp33 epitope (VVgp33) (Fig. 4A). Expansion of the memory $CD8^+$ T cells was followed in the blood of mice that had received cells from PR8-, 73-, 113-, 126-, or ΔNS1-immune mice (Fig. 4B). Expansion peaked around day 16 postinfection. Interestingly, memory cells from mice infected with the NS1 mutant viruses expanded slightly better than those primed by wild-type PR8 infection. Together, this demonstrated that the attenuated influenza viruses containing truncated or deleted NS1 proteins primed functional memory $CD8^+$ T cells capable of responding and expanding upon rechallenge.

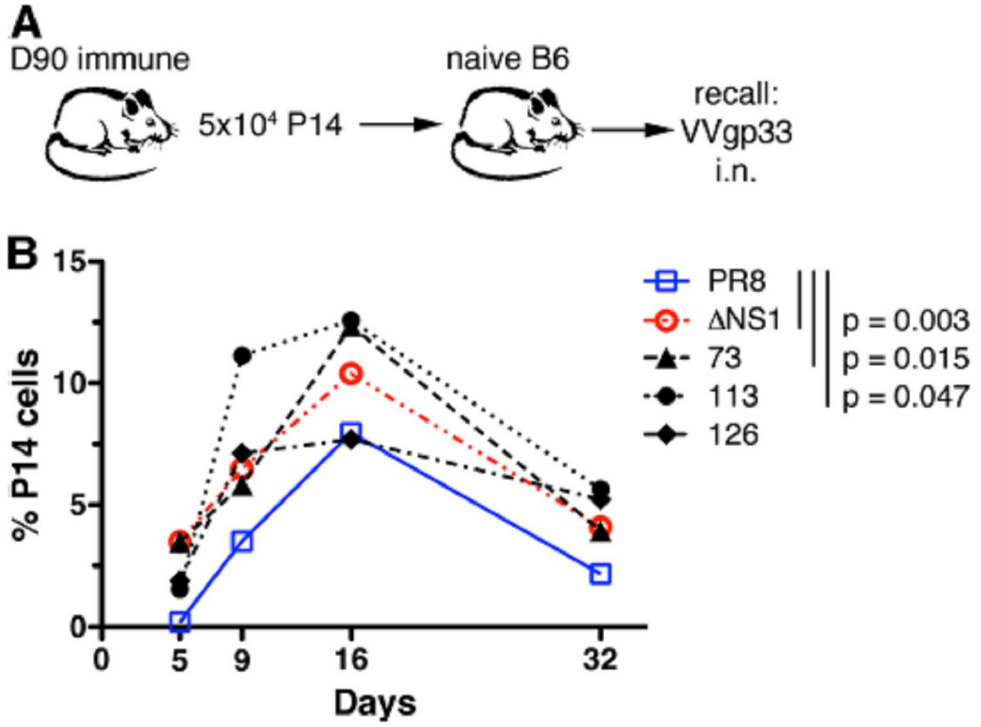

FIG. 4. Recall of memory P14 cells primed by NS1 mutant viruses. (A) Mice were given 5 x 10^4 memory P14 cells from mice initially primed with the NS1 viruses 3 months earlier and challenged with VVgp33 intranasally. (B) Expansion of the $CD8^+Thy1.1^+$ memory cells was monitored in the blood over time.

Rapid prime-boost responses after immunization with NS1 mutant influenza viruses. Although the mutant NS1 influenza viruses primed highly functional memory T-cell populations, the size of the memory pool was smaller than that induced after wild-type PR8 infection (see Fig. 3). However, infection of mice with the ΔNS1 virus induced long-lived memory T cells, despite a lack of detectable virus in the lungs and no significant malaise or weight loss in the mice (Fig. 1 and data not shown). Interestingly, effector $CD8^+$ T cells primed after ΔNS1 infection were less activated, and a greater proportion of the cells produced IL-2 as early as 8 days after infection (Fig. 2). The transition from an effector to central memory phenotype involves re-expression of CD62L and the capacity to produce IL-2 over time (23, 37). Our data indicated that stimulation of the P14 $CD8^+$ T cells after ΔNS1 infection may have resulted in a brief effector period, followed by more rapid transition to memory.

We sought to determine whether it was possible to increase the size of the memory T-cell pool generated by boosting the $CD8^+$ T cells only a short period after infection. To ascertain the capacity of the activated T cells to respond to an antigen boost soon after infection, P14 $Thy1.1^+$ $CD8^+$ T cells were isolated from mice infected with ΔNS1 9 days earlier, and transferred into naive mice (Fig. 5A and B). The mice were then infected with VVgp33 intranasally, and the expansion and protective capacity of the $CD8^+$ T cells was determined 5 days later. Expansion of the ΔNS1-primed $CD8^+$ T cells in the spleen and lungs after VVgp33 boost was significantly greater than that of cells primed by PR8 infection (Fig. 5C). The ΔNS1-primed and VVgp33-boosted effector cells mediated rapid

clearance of vaccinia virus from the lungs, although only slightly faster than that in mice containing WT PR8-primed $CD8^+$ T cells (Fig. 5D). This demonstrated that the cells remained responsive to antigen signals after primary immunization and were susceptible to boost vaccination to increase the size of the antigen-specific T-cell populations in the tissues.

We next wanted to more directly test the capacity of effector $CD8^+$ T cells primed with the mutant NS1 viruses to be boosted by a second, rapid immunization. Mice containing naive P14 $CD8^+$ T cells were infected with wild-type PR8 or ΔNS1 viruses and boosted with VVgp33 8 days later. The resulting populations of memory cells in the spleen and lungs of mice after boosting was significantly greater than that in mice immunized with just the primary dose of ΔNS1 (Fig. 5E). Similar results were also obtained in mice immunized with the NS1 truncated influenza viruses (NS1-73, NS1-113, and NS1-126) after boosting with VVgp33 (data not shown), suggesting that boosting was capable of improving the immune response to all mutant strains. This was in contrast to mice immunized with PR8, which did not demonstrate an increase in numbers of memory T cells in the spleen or lungs after VVgp33 boosting. Homologous boosting of the immune response with a second dose of the ΔNS1 virus also induced increased memory $CD8^+$ T responses in mice, which were significantly larger after two immunizations (Fig. 5F). Similarly, increased populations of endogenous LCMV gp33- and influenza virus np366-specific $CD8^+$ T-cell populations were also observed after ΔNS1 influenza virus prime-boosting in B6 mice that did not receive P14 cell transfers (data not shown).

To determine the protective capacity of these increased populations of antigen-specific memory $CD8^+$ T cells after prime-boost vaccination, mice were given two doses of ΔNS1 influenza virus 10 days apart and then rested for 8 weeks. The mice were then challenged by respiratory infection with the recombinant VVgp33 virus. Clearance of virus from the lungs was more rapid in mice containing ΔNS1-primed memory $CD8^+$ T cells (Fig. 5G). Similar results were observed in mice immunized with NS1-73, NS1-113 or NS1-126 viruses (data not shown). Thus, vaccination with attenuated influenza viruses expressing truncated or deleted NS1 proteins induced long-lived memory $CD8^+$ T-cell populations with the capacity to mediate rapid clearance of virus from the tissues.

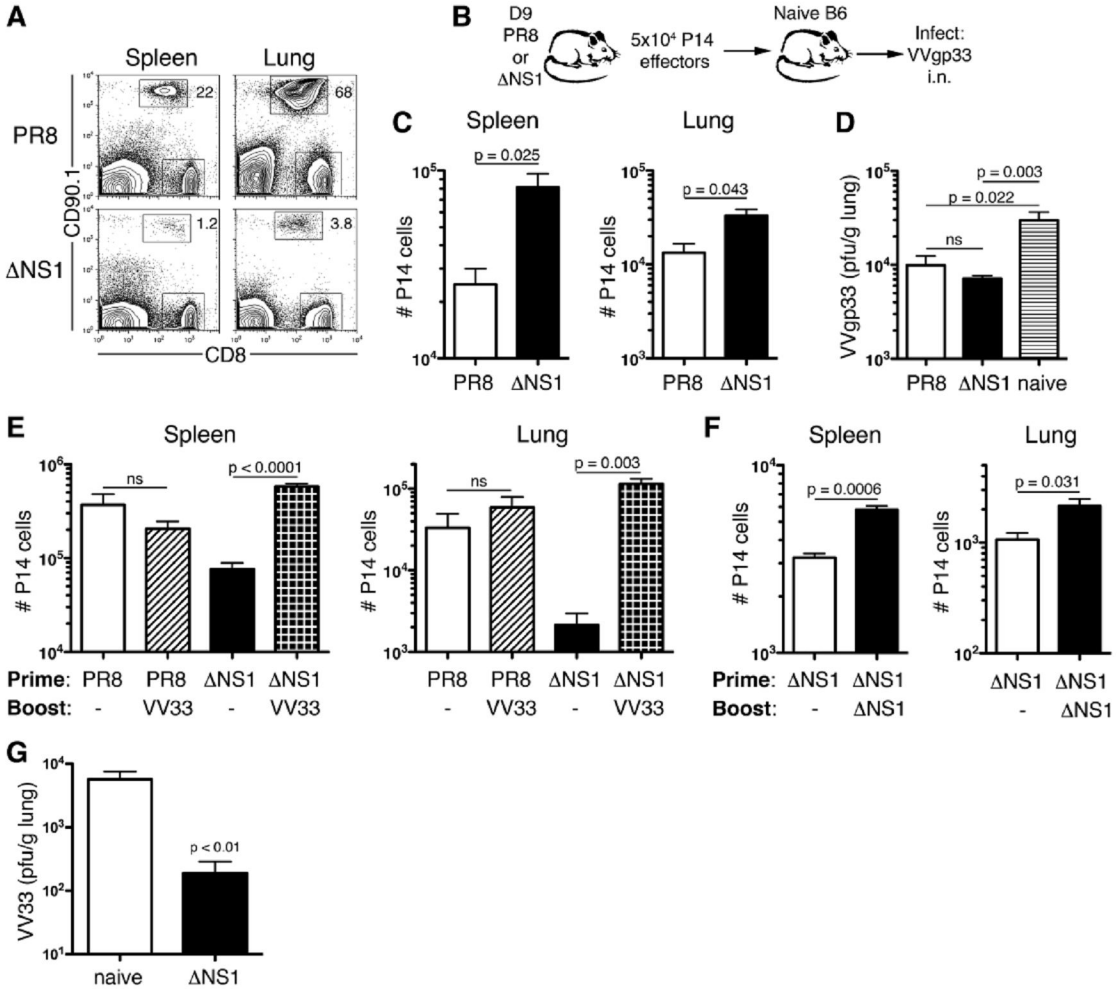

FIG. 5. Accelerated prime-boost responses soon after infection with NS1 mutant virus. (A) Example of effector P14 $CD8^+$ T-cell responses in the spleen and lungs 8 days after WT PR8 or ΔNS1 infection. (B) Day 9 P14 cells were isolated from mice infected with PR8 or ΔNS1 viruses and transferred into uninfected mice before intranasal boosting with VVgp33. (C) Expansion of the transferred CD8 T cells in the spleen and lungs. (D) Clearance of VVgp33 from the lungs, 5 days after infection. (E) Mice primed with PR8 or ΔNS1 were boosted 8 days later with VVgp33 as indicated, and the responses in the spleen and lungs were analyzed 6 weeks later. (F) Mice primed with ΔNS1 were boosted with ΔNS1 on day 8, and the memory responses were analyzed in the spleen and lungs. (G) Enhanced viral clearance from the lungs of mice immunized with ΔNS1 virus. Mice were immunized with ΔNS1 virus and boosted with ΔNS1 virus 10 days later, or control mice were given PBS. After 53 days, the mice were challenged with VVgp33 intranasally, and virus titers were measured in the lungs after 5 days (*n* = three to five mice per group). The results of one representative experiment of two to three are shown.

DISCUSSION

Influenza viruses can cause severe respiratory infection marked by high virus titers, fever, malaise, and weight loss. Recovery from infection is mediated by adaptive immune responses, which are generally very robust and long-lived. In contrast, immunization with vaccines composed of inactivated viral components, typically via the intramuscular route, may induce less effective cell-mediated immunity and require yearly administration to counter seasonal strains. The search for a universal influenza virus vaccine, which could generate long-lived immunity against heterologous virus strains, has recently focused on live attenuated viruses with the propensity to stimulate effective respiratory immunity without significant symptoms of disease. We generated live attenuated influenza A/PR/8/34 viruses expressing truncations or a deletion of the NS1 protein. The viruses were differentially attenuated in their growth both *in vitro* and *in vivo* in mice and yet all stimulated strong and specific $CD8^+$ T-cell immunity that was both long-lasting and capable of mediating faster clearance of virus from infected mice. Memory $CD8^+$ T-cell responses were primed in mice after infection with a ΔNS1 virus, which lacked expression of the NS1 protein and was undetectable in the lungs of mice after infection. Moreover, the attenuated influenza viruses induced T-cell responses that were amenable to very rapid boosting with heterologous or homologous viruses, thereby enabling the generation of enhanced, protective, memory populations.

Rapid prime-boost regimes may be beneficial for the administration of effective vaccines in humans since immunity could be generated fairly rapidly, with minimal time between doses. Boosting to increase immunity can require long intervals between immunizations to ensure effectiveness. If the T cells are not adequately rested, boosting during this period may have little effect (23). However, it has been shown that immunization with peptide-coated dendritic cells generated effector cells, or early memory cells, which were highly amenable to rapid boosting within 1 week (1). We demonstrate that immunization of mice with live attenuated influenza viruses with altered NS1 protein function was also able to induce T cells that were amenable to rapid boosting. This was particularly true of recombinant viruses with the least NS1 function (ΔNS1 and NS1-126). It also suggests that priming with more highly attenuated viruses, followed by a less attenuated strain, may be useful to induce strong immune memory, as well as minimizing detrimental symptoms from the vaccinating virus.

The search for new vaccine vectors for immunization against influenza viruses, particularly potential pandemic strains, is a topic of considerable research and debate. Recently, the use of live attenuated strains has been the focus of attention, particularly in relation to the current swine influenza outbreak (6). The NS1 mutant viruses utilized in the present study, in addition to having a reduced ability to replicate *in vitro* and *in vivo*, most likely also show reduced immunomodulatory effects due to their lack of NS1 function. NS1 functions, in part, as a negative regulator of host interferon responses, facilitating replication of the virus in IFN-I-producing cells. Lack of NS1 function in a viral vaccine vector

may have a number of benefits. Among these would be improved T-cell responses due to release of IFN-I (24). In addition, viruses with reduced NS1 function grow and spread considerably less aggressively, making them a safer option. However, if viral growth is too attenuated, antigen may be limiting. Nevertheless, despite showing a reduced ability to grow to high titers after infection in mice, the NS1 mutant viruses primed functional $CD8^+$ T-cell effector and memory responses. These responses provided protection against heterologous infections and were amenable to rapid prime-boosting to increase virus-specific memory T-cell populations. Together, these findings demonstrate the potential effectiveness of such attenuated viruses as influenza virus vaccine vehicles for generating cell-mediated immunity.

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FOOTNOTES

* Corresponding author. Mailing address: Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322. Phone: (404) 727-3571. Fax: (404) 727-3722. E-mail: rahmed@emory.edu

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†S.N.M. and W.A.L. contributed equally to this study.

‡Present address: Department of Microbiology and Immunology, The University of Melbourne, Parkville 3010, Australia.

§Present address: Department of Gene Therapy and Hepatology, CIMA, University of Navarra, Pio XII, 55 Pamplona 31008, Spain.

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Chapter 4

Induction of neutralizing antibody responses to anthrax PA using broadly-applicable influenza vectors: Implications for disparate immune system priming pathways

The work presented in this chapter has been submitted for publication in the *Journal of Virology*.

Induction of neutralizing antibody responses to anthrax PA using broadly-applicable influenza vectors: Implications for disparate immune system priming pathways

William A. Langley[1], Konrad C. Bradley[1], Mary Ellen Smith[2,3], Matthias J. Schnell[2,3*], and David A. Steinhauer[1*]

[1]Department of Microbiology and Immunology, Emory University Medical School, 1510 Clifton Road, Atlanta, GA 30322; Departments of Microbiology and Immunology[2], Medicine[3], Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA, USA.

[*]Correspondence:

D.A. Steinhauer

tel: 404-712-8542

email: dsteinh@emory.edu

M.J. Schnell

tel: 1-215-503-4634

email: Matthias.Schnell@jefferson.edu

running title: influenza vectors for priming immunity

ABSTRACT

Viral vectors based on influenza virus, rabies virus (RV), and vaccinia virus (vv) were used to express large polypeptide segments derived from the *Bacillus anthracis* protective antigen (PA). For the infectious influenza vector and recombinant vv constructs, the receptor binding domain (RBD or Domain 4) or the lethal and edema factor binding domain (LEF or Domain 1') were engineered into functional chimeric hemagglutinin (HA) glycoproteins. In the case of the RV vector, the viral glycoprotein (G) was used as a carrier for RBD in an inactivated form of the vector. These constructs were examined using multiple homologous and heterologous prime/boost immunization regimens in order to optimize the induction of α-PA antibody responses. Several immunization combinations were shown to induce high titers of antibody recognizing the anthrax RBD and LEF domains as well as the full-length PA protein in mice. The heterologous prime/boost immunization regimens that involved an initial intranasal administration of a live influenza vector, followed by an intramuscular boost with either the killed RV vector or the vv vector, were particularly effective, inducing antigen-specific antibodies at levels several-fold higher than homologous or alternative heterologous protocols. Furthermore, sera from several groups of the immunized mice demonstrated neutralization activity in an *in vitro* anthrax toxin neutralization assay. In some cases such toxin-neutralizing activity was notably high, indicating that the mechanisms by which immunity is primed by live influenza vectors may have beneficial properties.

INTRODUCTION

Bacillus anthracis (BA) is the causative agent of anthrax, a disease that can be fatal in humans, and a continuing concern regarding potential acts of bioterrorism (22). Presently, there is only one FDA approved vaccine against anthrax available in the United States, the anthrax vaccine absorbed (AVA). AVA is composed of culture filtrate of an avirulent non-capsulated form of BA, and it requires five or six immunizations over an 18 month period, as well as yearly boosts, to retain its effectiveness (39). One limitation of the AVA vaccine is that the intramuscular (IM) injection method of inoculation does not induce strong mucosal antibody responses and mucosal surfaces would likely be the sites where initial contact with BA would occur in the event of a deliberate release of the organism (5, 21). In addition, lot-to-lot variability in composition and adverse reactions to the vaccine have also been observed (26). For these reasons, the present vaccine is less than ideal, and would be impractical should anthrax reemerge in future acts of bioterrorism.

Protection against anthrax is thought to be mediated primarily by antibodies to the bacterial toxin, which is composed of three polypeptide subunits, the protective antigen (PA), the lethal factor (LF), and the edema factor (EF) (40). Most neutralizing antibodies target the PA subunit, which is responsible for binding to host cell receptors as well as the LF and EF toxin subunits. The PA protein is composed of 735 amino acids that fold into four structurally distinct domains (Figure 1A) (31). Two of these, domain 1 and

domain 4, have been shown to be specifically targeted by neutralizing antibodies (13, 40). Domain 1 consists of amino acid residues 1-258, the first 167 of which are cleaved by the protease furin after the binding of PA to the host cell receptor, leaving domain 1' (31). Domain 1' is responsible for binding the LF and EF subunits, and is therefore referred to as the lethal factor and edema factor binding domain, or LEF (43). Domain 4 is 140 amino acids in length and is designated as the receptor binding domain (RBD), as it contains the determinants for binding to the host cell receptors, tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) (6, 27, 31, 33). Over the past several years, a number of strategies have been explored to express or deliver PA, or components of it, in efforts to improve the costs, logistics, and efficacy of anthrax vaccines (for a review see (15)). However, to date, none of these strategies have demonstrated sufficient efficacy to replace the AVA.

In contrast to the AVA, influenza vaccines elicit strong mucosal and systemic responses as early as 2-6 days post-vaccination, and are produced annually on a large scale in both live and inactivated forms (8, 42). Both formulations have been used extensively in humans with minimal adverse events being reported (12). Additionally, responses to these vaccines have been shown to encompass both the humoral and cellular branches of the immune system (4, 7). The majority of neutralizing antibodies generated by influenza vaccines recognize the hemagglutinin glycoprotein (HA) on the viral surface and this protein has also been reported to have adjuvant-like effects when co-administered with virus-like particles (7, 24).

Similar to influenza vaccines, the RV vaccine also elicits strong humoral and cellular responses in vaccinated hosts (10-11, 28-29), and has a safe history of use in humans as an inactivated vaccine (9, 18, 37). The rabies virus encodes a single envelope glycoprotein (G) which has functions analogous to the influenza HA protein, and antibodies raised against the glycoprotein have been shown to be neutralizing and long-lasting (38).

With the goal of developing improved strategies for immunization against anthrax, studies were initiated to assess the antigenic properties of influenza and RV vectors expressing chimeric glycoproteins containing functional domains of the *Bacillus anthracis* PA. Initial studies demonstrated that chimeric HA proteins were able to express on cell surfaces in native oligomeric form and maintain HA functions required for host cell entry. Infectious influenza viruses containing these chimeric HA/PA proteins were also able to replicate to titers similar to WT viruses and the inserts were found to remain genetically stable over multiple rounds of replication. In addition, mice immunized with a single intranasal (IN) inoculum of these chimeric viruses generated high antibody titers specific for the inserted PA fragments. Furthermore, immunization with chimeric plasmids containing the recombinant HAs elicited stronger antibody responses against these domains than plasmids containing the anthrax domains alone suggesting that the HA does have an adjuvant effect (25).

Similarly, initial studies using chimeric RV G proteins containing the RBD domain from *Bacillus anthracis* PA showed that these chimeric proteins were able to incorporate into virus particles and that mice immunized with live or

inactivated forms of the vector mounted antibody responses against the inserted RBD domain. The humoral responses measured in mice after a single inoculation with this vector were similar to those seen in mice that were immunized with 150 times that amount of recombinant PA alone. This vector was also shown to stimulate a Th2 type response when given at doses of 50 ng or more (36). However, despite the induction of high levels of antibodies specific for PA by both the influenza and RV vectors, we were unable to detect anthrax toxin neutralization activity in the sera of immunized mice using *in vitro* assays when antigens were administered in homologous fashion.

Therefore, the purpose of the present study was to examine quantitatively and qualitatively, the antibody responses induced by these viral vectors using alternative prime/boost strategies. In addition to the influenza and RV vectors described above, recombinant vv was engineered to express the chimeric HA/PA proteins and used to optimize the immunization protocols and aid in defining our interpretations. We confirmed that each vector was capable of inducing specific antibody responses and that a second inoculation with the homologous constructs yielded moderate antibody boosting effects. However, we observed a striking increase in PA-specific antibody titers following heterologous prime/boost approaches when using the influenza vector as the primary immunogen. Furthermore, using an *in vitro* toxin neutralization assay, we found that the immunization protocols involving influenza priming and heterologous vector boosting induced particularly high neutralizing antibody titers against the inserted domains. These results suggest that immunization regimens involving a primary

immunization with live influenza vectors may induce initial immune response pathways that are advantageous for the production of antibodies directed against certain pathogens. Such broadly applicable approaches, and the mechanisms by which they operate, may warrant consideration for the design of novel vaccination strategies.

MATERIALS AND METHODS

Construction of plasmids and viral vectors. The plasmids used for constructing the viral vectors utilized in this study, as well as the generation and growth of these viral vectors, has been described previously (25, 36). Briefly, for the chimeric LEF/HA and RBD/HA constructs, the HA gene from the H3 subtype influenza A strain A/Aichi/2/68 was used as the backbone, and the *B. anthracis* domain 1' (LEF) and domain 4 (RBD) regions of the PA gene were constructed by single-chain oligonucleotide extension with codons optimized for mammalian usage (16). The chimeric genes were initially cloned into pRB21 to generate recombinant vaccinia viruses for protein expression and functional studies (3), and these vv vectors were subsequently used for immunization studies as well. Recombinant infectious influenza viruses were generated using the 17 plasmid system initially described by (30). For the rabies construct, anthrax PA D4 was amplified from pGEM PA63 and cloned into pBS-STS to yield pD4-ΔED (34). The RV ectodomain fragment was ligated into pD4-ΔED resulting in pD4-E51. The

recombinant RV expressing pD4-E51 was digested with *Bsi*WI and *Nhe*I and the D4-51 fragment was ligated to pSPBN, resulting in pSPBN-D4-E51. Recombinant rabies viruses were recovered using standard methods (14).

Immunizations. Female Balb/C mice (6 to 8 weeks old) were purchased from Jackson Laboratories. The mice were infected with $1x10^5$ pfu of the recombinant influenza viruses I.N., $3x10^6$ pfu of the recombinant vaccinia viruses I.P., or 10 μg of inactivated RV virus SPBN-D4-E51 i.m. (5 μg in each hind leg). For the mixed infections, the total pfu and routes of infection were the same. Influenza viruses for the IM immunizations were inactivated by treatment with β-propiolactone (23). The mice were bled via cheek bleed at the times indicated and the sera were collected after spinning at 13,000 RPM for ten minutes in a tabletop centrifuge. The sera were stored at -80° C until use.

ELISA. ELISA assays were performed as described previously (25). Briefly, NUNC MaxiSorp plates (cat # 442404) were coated with 2.5 μg per well of recombinant PA, RBD, or LEF (obtained from BEI Resources) or standard amounts of mouse IgG diluted in BSS buffer and kept at 4° C overnight. The following day, serum was added at different concentrations and the plates were washed three times with PBS 0.2% Tween 20 before HRP conjugated goat □-mouse IgG was added (Sigma cat # A4416). SigmaFast OPD tablets (cat # P9187) were used to develop the plates. The reactions were stopped with 0.1 N

H_2SO_4 and the plates were read at 490 nm. Standard curves were constructed from the IgG standards and antibody concentrations were calculated from these.

Toxin Neutralization Assay. Toxin neutralization assays were performed as previously described (20). Sera from the different groups of mice were pooled, serially diluted and pre-incubated with 250 ng/ml rPA and 50 ng/ml lethal factor (final concentrations). After 30 minutes, these were added to 96-well plates containing J774A.1 macrophage cells for three hours. 25 μl of 5 mg/ml MTT dissolved in PBS was then added to the wells for two hours at 37 C at which time DMF/SDS was added to each well to lyse the cells. The plates were incubated overnight and then read at 570 nm the next day. The concentration of serum that protected 50% of the cells was determined by comparing to wells that did not receive toxin.

RESULTS AND DISCUSSION

Design of chimeric proteins and generation of viral vectors. The constructs involved in the generation and expression of the chimeric PA proteins analyzed in this study are outlined in Figure 1. Figure 1a shows the structure of the PA protein and highlights the individual domains. Based on structural considerations, coding regions for 90 residues corresponding to the LEF domain, and 140 residues corresponding to the RBD domain were cloned for insertion

into chimeric proteins. These were inserted near the N-terminus of the HA protein (Figure 1b), as it has been shown that this region of the protein is able to accommodate large polypeptide insertions in both H3 and H7 subtype HAs (17, 25). The constructs were designed to express the inserted domains at the N-terminus of the HA ectodomain following cleavage of the signal peptide (Figure 1c). This site was selected because it is believed to best support the folding of the inserted polypeptide domains, as well as the HA backbone, into native structures based on structural observations.

Initially, the HA/RBD and HA/LEF proteins were expressed using recombinant vaccinia viruses to verify cell surface expression, folding, and functional properties of the chimeric proteins. These vv recombinants were also used for some of the immunization regimens described below. The same chimeric HA/RBD and HA/LEF proteins were also used to generate infectious recombinant influenza viruses, which were shown to remain genetically stable and replicate to high titers in cell culture or embryonated eggs (25). For the rabies virus vector used in this study, the RBD domain was inserted into the rabies virus glycoprotein as an N-terminal replacement for most of the ectodomain (Figure 1d). As mentioned previously, these chimeric proteins have been shown to incorporate efficiently into infectious virions (36).

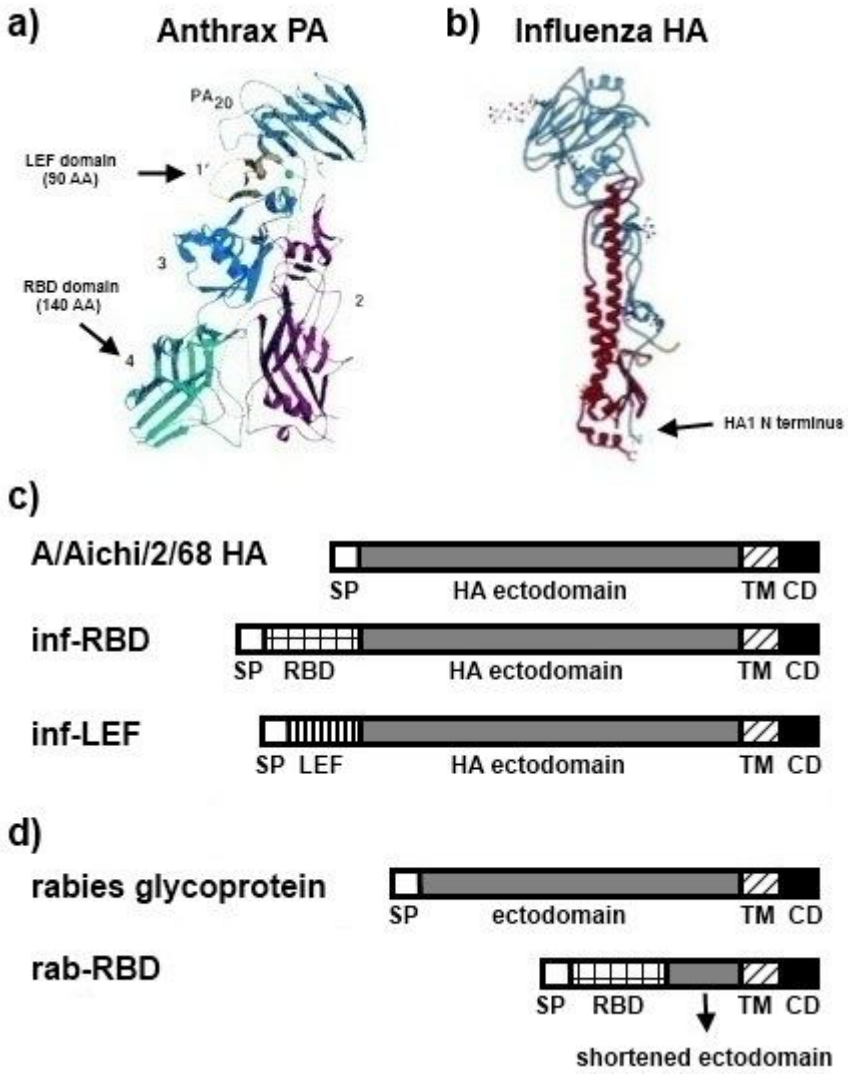

Figure 1. Bacillus anthracis protective antigen and vector constructs. a) PA structure showing the location and size of the LEF and RBD domains (31). b) Structural depiction of the influenza HA indicating the insertion site of the PA domains. c) Schematic diagram depicting the wildtype HA from A/Aichi/2/68 as well as the organization of the constructs with respect to location of the PA domains in the primary amino acid sequence. d) Schematic diagram depicting the wildtype rabies glycoprotein and the organization of the RBD domain in the truncated rabies G protein. For a more detailed description refer to (36). Inf = influenza, SP = signal peptide, TM = transmembrane domain, CD = cytoplasmic domain, rab = rabies

Immune responses using homologous or heterologous prime/boost regimens. For the experiments involving influenza viral vectors, mice were inoculated intranasally (i.n.), and for the experiments involving inactivated RV mice were inoculated intramuscularly (i.m.). Groups of six mice were pre-bled, then inoculated with the influenza vector containing the chimeric HA/RBD protein (inf-RBD), the influenza vector with the chimeric HA/LEF protein (inf-LEF), a mixture of the two recombinant influenza viral vectors (inf-MIX), the rabies virus containing the RBD domain (rab-RBD), or vaccinia viruses expressing either HA/LEF (vv-LEF) or a mixture of HA/LEF and HA/RBD (vv-MIX). Mice were bled at day 28, and bled, then boosted on day 42 with either the homologous vector or a heterologous vector. Following the boost on day 42, blood was taken weekly up to day 77 (35 days post-boost).

Sera from immunized mice were analyzed by ELISA to evaluate IgG reactivity to purified His-tagged LEF domain recombinant protein, His-tagged RBD domain recombinant protein, or complete recombinant PA protein. The data for these are shown in Figures 2 and 3. Specifically, Figure 2 shows the data for the influenza and RV prime/boost experiments. The data for reactivity to each antigen are displayed on separate graphs for homologous (Fig. 2a-c) or heterologous (Fig. 2 d-f) boost experiments to allow data points to be more easily distinguished. The data in Figure 2a show that the antibody titers against the RBD domain following the homologous prime/boost regimens were specific, as there is no titer observed for the mice inoculated with the LEF constructs. The immunizations with inf-RBD alone or a mixture of inf-RBD and inf-LEF gave

similar results, and provided for the higher initial antibody responses than the rab-RBD vector, as measured on days 28 and 42 following inoculation. However, the boost effect was greatest for mice primed with inf-RBD or inf-MIX and boosted by intramuscular inoculation with the RV vectors, which resulted in titers of about 10^4 ng/ml of IgG in sera following the boost. The reason for the rather moderate boost effect observed with the homologous influenza vector regimen is not known, but may result from immunity to the influenza virus generated during the priming phase leading to the inhibition of replication with this live virus vector during the boosting phase.

As shown in Figure 2b, all vectors were capable of inducing antibodies against purified recombinant PA protein. The results following homologous prime/boost regimens again show that the influenza vectors provided for higher initial responses, but the homologous RV protocol imparted a larger boosting effect. The α-PA data also highlight that the inf-LEF, as well as the inf-RBD, can induce significant antibody responses to intact PA. The ELISA data showing the reactivity of sera to purified His-tagged LEF protein (Figure 2c) indicate that the antibody response induced specifically to this domain are quite robust, even following a single inoculation. All influenza constructs also induced sizable antibody responses to the HA component of the chimeric protein (25) (data not shown).

The IgG antibody responses induced following heterologous prime/boost regimens are shown in Figures 2d, 2e, and 2f. The striking feature of these results involves the magnitude of the antibody responses induced by priming with

the influenza vectors followed by boosting with the RV vectors, which reached titers on the order of 10^5 ng/ml. This was approximately one log higher than those observed for the reciprocal RV/influenza regimen, and about four-fold higher than determined for homologous prime/boost experiments with the rabies constructs alone. This suggests that the influenza vectors are advantageous over the other vectors tested with respect to the mechanism of immune system priming. This could be due to several things, such as its properties as a live replicating vector, the route of inoculation in the respiratory tract, or the types of antigen presenting cells in these tissues. Single inoculations with the influenza-LEF viruses once again generated strong α-LEF responses, but, as expected, were not boosted by the rabies virus containing the RBD domain.

Although boosting with homologous viruses resulted in modest increases in antibody titers, the more striking results observed in influenza primed-rabies boosted mice may suggest that a heterologous boost is needed in order to avoid preexisting immunity to the original vector. This hypothesis is further supported by the ELISA data shown in Figure 3 that was obtained after boosting with the recombinant vaccinia viruses described above, which express chimeric HA/RBD or HA/LEF proteins equivalent to those in the influenza vectors. These data indicate that the vv vectors were able to boost the α-PA and α-RBD antibody titers to levels similar to those seen with the influenza primed-rabies boosted groups. The enhanced boost effect was also observed with the mice that were primed with inf-LEF and then boosted with vv-LEF, for which the serum titers were almost a log higher than those primed and boosted with inf-LEF alone (Fig.

3b). These results suggest that a heterologous boost is necessary in order to achieve these high antibody levels.

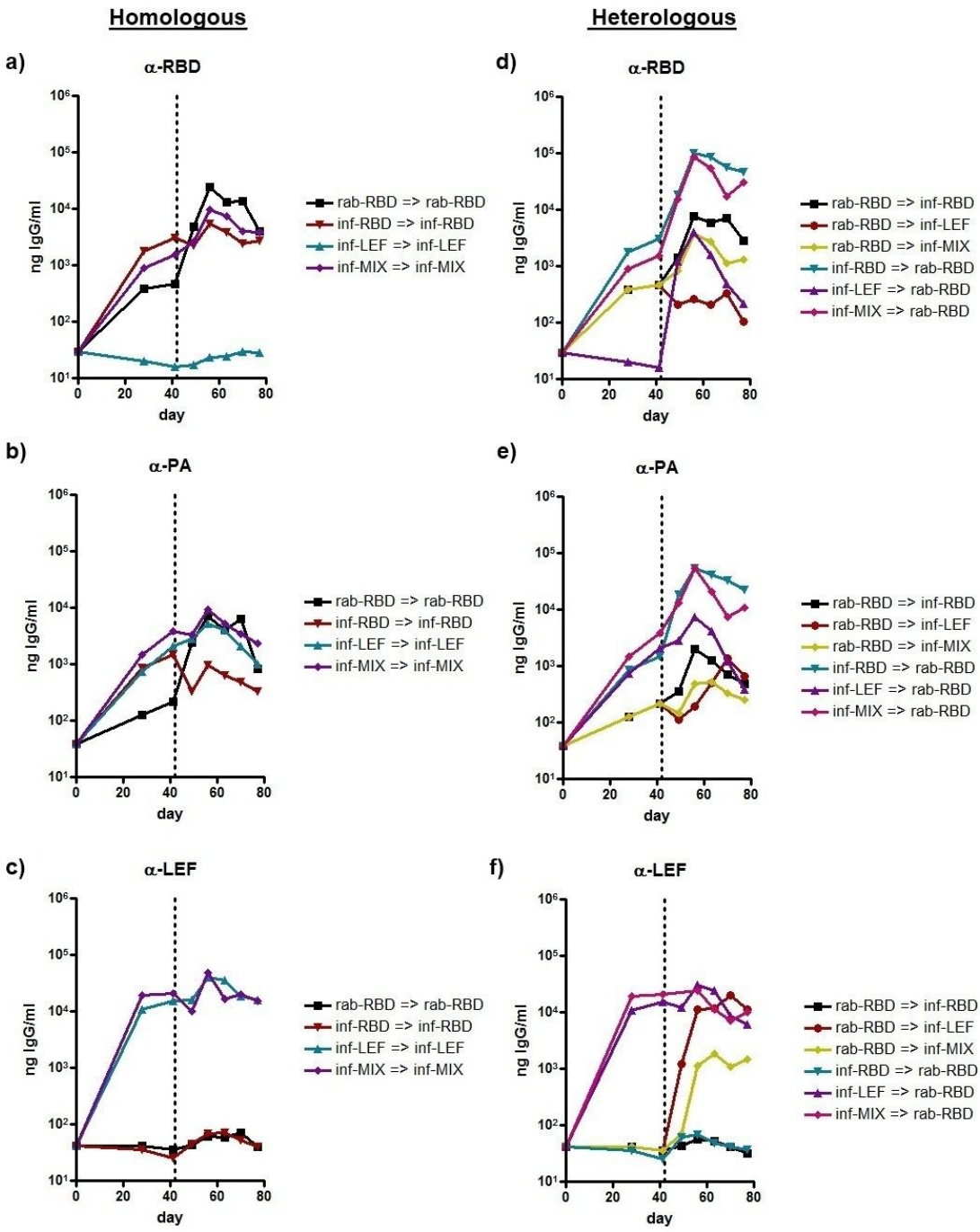

Figure 2. Antibody responses following immunization with viral vectors. ELISA titers determined using purified recombinant PA, or His-tagged RBD or LEF proteins as antigen. a) α-RBD, b) α-PA, or c) α-LEF antibody titers in mice before and following boosting on day 42 with homologous vectors. d) α-RBD, e) α-PA, or f) α-LEF antibody titers in mice before and following boosting on day 42 with heterologous vectors.

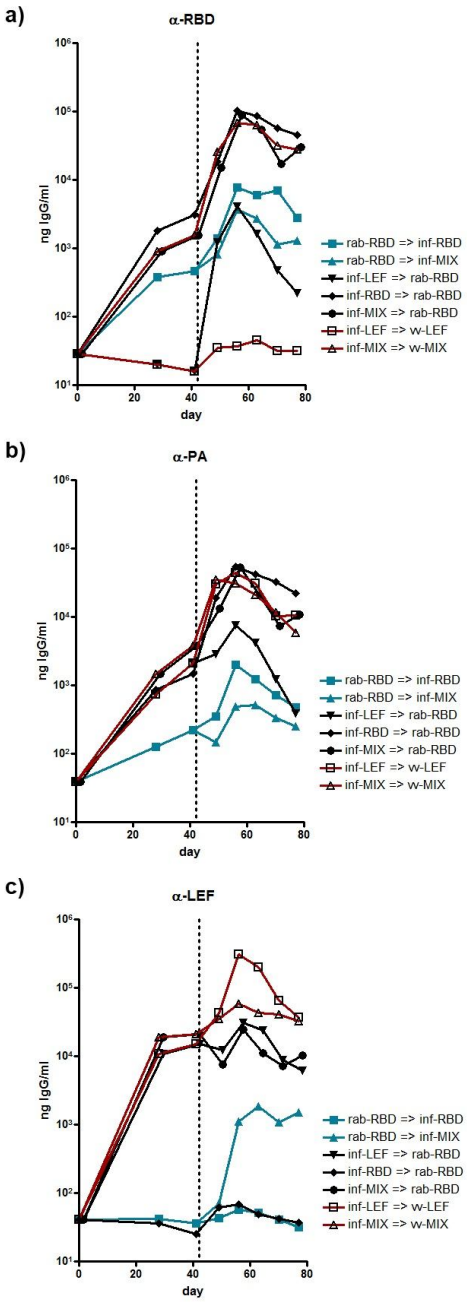

Figure 3. Antibody responses following heterologous boosting. a) α-RBD, b) α-PA, or c) α-LEF antibody titers in mice before and following priming and boosting with the indicated vectors.

Boost effects using alternative routes of inoculation. To examine whether the route of inoculation is responsible for the boost effects observed in Figure 2d-f and Figure 3, groups of mice were primed with inf-RBD IN or rab-RBD IM and then boosted IM with one of these two vectors on day 51. The results from these regimens are shown in Figure 4. Naïve mice immunized with either rab-RBD IM or β-propiolactone inactivated inf-RBD IM showed similar antibody titers against RBD, demonstrating that the influenza vectors are immunogenic when given by this route. However, antibody levels were only slightly increased in mice that were primed with rab-RBD IM or inf-RBD IN and then boosted with inf-RBD IM. In this experiment, high levels of α-RBD antibodies were again observed with the influenza IN/rabies IM regime, confirming the data from the experiments detailed in Figure 2. These data suggest that it is unlikely that the route of inoculation alone is responsible for the increased titers observed in the inf-RBD primed/rab-RBD boosted mice. Perhaps in inactivated form, the rabies vector displays the antigen more effectively, or persists for a longer period of time.

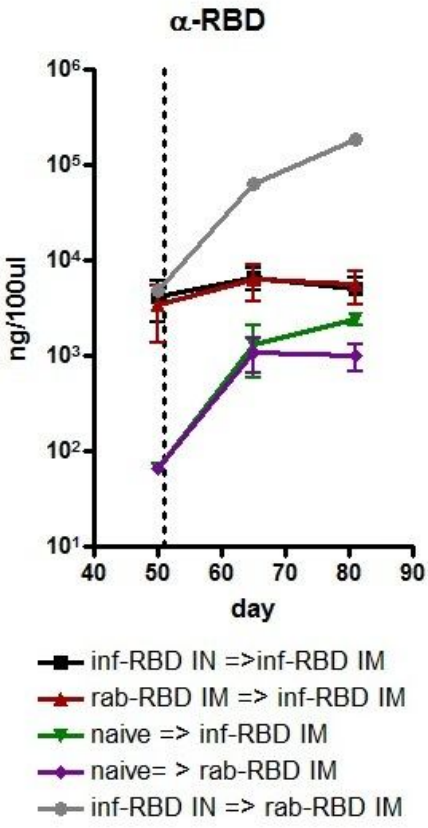

Figure 4. Antibody responses following intramuscular boosting. α-RBD antibody titers in mice before and following intramuscular boosts with the indicated vector on day 42.

***In vitro* toxin neutralization.** The ability of serum obtained in the experiments detailed in Figures 2 and 3 to neutralize anthrax toxin *in vitro* was assessed using an MTT cell viability assay with J774 macrophage cells (32). In this assay, a neutralization titer greater than 100 is considered positive and anything less than that is reported as <100. The results of this assay using pooled sera from different groups of mice taken at day 28 post-prime and days 56 or 77 (days 14 and 35 post-boost) were analyzed and are shown in Table 1. No neutralization was observed for mice that were primed with rab-RBD regardless of the time-point or immunization protocol. Similarly, no neutralization was seen in mice that were primed with the inf-LEF vector, even though the mice that were boosted with vv-LEF displayed very high α-LEF titers (Figure 3). This suggests that α-LEF antibodies alone may not be neutralizing in a BALB/c mouse background. In contrast, neutralizing activity was detectable with mice that were primed with a mix of inf-LEF and inf-RBD (inf-MIX) and boosted with either rab-RBD or a mix of vv-LEF and vv-RBD (vv-Mix). The only group that demonstrated any neutralization activity prior to being boosted was the inf-RBD primed group. This is not surprising as it correlates with the slightly higher antibody titers observed in these mice by ELISA at this time point (Figures 2, 3, and 4). Neutralizing activity remained detectable at day 14 following a homologous boost with inf-RBD, but had waned by day 35. The most striking results can be seen in the mice that were primed with inf-RBD and then boosted with rab-RBD. These mice had a neutralizing titer of 1038 on day 14 post-boost, and this increased to 7039 by day 35. The observation that the neutralization titers increased for this

group, while the total specific antibody detectable by ELISA decreased (Figure 2) is noteworthy. Potential explanations for this may involve B cell affinity maturation or somatic hypermutation in germinal centers during this time. As noted above, it is also possible that persistence of antigen with the RV vector could play a role as well.

Although the α-LEF antibodies were not shown to be neutralizing in these experiments, the correlates of toxin neutralization from challenge by live BA appear to vary with the animal model and genetic background. Indeed, α-LEF antibodies have been shown to be protective in C57BL/6 mice immunized with a purified form of this domain, and two mAbs with toxin-neutralizing activity were shown to recognize distinct epitopes within this domain (2). The lack of neutralizing ability with α-LEF sera observed here could be attributed to the use of BALB/c mice, as the responses to all domains have been demonstrated to be variable depending on the genetic background of immunized mice (1). Using a killed, but metabolically active (KBMA) form of BA, Skoble et al. demonstrated protection of mice against live spore challenges even though the □-PA antibodies in the sera were measured at levels of approximately $1x10^3$ ng/ml (35). This concentration is considerably lower than the antibody titers generated in our study, although other factors were also likely to be involved in the protective effects of the KBMA vaccine. Other studies suggest that the mouse model utilized here is not ideal for animal protection studies, particularly with regard to the role of α-PA responses (19, 41). Further examination of our constructs and immunization protocols in alternative animal models, for both the induction of

toxin neutralization activity as well as protection from a BA spore challenge, are being planned.

Importantly, this study demonstrates that simultaneous immunization with two homologous vectors, one containing the LEF domain and the other the RBD domain, results in antibodies against both domains and that both responses can be boosted by heterologous vectors (Figure 3). This could prove beneficial when immunizing more heterogeneous populations, including humans who have multiple MHC alleles. As with any vector-based immunization approach, preexisting immunity to the vector must be considered. Rabies has low seroprevalence in humans, and the expanding structural knowledge of the 16 HA subtypes should make it possible to construct influenza vectors with subtypes to which humans have no preexisting immunity. Indeed, pre-existing immunity to influenza vectors may not pose the problems that are envisaged, as drift strains appear capable of re-infecting individuals despite minimal changes to antigenic sites of HA. Nonetheless, the efficacy of alternative constructs in mice that have been previously infected with different influenza subtypes is ongoing.

It is interesting to note that none of the sera from mice primed with rab-RBD demonstrated neutralization capacity, regardless of the immunization protocol. This suggests that the mechanism by which intranasal infection by live influenza viruses prime the immune system in this mouse model provides a desirable effect. Several factors could be responsible for this, including the properties of a live vector versus an inactivated vector, or possibly the species or environment of antigen presenting cells encountered in the respiratory tract.

These issues merit further examination, as they may be relevant for the design of immunogens unrelated to those examined in this study. Furthermore, the utility of employing chimeric influenza and rabies virus vectors such as those described here, is not limited to a single pathogen, but can be broadly applicable.

Anthrax toxin neutralizing titers

Priming virus (day 0)	day 28 titer	Boost virus (day 42)	day 56 titer	day 77 titer
rabies-RBD	<100	rabies-RBD	<100	<100
		inf-RBD	<100	<100
		inf-LEF	<100	<100
		inf-mix	<100	<100
inf-RBD	166	rabies-RBD	1038	7039
		inf-RBD	163	<100
inf-LEF	<100	rabies-RBD	<100	<100
		inf-LEF	<100	<100
		vv-LEF	<100	<100
inf-mix	<100	rabies-RBD	165	241
		inf-mix	<100	<100
		vv-mix	165	<100
WT H3N1	<100			
Naïve	<100			

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Chapter 5

The effects of preexisting immunity to influenza on responses to influenza vectors in mice

The work presented in this chapter has been submitted for publication in *Vaccine*.

The effects of preexisting immunity to influenza on responses to influenza vectors in mice

William A. Langley [a], Zhu-Nan Li [a,b], Konrad C. Bradley [a], Ganesh R. Talekar [a], Summer E. Galloway [a], David A. Steinhauer [a*]

[a]Department of Microbiology and Immunology, Emory University Medical School, 1510 Clifton Road, Atlanta, GA 30322

[b]Current address: Influenza Division, National Center for Immunization and RespiratoryDiseases, Centers for Disease Control and Prevention, Atlanta, GA 30333

[*]Correspondence:

D.A. Steinhauer

tel: 404-712-8542

email: dsteinh@emory.edu

Abstract

The use of viral vectors as vaccine candidates has shown promise against a number of pathogens. However, preexisting immunity to these vectors is a concern that must be addressed when deciding which viruses are suitable for use. A number of properties, including the existence of antigenically distinct subtypes, make influenza viruses attractive candidates for use as viral vectors. Here, we evaluate the ability of influenza viral vectors containing inserts of foreign pathogens to elicit antibody and $CD8^+$ T cell responses against these foreign antigens in the presence of preexisting immunity to influenza virus in mice. Specifically, responses to an H3N1-based vector expressing a 90 amino acid polypeptide derived from the protective antigen (PA) of *Bacillus anthracis* or an H1N1-based vector containing a $CD8^+$ T cell epitope from the glycoprotein (GP) of lymphocytic choriomeningitis virus were evaluated following infections with either homosubtypic or heterosubtypic influenza viruses. We found that mice previously infected with influenza viruses, even those expressing HA and NA proteins of completely different subtypes, were severely compromised in their ability to mount an immune response against the inserted epitopes. This inhibition was demonstrated to be mediated by $CD8^+$ T cells, which recognize multiple strains of influenza viruses. These $CD8^+$ T cells were further shown to protect mice from a lethal challenge by a heterologous influenza subtype. The implication of these data for the use of influenza virus vectors and influenza vaccination in general are discussed.

1. Introduction

Vaccination strategies involving viral vectors have continued to expand and evolve over recent years to target many pathogens, including some for which vaccine development has otherwise proven to be problematic. Replicating vectors elicit immune responses, both humoral and cellular, that are greater in magnitude and longer lasting when compared to many non-replicating vaccines [1-4]. This enhanced immunity is likely due to several factors, including the ability of viral vectors to display the antigens in their native state on viral surfaces, desirable for B cell responses, and the ability of host antigen-presenting cells (APCs) to display viral epitopes derived from proteins synthesized in their cytoplasm in the context of major histocompatibility complex (MHC) molecules, a requisite for effective $CD8^+$ T cell responses [5].

A wide variety of viruses are currently being developed for use as vaccine vectors, including adenovirus, canarypox virus, rhabdoviruses, measles virus, and modified vaccinia virus Ankara, to name just a few [6-10]. A number of factors must be taken into account when choosing which viruses may be optimal to develop as vectors. Viral vectors must be safe, genetically stable, immunogenic, and efficiently targeted to the appropriate tissue, whether directed by vector tropism or the route of inoculation by which it is administered. Another fundamental consideration for vaccine vector suitability is preexisting immunity to the vector itself. Populations are likely to have immunity to viral vectors that they have been exposed to by natural infection or by vaccination, and this preexisting

immunity is likely render these vectors ineffective or at least less effective than those to which no prior immunity exists.

Influenza virus has many properties that make it a viable candidate for use as a viral vector. Infection with influenza virus elicits robust humoral and cellular immune responses in hosts [2, 11-13]. Influenza vaccines have an extensive record of effectiveness and safety in the human population and are manufactured in both live and inactivated forms annually [14-16]. A reverse genetics system has been developed for influenza which facilitates the manipulation of individual viral segments and allows for the generation of recombinant influenza strains [17]. In addition, extensive knowledge of both the structure and function of the hemagglutinin (HA) glycoprotein exists [18-21] and we have previously shown that it is able to accommodate large insertions of foreign proteins while maintaining its normal functions. When expressed in recombinant influenza viruses, these chimeric proteins are able to elicit antibodies against the inserted fragments [22]**.** Furthermore, the HA protein has been reported to have an adjuvant effect when administered as a component of virus-like particles containing epitopes derived from simian immunodeficiency viruses [23].

Although influenza infections are common in humans, infecting 5-20% of Americans each year [24], immunity to influenza viruses is largely mediated by antibodies against the HA and neuraminidase (NA) glycoproteins, both of which continually undergo genotypic and phenotypic changes in response to host selective pressures. This progressive modification of the antigenic properties of

viral surface proteins often necessitates reformulation of influenza vaccines in order to maximize immune protection.

Based on non-overlapping reactivity with polyclonal antisera, sixteen different HA subtypes and nine distinct NA subtypes have now been identified to circulate in waterfowl, the natural host of the virus. Importantly, immunity against one subtype does not confer immunity to the others [25]. Of the existing HA and NA subtypes, only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have become established in humans during the past century, suggesting that it might be possible to develop influenza vectors for use in humans that express HA and NA subtypes to which preexisting immunity would not be present [25-26].

Although a comprehensive analysis of data from the recent pandemic of H1N1 viruses may provide new insights when fully developed, the extent to which subtype cross reactivity can protect or ameliorate disease symptoms in humans is not well understood. It is known that individuals are susceptible to repeated infection, not only with novel subtypes, but with viruses of the same subtype that have undergone antigenic drift. In efforts to understand this, and to evaluate vaccine candidates in general, a number of animal models have been utilized for studies on influenza virus replication and immune responses. Among these, ferrets have long been considered the best models, and Guinea pigs and hamsters have been proposed as preferable alternatives for some purposes [27-28]. However, due to their size, expense considerations, and the detailed knowledge of the genetics of immune responses, the mouse model has been utilized extensively [29]. A more detailed understanding of the effects of

preexisting immunity to influenza viruses on subsequent infections with the virus, and on the capacity of influenza vectors to present antigen in the face of it, will be useful for animal models as well as humans.

Previous work from our group showed that recombinant influenza viruses expressing chimeric HA proteins containing the lethal and edema factor binding domain (LEF) from the protective antigen (PA) of *Bacillus anthracis* toxin induced robust antibody responses in mice that were specific for the inserted domain [22]. A more recent study demonstrated that the responses generated by these vectors were not significantly enhanced following a second inoculation with the homologous vectors, but could be markedly boosted by heterologous rabies or vaccinia vectors (Langley et al., submitted). In addition to our studies, there have been numerous other reports highlighting the effectiveness of influenza virus vectors as vaccine candidates for other pathogens [22, 30-33]. None of these, however, to our knowledge, have examined the effect of preexisting immunity to heterosubtypic influenza viruses on their ability to elicit immune responses. In the present study, we examine the effect of preexisting homosubtypic or heterosubtypic immunity to influenza virus on the ability to mount an immune response to an influenza vector with a chimeric HA protein containing the 90 amino acid LEF domain from the protective antigen of *Bacillus anthracis*. Our results show that infection of mice with influenza viruses, regardless of the subtype, severely inhibits their ability to generate an immune response against the inserted LEF domain. In addition, the data demonstrate that $CD8^+$ T cells recognizing conserved epitopes within the influenza virus mediate this inhibition,

and that these cross-reactive $CD8^+$ T cells are able to protect mice from a lethal challenge with a heterosubtypic influenza virus.

2. Materials and methods

2.1. Viruses, influenza vectors, mice, and infections

The influenza viruses utilized in this study were H1N1 (mouse adapted A/PR/8/34), H3N2 (mouse adapted A/Aichi/2/68), H3N1 (A/WSN/33 NA and internal genes and A/Aichi/2/68 HA), and H1N2 (A/WSN/33 HA and internal genes and A/Aichi/2/68 NA). The inf-LEF and the PR8-33 vectors utilized in this study, as well as the generation and growth of these vectors, have been described previously [22, 34]. Briefly, inf-LEF consists of A/WSN/33 NA and internal segments and a chimeric A/Aichi/2/68 HA protein into which the lethal and edema factor binding domain (LEF) from the *Bacillus anthracis* protective antigen has been inserted. The PR8-33 vector is a wild type A/PR/8/34 virus into which the LCMV GP_{33-41} epitope (KAVYNFATM) has been inserted into the NA stalk at residue 42. Influenza was inactivated by treatment with β-propiolactone as previously described [35].

Female C57BL/6J mice (6 to 8 weeks old) were purchased from Jackson Laboratories. All mice were inoculated IN with the following amounts of virus or viral vectors, which were determined experimentally in previous studies with

these viruses: H3N1, H1N2 and inf-LEF = 1 x 10^5 pfu; H1N1 = 100 pfu; H3N2 = 50 pfu; PR8-33 = 1000 pfu. Mice were infected with 2x10^5 pfu IP for the LCMV-Armstrong infections. Serum was collected by cheek bleeds at the indicated times.

2.2. Lymphocyte isolation

Lymphocytes were isolated from lungs by treatment with 1.3 mM EDTA in HBSS (30 min at 37°C, shaking at 200 rpm), followed by incubation with 100 U/ml collagenase (Invitrogen Life Technologies) in 5% RPMI 1640 supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (60 min at 37°C, shaking at 200 rpm). Single cell suspensions were obtained by pushing spleens, lymph nodes or digested lungs through 70 μM nylon mesh filters (Becton Dickinson). Lymphocytes from lungs were purified by centrifuging on a 44/67% Percoll gradient (800 × g for 20 min at 20°C).

2.3. ELISA and flow cytometry

ELISA assays were performed as described previously [22]. Briefly, NUNC MaxiSorp plates (cat # 442404) were coated with 2.5 ug per well of recombinant LEF (obtained from BEI Resources) or standard amounts of mouse IgG diluted in BSS buffer and kept at 4 C overnight. The following day, serum was added at different concentrations and the plates were washed three times with PBS/0.2%

tween before HRP conjugated goat α-mouse IgG was added (Sigma cat # A4416). SigmaFast OPD tablets (cat # P9187) were used to develop the plates. The reactions were stopped with 0.1 N H_2SO_4 and the plates were read at 490 nm. Standard curves were constructed from the IgG standards and antibody concentrations were calculated from these curves.

For flow cytometry analysis, single cell suspensions of lymphocytes were stained with anti-CD8a-PE, IFN-γ-FITC, or TNF-α-APC (BD Pharmingen). Intracellular staining for IFN-γ and TNF-α after 5 h *in vitro* stimulation with 0.1 μg/ml NP_{366-74} peptide (ASNENMDAM) was performed using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Samples were analyzed using a Becton Dickinson FACScaliber®. GP33 tetramers were made and used as previously described [36].

2.4. Hemagglutination inhibition assay

One part serum was added to three parts receptor destroying enzyme (RDE) (Accurate Chemical & Scientific) and incubated at 37°C overnight. The RDE was inactivated the following morning by incubating the samples at 56°C for one hour. Samples were then serially diluted with PBS in 96 well v-bottom plates and eight hemagglutination units (as determined by incubation with 0.5% turkey RBCs in the absence of serum) of influenza virus was added to each well. After 30 minutes at room temperature, 50 ul of 0.5% turkey RBCs (Lampire Biological Laboratories) suspended in PBS/0.5% BSA was added to each well and the

plates were shaken manually. After an additional 30 minutes at room temperature, the serum titers were read as the reciprocal of the final dilution for which no hemagglutination was observed.

3. Results

3.1. The effect of preexisting immunity to influenza on antibody responses to influenza vectors

To examine whether preexisting influenza immunity affects the antibody response to an influenza virus vector, we utilized the previously characterized influenza vector, inf-LEF [22]. This vector is a recombinant influenza virus containing the HA protein from A/Aichi/2/68 (H3N2) that has been engineered to express the 90 residue LEF domain from the protective antigen of *Bacillus anthracis* at the amino terminus of the HA ectodomain. The remaining seven gene segments all come from the A/WSN/33 (H1N1) influenza strain, making the vector an H3N1 subtype virus. Figure 1A depicts the protocol for the first series of experiments. In these, mice were initially infected with influenza viruses having homologous HA and NA (H3N1), heterologous HA (H1N1), or heterologous HA and NA (H1N2), with respect to the inf-LEF vector (H3N1). Forty-two days after the initial infection, the mice were infected with inf-LEF and the antibody responses to the different viral components were followed in the serum (Fig. 1A).

As expected, only the mice that had been immunized with H1N1 or H1N2 had antibody responses against the H1 HA, as measured by hemagglutinin inhibition (HAI), and HAI titers specific for H1 were not boosted following infection with inf-LEF (Fig. 1C). Likewise, only the mice that were immunized with H3N1 showed HAI specific for the H3 HA prior to infection with inf-LEF (Figure 1B). Upon infection with the inf-LEF vector, none of the previously infected mice, including the H1N2 group, had a significant increase in anti-H3 responses, while age-matched naïve mice receiving the inf-LEF vector generated robust anti-H3 responses (Fig. 1B). Although the inf-LEF vector was able to elicit low levels of anti-H3 antibody production in the H1N1 immunized mice, these levels were below the 1:40 titer that is considered to be protective [37].

Antibody responses against the LEF domain of inf-LEF are shown in Figure 1D. Confirming previous studies [22], the vector elicited robust anti-LEF antibody responses when given to naïve mice. In contrast to this, the anti-LEF titers were significantly lower in mice previously infected with any of the influenza viruses, even those that had received the completely heterologous H1N2 virus. Mice that had been previously infected with H1N1 virus, which differs from the inf-LEF vector only by the HA protein, had an undetectable anti-LEF antibody response. Surprisingly, mice that had preexisting immunity to the homologous H3N1 virus generated greater anti-LEF titers than the H1N1 immunized mice, although these antibody levels were just above the limit of detection. The H1N2 infected mice generated the highest anti-LEF titers of the immunized mice, but the antibody levels were still more than a log below those seen in mice with no

prior exposure to influenza virus (Fig. 1D). These results indicate that previous influenza virus infection in mice, even by a different virus subtype, significantly decreases the quantity of antibodies generated by influenza vectors.

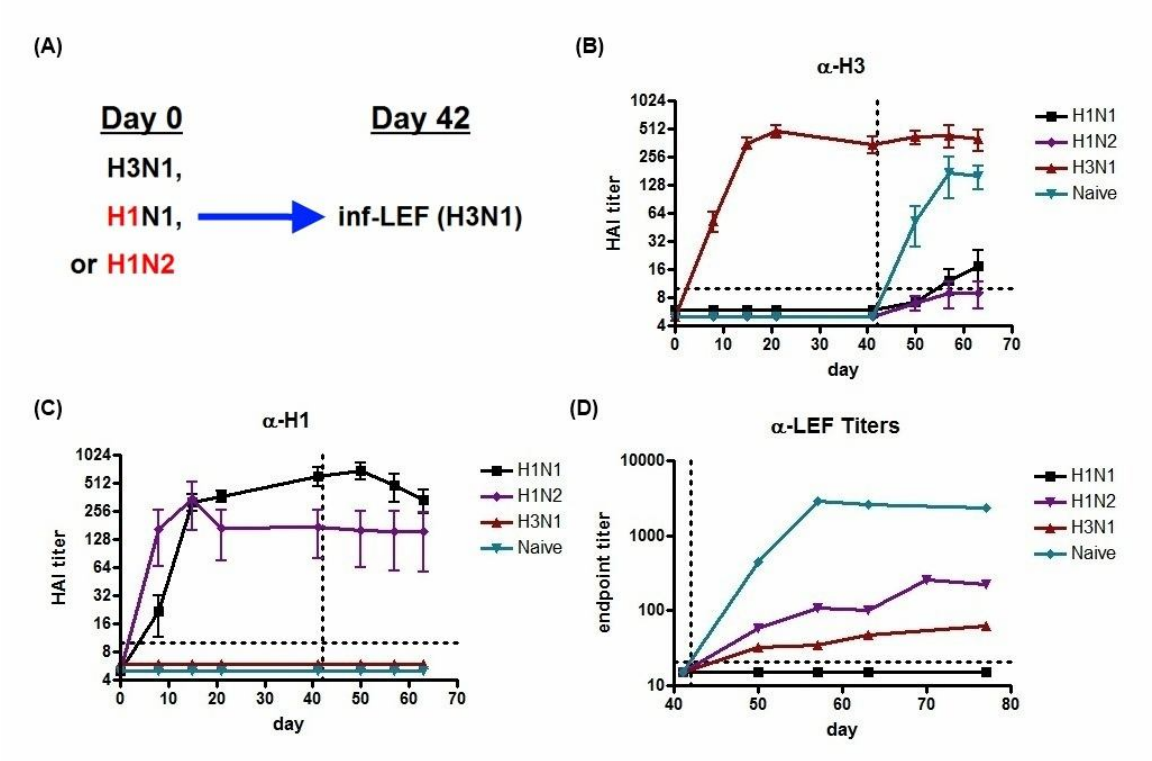

Fig. 1. The effect of previous exposure to influenza on antibody responses to influenza vectors. Groups of mice were first infected with different influenza subtypes and then administered the inf-LEF vector on day 42 (A). The antibody titers against H3 (B) or H1 (C) subtype virus were measured by HAI and the anti-LEF titers (D) were measured by ELISA. Red lettering indicates HA or NA proteins that are heterologous compared to the vector.

3.2. The effect of preexisting immunity to influenza virus on CD8$^+$ T cell responses to epitopes expressed by influenza vectors

Although influenza vectors are able to induce antibody responses, influenza viruses are also known to elicit strong cellular responses, and several vectors have been designed and tested to exploit this property [30-32]. Therefore, we wanted to determine whether the lack of response that we described in Figure 1 was specific for B cell responses, or if the same results would also be seen with a CD8$^+$ T cell epitope. We initially screened a library of peptides covering the entire LEF domain from the *Bacillus anthracis* protective antigen, but found no CD8$^+$ T cell epitope that was robust enough to be detected reliably (data not shown). Therefore, we utilized a previously described influenza vector, PR8-33, that was known to elicit CD8$^+$ T cell responses in mice. The PR8-33 vector is a wild-type A/PR/8/34 influenza virus into which the lymphocytic choriomeningitis virus (LCMV) H2Db-restricted GP_{33-41} CD8$^+$ T cell epitope (KAVYNFATM) has been inserted in the NA stalk [38]. As in the previous experiment, mice were infected with influenza viruses having homologous HA and NA (H1N1), heterologous HA (H3N1), or heterologous HA and NA (H3N2), relative to the PR8-33 vector (H1N1). After 42 days, the mice were re-infected with the PR8-33 vector and GP33-specific CD8$^+$ T cells in the blood were followed by tetramer staining (Fig. 2A). As seen in previous studies, the PR8-33 vector elicited the production of GP33 specific cells in naïve mice [38]. However, CD8$^+$ T cell responses were absent in mice that had been immunized with

influenza virus, even those that had received the heterologous HA/NA combination (H3N2) (Fig. 2B, C, D, and E). Similar to the antibody responses described in Figure 1, these results show that prior infection with influenza virus, regardless of the subtype, substantially diminishes the $CD8^+$ T cell response against an influenza virus vector in mice.

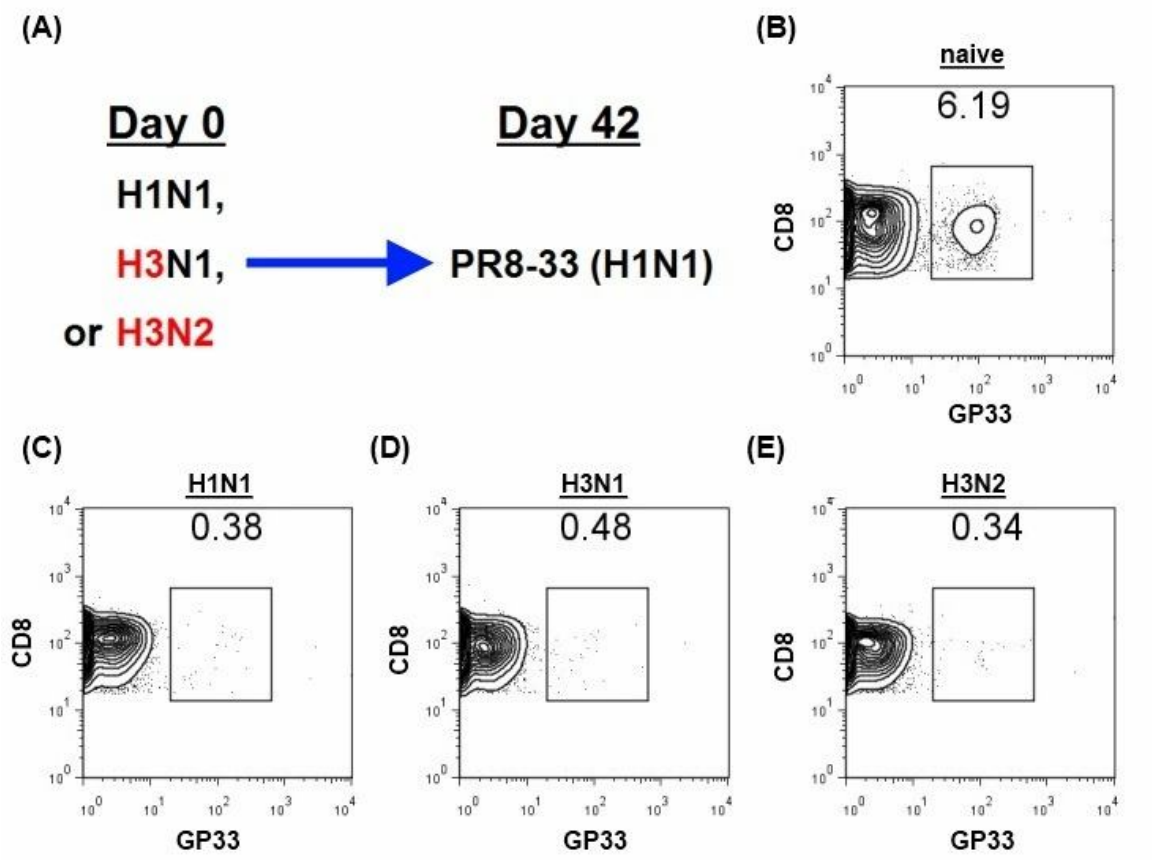

Fig. 2. The effect of previous exposure to influenza on $CD8^+$ T cell responses to influenza vectors. Groups of mice were initially infected with different influenza subtypes and then given the H1N1 PR8-33 vector on day 42 (A). The GP_{33-41}-specific $CD8^+$ T cell response was measured by tetramer staining and flow cytometry in the peripheral blood of the naive group (B), the H1N1 immunized group (C), the H3N1 immunized group (D), and the H3N2 immunized group (E). Red lettering indicates HA or NA proteins that are heterologous compared to the vector.

3.3. $CD8^+$ T cell responses to influenza viruses in lungs and spleen

The observation that immune responses elicited against influenza virus vectors with chimeric surface glycoproteins that express antigenic epitopes are diminished, even in mice that have been exposed to completely heterotypic surface antigens, led us to hypothesize that conserved T cell responses to internal influenza proteins were responsible for this phenomenon. To test this, mice were first infected with H3N1 or H1N1 followed by infection with the H3N1 inf-LEF vector 42 days later. Mice were then sacrificed on days eight, 41, and 50, and the influenza-specific $CD8^+$ T cells in both the lungs and spleens were quantified.

Influenza-specific $CD8^+$ T cells recognizing the H2Db-restricted epitope $NP_{366-374}$ were chosen for analysis in these experiments because this is the dominant $CD8^+$ T cell epitope generated during repeated influenza virus infections in C57BL/6 mice [39]. Furthermore, $CD8^+$ T cells recognizing this epitope express both interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) upon stimulation with purified $NP_{366-374}$ peptide *in vitro*, making the cells quantifiable by flow cytometry. Using this peptide stimulation assay, $NP_{366-374}$-specific $CD8^+$ T cells were detected in both the lungs and spleens of infected mice eight days after infection with the H3N1 or H1N1 influenza viruses and they were still detectable on day 41, one day before the mice received the inf-LEF vector, although at a lower frequency (Fig. 3A). In fact, $NP_{366-374}$-specific cells

could be detected in the lungs of mice for at least 400 days post-infection (data not shown).

Eight days after mice were infected with the inf-LEF vector (day 50), the percentage of $NP_{366-374}$-specific cells in both the lungs and spleens had increased, indicating that the vector was able to stimulate the memory T cells (Fig 3A). These data also provide further evidence that cross-reactive antibodies did not neutralize the inf-LEF vector and prevent it from initiating an infection in the mice. In addition, the percentage of $CD8^+$ T cells recognizing the $NP_{366-374}$ epitope was higher in the lungs of the previously infected mice than in age-matched naïve mice, further suggesting that these were memory cells that expanded rapidly during the secondary infection (Fig. 3B). The percentage of $NP_{366-374}$-specific cells in the spleens, however, was comparable between the groups (Fig. 1D). This can be explained by the fact that influenza virus infections are localized to the lungs and do not typically spread systemically.

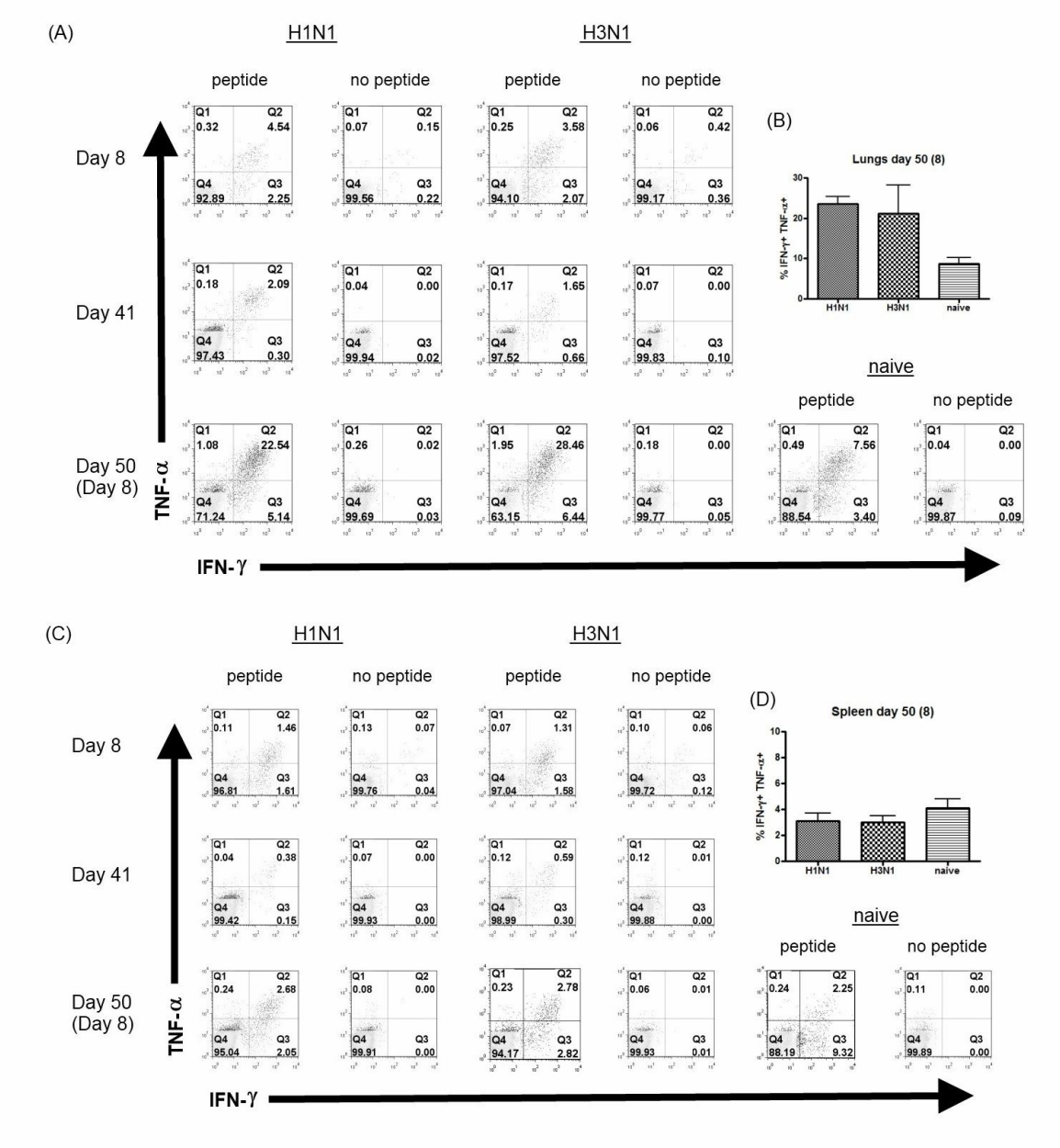

Fig. 3. $NP_{366-374}$ $CD8^+$ T cell responses in previously infected mice prior to and following administration of the inf-LEF vector. The percentage of $NP_{366-374}$-specific $CD8^+$ T cells of total $CD8^+$ T cells was measured by peptide stimulation and flow cytometry in the lungs (A and B) and the spleens (C and D) of H1N1 or H3N1 immunized mice on days eight and 41 post-infection and on day eight after receiving the inf-LEF vector. The responses in naive mice are also shown for day eight post-vaccination. Representative flow plots are shown for the lungs (A) and spleen (C) as well as the means on day 50 (B) and (D).

3.4. Antibody responses to influenza virus vectors in the absence of CD8$^+$ memory cells

The finding that all of the influenza viruses tested elicited the same CD8$^+$ T cell response led us to hypothesize that these cross-reactive cells were responsible for the lack of immune response seen to influenza vectors in the previously infected mice. In order to test this, the vector was given to mice that did not have preexisting CD8$^+$ T cells. Inoculation of mice with inactivated influenza virus or with live virus by the IM route has been demonstrated to lead to poor CD8$^+$ T cell responses [11]. This is likely due to reduced presentation of viral epitopes on the MHC I complex by APCs, as little or no virus replication would take place under these conditions. Mice were infected with live or inactivated H1N1 influenza virus IN or IM, or with LCMV as a control. All groups that received influenza virus demonstrated protective HAI titers 50 days post-infection, indicating that protective antibody responses had been generated (Fig 4A).

On day 51, all mice were administered the inf-LEF vector (live, IN) and the serum antibody levels specific for LEF were measured fourteen and thirty days later (days 65 and 81). As expected, the control mice that had been immunized with LCMV generated robust anti-LEF responses, indicating that the lack of response to the inf-LEF and PR8-33 influenza vectors seen in the above experiments was influenza-specific (Figure 4B). The mice that had been immunized with live influenza virus IN had anti-LEF responses more than a log

lower than the LCMV control mice on day 65 (354 vs. 4169), confirming the results shown in Figure 1. While slightly lower anti-LEF responses were seen in mice that had been immunized with inactivated virus or live virus IM, the levels obtained were at least five times higher than those seen in the mice that had received live virus IN (Fig 4B). These results implicate preexisting $CD8^+$ T cells as being responsible for the lack of response to inserted epitopes when mice have been previously infected with an influenza virus.

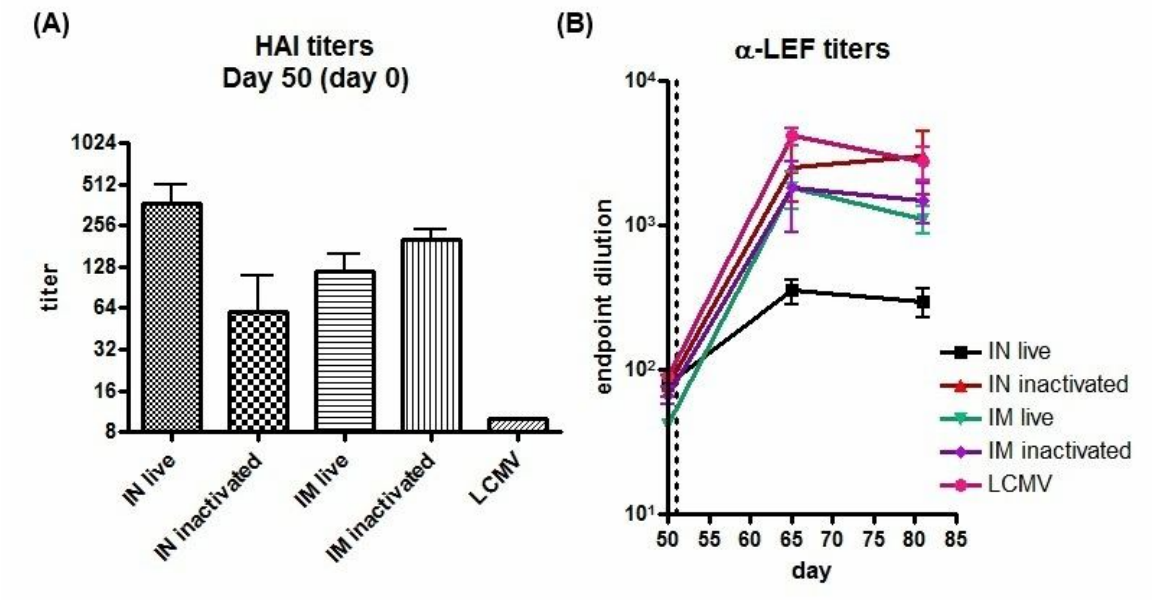

Fig. 4. The effect of the absence of preexisting influenza-specific $CD8^+$ T cells on the antibody response to the inf-LEF vector. Mice were vaccinated with live or B-propiolactone inactivated influenza viruses IN or IM, or infected with LCMV IP, and the antibody responses were measured by HAI on day 50 (A). The LEF specific antibodies were measured prior to and following administration of the inf-LEF vector (B).

3.5. Protection from heterologous challenge

Having demonstrated that $CD8^+$ T cells recognizing conserved epitopes within the influenza viruses were responsible for the lack of immune responses to viral vectors, we next wanted to assess the ability of these cross-reactive cells to protect mice from a lethal challenge by a heterosubtypic influenza virus. To address this, mice were first infected with sub-lethal doses of H1N1, H3N1, or H3N2 influenza viruses and then challenged with 5 LD50 of H1N1 on day 50. All of the naïve mice had steady weight loss and succumbed to the virus within seven days. The mice that had been immunized with the homologous virus showed no weight loss and survived the challenge infection. Despite being previously infected with different subtypes from the challenge virus, the H3N1 and H3N2 groups both had minimal weight loss and only one mouse from the H3N1 group died (Fig 5A and B).

A subset of the previously infected mice were sacrificed three days after being challenged and the numbers of $NP_{366-374}$-specific $CD8^+$ T cells in their lungs were quantified by peptide stimulation. The naïve mice had very low levels of $NP_{366-374}$-specific $CD8^+$ T cells in their lungs at this time. In contrast, the mice previously infected with H1N1, H3N1, or H3N2 subtype viruses all had measurable $NP_{366-374}$ responses, even at this early time point, indicating that they were memory cells that had been formed during the previous infection (Fig 5C). These cells were present despite the fact that the H3N1 and H3N2 mice had previously been infected with heterosubtypic influenza viruses.

The viral titers in the lungs of infected mice were calculated on days three and six post-challenge to determine if the memory T cells were contributing to viral clearance at these early time points. As expected, high viral titers were observed in the naïve mice at both time points (Fig 5D). In contrast, virus was undetectable at both time points in the lungs of the mice receiving homologous challenge (Figure 5D). This is likely due to neutralization of the virus by preexisting antibody before it was able to infect host cells or spread. The H3N1 and H3N2 immunized groups both had virus detectable in their lungs at day three, but titers were reduced by approximately three logs relative to naïve mice, and no virus could be detected in their lungs on day six, indicating that it had been cleared (Figure 5D). The lower viral titers observed in the previously infected mice compared to the naïve mice indicates that the $NP_{366-374}$-specific T cells may have ameliorated the infection, even as early as day three, which also correlates with the weight loss and survival data (Figures 5A and B). These results show that a cross-reactive $CD8^+$ T cell response, in the absence of a cross-reactive antibody, is able to protect mice from lethal challenge by heterologous influenza subtypes.

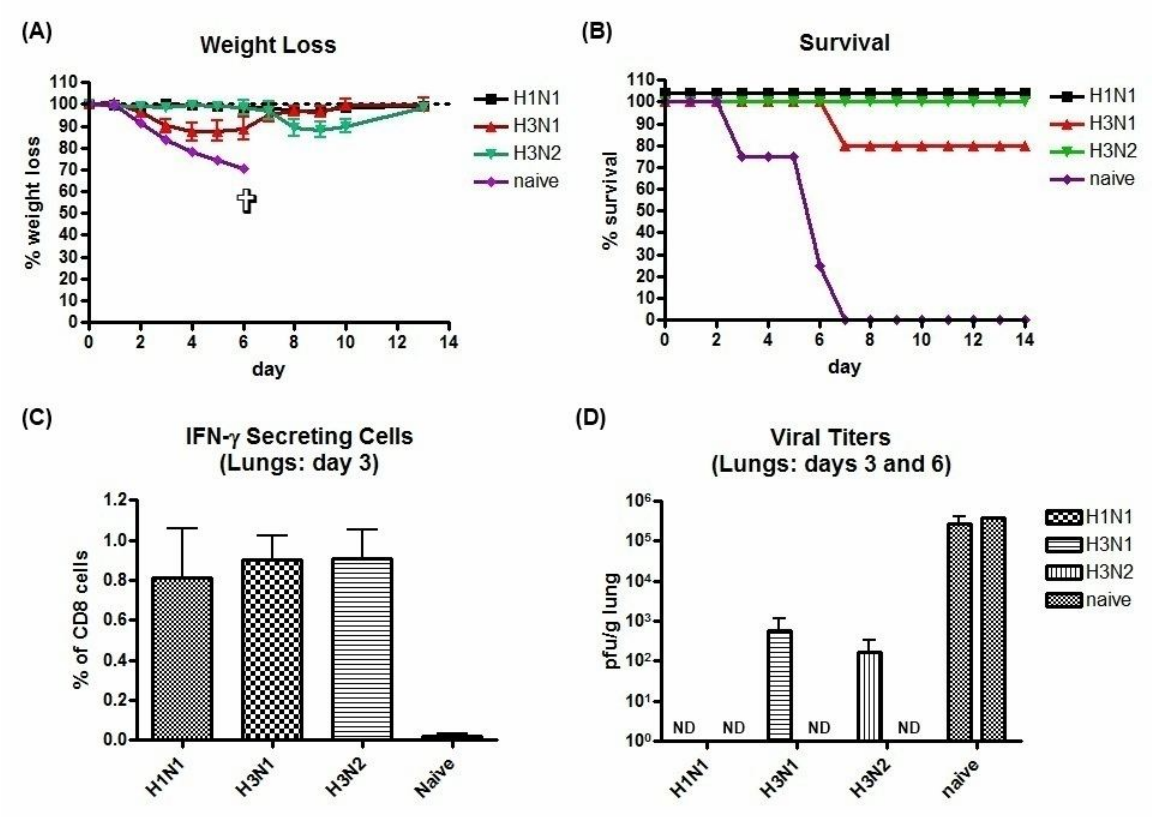

Fig. 5. The effect of previous infection with homologous or heterologous influenza subtypes on mice receiving a lethal challenge of H1N1. Mice were immunized with H1N1, H3N1, or H3N2 and then challenged with a 5LD50 dose of H1N1 on day 50. Weight loss (A) and survival (B) were monitored. The percentage of $NP_{366-374}$ $CD8^+$ T cells of total $CD8^+$ T cells in the lungs on day three post-challenge was measured by peptide stimulation (C) and the influenza viral titers in the lungs were measured on days three and six post-challenge (D). ND = none detected

4. Discussion

A fundamental issue regarding viral vectors centers on potential limitations due to preexisting immunity to the vector. In some cases it might be possible to circumvent such concerns by using alternative routes of administration, or related vectors with different antigenic properties. The fact that 16 subtypes of HA and 9 subtypes of NA exist for influenza A viruses makes it reasonable to conceive that vectors with antigenically distinct surface proteins might be of practical use, particularly as these are the principle target for neutralizing antibody responses. By contrast, the internal proteins of influenza A virus are much less divergent, and these contain a number of well documented, highly conserved epitopes recognized by $CD8^+$ T cells. The data shown here indicate that previous influenza infections in mice, regardless of the subtype, severely inhibit the responses elicited upon infection with an influenza viral vector expressing antigenic epitopes in mice. Our data show that this inhibition is at least partially mediated by cross-reactive $CD8^+$ T cells, which can also mediate protection of mice from a lethal influenza virus challenge.

There are several ways to potentially overcome the effect of the preexisting $CD8^+$ T cells. Administration of the vectors via different routes could prove effective in this regard by limiting replication, and thus availability of the conserved T cell epitopes to memory cells. It has been shown that H1N1 influenza vectors containing anti-tumor epitopes were still able to elicit protective responses against tumors in mice that had been previously infected with an

H3N2 influenza virus [40]. However, administration of influenza vectors by routes other than IN may be less effective at inducing mucosal responses, eliminating one of the major advantages of this vector. Clearly, the effects of preexisting immunity will differ depending on the antigen and system utilized.

A study examining the sequences of influenza viruses covering several different subtypes found that the $NP_{366-374}$ $CD8^+$ T cell epitope followed in this study was very highly conserved (78-100%) in all of the viruses sequenced [41]. Engineering an influenza viral vector lacking this epitope could prove useful in making these vectors more effective, but it is likely that many of the conserved subdominant epitopes would exert an effect and yield similar results [39]. For example, the second most prominent $CD8^+$ T cell epitope recognized by C57BL/6J mice is $PA_{224-233}$, which is present in 80-100% of the viruses sequenced [41]. The conservation of $CD8^+$ T cell epitopes is not just a C57BL/6J mouse-specific phenomenon, as the same study found that $NP_{147-155}$, the dominant $CD8^+$ T cell influenza epitope in Balb/C mice is 100% conserved throughout all of the influenza viruses sequenced. Another study found that while some human influenza $CD8^+$ T cell epitopes are able to mutate and escape cellular responses, others, including $M1_{58-66}$ are conserved, despite their immunodominance, due to functional constraints, and others required co-mutations in order for the viruses to remain viable [42-43].

Heterosubtypic immunity to influenza virus in mice has been reported before. In one study, mice previously infected with an H3N2 subtype showed enhanced recovery when challenged with H1N1 compared to naïve mice [44].

Another study found that when mice were infected with a sub-lethal dose of H3N2 and subsequently challenged with a lethal dose of H1N1, the H3N2 immunized mice cleared the challenge virus faster, displayed fewer clinical signs of morbidity, had fewer lesions in their lungs, and survived at higher rates than naïve mice. This outcome was also correlated with the shared $NP_{366-374}$ $CD8^+$ T cell response [45]. Finally, T cells have also been shown to increase resistance to influenza virus in B cell knockout mice [46].

Heterologous immunity in humans has been more controversial as humans are known to be re-infected by different influenza subtypes and even by the same subtype once it has undergone genetic drift. However, many conserved epitopes have also been found in influenza viruses that are recognized by human HLA alleles [41]. In one study, polyclonal T cells from healthy individuals with a history of having been previously exposed to H3N2 influenza viruses were shown to react with H5N1 infected cells. It was concluded that, apart from some individual epitopes that displayed amino acid variation between H3N2 and H5N1 viruses, considerable cross-reactivity exists between them [47]. Other findings indicate that the human memory CTL response to influenza A virus is broadly directed to epitopes on a wide variety of highly conserved internal proteins [48]. It was also reported that during the 1957 H2N2 pandemic, only 5.6% of adults that had previously been infected with influenza developed influenza during the pandemic while 55.2% of children that had previously been infected contracted it again. These observations were suggested to be the result of accumulated heterosubtypic immunity in the adults over their lifetime [49]. However, this could

also be explained by a similar virus having circulated at some point in the past before the children were born. One possible reason for the disparity between mice and humans is the high level of polymorphism among human HLA alleles, epitopes recognized by some people are not universally recognized by others.

The results here may illustrate the fact that mice and humans can have very different responses to the same viruses and that any results reported in mice, particularly with regard to influenza, will not necessarily reflect the situation in humans or other animals. While these results should not be taken as a direct indictment of influenza viruses as a vector, they do illustrate that a simple exchange of surface antigens may not suffice to overcome pre-existing immunity. While the issues might be different for humans, and vectors that replicate poorly in the face of pre-existing immunity might even be advantageous, the $CD8^+$ T cell responses will need to be taken into account.

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Chapter 6

The role of memory B cells in maintaining long-lived humoral immunity

The work presented in this chapter is still in preparation for submission.

The role of memory B cells in maintaining long-lived humoral immunity

William A. Langley[1], Scott N. Mueller[1,2], Rustom Antia[3], Muhammed A ur Rasheed[1], Mark J. Shlomchik[4], Rafi Ahmed[1,*].

[1]Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322.

[2]Present address: Department of Microbiology and Immunology, The University of Melbourne, Parkville 3010, Australia.

[3]Department of Biology, Emory University, Atlanta, GA, 30322

[4]Departments of Laboratory Medicine and Immunobiology, Yale University, New Haven, CT 06510

[*]Corresponding author

email: rahmed@emory.edu

ABSTRACT

Humoral immunity is a major component of the protective immune response against many pathogens and has been shown to be long-lived in the absence of re-exposure to the immunizing antigen. However, the mechanisms behind long-term B cell and antibody memory remain unclear. A particular question that remains unanswered is whether plasma cells are able to persist long-term on their own, or if memory B cells are needed in order to maintain them. In these experiments, we measure the longevity of plasma cells specific for influenza, LCMV, or VSV in the absence of memory B cells. We show that memory B cell depletion by rituximab treatment of human $CD20^{+}$ transgenic mice results in a biphasic decline of virus-specific plasma cell numbers. The initial decline is demonstrated to be due to a subset of plasma cells that expresses hCD20 while the second phase of the decline is attributable to plasma cell decay and was used to estimate the half-lives of these cells following viral infections. These data indicate that plasma cells are intrinsically long-lived, but that some re-seeding by memory B cells may be necessary to maintain their numbers over a lifetime. These data offer insights into plasma cell longevity and hold implications for human rituximab treatment.

INTRODUCTION

Humoral immunity has been demonstrated to be the most protective branch of the immune system against many pathogens and antibody titers are the correlate of protection for most currently licensed vaccines. Humoral immunity to most acute infections and vaccinations has been shown to be long-lived, even in the absence of re-exposure to the original antigen by natural occurrences or vaccine boosting. In 1846, it was observed that adults who had survived a measles outbreak on the isolated Faroe Islands were protected from the virus when it reemerged 65 years later, despite the fact that no outbreaks had occurred during the intervening years (28). More recently, it was demonstrated that smallpox-specific memory B cells can persist for more than 50 years in immunized individuals (12) and in 2008, memory B cells recognizing the 1918 H1N1 influenza strain were able to be isolated from the blood of people who survived the pandemic 90 years earlier (37). Another study estimated the half-lives of the humoral response to some viral antigens in humans to be more than 200 years (3). Following lymphocytic choriomeningitis virus (LCMV) infection in mice, antibodies secreted by plasma cells, residing mainly in the bone marrow, have been shown to persist at relatively constant levels for the lifetime of the mice (32). However, the half-life of antibodies is known to be less than three weeks, leading to the conclusion that plasma cells must somehow be maintained for long periods of time in hosts in the absence of re-exposure (16, 33, 35).

Currently, the mechanisms behind long-term plasma cell maintenance are unclear. Several models have been suggested by which this persistence may be accomplished. One theory is that plasma cells are long-lived and do not need to undergo continued renewal (1, 14, 24). Another theory is that memory B cells are needed to re-seed the plasma cell population. Evidence supporting this includes the fact that memory B cells have been shown to be long-lived and to undergo homeostatic renewal (4, 30). Memory B cells can also be non-specifically stimulated *in vitro* and transformed into plasma cells, however, the occurrence of this *in vivo* is more controversial (8, 14). One school of thought is that antigen or antigen-antibody complexes are retained for long periods of time on follicular dendritic cells (FDCs) and memory B cell interactions with these are required in order for the memory B cells to be transformed into plasma cells (6, 27). Other work suggests that non-specific stimuli, including bystander T cell help and toll-like receptor signaling, stimulates memory B cells and can transform them into plasma cells (8).

In an attempt to elucidate the mechanism behind the maintenance of plasma cells, our laboratory demonstrated that sub-lethally irradiating LCMV-immune mice to deplete memory B cells resulted in a gradual decline of LCMV-specific antibodies over time and concluded that although plasma cells are long-lived, with an estimated half-life of 138 days, they do require memory B cells in order to be maintained (31). However, questions about this study, specifically regarding the effects of radiation on the plasma cells and the bone marrow

stromal cells, have been raised. It is possible that the irradiation process could have damaged these cells leading to their eventual death.

In these studies we attempt to determine the longevity of plasma cells following viral infections in mice depleted of their memory B cells by antibodies. To accomplish this, we utilize transgenic mice expressing the human CD20 (hCD20) molecule. In these mice, hCD20 is expressed exclusively on naïve and memory B cells, allowing for the specific depletion of these cells by treatment with rituximab (anti-CD20), while sparing the plasma cells, which have down-regulated the expression of hCD20 on their surfaces (17, 29, 34). We infected these $hCD20^{+}$ mice with influenza virus, LCMV, or vesicular stomatitis virus (VSV) and then depleted B cells at memory time points. We show that depletion of naïve and memory B cells results in a biphasic decline of virus-specific antibody levels and that this correlates with drops in plasma cell numbers. We also show that the biphasic decline is due to a subset of plasma cells that have not down-regulated hCD20 expression on their surfaces. These data indicate that plasma cells are intrinsically long-lived, but that some re-seeding by memory B cells may be necessary to maintain their numbers over time. In addition to adding insight into the mechanisms behind plasma cell longevity and offering an estimate of the half-life of plasma cells following viral infections, these data also have implications for human rituximab treatment.

RESULTS

1. Rituximab treatment depletes circulating B cells in hCD20$^+$ mice

Rituximab treatment of hCD20$^+$ mice has been previously characterized and shown to specifically deplete naive and memory B cells, but not plasma cells or non-B cells (2, 17) and we confirmed this finding in naïve mice bred at our facility (data not shown). In the first set of experiments presented here, hCD20$^+$ mice were infected with influenza intranasally (IN), LCMV interperitoneally (IP), or VSV intravenously (IV). Prior studies in our laboratory have demonstrated that memory B cell responses to LCMV reach the levels at which they are maintained long-term around day 40 post-infection in mice (31) and we wanted to study the effects of B cell depletion at memory time points, so we waited until after this to begin treating the mice with rituximab. On day 62, one half of the mice from each of the infected groups were treated with the human dose equivalent of rituximab (1 mg/mouse) while the other half received PBS. The mice were subsequently treated weekly for the remainder of the experiment and the B cell levels in their peripheral blood (PB) were monitored by flow cytometry at the indicated time points (Figure 1). The flow data indicate that B220$^+$ CD19$^+$ CD3$^-$ cells were efficiently depleted in the PB of influenza (Figure 1A), LCMV (Figure 1B), and VSV (Figure 1C) infected mice following rituximab treatment. The B cell numbers declined sharply after just one week of treatment, however, maximum depletion was only achieved after about a month of treatment and B cell numbers in these

mice remained at these low levels for the duration of the experiments. It should be noted, however, that B cells were not depleted by rituximab treatment in non-transgenic LCMV immune mice (Figure 1C).

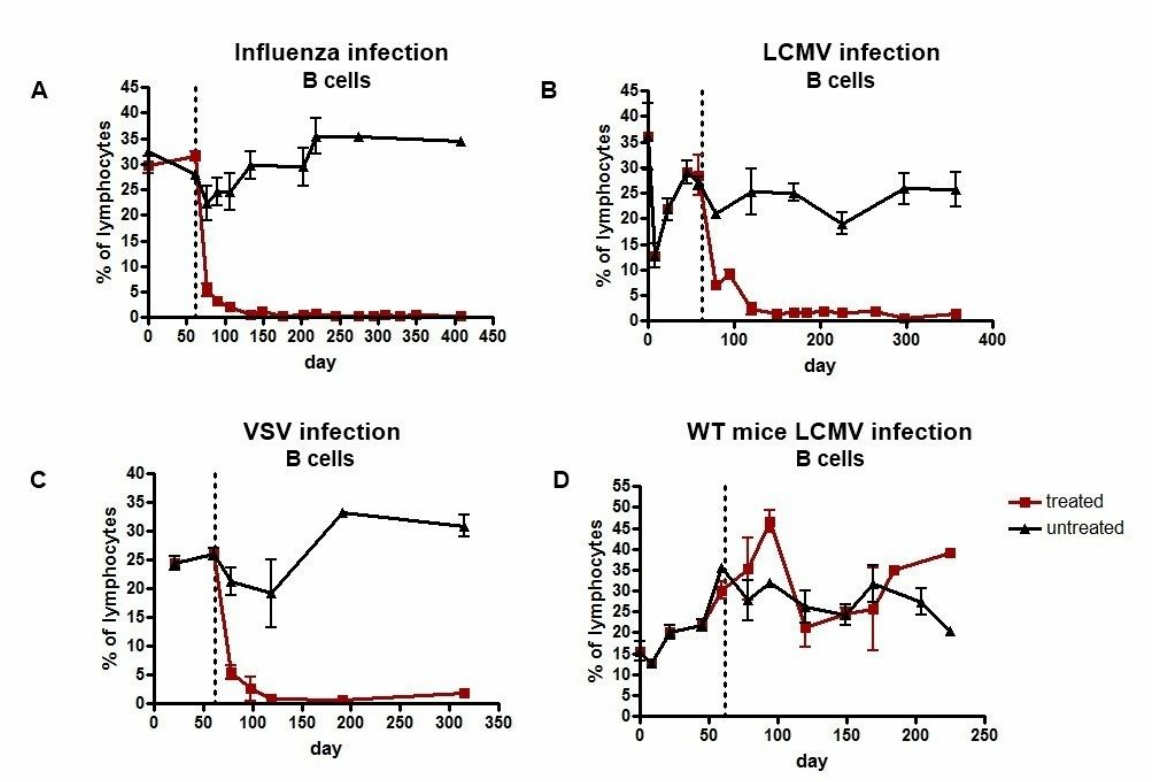

Figure 1. B cell depletion in PB of rituximab treated mice. Mice were infected with influenza virus (A), LCMV (B), or VSV (C) and treated with rituximab (red lines) or PBS (black lines) starting on day 62 post-infection. B cells (B220+ CD19+ CD3-) were monitored by flow cytometry at the indicated times. (D) Rituximab treatment of non-transgenic LCMV infected mice.

2. Rituximab treatment depletes memory B cells in immunized hCD20^{+} mice

After determining that B cells in the PB were depleted by rituximab treatment, we wanted to find out if the treatment was also effective at depleting memory B cells in the spleen. The different groups of mice were sacrificed between 247 and 408 days post-infection and memory B cell numbers were quantified. The assay used to detect memory B cells is an *in vitro* assay that relies on the fact that stimulated B cells can be transformed upon non-specific stimulation into plasma cells and detected by ELISPOT assay (Figure 2A). We chose to look for memory B cells in the spleens because this has been shown to be the major reservoir of these cells in mice (31). With each infection virus-specific memory B cells were detected in the untreated mice by this assay (Figure 2B, C, D). In contrast to this, the numbers were below the level considered positive for all three of the groups that had been treated with rituximab. These results indicate that memory B cells are efficiently depleted in mice that have been treated long-term with rituximab.

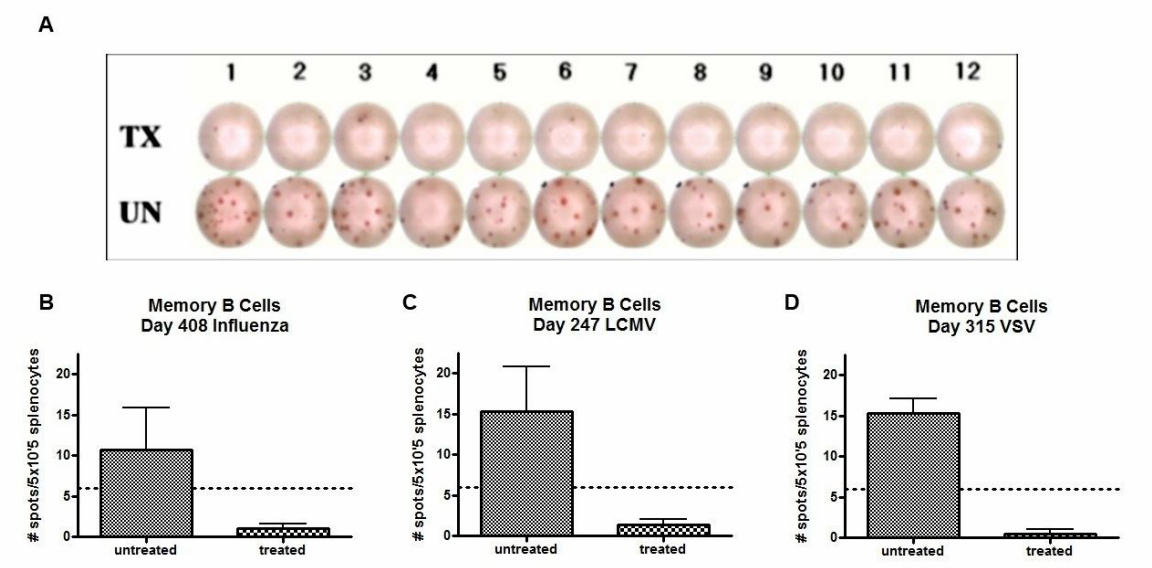

Figure 2. Memory B cell depletion in rituximab treated mice. Memory B cell numbers were quantified in the spleens of rituximab treated or PBS treated mice. (A) Representative results from rituximab treated (TX) or PBS treated (UN) LCMV immune mice. In influenza (B), LCMV (C), and VSV (D) immune mice memory B cells were detected in the PBS treated, but were below the cutoff point in the rituximab treated groups.

3. Naïve and memory B cell depletion in immunized hCD20^{+} mice leads to a decrease in serum specific antibody levels

After confirming that rituximab treatment results in the depletion of memory B cells, we next wanted to determine the effect this had on virus-specific antibody levels. At the same time that PB was collected to monitor PB B cell levels, serum was also collected and the virus-specific IgG antibody levels were determined by ELISA (Figure 3A, B, C, D). In mice infected with any of the three viruses, there was a sharp drop within the first two weeks after treatment was initiated, followed by a much slower decline over time, resulting in a biphasic pattern (Figure 3 F, G, H). These declines were not seen in PBS treated mice or in WT mice that were treated with rituximab, indicating that the observed decreases were a consequence of hCD20^{+} cell depletion and not a side-effect of the drug (Figure 3E).

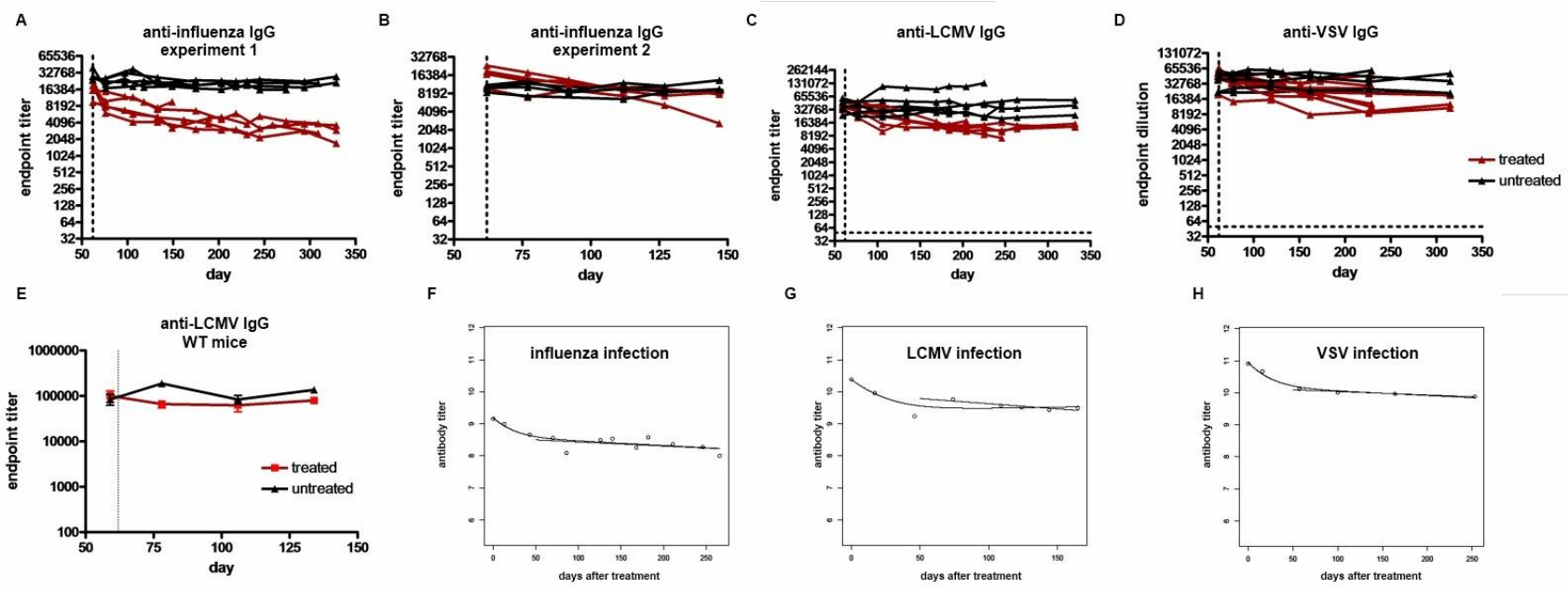

Figure 3. Anti-viral IgG titers in rituximab treated mice. Virus-specific IgG was measured by ELISA in the serum of rituximab treated (red lines) and PBS treated (black lines) mice after infections with influenza (A and B), LCMV (C), or VSV (D). (E) The means of LCMV-specific IgG titers in non-transgenic mice following treatment with rituximab (red lines) or PBS (black line). Representative plots showing the biphasic decline of antibody titers are shown for influenza (F), LCMV (G), or VSV (H) immune mice treated with rituximab.

4. The initial drop is due to a subset of plasma cells that express hCD20

Prior data indicates that humoral responses should have been fully developed at the time in which rituximab treatment was initiated, however, it was still possible that the initial decline observed in the treated mice could be attributed to the depletion of early plasma cells from ongoing germinal center reactions that had not yet down-regulated hCD20 levels. To test this, mice that had been immunized for a much longer time were treated to see if the same initial antibody decline would be seen. Mice that had been infected with LCMV 274 days prior were treated for two weeks with rituximab and their antibody titers were measured both before and after the treatment. The mice that were treated all showed sharp antibody declines during this short period, similar to those seen in the day 62 treated mice, while no decline was observed in the untreated mice (Figure 4A). In a separate experiment, a mouse immunized with VSV 425 days prior was treated for two weeks and, once again, a sharp decline in antibody levels was seen over this two week period in this mouse, but not in the untreated counterpart (Figure 4B). These results indicate that residual germinal centers are not the cause for the initial drop in antibody titers.

To determine the cause of this initial decline, bone marrow was taken from immune mice and lymphocytes were analyzed by flow cytometry for hCD20 expression. Staining showed that 12-20 percent of the $CD138^{+}$ cells in LCMV immune mice expressed hCD20 on their surface (Figure 4C, D). These cells were significantly reduced in mice that had been treated for two weeks with

rituximab (p=0.044) (Figure 4E, F). A second experiment was performed in which hCD20 positive and negative cells were sorted and it was calculated that 11.6 percent of the virus-specific plasma cells sorted with the hCD20 positive fraction (Data not shown). These results both indicate that a subset of plasma cells expresses hCD20 and that these cells are depleted in rituximab treated mice leading to the initial drop in antibody titers that is observed.

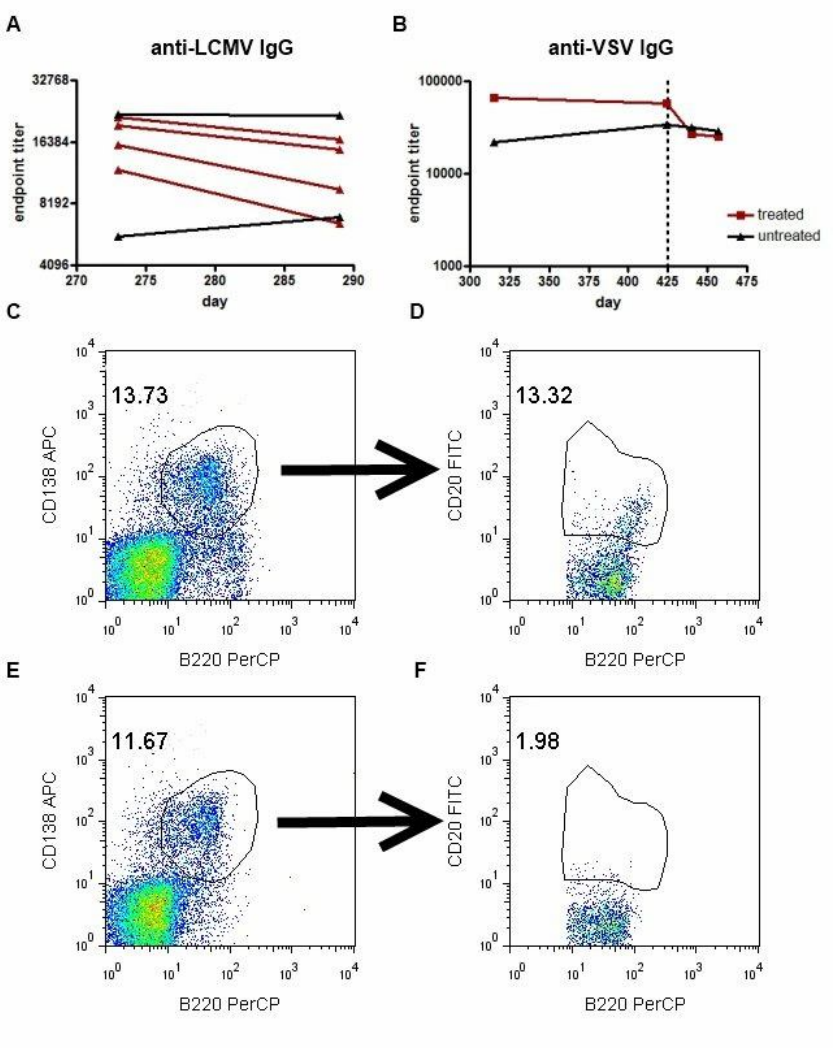

Figure 4. Antibody titers and plasma cell numbers following rituximab treatment in aged immune mice. LCMV (A) or VSV (B) immune mice were treated with rituximab (red lines) or PBS (black lines) 273 or 425 days after the initial infections, respectively, and virus-specific IgG was measured by ELISA. (C and E) Representative plots showing percentage of total lymphocytes expressing CD138 and B220 in the bone marrow of untreated (C) or two week-treated LCMV immune mice. (D and F) Percentage of plasma cells expressing hCD20 in untreated (D) or two week-treated mice.

5. The antibody decreases in naïve and memory B cell depleted mice correlate with functional assays

Following the finding that antibodies were declining in the rituximab treated mice, we wanted to determine if this drop correlated with a loss in functional ability of the serum in the mice. Specifically, were the antibodies that were disappearing affecting the ability of serum to neutralize virus or were the less functional antibodies being lost preferentially over the more effective ones?

In order to test this in influenza virus infected mice, hemagglutination inhibition (HAI) assays were performed (Figure 5A). The results show that the loss of antibodies in the treated mice over time correlates with the HAI titers as well. The same initial drop followed by a much slower decline can be seen. Similarly, VSV neutralization titers also show this same decline (Figure 5B). Titers drop from an average of 640 on the day before treatment to 320 one-hundred days post-treatment. These results indicate that the decrease in antibody titers observed in rituximab treated mice is not preferentially directed at the antibodies that are the least effective, but that the drop correlates with loss of function in the treated mice.

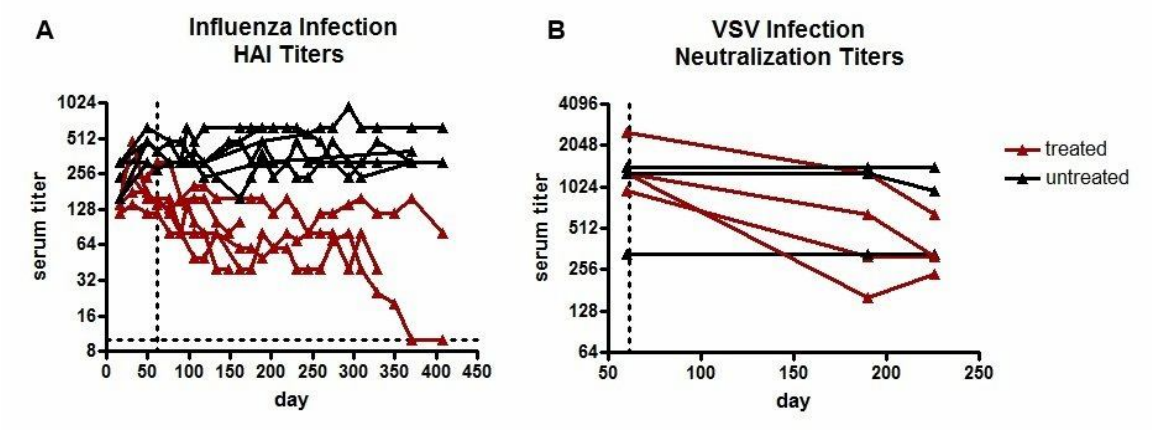

Figure 5. HAI and VSV neutralization serum titers. HAI titers of the serum from influenza immune mice (A) and neutralization titers from VSV immune mice (B) were measured in rituximab treated (red lines) or untreated (black lines) groups.

6. Memory and naïve B cell depletion in immunized hCD20^{+} mice leads to decreases in plasma cell numbers

We next wanted to determine if the loss of memory B cells and the decreases in antibody titers correlated with plasma cell numbers in the bone marrow of rituximab treated mice. The numbers of virus-specific plasma cells were quantified at late time points by ELISPOT (Figure 6). For LCMV infections, mice were sacrificed either 185 days or 295 days after treatment with rituximab had been initiated. At the earlier time point, treated mice were found to have roughly 33 percent as many plasma cells compared to the untreated group. By day 295, the numbers had dropped to around 25 percent of those present in untreated mice. Similarly, the plasma cell numbers in influenza immunized mice had dropped to less than 25 percent of the number seen in untreated mice 346 days after treatment was initiated and the numbers in VSV immunized mice had dropped to around 35 percent 253 days after treatment was started. Total IgG plasma cell numbers were also decreased to similar percentages in the treated mice compared to the untreated mice (data not shown). These results indicate that plasma cell numbers significantly decline in mice that are continuously treated with rituximab. The results from the LCMV mice at two different time points clearly indicate that the plasma cells continue declining after the initial hCD20 expressing cells are depleted.

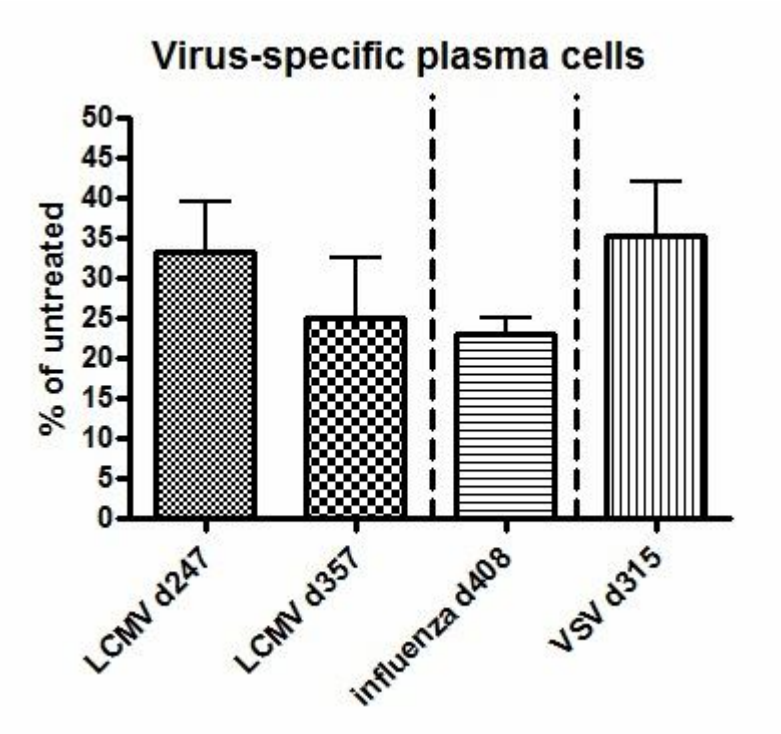

Figure 6. Plasma cell numbers in rituximab treated versus untreated mice. Plasma cell numbers were quantified in the bone marrow of rituximab treated mice and are shown as a percentage of those present in the untreated counterparts.

7. The decrease in plasma cell numbers after the initial decline is due to the lack of memory B cells.

Following the finding that plasma cell numbers continue to decline following the initial treatment, we wanted to determine if this slow decline was due to the continuous treatment with rituximab, or if it was due to the absence of memory B cells. In an attempt to answer this question, mice were infected with VSV and then separated into three different groups. The first group received rituximab weekly starting on day 62, the second group received treatment starting on day 62, but treatment was discontinued one month later on day 92, and the third group only received PBS. B cells were depleted to similar levels in the PB of mice from both treatment regimens, however they returned in the one month treated group after treatment was stopped (Figure 7A). As seen in earlier experiments, both the continuously treated and the one month treated groups showed the same initial decline upon initiation of rituximab treatment (Figure 7B). Interestingly, despite the return of B cells in the one month treated group, the antibody levels continued to decline at a rate comparable to the mice that were continuously treated (Figure 7B). In fact, the p value for the difference between these two groups was calculated to be 0.96, indicating that they were virtually identical (Figure 8B). Furthermore, the VSV-specific plasma cell numbers in these two groups of mice were similar at day 250 (Figure 7C) and memory B cells could not be detected in either group (data not shown).

A second experiment was performed with LCMV infected mice, but the mice in the stop treatment group were only treated for three weeks. Similar to the previous experiment, the B cell numbers in this group returned to WT levels upon cessation of treatment (Figure 7D). In these mice, however, the antibody levels initially declined, but began to increase after treatment was discontinued (Figure 7E). This increase correlated with plasma cell numbers (Figure 7F) and memory B cells were detected in the three week treated group, but not in the continuously treated group, at the time of sacrifice (data not shown). These results indicate that the lack of memory B cells leads to the decline in plasma cell numbers during the second phase of decline, not the continuous treatment with rituximab.

Lastly, the antibody data were used to calculate the rate of decay of plasma cells in treated mice and to estimate their half-lives following the three viral infections (Figure 8A). From these data, the half-life of influenza-specific plasma cells was estimated to be about 245 days. The half-life of LCMV- and VSV specific plasma cells was found to be longer than those of the influenza infection at 360 days and 370 day, respectively.

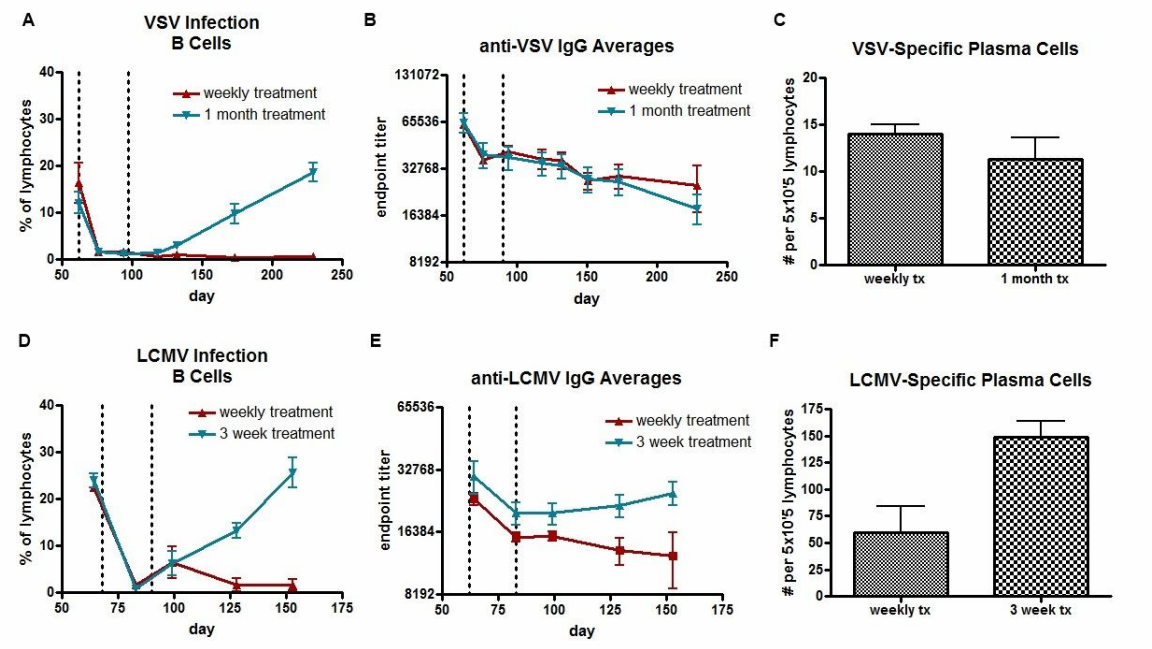

Figure 7. B cell numbers and antibody titers following short treatment regimens. VSV immune mice were treated with rituximab weekly for the duration of the experiment or for only one month and their B cell numbers (A), virus-specific IgG titers (B), and plasma cell numbers (C) were quantified. In a second experiment, LCMV immune mice were treated either continuously or for three weeks with rituximab and their B cell numbers (D), anti-LCMV IgG antibody titers (E), or plasma cell numbers (F) were quantified.

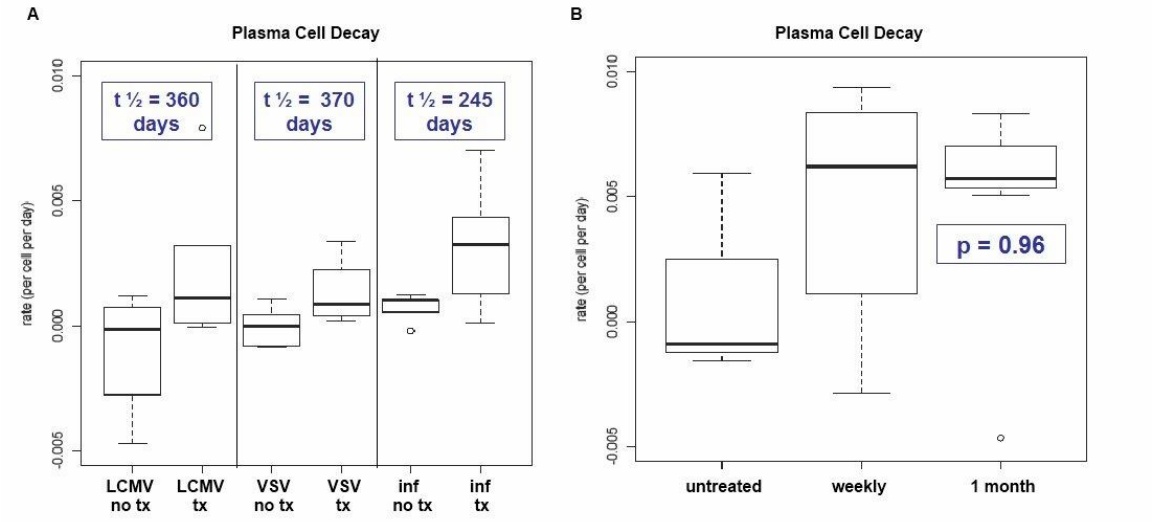

Figure 8. Plasma cell decay rates. (A) Plasma cell decay rates in rituximab treated or untreated mice and half-life estimates are shown for the three different viral infections. (B) The decay rate between untreated, weekly treated, and one month treated VSV immune mice is shown.

DISCUSSION

In these studies, we show that depletion of B cells with rituximab in immunized mice results in a biphasic decline of virus-specific antibody levels. This decline correlates with drops in plasma cell numbers in mice immunized with any of the viruses tested, declines in HAI titers in influenza immune mice, and decreases in viral neutralization titers in VSV immune mice. Plasma cell decay rates were calculated from these data and estimates of their half-lives were made. These data indicate that plasma cells are intrinsically long-lived, but some re-seeding by memory B cells is probably necessary to maintain their numbers over time.

Recently, two studies have been published reporting no decline in plasma cell numbers in mice immunized with the hapten 4-Hydroxy-3-nitrophenyl acetyl (NP) in the absence of memory B cells (1, 14). The disparity in these findings, compared to our own, could be explained by several factors. One difference between these studies is that we utilized viral infections, which may stimulate the immune system differently than immunization with haptens. Another major difference is that we treated mice long-term with anti-CD20 antibodies while the other studies treated for no longer than two weeks. Previously published data reported that although a seven week treatment with anti-hCD20 resulted in significant B cell depletion, treatment for ten weeks resulted in greater depletion of B cell numbers in all tissues as well as a larger decline in serum antibody levels (2). It has also been demonstrated that B cells residing in the peritoneal

cavity are not effectively depleted by anti-CD20 treatment, but that they can be depleted upon migration to other lymphoid organs (18). We reason that the continuous treatment with rituximab in our experiments not only depletes the naïve and memory B cells in lymphoid organs, but also depletes any memory B cells that may be residing in the peritoneal cavity that migrate to other organs at late time points or any newly formed plasma cells that could arise from these cells during the experiments. The experiments presented in Figure 7 provide evidence that although transient depletion may be able to deplete the levels of memory B cells, the residual cells are capable of replenishing the plasma cell population and may lead to the conclusions drawn in these other studies.

The initial decline observed in rituximab treated mice was shown to be due to depletion of a subset of plasma cells that expresses hCD20. The studies in aged mice (Figure 4) argue against the notion that these are newly formed cells from germinal center reactions, as the mice were infected at least six months before depletion was initiated. It has been reported that memory B cells stimulated *in vitro* to become plasma cells express CD20 on their surface (14), so it is possible that these cells are newly formed plasma cells from memory B cells. It is also possible that these cells represent a subset of plasma cells that do not down-regulate hCD20 expression.

In humans, rituximab is used for the treatment of many lymphomas, leukemias, and some autoimmune disorders. Many human studies appear to support the data that we report here. Although studies reporting no significant decreases in serum IgG levels following rituximab treatment can be found (5),

several papers have been published reporting panhypogammaglobulinemia lasting for months or even years following treatment (19, 21-22, 25). One study found that serum antibodies continued decreasing even though rituximab treatment had been discontinued over thirty months earlier (26). This decrease continued despite the return of B cell numbers to normal levels and correlated with very few, if any, memory B cells being detected in these patients (as measured by CD27 expression). Another study reported that upon initiation of rituximab treatment, anti-tetanus antibody levels in patients decreased by 22.7% and did not return to original levels when treatment was stopped (9). These results mirror the data that we observed in our mice experiments. It should be noted, however, that another study found only modest decreases in anti-tetanus titers after rituximab treatment and that the levels returned to near baseline levels at 3-4 months post-treatment (10), while others have reported no significant decreases at all in anti-tetanus titers following B cell depletion (15, 23).

Several complications have been attributed to rituximab treatment in humans. Abnormal T cell responses, possibly caused by lack of B cells, resulted in three patients developing psoriasis following rituximab treatment (13) and a patient died from a varicella-zoster virus (VZV) infection following rituximab treatment, possibly due to a decline in anti-VZV antibodies (7). The results presented here indicate that antibody levels against vaccine antigens in humans treated with rituximab should be monitored closely, especially in those that are treated long-term.

MATERIALS AND METHODS

Mice

The generation and characterization of hCD20^{+} mice has been described previously (2). Briefly, the hCD20 locus was incorporated into bacterial artificial chromosomes and incorporated into pronuclei of F_2 embryos by the Yale Genomics Transgenic Mouse Service. Mice expressing hCD20 were further characterized and backcrossed to a Balb/C background. A breeding pair was obtained from Mark Shlomchik (Yale University) and were then maintained and bred at Emory University.

Infections, bleeding, and rituximab treatment

All mice were infected at the age of 6-8 weeks of age. For influenza infections, anesthetized mice were administered $5x10^3$ pfu of A/WSN/33 (H1N1) diluted to 30 μl in PBS IN. LCMV-Armstrong infections were accomplished by injecting $2x10^5$ pfu diluted to 500 μl in PBS IP, and $2x10^6$ pfu of VSV-Indiana diluted to 500 μl in PBS was given IV for the VSV infections. Peripheral blood was collected at the indicated times via cheek bleeds. Rituxan® (rituximab)(Biogen Idec Inc. and Genentech USA, Inc, NDC 50242-051-21) was obtained from the Emory University Hospital pharmacy and treatment consisted of injecting the mice IP with 1 mg/mouse on days one, three, and five, followed by weekly injections of 1 mg, except for the experiments where it is indicated that treatment was stopped at earlier timepoints.

Hemagglutinin Inhibition Assay

One part serum was added to three parts receptor destroying enzyme (RDE) (Accurate Chemical & Scientific) and incubated at 37°C overnight. The RDE was inactivated the following morning by incubating the samples at 56°C for one hour. Samples were then serially diluted with PBS in 96 well v-bottom plates and eight hemagglutination units (as determined by incubation with 0.5% turkey RBCs in the absence of serum) of influenza virus was added to each well. After 30 minutes at room temperature, 50 μl of 0.5% turkey RBCs (Lampire Biological Laboratories) suspended in PBS-0.5% BSA was added to each well and the plates were shaken manually. After an additional 30 minutes at room temperature, the serum titers were read as the reciprocal of the final dilution for which no hemagglutination was observed.

VSV Neutralization Assay

Serum was heat inactivated at 56° C for 30 minutes before being diluted 1:40 in serum free DMEM. Serial dilutions were performed and mixed with 100 pfu VSV-Indiana at 37° C for one hour. These were then placed on confluent Vero cells in 6-well plates and incubated at 37° C for 45 minutes. After the incubation, the cells were covered with an agar overlay supplemented with 10% DMEM and incubated at 37° C. After two days, the overlay was removed and the cell monolayers were stained with 1% crystal violet. The neutralization titer was reported as the reciprocal of the dilution that inhibited 50% of the virus from forming plaques.

Lymphocyte Isolation and Cell Sorting

Lymphocytes were isolated from lungs by treatment with 1.3 mM EDTA in HBSS (30 min at 37°C, shaking at 200 rpm), followed by incubation with 100 U/ml collagenase (Invitrogen Life Technologies) in 5% RPMI 1640 supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (60 min at 37°C, shaking at 200 rpm). Single cell suspensions were obtained by pushing spleens, lymph nodes or digested lungs through 70 μM nylon mesh filters (Becton Dickinson). Lymphocytes from lungs were purified by centrifuging on a 44/67% Percoll gradient (800 × g for 20 min at 20°C). hCD20 positive and negative cells were sorted using magnetic beads and a MACS column per the manufacturer's instructions (Mitenyi Biotec, catalog # 130-091-104).

Antibodies and Flow Cytometry

For flow cytometry analysis, single cell suspensions of lymphocytes were stained with with anti-CD3-APC (53-6.7), B220-PerCP (CD45R), CD19-PE (MEL-14), CD20-FITC, CD11c-PE-Cy7, or CD138-APC (BD Pharmingen) as previously described (36). Samples were analyzed using a Becton Dickinson FACScaliber®.

ELISPOT

ELISPOT assays were performed as previously described (11). Briefly, ELISPOT plates were coated with 1.5 ml viral lysate/plate for LCMV and VSV, 2.5 mg/well

for influenza, or 62.5 μl per well of goat anti-mouse IgG+M+A (Caltag Laboratories, catalog # M30900) for total plasma cell numbers. PBS was added to these so that a total of 100 μl could be plated/well. The plates were incubated at 4° C overnight. The following morning, plates were washed with PBS/0.1% tween and then PBS and they were blocked for two hours at RT with 10% RPMI. Lymphocytes were resuspended to $1x10^7$ cells/ml and 50 μl of this was added to the first well on each plate. Serial dilutions were and the plates were incubated at 37° C for five hours. Plates were washed with PBS followed by PBS/0.1% tween, and 100 μl of biotinylated anti-IgG diluted 1:1000 in PBS/0.1% tween/1% FCS was added to each well before incubating the plates overnight at 37° C. The following morning, plates were washed with PBS/0.1% tween and 100 μl/well of HRP-conjugated avidin-D (Vector) diluted 1:1000 in PBS/0.1% tween/1% FCS before incubating the plates for one hour at RT. Plates were washed with PBS/0.1% tween followed by PBS and 100 μl of the enzyme chromogen substrate (see below) was added to each well for ~8 minutes to develop the spots. Plates were counted manually. The enzyme chromogen substrate consists of 20 mg/ml 3 amino-9-ethyl-carbazole (Sigma) in dimethylformamide diluted 1:67 in 0.1 M Na-acetate buffer. This was filtered through an 0.2 μm membrane and 100 μl H_2O_2/ 10 ml of substrate was added immediately before use.

Memory B cell assay

Splenocytes were collected from infected mice and from 1 naïve mouse/96-well plate to irradiate as feeder cells. A master mix was made as follows (amount per

plate is listed: 60ul of 1mg/ml R595 LPS (ALX-581-008-L002, Alexis Biochemicals), 2ml ConA stimulated supernatant (see below), 3ml RPMI-10% FCS, 10ml 1E7 cells/ml irradiated naïve splenocytes (sex-matched) 1200 rad irradiation. To each well 150ul of stimulant master mix and 50ul of diluted splenocytes were added. Splenocytes were diluted as follows: 4 cell concentrations with 12 wells/ concentration (across one row of plate). The first dilution was 2.5E5 splenocytes/well and then followed by three, 5-fold dilutions (5E4, 1E4, 2E3/well). The plates were incubated at 37°C with 5%CO_2 for 5 days. On the fourth day ELISPOT plates were coated as described above. On day 5, the plates were blocked as described above. The cultured cells were transferred to 96-well round bottom plates and centrifuged at 800RPM for 3 minutes three times. Cells were resuspended in 200ul RPMI+1%FCS. Cells were transferred to ELISPOT plates and incubated overnight at 37°C with 5%CO_2. The remainder of the procedure is identical to the ELISPOT assay described above. ELISPOT wells were scored positive for Memory B cells if there were greater than 3 plasma cell spots at a dilution where the day 0 ELISPOT assay revealed less than or equal to 1 plasma cells.

Concanavalin A (ConA) supernatant was prepared as follows: Splenocytes were isolated from a naïve mouse and suspended at 1.25 x 10^6/ml in DMEM-5% FCS medium. Cells were cultured in a T-75 flask at 20 ml volume with 2.5μg/ml ConA (C 5275, Sigma) and 20 ng/ml PMA (P1585, Sigma) for 48 hours at 37^{o}C. The supernatant was transferred to 50ml tubes and spun at 1,500 rpm for 10 minutes

to remove the cells. The supernatant was sterilized by filtering through a 0.2μM syringe filter and aliquotted and frozen at -80^{o}C.

ELISA

ELISA assays were performed as described previously (20). Briefly, NUNC MaxiSorp plates (cat # 442404) were coated with 2.5 μg per well of A/WSN/33, LCMV lysate, or VSV lysate diluted in PBS and kept at 4° C overnight. The following day, serum was added at different concentrations and the plates were incubated at 37° C for one hour. Plates were then washed three times with PBS/0.2% tween before HRP conjugated goat α-mouse IgG was added (Sigma cat # A4416). SigmaFast OPD tablets (cat # P9187) were used to develop the plates. The reactions were stopped with 0.1 N H_2SO_4 and the plates were read at 490 nm. The antibody concentrations were determined by endpoint titers.

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Chapter 7: Discussion and future directions

The studies presented in this dissertation, although seemingly disparate, all share the common goal of gaining insight into the biology of and immune responses generated by influenza and other RNA viruses. Specifically, the results presented in Chapters 2 and 3 could aid in the development of improved drugs and vaccines to combat influenza viruses of multiple subtypes. The work presented in Chapters 4 and 5 could lead to the development of novel vaccine vectors that could be used against a wide array of pathogens for which vaccine design has otherwise proven to be problematic, and may have implications for influenza vaccine strategies in humans. Finally, the work presented in Chapter 6 contributes to our understanding of the mechanisms involved in long-term humoral immunity following viral infections and may have implications for rituximab treatment in humans.

The work presented in Chapter 2 focuses on the structural constraints of the influenza virus HA protein fusion peptide. Our results with deletion mutants suggest that structural elements along the length of the fusion peptide, or the spacing or orientation of such elements relative to one another, are critical for the association of the fusion peptide with target membranes during the fusion process. These data suggest that the length of the fusion peptide domain of influenza HA can influence the functional properties of the molecule and the finding that single residue deletions within this domain can have such a large impact on viral fitness has implications for anti-influenza drug design.

In contrast to the targets of currently licensed drugs that are available to combat influenza infections, where escape mutants are common, our results indicate that drugs that are able to effectively target the fusion peptide may be less likely to generate escape mutants due to the structural rigidity of this region. Furthermore, the high level of homology seen in this region between different subtypes of influenza (Chapter 2, Figure 1A) indicates that drugs targeting this area may be active against multiple influenza subtypes. As mentioned in the introduction, one such drug that inhibits the low-pH conformational change from occurring, TBHQ, has been demonstrated to have some effect on influenza infectivity and other drugs aimed at targeting this area are currently being developed [313-314].

Indeed, most, if not all, enveloped viruses encode glycoproteins containing viral fusion proteins (VFP). In addition to members of the orthomyxoviridae family, members of the paramyxoviridae and retroviridae families, among others, are also known to have fusion peptides that are released following proteolytic cleavage of the VFP [419-420]. Although, these fusion peptides do not share direct homology with those of influenza viruses, they are also rich in hydrophobic residues and contain several interspersed glycines, indicating that these fusion peptides may be under similar constraints to those reported in influenza and may also be potential targets for anti-viral drugs [121].

The work presented in Chapter 3 evaluates the ability of candidate influenza vaccines containing partial or complete deletions of the NS1 proteins to generate memory $CD8^+$ T cells. The lack of NS1 function in an influenza vaccine

may have a number of benefits. Among these are the ability to elicit improved T-cell responses due to increased stimulation of IFN-I responses (24) and increased safety due to the inability of these viruses to grow and spread as efficiently as WT strains.

We found that, despite significantly reduced viral replication in the lungs compared to that seen with WT influenza strains, intranasal infection of mice with the NS1 deletion viruses were still able to prime long-lived T and B cell responses. The antigen-specific $CD8^+$ T cells present after vaccination with these attenuated viruses were shown to expand rapidly upon re-challenge, demonstrating that, despite the attenuation of the vaccines, potent memory responses were still generated. Importantly, the attenuated viruses generated larger protective memory T cell populations after boosting than WT viruses and were able to be boosted more readily at early time points. This suggests that priming with more highly attenuated viruses, followed by boosting with a less attenuated strain, could be a way to induce strong immune memory quickly and efficiently.

Finally, the memory T cells generated by these viruses were demonstrated to be capable of clearing heterologous viruses in adoptive transfer experiments [230]. Prior studies with these attenuated viruses had also shown them to be safe and effective influenza vaccines in several animal models [229, 231-233], however, none of these studies looked at the quality of T cells that these viruses are capable of generating. Our study offers insight into this aspect of the immune response against these vaccine candidates and confirms that

NS1-modified viruses could be useful for the design of a new generation of influenza vaccines and that they may be able to target multiple subtypes of the virus through the generation of T cells recognizing conserved epitopes.

Chapters 4 and 5 explored the potential for influenza viruses to serve as vaccine vectors. Although the techniques and principles gleaned from these studies could potentially be applied to a wide range of pathogens, we chose to focus on the anthrax model. The experiments in Chapter 4 demonstrate that, although chimeric influenza viruses containing large pieces of the protective antigen from anthrax are able to elicit immune responses against these inserted domains, these responses are able to be increased substantially by boosting with heterologous viruses. We demonstrate that priming with influenza virus vectors followed by boosting with either recombinant rabies or recombinant vaccinia viruses containing the same inserted anthrax domains both result in these large increases. A scenario where this type of prime-boost regimen would be allowed in humans is unlikely, however, it is not unheard of. The recently reported HIV vaccine success was accomplished by priming with a canarypox vector followed by boosting with a recombinant glycoprotein 120 subunit vaccine [421].

Importantly, we found the order of the priming and boosting of our heterologous vectors to be significant. Mice primed with the inactivated rabies virus and then boosted with the recombinant influenza vector did not generate the same levels of antibodies as those that were given the two vectors in the reverse order. These results suggest that priming the immune system with a live influenza vector is advantageous over the other vectors tested with respect

immune system priming. This could be attributed to replication of the influenza vector stimulating the immune system in a different way from inactivated vectors, perhaps leading to better presentation of antigen by antigen presenting cells. Another factor could be the route of inoculation leading to increased systemic responses. Whether these reasons, or others, are the cause of the increased responses remains to be elucidated.

Other work that could be followed up on from Chapter 4 is the ability of the antibodies generated by these vectors to protect mice from an actual anthrax spore challenge. We were able to elicit significantly higher neutralization titers in our heterologously prime/boosted mice (Chapter 4, Table 1), however, this did not translate to greater protection in spore challenges (Figure 1). This could be a problem with the mouse model, as several studies have suggested that the mouse model is not ideal for animal protection studies, particularly with regard to the role of α-PA responses [422-423]. Further examination of our constructs and immunization protocols in alternative animal models, for both the induction of toxin neutralization activity as well as protection from a BA spore challenge could provide more human-relevant results. We were also perplexed to see no difference in protection between the influenza primed-influenza boosted group and the influenza primed-rabies boosted group, despite our findings in repeated experiments that antibody levels are significantly increased in heterologously vaccinated mice. In fact, the neutralization titers in the heterologously vaccinated mice in this spore challenge experiment were not increased over the

homologously vaccinated counterparts, indicating that the rabies-RBD may not have been properly administered during this trial.

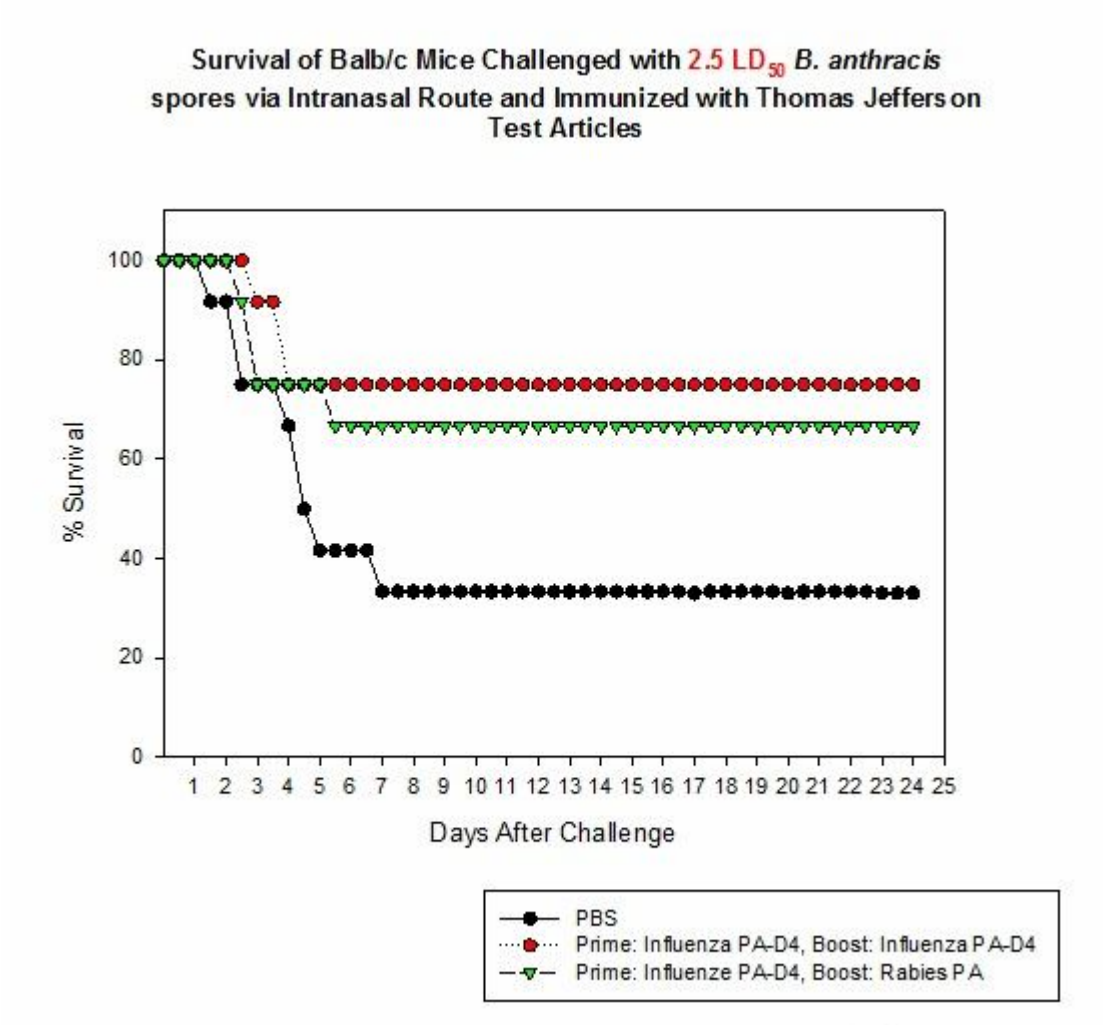


Figure 1. Survival of anthrax spore challenge. Mice were immunized with inf-RBD on day 0 and then boosted with either inf-RBD or rab-RBD on day 42. Mice were challenged on day 77 with 2.5 LD_{50} *B. anthracis* spores and survival was monitored for 24 days. PA-D4 = RBD domain.

Another interesting finding from the work presented in Chapter 4 was the inability to increase anti-PA antibody levels in mice primed with influenza vectors by boosting at later time points with the same influenza vector. We wanted to see if we could overcome this block in boosting by infecting mice with heterosubtypic influenza vectors. The experiments looking at this are described in Chapter 5 and the findings indicate that preexisting immunity to influenza virus, even to viruses containing completely heterologous HA an NA subtypes, leads to a significantly diminished response to influenza vectors in mice. This was surprising, as we know that humans can be infected by multiple influenza subtypes, and even the same subtype that has undergone genetic drift. The finding that responses to the vectors are being diminished by $CD8^+$ T cells recognizing conserved influenza epitopes and that these cells are able to mediate protection against heterosubtypic influenza challenges has implications for influenza vectors as well as influenza vaccination strategies in humans. Though humans can be infected by influenza multiple times, it is hard to determine if prior exposure to influenza viruses is able to ameliorate the effects of subsequent infections, although anecdotal evidence of this does exist [424] and, as discussed in the introduction, the ability to elicit T cell responses is thought to be an advantage of the LAIV over the inactivated vaccine and many influenza vaccines currently in development are designed to elicit both B and T cell responses.

As discussed in Chapter 5, one way to potentially overcome the inability of influenza vectors to elicit immune responses in hosts previously infected with influenza would be to eliminate the cross-reactive epitopes. This would be

difficult to do in mice, as the second most dominant $CD8^+$ T cell epitopes in both B6 and Balb/C mice are also highly conserved, as is the third most dominant, and many of these epitopes are critical for viral fitness. This would be even more difficult to accomplish in humans that have a high amount of heterogeneity in their HLA alleles.

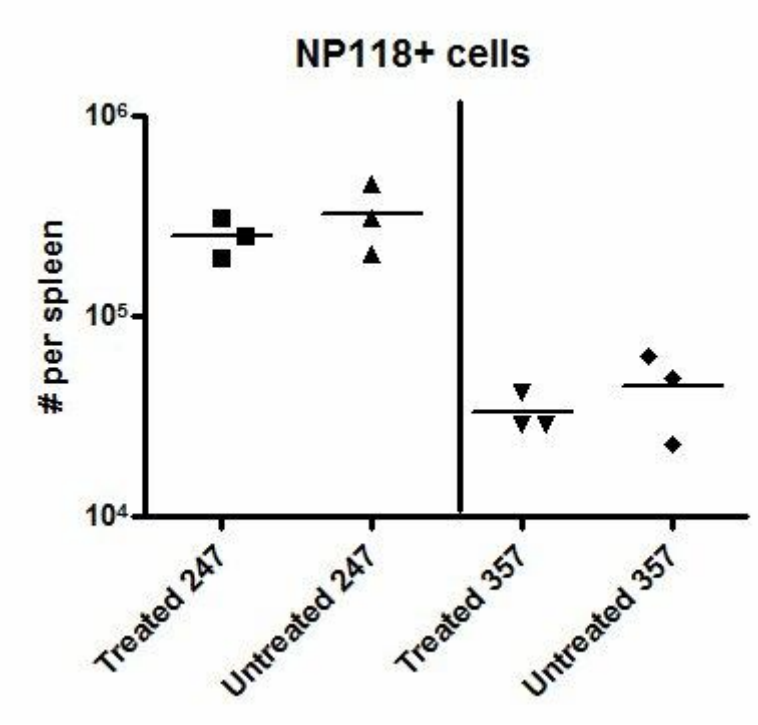
Another potential way to overcome this could be to vaccinate with inactivated influenza vectors. Inactivated vectors should still be able to elicit antibody responses, although T cell responses to these vectors would likely be greatly diminished, as would mucosal responses. In anticipation of immunizing with inactivated vectors, our laboratory has explored inserting foreign antigens into membrane distal sites on the HA protein in order to maximize the ability of the antigen to be recognized by B lymphocytes. We have found several sites, in addition to the amino terminus, into which these foreign domains can be inserted without interfering with viral replication. These constructs have yet to be tested for their ability to elicit immune responses and could merit future studies.

The work presented in Chapter 6 explores plasma cell longevity in the absence of memory B cells. We report that a subset of plasma cells expresses hCD20 and that these cells are depleted during rituximab treatment in hCD20 mice. It is possible that these cells are newly formed plasma cells from memory B cells, as memory B cells stimulated *in vitro* to become plasma cells have been reported to express CD20 on their surface [408]. These cells being a subset of plasma cells that do not down-regulate hCD20 expression is also a possibility

and future studies to distinguish between these two possibilities may be warranted.

A number of studies following antibody levels in humans treated with rituximab were discussed in Chapter 6 and seem to correlate with our findings. A problem with these studies is the fact that most of the patients treated with rituximab had underlying immunological disorders, such as leukemia or rheumatoid arthritis, and a number of them were also taking multiple medications. A further problem with interpreting these studies is that they usually follow low numbers of subjects and the rituximab treatment regimens are inconsistent. Our results indicate that rituximab treatment could have serious consequences in humans over the long-term and that antibody levels against vaccine antigens in these patients should be carefully monitored.

Another area of interest that could be addressed by these hCD20 mice is the fate of T cells in the absence of B cells. A prior study found that LCMV-specific memory CTL responses were not affected by the absence of B cells in B cell-deficient mice and that these mice were able to clear virus faster than naïve mice upon re-challenge with LCMV [425]. However, another paper found that IgG secreting B cells are necessary for the development of memory T cells in a murine model of Graves' disease [426]. In preliminary studies, we have found that LCMV-specific $CD8^+$ T cells do not decline when B cells are depleted at memory time points (Figure 2). Experiments looking at the effects of B cell depletion on $CD4^+$ and $CD8^+$ T cells during all phases of the immune response are either underway or have been planned.



Figures 2. LCMV-specific CD8$^+$ T cells following B cell depletion. Mice were infected with LCMV on day 0 and B cells were depleted with rituximab starting on day 62. The numbers of NP118$^+$ CD8$^+$ T cells in the spleen were quantified by staining and FACS analysis.

Other interesting questions that were not addressed by these studies include the longevity of plasma cells in memory B cell deficient mice that have been immunized with protein antigens or infected with chronic viruses. The finding that antibody levels recognizing tetanus and diphtheria toxins in humans decline much more rapidly than those generated by viral antigens leads to the hypothesis that protein-specific plasma cells may have shorter half-lives compared to virus-specific cells [320]. If this hypothesis is true, the development vaccines and vaccine strategies aimed at overcoming this may be possible. Similarly, it would also be interesting to determine what happens to antibody levels in mice infected with chronic viruses when B cells are depleted and what the consequences of this might be for viral control. Finally, immunizing mice with one antigen, depleting the memory B cells at memory time points, and then re-infecting with a second antigen could give valuable insight into how plasma cells are maintained in humans despite new infections constantly resulting in the influx of new plasma cells recognizing different antigens.

The data presented in this dissertation answer questions spanning many different aspects of influenza A virus, from structural constraints in the fusion peptide to immune responses against influenza vectors and influenza-specific plasma cell longevity. These studies provide insights into these subjects, but they also raise many more questions that will need to be addressed in future studies.

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