Tailored influenza virus vaccines for both the young and old: Vaccine Efficacy of Whole Inactivated Vaccines bearing Immunomodulatory Adjuvants or Multimeric peptides

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biomedical and Veterinary Sciences

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June 5, 2012

Blacksburg, Virginia

Keywords: influenza, virus, vaccine, cytokine, M2e
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By

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(ABSTRACT)

Influenza epidemics and pandemics remain a significant burden to world health and economy. Low efficacy of current inactivated influenza vaccines in the elderly and immunocompromized and the inability to protect against antigenically drifted or shifted strains of influenza virus are the two major problems in influenza vaccine research. To overcome these hurdles, we have utilized an in vitro cell culture vaccine platform, which results in whole inactivated influenza vaccine (WIV) bearing bioactive membrane-anchored immunomodulatory proteins such as cytokines on the virion surface, collectively known as CYT-IVACs (Cytokine bearing-Inactivated Vaccine). In addition, we tested whether a multimeric M2e peptide presented on WIV can serve to enhance immunogenicity and augment protective efficacy of whole virus vaccines. Our panel of cytokines includes IL-2, IL-4, IL-12, IL-23, and Flt3L as well as the multimeric M2e peptide, all fused to the membrane anchoring regions of influenza virus hemagglutinin protein and constitutively expressed in virus permissive MDCK cell line. Subsequent infection with influenza virus results in incorporation of fusion constructs directly into budding progeny virions that are harvested, purified and inactivated to generate distinct CYT-IVAC formulations. Following validation of immunomodulator incorporation, vaccines were tested for in vivo efficacy in either “young adult” or “aged” female Balb/c mice. Our results demonstrate that our CYT-IVAC~IL-12/HA and CYT-IVAC~IL-23/HA serve as potent mucosal adjuvants in young adult mice elicited significantly high levels of mucosal IgA antibodies and afford superior protection against lethal virus challenge. Our Flt3L/HA formulation was the most effective stimulator of systemic anti-viral antibody levels. In “aged” mice a single dose formulation of IL-12 bearing CYTIVAC was superior at affording protection against lethal homotypic virus challenge. Finally, administration of multimeric M2e molecule co-presented on
WIV elicited prolonged antibody responses in “young adult mice” and provided cross-protection from challenge with the heterologous influenza A pandemic strain 2009 H1N1. In conclusion, the CYT-IVAC approach represents a novel tailored advancement to current WIV approaches that has the potential to elicit both potent mucosal and systemic immune responses in young and old.
Dedication

To My Parents
Acknowledgements

I feel truly blessed to be given opportunity to pursue my graduate studies in state of art Virginia Polytechnic Institute and State University. I express my humble gratitude to all the people I met here in Virginia Tech and United States.

To my mentor, Dr. Chris Roberts, I am highly grateful to him for all his encouragement, guidance and mentorship through my graduate career. I learnt numerous molecular, virological and immunological and lab animal related techniques under his supervision. His invaluable advice always helped me whenever I was stuck in research problems and always gave me new ideas to find solutions of any problem I encountered. He helped me think independently and developed confidence in me and showed me how to interpret scientific data in different ways. I am highly honored to have such a fulfilling learning experience I had in his laboratory.

To my esteemed committee, I express my sincere gratitude to Dr. X.J. Meng, Dr. Sharon Witonsky, Dr. Lijuan Yuan and Dr. Josep Bassaganya-Riera for all the intellectual discussions and their inputs and suggestions were very helpful in designing experiments. Special thanks to Dr. Kevin High for all his advice and assistance in carrying research.

I am highly grateful to Dr. Roop Mahajan for selecting me in prestigious ICTAS (Institute for Critical Technology and Applied Science) doctoral scholar program. This program fully supported my graduate research assistantship for a period of four years, along with travel grant support and I was able to interact with eminent people in different fields of science and technology. I am equally grateful to VMRCVM for my graduate research assistantship in last year of my program.

I am thankful to all members of our research group especially Dr. Andrew Herbert, Lynn Heffron, Amanda Gasser and Courie Cohen. Andrew’s guidance, lab notes and thesis helped me tremendously in all stages of my research. Special thanks to Lynn for her help in all of my animal experiments, collection of tissues and processing of samples. You have a special place in my heart and your friendship, generosity and kindness will always be with me for whole life. Thank you for running lab so efficiently. I was lucky to meet and learn from Dr. Binu Velayudhan and Alana Barnes during initial years of my degree.

I feel obliged to all those people who were involved in making this project possible including lab animal care staff, lab animal veterinarian, glassware cleaning services personnel and above all the lives of experimental mice that were used in this dissertation.

I am very indebted to my friends Abeera, Uroosa, Saimeen, Abeer, Nadia, Anum, Naresh, Pratyusha, Vyas, Abhranil and Sachi here in Blacksburg and all those in India for all their good wishes and special moments we shared together that made my stay here pleasant and enjoyable. I express my sincere gratitude to aunties Mrs. Nikhat Rizvi and Mrs. Najma Mazahir for all their prayers and special affection for me.

I dedicate this dissertation to my father Dr. Abdul Qayyum Khan and my mother Mrs. Shehla Khan for their unconditional love, support, encouragement, sacrifice and invaluable prayers
throughout my life. It is because of them I am here at this stage of life. I owe my deepest gratitude and also dedicate this dissertation to my maternal grandparents Late Chaudhary Mohammad Arif and Late Mrs. Shahjahan Begum and paternal grandparents Late Zafar Alam Khan and Late Mrs. Maqsoodan Khan for their sacrifices and prayers throughout their life which has a very big role in making this journey possible. Special thanks are reserved to my sister Dr. Yusra Khan, brother-in-law Dr. Aslam Baig and Brothers Kunwar Hussain, Kunwar Taha and niece Amna for their constant love and wishes. I was very fortunate of having my family so nearby which very few international students get this opportunity. I express my sincere gratitude to my sister Deena Khan, brother-in-law Syed Makhmoor Mazahir and adorable niece Sarah Syed for all their untimely help, support and guidance in every aspect of personal and professional life here in Blacksburg. Thank you for making my life so comfortable, easy and enjoyable. This journey would not have been possible without the support of my husband Mohammad Rabius Sunny who stood by me through the good and bad times and supported me constantly with his unconditional and undemanding love and care. Lastly, I would like to thank my father-in-law Mr. Abdus Sattar, mother-in-law Mrs. Ruba Begum and brother-in-law Mehboob Rabbani for all their prayers, wishes and support.
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<tr>
<td>3</td>
<td>M2e</td>
<td>Ectodomain of M2 protein</td>
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<tr>
<td>4</td>
<td>A/PR/8/34 or PR8</td>
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Chapter 1. Introduction

Background and Significance
Influenza is an ancient human disease that causes acute respiratory infection and is responsible for seasonal epidemics and pandemics in human population. In recent years it has been reported that the frequency of emerging and reemerging diseases by influenza A viruses in humans and animals has increased [1]. Influenza illness is a major cause of outpatient visits, influenza related hospitalizations and deaths in the community. On average, each year seasonal epidemics account for three to five million cases of illness and 250,000 to 500,000 deaths worldwide [2]. In the United States alone, 5-20% of the population is affected annually resulting in 226,000 hospitalizations and 3,000-49,000 deaths estimated by Centers for Disease Control and Prevention [3]. These epidemic events result in substantial socio-economic losses to the world economy, inspite of widespread vaccination efforts and eradication programs. Hospitalization and health care costs pose a significant economic burden and in United States alone annual costs are estimated at upwards of $167 billion dollars [4, 5]. Elderly people are disproportionately affected by influenza and influenza associated complications. On average, in United states alone, 90% of influenza associated deaths and more than 60 percent of seasonal flu related hospitalizations occur in people of age 65 years or older [6]. The likelihood of deaths from influenza-related illness and influenza associated pneumonia among people of age more than 85 years is 16 times and 32 times respectively, more than that of people of age 65 to 69 [6]. Influenza related hospitalizations are also seen to be three times more in elderly people of age more than 65 years as compared to young healthy adults [7].

Influenza pandemics are a great threat to global health. However, the severity of disease and deaths in pandemics of the last century was not always greatest in the elderly. The pandemic 1918 Spanish influenza virus mainly affected young adults aged between 15-34 years [8]. This is postulated to be due to existing pre-existing immunity from pre-exposure of pandemic like virus in aged people during their early life. The recent pandemic influenza A H1N1/09 virus affected mostly people of age <60 years(mortality peaks <20 years of age), which is in contrast to seasonal epidemics which disproportionately affect elderly people (≥65 years of age) [9]. The increased vulnerability of older adults (≥65 years of age) has been attributed to waning immune
systems or immunosenescence with decreased ability to fight against infection, impaired overall immune responses and risk factors that lead to severe cases of disease and complications [10]. Other factors like underlying chronic diseases such as ischemic heart disease, stroke and others impact the outcome of influenza infection and effectiveness of vaccination in elderly [11]. Immunosenescence is associated with impaired innate and adaptive immune responses, decreased antibody production to vaccines, decline in production of mature naïve T cells, decline in number of Langerhans cells (dendritic cells of the skin and mucosa), reduced capacity of dendritic cells, impaired Toll-like receptor expression and function, reduced MHC class I and MHC class II production and decreased number of naïve T cells in peripheral blood [10, 12-14]. Therefore, older adults and people with associated medical conditions are among the highest recommended groups for annual influenza vaccination.

**Influenza Virology**

Influenza is an acute respiratory disease caused by influenza viruses classified within the Family *Orthomyxoviridae*. *Orthomyxoviridae* family members are RNA viruses with single stranded negative sense segmented RNA genomes [15]. Influenza viruses are further classified into three different serotypes namely A, B and C. Serotypes A and B are commonly associated with influenza disease. Influenza epidemics were first reported in 1173-4 [16], with definitive reports in 1694AD [17]. Type A and B influenza viruses have eight genomic segments while type C viruses have seven segments in their genome. Influenza A viruses can infect a variety of species namely human, pigs, horses, birds and many others. Influenza B viruses have limited host range that is largely restricted to humans and seals [18, 19]. Recently influenza B virus transmission was reported in a guinea pig animal model [20]. Serotypes A and B are commonly associated with influenza disease and are mainly responsible for epidemics. Influenza A viruses have resulted in pandemics. Influenza C viruses infect humans and pigs. However, human infections caused by type Influenza C viruses are rare as compared to Influenza A and Influenza B viruses. The influenza A virus genome codes for 11 viral proteins, namely hemagglutinin (HA), neuraminidase (NA), matrix protein 2 (M2), nucleoprotein (NP), RNA polymerase basic protein 1 (PB1), the small PB1-F2 protein, polymerase basic protein 2 (PB2), RNA polymerase acidic (PA) protein, matrix protein 1 (M1), nonstructural protein 1 (NS1), and the nonstructural protein 2 (NS2), also known as nuclear export protein (NEP). Hemagglutinin (HA) and Neuraminidase
(NA) are the two most abundant structural proteins expressed on the surface of influenza virus and are required for viral entry and exit from the host cell respectively. Based on the genetic and antigenic differences in HA and NA, influenza A viruses are further subtyped. There are currently 17 known HA and 10 known NA subtypes [21, 22].

HA is the viral attachment protein that binds to sialic acid residues on the host epithelial cells and mediates fusion of the viral envelope with lyso-endosomal membranes in a low pH dependent manner allowing the release of viral genomic segments into the host cell cytoplasm [23-25]. Neuraminidase is a sialidase that cleaves the sialic acid residues or glycosidic linkages on the glycoproteins on the host cells and helps facilitate virus penetration through the mucin layers coating the surface of the respiratory tract. This aids in viral spread and also allows the release of newly formed virions by removing HA binding glycoconjugates from the surface of infected cells. The viral matrix protein (M1) is located inside the viral envelope providing structural support. It also plays a crucial role in regulating transport of vRNPs from cytoplasm to nucleus, export of RNP from nucleus and in assembly and budding of virus by mediating transport of RNPs to the cytoplasm, where they can subsequently associate with the membrane and other viral assembly proteins [26-28]. The M2 protein is an integral viral envelope protein found in low abundance in virions that functions as a proton selective ion channel and plays a crucial role in viral uncoating, assembly and budding. In the endocytotic vesicles, its activity serves to pump H+ ions inside the virion. This acidifies the virion interior leading to pH dependent conformational changes in the viral matrix protein (M1) and dissociation of M1 from viral ribonucleoprotein (vRNP) complexes, which following HA-mediated fusion of the viral envelope with endosomal membranes, are free to traverse to the nucleus where viral transcription and replication takes place. The nucleoprotein (NP) encapsidates the viral genome and is involved in viral RNA transcription, replication and packaging [23]. Genomic segments associate with the three polymerase proteins (PB1, PB2 and PA proteins) along with NP to form ribonucleoprotein complexes [23]. Viral RNA replication and transcription takes place inside the nucleus of the host cell. Export of progeny vRNPs from the nucleus to cytoplasm depends on viral and cellular proteins which includes M1, NP and viral non-structural protein 2 (NS2) or (NEP) and cellular transport receptors such as chromosome region maintenance 1 (CRM1) in the presence of Ran~GTPs [29-32]. However, the exact mechanism of M1 and NS2 in nuclear export is still unclear. Recently it has been shown that C-terminal domain of NS2 protein is
essential for M1 binding and nuclear export [33]. PB1, PB2 and PA form a heterotrimeric RNA polymerase complex that catalyzes viral RNA transcription to mRNA and viral RNA replication via a cRNA intermediate and from cRNA to vRNA in the nucleus of infected cells. Other functions of the complex include endonuclease activity to cleave cellular pre-mRNAs, cap snatching of host cell pre-mRNAs that serve as primers to initiate transcription and polyadenylation activity for viral mRNAs [34]. Recently the PB1-F2 protein, which is derived from a +1ORF shift during transcription of the PB1 gene, was identified in numerous strains of influenza A viruses. The PB1-F2 protein can initiate the apoptotic pathway in cells acting through the mitochondrial adenine nucleotide translocator 3 (ANT3) and the voltage-dependent anion channel 1 (VDAC1) proteins [35]. It has also been reported to have pro-inflammatory activity by inhibition of the type I interferon (IFN) signaling pathway during influenza and secondary bacterial infections; hence, it can contribute significantly to exacerbation of viral pathogeneses [36, 37]. The NS1 protein is a non-structural protein, which inhibits host innate and adaptive immunological responses and allows the virus to establish infection. Specifically, it inhibits the type I interferon signaling system (IFN) and antiviral activities of IFN-induced proteins like dsRNA-dependent protein kinase R (PKR) and 2′5′-oligoadenylate synthetase (OAS) and RNaseL ([38-40]). NS1 also inhibits host replication and translation by binding and sequestering dsRNA, interferes with host mRNA processing and increases viral mRNA translation. It is known to inhibit host apoptosis by the activation of phosphoinositide 3-kinase (PI3K)/Akt pathway [41-43].

The glycoproteins, HA and NA, are the two major immunogenic proteins of influenza virus. HA is an essential protein required during early stages of influenza virus infection with multiple functions including binding to receptors on host cell surface, viral entry in the host cell and fusion of viral and host membranes [44]. HA is synthesized in host cells as an HA0 precursor that is subsequently cleaved into the two covalently bound subunits HA1 and HA2 as it is transported to the cell surface of infected cells. Cleavage of HA is important in establishing viral infection and determines viral pathogenicity and host tissue tropism [45]. Seasonal human influenza viruses have a single arginine (basic) amino acid at the cleavage site with the recognition sequence Q/E-T/X-R. This is recognized and cleaved by extracellular proteases such as serine proteases which includes Clara cell derived tryptase [46], pancreatic trypsin [47], calf and chicken serum plasmin [48], blood clotting factor Xa isolated from chick embryos [49],
mini-plasmin and ectopic anionic trypsin from rat lungs [50, 51], and the tryptase isolated from porcine mast cells [52], as well as tryptase TC30 from porcine lungs [53], all identified in experimental animal models of influenza [46]. Recently, transmembrane protease serine (TMPRSS) 2 and type II membrane protein human airway trypsin-like protease (HAT) were also identified [54]. Due to their dependence on extracellular cleavage, the spread of human viruses are restricted to those host tissues in which these proteases are present such as the intestinal tract in birds and the respiratory tract in birds and mammals [55].

On the other hand, highly pathogenic viruses like H5 and H7 subtypes have multiple basic residues R-K/R-(K/R/X)-R at the cleavage site and are cleaved by intracellular proteases such as furin a subtilisin-like endoprotease, proprotein convertases (PCs)5/6, which are present in the trans Golgi network and are ubiquitously expressed in many tissues [56-58]. The K-X-K/R-R consensus site motif was recently identified in HPAI viruses and is cleaved by serine proteases within or localized to the cellular membrane, mosaic serine protease large form hMSPL and its splice variant hTMPRSS13 [59]. The presence of a multiple basic cleavage is typically an indicator of systemic infections and higher pathogenicity within these viruses [60].

Influenza virus attaches to host cell by binding of HA viral surface glycoprotein to sialic acid residues present on glycolipids and glycoproteins on the host epithelium. Receptor binding leads to entry of virus into endosomal vesicles via clathrin-mediated endocytosis [61, 62] and macropinocytosis serving as an alternative entry pathway [63]. Endosomes have acidic pH of 5-6, which facilitates the fusion of viral and endosomal membranes by mediating an irreversible conformational change in the HA which leads to exposure of the viral fusion peptide from its buried position in the interior of HA2 resulting in fusion of viral and endosomal membranes [64, 65]. Low pH also leads to triggering of the proton channel activity of M2 protein, which leads to acidification of the viral core, disassociation of vRNP and M1 proteins and release of vRNPs into the host cell cytoplasm. The genetic material is imported into host nucleus through nuclear pores where replication and transcription of viral genome take place (reviewed in [66, 67]). In the nucleus, the viral RNA dependent RNA polymerase complex (PA, PB1 and PB2) initiates and drives genome replication and transcription of viral proteins. Viral mRNAs are exported from nucleus to host cytoplasm for mRNA translation to viral proteins. Viral polymerases (PBA, PB1 and PB2) are translocated to the nucleus through their nuclear localization signal (NLS)
NP and M1 proteins, which also have an NLS, are also translocated into the nucleus, with NP associating with new viral genomic segments forming vRNPs and the M1 proteins associating with vRNPs. The latter associations together with help from the NS2 (NEP) protein, vRNP complexes are shuttled out of the nucleus in a CRM1 dependent manner [69-71]. HA, NA and M2 proteins enter the cells’ exocytic pathway and are transported via the golgi network to the plasma membrane, the site of virus assembly and release. Viral assembly or morphogenesis occurs at the apical regions of the plasma membrane of polarized epithelial cells [72]. Specifically HA and NA associate with lipid raft domains in the apical regions of membranes and this association is required to initiate virus assembly and budding [73]. The M2 protein accumulates within non-lipid rafts domains at the cell surface [74]. M1 protein plays an important role in viral assembly and budding. It binds both viral RNPs (a complex of negative sense vRNA, NP, polymerase complex and NS2) and the cytoplasmic tail domains of HA, NA and M2 proteins projecting from the plasma membrane and this serves to recruit the viral components to the budding sites in plasma membrane [75, 76]. During budding of virions, the M2, initially stabilizes the site of budding, alters the curvature of membrane at the neck of budding virion and helps facilitate membrane scission [77] resulting in virion release. The NA removes sialic-acid containing glyconjugates from neighboring regions on the cell surface, which aides in the final release of newly synthesized virions from the surface of infected cell [78].

**Influenza Epidemics and Pandemics**

Influenza is an ancient human disease that is responsible for seasonal epidemics and pandemics at irregular intervals. Epidemics are mainly due to Influenza A and Influenza B viruses while pandemics are caused only by Influenza A viruses. It has been suggested that over the past 500 years 13 pandemics occurred [79]. Seasonal epidemics occur as a result of gradual minor antigenic changes or antigenic drifts caused by point mutations in the structural glycoproteins of influenza virus, the HA and NA proteins. These epidemics usually occur in colder months with one wave each season affecting 5-30% of the population. Epidemics affect the whole population but highest risks are seen in children and elderly. Mortality rates are generally much lower during epidemic outbreaks as compared to pandemics and deaths follow a U shaped pattern with respect to age [80-82]. These seasonal epidemics are moderated by the use of antigenically matched vaccines and antiviral drugs. Pandemics are epidemics that occur in a large
geographical area at irregular intervals and are also referred to as global epidemics with high transmission rates that vary in the severity of disease. Pandemics occur as a result of major antigenic changes or antigenic shifts in the hemagglutinin and neuraminidase proteins of influenza virus. This typically results in the introduction of a new infectious subtype to which a significant portion of the human population has not been previously exposed or they are immunologically naïve to this new subtype [8]. This can occur when a virus from an animal species undergoes genetic reassortment with a human virus during co-infections, resulting in the emergence of a virus that can infect and spread rapidly in the human population. Gene reassortment occurs due to the segmented nature of the influenza virus genome, which allows packaging of segments from two or more different subtypes of influenza giving rise to new subtypes. Together with the constantly evolving changes due to antigenic drift, a new virus subtype in an animal species may adapt over time such that it acquires the ability to efficiently infect and transmit the virus among humans [80, 81, 83, 84]. Pandemics can occur in any season and are usually seen in multiple waves. For a pandemic virus, the new virus typically should not have circulated in the human population for at least one generation, it should have acquired the ability to infect and efficiently replicate in humans and it also should have the ability of efficient human-to-human transmission. The previous century experienced three major pandemics with devastating consequences: the H1N1 1918 (Spanish flu), the H2N2 1957 (Asian flu) and the H3N2 1968 (Hong Kong flu) pandemics [85]. Of these, the 1918-1919 pandemic or Spanish influenza was the most notorious and serious. It has been estimated that this pandemic had an attack rate ranging from 20-60% in most countries and a very high mortality rate of around 1% to 2.5% of world population resulting in 20-50 million deaths worldwide [86, 87]. This pandemic mainly affected young adults aged between 15-34 years [8]. Post-mortem examination of patients by bacteriologists and pathologists during that time revealed the presence of bacterial microbes in lungs [88]. Further examination of preserved lung samples of 1918 patients showed rapid destruction of airway epithelium and profound lung pathology [89]. Secondary bacterial pneumonia has been shown to be the main leading cause of death in this pandemic, although viral pneumonia was also seen in many patients [90]. The Spanish Flu virus H1N1 has been shown to be almost entirely derived from avian origin [91], while the 1957 H2N2 and 1968 H3N2 pandemic viruses were reassortant viruses between human and avian viruses. The recent A/H1N1/09 pandemic virus was of swine origin and has been shown to be a result of multiple
reassortments specifically of North American H3N2 and H1N1 swine viruses with Eurasian avian-like swine viruses [92, 93].

Aquatic birds of the orders Anseriformes (waterfowl) and Charadriformes (gulls and shorebirds) are the clinically asymptomatic reservoir of influenza viruses and serve as a source of diverse variants and genotypes of influenza viruses for all host species [21, 94]. Most of the avian influenza viruses replicate in the intestinal epithelium of birds and transmission is mainly fecal-oral. In contrast, human influenza viruses proliferate mainly in respiratory epithelium and transmission is airborne or by manual transmission of mucosal secretions. It was believed since 1976, that avian influenza viruses must have undergone prior adaption in a “mixing vessel” such as swine to acquire the ability to infect humans [95]. However, the outbreak of high pathogenic avian influenza virus (HPAI) H5N1 in 1997 changed this conceptual notion as this virus was able to directly jump species and infect humans without prior adaptation in swine [96]. HPAI H5N1 is a highly fatal virus in avian species that emerged in Southern China in 1997 and since then it has caused numerous outbreaks in Asia, Europe and Africa and killed millions of poultry [97] as well as sporadic cases in humans [98]. This virus has the ability to infect humans, albeit the efficiency of human-to-human transmission is inefficient. As of March 2012, WHO (World Health Organization) data suggests that HPAI H5N1 has killed only 349 humans across fifteen countries, although, it has killed millions of birds [99].

There are species-specific differences in influenza viruses, which determine virus tropism. Human influenza viruses preferentially bind to receptors containing sialic acids attached to galactose via an $\alpha_2$-$6$-linkage (SAalpha2,6-gal) [100], while avian influenza viruses recognize $\alpha_2$-$3$-linkages (SAalpha2,3-gal motif). The SAalpha2,6-gal receptors are highly abundant on the upper respiratory tract epithelium in humans, whereas SAalpha2,3-gal glycoconjugates are abundantly expressed on epithelial cells lining the intestinal tract of waterfowl [101, 102]. Thus, in order to efficiently infect humans, avian influenza viruses need to acquire the ability to bind human type receptors. HPAI H5N1 virus replicated in the human respiratory tract only to a limited extent which is mainly due to the presence of some SAalpha2,3-gal receptors in the lower respiratory tract in humans. This served to restrict and limit the ability of the virus to efficiently transmit among humans. Interestingly, the trachea of pigs has both kinds of glycoconjugates [103], which allows them to serve as a “mixing vessel” for new emerging influenza viruses.
Swine influenza viruses can bind both kinds of receptors [104, 105]. There are rising concerns that the H5N1 virus as it continues to mutate and adapt will eventually acquire the ability to efficiently bind human SAalpha2,6-gal receptors, which would lead to a highly transmissible and virulent new subtype. Recently, it has been shown that other animals, especially terrestrial poultry can also serve as intermediate hosts by expressing both SAalpha2,3-gal and SAalpha2,6-gal receptors [106].

While the world was speculating about the potential of H5N1 as a pandemic viral strain, the 2009 H1N1 virus of swine origin emerged in February 2009 in Mexico, and swept across the globe within a 6 month period. The WHO declared an influenza pandemic in June of 2009, as the virus had spread to a total of 74 countries and territories. Fortunately, this virus caused a mild disease for most individuals, although a significant number of young or middle age people required hospitalization and intensive care treatment, as they were suffering from acute respiratory distress syndrome [107]. The influenza A/H1N1/09 virus is derived from a quadruple reassortment between North American swine influenza, North American avian influenza, human influenza and two swine influenza viruses found in Asia and Europe [108]. Tracing back events in its evolution, it has been reported that in 1998, there was a triple reassortment event between classical swine H1N1, human H3N2 and avian influenza viruses that led to the establishment of a triple reassortant virus (H1) circulating in pigs of North America, which later reassorted in a second event with Eurasian swine viruses, ultimately giving rise to the pandemic A/H1N1/09 virus. The quadruple reassortant A/H1N1/09 virus combines PB2 and PA genes from North American avian viruses, PB1 gene from human H3N2 virus, HA, NP and NS genes from classical swine H1N1 viruses and NA and M genes belong to Eurasian swine viruses which were originally derived from avian species [92, 93]. In August 2010, WHO declared that the H1N1 virus was in the post-pandemic phase and removed the phase 6 alert level. Overall mortality associated with this pandemic virus was less than seasonal influenza virus; albeit deaths were more prevalent in young children compared to the elderly, which are typically more affected during past seasonal outbreaks [109].
Innate immune response to influenza virus infection.

Influenza virus infects epithelial cells that line the respiratory tract of the host, where it suppresses host cell gene expression and cell translation machinery and replicates to generate progeny virions that are released by a budding mechanism within 20-40 hours after infection [110-112]. The initial innate responses to infection include the activation of transcription factors and signaling pathways leading to synthesis and production of chemokines (RANTES, MIP-1α, MCP-1, MCP-3, and IP-10), pro-inflammatory cytokines (IL-1β, IL-6, IL-18 and TNF-α), and antiviral (IFN-α/β) cytokines that all help to establish an antiviral state in neighboring cells and limit and restrict viral replication [113]. Local chemokine production triggers the migration of blood leukocytes to the site of infection, resulting in a cascade of proinflammatory (Th1) cytokine production important in establishing an antiviral state and contributes to promoting both humoral and cellular adaptive immune responses [114-117]. Host apoptotic pathways are also initiated following influenza infection, resulting in partial denudation of virus infected respiratory epithelium. During this period, influenza virus also infects macrophages, which act as a first line of defense leading to production of high amounts of chemokines and cytokines that exert chemotactic and proinflammatory effects respectively, to clear infection with subsequent activation of adaptive immune pathways [113, 118-120]. Alveolar resident macrophages also become highly phagocytic following infection and phagocytose apoptotic virus infected cells limiting viral spread as well as secrete abundant cytokines that further activates innate and cellular immune responses. Early responses to influenza infection also include infiltration of neutrophils, which secrete reactive oxygen species, cationic peptides, eicosanoids, elastase and proteolytic enzymes that further serve to limit infection, but can also lead to widespread tissue damage if left uncontrolled [121].

Type I Interferons (IFN) induction and their function

Type I IFN (IFNα/β) are antiviral proteins produced by infected epithelial cells or macrophages that act as a first line of defense against viral infections and serve to inhibit virus replication. Plasmacytoid dendritic cells particularly produce high amounts of type I IFNs upon influenza infection [122]. During viral infection and subsequent replication, several pathogen-associated molecular patterns (PAMP) are synthesized such as cytoplasmic viral ssRNA [123] and dsRNA intermediates of viral replication. These PAMPs are recognized by pattern recognition receptors.
(PRR) present on the host cells resulting in the initiation of host antiviral signaling pathways. The antiviral response has been reviewed extensively by Randall et al [124]. Pattern recognition receptors that recognize influenza viral PAMPs include Toll-like receptors (TLR) (TLR 3 and 7), retinoic acid inducible gene I-like receptors (RIG-I) and NOD-like receptors. TLR7, present within the endosomes recognize influenza genomic ssRNA and lead to activation of MyD88 adaptor and inflammatory cytokine and IFN production [125]. TLR3 also present on endosomes recognize dsRNA intermediates produced during viral replication and requires Toll-interleukin-1 (IL-1) receptor domain-containing adaptor-inducing beta interferon ([IFN-β] TRIF) for its signaling [126]. TLR3 and TLR7 pathways do not appear to play a role in CD4 or CD8 T cell activation following influenza virus infection although TLR7 does influence B cell responses and antibody class switching [127]. TLR4 at the cell surface has also been reported to be involved in innate responses to highly pathogenic H5N1 influenza virus [128]. Genomic ssRNA in inactivated whole virus influenza vaccines can also activate the TLR7 pathway and induce innate antiviral IFN responses [125]. RIG-I, is an internal PRR abundantly expressed in cDCs, macrophages and pulmonary epithelial cells, where it can recognize cytoplasmic ssRNA bearing 5’ phosphates and signals through the adaptors MAVS or IPS-1. The latter leads to activation of IRF-3/IRF-7 and/or NF-kB signaling pathways that subsequently initiate transcription of IFNβ mRNA and/or proinflammatory cytokines, respectively [123]. The NS1 protein of influenza virus has been reported to interact with RIG-I and thereby suppress IFNα/β production in conventional DCs and lung epithelial cells [129]. Nod-like receptor is a third PRR that responds to influenza virus infection leading to the formation of Nucleotide-binding domain and leucine-rich-repeat-containing protein 3 (NLRP3) inflammasomes, activation of caspase-1 and cleavage of IL-1[β], IL-18, IL-33, and IL-17f precursors to their biologically active forms. Notably, IL-1β and IL-18 cytokines play important roles in recruitment of monocytes to the lungs during infection [130].

The cytokines and IFNs produced by TLR, RIG-I and NOD-like receptor signaling pathways all serve to modulate innate and adaptive immune responses to clear virus infection [131]. The type I interferons (IFNα/β) released by infected cells act by inducing transcription factor ISGF3 and regulate the synthesis of antiviral proteins like protein kinase R (PKR), RNAaseL/2’,5’-oligoadenylate synthetase (OAS), and orthomyxovirus resistant GTPase (Mx protein) in infected
These antiviral proteins serve to limit replication of virus and degrade viral RNA (OAS), inhibit host translation machinery (PKR), interfere with transcription (Mx1) and posttranslational activities (MxA) and thus inhibit viral replication [134]. Other functions of IFNα/β include increased anti-viral resistance in nearby uninfected cells, maturation of antigen presenting cells, recruitment of monocytes/macrophages and T cells to site of inflammation, T cell survival and enhanced IFNγ production from NK and T cells in synergy with IL-18 and IL-12 [113]. IL-1 and IFNα/β release are associated with fever and muscle aches in the body. IFNγ further stimulates macrophages to secrete cytokines like IL-12, IL-18 that in turn activate NK and T cells to secrete IFNγ creating a positive feedback loop. However, all these early responses are often insufficient to clear virus infection from the lungs and require adaptive immune responses to aide in recovery from viral infections.

Adaptive immune response to influenza virus infection

Resident dendritic cells (RDC) are professional antigen presenting cells (APCs) that are present in the respiratory tract at distinct sites either within or at the margin of the epithelium (CD103+) or in the submucosa or interstitium (CD11b high) and are the major sentinals for trapping foreign antigens in the airways. Two types of RDCs have been characterized based on their phenotype, function and anatomical distribution in lung. These are conventional DCs (CD11c+) and plasmacytoid DCs (CD11c-B220+). cDCs are further classified into CD103+CD11b+ (in epithelium), CD103-CD11bhigh (in pulmonary interstitium) and monocytic DCs [135, 136]. cDCs capture viral antigens mostly by direct infection [137] or by phagocytosis of virions or infected dying or dead cells. Viral infection leads to the maturation and activation of DCs and along with the inflammatory milieu to upregulation of the chemokine receptor CCR7 on the surface of DCs. This allows chemokine dependent migration of DCs from lungs through the afferent lymphatics to draining lymph nodes [135]. Activated DCs bearing viral antigen present antigen to naïve T cells through either the MHCI or MHCII pathway in draining the lymphnodes leading to activation of CD8+ or CD4+ T cells respectively, followed by their clonal expansion and differentiation into effector and memory virus specific T cells. Effector T cells subsequently migrate from the lymph node through the efferent lymphatics to the blood via the thoracic duct and finally reach the infected lung. Effector CD8+ T (cytotoxic Tcells, Tc) cells play major role in clearance of virus infected cells and lung recovery from infection [138]. Tc cells also secrete
IFNγ and tumor necrosis factor (TNF) which helps in inhibiting virus replication and further attract phagocytes that eliminate virus infected dead cells and clean virus debris. On the other hand, CD4+ T cells serve as helper T cells (Th) cells and are of different types including Th1 or Th2 or Th17 cells. Th1 immune responses involve both CD8+ T cells and IFNγ producing CD4 T cells both of which contribute to clearance of virus. Th2 cells promote B cell activation which is followed by their clonal expansion and generation of virus specific antibody secreting plasma cells and memory B cells [139]. Influenza virus specific antibodies bind to released virus in the medium and cause them to agglutinate, thereby neutralizing viral receptors and preventing virion attachment to host cells.

**Recovery from influenza infection.**
Influenza viral replication leads to the death of infected epithelial cells by direct cell lysis or by apoptosis resulting in denudation and destruction of the epithelium around day 5-7 following infection. Virus induced apoptotic bodies are cleared by phagocytes such as dendritic cells and macrophages [140, 141]. However, extensive destruction of alveolar epithelial cells may lead to respiratory failure due to pulmonary edema and poor exchange of gases since these cells are essential for maintaining airway function. Therefore, to maintain homeostasis, excessive tissue damage and inflammation needs to be controlled in the host in order to facilitate recovery. Recovery involves regeneration of the mucosal respiratory epithelium. A balanced Th1 and Th2 immune response is required to clear virus infection and facilitate recovery from influenza [142]. Th1 induced IFNγ and cytotoxic T cell responses in the absence of IL-4 (Th2) can lead to pronounced immunopathology despite clearance of virus from lungs [143]. On the other hand, enhanced Th2 (IL-4) responses may impair local CD8+ T cell responses [143, 144]. Therefore, there needs to be a balance of Th1 and Th2 responses working synergistically to clear infection and restore lung architecture. Major cells that control lung injury during recovery are regulatory T cells (Treg), effector T (Te) cells and epithelial cells [145]. Alveolar macrophages also serve as regulatory cells that modulate the induction of adaptive immune responses by secretion of anti-inflammatory and regulatory cytokines such as TGFβ, IL-10, and NO which help dampen and restore immune homeostasis during the healing process [146]. T effector (Te) cells also act to control excessive inflammation by producing IL-10, TGFβ, and CD200 [147, 148]. Treg cells also produce regulatory cytokines like IL-10 and TGFβ which inhibit or dampen effector cell
responses. Lung epithelial cells express CD200, which upon engagement of CD200R on macrophages serves to restrict their inflammatory function [149].

**Influenza Vaccines.**
The most effective and economic strategy to reduce severity of influenza infection and influenza-related hospitalizations is vaccination [150]. The evolution of influenza vaccination began in 1930 when influenza virus was first isolated from swine by Shope and Smith [151]. In 1933, Smith, Andrewes and Laidlaw first isolated influenza virus from humans [152], but it wasn't until the early 1940s, that embryonated hen eggs were utilized for propagation of influenza virus [153]. This subsequently led to the manufacture of the first influenza vaccine, which was a formaldehyde-inactivated whole virus vaccine developed by US armed services and made commercially available in 1945 in the United States [154]. This vaccine had the disadvantage of being highly pyrogenic and induced local injection site reactions due to incomplete removal of contaminants during the purification process [155, 156]. Later with the introduction of zonal ultracentrifugation in the 1960’s the viral purification process was significantly improved, and remains in place today as an integral part of current inactivated influenza vaccine production [157, 158].

There are currently two major formulations of influenza vaccines available commercially for humans. These are the live attenuated, cold-adapted influenza vaccine (LAIV) and inactivated vaccines. The latter can be formulated as inactivated whole virus vaccine, (whole virus vaccines), as split vaccine (influenza virus split into viral contents by the use of detergents or solvents) or as subunit vaccine in which the surface antigens HA and NA are purified and administered as proteins (subunit vaccines). All human seasonal influenza vaccines, both inactivated and live attenuated, are trivalent in nature composed of three viral strains that represent both influenza A and B strains currently circulating within the human population. In the past several decades, these include influenza A H1N1 and H3N2 strains as well as an influenza B strain. H2N2 strains have not circulated in humans since 1968. The WHO conducts global influenza surveillance each year and decides which viral strain will most likely be circulating in the coming year. Based on WHO recommendations a new trivalent vaccine combination is prepared each year to best match the currently circulating strains. For the year
2011-2012, trivalent vaccines contain A/California/7/2009 (H1N1)-like, A/Perth/16/2009 (H3N2)-like, and B/Brisbane/60/2008-like antigens [159]. The inactivated vaccine is administered intramuscularly, approved for use in persons 6 months and older and is safe to administer to high risk groups such as children with asthma, children 2-4 years of age with wheezing history, pregnant women, the immunosuppressed and those with chronic medical disorders, as well as residents of nursing homes and chronic care facilities. Importantly, it is contraindicated in persons having anaphylactic hypersensitivity to eggs or to other components of influenza vaccines.

Live attenuated influenza vaccine (LAIV) is a cold adapted, attenuated influenza virus vaccine marketed as Flumist®. It is administered intranasally and has been commercially available in United States since 2003. It was the first intranasal vaccine for humans in United States and is licensed only for individuals from 2 to 49 years of age. However, this vaccine has some biosafety issues, since it cannot be used in children less than 2 years as they develop wheezing which can result in respiratory illness and hospitalization [160]. The manufacture of LAIV involves serial passage of influenza type A strain (A/Ann Arbor/6/60 H2N2) and a type B strain (B/Ann Arbor/1/66) in specific pathogen-free primary chick kidney cells at sequentially lower temperatures [161]. These are the master donor viruses (MDV). Passaging of these viruses at lower temperatures result in the acquisition of multiple changes in viral genomic segments and different phenotypes such as cold-adapted (ca), temperature-sensitive (ts), and attenuated (att). These MDV viruses are used for the generation of reassortant strains of LAIVs and allow efficient replication of reassortant viruses in chicken embryonated eggs. Live attenuated influenza viral strains are generally 6:2 reassortant strains that contain six internal gene segments (PB1, PB2, PA, NP, M and NS1) from the cold adapted (ca) master donor viruses (caMDV A/Ann Arbor/6/60 H2N2) or cold adapted (ca) (B/Ann Arbor/1/66) viruses and two gene segments coding for HA and NA from circulating influenza viruses of that season. Due to continuous antigenic drifts each year to make a seasonal trivalent vaccine, master donor viruses (MDV) serve as backbone with conserved internal proteins and HA and NA genes are varied based on the currently circulating strains during that year to yield a new vaccine seed strain. Wild type viruses and MDVs are co-infected and 6:2 live attenuated reassortants are selected by genotyping. In 1998, a reverse genetics method was made available whereby 8 or 12 plasmid
cDNAs encoding influenza virus sense RNA and the polymerase proteins, PB1, PB2 and PA were transfected into cells to generate infectious virus. The cold-adapted (ca) phenotype allows the reassortant viruses to replicate efficiently at lower temperatures (25°C) of the upper respiratory tract and limits viral replication at higher temperatures (37°C) of lower respiratory tract. Thus LAIV administration does not lead to infection of the lower airways [162]. As noted this vaccine is restricted to healthy infants and adults aged 6 months to 49 years of age. It is not recommended for at risk groups, including infants less than 6 months old, the immune compromised, and older individuals (>50 years of age). As with the inactivated vaccines propagated in eggs, people with hypersensitivity to any component of LAIV and to eggs should avoid vaccination.

In general, it has been seen that current influenza vaccines are able to provide protection against severe infection and reduce symptoms from disease [163]. Protection is mainly mediated by inducing neutralizing antibodies against the viral surface glycoproteins HA and NA [164, 165]. These vaccines are propagated in eggs and are standardized based on the antigen content of the viral hemagglutinin. However, they possess several limitations. First, they have lower efficacy in high risk populations like the aged (17 to 53% effectiveness) while in young adults it is very effective (70 to 90% effectiveness). Second, these vaccines are not able to protect if the circulating virus strain is antigenically different from the vaccine seed strains. This happens periodically, when antigenic drift variants are numerous. The immunity induced is not broadly cross-protective. Moreover, frequent antigenic changes in circulating influenza virus strains require constant annual surveillance and vaccine compositional changes. Finally, these vaccines are produced in embryonated chicken eggs but this production method suffers from limitations of long production time, egg allergies in some individuals and limited production capacity. During high demands incurred by epidemic outbreaks and as a consequence of avian influenza outbreaks there is a potential risk of depletion of the egg supply [166]. Moreover, split vaccines are considered to be less immunogenic than whole virus vaccines in immunologically naive individuals [167, 168]. While exhibiting better overall immunogenicity, whole virus-inactivated vaccines were available in the market only until the 1980’s, as they suffered from increased side effects such as fever and headaches [169]. Trivalent inactivated vaccines are injectable and thus suffer from limitations due to poor induction of mucosal and cell-mediated immunity. Immunologically naïve individuals like children are more at risk in the event of a pandemic due
to poor immunogenicity of current vaccines [170]. Recently, a high dose injectable trivalent influenza vaccine (Fluzone High-Dose, Sanofi Pasteur) was licensed by the US FDA in December 2009 for persons ≥65 years of age [171]. This is a single intramuscular or intradermal formulation vaccine that contains high amounts (4 times) of HA antigen (total 180µg with 60µg from each of the 3 representative strains). However there are concerns about safety, reactogenicity and adverse effects like pain and inflammation at the injection site [172].

Therefore, more effective influenza vaccines are needed which are highly immunogenic and induce robust mucosal antibody and balanced Th1/Th2 immunity that is sustained. These vaccines should also enhance cytotoxic T cell memory responses that are broad and cross-protective as well as exhibit enhanced efficacy in high risk populations such as the elderly.

**Route of vaccination and immune responses following vaccination**

Following parenteral administration of influenza vaccine, viral antigen migrates from the muscle to the draining lymph nodes; the armpit of humans. Migration occurs either in the form of free antigen or as an opsonized antigen or taken up by local antigen presenting cells, mainly immature dendritic cells with high antigen uptake and processing ability [173]. Upon stimulation with vaccine, dendritic cells increase expression of class II MHC and CD54 molecules on their surface and start secreting co-stimulatory cytokines like IL-12 and TNFα [174] that leads to the maturation of dendritic cells. Mature dendritic cells present vaccine antigenic peptides through MHC class II to naive T cells in the draining lymph node along with the secretion of co-stimulatory molecules leading to Th cell activation which further activate B cells, and modulate their differentiation and antibody production. Follicular T (T<sub>FH</sub>) cells also regulate multiple stages of B cell immunity [175] which on contact with antigen loaded dendritic cells get activated and together with effector CXCR5+CCR7-T<sub>FH</sub> cells migrate to the follicular region of lymph nodes where effector T<sub>FH</sub> make contacts with B cells, which is followed by their migration to follicular regions and their expansion to secondary follicles [176]. Inactivated split and subunit inactivated virus vaccines induce a Th2 predominant response while whole inactivated virus vaccines (WIV) induce Th1 skewed immune responses as observed in murine experimental models [177]. Interestingly, in humans it was found that whole inactivated virus vaccine stimulated upregulation of co-stimulatory molecules on DCs and the secretion of Th1 cytokines like IL-12 and TNFα from PBMC, whereas subunit vaccines showed only moderate
effects on DC [178]. However, overall it has been suggested that subunit vaccines are stronger stimulators of T cell proliferation than whole inactivated vaccine [178]. Within 2-6 days following vaccination, influenza specific antibody levels, antibody secreting cells and peripheral blood lymphocytes can be detected in serum and tonsils [179, 180]. The highest levels of antibodies are seen at 2-3wk post-vaccination, albeit these levels subsequently decrease overtime with levels dropping two-fold by 6 months of vaccination [181]. Inactivated split or subunit virus vaccines induce protective antibodies in sera mainly against HA and NA glycoproteins as well as NP and M proteins [182]. Antibodies are also detected in saliva and are mainly of the IgA isotype compared to IgG and IgM in serum [180, 183]. There is also a rapid increase in influenza vaccine specific antibody secreting cells (ASCs) in tonsils and peripheral blood following parenteral immunization which are predominantly IgA and IgG respectively. However, inactivated vaccines are poor inducers of cellular immune responses [184].

LAIV are administered intranasally to the nasopharynx using a spray device [185]. The vaccine virus replicates in the ciliated epithelial cells of nasopharyngeal mucosa closely mimicking wild type virus infection leading to presentation of viral peptides via presentation on MHCI and MHCII molecules resulting in inactivation of T helper and cytotoxic T cells [186]. This ultimately leads to the induction of secretory IgA antibodies in the respiratory tract, neutralizing serum IgG antibodies and induction of cellular immunity [187]. As noted, LAIV administration does not lead to infection of the lower airways [162]. Mucosal IgA antibodies and cytotoxic T cells are the main immune correlates of protection induced by LAIV, although they are difficult to quantitate [188]. Serum IgA and IgM antibodies peak at 2 weeks following vaccination with LAIV declining by 4 weeks, whereas IgG levels peak at 4-12 weeks and are detectable for up to one year [173]. Secretory IgA levels in nasal secretions peak at 2-11 weeks following vaccination in adults and gradually wane over 6 months [189], or over 12 months in children [190]. Cytotoxic T cells typically recognize epitopes on internal viral proteins such as the matrix and nucleoprotein, which are more conserved among subtypes and this provides the basis of heterosubtypic immunity induced by LAIV [187, 191, 192].
Correlates of protection
Identifying correlates of protection from infection and following immunization are very important in vaccine design. It has been widely accepted that serum anti-hemagglutination antibodies (HAI) with a titer of 1/40 or above following immunization by inactivated influenza vaccine is protective [193-195]. However, the HAI test has low sensitivity and specificity issues [196]. Virus neutralizing antibodies are considered as a functional measure of humoral protective immunity as these antibodies prevent infection and help clear infection from infected lungs [197, 198]. Neutralizing antibodies are also assessed for their reactivity against antigenic drift variants as a measurement of the level of cross-protective humoral responses induced by vaccination [199]. Serum IgG antibodies protect by diffusion into nasopharynx and lung where they neutralize and dampen infection [200]. Of note, humoral responses can vary significantly with age. Serum IgG levels correlate well with protection in adults less than 50 years of age [194, 201, 202]. In contrast, cytotoxic T lymphocyte responses and Granzyme B production are considered as better correlates of vaccine protection in the elderly and older adults [203-207]. Serum IgG, secretory IgA and cellular immune responses all are correlates of protection that can be elicited by vaccination with LAIV [188, 208]. In addition, long lived influenza-specific memory CD4 T cells generated from infection and vaccination help modulate protective responses following challenge with influenza virus [209].

Adjuvants.
One attractive strategy to enhance vaccine immunogenicity and efficacy is through the use of adjuvants. Moreover, recent studies to develop vaccines effective against potential pandemic influenza strains like H5N1 have revealed that only with adjuvants can protective immunity be achieved against low immunogenic strains of influenza. Thus, development of novel adjuvants is an area of intense research focus. Adjuvants are compounds that are administered along with vaccines to help boost the immunogenicity of antigens, modulate the immune response and often reduce the requirement of high antigen doses [210]. Adjuvants may act by several different mechanisms including prolonging antigen release, acting as an antigen delivery system or through immunopotentiation. The mechanistic basis of immunopotentiation includes targeting of antigen to APCs, altering the local cytokine milieu, as well as enhancement of cross-presentation of antigen to cytotoxic T lymphocytes [211]. Adjuvants may also improve the antigen
presentation capacity of APCs through upregulation of surface MHC class I and class II molecules, enhanced antigen uptake, or upregulation of other APC maturation markers. Despite widespread research focus on influenza vaccine adjuvants, only very few adjuvants have been licensed for human use; these include Alum, in the form of Aluminium salts, and proprietary oil in water emulsions like MF59 (Fluad®) [212], and ASO3 (considered for pandemic H1N1/H5N1 vaccine) [213-215]. Importantly, the mechanism of action of these adjuvants is only marginally understood. Alum was approved in 1920’s, due to its adjuvant activity mainly by formation of a depot allowing for slow release of antigen from the injection site, promoting local inflammation and antigen phagocytosis. However, alum is a poor inducer of T cell immunity and Th1 responses and provides only marginal improvement to split or subunit vaccines [216-218]. MF59 was approved in 1997 in the European Union, as an adjuvant for human use in (Fluad™) seasonal influenza vaccines. It is also licensed for use in the elderly (>65 years of age) in the EU only. MF59 is an oil in water emulsion with squalene as the oil phase and has shown modest increases in antibody titers with seroprotection in the elderly and other age groups and cross reactivity to antigenic drift variants as compared to unadjuvanted vaccine [219-223]. MF59 adjuvant induces inflammation at the injection site stimulating the influx of macrophages, local production of chemokines and cytokines, which subsequently leads to leukocyte migration and activation of APCs and their subsequent migration to draining lymphnodes [224, 225]. However, the use of MF59 is also associated with local side effects like pain, erythema and enduration at the injection sites and does not lead to dose sparing [226]. A new adjuvant system ASO3 was developed recently by GlaxoSmith Biologicals that was incorporated into the pre-pandemic inactivated split H5N1 vaccine called Prepandrix [227] and in H1N1 2009 pandemic vaccine (Pandemrix, GSK) [228]. ASO3 is oil in water emulsion with good immunogenicity and dose sparing effect [229]. However, it does produce reactogenicity in the elderly (>65 years of age) [230] and adverse events like pain, swelling, irritability in children aged <5years of age [231]. Pandemrix has shown to induce narcolepsy as an adverse side reaction in children in Sweden and Finland and therefore was discontinued [232].

Other adjuvant systems include the particulate nature of the delivery vehicles or carriers like liposomes or virosomes, virus-like particles (VLPs), and immune stimulating complexes (ISCOMS). Virosomes are liposomes that act as an antigen carrier system or virus-like particles
with lipid membranes composed of immunogenic viral surface antigens (such as HA and NA glycoproteins of influenza) but usually devoid of internal genetic material, thus mimicking the native presentation of viral antigens without any pathological outcomes (infectious material) [233]. Mechanistically, they retain the receptor binding and membrane fusion activity as well as the repetitive arrangements of HA allows the virosomes to be recognized by immunoglobulin receptors on B cells leading to strong antibody responses (REF). Virosomes are also avidly taken up by dendritic cells and presented in both MHC class I and MHC class II context stimulating both Th1/Th2 cells and cytotoxic T cell responses [170]. Immunopotentiating reconstituted influenza viroso (IRIV) commercially available in the name of Inflexal® Vi is a trivalent viroso composed of three different influenza virus hemagglutinin and neuraminidase subtypes and is approved for human use [234]. However, studies comparing the immunogenicity of virosomes with that induced by nonadjuvanted split vaccines revealed varied results in different age groups and health status with superior [235], inferior [236] or similar [237] immune responses reported. A comparative study of MF59 adjuvant with viroso and split virus vaccines in elderly people with underlying chronic conditions demonstrated that MF59 adjuvant induced greater antibody responses against homologous and drifted strains of influenza viruses than conventional virosoal and split vaccine [221].

**Rationale for using immunomodulators as adjuvants**

Current influenza-inactivated vaccines mainly induce antibodies that are protective and neutralizing against circulating strains that are homologous to the vaccine strains. They induce only marginal cellular immune responses. Moreover, the efficacy of current inactivated split influenza vaccines in the elderly population over the age of 65 years of age is low ranging from 39%-69%, and varies significantly, depending on the study [238-240]. This has been postulated to result from age-associated immunosenescence, which is characterized by diminished T cell responses with increasing age. Since cellular immunity is required for clearance of viral pathogens and cross-protection from drifted influenza viral strains more potent and targeted adjuvants are needed to potentiate cell-mediated and mucosal immune responses.

Conserved antigens of influenza virus such as matrix protein (M1) and nucleoprotein (NP) induce cellular immunity in an infected host that is broad and cross-protective. The M gene segment of influenza virus codes for M1 and M2 proteins in overlapping reading frame. Both
these proteins are highly conserved including the ectodomain of M2. The ectodomain of M2 (M2e) is remarkably conserved in all influenza A viral strains. Moreover, anti-M2e antibodies have the ability to reduce replication of influenza virus in vitro [241] and in vivo [242]. Baculovirus expressed M2e induced serum antibodies against the amino and carboxy terminal M2e peptides that were presumed to be associated with protection against lethal homotypic viral challenge [243]. Cross protection associated with vaccination using M2e was first observed when M2e immunization provided protection against H3N2 challenge virus whose M2e sequence was identical or had one amino acid difference from the M2e vaccine [244]. Due to its highly conserved nature and broad immunity elicited through targeted vaccination, the M2e peptide has gained considerable interest as a broad-spectrum immunogen that can provide protection from antigenically drifted strains of influenza viruses. This approach may prove to be beneficial during a pandemic.

Cytokines, also referred as immunomodulators, are biomolecules that help in communication between cells playing a central role in activating and maintaining innate and adaptive immune responses. Numerous cytokines [245-248] and chemokines [249-252] have been used as adjuvants for infectious disease and cancer vaccines. Cytokines can impact numerous immunological pathways like maturation of APCs, T cell activation, Th1 or Th2 differentiation, activation of NK cells and cytotoxic T lymphocytes, as well as B cell responses [245, 253, 254]. A plethora of studies have evaluated the use of cytokines such as IL-2, IL-4, IL-6, IL-12, IL-15 and GM-CSF either in the form of plasmid DNA or as protein fusion constructs and have shown that they can substantially improve the immune responses and efficacy of vaccines [255-257]. Notably, the Interleukin-1 family of cytokines when administered with recombinant HA protein enhanced mucosal IgA and serum IgG antibody responses resulting in enhanced protection from lethal influenza virus infection [258].

Interleukin-2 (IL-2) is a principal cytokine that plays an important role in primary immune responses to foreign antigens and during establishment of immunological memory responses. IL-2 is produced when antigenic peptides presented along with MHC molecules engage with T cell receptors (TCR) on naïve T cells together with co-stimulatory signals induced by B7 and CD28 engagement that leads to T cell activation. This ultimately leads to IL-2 secretion and IL-2 receptor (IL-2R) expression. IL-2 in turn signals through its receptor IL-2R and promotes growth
of T cells [259], clonal expansion of antigen specific T cells [260] and differentiation and survival of effector T cells [261]. IL-2 also stimulates the proliferation of NK cells, B cells and macrophages [262, 263]. Interleukin-4 (IL-4) is another important cytokine that promotes the differentiation of naive T cells to Th2 cells helping to drive Th2 mediated immune responses, including antibody class switching and it also stimulates the proliferation of activated B and T cells [264, 265]. IL-12 is a heterodimeric cytokine produced by APCs, monocytes, macrophages, B cells, and DCs in response to antigen, bacteria, as well as intracellular parasites. IL-12 also referred to as NK cell stimulating factor (NKSF) induces robust proliferation of NK cells as well as Th1 and CTL cells [266]. During T cell differentiation in response to antigenic stimuli, it drives the differentiation of T helper cells (Th0 cells) towards a type 1 Th (Th1) phenotype and also exerts inhibitory effects on Th2 differentiation [267]. IL-12 signaling through its receptor (IL-12Rβ1/β2), induces the production of IFNγ by NK and T cells [268, 269]. Flt3L (Fms-related tyrosine kinase 3 ligand) is a hematopoietic growth factor produced by bone marrow stromal cells and other cells, which in conjunction with other cytokines can induce proliferation and expansion of different lineages from hematopoietic progenitor cells [270, 271]. Flt3L is a key cytokine for DC differentiation and maintenance. Flt3 or Flt3L gene knock out results in reduced DC progenitors, conventional DCs, plasmacytoid DCs and interstitial dermal DCs in mice [272]. Additionally, Flt3L administration in mice increases the number of DC of both lymphoid and non-lymphoid origin [273].

All of the above cytokines and growth factors have demonstrated adjuvant activity when combined with vaccines. IL-2 together with GM-CSF has been used as an adjuvant to peptide vaccines or in DNA-based vaccines [274-276] where it served to induce stronger immune responses, particularly T cell responses [255]. IL-2 linked to an immunodominant T cell epitope of influenza HA resulted in more robust T cell activation and antigen presentation in vitro [277]. IL-2 containing liposomes administered with low dose of inactivated influenza vaccine enhanced protective efficacy of the vaccine comparable to the high dose vaccine alone in aged mice [278]. IL-12 plasmid used together with an SIVgag DNA-based formulation in rhesus macaques enhanced both antibody and T cell responses [279]. IL-12 has been used to induce antigen specific IgA responses when administered intranasally in numerous experimental studies [280-282]. IL-12 also enhances IFNγ secretion when given nasally to mice [282]. IL-12 enhanced
antiviral antibody titers and provided complete protection from influenza lethal challenge when co-administered with influenza VLPs [283]. Soluble IL-12 with soluble HA and NA led to enhanced antiviral IgG2a and IgA levels in serum and BALF respectively and provided enhanced protective efficacy [284, 285]. Flt3L has been used as a mucosal adjuvant when administered in soluble form or in DNA form. Soluble Flt3L induced mucosal sIgA and systemic IgG antibody responses and increased the number of DCs in NALT [286, 287]. Flt3L in a plasmid DNA formulation or expressed by an Adenovirus expression vector together with ovalbumin antigen (OVA) administered intranasally induced robust mucosal IgA immune responses and induced migration of NALT DCs to effector sites and subsequent humoral and cellular responses [288]. Flt3L cDNA and CpG ODN administered together increased the number of plasmacytoid DCs and CD8+ DCs in the NALT of aged mice [289]. Interestingly, it has also been shown that Flt3L treatment increases the number of CD11c DCs in spleen which are also enriched in DEC-205 DC, which are specialized for cross-presentation in DC targeted vaccines [290].

It is important to note, that administration of recombinant cytokines in soluble formulations are expensive, they rapidly disperse from the application site, they have short half-lives and there is potential toxicity issues when administered systemically. Despite their demonstrated adjuvant potential, administration of soluble cytokines for immunomodulation can be associated with severe toxicity and side effects as reported in several studies targeting both cancer [291-293] and infectious diseases [294, 295]. Alternative strategies with reduced toxicity are clearly needed to take advantage of the adjuvant properties of cytokines. Results from numerous studies suggest a promising alternative is to deliver the cytokine(s) in particulate or membrane-bound form to improve the stability, shelf life and bioactivity. Babai. etal. prepared an influenza subunit vaccine composed of hemagglutinin/neuraminidase (H3N2) with IL-2 and GM-CSF encapsulated separately or together on liposomal vesicles, increased immune responses (2-3 orders of magnitude higher) were observed using the liposomal formulation [296]. They were also able to elicit broader, more cross-reactive antibodies against a variety of influenza virus strains, when they included multiple HA and NA subunits together with IL-2 and GM-CSF [297]. Another approach to keep the cytokine in close proximity to the antigen is to anchor or fuse the cytokine to the antigen itself. This will help to maintain the bioactivity and protect the protein from being
degraded at the site of administration. Nagarajan et al. expressed GPI-anchored IL-12 on the surface of tumor cells, which enhanced the generation of anti-tumor immune responses [298]. Wang et al. anchored the TLR5 ligand flagellin as an adjuvant onto influenza virus-like particles, which increased the breadth of elicited immune responses and also provided partial protection against heterotypic influenza virus challenge [299]. In a follow up study, they demonstrated that flagellin~VLPs when administered intranasally provided superior protection against both homotypic and heterotypic lethal influenza virus challenges [300].

Previously, in our lab a novel method was designed that incorporated bioactive, membrane-bound cytokines on the surface of the influenza virus particle itself [301]. These membrane-bound Cytokine bearing Inactivated Vaccines were referred to as CYT-IVACs. The CYT-IVAC technology involves construction of expression plasmids that encode for the mature form of cytokines fused to the regions coding for either the HA and/or NA membrane spanning domains. The expression plasmids are used to generate MDCK vaccine producer cell lines that constitutively express the membrane-bound cytokines at the cell surface. Initial studies in our laboratory focused on the expression of IL-2, IL-4 and GM-CSF as fusion constructs. All of these fusion proteins were actively incorporated into influenza virus particles following infection of the producer cell lines (MDCK) with several different influenza subtypes. Importantly, cytokine bioactivity was retained following inactivation of the virus. Hence, this approach capitalizes on the use of whole inactivated vaccine that bears bioactive immunomodulators in direct context with viral antigen. Our previous in vivo efficacy studies demonstrated that both the IL-2 and IL-4 CYT-IVAC formulations were superior to control, non-adjuvanted WIV in eliciting virus-specific antibody responses as well as affording protection against lethal challenge [80,167].

The presented body of work described herein, expands upon our earlier observations and focuses on the assessment of a novel panel of CYT-IVAC formulations that includes Flt3L, IL-12 and IL-23. In addition, we capitalize on the cross-protective potential of the M2e peptide and apply our CYT-IVAC approach to present a multimeric M2e formulation directly on WIV. The following chapters highlight the in vitro and in vivo assessments of these vaccine applications using the murine model of influenza vaccination and challenge. Importantly, we assess age-associated differences in vaccine responsiveness as well as the route of administration required
to elicit optimal protective responses. Finally, we discuss the future potential of our CYT-IVAC formulations in the establishment of more effective vaccines targeting all age groups in the human population.

**Specific Aims**
The goal of this dissertation study is to evaluate the adjuvant properties and mechanisms of protection induced across the adult life by membrane-bound immunomodulatory proteins that are incorporated directly into the influenza virus particle. This vaccine technology is cell culture based and is amenable to any influenza viral strain or subtype for culture in mammalian cells.

The central hypothesis for this doctoral dissertation is that whole inactivated influenza virus vaccines can be substantially improved through the use of immunomodulatory adjuvants that are directly co-presented in context with viral antigen. Specifically, we postulate that membrane-bound immunomodulators together with the adjuvant-like particulate nature of the whole virus particle and its associated viral RNA component can serve to boost protective immune responses to influenza. Further we theorize age associated decline in responsiveness to immunization can be overcome through the use of these vaccine formulations. We also postulate that some of the immunomodulators may preferentially stimulate mucosal versus systemic immune responses. Finally, we propose to capitalize on our vaccine platform and test the hypothesis that a multimeric M2e peptide co-presented on the surface of whole inactivated vaccine (WIV) will enhance the immunogenicity of M2e and elicit cross-protective antibody responses.

Our approach capitalizes on previous studies performed in our laboratory and has been referred to as the CYT-IVAC vaccine platform, Cytokine-bearing Inactivated Vaccines. In contrast to the co-administration of soluble forms of growth factors and cytokines, our approach directly incorporates the immunomodulator as a membrane-bound form directly into virus particles. To facilitate their incorporation, the immunomodulators are fused to the membrane-anchoring domain of the influenza virus hemagglutinin and constitutively expressed in the MDCK, virus permissive cell line. Upon infection with influenza virus, the virus assembly and maturation complex recognizes the cell surface expressed fusion constructs, which leads to their incorporation into progeny viruses as they bud and are released from the infected cell. Whole
inactivated viral vaccine is subsequently prepared by gradient purification of virus shed into the media of infected cells, followed by inactivation of infectivity with β-propiolactone, a common virus inactivating compound. Our cytokine-bearing inactivated influenza vaccines were subsequently evaluated for efficacy using a mouse influenza challenge model. To test our hypothesis and to accomplish the overall objective, we pursued the following specific aims:

Specific Aim 1: Establish vaccine producer cell lines that facilitate active incorporation of bioactive, membrane-bound immunomodulatory proteins into influenza A virus to serve as immune stimulators.

**Working hypothesis**: Infection of MDCK producer cell lines constitutively expressing immunomodulators fused to transmembrane and cytoplasmic tail domain of viral hemagglutinin or neuraminidase with influenza A virus will lead to the shedding of progeny virions that co-present membrane-bound immunomodulators projecting from their surfaces that maintain their bioactivity following virus inactivation. Here we have chosen to evaluate a panel of cytokines and growth factors that play important roles in immune modulation of innate and adaptive humoral and cell mediated responses. Specifically, we have selected interleukin-12p70 (IL-12), interleukin-23 (IL-23) and Flt3L as well as IL-2 and IL-4. All these cytokines and growth factors play well-defined roles in stimulation and regulation of immunological responses to vaccination. This aim will culminate with the production of individual CYT-IVAC formulations as well as non-adjuvanted whole inactivated vaccine, WIV.

Specific Aim 2: Determine the in vivo efficacy of inactivated influenza A virus bearing membrane-bound immunomodulators in young adult and aged mice.

**Working hypothesis**: Whole inactivated influenza vaccine bearing membrane-bound immunomodulators will serve to overcome vaccine non-responsiveness in “aged” animals as well as display site-specific immunomodulatory activity. Here both young adult and aged animals will be employed as well as different routes of immunization to assess in vivo efficacy of our vaccine formulations.

Specific Aim 3: Determine the in vivo efficacy of inactivated, influenza A virus bearing a multimeric, membrane-bound version of the M2e peptide in young adult mice.
*Working hypothesis:* A multimeric membrane-bound version of the highly conserved M2e peptide will enhance immunogenicity of the M2e peptide as well as when co-presented on whole inactivated vaccine serve to provide cross-protection against challenge with heterologous influenza virus strains.
REFERENCES


36. Varga, Z.T., et al., The Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon at the Level of the MAVS Adaptor Protein. Plos Pathogens, 2011. 7(6).


Chapter 2. Vaccines targeting the elderly using whole inactivated influenza vaccines bearing immunomodulators

Abstract
Seasonal influenza can affect all age groups but the elderly population is disproportionately affected with high morbidity and mortality from influenza and its complications. Current influenza vaccines are less effective in the elderly, which is largely attributed to a decline in immune function, or immunosenescence. Newer vaccine strategies targeting the elderly must aim to induce sufficient cell mediated immunity that provides protection from both existing and emerging strains of influenza viruses, but must also be cost-effective and exhibit lesser side effects than high dose vaccine formulations. We have previously shown that co-presentation of the immunomodulators IL-2 and IL-4 on the surface of inactivated influenza virus particles serves as a potent adjuvant that enhances humoral immune responses and affords better protection from lethal homotypic viral challenge and at lower doses of antigen than conventional non-adjuvanted whole inactivated vaccine. Here, we evaluated the efficacy of these vaccine formulations in “aged” Balb/c mice together with the addition of a membrane-bound IL-12 vaccine formulation. Our investigations found that a single low dose vaccination with inactivated whole influenza vaccines co-presenting IL-12 was sufficient to provide enhanced protection from subsequent influenza challenge as compared to non-adjuvanted whole inactivated vaccine. Our results indicate that incorporation of cytokines such as IL-12 in a membrane-bound formulation may provide a means to lower the vaccine dose while eliciting enhanced protective responses in the elderly, an age group that responds poorly to current vaccination regimens.

Introduction
The elderly population, which encompasses people of age 60 years or older, is steadily increasing and is estimated to double from the current 11% to 22% by the year 2050 worldwide [1]. Considering the significance of this segment of the population in the coming years and to meet the increasing demands of health care facilities, vaccines and drug production costs, newer strategies must be developed to reduce the production time and costs of vaccines and anti-viral drugs without compromising efficacy. Improving on existing viral vaccine platforms while maintaining broad efficacy across the lifespan of individuals would greatly reduce the costs associated with yearly vaccination regimens.
Influenza is a significant cause of morbidity and mortality in humans, typically resulting in 226,000 hospitalizations and an average of 36,000 deaths annually in United States [2]. Importantly, 90% of influenza related deaths occur in the 65 years and above age group [3], which is also the risk group most likely to require hospitalizations due to influenza-associated secondary complications. There are increasing trends in hospitalization rates for pneumonia following influenza in the elderly with a 20% increase reported from 1988-2002 despite widespread vaccination efforts [4]. Influenza related hospitalizations and outpatient treatment costs of elderly represent almost 64% of the total influenza economic burden [5].

Commercially available influenza vaccines indicated for the elderly include the seasonal trivalent inactivated split or subunit influenza vaccine (TIV) and the recent Fluzone® High-Dose vaccine approved in 2009. The latter vaccine contains four times (60µg HA per subtype) the amount of antigen as compared to regular flu vaccines (15µg HA/subtype) [6]. Despite superior induction of anti-viral antibody responses following Fluzone® High-Dose vaccination, more adverse side effects were noted and it remains unclear whether it provides superior protection against influenza illness as compared to TIVs [7]. Trivalent inactivated vaccines are successful in the young with vaccine efficacies greater than 90% and they prevent 70-90% of healthy adults from laboratory confirmed influenza illness in randomized controlled trials during antigenic mismatch between vaccine and circulating viral strains [8]. In contrast, the efficacy of current vaccines in the older adults over the age of 65 is low, ranging from 39%-69%, and varies significantly, depending upon the study [9-11], including the parameters that are used as correlates of protection [12, 13]. Nichol et.al determined the occurrence of influenza and associated complications in the community dwelling elderly population over 64 years of age and which were already suffering from co-existing illnesses [14]. The latter study, found that influenza vaccination for at least three seasons was associated with reductions in hospitalizations for pneumonia and influenza by 48-57%, chronic respiratory conditions (27-39%) as well as for congestive heart failure (37%) [14]. However, there is a paucity of data from randomized controlled trials and most of the observational studies suffer from selection bias problems that lead to overestimation of the benefits of vaccination [15-18].

Evaluating the vaccine efficacy and identifying correlates of protection in older individuals is difficult and often complicated by the various co-morbidites found. The current available
influenza vaccines licensed for use in the elderly are designed to induce robust anti-viral antibody responses, which are generally vaccine strain specific and are not cross-protective against antigenic drift variants of influenza viruses that may arise suddenly between seasons. Importantly, vaccine-induced antibody responses are typically lower in the elderly when compared to younger individuals [19]. Recently, it was shown that low production of vaccine antigen specific antibodies was responsible for diminished efficacy of influenza vaccines in elderly [20]. There is only marginal information about the influenza vaccine induced T cell specific responses in older individuals as the current vaccines elicit generally poor cellular immune responses. However, cell-mediated immunity is considered to be a more appropriate correlate of protection in the elderly as cytotoxic T cells are involved in clearance of virus from the respiratory tract and provide clinical protection from disease [21-26]. Hence, new vaccine strategies must include ways to activate both humoral and cellular immune responses in the elderly, either through the use of more immunogenic vaccines or by using potent adjuvants and other immune modulators to enhance host immunity [27].

Recent advances in vaccine strategies include the use of novel adjuvant formulations such as MF59 and AS03 [28-30], toxin-based adjuvants [31], virosomes [32, 33], virus-like particles [34], recombinant vaccines [35], DNA vaccines [36], alternative routes of vaccination [37, 38], high-dose TIV vaccine [6, 39], as well as universal vaccine formulations targeting either the multiple cleavage site of the HA2 subunit, the highly conserved ectodomain of the M2 protein or conserved domains within the nucleoprotein [40-42] to boost influenza vaccine efficacy. Adjuvants hold considerable promise as they serve to increase the breadth of the elicited immune response and limit the dose of antigen, which will likely lower the overall production costs of vaccines. Several adjuvants have been investigated in either the elderly such as MF59 [43, 44] FLU-ISCOMS [45, 46] and TLR like agonists [3, 47-49] using aged animal models.

Our laboratory has previously demonstrated that membrane-anchored immunomodulators co-presented on whole inactivated influenza virus particles (WIV) were able to serve as bioactive moieties stimulating humoral immune responses and providing superior protection against lethal challenge when used at low doses compared to traditional non-adjuvanted WIV in young adult mice [50]. Our Cytokine bearing Inactivated Vaccine (CYT-IVAC) approach provides a novel vaccine platform to incorporate bioactive immunomodulators or cytokines on purified
inactivated influenza virus produced by cell culture technology, ruling out the potential need of additional adjuvant substitutions. These vaccines may provide a means to significantly reduce the antigenic dose required to achieve protective immunity in the elderly. Here, we have tested the hypothesis that membrane-bound immunomodulators (IL-2, IL-4 and IL-12) presented in direct context with WIV will serve as adjuvants and elicit superior protective immune responses in "aged" mice, defined as greater than 17 months of age.

Materials and Methods

Construction of expression plasmids
The murine interleukin 2 and 4 (mIL-2, mIL-4) genes fused inframe to a short stalk, transmembrane, and cytoplasmic tail encoding domain derived from the hemagglutinin gene of influenza A/WSN/33 hemagglutinin (HA) has been described previously [50, 51]. The murine IL-12 gene was amplified from pORF-mIL-12(p35p40) plasmid (Invivogen™) to generate full length mIL-12(p35p40) coding region (1623bp) by Platinum Pfx Polymerase (Invitrogen™) using forward primer (5' CCCAAGCTTCACCATGGTGTCGCTA 3') and reverse primer (5' CCCGGATCCTCCGATCGGACCCTGC 3') and to insert HindIII and BamHI restriction enzyme sites at 5’ end and 3’ end respectively and to remove a premature stop codon. Purified PCR products were digested with restriction endonucleases HindIII and BamHI (Invitrogen™) and subcloned into the HindIII/BamHI site within the multiple cloning site of pcDNA3.1~HA1513 [50] using standard molecular subcloning procedures and transformed into One shot DH10-B Maximum efficiency chemically competent E.coli cells (Invitrogen™) according to manufacturer’s instructions.

Generation of CYT-IVAC producer cell lines
The establishment of Madin-Derby Canine Kidney (MDCK) cell lines constitutively expressing mIL-2/HA and mIL-4/HA at the cell surface has been described previously [50, 51]. MDCK cell line constitutively expressing the mIL12/HA was established in a similar fashion. Briefly, MDCK cells were transfected with the pCDNA3.1/mIL12~HA1513 expression plasmid using Lipofectamine 2000 (Invitrogen™) in DMEM media without serum and antibiotic in a six well tissue culture plate. Stable transfectants were obtained by drug selection following growth in DMEM supplemented with 10% FBS (Atlanta biologicals©) and Geneticin @ 1.4mg/ml
Vaccine producer transfectants were subsequently cloned for high surface expression of IL12/HA by positive magnetic sorting using biotinylated anti mIL-12 p40 antibody (ebioscience®) and Streptavidin conjugated magnetic microbeads (Miltenyi Biotec®) according to manufacturer’s instructions. Cells were maintained in G418 until infection with influenza viruses, at which time G418 was omitted from the culture media.

**Indirect Immunofluorescence Microscopy**

Cell surface expression of immunomodulators on vaccine producer MDCK cell lines was verified by indirect immunofluorescence microscopy. Briefly, cells were seeded and cultured on glass coverslips to 80-100% confluency. Cells were subsequently fixed in 3% paraformaldehyde in PBS, quenched in 50mM glycine in PBS and blocked in 2% chicken serum. Immunostaining was performed using sequential incubations with cytokine specific primary antibodies followed by fluorophore-conjugated secondary antibodies [50].

**Viral infection, purification, and inactivation**

MDCK producer cell lines were washed twice in PBS and infected with Influenza A (IA/PR/8/34) at a multiplicity of infection (MOI) of 2. Viral supernatants were harvested following 36-48 hour of infection and precleared by centrifugation at 1500 rpm for 15 minutes at 4°C. Viruses were two times purified through 10%-26% iodixanol (Optiprep™, Axis-Schield) continuous gradients by ultracentrifugation at 18000rpm for 45 minutes at 4°C, and chemically inactivated by β-propiolactone (Acros Organics) for 30 minutes as described in detail previously [50]. Total protein concentration in the purified vaccine was determined by lyses of vaccine in RIPA buffer with SDS and analyzed using bicinchoninic acid protein kit (Pierce Biotechnology). Inactivation of purified vaccine was verified by culturing 5µg of vaccine in MDCK cells for 3-4 days in the presence of DMEM with TPCK treated trypsin (Sigma-Aldrich) as described previously [50].

**Western blot analysis**

Immunomodulator incorporation on inactivated purified vaccine was verified by western blot analysis of whole viral lysates. Vaccines were diluted in laemelli buffer (Bio-Rad) containing β-mercaptoethanol (Bio-Rad) and run on 12% SDS-PAGE followed by western blotting on PVDF membrane (Bio-Rad). Membranes were blocked in block solution (5% skim milk in PBST) for 2
hours shaking at room temperature, washed thrice in TBST (TBS with 0.05% Tween 20) and probed for rat anti mouse IL-12/IL-23 p40 antibody (ebioscience®) or rat anti mouse IL-2 antibody (BD Biosciences) or anti influenza A HA1 antibody (Life Science®) followed by goat anti-rat IgG HRP (Santa Cruz®) or donkey anti-mouse IgG IRDye800 (Rockland™). Membranes were incubated with Pierce ECL western blotting substrate or Super signal west Femto chemiluminescent substrate as per manufacturer’s instructions and visualized under Chemidoc machine (Bio-Rad).

**Hemagglutination assay and Cytokine quantitation**

Hemagglutination assay on purified vaccines was performed using chicken red blood cells as described previously [52]. Serial two fold dilutions of vaccine preps were prepared in PBS starting from 2µg and mixed with equal volume of fresh 0.5% chicken red blood cells and incubated at room temperature for agglutination of red blood cells for 30 minutes. Reciprocal of the last dilution showing agglutination is HAU per µg of total viral protein. Cytokine concentrations in our CYT-IVACs was obtained by lysing the vaccine in 1% Triton in the presence of protease inhibitor and run through Flowcytomix kit (ebioscience®) along with kits standards as per manufacturer’s instructions.

**Bioassay of membrane bound cytokines**

Bioactivity of membrane bound IL-2 on inactivated virus was performed using recombinant mIL-2 dependent cell line such as CTLL2 by in vitro bioassay. Same procedure was followed as described previously [50]. For IL-12, a cell culture based bioassay was performed as described previously with some modifications. Splenocytes were collected from naïve Balb/c mice and cultured in the presence of ConA (5µg/ml, Sigma-Aldrich) in 24 well tissue culture plate at 7.5x10⁶ cells/2.5ml/well and cultured at 37°C for 5 days. Activated splenocytes were washed twice in cRPMI and seeded @ 1.5x10⁵ cells/well in 100µl volume in triplicates on confluent MDCK cell lines and MDCK cells expressing membrane bound IL-12 in 96 well flat bottom tissue culture plates. Media alone (without splenocytes) served as negative control. Plates were incubated at 37°C for 48hrs; supernatant media was collected from MDCK cell lines and seeded in a fresh 96 well flat bottom plate. Alamar blue® was added in all wells, cultured for 18 hours at 37°C and absorbance was measured at 570nm wavelength and 600nm reference.
Vaccination and challenge studies

Seventeen month old female Balb/c strain of mice (*Mus musculus*) were purchased from National institute of aging (NIA) and all animal experiments were performed based on the guidelines of NIH and approval of Institutional Animal Care and Use Committee (IACUC) of Virginia Tech. After one week of acclimatization in animal facility, animals were anaesthetized by administering Ketamine (75mg/kg BW) and Xylazine (7.5mg/kg BW) intraperitoneally (i.p.). Mice were immunized with whole inactivated unadjuvanted virus vaccine WIV or A/PR/8, WIV~mIL-2/HA1513, WIV~mIL-4/HA1513 and WIV~mIL-12/HA1513 (n=22 mice/group) 1µg dose in 100µl volumes of sterile PBS intramuscularly (I.M) in right hind quarter. PBS served as negative control (n=22 mice/group). On day 21 post vaccination, animals were vaccinated with booster dose (0.3µg) of same vaccines intramuscularly in 100µl volumes in opposite leg. Serum was collected pre and post booster vaccination on day 16 and day 28 respectively, via retro-orbital sinus under Ketamine/Xylazine anaesthesia. At day 45 post vaccination, animals (n=6 mice/group) were challenged intranasally with mouse adapted IA/PR/8/34 (1000 TCID50/mouse) in 50µl volume of PBS under Ketamine/Xylazine anaesthesia. All animals were euthanized at day 5 following challenge using Pentobarbital sodium (100µl i.p.) and blood, spleen and lung tissues were collected. Remaining mice were challenged with same dose of IA/PR/8/34 on day 65 post vaccination and monitored for body weights and sickness for 16 days and some were sacrificed at day 5 post challenge and rest mice were left for survival.

A single dose vaccination regimen was also performed on a different set of Balb/c mice of same age group. Here, mice were vaccinated with 0.5µg of inactivated WIV, WIV~mIL-4/HA1513 and WIV~mIL-12/HA1513 in 100µl volumes of sterile PBS intramuscularly in right hind quarter without anaesthesia (n=23 mice/group). Serum was collected on day 21 by submandibular bleeding without anaesthesia. Animals were challenged with mouse adapted IA/PR/8/34 (1000 TCID50/mouse) in 50µl volume on day 35 post vaccination and sacrificed at day 5 post challenge. Remaining mice from each group were challenged on day 100 post-vaccination with same dose of mouse adapted IA/PR/8/34 and monitored for a period of 16 days for reduction in weight loss and percent survival.
Enzyme-Linked Immunosorbent Assay (ELISA)

Influenza virus (A/PR/8) specific IgG, IgG1, IgG2a levels were determined by ELISA. Briefly, purified whole inactivated PR8 virus were diluted (100HAU/ml) in ELISA coat buffer (Na₂CO₃ (150mM), NaHCO₃ (350mM), NaN₃ (130mM)) and seeded in 100µl volumes in 96 well immunosorbent plate (Nunc, 475094). Plates were spun at 4000rpm for 15 minutes at 4°C and left overnight at 4°C. Coated plates were washed once in ELISA wash buffer (PBST) using plate washer and blocked with blocking buffer (2% BSA in PBST) 100µl/well for 2 hours at room temperature. Serum samples were diluted 1:100 in blocking buffer in duplicates and incubated overnight at 4°C on shaker. Plates were washed three times and incubated with secondary anti mouse IgG-HRP or IgG1-HRP or IgG2a-HRP in 100µl volumes in all wells and incubated for 1.5 hours at room temperature on shaker. Plates were washed three times and incubated with ABTS substrate (Rockland™) in 50µl volumes for 30 minutes at room temperature in dark and reaction was stopped by the addition of equal volume of 1% SDS. Absorbance was measured with blank reduction at 405nm.

Virus Neutralization Assay

Vaccination induced virus neutralizing antibodies in serum collected at day 28 post vaccination were determined as described previously [50]. Briefly, serum was heat inactivated at 56°C for 30 minutes and serially diluted 10 fold in PBS. A/PR/8/34 virus was diluted (2000TCID₅₀/ml) in PBS and 50µl volumes were mixed with 50µl of serum dilutions and incubated for one hour at room temperature on shaker. After one hour incubation, serum-virus mixture was added on confluent MDCK cells washed twice with PBS and incubated for one hour at 37°C. Serum-virus mixture was taken off; cells were washed once and incubated with DMEM media with TPCK for 72 hours at 37°C.

ELISPOT Assay

Immunized Balb/c mice were challenged with mouse adapted IA/PR/8/34 as described above [50] and at day 5 post challenge spleens were harvested aseptically. ELISPOT plates (Millipore, Cat. No. MAIPS4510) were coated with capture antibodies for mIFNγ (BD Bioscience, 551881), mIL-2 (ebioscience®, 88-7824), mIL-4 (ebioscience®, 88-7844) and mIL-17A (ebioscience®, 88-7370) overnight at 4°C according to manufacturer’s instructions. Freshly isolated splenocytes (4x10⁶ cells/ml) were cultured with inactivated purified A/PR8 antigen or VSV antigen (10µg/ml)
in triplicates and incubated at 37°C for 24-36 hours. Media alone and ConA/PMA/Ionomycin (at 20µg/100ng/1µg per ml respectively) served as negative and positive control respectively. Plates were washed according to manufacturer’s instructions and incubated with detection antibody for 2 hours at room temperature followed by washing and incubation for one hour with Streptavidin-HRP (BD™ ELISPOT 557630) for IFNγ ELISPOT and Avidin-HRP (ebioscience®) for mIL-2, mIL-4 and mIL-17A ELISPOT respectively. Plates were washed and incubated with 100µl of AEC Substrate/well (BD™ ELISPOT 551951) for 60 minutes in dark and reaction was stopped by DI water. Spots were read on AID ELISPOT plate reader (AID EliSpot).

Quantitation of viral loads in lung tissue

Lungs were collected in DMEM from vaccinated mice at day 5 following challenge, weighed and flash frozen in liquid nitrogen. Viral titers on homogenized lung samples were determined as described previously [50]. Frozen lung samples were thawed on ice, homogenized and cell debris removed by pelleting at 6000rpm for 10 minutes. The supernatants were collected and the volume was adjusted to 1ml total volume with DMEM. Serial 10-fold dilutions of lung homogenates prepared in PBS were incubated with confluent MDCK cells in a 96-well tissue culture plate for 1hr adsorption at 37°C. Viral supernatants were aspirated off and plates were cultured for the development of cytopathic effects (CPE) in the presence of DMEM with L-(tosylamido-2-phenyl) ethyl chloromethylketone TPCK- treated trypsin (1.5µg/ml; Sigma-Aldrich®) for 72 hour at 37°C. Cytopathic effects were recorded and 50% tissue culture infectious dose units (TCID₅₀/ml) were determined by the Reed-Muench method [53]. TCID₅₀/ml was further divided by individual lung weights to obtain TCID₅₀/gram of lung tissue.

Intracellular cytokine staining of lung lymphocytes

Lung samples collected following challenge from each vaccine group were pooled together (N=6) and digested in digestion buffer (1.8mg/ml Collagenase type 4 (Worthington Biochemical) 0.1mg/ml DNaseI (Sigma-Aldrich®), 10% FBS in GKN buffer) at 37°C for 30 minutes. Following digestion, lungs were mashed against metal screen with forceps forcing the single cell suspension through the screen into 100mm dish. Cell suspension was passed through 70µm cell strainer followed by 40µm cell strainer, pelleted, lyzed in RBC lysis buffer and washed. Cells were cultured (3x10⁶ cells/ml) with PMA/Ionomycin (Sigma-Aldrich®) (50ng/ml/10µg/ml,
respectively) or inactivated PR8 antigen (6µg/ml) for 6 hours in 24 well plate in the presence of golgi stop Brefeldin A (ebioscience®, 00-4506) at 37°C. Cells were collected, washed in flow staining buffer (ebioscience®, 00-4222) and stained with fluorescently labeled antibodies for lymphocyte surface markers such as CD3-APC, CD4-PECy7 and CD8-Pac blue (ebioscience®) for 20 minutes in dark at 4°C. Cells were washed and fixed in intracellular fixation buffer (ebioscience®, 00-8222) for 15 minutes at room temperature, washed and permeabilized in permeabilization buffer (ebioscience®) and stained with PE conjugated intracellular cytokine antibodies such as IFNγ (ebioscience®) and Granzyme B (Invitrogen™) at a 1:100 dilution in 2X permeabilization buffer for 20 minutes in dark. Cells were washed in PBS and analyzed in BD FACS Aria.

Statistics
The significance of differences between vaccine and control group was assessed using GraphPadInstat version 3.0a for Macintosh (GraphPad Software). ELISA and comparison of lung titers were analyzed by One-way Analysis of Variance (ANOVA) using Bonferroni’s Multiple Comparisons Test with PBS group as control. Microneutralization titers were analyzed by Kruskal-Wallis Test (Nonparametric ANOVA) using Dunn’s multiple comparison test. ELISPOT data was analyzed by One-way ANOVA Tukey-Kramer multiple comparison test or Bonferroni’s multiple comparison test.

Results

Construction of an IL-12/HA fusion gene and establishment of a virus permissive vaccine producer cell line expressing membrane-bound IL-12
Our previously described CYT-IVAC vaccine platform [50, 51] utilizes virus-permissive, mammalian-based vaccine producer cell lines that express the membrane-bound version of specific immunomodulators, such as cytokines, on the surface of cells. During subsequent infection of these cells with influenza virus, the immunomodulator is actively incorporated into newly formed virions as they assemble and bud from the infected cell. The virions are concentrated, purified and inactivated resulting in a vaccine formulation comprised of inactivated, whole virus particles that co-present a bioactive immune modulator on the particle surface. Importantly, full-length viral glycoproteins must also be incorporated to ensure vaccine
efficacy, as they are the major targets for the induction of neutralizing anti-viral antibodies. To overcome the weakened immune responses induced in the elderly with current vaccine formulations, we hypothesized that our CYT-IVAC formulations may provide a more robust protective response in the elderly and potentially provide a means to lower the vaccine dose without compromising efficacy and reduce potential side effects due to high antigen dosing. Previously, we evaluated our CYT-IVAC approach in young adult mice using the immunomodulator IL-2 and IL-4 [50]. To expand on our previous panel of CYT-IVAC formulations, we describe here the generation of a CYT-IVAC formulation co-presenting a membrane-bound form of the cytokine IL-12.

The establishment of the IL-2 and IL-4 CYT-IVAC producer cell lines has been reported elsewhere. In contrast to IL-2 and IL-4, IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits that are encoded by separate genes. In order to ensure that both subunits assemble correctly in our virus producer cell lines, we chose to utilize an IL-12 fusion construct in which the two subunits are expressed as a full-length intronless open reading frame, albeit the two subunits are separated by a hydrophobic valine-proline-glycine polylinker encoding sequence that allows the subunits to fold and assemble into a biologically active molecule [54]. Importantly, the full-length IL-12p35p40 construct has been reported to exhibit comparable bioactivity to recombinant IL-12 as well as has been used to elicit enhanced in vivo anti-tumor immune responses [55, 56]. Moreover, IL-12 linked to glycophashtidylinositol (GPI-IL12) and expressed on the surface of tumor cells was bioactive, induced IFNγ secretion from T cells and prevented tumor growth in mice [57]. To facilitate membrane-incorporation and active incorporation into budding influenza virions, the full-length IL-12p35p40 construct was further fused in frame to the transmembrane and cytoplasmic tail (collectively referred to as TM domain here) encoding domain of the influenza virus hemagglutinin gene, IL-12/HA (Figure 1A) and placed under control of a CMV promoter element in plasmid pcDNA3.1 as described previously for IL-2 [50]. Figure 1 depicts the membrane-bound cytokine constructs that were evaluated in this study.

Stable MDCK transfectants expressing the membrane-bound form of IL-12/HA at the cell surface were established following drug selection (G418) and magnetic sorting of IL-12 expressing cells using IL-12 specific antibodies. As depicted in Figure 2, stable transfectants
readily expressing IL-12/HA at the cell surface were validated by surface immunofluorescent staining using a rat anti-mouse IL-12 p70 antibody (Figure 2B). MDCK control cells did not stain positive for surface IL-12 (Figure 2A).

**Validation of immunomodulator incorporation and inactivation of vaccines**

CYT-IVAC formulations are prepared by infection of membrane-bound cytokine-expressing producer cells with influenza virus and harvesting virions from the supernatants of infected cells. As viral assembly and budding is initiated by the M1 protein of influenza binding to the cytoplasmic tail domains of HA and NA, both viral encoded, full length HA, NA and M2 proteins together with cytokine-linked HA-TM containing proteins are packaged into budding virions, albeit the latter are incorporated at much lower levels than full length proteins [50]. Virions were subsequently gradient purified and inactivated using β-propiolactone (43). Virus inactivation was verified by culturing with MDCK cells in the presence of TPCK-treated trypsin and monitoring for the development of virus-induced cytopathic effects (CPE) for a period of at least 5 days. The lack of CPE confirmed that all of our CYT-IVAC formulations were inactivated prior to use.

To confirm the bioactivity of membrane-anchored IL-12/HA on the surface of MDCK cells an *in vitro* splenocyte proliferation assay was performed by co-culturing fixed MDCK IL-12/HA expressing cells or vector control MDCK cells with ConA-activated splenocytes. As depicted in Figure 2F, ConA-activated splenocytes only proliferated upon co-culture with MDCK IL-12/HA cells and not in response to control MDCK cells, confirming that the IL-12/HA fusion construct is expressed as a bioactive surface protein. To validate the incorporation of membrane-bound cytokine into virions and assess whether the bioactivity of the incorporated cytokines was maintained following virus inactivation, vaccine formulations were subjected to western blot analysis and specific bioactivity assays as described previously [50]. Figure 2C depicts western blot analysis of CYT-IVAC formulations probed with antibodies specific for IL-2 or IL-12. The CYT-IVAC co-presenting mbIL-12 revealed multiple bands ranging from 48kDa to 75kDa in molecular weight when probed with anti-IL-12 specific antibody. The 75kDa band represents the full-length IL-12/HA heterodimer (p35-p40/HA). The predicted size of the mbIL-12 construct is 68.93kDa. Since, IL-12 is a glycoprotein with p35 and p40 subunits having three to four putative N-linked glycosylation sites respectively, these bands may reflect heterogeneity in the
glycosylation status of the incorporated IL-12. The CYT-IVAC co-presenting mbIL-2 revealed a specific IL-2 band with a molecular weight of approximately 25kDa which represents the predicted size of the IL-2/HA fusion construct (27kDa) (Figure 2D). We also controlled for the incorporation of full length HA (ca. 76kDa band) in all vaccine formulations by western blot analysis (Figure 2E). HA incorporation was dose dependent with levels gradually decreasing with gradient doses of all vaccine formulations (Figure 2F). The presence of full-length HA content was also determined by hemagglutination assay as depicted in Table 1. Only those CYT-IVAC formulations were used which exhibited high levels of immunomodulator incorporation without compromising the incorporation of full length HA. The amount of cytokine incorporation per µg of vaccine was calculated using Flowcytomix kit (ebioscience®) as depicted in Table 1.

**High dose Prime/Boost Vaccination Regimen and Efficacy in “Aged” Mice**

**Prime/Boost Vaccination with WIV induces high levels of anti-viral antibodies.** Studies have suggested that protective immunity in "aged" animals may require high antigen dosage or administration of booster doses of vaccines [39, 58, 59]. Hence, our initial investigations into efficacy of our CYT-IVAC formulations in the "aged mouse model" utilized higher doses of CYT-IVAC compared to our previous studies in young adult mice [50], and included a booster dose of vaccine. Here we utilized mice obtained from the aged mouse colonies established by the National Institute on Aging (NIA) at the NIH. These animals are nulliparous and were aged to 17 months prior to vaccination. Specifically, animals were primed by immunization with 1µg of CYT-IVAC or non-adjuvanted WIV followed by a low booster dose (0.3µg total viral protein) given at day 17 both administered intramuscularly (IM). Sera were collected on day 16 (pre-boost) and day 28 (post-boost) to evaluate the level of influenza virus specific IgG, IgG1 and IgG2a antibodies. Immunized mice were subsequently challenged with 100 LD50 of mouse-adapted influenza A/PR/8/34 at day 45 and sacrificed 5 days later to determine viral loads in the lung tissues and cellular responses in lungs and spleens. In addition, mice were again challenged on day 65 to evaluate the level of protection afforded by our CYT-IVAC formulations. To evaluate the humoral antibody responses following vaccination, influenza specific serum IgG levels were determined following primary vaccination (Figure 3A) and booster vaccination (Figure 3B). The dosing regimen used led to high seroconversion rates.
in all animals regardless of whether CYT-IVACs or WIV was administered after the initial priming dose of vaccine (Figure 3A). High levels of influenza specific IgG antibodies were observed in all groups of vaccines as compared to the mock (PBS) vaccinated animals (Figure 3A), albeit the IL-12 CYT-IVAC group exhibited the highest mean antibody levels. Post-boost antibody levels were only marginally enhanced and restricted to individual animals within each group. There was no direct indication as to why some animals responded better than others (Figure 3B). These primary antibody responses were surprisingly high taking into consideration the age of the mice and reported weakened immune systems [19, 60]. Importantly, there were a few non-responders in all vaccine groups that did not respond to either the priming or boosting vaccination dose (Figure 3). In addition, the levels of circulating IgG actually went down post-boost in some animals. The latter illustrated in Figure 4, depicts anti-viral IgG levels on an individual animal basis pre- and post-boost. The overall trend in animals vaccinated with IL-12 and IL-2 CYT-IVACs showed an enhancement of serum antibodies following booster vaccination as compared to IL-4 CYTIVAC and WIV immunized animals, both of which displayed only marginal increases or decreases in antibody levels post-boost (Figure 4).

To further characterize the quality of the elicited antiviral antibody responses induced by the individual CYT-IVAC formulations, we compared the influenza specific IgG2a and IgG1 antibody levels post-boost (Figure 5) as well as determined the levels of virus neutralizing antibodies present in the serum of vaccinated animals (Figure 6). Interestingly, we found higher levels of IgG2a antibodies (Figure 5B) as compared to IgG1 (Figure 5A) in all inactivated vaccine groups with the IL-12 CYT-IVAC group displaying slightly higher mean IgG2a (Figure 5B) and neutralizing antibody levels following vaccination compared to control WIV (Figure 6). As anticipated, IgG2a, IgG1 and neutralizing antibodies were not detected in any of the PBS vaccinated animals (Figure 5 and Figure 6).

Prime/Boost vaccination reduces viral titers in lung tissue and induces enhanced splenic and lung cellular responses following lethal challenge of aged mice

To assess the level of protection afforded by our CYT-IVAC formulations, vaccinated mice were challenged with a high lethal dose (100 LD_{50}) of homotypic mouse-adapted influenza A/PR/8/34 virus on day 45 post-vaccination. At day 5 post-challenge, animals (n=6/group) were sacrificed and lung tissues collected and as a measure of protection were assessed for a reduction of viral
replication in the lungs of mice following lethal viral challenge. The viral loads at day 5 post-challenge were reduced in all immunized groups compared to control (sham) animals, suggesting that this dosing regimen using whole inactivated vaccine is sufficient to confer protection in "aged" animals (Figure 7). Although there was no significant protective advantage induced by our vaccine formulations compared to non-adjuvanted WIV, subtle differences were evident. As expected the PBS-sham vaccinated group exhibited the highest levels of virus at day 5 post-challenge, followed by the non-adjuvanted WIV-PR8 group and the IL-12 CYT-IVAC vaccinated groups. Interestingly, even though antiviral antibody levels were lower in the IL-2 and IL-4 CYT-IVAC groups (see Figure 3 and Figure 4), these animals exhibited lowest viral titers with the IL-4 CYT-IVAC group displaying the lowest viral lung burden among the vaccinated groups (Figure 7). Importantly, there was high degree of variation in the viral titers with 3 out of 6 animals showing the lowest limit of detection ($10^3$ TCID$_{50}$/gm tissue) in IL-12 CYT-IVAC and one animal in IL-4 CYT-IVAC group, while other animals showed higher titers ($\sim10^5$TCID$_{50}$). This variation may be due to age and it affects the overall mean titers of whole group. There is a need to increase cohort size to have significant differences between the groups.

To further assess the nature of the immune response elicited by our vaccines, we screened splenocytes isolated from individual animals within each group for virus specific IFN$\gamma$ (Th1 cytokine), IL-2 (T cell stimulatory cytokine), IL-4 (Th2 cytokine) and IL-17A (Th17 cytokine) cellular responses by ELISPOT assay. Interestingly, there appeared to be a hierarchal influenza specific IFN$\gamma$ cellular response elicited by our vaccines with the most robust response elicited by the IL-4 CYTIVAC, IL4 CYT-IVAC>IL2 and IL12 CYT-IVACs>non-adjuvanted, control WIV (Figure 8). All vaccine groups elicited lower levels of IL-2 and IL-4 cellular responses as compared to IFN$\gamma$ responses in the spleens (Supplemental Figure 17). There were no significant differences in IL-2 and IL-4 responses between the non-adjuvanted WIV group and other CYT-IVACs. We were unable to detect any anti-viral specific IL-17A cellular responses in the spleens of vaccinated animals.

A subset of animals (n=6) within each group was additionally challenged with 100LD$_{50}$ of mouse-adapted influenza A/PR/8/34 on day 65 post-vaccination to gain further insights on the nature of the protective cellular responses in the lung following challenge. Lung intracellular cytokine staining was performed following global stimulation for 6 hours with PMA/Ionomycin
(Table 2). We theorized that the dominant recall responses present in the lungs at day 5 post-challenge would be indicative of the type of cellular response elicited by each vaccine formulation. Although this assay, which utilized pooled lung samples, is not quantitative as such, it does offer insights into the type of T cell responses induced in vaccinated versus non-vaccinated animals upon challenge. Global stimulation of lung lymphocytes with PMA/Ionomycin increased the IFN\(\gamma\) production capacity of inclined CD4+ and CD8+ T cells in all challenged animals. Notably, there were higher levels of IFN\(\gamma\) expressing CD4/CD8 cells in the lungs of IL-12 and IL-4 CYT-IVAC immunized mice compared to the other groups including the non-adjuvanted, control WIV group. Almost 75% of the CD4 and CD8 T lymphocytes in the IL-12 CYT-IVAC challenged mice group were induced to express IFN\(\gamma\) upon PMA/Ionomycin stimulation, compared to 50-56% in the IL-4 CYT-IVAC group and only 30-40% in the control WIV and IL-2 CYT-IVAC groups. The level of granzyme B (GrB) expressing CD8 T cells in the lungs was high even at basal levels (media control), suggesting that resident lung CD8 T cells may be inherently activated to express granzyme B (GrB) as a consequence of viral infection. It should be noted that all vaccinated animals regardless of the formulation exhibited higher basal levels of GrB-expressing CD8 T cells in the lungs compared to unvaccinated animals (PBS group). Almost 50% of the CD8 T cells in the lungs of the IL-12 and IL-2 CYT-IVAC animals exhibited GrB expression, compared to the 26% observed in PBS challenged animals, and 36% in the control WIV and IL-4 CYT-IVAC vaccinated animals. Global stimulation with PMA/ionomycin led to marginal increases in the GrB-expressing CD8 populations, suggesting that these populations were already recruited and inclined to express GrB.

**Single dose vaccination regimen and efficacy in the “aged model”**

**Single dose vaccination of whole inactivated influenza vaccines induces seroconversion following vaccination**

Since the prime/boost vaccination strategy led to high antiviral antibody levels in serum and induced protective responses in lung tissues following challenge in all groups of vaccines (non-adjuvanted WIV and CYT-IVAC), we decided to investigate whether a single dose of CYT-IVAC afforded enhanced protection compared to non-adjuvanted WIV in aged animals. Here, groups of female mice aged 17 months were vaccinated (0.5\(\mu\)g total viral protein) with control WIV–PR8, IL-4 CYT-IVAC, IL-12 CYT-IVAC, or PBS administered intramuscularly. Sera
were collected on day 21 post-vaccination and evaluated for the presence of influenza specific IgG, IgG1 and IgG2a antibodies. Similar to the prime/boost vaccination regimen most of the vaccinated animals seroconverted and had high levels of serum IgG (Figure 9) predominantly of the IgG2a isotype compared to IgG1 (Figure 10A and Figure 10B). Notably, there were several non-responders in each vaccine group, which likely reflects age-associated non-responsiveness, since our previous studies in young adult animals typically results in 100% seroconversion rates [50].

For determination of protective responses by single dose vaccination regimen, animals (n=6 per group) were subsequently challenged on day 51 post-vaccination with 100 LD$_{50}$ of mouse adapted A/PR/8/34, and sacrificed five days later to evaluate lung viral titers and cellular immune responses. As expected the PBS (sham) group displayed the highest levels of viral replication in the lungs following challenge (Figure 11). There was only marginal reduction in the viral loads in IL-12 CYT-IVAC and IL-4 CYT-IVAC groups compared to unvaccinated animals, suggesting that the cytokines were not boosting vaccine efficacy at this dose. In contrast, the animals that received the non-adjuvanted, control WIV exhibited significantly reduced viral lung loads upon challenge (Figure 11). To further examine the cellular responses induced following lethal challenge we evaluated IFN$\gamma$ secreting T lymphocyte responses in spleens (Figure 12) and percentage of granzyme B producing CD8+ T cells in lungs at day 5 following challenge (Table 3). Splenocytes were cultured with inactivated VSV antigen or PR8 antigen or pool of MHC Class I peptides or MHC Class II peptides. PBS group did not show much of IFN$\gamma$ T cell response. IL-12 CYT-IVAC and WIV group showed enhanced IFN$\gamma$ splenocyte responses with PR8 and MHC I peptides although the differences were not significant. These levels were lower than prime-boost vaccination regimen (Figure 8). It should be noted that in this assay background (VSV treatment) was high IFN$\gamma$ response observed in all groups of animals unvaccinated as well as vaccinated animals regardless of the type of vaccine. However, IL-4 CYT-IVAC group showed inferior responses with all antigens, which is in contrast to high IFN$\gamma$ responses observed in prime-boost vaccination regimen (Figure 8). In addition, we also measured splenocyte proliferation with influenza antigen and did not find any statistical differences between the groups (Supplemental Figure 19). It may be likely that whole inactivated vaccines require two doses to induce cellular responses in spleens.
Single low dose of IL-12 CYT-IVAC provides superior protection against lethal Influenza A/PR/8 challenge

To evaluate the protective efficacy of membrane bound IL-12 and IL-4 CYTIVAC’s in a single low dose vaccination regimen, animals from all groups (n=12-13/group) were challenged with a high lethal dose (100LD_{50}) of mouse-adapted A/PR/8/34 virus on day 100 following vaccination. This was a homotypic lethal viral challenge with same dose as used in high dose prime/boost vaccination and at this time animals were 21 months old. We challenged on day 100 since we wanted to investigate the longevity of protective responses induced by our CYT-IVAC formulations. Reduction in body weights and percent survival was monitored for a period of 16 days as a measure of protection. The PBS group of mice reached established end points (body weight $\geq 25\%$ of original body weight) by day 7 post-challenge and were euthanized (Figure 13A). Vaccinated group of animals exhibited weight loss and did exhibit clinical signs of disease following challenge and after day 9 mice from all groups started gaining weights and thus recovered from infection (Figure 13A). IL-12 CYT-IVAC and WIV groups were most effective in reducing weight loss following lethal challenge and their body weights were greater than IL-4 CYT-IVAC and PBS group with statistical differences (Figure 13A). Reduction in weight loss indicates protection afforded by vaccine formulations. Although, robust antibody responses were observed in all groups of vaccines, only the IL-12 CYT-IVAC demonstrated enhanced protective responses to lethal challenge. Figure 13B and Table 3 depicts that PBS group was unprotected from challenge, while non adjuvanted WIV vaccine protected only (3/13) 23% of challenged animals. Interestingly, our membrane bound IL-12 CYT-IVAC formulation afforded protection in (7/12) 58% of animals. However, the membrane bound IL-4 CYT-IVAC was not effective as a single dose formulation with only 8% of the animals surviving. This suggests that our membrane anchored IL-12 bearing inactivated influenza vaccine can provide better protection in aged mice with a single low dose (0.5µg) vaccination.

Importantly, we also collected sera from the challenged mice as they reached end points in body weights over a period of 16 days and also from animals that survived to determine the type of antibody responses induced upon challenge (Figure 14A and B). We found very high levels of both IgG1 and IgG2a antibodies that were higher than those observed following single dose vaccination (see Figure 10), indicating expansion of memory responses in vaccinated mice as a consequence of challenge. All of the IL-12 CYT-IVAC vaccinated animals displayed higher...
levels of IgG2a antibodies compared to other vaccine formulations. However, 5 of 12 mice in the IL-12 group succumbed to challenge despite these high anti-viral levels, suggesting that other protective factors may be absent in these animals.

Discussion
Influenza virus causes severe respiratory disease in the elderly population with 90% of influenza related deaths found in this age group [3]. The increased vulnerability of this age group is likely due to a deterioration or waning of immunity during the aging process, which results in decreased ability to mount effective innate and cellular immune responses to fight against infections [61] and is also associated with lower effectiveness of current vaccines in elderly [49], leading to more serious outcomes due to infection [62]. Therefore, newer strategies need to be designed with the goal of augmenting the immunogenicity and hence efficacy of vaccines in this age group. Whole virus inactivated vaccines (WIV) are an attractive alternative to split/subunit vaccines, particularly since they are considered to be more immunogenic than latter. WIV induce a predominant IgG2a type immune response in mice, an indicator of Th1 type responses, and a quicker and higher neutralizing antibody response while subunit vaccines induce predominant Th2 type response [63, 64]. These enhanced responses are thought to be elicited by the inherent particulate nature of the whole virus particle and via stimulation of TLRs by the viral RNA genomic component present in WIVs, of which the latter is otherwise degraded or absent in split and subunit vaccines [65].

To improve the vaccine efficacy, it has been suggested that there is a need to include stable, safe and non-toxic adjuvants in vaccine formulations to enhance immunogenicity and to reduce antigen dosage and its associated side effects. Here, we hypothesized that anchoring cytokines or immunomodulators on the surface of whole inactivated influenza virus will serve as potent adjuvants to modulate immune responses and enhance protective efficacy of whole virus vaccines in aged mice. This approach takes advantage of the β-propiolactone inactivated, whole virus vaccine that has been shown to provide better stimulatory signals during antigen priming and couples it with bioactive cytokines on the viral surface that will all serve to increase stimulatory signals for better induction of immune responses.
We have previously demonstrated the adjuvant potential of cytokines co-presented in a membrane-anchored form on whole inactivated virus vaccines in young adult mice and chickens [50, 51]. These vaccines are produced in cell culture and this approach is applicable to any vaccine strain that grows well in MDCK cells. In addition, the vaccine platform can easily be established in other vaccine suitable cell lines such as the Vero cells. However, to make this platform suitable for humans, immunomodulators will have to be human-derived to limit species incompatibility and immunogenicity of the cytokines. Besides IL-2 and IL-4, we expanded our panel of immunomodulators and included a single chain interleukin-12p70 (IL-12) vaccine formulation. Importantly, we decided to test whether our CYT-IVAC formulations could overcome age-associated vaccine non-responsiveness.

In a previous pilot study performed in our laboratory two low dose subcutaneous immunizations in 14 month old retired breeder mice with CYT-IVACs bearing IL-2 and IL-4 provided superior protection against lethal influenza challenge [66] unpublished observations, supplementary Figure 16). In an attempt to further evaluate the adjuvanticity of the co-presented cytokines, we conducted an additional study using aged, nulliparous animals (17 months of age) using higher doses of vaccine using a prime/boost immunization regimen. Unfortunately, the dosing regimen used was too high to clearly differentiate effects due to the co-presented cytokines. However, we were surprised that the low dosing regimen employed (1µg vaccine followed by a 0.3µg booster dose) was sufficient to provide significant protection against challenge in all groups of vaccinated animals, except the IL-4 CYT-IVAC group, in which vaccination only protected 20% of the animals (see Supplemental Figure 18). This suggests that inclusion of IL-4 in this formulation is detrimental to “aged” mice. It also suggests that we need to revisit the use of WIVs, particularly as a means to stimulate immunity in the elderly. Of note, there were some non-responders in each vaccine cohort, which correlates well with clinical observations in the elderly [67]. The ELISPOT assay is a highly sensitive assay for evaluation of cell mediated immune (CMI) responses elicited by vaccination [68, 69]. All vaccine groups exhibited high viral specific splenocyte IFNγ responses (Figure 8). Viral specific splenic IL-4 responses were also observed but IL-2 responses were only marginally detected (Supplemental Figure 17). The latter correlates well with diminished IL-2 clinical cellular responses to influenza vaccination observed in the elderly [70]. Interestingly, the IL-4 CYT-IVAC induced the highest splenic IFNγ cellular responses as compared to other vaccine groups, which is somewhat counterintuitive as
IL-4 is known to drive development of Th2-type responses and suppress Th1. However, there are reports that the presence of IL-4 in highly contained microenvironment or in when presented as membrane-bound form can lead to stimulation of IFNγ and IL-12 from antigen presenting cells such as plasmacytoid dendritic cells priming Th1 responses [71-75]. It is important to note that during the latter part of the prime/boost study, many of the animals developed co-morbidities that included cancer, respiratory illness and eye infections, which resulted in sudden deaths unrelated to vaccination. We did challenged surviving animals with influenza virus on day 65 post-infection and noted variable levels of protection (Supplemental Figure 18). In addition, necropsy of sacrificed animals revealed that most of the animals had developed lung tumors. Thus, we were unable to accurately assess survival based on immunization and subsequent challenge. This does highlight the difficulty of conducting immunization studies with “aged” animals.

Due to the complications associated with the prime/boost vaccination study described above and due to lack of clarity in the contribution of the cytokine to the vaccine formulations, we conducted an additional vaccine efficacy using a single low dose immunization. Single dose vaccine induced high humoral immune responses with high influenza specific IgG (Figure 9), predominantly of the IgG2a isotype (Figure 10). Our results suggest that our WIV formulations with and without anchored cytokines were sufficient to induce type 1 humoral immune responses. Similar to our prime/boost vaccination study, we noted that in each cohort there were animals that responding poorly to vaccination regardless of whether a cytokine was co-presented. In our single dose study, we conducted two challenge experiments at different time points post-vaccination to assess long-term potential benefits of our CYT-IVAC formulations; each time using a randomly selected subset of animals from each vaccine cohort. However, conflicting data was obtained. At day 51, challenged animals (n=6) were sacrificed at day 5 post-challenge and viral lung burden was compared to sham vaccinated animals as a correlate of vaccine afforded protection. Here, only the non-adjuvanted control WIV cohort displayed reduced viral lung burden post-challenge (Figure 11). This was in part explained by whether these animals were low responders or not. In our later challenge study, performed 3 months post-vaccination, the CYT-IVAC bearing IL-12 was superior in protecting animals against lethality and establishment of clinical disease. In order to gain insights into the nature of the protective responses elicited, we collected sera from all animals either post-survival or at the time of sacrifice. The majority of
animals still exhibited high levels of viral specific IgG, hence the presence of high levels of antiviral antibody does not correlate with protection. Of note, surviving mice started gaining weight at day 9 post-challenge (Figure 13A). Interestingly, peak CD8 T cell responses are delayed in aged mice (d14) compared to young adult animals, which peak at d10 post-infection [76, 77]. The earlier and enhanced recovery of our IL-12 CYT-IVAC cohort, suggests that IL-12 may provide for superior recall responses. The IgG2a and IgG1 levels were further increased upon challenge especially the IgG2a levels in the IL-12 CYT-IVAC group supporting the role of IgG2a in protection (Figure 14). The induction of IgG2a by influenza vaccines has been associated with protective efficacy [63, 78, 79]. This is due to its role in complement activation [80] or activation of Fc receptor mediated phagocytosis and subsequent clearance of virus infected cells during challenge [81, 82]. Together the data, while not conclusive, suggest that an IL-12 CYT-IVAC formulation does have the potential to induce protection at lower antigen dosage when administered parenterally, but additional studies are needed. This would represent a significant benefit in mitigating adverse side effects associated with high antigen dosage. Alternatively, based on our observations in young adult mice (see chapter 3), CYT-IVAC formulations administered by the intranasal route may prove to be the most beneficial means to boost vaccine efficacy in the elderly. These studies are currently being planned.

In conclusion, the CYT-IVAC approach offers several advantages over current egg-based vaccine production methodologies: it is cell culture based and can be tailored to elicit specific responses depending on the cytokine used as well as the route of vaccine administration. Further, the cytokine is anchored on the membrane of virus particle and provides a stimulatory signal in direct context with viral antigen. Our data also suggest that WIV formulations need to be revisited for the elderly and together with suitable adjuvants may allow for administration of lower antigen dosing providing protection in this highly vulnerable age group. Future studies that incorporate more robust cytokines, use combinatorial presentation of cytokines or more targeted cytokines targeting recall and memory responses such as IL-15 may provide for more efficacious vaccines tailored for the elderly. Our vaccine platform can readily be utilized as a rapid screening platform to evaluate different cytokines as well as combinatorial use of cytokines.
Figure 1: Construct diagram of Mouse derived Immunomodulators.

IL-2, IL-4 and IL-12p70) were fused with 71 amino acid long membrane anchoring regions of hemagglutinin (HA) protein of Influenza A/WSN/33 that include short stalk, transmembrane and cytoplasmic tail regions of HA protein (HA1513). This was achieved by expressing under pCMV promoter in pCDNA3.1 cloning expression vector.
Figure 2: Validation of immunomodulator expression on the surface of transfected cells

(A and B). Indirect immunofluorescent staining on MDCK cells stably transfected with plasmid mIL-12-HA1513-pCDNA3.1 (B). Paraformaldehyde fixed cells were probed for surface expression of IL-12 using primary antibodies against mIL-12 followed by chicken anti mouse Alexa Fluor® 488 secondary antibody. Wild type MDCK cells stained with same antibodies (A) show background. Western blot analysis of whole inactivated gradient purified virus WIV IL-12/HA1513, mIL-2/HA1513 WIV mIL-4/HA1513 or wild type MDCK cells (C, D and E). Whole viral lysates were run on 12% SDS-PAGE gel, blotted on PVDF membrane and incubated with IL-12p40 antibody (C) and IL-2 antibody (D) and HA antibody (E) followed by antispecies secondary antibodies. Different dilutions of CYT-IVAC-IL-12/HA were run on gel to validate dose dependent incorporation starting from 10µg to 0.5µg (C). Two different preps of PR8 (lane 1, 2) (5µg) and CYT-IVAC-IL-2/HA (lane 3, 4) (5µg) were run on gel in (D). Membrane was visualized under Chemidoc. Different dilutions of all vaccine constructs were run to validate dose dependent HA incorporation (E). Invitro cell based bioassay of IL-12 (F). Naïve splenocytes were stimulated in the presence of ConA for 4 days followed by culture with washed MDCK or MDCK mIL-12HA1513 cells for 48 hours. Splenocytes were collected and Alamar blue® dye was added in last 18 hours of incubation to measure cell proliferation.
Table 1 Hemagglutination units (HAU) and cytokine content in purified whole virus vaccine.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>HAU/µg of vaccine</th>
<th>Total Cytokine (pg/µg of vaccine)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIV (PR/8/34)</td>
<td>512</td>
<td>N/A</td>
</tr>
<tr>
<td>WIV IL-2/HA</td>
<td>256</td>
<td>38</td>
</tr>
<tr>
<td>WIV IL-4/HA</td>
<td>256</td>
<td>49</td>
</tr>
<tr>
<td>WIV IL-12/HA</td>
<td>256</td>
<td>23</td>
</tr>
</tbody>
</table>

* Quantitation of virus-incorporated cytokine based on the cytokine quantitation kit (pg of cytokine per µg of vaccine)

Figure 3: Inactivated influenza vaccines with or without immunomodulators enhance serum anti-viral IgG antibody titers

(A) pre (B) and post booster vaccination. Aged Balb/c mice (17 month old) were vaccinated intramuscularly (I.M.) with 1µg of A/PR/8/34 or A/PR/8/34 bearing membrane bound mouse IL-2 (N=22), IL-4 (N=22) or IL-12 (N=22) followed by booster vaccination with 0.3µg at day 21. PBS served as negative control (N=22). Sera was collected at day 16 and day 28 post vaccination and antibody titers for virus specific IgG were determined by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. (***p<0.001 compared to PBS IM group, One way ANOVA, Bonferonni’s multiple comparison test.)
Figure 4: Comparison of antiviral serum IgG antibodies pre-boost and post-booster vaccination.

Sera collected pre-boost (day 16 post vaccination) and post-boost (day 28 post vaccination) vaccination were analyzed for IgG titers by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. The blank reduced absorbance values of each mice pre-boost and post-boost are plotted as a comparison and values joined together.

Figure 5: Whole inactivated influenza vaccines with or without immunomodulators enhance serum anti-viral IgG2a levels as compared to IgG1.

Sera was collected from vaccinated animals (N=8-10) on day 28 post vaccination and antibody titers for virus specific IgG1 (A) and IgG2a (B) were determined by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. (*p<0.05, **p<0.01, ***p<0.001 compared to PBS IM group, One way ANOVA Bonferroni’s Multiple Comparison Test).
Sera collected on day 28 post vaccination (n=18) was heat inactivated at 56°C for 30 minutes, serially 10 fold diluted and incubated with 100TCID$_{50}$ of A/PR/8/34 for an hour at room temperature. Serum-virus mixtures were incubated with MDCK cells for 1 hour and subsequently cultured for 3 days in DMEM media supplemented with TPCK treated Trypsin (1.5µg/ml). Microneutralization titer is presented as the serum dilution that was still able to neutralize and inhibit PR8 induced cytopathic effect. Note that a serum dilution of 1:5 is considered the lowest detectable dilution at which no neutralization (CPE) was observed. (*p<0.05, **p<0.01, ***p<0.001 compared to PBS I.M., Kruskal-Wallis Test, Dunn’s Multiple comparison test).

Figure 6: IL-12 CYT-IVAC vaccination induces virus specific microneutralizing antibodies in serum post booster vaccination

Figure 7: Inactivated vaccines reduce viral loads in lung tissue at day 5 following lethal challenge

Aged mice vaccinated with wild type inactivated vaccine (WIV) or with immunomodulators followed by booster on day 21, were challenged with a lethal dose (100 LD$_{50}$) of mouse adapted A/PR/8/34 on day 45 post vaccination. (A) Body weights were monitored for 5 days following challenge and plotted as percent original weight (B) Lung tissues were collected on day 5 post challenge, homogenized and viral titers determined by tissue culture infectious dose
assay (calculated by Reed and Muench formula). Data represents TCID$_{50}$/gram of lung tissue. Data not statistically significant.

Figure 8: Cellular immune response to CYT-IVAC vaccination.

(A) IFN$\gamma$ ELISPOT assay on spleens from vaccinated mice challenged with A/PR/8 on day 45 post-vaccination and sacrificed 5 days following challenge. Data shows average number of IFN$\gamma$ spots from each group of mice (N=6) stimulated with VSV antigen or PR8 antigen. (**p<0.001 compared to WIV (PR8), One way ANOVA, Tukey-Kramer multiple comparison test) (B) IFN$\gamma$ ELISPOT showing average of reduced values of spots (PR8 Spots-VSV Spots). Error bars indicate standard errors (*p<0.05 compared to WIV IM, One way ANOVA, Bonferroni’s multiple comparison test).

Table 2 CYT-IVACs increase the recruitment of Granzyme B producing T cells in lung following lethal viral challenge.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD8+ IFN$\gamma$+</th>
<th>IFN$\gamma$+</th>
<th>Granzyme B+</th>
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</thead>
<tbody>
<tr>
<td>PBS LML</td>
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<td>4.20</td>
<td>26.41</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PMA/Ionomycin</td>
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<td>40.54</td>
<td>36.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS LML</td>
<td>Media</td>
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<td>2.24</td>
<td>36.07</td>
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<tr>
<td></td>
<td>PMA/Ionomycin</td>
<td>35.49</td>
<td>31.06</td>
<td>40.00</td>
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<td></td>
</tr>
<tr>
<td>IL-2 LML</td>
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<td>3.77</td>
<td>4.67</td>
<td>50.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMA/Ionomycin</td>
<td>74.41</td>
<td>79.06</td>
<td>80.76</td>
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<td></td>
</tr>
<tr>
<td>IL-4 LML</td>
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<td>1.17</td>
<td>51.20</td>
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<tr>
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<tr>
<td></td>
<td>PMA/Ionomycin</td>
<td>49.79</td>
<td>56.38</td>
<td>48.94</td>
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</tr>
</tbody>
</table>

Table shows lung intracellular cytokine staining data on pooled lungs collected from mice (N=6) challenged with A/PR/8 at day 5 post challenge. Lung cells were cultured for 6 hours with media alone or PMA/Ionomycin in the presence of golgi stop (Brefeldin A). Data represents the percentage of CD3+, CD4+ or CD8+ T cells expressing IFN$\gamma$ and Granzyme B.
Figure 9: Single dose of inactivated influenza vaccines enhance serum anti-viral IgG antibody titers.

Aged Balb/c mice (17 month old) were vaccinated intramuscularly (I.M.) with 0.5µg of WIV (N=23) or WIV bearing membrane bound mouse IL-4 (N=23) or IL-12 (N=23). PBS served as negative control (N=23). Sera was collected on day 21 post vaccination and antibody titers for virus specific IgG were determined by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. (**p<0.001 compared to PBS IM group by One way ANOVA, Bonferroni’s multiple comparison test).

Figure 10: Single dose immunization of inactivated influenza vaccines enhance serum anti-viral IgG1 and IgG2a antibody titers.

Aged Balb/c mice (17 month old) were vaccinated intramuscularly (I.M.) with 0.5µg of WIV (N=23) or WIV bearing membrane bound mouse IL-4 (N=23) or IL-12 (N=23). PBS served as negative control (N=23). Sera was collected on day 21 post vaccination and antibody titers for virus specific IgG1(A) and IgG2a (B) were determined by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. (*p<0.05, ***p<0.001 compared to PBS IM group by Bonferroni’s multiple comparison test).
Vaccinated animals (N=6 mice/group) were challenged with 100LD$_{50}$ of mouse adapted A/PR/8/34 on day 51 following vaccination and sacrificed 5 days later. Lung tissues were collected on day 5 post challenge, homogenized and viral titers were determined by tissue culture infectious dose assay (calculated by Reed and Muench formula). Data represents TCID$_{50}$/gram of lung tissue. (*p<0.05 compared to PBS IM, One way ANOVA Bonferroni’s multiple comparison test)

Figure 12: T cell responses to single dose vaccination with CYT-IVACs

(A) IFN$\gamma$ ELISPOT assay on spleens from vaccinated mice challenged with A/PR/8/34 on day 51 post-vaccination and sacrificed 5 days following challenge. Data shows average number of IFN$\gamma$ spots from pool of 2 mice group of mice (N=6) stimulated with VSV antigen or PR8 antigen or MHC Class I peptide pool or MHC Class II peptide pool. Error bars indicate standard errors. Data is not significantly different.
Figure 13: IL-12 CYT-IVAC vaccination protects mice from lethal homotypic viral challenge.

Aged Balb/c mice vaccinated with wild type inactivated vaccine or with immunomodulators were challenged with a lethal dose (100 LD$_{50}$) of mouse adapted A/PR/8/34 on day 100 post vaccination. Mice were monitored daily for percent reduction in body weights and survival following challenge. Data represents percent original/start weight monitored for 14 days following challenge (A) and percent survival following challenge (B). (A)*p<0.05 compared to IL-4 PR8 IM on day 2 and ***p<0.001 compared to PBS I.M. on day 3, 5 and 6 by One way ANOVA (Bonferroni’s Multiple comparison test), (B) ***p<0.001 compared to PBS I.M., Logrank test.

Table 3 Percent survival following lethal viral homotypic viral challenge of mouse adapted A/PR/8/34.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. of mice</th>
<th>No. survived</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
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<td>0</td>
</tr>
<tr>
<td>WIV IM</td>
<td>13</td>
<td>3</td>
<td>23.07</td>
</tr>
<tr>
<td>WIV IL-4 IM</td>
<td>12</td>
<td>1</td>
<td>8.33</td>
</tr>
<tr>
<td>WIV IL-12 IM</td>
<td>12</td>
<td>7</td>
<td>58.33</td>
</tr>
</tbody>
</table>

Table shows number of subjects at risk and their percent survival during the study.
Figure 14: IgG1 and IgG2a antibodies are increased upon homotypic lethal viral challenge.

Animals (N=12-13) were challenged with 100LD$_{50}$ of A/PR/8 on day 100 post vaccination, and sera was collected over a period of 14 days as animals reached end points or from survived mice sacrificed at day 14 following challenge and evaluated for IgG1 (A) and IgG2a (B). Data represents difference in absorbance values (405nm-Blank). ***p<0.001 compared to PBS, One way ANOVA, Bonferroni’s multiple comparison
REFERENCES


74. Ebner, S., et al., *Production of IL-12 by human monocyte-derived dendritic cells is optimal when the stimulus is given at the onset of maturation, and is further enhanced by IL-4*. Journal of Immunology, 2001. **166**(1): p. 633-641.


Figure 15: IL-2 Bioassay.

CTLL-2 cells, were incubated with different concentrations of inactivated whole virus vaccine (WIV) or WIV IL-2/HA. Recombinant murine IL-2 served as positive control and cell proliferation was measured by reduction of indicatory dye Alamar blue®. Data represents difference in absorbance at 570nm measurement and 600nm reference.
Figure 16: Two low doses of inactivated influenza vaccines bearing IL-2 or IL-4 protect aged mice against lethal challenge

14 month old Balb/c mice (N=7) were vaccinated s.c with 0.375µg of inactivated A/PR/8/34 wild type or A/PR/8/34 bearing membrane-bound IL-2 or IL-4. Mice were boosted with same dose on day 21 then challenged with 100LD50 of mouse-adapted A/PR/8/34 on day 110 post primary vaccination. PBS served as negative vehicle control. Percent weight change (A) and survival (B) were monitored overtime.

In a previous study, we evaluated the protective efficacy of our CYT-IVACs in a “young adult” mouse model and demonstrated that a single low dose vaccination with IL-2 and IL-4 bearing CYT-IVACs induced superior protective immune responses as compared to non-adjuvanted influenza A/PR/8/derived WIV. To expand these observations, we assessed whether these CYT-IVAC formulations could also enhance protection in the elderly using an “aged mouse” model. In initial studies using retired breeder, multiparous mice, we found that 2 low doses (0.375µg) of CYT-IVACs co-presenting membrane bound IL-2 or membrane bound IL-4 administered subcutaneously (S.C.) could provide enhanced protection from lethal homotypic viral challenge.
in 14 month old female Balb/c mice compared to non-adjuvanted WIV at day 110 following primary vaccination (supplementary Fig. 16 and ).

**Figure 17: IL-2 and IL-4 responses from spleens following challenge.**

IL-2 and IL-4 ELISPOT assay on spleens from mice vaccinated prime and boost and challenged with A/PR/8/34 on day 45 post-vaccination and sacrificed 5 days following challenge. Data shows average number of IL-2 spots (A) and IL-4 spots (B) from each group of mice (n=6 mice/group) stimulated with VSV antigen or PR8 antigen. Error bars indicate standard errors. IL-2 ELISPOT data was not statistically different. *p<0.05 compared to PBS group (PR8 Antigen), Kruskal-Wallis Dunn’s multiple comparison test.
Figure 18: Lethal Challenge study in Prime/Boost Vaccination regimen.

Aged Balb/c mice vaccinated with wild type inactivated vaccine or with immunomodulators were challenged with a lethal dose (100 LD$_{50}$) of mouse adapted A/PR/8/34 on day 65 post vaccination. Mice were monitored daily for percent reduction in body weights and survival following challenge. Data represents percent original/start weight monitored for 16 days following challenge (***p<0.001 compared to PBS IM, One way ANOVA Bonferroni’s multiple comparison test)(A) and percent survival following challenge (B) with Table depicting number of animals and percent survival.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. of mice</th>
<th>No. survived</th>
<th>% Survival</th>
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<td>WIV IM</td>
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<tr>
<td>WIV/IL-12 IM</td>
<td>8</td>
<td>5</td>
<td>62.5</td>
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</table>

Figure 19: Splenocyte Proliferation Assay following lethal challenge.

Splenocyte proliferation on mice vaccinated with a single dose of vaccine and challenged with a lethal dose of mouse adapted A/PR/8/34 on day 51 post vaccination and sacrificed 5 days later. Data shows culture of splenocytes
with inactivated PR8 antigen or Class I peptide pools or Class II peptide pools for 72 hours. ConA was positive control. Cell proliferation was measured by reduction of indicator dye Alamar blue®. Data shows difference in absorbance values measured at 570nm and 600nm reference. Data not significantly different among the vaccine groups.
Chapter 3. Efficacy of membrane-bound IL-12, IL-23 and Flt3L bearing CYT-IVACs in young adult Balb/c mice

Abstract

Potent and safe adjuvants are needed to improve the efficacy of parenteral and mucosal vaccines. Cytokines, chemokines and growth factors have all proven to be effective immunomodulatory adjuvants when administered with a variety of antigens. We have previously evaluated the efficacy of membrane-anchored cytokines interleukin-2, interleukin-4 and interleukin-12 in an aged mouse model of infection. Here, we describe studies evaluating the efficacy of mouse-derived FMS like tyrosine kinase 3 ligand (Flt3L), interleukin-23 (IL-23) and interleukin-12 (IL-12) co-presented on whole influenza virus vaccine formulations in young adult Balb/c mice. Mice were vaccinated intramuscularly or intranasally followed by an intramuscular booster vaccination. The parenteral route of vaccination elicited higher influenza-specific IgG antibodies in serum as compared to the intranasal route with the highest levels obtained with the Flt3L CYT-IVAC formulation. Mucosal immunization using IL-12 and IL-23 formulations was more effective at eliciting protective responses as evident by a reduction in lung viral burden compared to control WIV. Both IL-12 and IL-23 CYT-IVACs elicited the highest anti-viral sIgA levels. This study highlights the potential of IL-12 and IL-23 CYT-IVAC formulations in eliciting mucosal immune responses and protective immunity. In addition, the co-presentation of immunomodulators in direct context with viral antigen may provide a means to lower the dose of vaccine without compromising protection.

Introduction

Influenza epidemics and pandemics remain a significant burden on world health and economy. Current influenza vaccines are available in the form of inactivated split or subunit vaccines or as live attenuated vaccines, all of which are typically propagated in embryonated chicken eggs. The presence of multiple antigenically different circulating strains of influenza viruses (H1N1, H3N2 and influenza B virus), the emergence of antigenic drift variants and introduction of new pandemic strains of virus are all factors that contribute to annual reformulation of vaccines targeting influenza. Insufficient mucosal immunity or protection against heterosubtypic strains of
influenza viruses, low efficacy in young children and elderly and limited production capacity of egg based production of influenza vaccines necessitates the development of new strategies in vaccine design and production. The use of novel adjuvants, non-replicating virus-like particles (VLP), replication defective vaccines, T-cell targeting vaccines, whole inactivated influenza vaccines, γ ray inactivated virus vaccines, universal vaccines, alternative routes of immunization and others are potential approaches to address current influenza vaccine challenges [1, 2]. Interestingly, in mice, whole-virus inactivated vaccines (WIV) have been shown to be more immunogenic and induce superior mucosal IgA responses when administered intranasally compared to split vaccines [3]. They also induce higher hemagglutination-inhibition titers and virus neutralizing antibodies compared to virosome or subunit vaccines when administered parenterally in mice [4-6] with similar responses in humans [7-9]. The enhanced induction of innate immune responses through Toll-like receptor 7 (TLR7) signaling and the skewing towards Th1-type cellular immune responses has been implicated in the superior immunogenicity of WIVs compared to subunit vaccines [4, 6, 10]. Interestingly, intranasal administration of WIV can lead to B cell dependent heterosubtypic immunity that is associated with elevated lung and serum sIgA and IgG levels [11]. The use of novel adjuvants in combination with WIV will likely serve to modulate and enhance both mucosal and systemic immune responses with the potential benefit of lowering the antigen dose and costs associated with vaccine production [12].

Cytokines have great potential to serve as potent and specific adjuvants when co-administered with antigen [13-16]. They are currently being actively evaluated for use as adjuvants for intranasal vaccine formulations [17]. Notable cytokines under active investigation include interleukin 12 (IL-12), interleukin 23 (IL-23), and FMS-like tyrosine kinase 3 ligand (Flt3L). IL-12 is a Th1 polarizing cytokine that promotes the differentiation of naïve CD4+T helper cells towards a Th1 phenotype and stimulates both NK cells and T cells to secrete the immunomodulatory cytokine, IFNγ [18, 19]. Direct effects of IL-12 in the growth of activated B cells have also been shown previously [20]. Interestingly, it has also been shown that IL-12 produced by activated DCs induces the differentiation of naïve T cells to IL-21 producing T follicular helper Tfh cells and also regulated development of antibodies [21]. Co-administration of IL-12 during intranasal vaccination has proven to be efficacious in stimulating enhanced immune responses to pneumococcal [22] and HIV antigens [23] as well as the induction of systemic and mucosal CTL responses [24]. Soluble IL-12 when administered with influenza
virus-like particles also led to enhanced antibody responses [25]. When co-administered in a DNA formulation together with HA and GM-CSF, IL-12 led to elevated neutralizing antibody responses [26] and induced robust T cell memory responses in mice [27]. In addition, membrane-anchored IL-12 expressed on the surface of tumor cells stimulated potent anti-tumor activity and induced T cell proliferation in vitro [28]. Recently, Pan et al [29] anchored IL-12 to the B7-1 transmembrane and cytoplasmic domains on the surface of adenocarcinoma cells, which significantly inhibited growth of subcutaneous tumors and lung metastasis. Further, an adenoviral vector encoding this form of IL-12 when administered intratumorally in mice induced complete tumor regression of local tumors and generated strong memory responses with minimal levels of IL-12 in circulation [29].

IL-23, a related IL-12 family member is another important mucosal cytokine and shares the common p40 subunit with IL-12 covalently linked to the IL-23 specific p19 subunit [30]. Interestingly, the receptor for IL-23 (IL-23R), which is rarely expressed on naïve T cells, is expressed on memory T cells, NK and NKT cells, monocytes as well as dendritic cells; hence, IL-23 can target diverse cell types [31, 32]. Deletion of IL-23p19 gene in mice resulted in severely compromised T cell dependent antibody responses, but did not affect normal T cell-independent B cell responses, suggesting that IL-23 plays a significant role in regulation of B cell responses [33]. IL-23 also plays a crucial role in the development, maintenance and homeostasis of T helper 17 (Th17) cellular responses [34, 35]. As an adjuvant, IL-23 enhances hepatitis C virus specific cytotoxic T cells responses when co-expressed in a DNA-based formulation with the hepatitis C virus (HCV) core protein [36].

Flt3L is another immunomodulator of interest that can potentially be exploited as an adjuvant in vaccine studies. It is a hematopoietic growth factor that regulates the development of hematopoietic progenitor cells, conventional as well as plasmacytoid dendritic cells [37, 38], and enhances the accumulation of antigen presenting cells, including plasmacytoid DCs in the lungs [39]. Flt3L plays a role in mobilizing and stimulating dendritic cells and progenitor cells of lymphoid and myeloid origin [40-43]. The peptide pulsed dendritic cells transfected with Adenovirus expressing Flt3L when administered subcutaneously has shown to activate Th1 and tumor specific cytotoxic T cell and NK cell immune responses and anti tumor activity in mice [44]. Flt3L has been used as a mucosal adjuvant, when administered intranasally combined with
CpG oligonucleotides [45] or expressed by adenovirus [46], induced the migration of mature dendritic cells in NALT thus elevating mucosal immune responses. In addition, it has been found that Flt3L increases plasmacytoid DCs in mucosal tissues [47, 48]. Flt3L in combination with TLR9L as adjuvant enhanced cellular immunity to SIV including antigen specific CD8+ T cells [49].

Due to their diverse roles in the initiation and the maintenance as well as linking innate and adaptive immune responses, cytokines are utilized as adjuvant to create a more immunogenic vaccine that will serve to augment immune responses and lower the doses of antigen. Moreover, the superior immunogenicity of whole inactivated vaccines with a Th1 predominant immune responses due to the presence of viral RNA in contrast to split or subunit influenza vaccines [4, 10, 50] encouraged us to utilize whole inactivated virus bearing cytokines as a more immunogenic influenza vaccine. Previous studies in our laboratory have evaluated the adjuvant potential of membrane-anchored cytokines directly incorporated on the inactivated influenza virus particles in mice and chickens [51, 52]. Transfection of fusion gene constructs into MDCK cell lines led to the constitutive surface expression of cytokines in a membrane-bound formulation. Subsequent infection of these producer lines with influenza A virus led to the release of progeny virions bearing the cytokine on their surface. Following gradient purification and inactivation of virus infectivity, these Cytokine bearing Influenza Vaccine (CYT-IVAC) formulations were assessed for immunogenicity and protective efficacy using a murine model of influenza virus challenge. Importantly, we were able to demonstrate that a single dose of IL-2 and IL-4 CYT-IVACs administered subcutaneously in young Balb/c mice provided enhanced protection from lethal homotypic viral challenge and generated a balanced Th1/Th2 humoral immune response [51]. Here, in this study we expanded our panel of immunomodulators to include IL-12, IL-23 and Flt3L and evaluated their immunogenicity and protective efficacy by different routes of immunization in young adult mice.

**Materials and Methods**

**Construction of expression vectors**

Mouse derived immunomodulators such as IL-12, IL-23 and Flt3L were fused inframe to the membrane anchoring regions of the hemagglutinin (HA) of A/WSN/33 influenza virus as
previously described [51]. Membrane anchoring regions of HA gene consists of nucleotides from positions 1521-1730 that encodes for the 71 amino acid region encompassing short membrane proximal extracellular stalk domain, transmembrane domain and cytoplasmic tail regions termed as HA1513. HA1513 region was amplified by PCR using primers (Table 1) to introduce restriction enzyme sites BamHI and EcoRI at 5’ and 3’ end respectively, and subsequently cloned in pcDNA3.1 expression vector (Invitrogen®) using same restriction sites. The mFlt3L and mIL-12 genes were derived from pORF9-mFlt3L (InvivoGen®) and pORF-mIL-12 expression plasmid (InvivoGen) respectively. mIL-23~HA1513 gene construct was synthesized in vitro (GenScript®). IL-23 is a heterodimeric cytokine composed of p19 and p40 subunits. Similar to the IL-12/HA construct, we expressed IL-23 gene as a single chain open reading frame of p19:p40 linked by a hydrophobic linker molecule. Flt3L is membrane anchored protein and to make the protein soluble in nature and facilitate membrane incorporation we truncated the juxtamembrane tether region to a 492bp soluble region. From these plasmids we amplified mFlt3L (492bp), mIL-12 (1623bp) and mIL-23(1563bp) gene coding region by Platinum Pfx Polymerase (Invitrogen®) using cytokine specific forward primer and reverse primer to insert restriction enzyme sites at 5’ end and 3’ end respectively and to remove stop codon (Table 1). PCR amplicons were digested with restriction enzymes such as KpnI and BamHI (mFlt3L gene) or HindIII and BamHI (mIL-12p70 gene) or EcoRV and BamHI (mIL-23 genes) and subsequently ligated with pCDNA3.1~HA1513 vector using T4 DNA ligase (Promega) and transformed into One shot DH10-B Maximum efficiency chemically competent E.coli cells (Invitrogen®) according to manufacturer’s instructions.

**Generation of CYT-IVAC producer cell lines**

Madin Derby canine kidney cells (MDCK) were transfected with CYT-IVAC expression plasmids using Lipofectamine 2000 (Invitrogen) in DMEM media without serum and antibiotic (Penicillin/Streptomycin) in a six well tissue culture plate as described previously [51, 53]. pcDNA3.1 plasmid has CMV promoter that drives the expression of gene insert and a neomycin resistance gene for selection of cells containing gene of interest. Transfectants were cultured in drug selection medium with Geneticin® (1.4mg/ml; Gibco®), for selection of positive cells. MDCK transfectants were further purified for expression of immunomodulators by subcloning by limiting dilution in 96 well plates in the presence of Geneticin (1.4mg/ml) as described previously [51] or by magnetic activated cell sorter (MiltenyiBiotec®) by incubating the cells
with Biotinylated anti mIL-12/23 p40 antibody (ebioscience®) or biotinylated anti mFlt3-L (R&D Systems) followed by Streptavidin magnetic microbeads (MiltenyiBiotec®) according to manufacturer’s instructions (MiltenyiBiotec®).

**Cell surface expression of membrane-bound cytokines (Immunofluorescence Microscopy)**

MDCK cells expressing membrane bound cytokines were seeded at 2 x 10^5 cells on glass coverslips in a 24 well tissue culture plate. Upon reaching confluency, monolayers were washed with PBS, fixed for 10 minutes in 3% paraformaldehyde (250 mM HEPES), washed and unreactive groups quenched by incubation in 50mM glycine/PBS for 10 minutes. Coverslips were blocked for at least 30 minutes in 2% chicken serum prior to immunostaining. Cells were sequentially incubated with corresponding primary goat anti mFlt3L-IgG antibody (Santa Cruz Biotechnology,®Inc) or goat anti mIL-23p19antibody (R&D Systems) (1:100 in 2% chicken serum) for 20 minutes, and secondary chicken anti-goat IgG conjugated Alexa Fluor®488 antibody (Invitrogen) (1:300 in 2% chicken serum) for 20 minutes. Coverslips were washed in PBS and mounted on glass slides using ProLong Antifade (Invitrogen/Molecular Probes). Immunofluorescent staining was visualized using a Nikon E800 Epifluorescence Microscope.

**Generation of double gradient purified inactivated influenza A virus bearing immunomodulators**

Producer cell lines expressing specific immunomodulators were grown to 80% confluency in 150mm tissue culture dishes (Corning) and then infected with influenza A/PR/8/34 (H1N1) virus at a multiplicity of infection (MOI) of 2 for one hour adsorption at 37°C. Viral supernatants from infected monolayers were harvested following 36-48 hour of infection and pre-cleared at 1500rpm for 15 minutes at 4°C. Viruses were purified by centrifugation at 88,000 x g for 45 minutes at 4°C in an ultracentrifuge (Beckman Coulter). Viral pellets were resuspended in PBS (supplemented with calcium and magnesium) and laid over the top of 10%-26% iodixanol (Optiprep™, Axis-Schield) continuous gradients (55,000 x g for 45 minutes at 4°C) zonal bands were collected from the center of gradient and washed in PBS by centrifugation (88,000 x g for 45 minutes at 4°C). Purified virus was run over second 10%-26% iodixanol gradient followed by chemical inactivation with 15mM β-propiolactone (Acros Organics) for 30 minutes at 25°C followed by neutralization with sodium thiosulphate (40mM) for 15 minutes at 25°C. Inactivated
virus was pelleted again at 88,000 x g for 45 minutes at 4°C and final resuspension was done in PBS. Total protein concentration in inactivated double gradient purified virus was determined by treating the purified virus with RIPA buffer (Tris (20mM), Triton X-100 (1%), SDS (0.1%), deoxycholate (0.5%), EDTA (1mM), Sodium chloride (150mM) for 10 minutes in ice and analyzed using bicinchoninic acid protein assay kit (Pierce Biotechnology). Inactivation was verified by culturing double gradient purified virus (5µg) on confluent MDCK cells in a 96 well plate for 72 hours at 37°C in the presence of TPCK (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone) treated trypsin (1.5µg/ml; Sigma) and monitored for the development of cytopathic effects (CPE).

**Validation of immunomodulator incorporation into gradient-purified inactivated vaccine formulations**

Immunomodulator incorporation on inactivated vaccine was validated by western blot analysis of whole viral lysates. Vaccines were diluted in 2xlaemelli buffer (Bio-Rad) with β-mercaptoethanol (Bio-Rad) and heated at 100°C for 10 minutes. Whole viral lysates were run on 12%SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by transfer onto PVDF membrane (Bio-Rad) using semi-dry or wet method. Following blotting, membranes were blocked in block solution (5% skim milk in PBST) for 2 hours shaking at 25°C followed by washing three times in TBST (TBS with 0.05% Tween 20). Membranes were probed with primary rat anti mouse IL-12/IL-23 p40 antibody (1:1000; ebioscience) or goat anti mouse Flt3L antibody (1:1000; Santa Cruz Biotechnology,®Inc) or anti influenza A(HA1) antibody (1:1000; Meridian Life Science,® Inc.) diluted in block solution and incubated overnight with shaking at 4°C. Membranes were washed three times in TTBS and incubated with secondary goat anti-rat IgG horse-radish peroxidase antibody (SantaCruz Biotechnology,®Inc) or rabbit anti-goat IgG horse-radish peroxidase antibody (Bio-Rad) or goat anti-mouse IgG horse-radish peroxidase antibody (Bio-Rad) (all diluted 1:5000) for 1hr with shaking at 25°C. Membranes were washed and exposed to Pierce ECL western blotting substrate or super signal west Femto chemiluminescent substrate (Pierce) as per manufacturer’s instructions and visualized using Chemidoc XRS (Bio-Rad).
Validation of immunomodulator bioactivity following inactivation

Bioactivity of membrane bound cytokines was determined using cytokine specific in vitro bioassays. These assays were based on indicator cell lines that are growth factor or cytokine dependent and require cytokine for survival and or proliferation. The Flt3L bioassay was conducted with human acute myeloid leukemia cell line OCI-AML5 cells (GmbH, DSMZ) in Flt3L dependent survival bioassay. Briefly, OCI-AML5 cells were washed three times in α-MEM medium (without serum) and seeded at 5x10^4 cells/well in 0.1ml of α-MEM medium in 96-well flat bottom tissue culture plates. Non-adjuvanted whole inactivated virus vaccine, recombinant mouse Flt3L and media alone served as controls. Fivefold dilution of WIV and CYT-IVAC Flt3L/HA vaccines at 50µg/ml, 10µg/ml and 2µg/ml were added to wells in 10µl volumes per well and incubated at 37°C for 48-72 hours. Alamar Blue® (Sigma) was added to each well in last 18 hours of incubation. Alamar Blue® dye reduction was recorded by measuring absorbance at 570nm (measurement) and 600nm (reference). For IL-12, a cell culture based bioassay was performed as described in Chapter 2 with some modifications [54].

Animals, Vaccination and Virus Challenge

All animal experiments were performed based on the guidelines of NIH and approval of Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State University. 8-10 week old female Balb/c mice (Mus musculus) were purchased from Charles River Laboratories (CRL) and allowed to acclimate for at least 10 days prior to vaccination. Animals were lightly anesthetized using isoflurane inhalant and immunized with inactivated double gradient purified control WIV (A/PR/8/34), CYT-IVAC Flt3L/HA, CYT-IVAC IL12/HA and CYT-IVAC IL23/HA (n=5 mice/group) either intramuscularly (I.M.) (0.5µg in 100µl volume of sterile PBS) in the right hindquarter or intranasally (I.N.) (3µg in 10µl volume of sterile PBS) into the nostrils. PBS administered intramuscularly (I.M.) served as mock (n=5mice/group). On day 21, all mice were administered a booster dose of vaccine administered I.M. The I.M. groups all received 100ng administered in the left hindquarter, whereas the I.N. groups received 500ng administered to the right hindquarter while animals were lightly sedated using isoflurane inhalant anaesthesia. Blood was collected from the retro-orbital sinus on day 19 and day 35 post-vaccination. Sera were used for the determination of antiviral antibody levels. Animals were challenged intranasally on day 36 post-vaccination with mouse-adapted influenza
A/PR/8/34 virus, 1000 TCID\textsubscript{50}/50 µl volume of PBS (100LD\textsubscript{50}) while the animals were under light anesthesia (isoflurane inhalation). Body weights and clinical symptoms were monitored daily and at day 4 post-challenge, all animals were sacrificed. Sera, lungs, nasal washes and spleens were collected post-mortem for biological assays. Nasal wash specimens were collected by rinsing the nasal passage with 2 ml of PBS; PBS was passed through the posterior nasal passage out of the nares into a sterile collection tube. Lung tissues and nasal washes were flash frozen and stored at -80°C.

**Enzyme linked Immunosorbent Assay (ELISA)**

Quantitative levels of Influenza specific total IgG subtype antibodies in serum were determined by enzyme-linked immunosorbent assay, ELISA as described previously [51]. Briefly, 96-well immunosorbent ELISA plates (Nunc 475094) were coated with 100 HAU/ml of gradient purified inactivated influenza virus (100µl/well) in ELISA coating buffer (sodium carbonate 150mM, sodium bicarbonate 350mM, sodium azide 130mM) or for standard curve, goat anti-mouse Ig (H+L) (Southern Biotech) (10ug/ml in PBS). Plates were spun at 4000 rpm for 15 minutes and stored overnight at 4°C. Coated plates were washed once with PBS-T (phosphate buffered saline with 0.05% Tween 20) and blocked with blocking buffer (2% BSA in PBST) at100µl/well with shaking overnight at 4°C. Plates were washed three times with PBST. For standard curve generation, anti mouse IgG (SouthernBiotech) or anti-mouse IgA (SouthernBiotech) antibodies were serially diluted 2 fold from 160ng/ml to 0.03125ng/ml or 512ng/ml to 0.03125ng/ml respectively in blocking buffer and added (100µl/well) to wells coated with Ig (H+L) in triplicates. Serum samples were diluted 1:20 in blocking solution, added 100µl/well in duplicates and incubated with shaking overnight at 4°C. Nasal washes were undiluted for IgA antibodies and added 100µl/well in duplicates. Plates were washed three times with PBST and incubated with secondary anti mouse IgG or IgA-Horseradish peroxidase (SouthernBiotech) (1:4000) in blocking solution for 1.5 hours at RT followed by three wash and addition of substrate (2,2’-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid; Sigma) (50µl/well) and incubation in dark for 30 minutes at RT. Reaction was stopped by addition of equal volume of 1% sodium dodecyl sulfate (SDS). Absorbance was measured at 405nm using plate reader (SpectraFluor plus, Tecan). OD values were plotted against known standard curve to evaluate the concentration of influenza specific IgG or IgA antibodies per milliliter of serum or nasal wash.
**Quantitation of viral loads in lungs**

Vaccinated animals were challenged with mouse adapted A/PR/8/34 virus and at day four following challenge, mice were sacrificed and lung tissues were minced and collected aseptically in 0.5ml DMEM media. Lungs were weighed, flash frozen in liquid nitrogen and stored at -80°C. Frozen lung samples were thawed on ice, homogenized and cell debris removed by pelleting at 6000rpm for 10 minutes. The supernatant volume was adjusted to 1ml total with DMEM. Serial 10-fold dilutions of lung homogenates were prepared in PBS supplemented with calcium and magnesium and added on confluent MDCK cells in a 96-well tissue culture plate for 1hr adsorption at 37°C. Viral supernatants were aspirated off and plates were cultured for 72 hours for the development of cytopathic effects (CPE) in the presence of DMEM with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone TPCK treated trypsin (1.5µg/ml; Sigma). Cytopathic effects were recorded and 50% tissue culture infectious dose units (TCID\textsubscript{50}/ml) were determined by the Reed-Muench method [55]. TCID\textsubscript{50}/ml was further divided by individual lung weights to obtain TCID\textsubscript{50}/gram of lung tissue.

**Splenocyte Proliferation assay**

Animals were challenged with mouse adapted A/PR/8/34 virus and at day four following challenge, mice were sacrificed and spleens were harvested, minced in 10ml of Dulbecco Modified Eagle Medium and single cells filtered through a 60µm sterile wire mesh. Splenocytes were pelleted at 1500rpm for 10 minutes and contaminating red blood cells were removed by resuspension in RBC lysis buffer (3ml) for 3 minutes on ice, followed by the addition of RPMI (complete) (RPMI, FBS 10%, sodium pyruvate 2mM, HEPES 20mM, L-Glutamine 20mM, β-mercaptoethanol 2µM, penicillin/streptomycin) and recovery of the cells by centrifugation. Pellets were resuspended in cRPMI at a concentration of 2x10^6/ml and 100µl of cell suspension was seeded per well in a 96 well tissue culture flat bottom plate. Inactivated PR8 antigen was diluted in cRPMI (10µg/ml) and 100µl dilution was added to cells in triplicates. Media alone and Con A (5µg/ml) served as negative and positive control respectively. Plates were incubated at 37°C for 72 hours and during the last 18 hours of incubation the redox reduction dye Alamar blue® was added to all wells [16, 56]. Absorbance readings were taken at 570nm wavelength with 600nm reference after 8 hours and again at 18 hours and data is expressed as the difference between 570 and 600nm readings [57, 58].
Statistics
The significance of differences between vaccine and control group was assessed using Graph Pad Instat version 3.0a for Macintosh (GraphPad Software). IgG and IgA ELISA were analyzed by One-way Analysis of Variance (ANOVA) using Bonferroni’s Multiple comparison test with PBS group (IgG) and PR8 group (IgA) as control. Comparison of lung titers was also analyzed by One-way Analysis of Variance (ANOVA) using Bonferroni’s Multiple comparison test with PBS group as control. Splenocyte proliferation assay was analyzed by two way ANOVA with media treatment as control.

Results

Establishment of IL-23 and Flt3L vaccine producer cell lines for the production of Cytokine bearing inactivated Influenza Vaccines, CYT-IVACs

The CYT-IVAC vaccine platform utilizes virus-permissive, mammalian-based vaccine producer cell lines that express the membrane-bound version of specific immunomodulators, such as cytokines, on the surface of the cells. During subsequent infection of these cells with influenza virus, the immunomodulator is actively incorporated into newly formed virions as they assemble and bud from the infected cell. The virions are concentrated, purified and inactivated resulting in a vaccine formulation comprised of inactivated, whole virus particles that co-present a bioactive immune modulator on the particle surface. Importantly, full-length viral glycoproteins must also be incorporated to ensure vaccine efficacy, as they are the major targets for the induction of neutralizing anti-viral antibodies.

The membrane-bound cytokine constructs that were evaluated in this study are depicted in Figure 20. Flt3L (FMS like tyrosine kinase 3 ligand) is a α-helical type I transmembrane protein composed of an extracellular cytokine-like domain, transmembrane and cytoplasmic tail domain [59]. We truncated the transmembrane and cytoplasmic tail regions to Flt3L to make it soluble. To facilitate the active membrane incorporation into influenza virions we fused the coding regions for soluble form of Flt3L to the regions coding for transmembrane and cytoplasmic tail (TM/cyt.tail) domain of the HA derived from influenza virus A/PR/8/34 (H1N1) using standard PCR and recombinant DNA methodologies. Start codon and signal sequence of Flt3L were included and placed under the control of CMV promoter element in plasmid, pcDNA3.1. IL-23 is a heterodimeric cytokine belonging to the IL-12 cytokine family and is composed of a p40
subunit (common to both IL-12 and IL-23) and the IL-23 specific p19 subunit. Similar to the
generation of the IL-12/HA fusion construct described in chapter 2, we chose to use a single
chain full-length IL-23p19p40 molecule with both subunits joined by linker molecule instead of
using two separate subunits to ensure that both units assemble correctly in our producer cell
lines. Single chain IL-23 have been shown to be bioactive in vitro and in vivo [60]. Similar to the
Flt3L and IL-12 fusion proteins, we fused the IL-23 gene to the gene segment encoding the
membrane anchoring and cytoplasmic tail domains of the influenza HA gene. Stable MDCK
transfectants expressing the membrane-bound forms of Flt3L or IL-23 at the cell surface were
established following drug selection (G418) and magnetic sorting of Flt3L or IL-23 expressing
cells using cytokine specific antibodies conjugated to magnetic beads. As depicted in Figure 21,
stable transfectants constitutively expressing Flt3L/HA (Figure 21C) and IL-23/HA (Figure 21D)
at the cell surface were validated by surface immunofluorescent staining using Flt3L (Figure
21C) and IL-23 p19 (Figure 21D) specific antibodies, MDCK control cells did not stain positive
for surface Flt3L (Figure 21A) or IL-23 (Figure 21B).

**Validation of immunomodulator incorporation and inactivation of IL-23 and
Flt3L bearing CYT-IVAC formulations**

CYT-IVAC vaccine formulations were prepared by infection of membrane-bound cytokine-
expressing MDCK cells with influenza virus and harvesting virions from the supernatants of
infected cells. To maximize immunomodulator incorporation on the progeny virions, cells were
infected at a moi of 1 and supernatants containing released virions were collected at 36 hour
post-infection. As viral assembly and budding is initiated by the M1 protein of influenza, binding
to the cytoplasmic tail domains of HA and NA both full length HA, NA and M2 proteins
together with cytokine-fused HA proteins are packaged into budding virions, albeit the latter are
incorporated at much lower levels than the full length proteins [51]. Viruses were subsequently
gradient purified and inactivated using β-propiolactone. Complete virus inactivation was verified
by culturing aliquots of purified, β-propiolactone treated virus (5µg of total protein) with MDCK
cells in the presence of TPCK-trypsin for 3-5 days and monitoring the development of virus-
induced cytopathic effects (CPE). β-propiolactone completely abolished infectivity of all vaccine
formulations in MDCK cells.
To validate the incorporation of membrane-bound cytokine into virions and assess whether the bioactivity of the cytokines was maintained following virus inactivation, vaccine formulations were subjected to western blot analysis. Figure 22 depicts western blot analysis of CYT-IVAC formulations probed with antibodies specific for Flt3L or IL-23. A single band with a molecular weight of approximately 27kDa was identified in our CYT-IVACFlt3L/HA formulation using Flt3L specific antibodies (Figure 22A). The predicted molecular weight of the Flt3L/HA protein is 26.48kDa. Incorporation of full-length HA was also confirmed by western blot analysis using HA-specific antisera and is depicted in the upper panel in Figure 22A. Western blot analysis of CYT-IVACIL-23/HA revealed a prominent band of approximately 70kDa in molecular weight when probed with an anti-murine IL-12/IL-23p40 subunit antibody (Figure 22B). The predicted molecular weight of the IL-23/HA construct is 66.87kDa. The IL-12/HA fusion construct and establishment of an IL-12/HA expressing MDCK cell line is described in detail in Chapter 2. The cytokine specific bands were not detected in our control non-adjuvanted WIV formulation (PR8).

We also confirmed the incorporation of the full length HA (76kDa) in all vaccine formulations by western blot analysis (Figure 22C and Figure 22D). Together, these data confirm that our CYT-IVAC formulations display membrane-bound immunomodulators in direct context with full-length viral hemagglutinin and other virion associated proteins.

**Bioactivity of Flt3L/HA is maintained following β-propiolactone inactivation of vaccines**

Retention of the bioactivity of membrane-bound cytokines in our inactivated CYT-IVAC formulations is important for comparative efficacy studies; hence CYT-IVACs were further evaluated using cytokine specific *in vitro* bioassays when specific cytokine responsive cell lines were available. The OCI-AML5 cell line was employed to assess the bioactivity of CYT-IVAC^{Flt3L/HA} (Figure 23). This bioassay utilizes the reduction of Alamar Blue® as a quantitative indicator of cell proliferation and viability. In contrast to control, non-adjuvanted WIV (PR8), the CYT-IVAC bearing Flt3L stimulated the proliferation of OCI-AML5 in a dose dependent manner; CYT-IVAC^{Flt3L/HA} contained approximately 1ng of bioactive Flt3L per µg of total viral protein (Figure 23). Bioassays for IL-12 and IL-23 proved to be unreliable, hence we quantitated the amount of IL-12 and IL-23 incorporated using a quantitative IL12/IL23p40 specific bead assay.
Table 4. Cytokine content in purified whole virus vaccine.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Total Cytokine (pg/µg of vaccine)*</th>
</tr>
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<tbody>
<tr>
<td>WIV (PR/8/34)</td>
<td>N/A</td>
</tr>
<tr>
<td>CYT-IVAC IL-12/HA</td>
<td>23</td>
</tr>
<tr>
<td>CYT-IVAC IL-23/HA</td>
<td>156</td>
</tr>
</tbody>
</table>

* Quantitation of virus-incorporated cytokine based on the cytokine quantitation kit (pg of cytokine per µg of vaccine)

Vaccination and challenge

**CYT-IVACs elicit distinct anti-viral protective responses depending on the route of administration.**

Anti-viral antibody levels elicited by CYT-IVACs and control WIV were determined on sera collected on day 35 post-vaccination. Quantitation of antibody levels was determined following extrapolation from an IgG standard curve and is reported as ng/ml (Figure 24). Animals that were subject to the I.M. vaccination regimen exhibited higher total anti-viral IgG levels in the serum (range of 1984ng/ml to 50,440ng/ml) as compared to intranasally vaccinated animals (range of 5ng/ml to 960ng/ml) (Figure 24). Interestingly, the CYT-IVAC<sup>Flt3L/HA</sup> administered by the I.M. route elicited the highest levels of anti-viral IgG and these were significantly higher than that obtained by control WIV or the other CYT-IVACs (Figure 24A). Vaccination with the CYT-IVAC<sup>IL12/HA</sup>, or CYT-IVAC<sup>IL23/HA</sup> elicited only marginal increases in anti-viral IgG levels compared to the non-adjuvanted control vaccine. There were no significant differences detected in the serum anti-viral IgG levels following the I.N. vaccination regimen (Figure 24B).

To assess the levels of sIgA elicited by both vaccination regimens, we collected nasal washes from animals at day 4 post-challenge. This time point is insufficient to generate detectable sIgA or IgG responses in naïve animals and hence, can be used as measurement of the vaccine-induced mucosal response; anti-viral sIgA was not detected in nasal washes of naïve challenged animals at day 4 (PBS group,
Influenza specific sIgA antibodies were detected in all groups vaccinated by the intranasal route; albeit the control WIV and CYT-IVAC^{Flt3L/HAI23} elicited only marginal sIgA responses. Both the CYT-IVAC^{IL23/HAI23} and CYT-IVAC^{IL12/HAI23} vaccinated groups exhibited significantly higher levels of sIgA (p<0.001) compared to the control WIV. This suggests that both IL-12 and IL-23 serve as potent mucosal adjuvants when co-presented in a membrane-bound formulation in direct context with WIV. Unexpectedly, high sIgA levels were detected in the nasal washes of animals that received CYT-IVAC^{Flt3L/HAI23} and CYT-IVAC^{IL12/HAI12} by the I.M. route (Figure 25). This suggests that IL-12 and IL-23 in the membrane-bound formulation are able to stimulate mucosal responses when administered by the parenteral route as well.

All immunized animals were challenged on day 36 with 100 LD_{50} of mouse adapted influenza virus A/PR/8/34 (H1N1) virus and sacrificed four days later to compare viral lung loads. Pre-existing protective responses should be discernible by comparison of the viral lung burden at day 4 post-infection. Interestingly, despite high levels of anti-viral serum IgG, particularly in the CYT-IVAC^{Flt3L/HAI23}, there was no significant reduction in the lung viral burden of animals immunized using our low dose I.M. vaccination regimen (Figure 26A). However, there was a trend towards a reduction of viral burden in all the CYT-IVAC immunized groups compared to control WIV (Figure 26A). In addition, there was lowest reduction of body weights on mice from CYT-IVAC^{Flt3L/HAI23} administered IM monitored up to day 4 following challenge however differences among different vaccine groups in both routes of administration (data not shown). Interestingly, CYT-IVAC^{IL-12/HAI12} and CYT-IVAC^{IL-23/HAI23} elicited superior protection when administered mucosally as evident by the significantly reduced viral loads at day 4 post-challenge compared to control WIV (PR8 WT, Figure 26B). The reduced viral lung burden correlated well with the observed high levels of sIgA detected in the nasal wash of animals receiving either CYT-IVAC^{IL23/HAI23} or CYT-IVAC^{IL12/HAI23} intranasally. Of note, the fact that both CYT-IVAC^{IL23/HAI23} and CYT-IVAC^{IL12/HAI12} vaccines administered IM failed to afford significant protection despite eliciting high sIgA in nasal washes, suggest that there are fundamental differences in the specificity of mucosally induced anti-viral sIgA compared to sIgA induced at other sites.
Discussion

Influenza and pneumonia are ranked ninth among leading causes of death in the United States, and are associated with some 226,000 hospitalizations and 3,000-49,000 deaths annually [48, 49]. Despite the tremendous success of influenza vaccination programs in controlling the emergence of antigenic drift variants of influenza and providing protection in most age groups, there are still numerous deficiencies in vaccination strategies and efficacy that need to be addressed. These include the insufficient capacity to protect from antigenically divergent subtypes and emerging drift variants, low efficacy in high-risk group populations including elderly and very young, poor induction of cellular immunity and limited production capacity of egg-based production facilities. Egg-based manufacture is not practical, scalable and quick enough to respond to sudden emergence of a highly virulent pandemic strain of influenza [61, 62]. Other vaccine production methods like cell culture-based bioreactors may represent a better alternative over egg-based production and are currently under development. In addition current parenterally administered inactivated vaccines induce protective neutralizing serum antibodies yet are inefficient at inducing sufficient mucosal antibodies. In addition antibodies are less effective against heterologous influenza viral strains [63].

Mucosal immunity acquired by natural influenza viral infection acts as a first-line of defense and is mainly mediated by secretory IgA (sIgA) antibodies produced in respiratory tract. sIgA antibodies are synthesized by B cells in lamina propria and are dimeric in nature joined by the J chain. These antibodies bind to polymeric immunoglobulin Ig receptor in the basolateral surface of epithelial cells and are released by transcytosis from the apical surface of epithelium into the lumen of respiratory tract. Secretory IgA antibodies are present predominantly in the upper respiratory tract while in the lower respiratory tract IgG antibodies mediate protection [64, 65]. sIgA antibodies are more broadly cross-protective against heterologous strains of influenza as compared to serum antibodies induced by parenteral immunizations [63, 64, 66]. Hence, newer vaccination strategies are focused on eliciting both mucosal antibody and cell mediated immune responses, which should provide more cross-protective responses as well as accelerate recovery from influenza infection.

Intranasal immunization is an attractive alternative to parenteral immunization in terms of inducing superior mucosal and T cell responses. Live attenuated influenza vaccines (LAIVs)
contain attenuated influenza viruses, which replicate in the nasopharynx and induce protective mucosal immune responses. However, LAIVs induce lower serum antibodies as compared to inactivated vaccine and are not recommended for use in children < 2 years of age and in the elderly. In addition, adverse side effects in children have been reported and these include: rhinorrhea, runny nose or nasal congestion, wheezing, irritability, decreased activity, low fever, vomiting, muscle ache and abdominal pain [67-69]. Considering these side effects of LAIVs, whole virus inactivated vaccines administered intranasally might be a better alternative and their use should be revisited. Interestingly, intranasal administration of formalin inactivated whole virus vaccine but not split virus vaccine induced broad spectrum, heterotypic immunity in mice [3, 70]. Therefore, whole virus inactivated vaccines administered intranasally might be a potential alternative but still immune correlates, lower serum antibody response, cross-protection potential and safety issues need to be addressed. Further the immunogenicity of WIVs administered intranasally can be improved by addition of adjuvants which may potentially improve serum antibody responses and mucosal immune responses. Cross protective immunity can also be achieved by administration of vaccines intranasally with mucosal adjuvant like TLR3 [71].

The use of novel more robust adjuvants with whole inactivated vaccines should aim to modulate immune responses and possibly enable administration of vaccine at lower dosage; ultimately reducing the overall costs of the vaccine. To investigate novel adjuvants, we chose cytokines since they are potent immunomodulators and have been used extensively as soluble adjuvants co-administered with numerous vaccines [72-74], but also in a fused or linked form directly with antigen [14, 75-78]. The latter is particularly relevant to our vaccine design. Previously, we investigated the use of membrane-bound immunomodulators directly incorporated into influenza virus particle to boost vaccine efficacy [51]. Specifically, we examined the adjuvant properties of membrane-bound formulations of Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Granulocyte-Monocyte Colony-Stimulating Factor (GM-CSF) [51]. These vaccines are produced using a cell-culture based vaccine production technology that can incorporate any immunomodulator of interest and is applicable to any strain of influenza virus including highly pathogenic avian influenza virus and other enveloped viruses as long as they replicate well in virus permissive cell lines. Cell culture produced vaccines can be rapidly scaled up in a short amount of time, and are free from egg-based allergens.
Here, we expanded our previous investigations to include CYT-IVAC formulations co-presenting 3 different immunomodulators (Flt3L, IL-12 and IL-23) that we postulated may have superior mucosal and parenteral adjuvant properties when co-presented with influenza whole virus particles. In addition, we explored the differences in antibody responses of parenteral (IM) versus mucosal (IN) routes as the primary vaccination followed by a booster parenteral immunization. The vaccination regimen strategy of intranasal prime followed by an intramuscular boost was chosen to provide an initial stimulus for generation of mucosal responses as well as to boost systemic responses, which are often weakly elicited by intranasal vaccination of inactivated or subunit vaccines. Of note, parenteral boosting has been shown to clinically boost mucosal responses using the polio vaccine [79]. As anticipated, the intramuscular route of immunization induced higher serum IgG responses as compared to mucosal (IN) route. Of note, the Flt3L CYTIVAC administered by the intramuscular route elicited significantly higher influenza-specific IgG antibody levels compared to all vaccine groups (Figure 24A). This validates a previous study in which Flt3L when administered with hepatitis B virus in a DNA vaccine formulation served as a potent adjuvant increasing both humoral and cellular immune responses to hepatitis B virus [80]. Antiviral serum IgG antibody responses were lower in the intranasal group as compared to the intramuscular group (Figure 24B) supporting previous reports in both animal [81] and human [82] vaccine studies. Our observations are also in line with those examining immunization with trivalent inactivated influenza vaccine [83, 84]; High influenza specific IgG and lower IgA responses are induced by parenteral immunization. Similar to clinical observations [85-87], intranasal immunization with our vaccine formulations led to high influenza specific sIgA and lower serum IgG antibody levels (Figure 26).

Mucosal antibodies present in the respiratory tract are known to provide significant protection from influenza infection [88-90]. Hence, it was important to determine whether any of our vaccine formulations elicited superior mucosal responses. Notably, intranasal administration of both IL-12 and IL-23 CYT-IVACs induced significantly higher levels of influenza specific sIgA in nasal washes as compared to non-adjuvanted WIV demonstrating their potent mucosal adjuvant properties. This is the first time that the membrane-bound IL-12 or IL-23 co-presented directly on influenza WIV can induce robust mucosal immune responses. IL-12 as a nasal adjuvant is well documented [13, 91] and has been reported to stimulate mucosal sIgA and systemic IgG antibody as well as cellular responses (also reviewed extensively by Kunisawa,
Our data supports these observations, but also suggest that even low concentrations of IL-12 (pg) can be used in a membrane-bound formulation to boost mucosal sIgA levels. In contrast, how our IL-23 vaccine formulation is acting as a nasal adjuvant is unclear. IL-23 inducing mucosal antibody responses is an interesting and novel observation and clearly further work is required to address the mechanism. Based on previous reports of IL-17 influencing B cell biology including B cell activation [92] and germinal center development [93], the IL-23 CYT-IVAC could be acting by modulating Th17 responses. Recently, activation of lung Th17 cells were also found to induce the development of polymeric Ig receptor and elevate mucosal secretion of IgA antibodies [94]. Alternatively, common to both IL-12 and IL-23 is the p40 subunit, which may be eliciting the observed elevation in sIgA responses, when co-presented in a membrane-bound formulation. Whole inactivated vaccine administered intranasally was sufficient to induce sIgA antibodies; albeit at significantly lower levels than the IL-12 and IL-23 CYT-IVACs.

Figure 25). Therefore, the inclusion of IL-12 and IL-23 in direct context with WIV induced superior mucosal responses. Of note, we also found sIgA levels in nasal washes in animals immunized intramuscularly with IL-12 and IL-23 CYT-IVACs but not with WIV IM. This was an unexpected observation as mucosal antibodies are predominantly elicited by the mucosal route. However, the transcutaneous route of immunization coupled with mucosal adjuvants including bacterial toxins, chemical enhancers of cyclic AMP and the active form of Vitamin D3 has been reported to induce peripheral DC migration to Peyers patches (mucosal tissues) [95]. IL-12 and IL-23 co-presented with the particulate nature of the WIV and the TLR7 agonist of the associated vRNA, may be sufficient to redirect peripheral DCs to mucosal sites. Interestingly, hair follicle associated DCs targeted by transcutaneous prime immunization followed by a subcutaneous boost [96] or the migration of langerhans cells following epicutaneous immunization to draining lymph nodes both lead to subsequent priming of T and B cells for mucosal homing [97]. It is possible that our membrane anchored IL-12 and IL-23 adjuvants are activating dendritic cells in muscle tissue and targeting them to migration in draining lymph nodes, in a state that is conducive for priming T and B cells for homing to mucosal tissues. Regardless of the mechanism by which parenteral immunization elicits mucosal influenza specific sIgA responses, these are nonetheless inferior than those elicited by mucosal vaccination. Despite high levels of sIgA in the nasal washes of IL-12 and IL-23 CYT-IVAC...
immunized animals, there was no sufficient enhancement of protection when administered at these doses.

Intranasal administration of IL-12 and IL-23 CYT-IVACs led to more robust protective responses as evidenced by their ability to reduce viral titers in lungs as compared to non-adjuvanted WIV (Figure 26). A reduction in viral titers in lung tissue of vaccinated animals following homotypic or heterotypic viral challenge acts as a valuable correlate of protective efficacy of viral vaccines [98-103]. IL-12 has shown to enhance protective efficacy of co-delivered antigens against infectious challenge in several experimental vaccine formulations [13, 22, 104]. Besides IgA antibodies, we also cannot undermine the potential role of IgGs in BAL fluids in mediating protection from viral challenge in lungs from both intramuscular and intranasal groups. In addition, our vaccine formulations could be eliciting robust T cell responses that respond rapidly to infection. Of note, IL-23 was recently reported to play an important role in CD4+ T cell recall memory responses and protection from Mycobacterium tuberculosis intranasal challenge via IL-17 producing CD4 T cells [105]. IL-23 was also found to play role in protection from Listeria monocytogenes infection through IL-17 production from γδT cells leading to enhanced neutrophil recruitment and bacterial clearance [106]. In our study, we did not evaluate the cellular responses in great depth, however, comparable viral specific splenic lymphocyte responses were similar across all vaccine formulations irrespective of the route of administration or the cytokine adjuvant used (Supplemental Figure 27). Together our data suggests that IN vaccination followed by IM vaccination with IL-12 and IL-23 CYT-IVACs induces superior mucosal IgA responses and provides better protection from lethal homotypic viral challenge. A large scale study evaluating single dose vaccination with the IL-12 and IL-23 CYT-IVAC using the intranasal route of immunization is clearly warranted.

This approach of using cytokines in direct context with whole inactivated virus vaccines involves very low amounts of cytokine (picograms per microgram of vaccine) while still improving the immune responses to whole virus vaccines. This may potentially lower the dose of WIV needed to provide robust protection, which may also lower the incidence of adverse side effects due to the WIVs. In most studies recombinant IL-12 is given 1µg although the total number of doses and dosing schedules vary significantly depending on the study (reviewed extensively in [17]. Soluble Flt3L administered in 10µg doses [74] is clearly well in excess of the amounts utilized in
the present study, suggesting that the membrane-bound formulation in direct context with viral antigen may be superior and the dose lowering factor. Interestingly, 10µg of virus-like particles expressing membrane anchored flagellin administered twice at 4 week intervals was successful at providing protection against lethal challenge with 40LD_{50} [107]. This dose was much higher and challenge virus dose was also lower as compared to our vaccine study. Future studies using higher single dose formulations, particularly with the IL-12 and IL-23 CYT-IVAC formulations will allow us to fully assess the adjuvant potential of these vaccines. It should be noted that IL-23 has also been reported to have adverse effects such as inducing chronic inflammation and autoimmune disease [30, 35, 108]. Its reported anti-tumor activity has been controversial with studies indicating both antitumor and tumor promoting effects [109, 110]. This may be due to the context of how IL-23 is presented, particularly the cytokine milieu that is triggered, since Th17 cells and associated cytokines can have dual effects [111]. Therefore, safety issues and autoimmune inflammatory reactions from IL-23 containing vaccines will have to be addressed prior to these vaccines moving on to clinical trials. Finally, an in depth characterization of the isotype of sIgA and IgG in nasal washes and BAL fluid together with the type of T helper cell responses induced upon vaccination and expanded upon challenge will be required. In summary, this study represents the first report of the nasal adjuvanticity of both IL-12 and IL-23 in a membrane-bound formulation co-presented with whole inactivated vaccine. These formulations may prove beneficial in the elderly, where immunity is often waning and where protective immunization is highly variable.
Figure 20: Construct diagram of mFlt3L/HA1513, mIL-12/HA1513 and mIL-23/HA1513.
Table 5 CYT-IVAC constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number of nucleotide</th>
<th>No. of amino acids</th>
<th>Mol. Wt</th>
<th>Cloning enzymes in pCDNA3.1</th>
<th>Primar sequance</th>
</tr>
</thead>
<tbody>
<tr>
<td>mFlt31/HA1513</td>
<td>721bp</td>
<td>233aa; 26.48kDa</td>
<td>KpnI/EcoRI</td>
<td></td>
<td>Forward primer 5’ CCGGTACGAGCAGCTGCTGACCTGACC 3’ (KpnI) Reverse primer 5’ CCGGTACGAGCAGCTGCTGACCTGACC 3’ (BamHI)</td>
</tr>
<tr>
<td>mIL-12/HA1513</td>
<td>1862bp</td>
<td>611aa; 68.93kDa</td>
<td>HindIII/EcoRI</td>
<td></td>
<td>Forward primer 5’ TTTAGGATCCACCATTGCGTCAATCAGCTACC 3’ (32bp) Reverse primer 5’ TTTAGGATCCACCATTGCGTCAATCAGCTACC 3’ (23bp) BamHI</td>
</tr>
<tr>
<td>mIL-23/HA1513</td>
<td>1805bp</td>
<td>595aa; 66.67kDa</td>
<td>EcoRI/EcoRI</td>
<td></td>
<td>Forward primer 5’ CGGTATATCAAGCTTACGCTGATGTG 3’ (FseI) Reverse primer 5’ CGGTATATCAAGCTTACGCTGATGTG 3’ (EcoRI)</td>
</tr>
<tr>
<td>Influenza A/WSN/33HA (HA1513)</td>
<td>729bp</td>
<td>1aa; 8kDa</td>
<td>BamHI/FseRI</td>
<td></td>
<td>Forward primer 5’ CCGGTACGAGCAGCTGCTGACCTGACC 3’ (BamHI) Reverse primer 5’ CCGGTACGAGCAGCTGCTGACCTGACC 3’ (EcoRI)</td>
</tr>
</tbody>
</table>

Table includes size of construct in base pairs and number of coding amino acids, total predicted molecular weight of fusion protein, restriction enzymes used for cloning genes in pCDNA3.1 expression vector and primer sequences used to amplify primary cytokine genes and membrane anchoring regions of HA (HA1513).

Figure 21: Validation of immunomodulator expression on the surface of transfected cells.
Indirect immunofluorescent staining on MDCK cells stably transfected with plasmid mFlt3L-HA1513-pCDNA3.1 (C) and mIL-23-HA1513-pCDNA3.1 (D). Paraformaldehyde fixed cells were probed for surface expression of mFlt3L, mIL-12 and mIL-23 using primary antibodies against mFlt3L(R&D) and mIL-23p19(R&D) followed by chicken anti species Alexa Fluor® 488 secondary antibody. Wild type MDCK cells stained with same antibodies (A)and (B) show background.

Figure 22: Western blot analysis of CYT-IVAC mFlt3L/HAl513, mIL-23/HAl513 or WIV.

Whole viral lysates were run on 12% SDS-PAGE gel, blotted on PVDF membrane and incubated with Flt3L (A) and IL-23 antibody (B) followed by antispecies secondary antibodies conjugated to HRP. Different dilutions of CYT-IVAC~Flt3L/HAl (A) were run on gel to validate dose dependent incorporation starting from 10µg to 0.25µg (A). Two different preps of CYT-IVAC~IL-23/HAl (lane 2, 3) (5µg) and PR8 (lane 1) (5µg) were run on gel in (B). HA incorporation was validated by probing CYT-IVACs with Anti HA (H1N1) antibody. Dose dependent incorporation of HA were measured on CYT-IVAC~Flt3L(A) and on PR8 and CYT-IVAC~IL-2, IL-12 and IL-23 (5µg) (C). Membrane was visualized under Chemidoc.
Figure 23: In vitro bioassay for Flt3L using OCI-AML5 cells (Flt3L indicator cell line).

OCI-AML5 cells were cultured with different doses of recombinant mouse Flt3L in triplicates or gradient purified inactivated influenza vaccine (WIV) with and without membrane bound Flt3L for 48 hours. Cell survival and proliferation was measured by Alamar blue assay. Data represents absorbance values at 570nm (measurement) and 600nm (reference).

Figure 24: Serum antiviral IgG antibody responses following booster vaccination in young mice

Serum was collected on day 35 post-booster vaccination and antibody titers for influenza virus specific IgG were determined by ELISA. Data is displayed as the mean ± SEM. (A)*p<0.05 compared to WIV (B) and *p<0.05 compared to PBS by One way ANOVA, Bonferroni’s multiple comparison test).
Mice vaccinated with either wild-type vaccine or CYT-IVACs were challenged with 100LD_{50} dose of mouse adapted A/PR/8/34 on day 36 following vaccination. At day 4 following challenge, animals (n=5 mice/group) were sacrificed, nasal washes were collected in PBS (2ml total volume) were collected and flash frozen. Quantitative levels of Influenza specific sIgA antibodies were determined by IgA ELISA with IgA standard curve extrapolation. Data represents mean concentration ±SE. (***(p<0.001 compared to WIV, One way ANOVA Bonferroni’s multiple comparison test).
Mice vaccinated with either wild-type vaccine or CYT-IVACs were challenged with 100LD_{50} dose of mouse adapted A/PR/8/34 on day 36 following vaccination. At day 4 following challenge, animals (N=5) were sacrificed, lung tissues were collected and flash frozen. Viral loads from homogenized lung tissue (N=5) were determined by tissue culture infectious dose assay. Data is expressed as TCID_{50} per gram of lung tissue. (*p<0.05, **p<0.01, ***p<0.001 compared to PBS, one way ANOVA, Bonferroni’s multiple comparison test.)
REFERENCES


Figure 27: Whole inactivated influenza virus vaccines boost influenza virus splenic T cell repertoire in mice.

Spleens were collected on Day 4 post challenge (n=5) and Splenocyte Proliferation assay was performed using Alamar Blue. Data is represented as a mean of difference in Absorbance at 570nm (measurement) and 600nm (reference) as an indicator of dye reduction and cell proliferation. (**p<0.001 compared to media treatment by Two way ANOVA.)
Chapter 4. Vaccine Efficacy of Multimeric M2e co-presented directly on the surface of inactivated influenza virus particles

Abstract

Influenza epidemics and pandemics have been a significant cause of human hospitalizations and death. Inactivated split or subunit flu vaccines are efficient in protecting against antigenically matched strains of influenza virus but fail to provide immunity to emerging new strains of influenza viruses, thus, requiring annual reformulation of strain specific vaccines. Immunity induced by conserved proteins is often broad and cross-protective. The small matrix protein, M2 is highly conserved among all influenza A subtypes. The ectodomain of M2, the M2e peptide is an attractive target for vaccine development due to its high conservation among virtually all influenza A viruses. To overcome the low immunogenicity of the M2e peptide alone, fusion peptides, carrier or multimeric formulations of the M2e peptide have been investigated. Here we describe, a novel method to incorporate a multimeric M2e peptide directly into whole inactivated vaccine and describe our initial “proof of concept” vaccination efforts. Multimeric M2e peptides were fused inframe to the membrane anchoring regions of the HA protein of influenza virus (mM2e/HA) and constitutively expressed on the surface of the virus permissive MDCK cell line, MDCK~mM2e/HA. Recombinant multimeric mM2e/HA was readily incorporated into progeny virions following influenza infection of MDCK cells and whole inactivated vaccine bearing mM2e/HA, WIV~mM2e/HA was subsequently evaluated for in vivo efficacy in young adult Balb/c mice. In vivo experiments in young Balb/c mice were performed to evaluate the protective efficacy of vaccine against different strains of influenza virus. Two doses of inactivated vaccine (WIV) with and without mM2e/HA administered intramuscularly (IM) and intranasally (IN) induced superior PR8 specific antibody responses and provided 100% protection against lethal homotypic influenza A/PR/8 challenge. In addition, higher influenza antigen specific IFNγ and IL-4 secreting T cell responses were found in splenocytes of WIV~M2e/HA IN group as compared to WIV IN following homotypic A/PR/8 challenge. Interestingly, WIV~M2e/HA IM vaccine provided enhanced protection from lethal challenge of heterotypic H1N1/09 virus as compared to WIV.
Introduction

Commercially available influenza vaccines are mainly based on the structural viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA). These vaccines provide protection mainly by inducing neutralizing antibodies against the hemagglutinin (HA) and neuraminidase (NA) proteins of the vaccine strain or antigenically matched circulating strains [1]. However, they typically do not induce broad cross-protective humoral and cell-mediated immunity against newly emerging strains of influenza virus with different subtypes of HA and NA. Moreover, continuous antigenic drift variants require annual reformulation of influenza vaccines as suggested by the World Health Organization based on the circulating strains that are prevalent during a given season [2]. An attractive approach to overcome this limitation is to design a vaccine that targets genetically conserved antigens common to multiple influenza strains and subtypes such as the nucleoprotein (NP), the matrix protein (M1), fusion peptide domains of hemagglutinin (HA) or the ectodomain of the M2 protein (M2e). Targeting these antigens provides protection against antigenically drifted viruses and induces strong long lasting heterosubtypic immunity [3-8]. Cross-protective, heterosubtypic immunity is also often elicited following natural infections [5, 6, 8], vaccination with inactivated whole virus particles [9], influenza virus-like particles [10], or as noted highly conserved antigens or adjuvanted antigen formulations [11, 12]. Inactivated whole influenza virus vaccine (WIV) has been shown to elicit robust anti-NP cytotoxic T cell responses [13]. Budimir et al. recently demonstrated that β-propiolactone inactivated WIV undergoes receptor mediated endocytosis in antigen presenting cells (e.g., dendritic cells) and following fusion viral antigens are cross-presented through the MHC class I pathway stimulating virus specific cytotoxic T cells [13]. Importantly, the latter studies revealed that membrane fusion activity of inactivated whole virions is highly dependent on the method of inactivation; formalin was found to disrupt the membrane fusion activity of the virus hemagglutinin [13]. β-propiolactone-inactivated WIV(H5N1) provided solid protection against heterosubtypic (H1N1) lethal challenge[14], which was mediated by influenza specific CTL activity. In contrast, split or subunit vaccines in the latter study did not protect mice from heterosubtypic challenge [14].

The ectodomain of the M2 protein and the fusion active subunit of HA (HA2) are potent candidates for heterosubtypic vaccines due to their highly conserved nature. The M2 protein of
influenza virus is highly conserved among type A influenza viruses and is an attractive target for broad spectrum vaccines [15]. The M2 protein, a type III integral membrane protein of influenza virus is a translation product of spliced mRNA from genomic segment7 [16]. It functions as a pH-dependent proton-selective ion channel [17-19] during the process of viral entry and uncoating inside the host cell. M2 is also involved in viral morphogenesis [20] and assembly especially its cytoplasmic tail domain [21]. Structurally the M2 protein, which is composed of 96 amino acids, can be divided into three domains: the amino terminal, non-glycosylated extracellular domain (M2e, a 23 amino acid polypeptide), the transmembrane domain (19 amino acids in length) and the long cytoplasmic tail domain (54 amino acid residues). The ectodomain of the M2 protein (M2e), is highly conserved [22] and its sequence has not changed considerably since the 1918 influenza pandemic despite numerous influenza epidemics and pandemics during the last century [23]. Its sequence is also conserved in all human influenza A viral strains. Recent studies have shown that the M2e peptide provides better heterotypic protection as compared to conserved fusion peptide of the HA protein [24]. It is noteworthy, that very few M2 molecules (23-60) are embedded in the virus particle itself [25], whereas it is highly expressed on the surface of infected cells at a ratio of approximately two M2 molecules per HA trimer in CV-1 cells [26]. Interestingly, anti-M2e monoclonal antibodies are very effective at inhibiting virus replication in cell culture [25] and reduce virus burden in the lungs when passively administered to infected animals [27]. However, the M2e peptide does not appear to be very immunogenic when presented in either current vaccine formulation or during natural infections, as M2e-specific antibodies are only present at very low concentrations in human serum [28-30]. Alternatively, access to the M2 protein may be masked by the HA and NA or its low copy number may not be sufficient to elicit humoral immune responses [31]. The highly conserved nature of M2 and the induction of protective antibodies makes M2 especially the ectodomain of M2 makes it an attractive target for a broad spectrum influenza vaccine. Numerous formulations have been applied to make the M2e peptide more immunogenic, these include, linking the M2e genetically or chemically to different carrier conjugates [11, 32-36], or fusion proteins [11, 12], presenting it on virus-like particles [12, 32, 37-43], expression via viral vectors [33], liposomal mixtures[44, 45], or co-administration with complex adjuvants such as toll like receptors ligands [46], or chitosan [47]. However, many of these formulations did not provide complete protection as clinical symptoms and significant
weight loss were frequently observed in immunized animals [35, 41, 46, 48, 49]. Other M2e vaccine formulations tested include: aM2e single peptide vaccine [50], synthetic multiple antigenic M2e peptides [51-53], DNA-based expression vectors [33, 54-56], baculovirus expressed M2 and passive monoclonal M2 antibody [57, 58]. Most of the M2e based animal studies have been conducted in either mouse or ferrets and focused on homotypic and heterotypic influenza virus challenge using both human and avian strains of virus; albeit with varying level of protection afforded. Apart from studying different M2e vaccine formulations, recent studies have also evaluated the route of immunization as a means to boost immunogenicity and efficacy [50]. All of these studies suggest that anti-M2e antibodies can be elicited to play a more active role in protection. The mechanism of protection mediated by M2e antibodies is still unclear and was originally postulated to involve antibody dependent cell cytotoxicity mediated by NK cells [59], but this was later disputed as NK cell depletion studies did not support this observation [48]. There are also findings supporting that anti-M2e antibodies act in a non-neutralizing fashion [45, 48]. Besides antibodies, there CD4T cell responses have been implicated in the anti-M2e mediated responses [58, 60].

The compilation of the available data suggests that increasing the number of presented epitopes or copies of M2e and co-administering them with adjuvants or antigen carriers will greatly improve the immunogenicity and immune responses targeting the M2e. In addition, increasing the M2e-epitope density may also provide superior protection against lethal influenza A viral challenge. Whole inactivated influenza viruses (WIV) are highly immunogenic influenza vaccine formulations that provide superior protection against lethal heterotypic viral challenge as compared to conventional inactivated split and subunit vaccine formulations [14]. Here, we describe our investigations of an alternative approach to utilize M2e in a vaccine formulation. Specifically, we postulated that an increase in the density of M2e expression on the surface of whole virus particles would serve to not only elicit high levels of anti-M2 antibody, but also serve to provide robust anti-viral humoral and T cell responses at the same time. An all in one influenza vaccine formulation that affords robust protection against homosubtypic and heterosubtypic influenza viruses mediated by induction of both humoral anti-M2, -HA and -NA antibodies as well as cellular immune responses to internal virus proteins. Our approach is based on the use of a fusion construct in which four copies of the M2e peptide are fused to the hemagglutinin short stalk, transmembrane and cytoplasmic domains. It was envisioned that the
hemagglutinin containing domains would lead to more active incorporation into budding virus particles as they leave the infected cell. Hence, this approach utilizes inactivated WIV that co-presents multimeric M2e on the surface of the particle at a higher density. As proof of concept, we describe our initial studies evaluating the \textit{in vivo} efficacy of inactivated WIV bearing multimeric M2e on the virion surface.

\textbf{Materials and Methods}

\textbf{Construction of mM2e/H\textit{A} construct}

Recombinant expression DNA plasmid encoding four copies of M2e (M2e(I-IV)) or multimeric M2e (mM2e) including M2e conserved consensus sequence followed by M2e sequence from three different strains of Influenza A virus were fused inframe to regions coding for short extracellular stalk domain, transmembrane (TM) and cytoplasmic tail (Cyt.) domains of hemagglutinin (HA) protein of influenza A (A/WSN/33) virus (Figure 28: \textit{Construct diagram of M2e(I-IV)/HA1513}).

NheI and BamHI restriction enzymes at 5’ and 3’ end respectively were used to clone M2e(I-IV) or mM2e region in pCDNA3.1 vector (Invitrogen) expressing HA1513 under pCMV promoter.

Therefore the entire construct consists of

5’- KpnI - BglII - Kozak sequence - mIL-15 Optimized signal sequence - 9 nucleotides-M2e (I) - M2e (II)–M2e (III) - M2e (IV) – HA1513-3’

\begin{verbatim}
GCCGGTACCAGATCTCACCTGACCTGGATCTCTGTCCTGTCGTCGGCGCGCCGC
CACACGGGTGACAGCAACTGGATCAATGTGATCTCCTCTGCTGACCCGAGGTGGAGA
CACCCATCCGCAACGAGTGGGCTGACAGGTGTAATGATAGCTCCGACAGTCTCTCA
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\end{verbatim}
Table 6. Amino acid sequence of mM2e/HA construct.

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<th>Sequence region</th>
<th>Peptide sequence</th>
<th>Nucleotide position on construct</th>
<th>Number of coding amino acid</th>
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Generation of M2e expressing cell line.

Positive clone of plasmids pCDNA3.1~mM2e/HA were used to transfect Madin Derby canine kidney cell lines (MDCK) using Lipofectamine 2000 (Invitrogen) in DMEM media without serum and antibiotic (Penicillin/Streptomycin) as described previously [61, 62]. pcDNA3.1
plasmid (Invitrogen) has a CMV promoter that drives the expression of gene insert and a neomycin resistance gene for selection of cells containing gene of interest. Cells were grown under the drug Geneticin® (1.4mg/ml; Gibco®), for the selection of positive cells and further enriched by subcloning by limiting dilution as described previously [61].

**Cell surface expression of membrane bound M2e (Immunofluorescence Microscopy)**

MDCK cells expressing membrane bound M2e (mM2e/HA) were seeded (2 x 10^5 cells) on glass coverslips in a 24-well tissue culture plate. Upon reaching confluency, monolayers were washed with PBS, fixed for 10 min in 3% paraformaldehyde (250mM HEPES), washed and unreactive groups quenched by incubation in 50mM glycine/PBS for 10 minutes. Coverslips were blocked for at least 30 minutes in 2% chicken serum prior to immunostaining. Cells were sequentially incubated with diluted primary goat anti M2e IgG antibody (1:100 in 2% chicken serum, Thermoscientific Pierce) for 20 minutes, and secondary chicken anti-mouse IgG conjugated Alexa Fluor®488 antibody (1:300, Invitrogen) for 20 minutes. Cells were washed in PBS and mounted on glass slides using ProLong Antifade (Invitrogen/Molecular Probes). Immunofluorescent staining was visualized using a Nikon E800 Epifluorescence Microscope.

**Generation of double gradient purified inactivated influenza A virus bearing mM2e/HA**

Vaccine producer cell lines expressing specific mM2e/HA were grown in 150mm tissue culture dishes (Corning), to 80% confluency and then infected with Influenza A A/PR/8/34 (H1N1) at a multiplicity of infection (MOI) of 1 for one hour adsorption at 37°C. Viral dilutions were aspirated off and cultured in maintenance media (DMEM with 2% fetal bovine serum). Viral supernatants from infected monolayers were harvested following 36-48 hour of infection and pre-cleared by centrifugation at 1500rpm for 15 minutes at 4°C. Viruses were concentrated by centrifugation at 88,000 x g for 45 minutes at 4°C in ultracentrifuge (Beckman Coulter). Viral pellets were resuspended in PBS (supplemented with calcium and magnesium) and laid over the top of 10%-26% iodixanol (Optiprep™, Axis-Schield) continuous gradients (55,000 x g for 45 minutes at 4°C). Viral zonal bands were collected from the center of gradient, washed in PBS by centrifugation (88,000 x g for 45 minutes at 4°C) and run over second 10%-26% iodixanol gradient. Double gradient purified virus was chemically inactivated by treating with 15mM β-
propiolactone (Acros Organics) for 30 minutes at 25°C followed by neutralization with sodium thiosulphate (40mM) for 15 minutes at 25°C. Inactivated virus was diluted in PBS and pelleted at 88,000 x g for 45 minutes at 4°C. Purified virus was re-suspended in PBS. Total protein concentration in the purified virus was determined by treating the virus with RIPA buffer (Tris (20mM), Triton X-100 (1%), SDS (0.1%), deoxycholate (0.5%), EDTA (1mM), Sodium chloride(150mM) for 10 minutes in ice and analyzed using bicinchoninic acid protein assay kit (Pierce Biotechnology Inc.). Viral inactivation was verified by culturing double gradient purified virus (5µg diluted in PBS) on confluent MDCK cells in a 96 well plate for 72 hours at 37°C in the presence of DMEM with TPCK (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone) treated trypsin (1.5µg/ml; Sigma-Aldrich) and monitored for the development of any cytopathic effects (CPE).

**Validation of mM2e/HA incorporation in gradient-purified inactivated vaccine formulations**

mM2e/HA incorporation on purified, inactivated vaccine was validated by western blot analysis of whole viral lysates. Vaccines were diluted in 2xlaemelli buffer (Bio-Rad) with β-mercaptoethanol (Bio-Rad) and heated at 100°C for 10 minutes. Viral proteins were separated on 12% SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by transfer onto PVDF membrane (Bio-Rad). Membranes were blocked in block solution (5% skim milk in PBST) for 2 hours shaking at 25°C followed by three washes in TBST (TBS with 0.05% Tween 20) and probed with anti mouse Anti-influenza A M2 monoclonal antibody (14c2) (Abcam®) (1:1000) overnight shaking at 4°C. Membranes were washed and incubated with secondary goat anti-mouse IgG horseradishperoxidase antibody (1:5000; Bio-Rad) for 1hr with shaking. Membranes were washed and exposed to Pierce ECL western blotting substrate or super signal west Femto chemiluminescent substrate (Pierce) as per manufacturer’s instructions and visualized using Chemidoc XRS (Bio-Rad).

**Hemagglutination assay**

To determine hemagglutination units (HAU) in the purified M2e and wild type PR8 vaccine, Hemagglutination assay was performed on chicken red blood cells. Serial two fold dilutions of vaccine preps were prepared in PBS in 96 well V bottom plate starting from 2µg in 50µl volume. PBS alone served as negative control. Equal volume of fresh 0.5% chicken red blood cells was
added to all wells and incubated at room temperature for agglutination of red blood cells for 30 minutes. Reciprocal of the last dilution showing agglutination is HAU per µg of total viral protein.

**Animals and Vaccination**

All animal procedures were performed based on the NIH guidelines and approval of Institutional Animal Care and Use Committee of Virginia Tech (IACUC) of Virginia Polytechnic Institute and State University. 6-8 week old female Balb/c mice (*Mus musculus*) were purchased from Charles River Laboratories (CRL) and were allowed to acclimate for at least 10 days prior to vaccination. Animals were anaesthetized using Ketamine (75mg/kg BW) and Xylazine (7.5mg/kg BW) anaesthesia with double gradient purified whole inactivated virus (WIV~A/PR/8/34) vaccine (n=36) or WIV~M2e/HA (n=36 mice/group) vaccines (0.75µg/mouse in 100µl volume of PBS) intramuscularly (IM) in right hind quarter or intranasally (IN) (7µg/mouse in 50µl volume of PBS). PBS IM (n=36 mice/group) served as mock. Animals were administered with same dose of booster vaccines on day 28 post primary vaccination, using same route of administration as the primary vaccines. On day 21 and 45 post primary vaccination, sera was collected and evaluated for influenza specific total IgG antibody titers prebooster vaccination and post boost vaccination respectively. On day 65 post vaccination, four mice from each group (n=4 mice/group) were sacrificed using pentobarbital sodium (100µl/mouse; Intraperitoneally i.p.) to evaluate IgG and secretory IgA antibody responses in nasal wash and BAL fluids following two vaccinations (prior to challenge). Rest of the mice (n=32 mice/group) were used for challenge studies.

**Challenge experiments**

**Homologous challenge**

To determine protection from the homologous influenza A (A/PR/8/34) virus, at day 74 and day 102 post-primary vaccination mice from IM group (n=12 mice/group) and IN group (n=12 mice/group) respectively, were challenged with 1000TCID50 (100LD50) of mouse-adapted A/PR/8/34 intranasally under Ketamine/Xylazine anaesthesia. Some mice (n=6 mice/group) were sacrificed at day 4 following challenge and rest of the mice (n=6 mice/group) were left for survival. At day 4 following challenge, Bronchoalveolarlavage fluid (BAL), nasal washes and
lungs were collected and flash frozen for further use. Serum samples were collected via cardiac puncture. Spleens were collected and processed for cellular assays. Percent weight loss, sickness and survival for rest of the mice was monitored over a period of 14 days following challenge.

**Heterologous challenge.**

A/California/07/2009 (H1N1) To determine long term cross-protection, on day 119 (~3.75 month) and day 221(~7 month) post primary vaccination, vaccinated mice from IN group (n=6 mice/group) and IM group (n=6 mice/group) respectively, were challenged with A/California/07/2009 (H1N1/09) virus (1000 TCID50/50µl volume of PBS) intranasally under Ketamine/Xylazine anaesthesia. Percent weight loss and survival were monitored for a period of 14 days.

**Enzyme Linked Immunosorbent Assay (ELISA)**

Quantitative levels of Influenza specific total IgG subtype in serum were determined by enzyme-linked immunosorbant assay, ELISA as described previously [61]. Briefly, 96-well immunosorbent ELISA plates (Nunc 475094) were coated with 100 HAU/ml of gradient purified inactivated influenza virus (100µl/well) in ELISA coating buffer (sodium carbonate 150mM, sodium bicarbonate 350mM, sodium azide 130mM). Plates were spun at 4000 rpm for 15 minutes and stored overnight at 4°C. Coated plates were washed once with PBS-T (phosphate buffered saline with 0.05% Tween 20) and blocked with blocking buffer (2% BSA in PBST) at100µl/well for 2 hours at room temperature, washed three times. Serum samples were diluted 1:100 in blocking solution, added 100µl/well in duplicates and incubated with shaking overnight at 4°C. Bronchoalveolar lavage (BAL) fluids and nasal washes used were diluted 1:5 in blocking solution for IgA and IgG antibodies and added 100µl/well in duplicates. Plates were washed three times and incubated with secondary anti mouse IgA or IgG-Horseradish peroxidase (SouthernBiotech) (1:4000) in blocking solution for 1.5 hours shaking at room temperature. Plates were washed three times and incubated with substrate (2,2’-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid; Sigma) (50µl/well) in dark for 30 minutes at RT. Reaction was stopped by the addition of equal volume of 1% sodium dodecyl sulfate (SDS). Absorbance was measured at 405nm using plate reader (SpectraFluor plus, Tecan). OD values were plotted against known standard curve to evaluate concentration of influenza specific IgG or IgA per milliliter of serum.
Quantitation of viral loads in lungs

At day four following challenge, mice were sacrificed and lung tissues were minced and collected aseptically in 0.5ml DMEM media. Lungs were weighed, flash frozen in liquid nitrogen and stored at -80°C. Frozen lung samples were thawed on ice, homogenized and cell debris removed by pelleting at 6000rpm for 10 minutes. The supernatants were collected and the volume was adjusted to 1ml total volume with DMEM. Serial 10-fold dilutions of lung homogenates prepared in PBS were incubated with confluent MDCK cells in a 96-well tissue culture plate for 1hr adsorption at 37°C. Viral supernatants were aspirated off and plates were cultured for 72 hour at 37°C for the development of cytopathic effects (CPE) in the presence of DMEM with L-(tosylamido-2-phenyl) ethyl chloromethylketone TPCK- treated trypsin (1.5µg/ml; Sigma). Cytopathic effects were recorded and 50% tissue culture infectious dose units (TCID₅₀/ml) were determined by the Reed-Muench method [63]. TCID₅₀/ml was further divided by individual lung weights to obtain TCID₅₀/gram of lung tissue.

Virus Microneutralization Titers

Serum samples collected following booster vaccination were heat inactivated at 56°C for 30 minutes. Serial ten-fold dilutions of sera were made in PBS in 50µl total volumes in a sterile 96 well round bottom plate starting from 1:10 to 1:5120 for A/PR/8/34 microneutralization and 1:10 to 1:80 for A/California/07/09(H1N1/09) and A/Aichi/2/68 (X31) (HKx31) viruses. Serum dilutions were mixed with 100TCID₅₀/50µl (2000TCID₅₀/ml) of live influenza A virus strains such as A/PR/8/34 or A/California/07/09 or A/Aichi/2/68(x31) in 96 well round bottom plate and incubated for 1hour at room temperature on shaker and then added on washed confluent MDCK cells in a 96 well tissue culture plate. Plate was incubated for 1hour adsorption at 37°C, followed by aspiration of mixture and cultured in DMEM media with TPCK treated trypsin at 37°C for 72 hours for the development of cytopathic effects. Microneutralization titer is the reciprocal of the highest dilution at which virus infection or cytopathic effects are blocked. Data is plotted as the reciprocal of highest dilution for individual mice.

T cell assays

At day four following challenge, mice were sacrificed and spleens were minced and collected aseptically in 0.5ml DMEM media ELISPOT plates (Millipore, Cat. No. MAIPS4510) were coated with capture antibodies for mIFNγ (BD Bioscience, 551881), mIL-4 (ebioscience, 88-
7844) overnight at 4°C according to manufacturer’s instructions. Freshly isolated splenocytes were cultured at 4x10^6 cells/ml with 10µg/ml of inactivated purified A/PR8 antigen or VSV antigen or MHC Class I/II peptide pool (20µg/ml) or M2e peptide (20µg/ml) in triplicates and incubated at 37°C for 24-36 hours. Media alone and ConA/PMA/Ionomycin (at 20µg/100ng/1µg per ml respectively) served as negative and positive control respectively. Plates were washed according to manufacturer’s instructions and incubated with detection antibody for 2 hours at room temperature followed by washing and incubation for one hour with Streptavidin-HRP (BD™ ELISPOT 557630) for IFNγ ELISPOT and Avidin-HRP (ebioscience) for, mIL-4 ELISPOT respectively. Plates were washed and incubated with 100µl of AEC Substrate/well (BD™ ELISPOT 551951) for 60 minutes in dark and reaction was stopped by DI water. Spots were read on AID ELISPOT plate reader (AID EliSpot). For splenocyte proliferation 4x10^6 cells/ml were cultured with same antigens in 96 well tissue culture plate at 37°C for 72 hours and Alamar blue ® dye was added in last 18 hours of incubation. Absorbance readings were taken at 570nm wavelength and 600nm reference after 19 hours of addition using Tecan plate reader.

Statistics
The significance of differences between vaccine and control group was assessed using GraphPad Instat version 3.0a for Macintosh (GraphPad Software). ELISA and Microneutralization titers were analyzed by One-way Analysis of Variance (ANOVA) using Bonferroni’s or Tukey-Kramer Multiple Comparison Test. Comparison of mucosal immune responses post booster vaccination was analyzed by Kruskal-Wallis Test (Nonparametric ANOVA) using Dunn’s multiple comparison test.

Results
Establishment of an MDCK cell line constitutively expressing membrane-bound mM2e/HA at the cell surface.
Since M2e is a short peptide and weakly immunogenic, we theorized that four copies of M2e along with membrane anchoring regions of the hemagglutinin (HA) protein will make it a larger more immunogenic macromolecule, particularly when co-presented in the context of the whole virus particle. This approach capitalizes on our success at incorporating immunomodulators
directly on the influenza virion in a membrane-bound form fused to the transmembrane spanning and cytoplasmic tail domain of the viral hemagglutinin [61]. The latter allows the fusion construct when stably and constitutively expressed on the cell surface of virus permissive cells to be incorporated into the budding progeny virions during the infection process. Using this same approach, we created a fusion construct in which a tetrameric M2e peptide was fused to the transmembrane spanning and cytoplasmic domains of the influenza A virus hemagglutinin (A/WSN/33). An optimized signal sequence from IL-15 gene was included at the N terminus to facilitate entry into the exocytotic secretory pathway that ultimately leads to expression of the multimeric M2e/HA (mM2e/HA) protein on the surface [64].

The tetrameric M2e domain includes one epitope of the M2e consensus sequence (SLLTEVETPIRNEWGCRCNDSSD) that is conserved in most human influenza A viruses of H1, H2 or H3 subtypes [12, 65], as well as M2e variant peptides derived from other influenza strains including A/Hongkong/156/97 (H5N1) [33], A/FM/1/47 and the recent A/California/14/2009 (H1N1/09) virus (Table 5). Influenza A/FM/1/47 (FM) was associated with an influenza outbreak in Fort Mammoth New Jersey in 1947 and its virulent mouse adapted (A/FM/1/47-MA) variant is used in many mouse models [66, 67]. The M2e peptide from influenza A/FM/1/47 (FM) deviates from the M2e consensus sequence at 3 sites (Error! Reference not found.). Influenza A/California/14/2009 (H1N1/09) is representative of the 2009 pandemic virus, whose M2e peptide differs from human strains at 4 amino acid residues [68]. Influenza A/Hong Kong/256/97 has >99% identity with avian H5N1 virus but its M2e sequence deviates from the M2e consensus sequence at four sites [69] and was included to provide protection from H5 subtype viruses. Hence, our mM2e/HA construct is designed to induce maximal anti-M2e humoral responses that are broadly reactive and protective against various subtypes of influenza.

In order to facilitate incorporation into influenza particles, the mM2e/HA fusion construct was first stably expressed in MDCK cells, an influenza virus permissive cell line. Stable transfectants constitutively expressing the mM2e/HA fusion construct were established following plasmid transfection and drug selection (G418, Geneticin). Positive surface expression of the mM2e/HA fusion protein was verified by indirect immunofluorescent staining using the M2e specific monoclonal antibody, 14C2. As depicted in (Figure 30A), positive surface expression of
mM2e/HA was readily visualized by immunofluorescence staining, whereas, control MDCK cells did not display positive staining with the 14C2 antibody (Figure 30B). High expressing subclones were obtained by limiting dilution subcloning and used to propagate the multimeric M2e/HA whole virus vaccine (WIV~M2e/HA). The expression of the mM2e/HA fusion protein was also verified by western blot analysis of mM2e/HA cell lysates (Figure 31A). The mM2e/HA fusion protein, which was estimated to have a molecular weight of 21kDa migrated as a doublet with molecular weight of approximately 30-34kDa. This may be reflective of differential glycosylation of the multimeric M2e fusion protein.

Validation of M2e incorporation into influenza virus particles and vaccine inactivation

We were able to obtain MDCK transfectants expressing the mM2e/HA fusion construct at the cell surface. Next it was important to determine whether the mM2e/HA, following virus infection could be recognized by the virus maturation and assembly complex and incorporated into budding virions. To this end, MDCK cells stably expressing M2e/HA were infected with influenza A/PR/8/34, virus was harvested from the supernatants, purified by gradient ultracentrifugation and inactivated with β-propiolactone as described previously [61]. This resulted in a vaccine formulation comprised of inactivated, whole virus particles that co-present mM2e/HA on the particle surface (WIV~M2e/HA). Control WIV was derived from A/PR/8-infected control MDCK cells that did not express the fusion construct, WIV. Viral inactivation was verified by culturing the WIVs (5µg total viral protein) in MDCK cells for 3-5 days and monitoring the development of live virus-induced cytopathic effects (CPE). No cytopathic effects (CPE) were observed in MDCK cells following culture with WIVs in the presence of TPCK-trypsin, thus, verifying viral inactivation of the vaccines.

Validation of M2e incorporation into virions was confirmed by western blot analysis of gradient purified, inactivated WIVs using M2e specific monoclonal antibodies (Figure 31B). The WIV co-presenting the multimeric M2e/HA fusion protein (WIV~M2e/HA) revealed 14C2 positive bands of approximately 37kDa and 15kDa molecular weight. The latter band (doublet) was also present in the control WIV and represents the full-length M2 protein encoded by the virus. The 37kDa band representing mM2e/HA may reflect differential glycosylation of the fusion construct. It is important to note that we also confirmed that incorporation of, full-length viral
glycoproteins HA and NA was not compromised using this vaccine approach. HA incorporation was monitored western blot analysis of WIV~M2e/HA using HA specific antibody (Figure 31C). In addition, the WIV bearing mM2e/HA as well as the control WIV displayed equal hemagglutination units (512HAU/µg of vaccine), confirming that full-length HA was present at equal levels. Prime/Boost Vaccination with a high dose of WIV elicits robust and sustained influenza specific antibody levels.

**Prime/Boost Vaccination with a high dose of WIV elicits robust and sustained influenza specific antibody levels**

To evaluate whether the co-presentation of mM2e/HA on whole inactivated vaccine potentiates or reduces anti-viral antibody responses, young adult naïve Balb/c mice were vaccinated intramuscularly or intranasally with WIV (A/PR/8), WIV~M2e/HA or PBS (sham). Mice received a booster dose of vaccine administered by the same route on day 28 post-vaccination. Sera were collected on day 45 (post-boost sera) post-vaccination from all animals to determine the influenza specific humoral antibody responses. High levels of influenza specific IgG antibodies were detected in the sera following booster vaccination across all groups of vaccines both intramuscular groups and intranasal groups (Figure 32A). Using this dosing regimen, both WIV and WIV~M2e/HA resulted in comparable seroconversion rates (100%) and high antibody levels in the sera. The PBS group (Sham) did not show any detectable antibody response to viral antigen. The levels of antibodies were increased and were maintained at high levels up today 234 (~7.5 months) post-vaccination, which was the designated endpoint of the study. These results suggest that two doses of WIV are sufficient to induce long-term humoral antibody responses when administered parenterally or intranasally (Figure 32B). Further, they confirm that the co-presentation of the M2e/HA does not interfere in the production of influenza specific antibody.

We next asked whether the antibodies following booster vaccination are neutralizing and sufficient to inhibit virus replication. Detection of virus neutralizing antibodies are considered a functional measure of humoral protective immunity, as these antibodies prevent infection and help clear infection from infected lungs [70, 71]. High neutralization titers were found against A/PR/8/34 virus in all vaccine groups with an overall higher mean in WIV (IM and IN) groups.
than WIV-M2e/HA group (~1:700-1:1100) (Figure 33A). PBS control group did not induce any detectable level of neutralizing antibody. In contrast, neutralizing antibodies against heterologous A/Aichi/2/68, X-31 (HKx31) and heterosubtypic A/California/07/09 (H1N1/09) virus were found to be low with mean values <1:40 dilution (Figure 33B and Figure 33C). This confirms to previous M2e based studies which suggest that elicited M2e antibodies are non-neutralizing [36, 59, 72].

High dose of WIV and WIV-M2e/HA PR8 provide complete protection against lethal challenge of A/PR/8

Protective efficacy of any viral vaccine is tested by challenging the vaccinated animals with the virus. Here, mice were challenged on day 74 (~2.5 months) post vaccination with a high lethal dose (100LD$_{50}$) of the homologous mouse-adapted influenza A/PR/8/34 virus. On day 4 following challenge, 6 mice from each group were sacrificed to determine infectious viral titers and mucosal antibody responses in nasal washes, bronchoalveolar lavage fluids (BAL) and lungs. Remaining mice were monitored daily for percent reduction in the body weights and survival following challenge for a period of 15 days. All of the animals in PBS sham group became sick (lost weight rapidly) following challenge and died by day 5 (Figure 34A and Figure 34B). In this group, when some animals were sacrificed on day 4, we found they had high viral loads in BAL fluid and lungs at day 4 (1x10$^5$ -1.58x10$^6$ TCID$_{50}$/gram of lung tissue and 1x10$^3$ -3.7x10$^4$ TCID$_{50}$/ml of BAL) suggesting lack of protective immune responses that allowed virus to be replicated (Figure 35). As depicted in Figure 36, mucosal influenza specific sIgA and IgG antibody responses in nasal wash and BAL fluid (Figure 36), were absent in sham vaccinated mice. Thus, the high levels of infectious virus in the respiratory tract and the lack of mucosal antibody responses is a consequence of the lack of pre-existing memory responses. Hence, the sIgA and IgG responses in vaccinated animals are directly due to prior vaccination-elicited responses. Interestingly, the WIV-M2e/HA vaccine, when administered intramuscularly, was superior at inhibiting early clinical disease (weight loss) compared to its WIV control counterpart; animals vaccinated IM experienced a 10% weight loss during the first 2 days post-challenge with A/PR/8/34 (Figure 34A). Although the latter animals rapidly recovered, this may reflect differences in the quality of the humoral antibody responses
induced by the two vaccine formulations. Despite the discrepancies in body weights during the initial 2 days post-challenge, both WIV and WIV-M2e/HA vaccine groups were completely protected with 100% of the animals surviving (Figure 34B). Interestingly, we were unable to detect virus in the lungs and bronchoalveolar lavage fluids (BAL) in any of the vaccine groups (WIV IM, WIV-M2e/HA IM, WIV IN and WIV-M2e/HA IN) at day 4 following challenge (Figure 35). The detection limit of the TCID$_{50}$ assay was $1 \times 10^3$ TCID$_{50}$/gm of lung tissue. The lack of detectable virus replication in lungs of challenged mice reflects the induction of robust protective immune responses by both WIV and WIV-M2e/HA vaccines. Even though PR8 neutralizing antibodies in serum following vaccination were higher in WIV vaccine group as compared to WIV-M2e/HA, the solid protection afforded by WIV-M2e/HA vaccine indicates that it is not just the serum neutralizing antibodies that are involved in providing protection from challenge.

Mucosal secretory IgA antibodies function to prevent initial virus infection in the upper respiratory tract while IgG antibodies help in treating virus infection and limit spread [73, 74]. IgA levels in mucosal tissues were determined at 4 days post-challenge; a time point when only pre-existing responses should be elevated or activated. As expected only the intranasal vaccinated animals had high antiviral sIgA levels in the nasal wash and BAL fluid (Figure 36). Low levels of anti-viral IgG were also detected in the nasal washes of these animals. Likewise, parenteral (I.M.) vaccination led to anti-viral IgG levels in the BAL fluid, but not in the nasal wash fluid. Overall intranasally vaccinated animals had higher levels of sIgA and IgG antibodies in nasal wash and especially high in BAL fluid with WIV IN leading than WIV-M2e/HA. Mean IgG levels were higher than sIgA in both nasal washes and BAL fluid. Higher IgG levels in BAL fluid indicate serum IgG transfusion into lungs. We also observed mucosal responses prior to challenge in some animals and found overall similar trend with no significant differences in sIgA antibody responses and somewhat lower levels of IgG in BAL fluid as compared to following infection (Supplemental Error! Reference source not found.).

**Intranasal whole inactivated virus vaccines induce T cell responses in spleens**

To determine cellular responses elicited by WIV-M2e/HA, we collected spleens from mice of intranasal group challenged with A/PR/8/34 at day 4 following challenge. We observed that both vaccine groups led to significant induction of IFN$_{\gamma}$ cellular responses particularly influenza
antigen specific and MHC ClassI/Class II peptide pool specific (Figure 37A). However, we did not observe anti M2e peptide specific T cell responses. IL-4 responses were lower than IFN\(\gamma\) although overall trend of IL-4 secretion was slightly higher in WIV~M2e/HA as compared to WIV IN (Figure 37B). We also observed splenocyte proliferation in response to influenza antigen or MHC ClassI/Class II peptide pool or M2e peptide in both vaccine groups although there were no statistical differences in between them (Figure 37C). This suggests both WIV and WIV~M2e/HA IN induced the development of influenza specific T cell repertoire in spleen of mice.

**WIV~M2e/HA IM provides better protection against heterologous H1N1/09 challenge**

After observing complete protection from homologous virus challenge, we also wanted to investigate the cross-protective ability of our WIV~M2e/HA vaccine, for which we challenged mice (n=6 mice/group) with heterologous H1N1/09 virus (1000 TCID\(50/\)mouse; 2 LD\(50\)) on day 119 (~3.75 month) post-vaccination (IN group) and day 221 (~7 months) (IM group) post-vaccination.

Figure 38 shows the combined challenge data. Mice were monitored daily for clinical signs of disease, including weight loss (Figure 38A) and survival for up to 14 days (Figure 38B). All animals displayed initial weight loss indicating that the level of protection afforded was not sufficient to inhibit the onset of clinical disease as was observed for homologous challenge. Mice from intranasal group both WIV and WIV~M2e/HA did not lose much weight as compared to intramuscular group which showed sharp drop in body weights starting from day one following challenge (Figure 38A). Both WIV and WIV~M2e/HA provided complete protection from lethal H1N1/09 challenge when administered by the intranasal route (Figure 38B). All mice in the PBS control group succumbed to infection and had to be euthanized upon reaching endpoints. Interestingly in the parenteral group (IM), 33% (2 out of 6) of the animals vaccinated with WIV~M2e/HA formulation survived lethal challenge, whereas none of
the control WIV (without M2e) vaccinated animals survived when challenged 7 months following primary vaccination. As noted earlier, both vaccines elicited very low (<1:40) neutralizing antibodies against H1N1/09 virus (Figure 33) hence the complete protection afforded by the intranasal administered vaccines indicates the cross-protective responses are mediated via other mechanisms.

**Discussion**

Current trivalent inactivated and live attenuated influenza vaccines afford sufficient protection from antigenically matched circulating strains of influenza viruses in most age groups. However, due to point mutations in the viral genomes, the epitopes or antigenic regions of hemagglutinin (HA) and neuraminidase (NA) undergo continuous mutations resulting in antigenically drifted strains [75, 76]. Immunization often does not provide complete protection from these mutated strains, which necessitates annual reformulation of influenza vaccines.

Formalin inactivated whole virus influenza vaccines WIV were the first influenza vaccines developed in the US in the 1930’s [77]. The vaccine was administered parenterally and induced systemic antiviral antibody responses and afforded protection. Interestingly, in the 1940s, it was noted that in some individuals parenteral immunization of WIV afforded protection even with low systemic influenza antibody levels [78]. This correlated with other virus inactivating substances later identified as sIgA [79]. During the 1960’s, WIV was discontinued due to its reported high reactogenicity and adverse side effects observed in children [80, 81]. This led to the advent of inactivated split or subunit vaccines which are less reactogenic [82]. However, comparative studies of whole inactivated influenza vaccines WIV with other formulations such as inactivated split or subunit or virosomal influenza vaccines have found superior responses of WIV [82, 83]. In humans, WIVs are found to be more immunogenic in naïve individuals due to the induction of systemic antibody responses and priming for cytotoxic T cell response [84]. The immune sera from mice immunized with UV inactivated whole virus vaccine, protects naïve mice from lethal influenza viral challenge. They also induce higher hemagglutination-inhibition titers and virus neutralizing antibodies than virosome or subunit vaccine when administered parenterally in mice [83, 85-89]. This confirms findings in our study, two high doses of WIV intramuscularly or intranasally induced seroconversion with high antiviral antibodies in sera following booster vaccination.
Figure 32) which were highly neutralizing against homotypic strain (A/PR/8/34) of virus (Figure 33). Besides systemic IgGs, we also found antiviral mucosal antibodies in intranasal WIV group. Two doses were completely protective against highly lethal homotypic viral challenge.

Interestingly, some studies report heterosubtypic immunity induced by whole inactivated influenza vaccines administered intranasally [90, 91] or when co-administered with adjuvants [92, 93]. UV inactivated whole influenza virus vaccine induces potent antibody response mainly against surface glycoproteins HA and NA and very low amounts against M2eA antigen [44, 94]. Therefore, a combination of whole inactivated vaccine with other target antigens or potent immune stimulators are needed to further boost the effectiveness and immunogenicity of whole inactivated influenza vaccines especially in terms of protection from the heterologous strains of influenza viruses. One emerging strategy is the use of conserved antigens that are conserved across most strains of influenza virus and are not readily susceptible to antigenic shifts and antigenic drifts such as M2 protein especially its ectodomain which is highly conserved across different epidemic and pandemic strains of influenza viruses and is a highly regarded target for influenza vaccine development. Studies have shown that four copies of the M2e consensus sequence fused to flagellin, a ligand for toll like receptor 5 [STF2.4xM2e] [46], or fused to glutathione-S-transferase as a single molecule formulation [34] resulted in higher antibody levels and improved protection, suggesting that high densities of M2e epitopes may be required for optimal induction of protective immune responses. In the present study, we investigated the potential of membrane anchored multimeric M2e on the surface of inactivated influenza virus particle. We hypothesized that anchoring M2e arising from three different strains of influenza viruses plus conserved M2e on the surface of inactivated influenza virus will serve as conserved antigenic target and will provide better heterosubtypic immunity. Membrane anchored M2e on the surface of VLP supplemented along with inactivated influenza vaccines have been used in past that provided complete cross-protection against multiple subtypes of influenza viruses [95]. M2e was readily incorporated from infected MDCK cell lines expressing multimeric M2e/HA (Figure 30 and Figure 31). Two doses of WIV M2e/HA vaccine administered intramuscularly or intranasally induced protective antibodies in serum (Figure 32) and provided complete protection from homologous lethal PR8 challenge (Figure 34). This confirms previous studies of hepatitis B virus core protein (HBVc) antigen carrier linked
with multiple M2e sequences in tandem that provided complete protection from challenge [32, 40]. M2e based vaccines mediate protection mainly through the induction of protective antibodies. Mucosal administration of WIV or WIV-M2e/HA induced the development of IgA and IgG antibodies in nasal washes and BAL fluid which were higher than intramuscular vaccines (Figure 36). As demonstrated in humans, IN group is effective in inducing mucosal IgA responses than IM vaccine [96-98]. Mucosal antibodies provide better protection from influenza infection than systemic antibodies [99-101]. The improved response of mucosal vaccination is because they closely mimic the natural infection. These local secretory IgA antibodies produced in the respiratory tract provide cross-protection against multiple influenza virus subtypes. To evaluate the long term cross protective efficacy of our WIV-M2e/HA vaccine, mice were challenged with heterologous H1N1/09 virus 7 months following vaccination and found complete protection in intranasal group and superior protection afforded by WIV-M2e/HA IM vaccine as compared to WIV IM highlighting its potential in inducing cross protective immunity (Figure 38).

Therefore, this study demonstrates that prime-boost vaccination of WIV-M2e/HA vaccine in young mice induced strong long term homologous immunity and long-term cross protective immunity against heterologous influenza viral strains. This confirms previous mice studies that have shown that whole intranasally administered inactivated vaccines induced cross-protective immunity [9, 90, 91, 102]. Several M2 vaccines have shown cross-reactive protective immune responses in animal models that reduced severity of disease mainly by M2e antibodies [31, 44]. However, here in this study we were unable to detect anti-M2e antibodies in serum using M2e peptide ELISA and Western blots. This may be due to membrane anchored mM2e/HA protein in low copies as compared to HA molecules on the surface of virions and M2e may be sequestered under HA trimeric molecules leaving to unaccessible to immune system. It is possible that M2e may be opening up sites within the HA for recognition by B cells especially the conserved HA2 region that are recognized by B cells for generation of cross-protective anti-HA2 antibodies. This region is normally masked by membrane distal HA head region. Our mM2e/HA fusion construct includes small region of stalk domain of HA which is known to induce broadly cross-reactive
antibodies [103]. Recent studies of immunization of mice with headless HA elicited broadly cross-reactive antibodies and provided protection upon lethal challenge [104].

Besides antibodies, we cannot rule out the possibility of memory T cell responses in controlling infection. We found strong influenza PR8 antigen specific splenic IFN\(\gamma\) responses in both WIV and WIV~M2e/HA IN at day 4 following challenge. IL-4 responses were slightly high in WIV~M2e/HA IN vaccine as compared to WIV IN (Figure 37). These were recall memory T cell responses elicited by our vaccines. Moreover, it has been found that protective heterosubtypic immune responses are mediated by robust T cell responses, including both CD8+ cytotoxic T cell (Tc) [105, 106] and CD4+ T cells [8]. In previous study, \(\beta\)-propiolactone-inactivated WIV (H5N1) provided solid protection against heterosubtypic (H1N1) lethal challenge [14], which was mediated by specific CTL activity. In contrast split or subunit vaccines did not protect mice from heterosubtypic challenge [14]. Cytotoxic T cell (CTL) mediated immunity plays a crucial role in clearance of virus following infection and recovery [106-109]. Here, our vaccines are also \(\beta\)-propiolactone-inactivated and we found following lethal PR8 challenge a sharp drop in body weights in WIV IM group as compared to WIV~M2e/HA group. This weight loss may be indicative of early memory T cell responses at day 2 induced by WIV~M2e/HA vaccination. Moreover intranasally administered WIV or WIV~M2e/HA provided complete protection from H1N1/09 challenge indicating the superior efficacy of intranasal whole inactivated vaccine. T cell responses to M2 protein has been observed previously [58] and immunization of mice with M2 DNA followed by M2-Ad DNA induced development of M2 peptide specific T cells that play role in M2 mediated protection from challenge [33].

Figure 28: Construct diagram of M2e(I-IV)/HA1513

Nucleotides encoding for four copies of M2e peptide arising from four different influenza A viruses were fused and designated as domain (I-IV). This M2e(I-IV) gene construct was fused with regions encoding membrane anchoring
regions of hemagglutinin (HA) of influenza A (A/WSN/33) virus including HA stalk, transmembrane (TM) and cytoplasmic tail (Cyt.) domain by standard PCR and recombinant DNA technologies.

Figure 29: Plan of immunization and challenge studies

Figure 30: Validation of M2e expression on the surface of transfected MDCK cells.

Indirect immunofluorescent staining on MDCK cells stably transfected with plasmid M2e(I-IV)/HA1513-pCDNA3.1 (A). Paraformaldehyde fixed cells were probed for surface expression of M2e using primary antibodies against M2e(14c2) followed by chicken anti mouse Alexa Fluor® 488 secondary antibody. Wild type MDCK cells stained with same antibodies (B) show background.
Figure 31: Western blot analysis of mM2e/HA.

A/PR/8/34 harvested from the supernatants of infected MDCK or MDCK-M2e/HA was gradient purified. MDCK cell lysates with and without M2e/HA expression (A) and purified whole viral lysates (B) AND (C) was separated by SDS-PAGE. MDCK and MDCK mM2e/HA cells were lysed in lysis buffer with β-propiolactone and boiled for 10 minutes. Western Blot analysis was performed probing for M2e/HA using anti M2e monoclonal antibody (14c2) and goat anti mouse IgG HRP secondary antibody. Western blot analysis was performed probing for HA incorporation using anti HA antibody and donkey anti mouse IRDye800 secondary antibody.
Young Balb/c mice were vaccinated intramuscularly (I.M.) or intranasally (I.N.) with 0.75 µg and 7µg respectively, of wild type inactivated influenza WIV vaccine (n=36 mice/group) or WIV-M2e/HA vaccine (n=36 mice/group). PBS I.M. (n=36 mice/group) served as negative control. Sera (n=11) was collected on day 45 (1.5 month) (A) and day 234(n=7-13) (7.5 month) (B) post vaccination, diluted 1:100 in ELISA blocking buffer and IgG antibody titers for Influenza A/PR8/32 were determined by ELISA. Data is displayed as blank reduced absorbance values (405nm) for each group. (**p<0.001 compared to PBS IM group, One way ANOVA, Bonferroni’s multiple comparison test).
Figure 33: Microneutralizing antibodies against different influenza strains following booster vaccination

Sera collected on day 45 post vaccination (n=6 mice/group) was heat inactivated at 56°C for 30 minutes, serially 10 fold diluted and incubated with 100TCID$_{50}$ of A/PR/8/34 (A), A/California/07/09 (B) or A/Aichi/68X31 (C) for an hour on room temperature shaker. Serum-virus mixtures were incubated with MDCK cells for 1 hour and subsequently cultured for 3 days in DMEM media supplemented with TPCK treated Trypsin (1.5 µg/ml). Data is displayed as microneutralization titer as the serum dilution that is still able to neutralize and inhibit virus induced cytopathic effect. Note that a serum dilution of 1:5 is considered the lowest detectable dilution at which no neutralization (CPE) was observed. (*p<0.05, **p<0.01 compared to PBS IM group, One way ANOVA, Bonferroni’s multiple comparison test).

Figure 34: Two high dose of WIVs completely protect mice from lethal homotypic challenge.

Vaccinated mice were challenged with 100 LD$_{50}$ of mouse adapted A/PR/8/34 on day 73 post first vaccination. Challenge mice (n=6 mice/group) were monitored for weight loss (A) and percent survival (B) over a period of 14 days.
Vaccinated mice were challenged with 100 LD$_{50}$ of mouse adapted A/PR/8/34 on day 73 post first vaccination. Challenged mice (n=6 mice/group) were sacrificed on day four following challenge and lungs and bronchoalveolar lavage fluids were collected. Lung tissues were homogenized (A), bronchoalveolar lavage fluid (B) were spun down and viral titers were determined by tissue culture infectious dose assay (calculated by Reed and Muench formula). Data represents TCID$_{50}$/gram of lung tissue (A) and TCID$_{50}$/ml of BALF respectively.
Figure 36: Mucosal immune responses induced following lethal A/PR/8/34 challenge.

Vaccinated mice were challenged with 100LD$_{50}$ of mouse adapted A/PR/8/34 on day 73 post first vaccination. Challenged mice (n=6 mice/group) were sacrificed on day four following challenge and nasal wash and bronchoalveolar lavage (BAL) fluids were collected. Influenza A/PR/8 specific IgA antibodies in nasal washes (A) and bronchoalveolar fluids (B) were determined by IgA ELISA. Influenza A/PR/8 specific IgG antibodies in nasal washes (C) and bronchoalveolar fluids (D) were determined by IgG ELISA. Nasal washes and BAL fluids were diluted 1:5 in ELISA blocking buffer. Data is displayed as blank reduced absorbance values (405nm) for each group. (***p<0.001 compared to PBS IM group, One way ANOVA, Bonferroni multiple comparison test).
Figure 37: Cellular immune response following lethal A/PR/8 challenge.

Spleens from mice challenged with A/PR/8/34 on day 73 post-vaccination and sacrificed 4 days following challenge and subjected to ELISPOT and Proliferation assays. (A) and (B). IFNγ ELISPOT (A) and IL-4 ELISPOT (B) assay shows average number of IFNγ or IL-4 spots from each group of mice (n=6) stimulated with VSV antigen (10µg/ml) or PR8 antigen (10µg/ml) or MHC Class I/II peptide pool (20µg/ml) or M2e peptide (20µg/ml). Error bars indicate standard errors. (C) Splenocyte proliferation assay determined by Alamar blue® dye reduction. Data shows difference in absorbance values at 570nm and 600nm reference wavelength.
Figure 38: WIV–M2e/HA administered intramuscularly provide superior protection from heterologous H1N1/09 challenge.

Vaccinated mice (n=6 mice/group) were challenged with (1000 TCID50/mouse) A/California/14/09 (H1N1/09) virus on day 119 (~3.75 month) (IN group) and day 221 (approximately 7 months) (IM group) post primary vaccination. Mice were monitored daily for percent reduction in their body weights (A) and percent survival (B) following challenge for a period of 14 days. Data is combined in weight loss and percent survival for both IM and IN groups.
REFERENCES


Kutzler, M.A., et al., *Coimmunization with an optimized IL-15 plasmid results in enhanced function and longevity of CD8 T cells that are partially independent of CD4 T cell help*. Journal of Immunology, 2005. 175(1): p. 112-123.


Supplemental Figures

Figure 39: Mucosal immune responses induced following booster vaccination

Vaccinated mice (n=4 mice/group) were sacrificed on day 65 following primary vaccination and nasal wash and bronchoalveolar lavage fluids were collected. Influenza A/PR/8 specific IgA antibodies in nasal washes (A) and bronchoalveolar fluids (B) were determined by IgA ELISA. Influenza A/PR/8 specific IgG antibodies in nasal washes (C) and bronchoalveolar fluids (D) were determined by IgG ELISA. Nasal washes and BALF were diluted 1:5 in ELISA blocking buffer. Data is displayed as blank reduced absorbance values (405nm) for each group. (**p<0.01 compared to PBS IM group, Kruskal-Wallis, Dunn’s multiple comparison test).
Chapter 5. Conclusions

Vaccination remains the most effective means for protection against influenza. Current inactivated vaccines are quite successful, affording protection in 70-90% of healthy adults [1-4], whereas they have suboptimal efficacy ranging from 39%-69% in the elderly depending on the year evaluated (reviewed in [5-8]). In addition, they do not typically elicit broad cross-protective immunity against emerging antigenically different strains of influenza viruses. The vulnerability of the elderly to influenza-associated co-morbidities predicates a significant need to focus research efforts on the development of a more efficacious vaccine for the elderly population. In primed individuals whole inactivated vaccines, split and subunit vaccines elicit similar immune responses [9]. Interestingly, whole inactivated influenza vaccines (WIV) appear to be more immunogenic when compared to subunit or split virus vaccines in antigen naïve, unprimed individuals [9-11]. Currently available inactivated split/subunit influenza vaccines induce predominantly Th2 type immune responses [7], with only marginal development of sustained Th1 type responses; both responses are thought to be a prerequisite for balanced immune responses that provide superior protection against infection and subsequent serious complications [12]. Interestingly, whole inactivated vaccines shift the balance towards a favored Th1-type immune response through co-activation of the TLR7 signaling pathway [7, 13].

Based on these findings, we and others have theorized that the use of novel adjuvants in combination with whole inactivated vaccines will further serve to modulate and enhance the immunogenicity of WIVs and reduce the effective dose as well as the overall vaccine production costs. Many research studies have adopted the use of chemokines and cytokines to enhance the immunogenicity of antigens, administered either in the linked form or in close proximity to antigen [14-16]. Here, we have used mammalian cell culture methodologies to generate whole inactivated influenza vaccines (WIV) that co-present membrane-anchored cytokines or a multimeric M2e construct directly on the virion surface. This method allowed us to express the bioactive immunomodulators in direct context with viral antigen. This approach can be applied to virtually any immunomodulator of interest by fusing the immunomodulator coding regions to the membrane-anchoring regions of either the viral hemagglutinin or neuraminidase glycoproteins of influenza A virus. This allows for presentation of the immunomodulator or
foreign peptide as either a type I or type II membrane protein, depending on the orientation of the bioactive site of the immunomodulator. We opted to anchor the immunomodulator as a fusion with the HA or NA transmembrane/cytoplasmic tail domains to maximize incorporation of our fusion constructs into budding virions. This takes advantage of the specificity of the viral budding machinery that recognizes and interacts with the cytoplasmic portions of the glycoproteins during the assembly and budding process. Of note, this approach can also be applied to any enveloped virus or virus-like particles vaccine platform. In fact, ongoing studies in our laboratory have successfully incorporated membrane-bound cytokines into Ebola and Marburgvirus based VLPs, which further highlight the utility of this vaccine platform (personal communication from Dr. Paul C. Roberts). Using the CYT-IVAC platform, we have generated CYT-IVACs co-presenting membrane-anchored murine interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 12 (IL-12), interleukin 23 (IL-23) and FMS-like tyrosine kinase 3 ligand (Flt3L). These immunomodulators were chosen in part due to their reported success in modulating anti-tumor immune responses (19-25). We also tested whether this vaccine platform could be exploited to co-present a multimeric, membrane-bound formulation of the ectodomain of the M2 protein, the M2e peptide. The latter, a so-called “universal vaccine” target [17] is important due to its ability to provide cross-protective responses against antigenically divergent strains of influenza viruses. The M2e peptide has been investigated using a plethora of formulations including fusion proteins, virus-like particles, liposomes, carrier conjugates [18-23]; all of which generate antibody-mediated immunity that is broadly cross-protective against different influenza viral strains [17]. This represents the first report of its co-presentation as a multimeric, membrane-bound fusion peptide directly incorporated into a WIV formulation. The latter also highlights the applicability of this approach to express virtually any multimeric epitope of influenza (e.g. HA fusion peptide or T cell epitopes) as well as foreign peptides. Future studies combining the M2e WIV formulation with our CYT-IVAC approach may induce more robust cross-protective responses. Particularly, an IL-12 or IL-23 CYT-IVAC co-presenting the multimeric M2e fusion peptide may provide strong cross-protective mucosal responses.

To evaluate the adjuvant potential of membrane-bound cytokines as immune modulators co-presenting with WIVs (our CYT-IVAC formulations), we conducted three vaccine efficacy studies in BALB/c mice. Importantly, we included a study in “aged” mice to assess whether our CYT-IVACs could overcome aged-associated dysfunctional responses to vaccination.
In our young adult efficacy study, we evaluated not only whether our CYT-IVACs enhanced anti-viral antibody responses, but also determined whether the route of administration influenced the type of protective responses elicited. In young adult animals, we observed strong influenza specific mucosal IgA immune responses in nasal washes when CYT-IVACs bearing IL-12 or IL-23 were administered by the intranasal route. IL-12 has been used as a mucosal adjuvant inducing elevated mucosal sIgA responses when administered in a soluble formulation [24, 25]. Importantly, the elevated influenza-specific sIgA responses elicited upon intranasal CYT-IVAC\textsuperscript{IL12/HA} and CYT-IVAC\textsuperscript{IL23/HA} administration correlated directly with protection as evidenced by the reduced viral lung loads determined at d4 post-challenge. Hence, our study is the first to demonstrate that membrane-bound IL-12 and IL-23 co-presented directly on influenza WIVs serve as potent mucosal adjuvants. Notably, our data also infers that inclusion of these mucosal adjuvants elicits protective immunity at lower doses than required by control non-adjuvanted WIV. Hence, our CYT-IVAC approach may provide a means to provide protection against influenza using lower doses of viral antigen. More importantly, this study may be highly relevant as a vaccine platform targeting the elderly, where immunity is often waning and vaccination is only marginally effective or requires extremely large antigen dosing to achieve protection. Future studies evaluating the efficacy of CYT-IVACs bearing IL-12 and IL-23 administered by the intranasal route are clearly warranted. Our data is also in line with recent studies evaluating SIV vaccines that demonstrate the superior immunogenicity and protective efficacy of mucosal or intranasal vaccines versus parenteral administered vaccines [26].

Interestingly, we found that the parenteral administration (IM) of our CYT-IVAC\textsuperscript{IL-12/HA} and CYT-IVAC\textsuperscript{IL-23/HA} formulations induced elevated sIgA antibodies. This was not intuitive and based on the literature is mostly unprecedented. Of note, the sIgA antibodies induced by IM vaccination, though elevated, were qualitatively inferior compared to those elicited by intranasal immunization. In general, WIVs do not lead to IgA responses when administered IM [27]. However, there are reports of transcutaneous immunization inducing mucosal IgA responses which is thought to involve the migration of skin dendritic cells to regional lymph nodes where they prime B and T cells in a manner that results to their homing to mucosal sites [28, 29]. Our CYT-IVAC\textsuperscript{Flt3L/HA} was the most efficient vaccine formulation at eliciting high levels of antiviral antibodies when administered by the parenteral IM route, yet failed to elicit mucosal antibodies when administered IN. Flt3L dependent responses at mucosal sites may require higher doses to
be effective. In support of the latter, several studies using plasmid-based delivery of Flt3L to nasal mucosa or an adenovirus expressing Flt3L had to employ multiple dosing regimens to achieve responses [30, 31], whereas in our study we used only 3 and 0.1 µg doses of vaccine administered as a prime/boost regimen, respectively. In addition, there is one report in which no significant increases in mucosal IgA responses were observed upon administration of 50µg Flt3L-plasmid with 100 µg OVA [32]. Our study did lack sufficient animal numbers to accurately assess in a statistical fashion the efficacy of the CYT-IVAC\textsuperscript{Flt3L/HA}. However, as a “proof of concept” study, these results provide invaluable insights into how distinct immunomodulators can be used to influence immune responses in a site specific manner.

In animals 17 months of age, “aged animals”, we decided to only evaluate the efficacy of our CYT-IVAC formulations using the intramuscular route administration, since this is currently the route of administration approved for the elderly using the Fluzone® High-Dose vaccine. Here, we found that all whole inactivated vaccines (WIV) with or without cytokine induced high levels of influenza-specific IgG; predominately of the IgG2a isotype. Interestingly the CYT-IVAC\textsuperscript{IL-12/HA} was the most effective at inducing IgG2a immune responses in our ‘aged” study, which correlates well with the role of IL-12 in upregulation of Ig-isotype switching as described previously [33]. In agreement with previous reports [34-36], our results highlight the ability of WIVs to skew the immune response towards a Th1-type response that is characterized by high IgG2a antibody levels and protection against lethal homotypic challenge. Our data also suggests that co-presentation of membrane-bound IL-12 has the potential of expanding these responses. Inclusion of the IL-12 may offer the added advantage of eliciting robust responses at lower vaccine doses, without compromising protection against challenge. The latter is supported by our single dose study, in which only the IL-12 bearing CYT-IVAC was able to provide any enhanced protective responses when compared to non-adjuvanted WIV. Of note, the CYT-IVAC\textsuperscript{IL12/HA} also increased the number of inclined Granzyme B and IFN\(\gamma\) producing T cells in lungs following lethal challenge. WIVs regardless of the presence of the cytokines led to predominant IFN\(\gamma\) cellular responses followed by IL-4 and IL-2 splenocyte responses, with virus specific IL17 responses being undetectable. Interestingly, the CYT-IVAC formulation bearing IL-4/HA elicited the most robust antiviral IFN\(\gamma\) responses in splenocytes; albeit this did not translate to
protection, as immunized animals all succumbed to challenge. This is of interest, since it suggests that there are distinct age-associated differences in the responses elicited by our CYT-IVAC formulations. Previous studies in our laboratory indicated that a single low dose of the CYT-IVAC\(^{\text{IL4/HA}}\) was superior at providing protection in young adult mice compared to control WIV or CYT-IVAC\(^{\text{IL2/HA}}\). The lack of efficacy in “aged” mice likely reflects non-responsiveness to IL-4 stimulation during initial priming of the immune response, but this will require further studies. We observed high levels of antiviral antibodies in all vaccine groups irrespective of immunomodulator using the prime/boost regimen. This was likely due to high dose of vaccine that made it difficult to fully evaluate the contribution of the immunomodulators co-presenting on the CYT-IVACs. Future studies are needed to evaluate a minimum immunomodulatory dose that will provide protection in aged animals while still maintaining antibody responses and/or cellular responses that are protective.

Together our data suggests that cytokines co-presented in a membrane-bound formulation in direct context with WIVs can, depending on the route of administration and the immunomodulator serve as robust adjuvants that expand WIV elicited immune responses. Additional studies investigating the mechanistic basis of cytokine adjuvants particularly regarding the elicited T cell responses are clearly warranted to optimize both the formulation and the route of administration. Combinatorial use of our CYT-IVAC approach utilizing multiple membrane-bound cytokines or with other adjuvants should also be explored including MF59, monophosphoryl lipid A (MPL). Our results also suggest that the CYT-IVAC formulation requires considerably less cytokine (pg) compared to the use of soluble cytokines (100-500 ng) co-administered with vaccine antigens [25, 37]. Additional studies will be needed to determine the minimal immunomodulatory dose required to achieve maximal antiviral protective responses in both young and aged mice. Our data further highlights that CYT-IVAC formulations may have to be tailored to distinct age groups to overcome age-associated non-responsiveness to vaccination. Potential toxic or autoimmune effects will also have to be addressed prior to utilization of the CYT-IVAC technology in humans. The low dose of immunomodulator employed may be insufficient to stimulate autoimmune responses to the immunomodulator itself, however, this needs to be evaluated using high doses of vaccine (25-100 ug) to rule out adverse side effects. Human-derived immunomodulators will also have to be employed when CYT-IVACs are tested in non-human primates or progress to clinical testing. The strong mucosal
adjuvant potential of IL-12 and IL-23 highlights their potential for further study and development. Future studies should evaluate whether these formulations are also highly effective in the elderly when administered by the intranasal route. Alternative immunomodulators including IL-7, IL-15, Type I IFN, IL-18, and RANTES should also be actively investigated using the CYT-IVAC platform, since they may allow for a more targeted approach in primed individuals facilitating further stimulation of virus-specific immune memory responses.

To further develop an influenza vaccine that protects against different strains of influenza A viruses, we chose to use the ectodomain of M2 (M2e) protein due to its widely conserved consensus sequence and its reported induction of cross-protective immune responses [38, 39]. Here, we described a novel method to incorporate a multimeric, membrane-bound M2e fusion protein directly on whole inactivated influenza vaccine, WIV\(^{M2e(I-IV)/HA}\). This vaccine formulation was based on the inherent immunomodulatory advantages of the WIV, an increase in the copy number of the presented M2e peptide and co-presentation of the M2e in a more immunogenic multimeric format. Recently, M2e bearing virus-like particles (VLP) (10µg) administered with 2µg of inactivated virus (A/PR/8) vaccine intranasally afforded complete protection against challenge with both heterologous and heterosubtypic influenza viruses [40]. Here in our study, mice were administered 7µg dose WIV\(^{M2e(I-IV)/HA}\) or WIV intranasally or 0.75µg intramuscularly. Our study validates some of those observations, but also suggests that the inherent immunogenicity of the WIV is sufficient to confer complete protection against homotypic viral challenge, including protection against clinical manifestations of disease. The WIV\(^{M2e(I-IV)/HA}\) IM formulation provided enhanced protection against challenge with influenza A/California/07/09 (H1N1/09). Importantly, cross-protective responses elicited by whole inactivated vaccine were sustained for at least 7 to 8 months post-immunization. Again, this suggests that WIV formulations need to be revisited in the clinical setting. We did not determine whether cellular CD8+ T cells or CD4+ T cell specific responses [41, 42], contributed to the cross-protective responses, but it is highly likely due to the ability of ß-propiolactone WIV to induce cross-presentation of antigen inducing both robust CD4 and CD8 cellular responses. Although we did observe very high neutralizing antibody levels against the immunizing influenza A/PR/8 strain, neutralizing antibody levels were low to non-detectable against heterologous strains such as influenza A/California/07/09.
Despite the observed cross-protective responses, we were unable to detect significant antibody responses to the targeted M2e conserved region. This may be due to a flaw in our assay design, since we were evaluating whether serum antisera recognizes the monomeric M2e peptide in our ELISA assay. Antibodies may be only recognizing the intact M2e found on mature virions or on the surface of infected cells. However, we were able to detect specific M2e responses using western blot analysis, when we probed sera against a bacterial expressed multimeric form of the M2e peptide, but we were unable to reliably quantitate differences between the groups. 

Alternatively, the oligomeric nature of M2e(I-IV) fused to the HA may through conformational alterations mask epitopes, or the epitopes recognized are only conformational-specific antibodies that don’t recognize the denatured monomeric M2e peptide. It is also possible that HA trimers and/or NA tetramers were masking the multimeric M2e/HA fusion protein and muted the anti-M2e response. Interestingly, cross-protective antibody responses have also been elicited by using conserved domains within the HA stalk domain and the association of M2e(I-IV)/HA may serve to expose or unmask these sites on the virion surface. Recently, it was shown that vaccination of mice with VLPs bearing an HA that lacks the globular head domain provided protection against a lethal influenza virus challenge and induced broad cross-reactive antibody levels [43]. Our M2e fusion peptide may be working in an analogous fashion to disrupt trimer interactions exposing more epitopes within the HA stalk region. Finally, it is possible that our WIV formulation did not incorporate sufficient levels of the M2eHA fusion peptide to elicit specific M2e responses. Further work needs to be done, to boost efficacy of the WIV\textsuperscript{M2e/HA} formulation. Combinatorial use of the IL12 or IL23 bearing CYT-IVACs may provide a means to boost anti-M2e mucosal responses.

In this study, we found long term maintenance of influenza antigen specific IgG antibodies in serum measured around 7 months following vaccination with WIV or WIV\textsuperscript{M2e/HA} irrespective of route of immunization. This indicates induction of long-lived plasma cells that maintain IgG levels in serum. In addition, this study highlights the long term cross-protective ability of WIV\textsuperscript{M2e/HA} which provided superior protection from heterologous A/California/07/09 virus challenge 8 months following primary vaccination. However, as noted, it is unclear whether this is due to anti-M2e specific responses or due to other non-neutralizing antibody responses such as those recognizing conserved epitopes within the influenza HA stalk domain.
To conclude, this dissertation has extended our knowledge on the potential of membrane-bound immunomodulators to serve as bioactive adjuvants on the surface of WIV. This study revealed the mucosal adjuvant potential of CYT-IVAC\textsuperscript{IL-12/HA} and CYT-IVAC\textsuperscript{IL-23/HA}, the potential of IL-12 bearing CYT-IVAC to overcome age-associated vaccine non-responsiveness, and the long term cross-protective ability of WIV\textsuperscript{M2e/HA}. This approach targets two urgent needs in the field of influenza vaccine development that includes a potent vaccine for elderly and a vaccine that provides cross-protection from antigenically drifted viruses. Future studies are needed to further assess the mucosal adjuvanticity of the CYT-IVAC\textsuperscript{IL-12/HA} and CYT-IVAC\textsuperscript{IL-23/HA} formulations in both young and aged animals. Further comparative studies of available adjuvants and our CYT-IVACs will provide more insights into what the adjuvants should be pursued in vaccine development. It would be interesting to do a combinatorial approach utilizing different cytokine constructs (CYT-IVACs) or CYT-IVAC\textsuperscript{IL-12/HA} and WIV\textsuperscript{M2e/HA} to augment immune responses that will serve to lower the doses. To further characterize the mechanisms elicited by CYT-IVAC\textsuperscript{IL-12/HA} and WIV\textsuperscript{M2e/HA}, it would be interesting to explore both humoral and cellular arms of immunity induced by our vaccines by adoptive transfer experiments. In addition, there is a need to characterize how our CYT-IVACs interact with players of innate immune system when administered \textit{in vivo} such as antigen presenting cells and their activation states.
REFERENCES


