Cytokine-bearing Influenza Vaccine: Adjuvant Potential of Membrane-bound Immunomodulators

Andrew Scott Herbert

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Paul Christopher Roberts	Committee Chair
Xiang-Jin Meng	Committee Member
Sharon G. Witonsky	Committee Member
Kevin Myles	Committee Member

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by

Andrew Scott Herbert

Paul Christopher Roberts, Committee Chair

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(Abstract)

Influenza epidemics continue to cause morbidity and mortality within the human population despite widespread vaccination efforts. This, along with the ominous threat of an avian influenza pandemic (H5N1), demonstrates the need for a much improved, more sophisticated influenza vaccine. Our group has developed an in vitro model system for producing a membrane-bound Cytokine-bearing Influenza Vaccine (CYT-IVAC). Numerous cytokines are involved in directing both innate and adaptive immunity and it is our goal to utilize the properties of individual cytokines and other immunomodulatory proteins to create a more immunogenic vaccine. Here we report methodologies for the construction of membrane-bound cytokine fusion constructs in which our cytokine of interest (mouse GM-CSF, mouse IL-2, mouse IL-4) was fused to the membrane anchoring regions of viral Hemagglutinin (HA). Progeny virions, produced from influenza infected MDCK cells expressing membrane-bound cytokines, readily incorporated membrane-bound cytokines during budding and these cytokines on the virus particles retained bioactivity following viral inactivation. In vivo vaccination studies in mice showed enhanced antibody titers and improved protection following lethal challenge in those mice vaccinated with IL-2 and IL-4-bearing CYT-IVAC's compared to the conventional wild-type vaccine without membrane-bound cytokines. In addition, the immune response induced by IL-2 and IL-4-bearing CYT-IVACs was skewed

toward T_h1 (cellular) mediated immunity compared to the T_h2 (humoral) dominated response induced with wild-type vaccination. Cellular mediated immunity afforded by IL-2 and IL-4 CYT-IVACs was manifested as enhanced influenza specific T cell proliferation and activation. In conclusion, we have developed a novel methodology to introduce bioactive membrane-bound cytokines directly into virus particles in order to augment the immunogenicity of inactivated, whole virus influenza vaccines.

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1. Introduction

Influenza epidemics continue to cause morbidity and mortality within the human population despite wide spread vaccination efforts. Yearly epidemics affect 5-20% of the population leading to 200,000 hospitalizations and 36,000 deaths annually in the United States [1]. The economic impact of influenza related illness costs the United States in upwards of $167 billion dollars per year [1]. Yearly epidemics occur as a result of mutations within the antigenic variable regions of the major viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [2, 3]. These antigenic drift events are the primary reason behind the need for yearly vaccinations [4, 5]. Pandemic influenza events occur when a new subtype of influenza, to which there is no pre-existing immunity, infects the human population and causes greater illness and death. This often occurs as a result of antigenic shift or gene reassortment. Since influenza viruses have segmented genomes, reassortment is an important mechanism for introducing diversity very rapidly; it primarily occurs among influenza A viruses in nature and is important for introducing pandemic strains to the human population [6]. During pandemic influenza events the number of afflicted individuals dramatically increases, compared to annual epidemics. This was best demonstrated by the influenza pandemic of 1918. It is estimated that the 1918 Flu pandemic resulted in 20 to 50 million deaths world wide with approximately 30% of the world population having been infected [7]. Aquatic birds serve as the natural reservoir for all subtypes of influenza and the next pandemic flu strain will likely evolve from those strains currently circulating among avian species. The gene pool of influenza A viruses necessary for the emergence of human pandemic influenza viruses are all maintained in aquatic wildfowl [6]. The recent emergence of Highly Pathogenic Avian Influenza (HPAI) H5N1 has raised new concerns regarding the origin of the next possible influenza pandemic strain and has challenged conventional wisdom regarding the need for a “mixing vessel” for the emergence of pandemic strains. The central dogma is that avian strains of influenza must first pass through a “mixing vessel” that permits simultaneous infections by both avian and human

influenza strains in order for avian strains to acquire the necessary characteristics for efficient human-to-human transmission [6, 8]. Pigs are thought to be this "mixing vessel" because receptors for both avian and human influenza strains are located in the trachea of pigs and furthermore, pigs are know to be infected with both human and avian strains of influenza [8-11]. Prior to 1997, it was thought that HPAI could not be transmitted from avian species to humans, directly. However, recent studies have documented how HPAI has been able to cross the avian-human species barrier and infect humans leading to disease and significant mortality (50%) without first going through a "mixing vessel" [12-14]. Furthermore, recent speculations of limited human-to-human transmission of H5N1 have heightened concerns that an H5N1 pandemic will occur [15, 16]. According to the World Health Organization, as of February 2009, a total of 404 human cases of avian H5N1 influenza had been confirmed resulting in 254 deaths in 15 countries across East Asia, Africa and the Middle East (fatality rate of 63%). While highly pathogenic H5N1 strains pose a significant threat for the next influenza pandemic, it should be noted that other avian influenza subtypes are known to infect humans and have pandemic potential. Documented cases of humans infected with H7N7, H7N3 and H9N2 have all been reported, although clinical symptoms associated with these infections were often mild and rarely fatal [17-20]. Still one more strain of influenza has the potential to cause a pandemic and some would argue that H2N2 subtypes pose the greatest threat for the emergence of the next great influenza pandemic event. This subtype was the cause of the "Asian flu" pandemic in 1957 but the last known cases of H2N2 infection were in 1968 after the emergence of the "Hong Kong flu" [21]. It is thought that anyone born after 1968 has no pre-existing immunity to H2N2 subtypes leaving them vulnerable to infection should these subtypes reemerge.

Influenza Life Cycle. Influenza viruses belong to the family *Orthomyxoviridae* and are subdivided into four genera, influenza A, B, C and thogotovirus. *Orthomyxoviridae* are enveloped viruses with a segmented single-strand RNA genome [22]. The RNA is of negative sense, meaning that viral messenger RNAs must first be transcribed from the viral RNA (vRNA) genomic

segments; vRNA is non-infectious. Influenza particles are spherical or pleomorphic, depending on the strain, and range from 80-120 nm in diameter with filamentous strains reaching several millimeters in length [23, 24]. Eight segments make up the genome of influenza A viruses encoding 11 viral proteins [25]. Segments 1, 2, and 3 encode for PB2, PB1, and PA and these proteins complex to make up the viral polymerase. Segment 2 encodes a newly discovered protein, PB1-F2 that traffics to the mitochondria and signals cell death [26]. Segments 4 and 6 encode for the major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) respectively. HA and NA are the major antigenic determinates and represent the means for classifying subtypes of influenza A viruses. Currently there are 16 known HA subtypes and 9 known NA subtypes. HA is responsible for binding sialic acid receptors on the host cell surface and mediates viral entry [27]. It is a type I transmembrane protein that functions as a homotrimer on the viral surface and it represents the major antigen of the virus against which neutralizing antibodies are produced [28]. NA has receptor destroying activity and is responsible for cleaving sialic acid from glycoproteins [29]. NA is a type II transmembrane protein that exists as a homotetramer on the viral surface. Segment 5 encodes the viral nucleoprotein that is responsible for binding and packaging viral RNA. This nucleoprotein and viral RNA complex is known as viral ribonucleoprotein (RNP). Segment 7 encodes 2 viral proteins, M1 and M2. M1 is the viral matrix protein that provides structural support and facilitates interaction between RNP and cytoplasmic tail domains of the viral glycoproteins during viral budding. M2 is an integral membrane protein with ion channel activity [30]. It functions to acidify the interior of the virus in the endosome by transporting protons to the interior of the virus particle [31]. Segment 8 encodes 2 viral proteins, NS1 and NS2. NS1 is a nonstructural protein that blocks host cell protein synthesis and inhibits the host cell innate immune response. NS2 is also a nonstructural protein that acts as a nuclear export protein to export viral RNPs with help from M1.

The course of infection begins with HA binding sialic acid containing receptors on the host cell surface. One important and fundamental difference between strains of influenza that infect avian species and those that infect humans

is their receptor specificity. Human cells of the respiratory mucosa primarily display sialic acid on glycoproteins with α2,6 linkages and these cells are preferentially infected by influenza strains that recognize α2,6 sialic acid linkages where as avian strains prefer α2,3 sialic acid linkages which are commonly found in the gut of avian species [32]. However, a subset of cells displaying α2,3 sialic acid linkages are known to populate the respiratory tract of humans and these cells are capable of being infected with avian strains of influenza, although viral replication and spread was shown to be limited [33-35]. Limited human-to-human transmission of avian influenza strains could be attributed to the scarcity and location of cells displaying α2,3 sialic acid linkages and the limited viral replication afforded by these cell types [33, 34, 36]. Receptor specificity is one characteristic that determines host range specificity and is one of the primary obstacles that must be overcome by avian influenza strains in order to efficiently infect humans. HA is cleaved into HA1 and HA2 by extracellular proteases prior to internalization. Cleavage of HA is critical as this event exposes the fusion peptide located within the HA protein that is required for fusion of viral and endosomal membranes [37]. This is a key event that must occur in order for the virus to be infectious. Influenza infections caused by epidemic human influenza strains are restricted to the lungs because extracellular proteases required for cleavage are present only in the respiratory tract, preventing the spread of the virus to other areas of the body. Restriction of infection to the respiratory tract is attributed to the nature of the cleavage site. Most influenza strains that infect humans have a single basic residue at the HA1/HA2 cleavage site and strains with this single basic cleavage site can only be cleaved by proteases located at the respiratory mucosa, thus restricting the spread of infection to the respiratory tract. However, the highly pathogenic avian influenza strains (H5N1) that have recently infected humans possess multiple basic residues at the cleavage site. These polybasic cleavage sites are cleaved by virtually any protease throughout the host, facilitating systemic spread of infection and exacerbating the severity of disease [38]. Receptor binding triggers receptor mediate endocytosis and the viral particle is internalized in an

endosomal vesicle [27, 39]. The vesicle acidifies and protons are quickly transported to the interior of the virus by the ion channel M2 [37]. Acidification of the virus interior causes the disassociation of viral RNP from M1 [37]. Meanwhile, the cleavage of HA and the low pH of the endosomal compartments exposes a fusion peptide that inserts into the endosomal membrane and facilitates fusion of the viral envelope with the endosomal envelope [40]. This fusion event allows for release of viral RNPs into the cytoplasm, which are then translocated to the nucleus [41]. Replication occurs in the nuclease of the host cell. The viral RNA-dependent-RNA-polymerase complex (PB1, PB2, and PA) produces viral mRNA for viral protein synthesis and copies the genome for packaging of progeny virions [42]. Newly synthesized glycoproteins HA, NA, and M2 are transported from the endoplasmic reticulum through the golgi network to accumulate at the apical

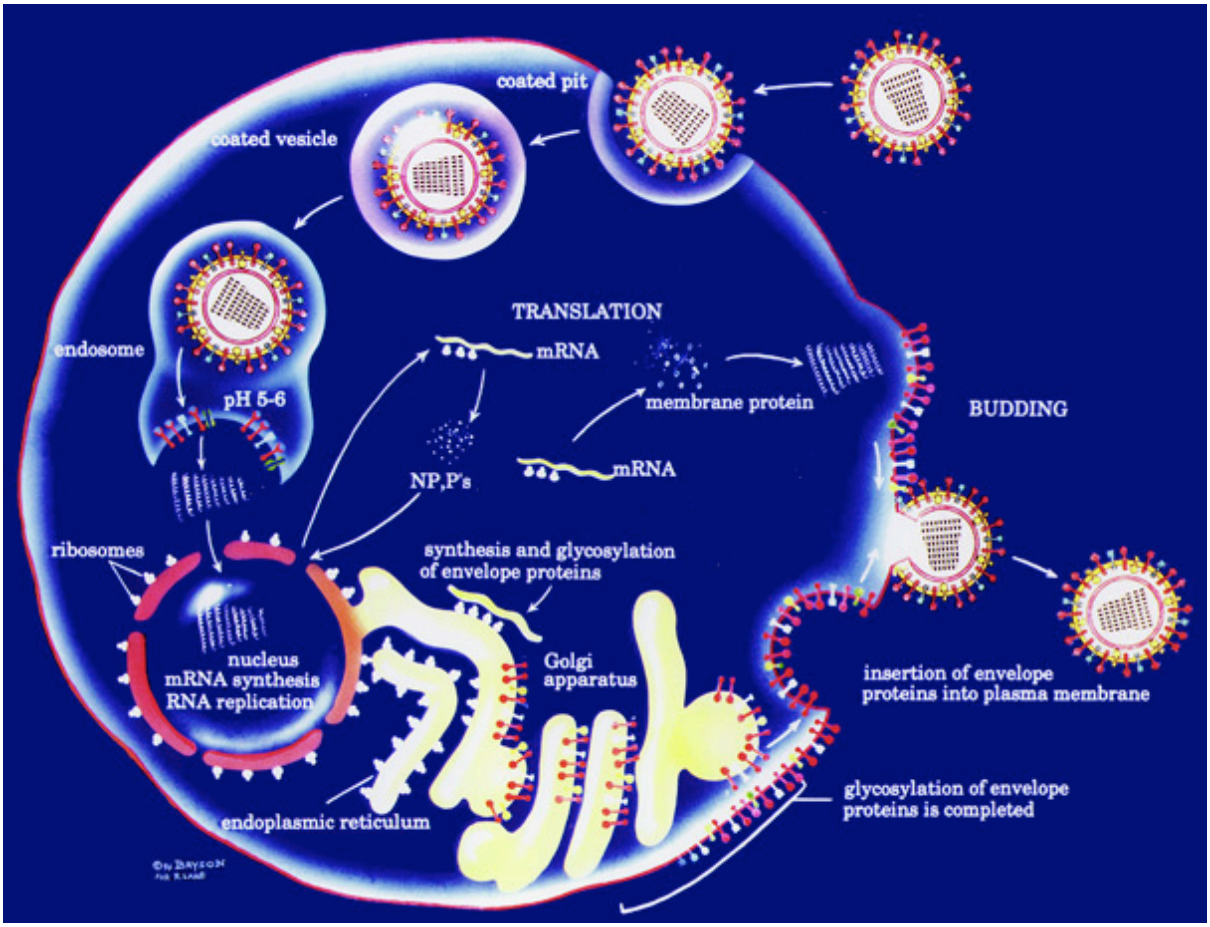


Figure 1. Influenza life cycle. (http://www.northwestern.edu/neurobiology/faculty/pinto2/pinto_flu.html)

surface of infected polarized epithelial cells [43-45]. Specifically, influenza viral glycoproteins preferentially associate with lipid raft regions within the host cell envelope and the raft associating residues are present in the transmembrane domain of HA and NA, although this has yet to be determined for M2 [46, 47]. Viral NP and M1 translocate to the nucleus where NP complexes with newly synthesized viral RNA to form RNP complexes [37, 48]. M1 associates with RNP in the nucleus and, with help from NS2, exits the nucleus and migrates to the apical surface [49-51]. M1, associated with RNP, binds the cytoplasmic tail domains of HA and NA at the apical surface and this interaction initiates viral budding [52-58]. The virus buds from the infected cell taking with it part of plasma membrane, which becomes the viral envelope. NA cleavage of sialic acid releases the progeny virion from the surface of the infected cell into the extracellular matrix [29, 59, 60]. The interaction between M1 and the cytoplasmic tail domain of the glycoproteins HA and NA (Figure 2, blue box) is instrumental in anchoring immunomodulators to progeny virions. Fusion of immunomodulators to the transmembrane and cytoplasmic tail domains of HA or NA facilitates viral incorporation of immunomodulators

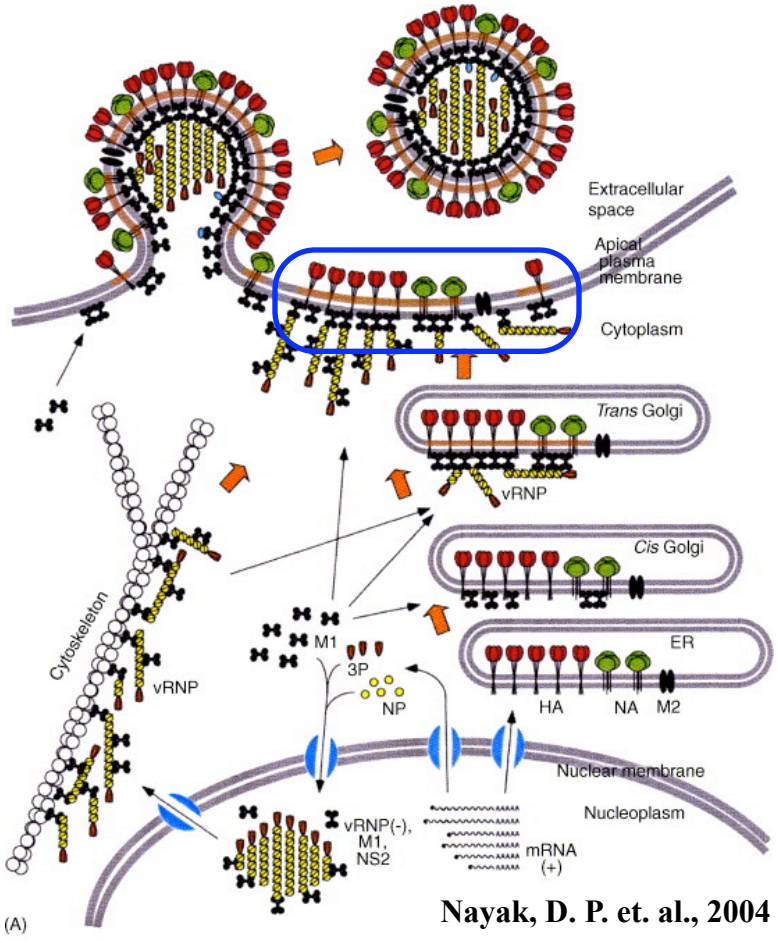


Figure 2. Influenza virus assembly at cell surface. Influenza matrix protien (M1) recognizes cytoplasmic tail domains of the viral hemaggutinin and neuraminidase (blue box). Binding of M1 to the cytoplasmic tail domains triggers budding of the virus from the infected cell, taking with it part of the host cell membrane that becomes the viral envelope.

through the association of M1 and the cytoplasmic tail domains of these chimeric proteins. This is because to the newly forming virion particle, the cytoplasmic tail domains of the fusion construct in no way differs from the cytoplasmic tail domain of the HA or NA produced during the course of infection. Therefore, budding viruses would have no way of distinguishing membrane-bound immunomodulators from HA or NA and would theoretically incorporate each at random.

Clinical Infection and Host Immune Response. Influenza viruses cause severe respiratory tract infections that are highly contagious and can be easily transmitted to naïve individuals. Influenza infects the respiratory epithelium and rapidly propagates to high titers 18-72 hours post infection [61]. Clinical signs involve both the upper and lower respiratory tracts and include nasal obstruction and discharge, sore throat, cough, breathing difficulties, fever, chills, headache, myalgias, malaise, and anorexia [61]. Healthy individuals generally recover from illness in 7-10 days with few long lasting complications. On occasion, influenza infection can lead to severe complications manifested as viral or bacterial pneumonia. Primary viral pneumonia occurs after a typical onset of influenza with rapid progression of dyspnea and cough leading to acute respiratory distress syndrome [62]. Secondary bacterial pneumonia occurs more frequently in the elderly and in patients with chronic pulmonary diseases [62]. The initial response to influenza infection, initiated by the innate immune system, is characterized by production of numerous cytokines and chemokines. Interferon production is initiated soon after infection and resident macrophages, infiltrating lymphocytes, and mucosal epithelial cells contribute to this production [63, 64]. In addition to interferon, macrophages secrete interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12), which activates natural killer (NK) cells [65]. NK cells in the lungs 48 hours post infection secrete interferon-γ and limit viral replication by virus-infected cell lysis [66-68]. The adaptive response to influenza infection is initiated by antigen presenting cells (APCs), primarily macrophages and dendritic cells. APCs present viral peptides on major histocompatability complex (MHC) molecules to T and B cells in the regional lymphnodes initiating clonal expansion of T helper cells (T_h0,1,2), cytotoxic T

lymphocytes (CTL) and B cells. Antibodies produced during the adaptive response, most importantly mucosal IgA, contribute to viral clearance by neutralizing virus through binding of the antibodies to viral antigens [69]. T_h1 cells contribute to viral clearance by secreting interferon-γ [70, 71]. CTL cells recognize viral infected cells by surveying MHC class I molecules and kill respiratory epithelial cells displaying viral peptides in the context of class I molecules [72, 73]. Naturally, an ideal influenza vaccine would induce an immune response that closely mimics the response initiated by a live infection without causing the clinical symptoms associated with a live influenza infection.

Current vaccination strategies against influenza. Immunization remains the most widely used prophylaxis against influenza infection. Two forms of influenza vaccines are commercially available in the United States, an inactive trivalent vaccine (TIV) that comes in three different formulations, and FluMist®, a live attenuated vaccine (LAIV). The inactive trivalent vaccine is composed of two strains of influenza A and one influenza B strain. The LAIV is also a trivalent vaccine composed of two strains of influenza A and one influenza B strain. This live vaccine is an attenuated, cold-adapted and temperature sensitive isolate developed to induce little or no flu-like symptoms and to eliminate lower respiratory tract replication [74]. Surveillance supported by the World Health Organization (WHO) helps to predict those strains that will most likely be circulating within the human population for a given year. Based on recommendations of the WHO, vaccine manufacturers alter the composition of yearly vaccines to best match circulating strains. The aforementioned vaccines are effective against epidemic strains of influenza, however each present their own unique inadequacies.

TIVs are effective at preventing influenza infection because they induce high antibody titers specific for influenza antigens, primarily hemagglutinin and neuraminidase. Vaccine manufacturers adjust the hemagglutinin and neuraminidase composition of yearly vaccines to antigenically match current circulating strains to provide optimal protection. For the influenza A components of TIVs, vaccine manufacturers use a “seed virus” comprised of the internal viral proteins of influenza A/PR/8/34 which allows for high-yield growth for maximal

vaccine production [75]. By using the PR/8/34 backbone and swapping in the hemagglutinin and neuraminidase of the current circulating strains, manufacturers can produce large amounts of vaccine with the best antigenic match [76, 77]. The high-yield influenza B component is obtained by serial passage of the currently circulating strain through embryonated eggs [77]. The humoral response elicited by TIVs is driven by T_h2 mediated immunity and is dominated by influenza specific IgG present in the serum of vaccinated individuals [78-82]. Mucosal antibodies, IgA and IgM play a minimal role in protecting those individuals vaccinated with TIVs, however IgA and IgM responses are advantageous for fighting any mucosal infection [81]. Neutralizing antibodies generated during vaccination are primarily responsible for limiting the spread of infection and it is widely accepted that an effective inactivated influenza vaccine is one that induces adequate neutralizing antibody titers. Because the T_h2 arm of adaptive immunity dominates immune responses to inactivated influenza vaccines, cellular immunity plays a minor role in protecting individuals from the onset of infection. This is problematic due to the fact that cytotoxic T lymphocytes are important for clearing influenza infections [83, 84]. TIVs are considered safe and effective for those individuals greater than 6 months of age, but they provide poor protection for the very young and elderly populations, two key demographics. Vaccine efficacy studies, in children 3-9 years of age, demonstrated a 56% efficacy rate and vaccine efficacy drops from 70-90% for healthy persons less than 65 years old to only 58% for persons greater than 65 years of age [74]. Immunity induced by TIVs provides poor protection against heterotypic challenge. The degree of protection after vaccination is dependent on the antigenic match between the vaccine strains and those strains circulating within the population [81]. As stated previously, HA and NA serve as the two major antigens to which immunity is acquired. Thus, these two proteins are under the greatest immunoselective pressure, therefore they mutate more rapidly (compared to internal viral proteins) leading to escape variants within the same subtype [85, 86]. Most neutralizing antibodies are directed at the highly mutated, antigenic variable regions within HA and NA and are specific for the variable regions of the vaccine strains. Antibodies generated during vaccination often do not recognize

these escape variants that have mutated their variable regions, allowing these strains to establish an active infection. This is the primary reason for the need to vaccinate against influenza on a yearly basis and protecting against these escape variants is a key focus for current influenza vaccine research.

The live attenuated influenza vaccine is administered intranasally where it mimics a live influenza infection without causing severe flu like symptoms. This vaccine is based on attenuated strains of influenza A and B developed by Hunein Maassab and Martin Bryant. The “Master” strains, influenza A/Ann Arbor/6/60-H2N2 and influenza B/Ann Arbor/1/66, were attenuated by adaptation of the virus for maximal growth at 25°C, i.e. cold-adapted, temperature sensitive [87]. Cold adapted virus growth is attenuated in vivo and replicates poorly in the lower respiratory tract lessening the severity of infection [88]. FluMist® is licensed by MedImmune and the yearly vaccine is manufactured using a reverse genetics system which creates a recombinant, live attenuated vaccine by combining 6 internal genes from the cold-adapted “Master” strain, or donor virus, with genes encoding the hemagglutinin and neuraminidase of currently circulating strains [89]. LAIVs stimulate influenza specific mucosal IgA and IgM as well as serum IgG. They also generate interferon-γ secretion and influenza specific cytotoxic T cells [81]. LAIV are 92% effective against antigenically similar strains of influenza and up to 86% effective when the vaccine and circulating strains are not an antigenic match [90]. While LAIVs provide great protection against viral infection, they are only approved for persons age 2-49 because of asthma concerns for young children and weakened immunity in the elderly population. Due to limited use of LAIV and weak immunogenicity of TIV, current vaccines fail to induce sufficient immune responses for the young and elderly population leaving them vulnerable to infection. Practical considerations for influenza vaccine development also have to be evaluated. Manufacturing capacities, the ability of candidate vaccine strains to grow well in eggs, and biological safety containment of parent strains for vaccine development are all concerns to be addressed [89].

Due to the inadequacies of the current inactivated vaccine and the limited use of the live attenuated vaccine, years of research have been dedicated to finding safe and effective adjuvants to augment the immunogenicity of inactivated influenza vaccines. Oil adjuvants, such as Freund's adjuvant, have long been known to boost immune responses to co-administered antigens [91]. Currently there are no licensed human influenza vaccines in the U.S.A which utilizing oil adjuvants because of concerns over adverse side effects. Recent studies have begun to evaluate other methods of boosting the immune response to influenza antigens by using adjuvants other than oils-based formulations. Flu-ISCOMs, an immune complex comprised of influenza antigen, cholesterol, lipid, and saponins, induced a better immune response based on their ability to provide homotypic protection following a single dose as well as provide heterotypic protection, where subvirion vaccines failed to elicit similar immunity [92]. Flu-ISCOMs have demonstrated protective immunity against avian influenza subtypes, even when vaccinated with an antigenicly different strain [93]. Other potent immunogens, such as cholera toxin B (CTB) subunit, have been evaluated for possible adjuvant properties when co-administered with influenza antigen. Intranasal immunization with the current vaccine, together with CTB, effectively provided cross-protection against variants within a subtype of influenza A viruses in the upper respiratory tract [94]. Adjuvants like alum, MF59, and Quil A have shown promising adjuvant like potential, albeit minimal enhancement of immunity [95]. Adjuvants have enormous potential to improve current influenza vaccines and may lead to an effective and practical vaccine against pandemic avian influenza strains.

Rationale for using cytokines/chemokines as adjuvants. Recently, investigators have begun to evaluate cytokines and chemokines for their ability to augment vaccine efficacy of numerous vaccine candidates. Cytokines have been investigated for their ability to enhance the efficacy of DNA vaccines, subunit vaccines, as well as whole cell/virus vaccines using both mucosal and systemic immunizations.

DNA vaccines employ a unique approach for vaccination by using host machinery to produce the desired antigen encoded within a plasmid to which the

host immune system can then mount a response. These plasmids are most often administered intramuscularly but have been shown to be effective when given intranasally, although it is generally thought that the best immune response is generated when DNA vaccines are administered systemically as opposed to mucosally. Because DNA vaccines are known to be less immunogenic compared to subunit or whole cell or whole virus vaccines, the DNA vaccine field has investigated cytokines and chemokines for their ability to boost the immune response to DNA encoded antigens [96]. Co-administration of plasmids encoding HIV antigens and plasmids encoding IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induced high levels of HIV-specific CTLs and an increase in delayed type hypersensitivity (DHT) when administered intranasally [97]. Plasmid derived interleukin-21 (IL-21) and interleukin-15 (IL-15), co-administered with plasmid encoding for HIV env protein, enhanced CD8+ T cell function, antibody dependent cellular cytotoxicity, and complement-dependent lysis of Env-expressing target cells [98]. Co-inoculation with a vector expressing GM-CSF enhanced the protective immunity against a PrV infection. This immunity was caused by the induction of increased humoral and cellular immunity in response to PrV antigen [99]. Plasmid encoded cytokine enhancement of influenza DNA vaccines has been evaluated. Mice that received either the IL-12 or the IL-6 gene had enhanced NP-specific CTL responses and co-administration of the IL-6 gene completely protected mice from a lethal challenge with influenza virus [100]. Co-injection of plasmid encoding a non-immunogenic mutant of influenza A NP(o) with GM-CSF and/or IL-12-expressing vectors restored near native NP-specific CTL responses [101]. Mice co-immunized with IL-12 encoding plasmid and plasmid encoded influenza HA antigen maintained a greater memory response than those immunized with the plasmid-encoded antigen alone which could be measured at least 6 months after vaccination [102]. Ongoing research continues to uncover the beneficial role cytokines play in enhancing immune responses to DNA vaccines [103-109].

Evaluations of cytokines and chemokines as potent adjuvants for peptide, subunit, and whole cell or whole virus vaccines have been promising. Kang et. al.

demonstrated that RANTES was able to boost immune responses to co-administered SIV virus-like particles in a T_h1 oriented manner as indicated by increased levels of gamma interferon producing lymphocyte and cytotoxic T-lymphocyte activities in both spleen and lymph nodes [110]. They also showed that RANTES was more effective than cholera toxin at increasing neutralizing titers of both serum and vaginal antibodies [110]. Co-injection of antigen with recombinant cytokine or chemokine proteins have been shown to be beneficial, however two major problems arise when utilizing this technique, (1) dispersion of the protein from the site of administration and (2) the short half life of the protein [111]. It has been suggested that immunomodulators would be better able to elicit their effect if they could be maintained in close proximity to antigens and remain in their bioactive state for a longer period of time [112-119]. To address this theory researchers have begun to investigate methodologies for maintaining antigen/ immunomodulator proximity. One method is to encapsulate the immunomodulator and antigen to diminish diffusion and breakdown of the adjuvants. Babai et. al. (1999) encapsulated HA and NA together with interleukin-2 (IL-2) and/or GM-CSF within a liposome. Encapsulated IL-2 and GM-CSF were more immunopotentiating than soluble cytokines in that they required less antigen and induced high HI titers at an earlier time post vaccination, which was sustained longer than titers boosted by soluble cytokines [112]. In a separate experiment Babai et. al. (1999) demonstrated that vaccines consisting of encapsulated antigen and cytokine, but not the free antigen, elicited a high titer of serum IgG_1, IgG_{2a}, IgG_3 and IgM antibodies and triggered delayed type hypersensitivity and cytotoxic T cell responses which suggest that their cytokine-supported liposomal influenza vaccines efficiently stimulate both T_h1 and T_h2 responses [113]. Interestingly, encapsulation of genetic material encoding for immunomodulators has also been demonstrated to increase vaccine efficacy. Oh et. al. (2004) produced an HPV virus like particle containing an IL-2 expression vector and demonstrated that encapsulated IL-2 VLP's induced the highest level of HPV specific mucosal IgA and serum IgG, IgG_1, and IgG_{2a} compared to non-encapsulated IL2 VLP's or VLP's alone [117]. Another method to diminish diffusion and degradation of

immunomodulators is to anchor the immunomodulator to the antigen itself. Yei et. al. (2002) observed that membrane-bound GM-CSF on the tumor cell surface retarded growth and induced protective immunity to subsequent wild-type tumor challenge more effectively than tumor cells secreting GM-CSF. They further demonstrated that membrane-bound GM-CSF B16.F10 cells can induce strong systemic immunity that protects against and therapeutically treats B16.F10 melanoma more effectively than analogous vaccines containing only GM-CSF secreting B16.F10 cells [118]. Nizard et. al. (2003) anchored IL-2 to the membrane of lymphoma cells by fusing it to the transmembrane domain of diphtheria toxin. The authors found that the fusion protein T-hIL-2 anchored to the surface of tumor cells retained its IL-2 activity and furthermore, vaccination of mice with these modified tumor cells induced a protective anti-tumor immunity mediated by tumor-specific cytotoxic T lymphocytes [116]. CD40-ligand anchored directly to a B cell lymphoma antigen greatly increased its immunogenicity and fully protected vaccinated mice from lethal tumor challenge while soluble CD40-ligand, administered with antigen proved to be less immunogenic and did not fully protect against tumor challenge [115]. Faulkner et. al. (2001) demonstrated that IL-2, linked to an immunodominant influenza HA peptide, resulted in enhanced T cell activation compared to HA peptide alone as well as unlinked IL-2 and HA. These findings were evaluated further and it was determined that the adjuvant properties of IL-2 were dependent on a functional IL-2 receptor [114]. These studies demonstrate that presentation of immunomodulators in close association with antigen greatly increases their immunogenicity and warrants further investigation.

Specific Aims. The central hypothesis for this doctoral dissertation is that inactivated whole virus influenza particles bearing membrane-bound immunomodulators will elicit a more robust and balanced immune response compared to non-adjuvanted, whole virus influenza vaccine. We chose to initially evaluate cytokines that are involved in diverse areas of the immune response and are well-defined immune modulators of both humoral and adaptive immunity. Granulocyte-macrophage colony stimulating factor (GM-CSF) is produced by many different cell types and stimulates the differentiation of granulocytes and

macrophages from progenitor cells. It is also intimately involved in many immune responses, including inflammation, dendritic cell and macrophage activation, and tumor immunity [120]. The adjuvant properties of GM-CSF are believed to be attributed to the recruitment and activation of dendritic cells which may lead to enhanced antigen presentation as well as macrophage, granulocyte and natural killer cell activation, all of which could augment immune responses to influenza vaccine [121-123]. Interleukin-2 (IL-2) is produced during immune responses to foreign antigens and functions to promote growth, differentiation and expansion of antigen specific T cells and is critically important for establishing memory-T cell responses [124-126]. IL-2 was included because of its T cell stimulatory properties in hopes that membrane-bound IL-2 would enhance influenza specific T cell immunity leading to better protection. Interleukin-4 (IL-4) promotes proliferation of activated B and T cells and is an important cytokine involved in antibody isotype switching and generating T_h2 mediate immune responses characterized by antibody mediated immunity [127-130]. IL-4 was included in initial studies because of its critical contribution to antibody-mediated immunity and to determine if membrane-bound IL-4 could boost influenza specific antibody titers and augment protection. By evaluating the immunostimulatory properties of numerous cytokines and immunomodulators we hoped to elucidate which candidates may generate a more robust immune response and would enhance protection against lethal challenge beyond that of conventional inactivated whole virus vaccines devoid of any immunomodulators. The following specific aims were designed to prove or disprove the central hypothesis and served as the foundation for this body of work.

Specific Aim 1: Establishment of CYT-IVAC producer MDCK cell lines for the production of Cytokine-Bearing Influenza Vaccines (CYT-IVACs). *Working Hypothesis:* Infecting MDCK cells expressing murine cytokines and chemokines, fused to the transmembrane and cytoplasmic tail domain of viral hemagglutinin and neuraminidase, will produce progeny virions that readily incorporate cytokines and chemokines directly into the virus particle. These cytokines and chemokines will retain their bioactivity following inactivation of the virus.

Specific Aim 2: Evaluation of CYT-IVACs ability to enhance protection against challenge and induce humoral and cellular immunity. *Working Hypothesis:* Immunostimulatory molecules on the surface of inactivated, whole virus influenza vaccine will generate an enhanced influenza specific humoral and cellular immune response, compared to conventional non-adjuvanted whole virus influenza vaccine, leading to greater protection against viral infection.

Specific Aim 3: Characterization of immunostimulatory properties of CYT-IVACs. *Working Hypothesis:* Membrane-bound immunomodulators will augment the stimulation of antigen presenting cells to better bridge the gap between innate and adaptive immunity leading to boosted humoral and cellular immunity. The immune response can be specifically directed with the use of appropriate immunomodulators.

2. Materials and Methods

2.1 Construction of expression plasmids.

Mouse derived granulocyte macrophage-colony stimulating factor (mGM-CSF) and interleukin 2 and 4 (mIL-2, mIL-4) were fused to the stalk, transmembrane, and cytoplasmic tail domain of influenza A/WSN/33 hemagglutinin (HA) using standard PCR methodologies. Primers, amplifying the carboxyl terminal 71 amino acids of WSN HA and the coding sequence of the cytokines, were designed to introduce the appropriate restriction sites. Nucleotides 1521-1730 coding for the 26 amino acid stalk region, the transmembrane domain, and cytoplasmic tail domain of the hemagglutinin, termed HA1513, were amplified using the forward and reverse primers (Table 1) to introduce restriction sites Bam HI and Eco RI (underlined), respectively. Primers specific for mGM-CSF, mIL-2, and mIL-4 were designed to remove stop codons and introduce Hind III (mGM-CSF) or Kpn I (mIL-2 and mIL-4) and BamHI endonuclease restriction sites on the 5' and 3' ends respectively. PCR products were generated using Platinum *Pfx* (Invitrogen) and GeneAmp PCR System 2400 (Applied Biosystems) per manufacturers instructions. Purified PCR products were subsequently digested and inserted into the respective restriction sites of pcDNA3.1 using T4 DNA Ligase (Invitrogen) according to the manufacturers protocol. Plasmid constructs, harboring the respective fusion constructs, were sequenced by the Wayne State University Sequencing Core (Applied Genomics Technology Center) to verify sequence and integrity of the constructs.

2.2 Generation of virus producer cell lines.

Madin-Darby canine kidney (MDCK) cells were maintained in complete growth media (DMEM/10% FBS) consisting of Dulbecco's Modified Eagles Media supplemented with 10% fetal bovine serum (Atlanta Biologicals) and the antibiotics penicillin/streptomycin (100 U/100 μg per ml). Cells were transfected with expression plasmids using Lipofectamine2000 (Invitrogen) as described previously

[131]. Stable transfectants were selected by growth in DMEM/10%FBS supplemented with Geneticin (1.5 mg/ml; Gibco). Geneticin-resistant cells were subcloned by limiting dilution in 96-well plates in the presence of Geneticin (G418™ Invitrogen, 1 mg/ml). Individual MDCK subclones were screened for cell surface expression and bioactivity of the respective membrane-bound cytokines.

2.3 Viral infection, purification and inactivation.

Wild-type and CYT-IVAC producer MDCK cells (90% confluent) were infected at an MOI of 1 with either influenza virus A/PR/8/34 (H1N1) or A/Udorn/72 (H3N2). Following virus adsorption (1 hr, 37°C), the inoculum was removed and DMEM/2% FBS was added. Supernatants from infected monolayers were harvested 24-36 hours post infection and cellular debris was pre-cleared at 400 x g for 15 minutes at 4°C. Virions were purified through a series of iodixanol (OptiPrep™, Axis-Schield) cushion and gradient centrifugation steps. Preps were first centrifuged through a 14% iodixanol cushion (SW41 rotor, 88,000 *x g*, 45 min at 4°C). Banded virus was collected and concentrated by centrifugation at 88,000 *x g* for 45 minutes at 4°C and subsequently re-suspended in phosphate-buffered saline (PBS). Purified virus was inactivated by treating with 15 mM ß-propiolactone for 15 minutes at 25°C. The reaction was neutralized by the addition of sodium thiosulfate (40 mM final concentration, 30 min, 25°C). To test the preservation of bioactivity following other inactivation methods, preps were also inactivated using heat, UV, and formalin. Heat inactivation of virus preparations was done by incubation at 56°C for 30 minutes. For virus inactivation using UV, preps were irradiated with 1500 *uW* /seconds/cm^2 *UV*, at 6 inches, for 15 minutes. To inactivate using formalin, preps were incubated with 1% formalin for 72 hours at 4°C. Following inactivation, viral preps were further purified over two sequential 10-26% iodixanol continuous gradients. Preps were loaded on top of the gradient and centrifuged at 55,000 x g for 45 minutes at 10°C. Banded virus was collected and run over a second gradient. Double gradient purified virus was diluted in PBS and concentrated with a final centrifugation step at 88,000 *x g* for 45 minutes at 4°C and subsequently re-suspended in PBS. Total viral protein concentration was

determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology). Inactivation was confirmed by monitoring cytopathic effect in MDCK cells treated with 5 µg of inactivated virus vaccine for a period of 3-5 days at 37°C in the presence of 1.5 µg/ml TPCK-treated trypsin (Sigma).

2.4 Cell surface expression and viral incorporation of membrane-bound cytokines (Immunofluorescence Microscopy).

Producer MDCK cells were grown to 90% confluency on glass cover slips in 24 well plates. Cells were washed with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde (PF) in 250 mM HEPES for 10 minutes at room temperature (RT). PF was removed and 50 mM glycine in PBS was added for 10 minutes at RT to quench any remaining PF. Cells were washed 2 times with PBS and blocked with 2% chicken serum in PBS for 30 minutes at RT. For immunostaining, cells were incubated sequentially with rat anti-cytokine specific antibody (BD Pharmagen) and chicken anti-rat IgG conjugated Alexa Fluor® 488 antibody (Invitrogen/Molecular Probes). All antibodies were diluted in PBS/2% chicken serum. Cover slips were mounted on slides using ProLong Antifade (Invitrogen/Molecular Probes). Immunofluorescent staining was visualized using a Nikon E800 Epifluorescence Microscope. Digital images were captured using a Roper CoolSnap FX digital camera and analyzed using MetaMorph Imaging Software (Universal Imaging).

To visualize viral incorporation of membrane-bound cytokines, CYT-IVAC producer cells, grown on cover slips, were infected with filamentous Influenza A/ Udorn/72 at an MOI of 1. The cells were fixed at 8 hr post-infection with 3% PF and blocked as described above. Cells were incubated with rat anti-cytokine specific primary antibody and Alexa Fluor® 488 conjugated secondary antibody as described above. Additionally, cells were incubated with goat anti-H3 antibody and secondary chicken Alexa Fluor® 594 conjugated anti-goat IgG (Invitrogen/ Molecular Probes). Cover slips were mounted and immunofluorescence was analyzed as described above.

2.5 Western blot analysis of CYT-IVACs.

Vaccines were solubilized in Laemmli Buffer (BioRad) (LB) and heated at 96°C for 10 minutes to denature proteins. Samples were separated on 12% PAGE-SDS and subsequently blotted to PVDF membrane. Membranes were probed by sequential incubation with rat anti-GM-CSF (BD Bioscience), followed by goat anti-rat IgG horseradish-peroxidase conjugated secondary antibody (Santa Cruz). Membranes were exposed to ECL or Femto solution per manufacturers (Pierce) instructions and membranes were visualized using Chemdoc XRS (BioRad).

2.6 Total cytokine and hemagglutinin quantitation by slot blot assay.

Serial dilutions of vaccines at 1, 0.5 and 0.25 µg (cytokine quantification) or 1, 0.2 and 0.04 µg (HA quantitfication) of total viral protein, as well as serial diluted recombinant cytokine (2000 ng to 1.95 ng) were blotted on PVDF membranes using a slot blot apparatus. Membranes were blocked with 5% milk solution and subsequently incubated sequentially with diluted primary antibody, specific for the respective cytokine (rat anti-GM-CSF, IL-2, or IL-4, BD Bioscience) or hemagglutinin (mouse anti-HA, Meridian Life Science,® Inc or rabbit, anti-H1N1/ Pan H1, Pierce®) followed by the respective horseradish-peroxidase conjugated secondary antibody (goat anti-rat IgG (Santa Cruz); goat anti-mouse IgG (BioRad) or goat anti-rabbit IgG (Sigma)). Membranes were exposed to ECL or Femto solution per manufacturers (Pierce®) instructions and chemiluminescent signals were recorded using a Chemdoc XRS (BioRad). Images were processed with ImageJ software (NIH freeware) and standard curves for each cytokine were generated using optical pixel densities. Total cytokine content for each vaccine preparation was extrapolated from standard curves and is expressed as the average of the three dilutions evaluated for each vaccine in nanograms (ng) of cytokine per microgram (µg) of total viral protein. The signal intensity of the HA specific signal for each vaccine was calculated for each dilution and the average pixel density per µg of total viral protein is given.

2.7 Hemagglutination assay

Hemagglutination units (HAU) were determined by agglutination of chicken red blood cells as previously described [132]. Briefly, serial diluted vaccine preparations were mixed with an equal volume of fresh 0.5% chicken red blood cells and incubated at room temperature for 30 minutes. Red blood cell agglutination was recorded and HAU per µg of total viral protein is expressed as the reciprocal of the last dilution of virus that resulted in agglutination.

2.8 Bioassay of membrane-bound cytokines.

Bone marrow (BM) cells, as indicator cells for mGM-CSF bioactivity, were prepared from the femurs of female Balb/c mice. Briefly, bone marrow was flushed from the femurs with RPMI and the cell suspension passed through a 70µm cell strainer. Red blood cells were lysed using RBC lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.01% EDTA). Cells were washed 2 times with RPMI and re-suspended in complete RPMI (10% FBS, 20 mM L-glutamine, 1 M HEPES, 100 mM Sodium Pyruvate, 55 µM 2ß-Mercaptoethanol, Penicillin/Streptomycin (100 units/ 100 µg/ ml)). For MDCK based bioassays, BM cells ($2x10^5$/well) were added to wells of a 96 well plate containing 90% confluent, mitomycin C (50 µg/ml) treated wild type or CYT-IVAC producer (mGM-CSF~HA) MDCK cells. For virus based bioassays and quantitation of viral incorporated bioactive GM-CSF, BM cells ($2x10^5$) or MPRO cells ($5X10^3$) [133], respectively, were added to wells of a 96 well plate containing inactivated A/PR/8/34 wild type or A/PR/8/34 mGM-CSF~HA. Recombinant GM-CSF was also used to establish a standard curve by which virus-incorporated bioactive GM-CSF could be quantitated. Plates were incubated at 37°C for 72 hours (BM) or 48 hours (MPRO). For the last 18 hours of incubation for the cell-based bioassay, cells were pulsed with ^{3}H-thymidine then harvested and counted using a scintillation counter. For the viral based bioassay, Alamar Blue® (Invitrogen) was added to each well at 10% of the total volume for the last 24 hours and Alamar Blue® reduction was determined from the absorbance values recorded at 570nm and 600nm after 72 (BM) or 48 (MPRO) hours.

CTLL-2 cells (a gift from Dr. Robert Swanborg, Wayne State University) were used as indicator cells for the bioactivity of mIL-2. Cells were maintained in complete RPMI supplemented with recombinant mouse IL-2 (10ng/ml). CTLL-2 cells ($5x10^3$) were added to 96 well plates containing mitomycin C treated cells (wild-type or mIL-2 CYT-IVAC producer cells) or inactivated virus (A/PR/8/34 wild-type or A/PR/8/34 mIL-2~HA) as described above. Recombinant IL-2 was also used to establish a standard curve by which virus-incorporated bioactive IL-2 could be quantitated. Plates were incubated at 37°C for 48 hours. For the last 18 hours of incubation for the cell-based bioassay, cells were pulsed with ^{3}H-thymidine then harvested and counted using a scintillation counter. For the virus particle based bioassay, Alamar Blue® was added to each well for the last 24 hours and absorbance was read at 570nm and 600nm after 48 hours.

CT.4s cells (gift from Dr. William Paul and Dr. Jane Hu-Li, Laboratory of Immunology, National Institute of Health) were used to determine mIL-4 bioactivity [134]. Cells were maintained in complete RPMI supplemented with recombinant mouse IL-4 (2ng/ml). CT.4s cells ($5x10^3$) were added to 96 well plates containing mitomycin C treated MDCK cells (wild-type or mIL-4 CYT-IVAC producer cells) or inactivated virus (A/PR/8/34 wild-type or A/PR/8/34 mIL-4~HA) as described above. Recombinant IL-4 was also used to establish a standard curve by which virus-incorporated bioactive IL-4 could be quantitated. Plates were incubated at 37°C for 48 hours. For the last 18 hours of incubation for the cell-based bioassay, cells were pulsed with ^{3}H-thymidine, harvested and counted using a scintillation counter. For the viral based bioassay, Alamar Blue® was added to each well for the last 24 hours and absorbance was read at 570nm and 600nm after 48 hours.

Standard curves for recombinant GM-CSF, IL-2 and IL-4 were deduced from the difference data of the 570nm and 600nm absorbance readings for each dilution of recombinant protein using Prism (GraphPad Software, Inc.). Difference data, collected from various dilutions of GM-CSF, IL-2, or IL-4-bearing CYT-IVAC preparations, was applied to their respective standard curve for quantitation of available bioactive membrane-bound cytokine for each CYT-IVAC on a per microgram of total viral protein basis.

2.9 Determination of Minimal Protective Dose 20 (MPD_{20}).

To establish the dose of wild-type vaccine required to protect 20% of vaccinated mice, groups (N=10) of female Balb/c mice (NCI, Charles, River Laboratories), 8-10 weeks old, were vaccinated with decreasing amounts of ß-propiolactone inactivated wild-type influenza A/PR/8/34. The MPD_{20} was determined for both single gradient purified vaccine preparations (crude preparations) as well as double gradient purified vaccine preparations (utra-purified preparations). To establish the MPD_{20} for crude preparations mice were vaccinated intranasally or subcutaneously with 10, 5, 2.5 or 1 µg of crude vaccine preparations. Groups of mice were vaccinated with 3, 2, 1 or 0.5 µg intranasally or 0.5, 0.25, 0.125 or 0.06125 µg subcutaneously of double gradient purified vaccines to determine the MPD_{20} for ultra-purified vaccines. On day 35 post-vaccination, mice were challenged with 100 lethal dose 50s of mouse-adapted Influenza A/PR/8/34. Weight loss and survival was monitored following challenge.

2.10 Vaccination studies and tissue collection (Appendix F and G).

Animal experiments were performed in accordance with NIH guidelines and with approval by the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University. Groups of 8-10 week old female Balb/c mice (NCI, Charles, River Laboratories) were immunized intranasally or subcutaneously with ß-propiolactone inactivated A/PR/8/34 wild-type, A/PR/8/34 mGM-CSF~HA, A/PR/8/34 mIL-2~HA, or A/PR/8/34 IL-4~HA diluted in PBS. Preliminary vaccine efficacy experiments were conducted using a single dose of 3 µg intranasally or 0.2 µg subcutaneously of single gradient purified vaccine preparations (crude preparations). Subsequent efficacy studies were completed using single doses of 1 µg intranasally or 0.375 µg subcutaneously of ß-propiolactone inactivated, double gradient purified vaccine (ultra-purified preparations). To assess cellular mediated immunity, mice were vaccinated with 5 µg intranasally or 1 µg subcutaneously. PBS alone acted as the negative vehicle control. Serum was collected on day 21 post-vaccination by retro-orbital bleeding.

Mice were challenged with mouse-adapted Influenza A/PR/8/34 (100 LD_{50}) on day 35 post-vaccination for efficacy studies and day 28 post-vaccination for cellular mediated immunity studies. For efficacy studies, weight loss and survival was monitored following challenge. For cellular mediated immunity studies, spleens, nasal washes, lungs and bone marrow were collected on day 4 post-challenge using aseptic technique. Nasal washes were collected by passing 1 ml of cold PBS through the posterior nasal passage out of the nares and into a collection tube. Bone marrow was collected by flushing the femurs as described above. Lungs and nasal washes were flash frozen in liquid nitrogen and stored at -80°C. Spleens and bone marrow were processed immediately as described below.

2.11 Enzyme linked immunosorbent assay (ELISA).

Antiviral antibody levels in sera of vaccinated animals were determined by a standard enzyme-linked immunosorbent assay using whole virus as the coating antigen. Briefly, Immuno Plates (Nunc) were coated with 10 hemagglutination units (HAU) of inactivated influenza A/PR/8/34 in coating buffer (sodium bicarbonate, pH 9.6) and blocked overnight at 4°C in PBST buffer (phosphate buffered saline with 0.05% Tween 20) supplemented with 2% BSA. Plates were washed 3 times with PBST buffer using a Hydro flex (Tecan). For end titer analysis, serial 2 fold dilutions of day 21 sera were added to wells of ELISA plates and plates were incubated with shaking for 4 hours at room temperature (RT). For quantitative analysis, serum samples were diluted in blocking buffer and added to wells of ELISA plates and incubated with shaking over night at 4°C. Plates were washed 3 times with PBST buffer using a Hydro flex (Tecan). Horseradish Peroxidase (HRP) conjugated secondary antibody (anti-mouse IgG, IgG_1, or IgG_{2a}; Southern Biotech), diluted in PBST with 2% BSA, was added and plates were incubated with shaking for 1.5 hours at RT. Plates were washed 3 times with PBST buffer and wells were incubated with substrate (2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid; Sigma) for 30 minutes at RT, followed by the addition of 1% SDS to stop the reaction. Absorbance was measured at 405 nm using a plate reader (SpectraFluor Plus, Tecan). End titers were determined to be

the reciprocal of the last dilution that was greater than background plus 2 times standard deviation (> background + (2 X s.d.)). For quantitative analysis, O.D. readings were plotted against a standard curve to determine the amount of influenza specific antibody per milliliter of serum.

2.12 Microneutralization assay.

Neutralizing antibody titers were determined for serum samples collected from mice on day 21 post-vaccination as described in the WHO Manual on Animal Influenza Diagnosis and Surveillance [135]. Briefly, two-fold serial dilutions of serum in PBS were incubated with 100 $TCID_{50}$ of influenza A/PR/8/34 for 1 hour at room temperature. The serum/virus cocktail was added to MDCK cells for 1 hour at 37°C. Serum/virus cocktail was removed and cells were incubated for 3 days at 37°C in the presence of 1.5 μg/ml TPCK-treated trypsin (Sigma). Neutralizing titer was determined to be the reciprocal of the last dilution of serum that protected MDCK cells from cytopathic effect.

2.13 Quantitation of viral loads in lungs.

Viral loads in the lung tissue of vaccinated mice were determined by collecting lungs from 3 or 6 mice per vaccine group on day 4 post-challenge. Lungs were weighed and flash frozen in DMEM with liquid nitrogen. Lung tissue was homogenized, pelleted and supernatants were collected. Lung homogenates were brought to equal volume with DMEM. Cellular debris was removed from nasal washes by pelleting and collecting the supernatants. Adding PBS to 1 ml total volume normalized nasal wash volumes. Viral titers of lung homogenates and nasal washes were determined from serial 10-fold sample dilutions incubated with MDCK cells for 1 hour at 37°C to allow for virus adsorption. Subsequently, cells were washed and incubated for 3 days at 37°C in the presence of 1.5 μg/ml TPCK-treated trypsin (Sigma) and cytopathic effects were recorded. Viral loads were reported as 50% tissue culture infectious dose units ($TCID_{50}$/ml) as determined by the Reed-Muench method [136].

2.14 Splenocyte proliferation assay.

Spleens were harvested from freshly euthanized mice on day 4 post-challenge and splenocytes were isolated by disruption of spleen against 60μm wire screen. Following lysis of the red blood cells as described above, splenocytes were resusupended in complete RPMI at $2X10^6$ cells/ml. Cells were added to a 96 well flat bottom plate at $1X10^5$ cells/well and stimulated with ß-propiolactone inactivated influenza A/PR/8/34 (1 μg/well) for 72 hours at 37°C/5% CO_2. Alternatively, cells were stimulated with influenza specific MHC I or MHC II (Appendix E) peptides (10 μg/ml) for 72 hours at 37°C/5% CO_2. Media alone or concanavalin A (10 μg/ml) served as negative and positive controls, respectively. Alamar Blue® was added to the culture media (10% final concentration) for the last 18 hours. Absorbance of Alamar Blue® was read at 570nm and 600nm and data is expressed as the difference between 570 and 600nm readings.

2.15 FLOW cytometric analysis of APCs.

Bone marrow derived dendritic cells (BMDC) used in FLOW cytometry experiments were generated in vitro as previously described with some modifications [137-139]. Briefly, femurs were collected from freshly euthanized female Balb/c mice (8-10 weeks of age) and disinfected with ethanol. Bone marrow was flushed from the femurs with DMEM and the cell suspension was passed through a 70μm cell strainer. Following lysis of red blood cells, cells were washed and resuspended in complete RPMI at $2.5X10^5$ cells/ml. Cells were seeded in 24 well plates at $2.5X10^5$ cells/well and media was supplemented with recombinant GM-CSF (20 ng/ml) and recombinant IL-4 (5 ng/ml). Cultures were incubated at 37°C for 6 days, changing half the media at days 2, 4, and 5 of culture. Non-adherent cells were collected, characterized by FLOW cytometry and proved to be >95% CD11c+, immature dendritic cells. BMDCs or MH-S cells were seeded in 24 well plates as $1.5X10^6$ cells per well. Cells were treated, in triplicate, with inactivated wild-type A/PR/8/34, A/PR/8/34 mGM-CSF~HA, A/PR/8/34 mIL-2~HA, or A/PR/8/34 IL-4~HA (1 μg/well), LPS (1 μg/well) or media alone for 48 hours

(BMDC) or 24 hours (MH-S). Cells were collected and washed two times in FLOW Staining Buffer (FSB) (eBioscience). Cells were then incubated for 10 minutes at 4°C with rat anti-mouse CD16/32 (BD Pharmingen) diluted to 1 μg per million cells in wash buffer. The following antibodies were used at a 1:100 dilution in FSB in various experiments in differing combinations, depending on cell type analyzed: CD11c-PE-Cy7 (BD Bioscience), CD11b-Pacific Blue, CD40-PE, CD80-PE, CD86-PE, CD70-PE, CD274-PE, CCR7-PE (eBiosciences). Cells were incubated with antibodies for 20 minutes at 4°C. Following incubation, cells were washed and resuspended in PBS and FLOW cytometric analysis was performed with BD FACSAria Cell Sorter. Data analysis was completed using FlowJo software.

2.16 Intracellular cytokine (ICC) FLOW cytometric analysis of splenocytes.

Splenocytes isolated from vaccinated animals, as described above, were stimulated ex vivo with inactivated wild-type A/PR/8/34. Freshly isolated spleen cells were plated in 6 well plates at $1X10^7$ cells per well and treated with inactivated wild-type A/PR/8/34 (10 μg/well). PMA/Ionomycin (50 ng/ml and 500 ng/ml respectively) treatment for 6 hours served as the positive control and media alone served as the negative control. Cells were incubated at 37°C for 5 days and Brefeldin A (3 μg/ml) (eBioscience) was added for the last 6 hours of culture. Cells were collected and washed two times in FSB (eBioscience) then incubated for 10 minutes at 4°C with rat anti-mouse CD16/32 (BD Pharmingen) diluted to 1 μg/ million cells in wash buffer. Cells were incubated with the following antibodies at a 1:100 dilution for 20 minutes at 4°C: CD4-APC, CD8-Pacific Blue, CD69-PE-Cy7 (eBioscience). Following two wash steps, cells were fixed with Fixation Buffer (eBioscience) for 20 minutes at 22°C. Cells were permeabilized with Permeabilization Buffer (eBioscience) per manufacturers instructions. Intracellular cytokine staining was performed by incubating the cells with the following antibodies at a 1:100 dilution for 20 minutes at 4°C: IL-4-PE, IFN gamma-PE, IL-17a-PE (eBioscience). Following incubation, cells were washed and

resuspended in PBS and FLOW cytometric anaylsis was performed with BD FACSAria Cell Sorter. Data analysis was completed using FlowJo software.

2.17 Statistics.

Statistical analysis using Prism software (Graphpad) was conducted with the help of Dr. Stephen Were (statistician for VA-MD Regional College of Veterinary Medicine). ELISA antibody titer data, lung and nasal wash viral loads, and splenocyte proliferation data were analyzed by One-way ANOVA on normalized log transformed data using Dunnett's multiple comparison test with PR/8/34 wild-type group as the control. Comparison of survival curves was analyzed using Fisher's exact test.

3. Results

Aim 1: Establishment of CYT-IVAC producer MDCK cell lines for the production of Cytokine-Bearing Influenza Vaccines (CYT-IVACs).

We have previously described an in vitro cell culture platform that allows for the direct incorporation of membrane-bound forms of chicken derived cytokines into virus particles [131]. Preparation of these cytokine-bearing influenza virus vaccines, or CYT-IVACs, requires that the cytokine or immunomodulator of choice be both anchored into the virion membrane, and efficiently packaged into virions as they are released from the infected host cell. Further, the membrane-bound immunomodulator must retain its bioactivity. To ensure successful membrane anchoring and virion packaging, a gene encoding for full-length cytokine (including its signal sequence) is fused inframe to a gene segment encoding a short extracellular stalk domain, the transmembrane spanning and the cytoplasmic tail domains of the influenza virus hemagglutinin derived from influenza A/WSN/33 (H1). Alternatively, genes encoding mature soluble forms of cytokines or chemokines can be fused inframe to the N-terminal encoding cytoplasmic tail, membrane-spanning and short stalk domains of the viral neuraminidase [131].

3.1 CYT-IVAC expression vectors.

For the present work, mouse derived IL-2, IL-4 and GM-CSF were fused inframe to the C-terminal portion of the influenza A/WSN/33 hemagglutinin (HA1513), nucleotides 1521-1730, and inserted into the mammalian expression vector pcDNA3.1 (Invitrogen) under control of the CMV promoter element, pcDNA3.1/mIL-2~HA, /mIL-4~HA and /mGM-CSF~HA respectively. Forward and reverse primers specific for the gene of interest were designed to introduce restriction sites used for 1) fusing the cytokine of interest to hemagglutinin and 2) cloning the entire fusion construct into pcDNA3.1 (Table 1). Expression vectors were sequenced to confirm correct gene sequences and orientation of fusion

constructs (Appendix A-D). Expression vectors were then used to establish stably transfected MDCK cells lines by selecting with geneticin and subcloning the surviving cells. The stably transfected MDCK cell lines were given the term CYT-IVAC producer cell lines as they were subsequently used as a cell line-based platform to produce Cytokine-bearing Influenza Vaccines.

Expression vectors encoding the cytoplasmic tail and transmembrane domains, as well as a stalk region of varying lengths, of influenza A/WSN/33 neuraminidase were also created for future use. Specifically, neuraminidase nucleotides 1-160, 1-184, or 1-259, encoding amino acids (aa) of the cytoplasmic tail domain (6 aa), transmembrane domain (29 aa) and 17, 25 and 50 amino acids of the stalk domain respectively, were cloned into pcDNA3.1 (Invitrogen). In addition to the abovementioned HA construct (HA1513), an expression vector encoding influenza A/WSN/33 hemagglutinin nucleotides 1599-1730 (HA1599) was also created which eliminated the stalk region. It was initially hypothesized that removal of the stalk domain may create a more stable construct, compared to the HA1513 construct, by removing potential proteolytic cleavage sites, however both constructs were equally stable and HA1513 was used for subsequent fusion constructs.

Table 1. CYT-IVAC Primers

Primer Name	Primer Sequence
mouse GM-CSF	
Forward Primer	5'-CC*AAGCTT*GGAGGATGTGGCTGCAGAA-3'
Reverse Primer	5'-GG*GGATCC*TTTTTGGACTGGTTTTTTGC-3'
mouse IL-2	
Forward Primer	5'- CC*GGTACC*AGCATGCAGCTCGCATCCTGTGTC-3'
Reverse Primer	5'- GG*GGATCC*TTGAGGGCTTGTTGAGATGA-3'
mouse iL-4	
Forward Primer	5'-CC*GGTACC*GCACCATGGGTCTCAACCCCCA-3'
Reverse Primer	5'-CC*GGATCC*CGAGTAATCCATTTGCATGATG-3'
Influenza A/WSN/33 HA	
Forward Primer	5'-CC*GGATCC*AATGGGACTTATGATTATCC-3'
Reverse Primer	5'-CC*GAATTC*TCAGATGCATATTCTGCACTGC-3'

3.2 Immunofluorescent assay of CYT-IVAC producer cells.

Following establishment of stably transfected MDCK cells, expression of fusion constructs at the cell surface was confirmed by immunofluorescence microscopy using cytokine-specific antibodies. As depicted in Figure 1, cell surface expression of mGM-CSF~HA, mIL-2~HA or mIL-4~HA could be readily demonstrated in MDCK cells stably transfected with the respective expression constructs (Fig. 1 D, E, and F respectively). Positive staining was absent in vector control MDCK transfected cells using each of the cytokine specific antibodies (Fig. 1 A, B, and C). Stable MDCK transfectants were subcloned by limiting dilution to ensure maximal surface expression of the fusion constructs and further selected based upon i) cell surface expression of the membrane-bound cytokines, and ii) cell surface bioactivity of the specific membrane-bound cytokines.

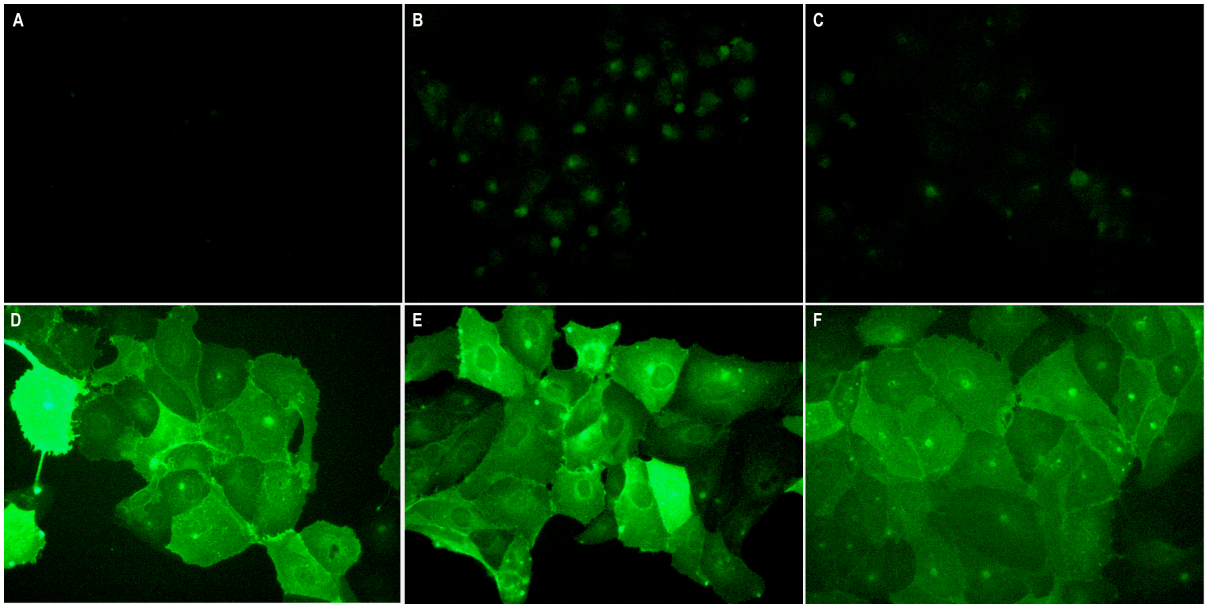


Figure 3. Cell surface expression of membrane-bound immunomodulator fusion constructs. Cell surface immunofluorescent staining of wild-type MDCK cells (A, B, C) and MDCK CYT-IVAC producer cells expressing membrane-bound mouse GM-CSF~HA (D), IL-2~HA (E), or IL-4~HA (F). Paraformaldehyde fixed cells were labeled using rat anti-GM-CSF (A, D), anti-IL2 (B,E) or anti-IL4 (C,F) specific antibodies followed by Alexa Flour® 488 conjugated secondary antibody.

3.3 CYT-IVAC producer cell bioassays.

Membrane-bound cytokine bioactivity was determined using specific cell-based bioassays in which MDCK transfectants, wild-type or subclones of membrane-bound cytokine producing cells, were incubated with cytokine specific indicator cells. Bioactivity or proliferation was based on the incorporation of ^{3}H-thymidine. All three stably transfected MDCK cell lines expressing either mGM-CSF~HA (Figure 4), mIL-2~HA (Figure 5), or mIL-4~HA (Figure 6) induced the proliferation of their respective indicator cell line at levels well above background (indicator cells alone). Vector control or wild-type MDCK cells failed to induce

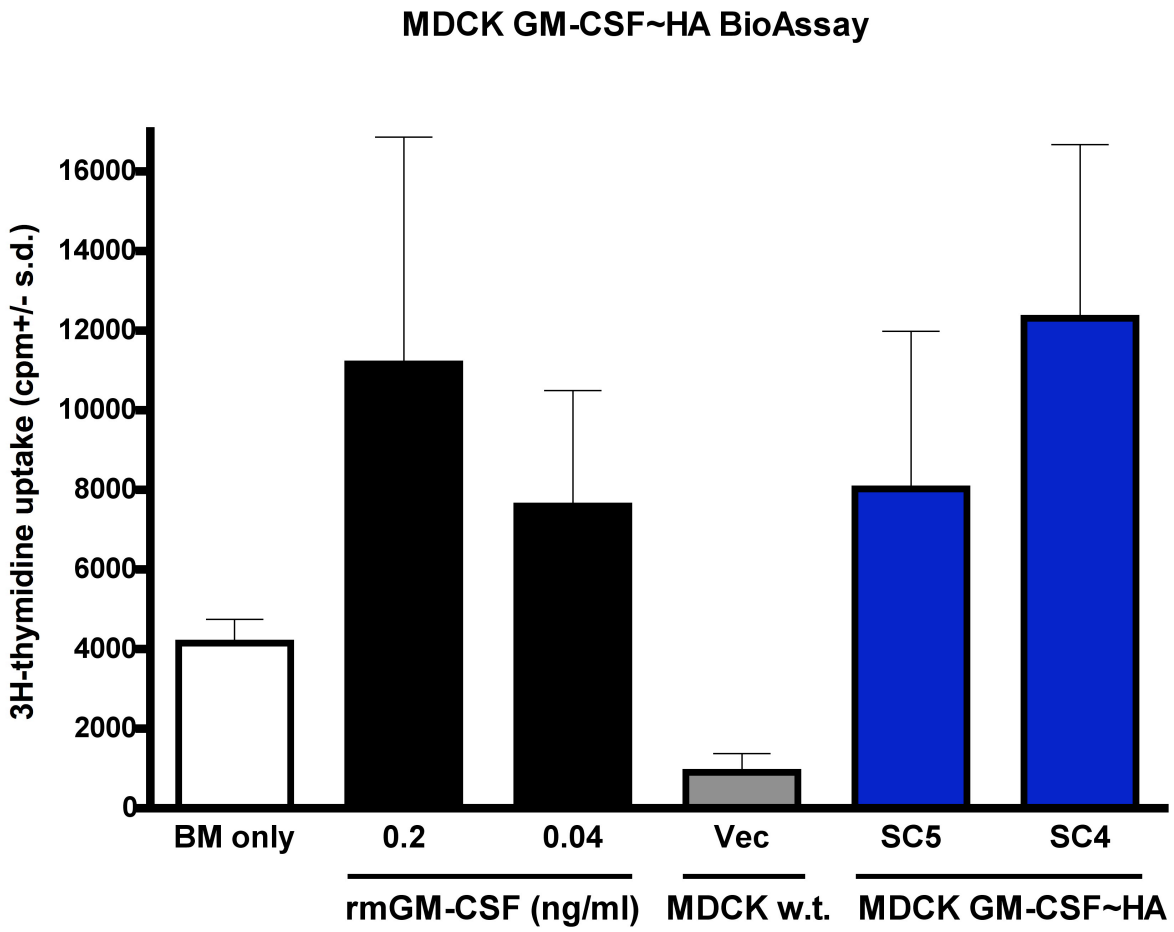


Figure 4. Membrane-bound immune-modulators are bioactive on the surface of MDCK CYT-IVAC producer cells. Mitomycin C treated sub-clones (SC) of CYT-IVAC producer cells expressing murine GM-CSF~HA or wild-type MDCK cells were co-cultured with bone marrow (BM) cells as GM-CSF specific indicator cells. Proliferation of bone marrow cells was measured by ^{3}H-thymidine incorporation. Recombinant murine GM-CSF was used as positive control.

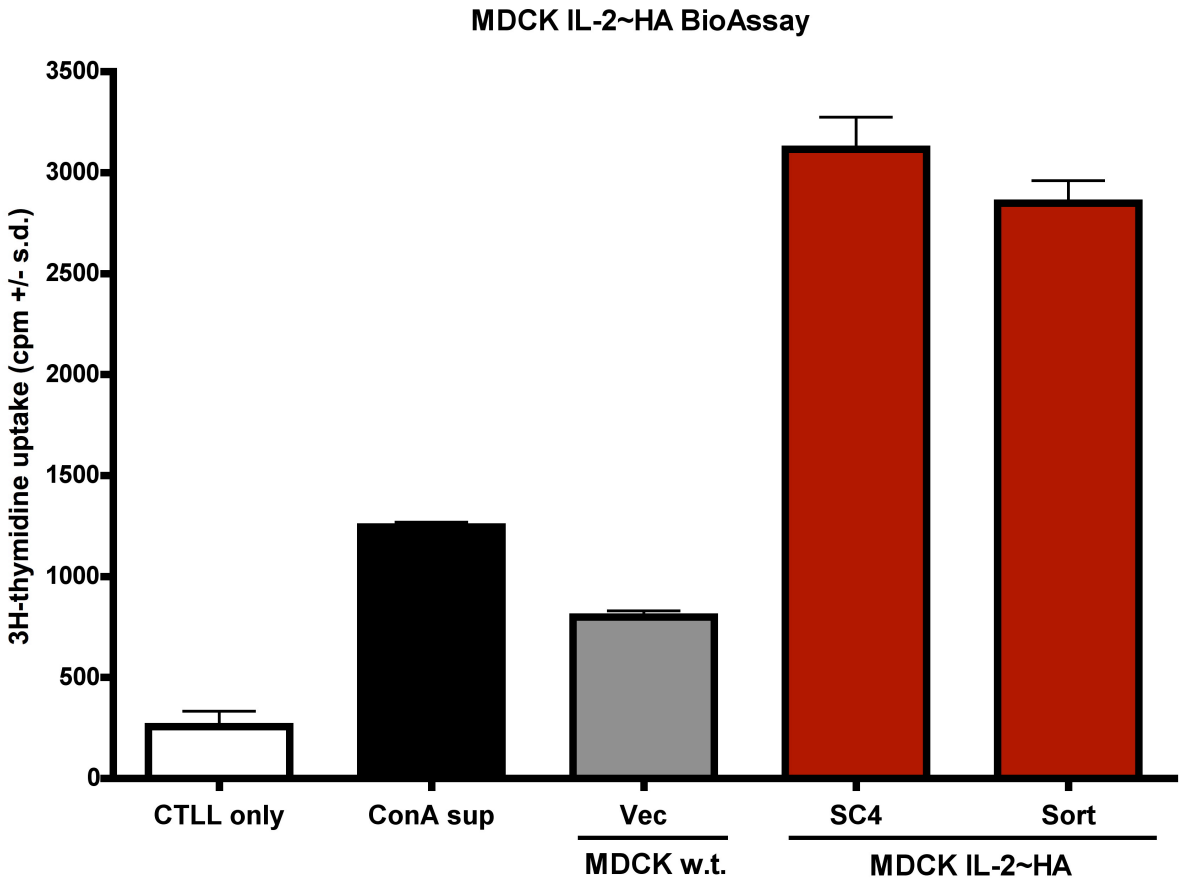


Figure 5. Membrane-bound immune-modulators are bioactive on the surface of MDCK CYT-IVAC producer cells. Mitomycin C treated sub-clones (SC) or FLOW sorted (Sort) CYT-IVAC producer cells expressing murine IL-2~HA or wild-type MDCK cells were co-cultured with CTLL-2 cells as IL-2 specific indicator cells. Proliferation of CTLL-2 cells was measured by ^{3}H-thymidine incorporation. Recombinant murine IL-2 was used as positive control.

significant proliferation of indicator cell lines. These results confirm that the mGM-CSF, mIL-2, and mIL-4 fusion constructs are expressed in a bioactive form on the surface of our CYT-IVAC producer cells.

In addition to the aforementioned constructs, constructs using murine derived Complement component 3d (C3d) or human derived IL-2 have been fused to influenza hemagglutinin as previously described. Specifically, the C3d construct is composed of a portion of the C3d gene encoding 28 amino acids responsible for CR2 receptor binding and adjuvant activity [140, 141]. A flag tag was introduced into the construct for easy detection of fusion construct expression. MDCK producer cells have been established using pcDNA3.1~mC3d 28mer Flag/HA and

pcDNA3.1~hIL-2/HA expressing membrane-bound murine C3d and human IL-2, respectively (data not shown)

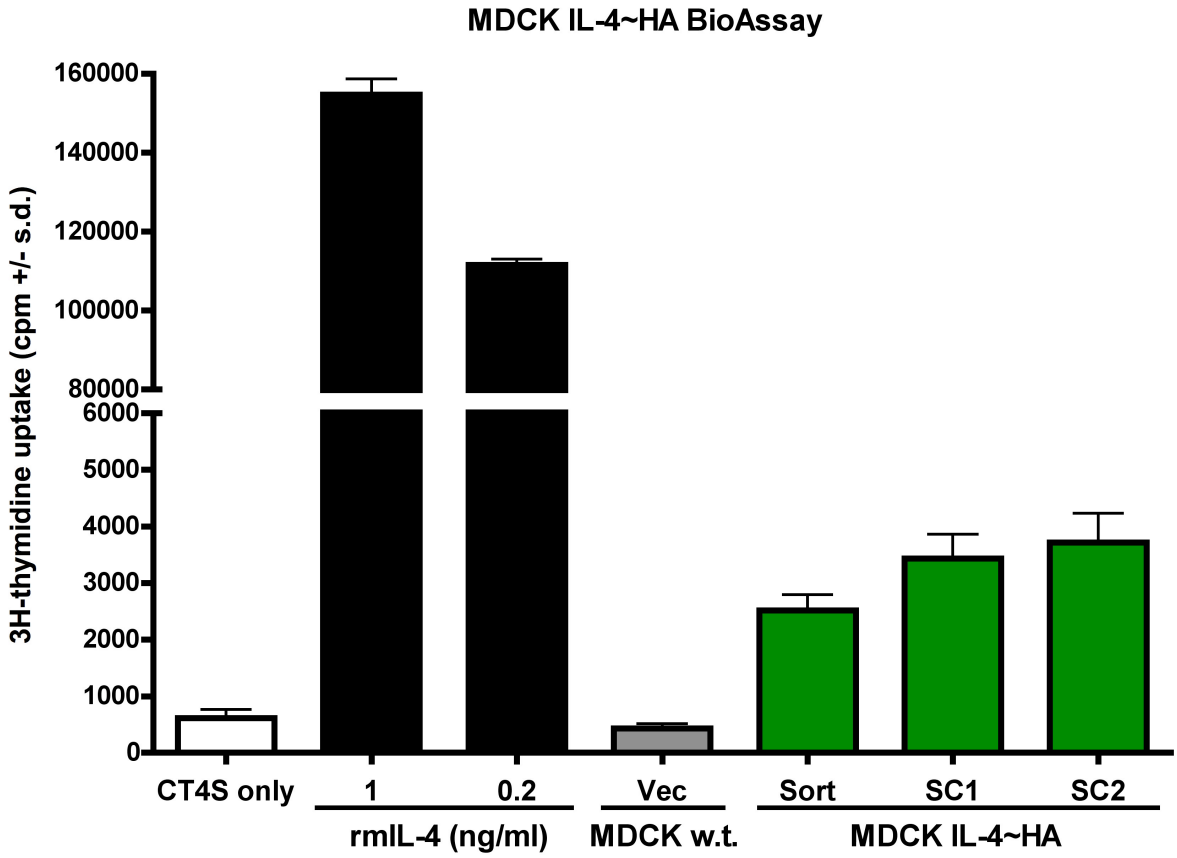


Figure 6. Membrane-bound immune-modulators are bioactive on the surface of MDCK CYT-IVAC producer cells. Mitomycin C treated sub-clones (SC) or FLOW sorted (Sort) CYT-IVAC producer cells expressing murine IL-4~HA or wild-type MDCK cells were co-cultured with CT.4s cells as IL-4 specific indicator cells. Proliferation of CT.4s cells was measured by ^{3}H-thymidine incorporation. Recombinant murine IL-4 was used as positive control.

3.4 Immunofluorescent assay of CYT-IVACs.

The purpose of this study was to produce inactivated whole virus vaccines, which exhibit immunopotentiating capacity compared to standard, non-adjuvanted influenza whole virus vaccine. In order for membrane-bound cytokines to serve as immunopotentiating adjuvants they must first be packaged efficiently into virions, and subsequently retain their bioactivity following inactivation of the virus particles. To confirm packaging of membrane-bound cytokines into virions, we initially took advantage of our experience with filamentous strains of influenza virus [142-144]. Filamentous strains allow for indirect immunomicroscopic visualization of virus

particles budding from infected cells or of virions released into the extracellular media. To assess whether membrane-bound cytokines at the surface of MDCK cells were incorporated into budding virions, CYT-IVAC producer cells were infected with filamentous Influenza A/Udorn/72 (H3N2) virus and at 8 hours post-infection, fixed and immunostained with antibodies specific for the respective cytokines or for the viral hemagglutinin glycoprotein (HA). As demonstrated in Figure 7 (A-D), budding filamentous virions clearly incorporated membrane-bound GM-CSF when propagated in infected MDCK GM-CSF~HA expressing cells. Co-localization (yellow fluorescence, Figure 7 C) was evident indicating that both membrane-bound GM-CSF and full-length, virally encoded HA were incorporated into budding viral filaments. Importantly, localization of GM-CSF~HA and full length HA was also confirmed on virions collected from the supernatants of infected producer cells (Figure 7 D).

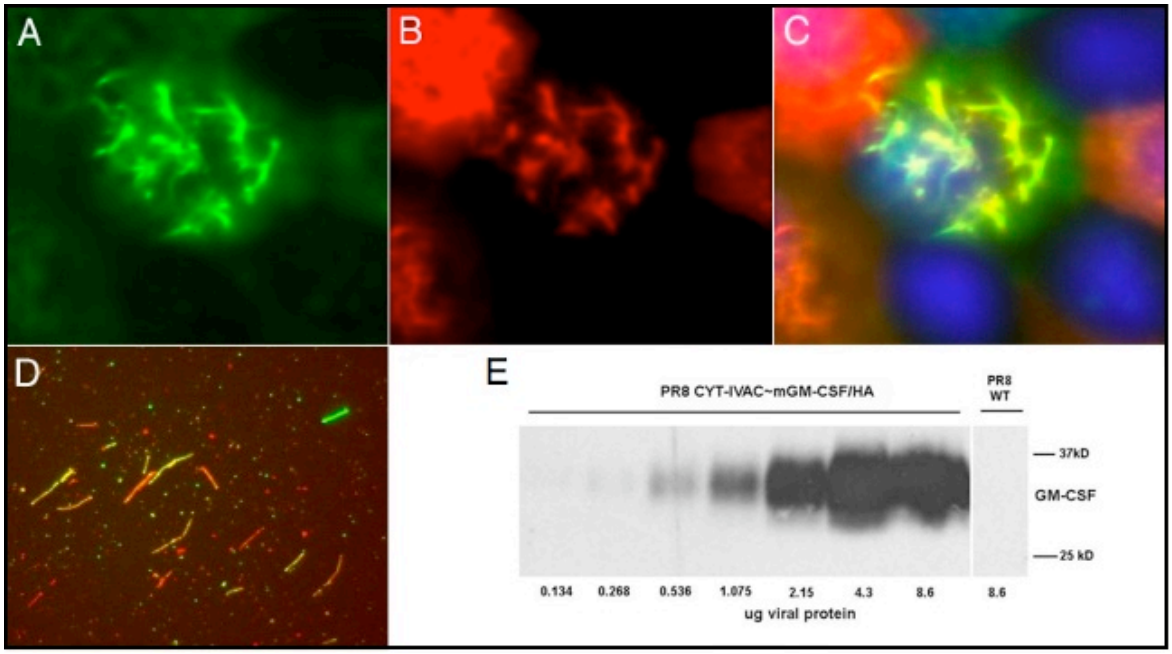


Figure 7. Membrane-bound immunomodulators are incorporated during budding and release of virions from influenza virus infected cells. MDCK CYT-IVAC producer cells infected with filamentous influenza virus A/Udorn/72 were stained at 8 hr post-infection with antibodies specific for mGM-CSF (A, green) and hemagglutinin (B, red). Images A and B are overlaid to depict co-localization of mGM-CSF and full-length HA to budding viral filaments (C). Free virus particles collected from supernatants of infected CYT-IVAC producer cells stained for GM-CSF and HA as described above (D). Western blot of gradient purified virus derived from GM-CSF~HA expressing MDCK cells or wild-type MDCK cells (E) and probed for the presence of GM-CSF.

3.5 Characterization of the cytokine and hemagglutinin content of CYT-IVACs.

To further characterize cytokine incorporation and evaluate full-length HA content, virus harvested from infected producer cells was double gradient purified and inactivated with ß-propiolactone. Complete virus inactivation was confirmed using a tissue culture infectious dose assay, which monitors virus induced cytopathicity or production of hemagglutinating virus particles. None of the inactivated CYT-IVACs (5 µg of purified virus) resulted in the production of hemagglutinating virus particles or cytopathic effect in wild-type MDCK cells over a five day monitoring period. Western blot analysis and slot blot assays were performed on gradient purified CYT-IVACs to further verify cytokine incorporation and to quantitate the total amount of virus-incorporated cytokine, respectively. In addition, the HA content of gradient purified wild-type and CYT-IVAC vaccine preparations was evaluated using slot blot and hemagglutination assays to rule out any potential adverse effects on packaging of full-length viral HA. The presence of mGM-CSF was detected only in progeny virions harvested from A/PR/8/34 infected mGM-CSF~HA producer MDCK cells and not in virions collected from A/PR/8/34 infected wild-type MDCK cells (Figure 7 E). GM-CSF was detectable in as little as 0.268 µg of total viral protein. The predicted molecular weight of the mGM-CSF~HA construct is 24 kilodaltons however, the actual molecular weight was determined to be approximately 32 kilodaltons. This divergence from the predicted molecular weight may be attributed to two known N-linked and one proposed O-linked glycosylation sites of murine GM-CSF [145, 146]. Using standard curves derived from slot blots of recombinant GM-CSF, IL-2 or IL-4, we were able to quantitate the amount of virus-incorporated cytokine for each CYT-IVAC (Table 2). The GM-CSF and IL-4-bearing CYT-IVACs incorporated relatively high levels of membrane-bound cytokines, 185 ng GM-CSF and 176 ng IL-4 per µg of vaccine respectively, compared to the IL-2-bearing CYT-IVAC, only 4.924 ng IL-2 per µg of vaccine. Due to lack of a suitable HA standard for A/PR/8/34 hemagglutinin, we were unable to precisely quantitate the viral HA content. However, we were able to

compare the relative HA amounts based on optical density scans of western or slot blot assays in which equal amounts of purified viral protein were loaded. Using this approach, the HA content across vaccine preparations did not differ significantly when equal amounts of viral protein were probed with either monoclonal or polyclonal antibodies specific for H1 hemagglutinin (Table 2). Additionally, hemagglutination units per μg of viral protein for wild-type and CYT-IVAC vaccines did not differ significantly, indicating comparable relative full-length and functional HA content for wild-type and CYT-IVAC vaccines (Table 2).

Table 2. Characterization of CYT-IVAC hemagglutinin and cytokine content

Vaccine	HA pixel density*	HAU/μg of vaccine	Total cytokine (ng/ug vaccine)**	Bioactive cytokine (pg/ug vaccine)***
PR/8/34 w.t.	5835.4	16	NA	NA
PR/8/34 GM-CSF~HA	6407.9	16	185±21	87.3
PR/8/34 IL-2~HA	5562.9	32	4.92±0.3	411
PR/8/34 IL-4~HA	6090.4	32	176±24	456

* Pixel density of HA specific chemiluminescent signal following equal loading of total viral protein

** Quantitation of virus-incorporated cytokine on protein level based on standard curve of recombinant cytokine (ng of cytokine per ug of vaccine)

*** Quantitation of virus-incorporated cytokine on bioactive level based on standard curve of recombinant cytokine (pg of cytokine per ug of vaccine)

In these latter studies, influenza virus A/PR/8/34, a spherical particle-producing virus, was used to prepare vaccines. Thus, incorporation of membrane-bound cytokine is neither restricted to a morphological phenotype nor a particular influenza virus subtype. Additional studies in our laboratory have further confirmed membrane-bound cytokine incorporation using H6N2 avian strains of influenza virus for the infection (data not shown).

3.6 CYT-IVAC bioassays.

Inactivated CYT-IVACs were subsequently analyzed by bioassay using the appropriate indicator cells. Wild-type inactivated virus harvested from vector control MDCK cells was used as a negative control and proliferation was monitored by reduction of Alamar Blue®. Alamar Blue® is a safe, non-radioactive alternative to ^{3}H-thymidine and it has been proven to be as sensitive and reproducible, in proliferation assays, as ^{3}H-thymidine [147]. As described by Ahmed et. al. (1994),

Alamar Blue® contains a oxidation-reduction indicator which proliferating cells reduce causing the media to change color from blue (oxidized) to red (reduced). Reduction of Alamar Blue® can be quantitated by measuring the absorbance of the media at 570 nm and 600 nm, reduced state and oxidized state respectively, and the difference between these two absorbencies correlates directly with proliferation. As depicted in Figures 8, 9 and 10 respectively, CYT-IVACs bearing mGM-CSF~HA, mIL-2~HA, and mIL-4~HA, all retained their bioactivity following ß-propiolactone inactivation inducing significant proliferation of their respective indicator cell lines compared to wild-type inactivated virus. In addition to the above-mentioned quantitation of virus-incorporated cytokine by slot blot assays, we

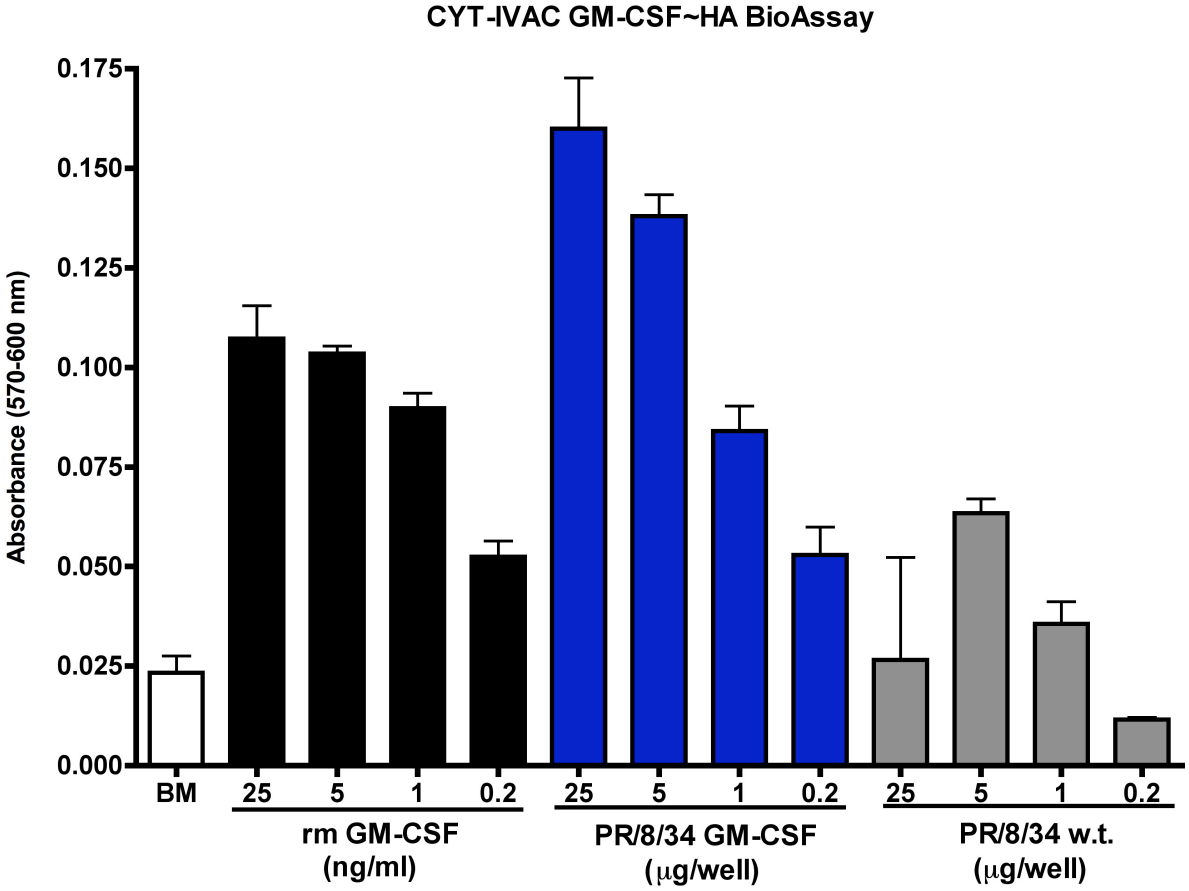


Figure 8. Membrane-bound immunomodulators retain bioactivity following viral inactivation. Bone marrow cells (BM), as GM-CSF specific indicator cells, were incubated with decreasing concentrations of ß-propiolactone inactivated wild-type vaccine or GM-CSF CYT-IVAC. Proliferation was determined by Alamar Blue® reduction. Recombinant murine GM-CSF was used as the positive control.

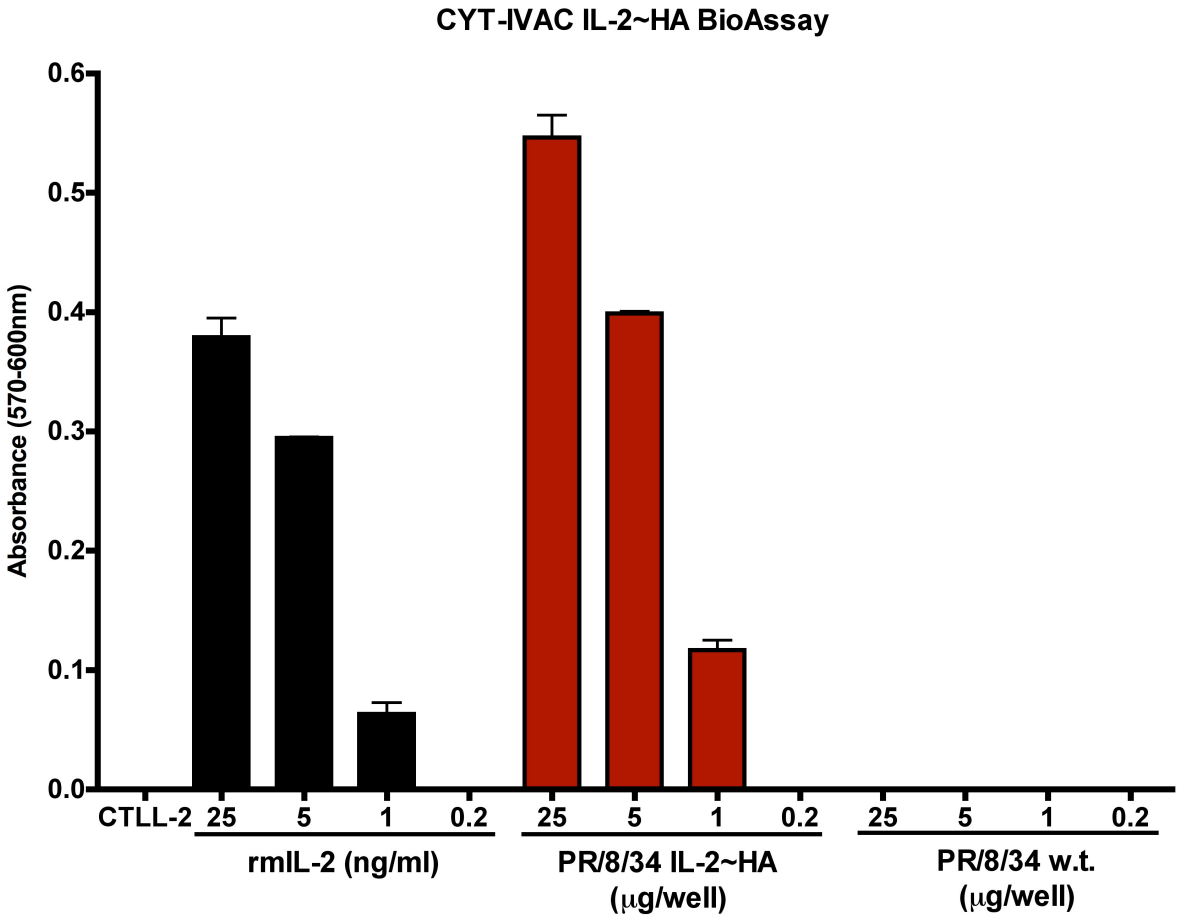


Figure 9. Membrane-bound immunomodulators retain bioactivity following viral inactivation. CTLL-2 cells, as IL-2 specific indicator cells, were incubated with decreasing concentrations of ß-propiolactone inactivated wild-type vaccine or IL-2 CYT-IVAC. Proliferation was determined by Alamar Blue® reduction. Recombinant murine IL-2 was used as the positive control.

thought it necessary to quantitate the biologically active membrane-bound cytokine to better indicate the dose of cytokine delivered during vaccination. Despite the relatively low level of virus-incorporated IL-2 compared to IL-4, the amount of biologically active IL-2 and IL-4 present in the respective CYT-IVACs was comparable at 0.411 ng IL-2 and 0.456 ng IL-4 per µg of vaccine, respectively (Table 2). In contrast, the amount of bioactive membrane-bound GM-CSF for the GM-CSF CYT-IVAC was considerably lower (87.3 pg per µg of vaccine) despite the relatively high level of virus-incorporated GM-CSF as determined by the slot blot assay (Table 2).

To verify that positive bioassays were due to the presence of bioactive cytokines we included non-specific CYT-IVACs and cytokine-neutralizing

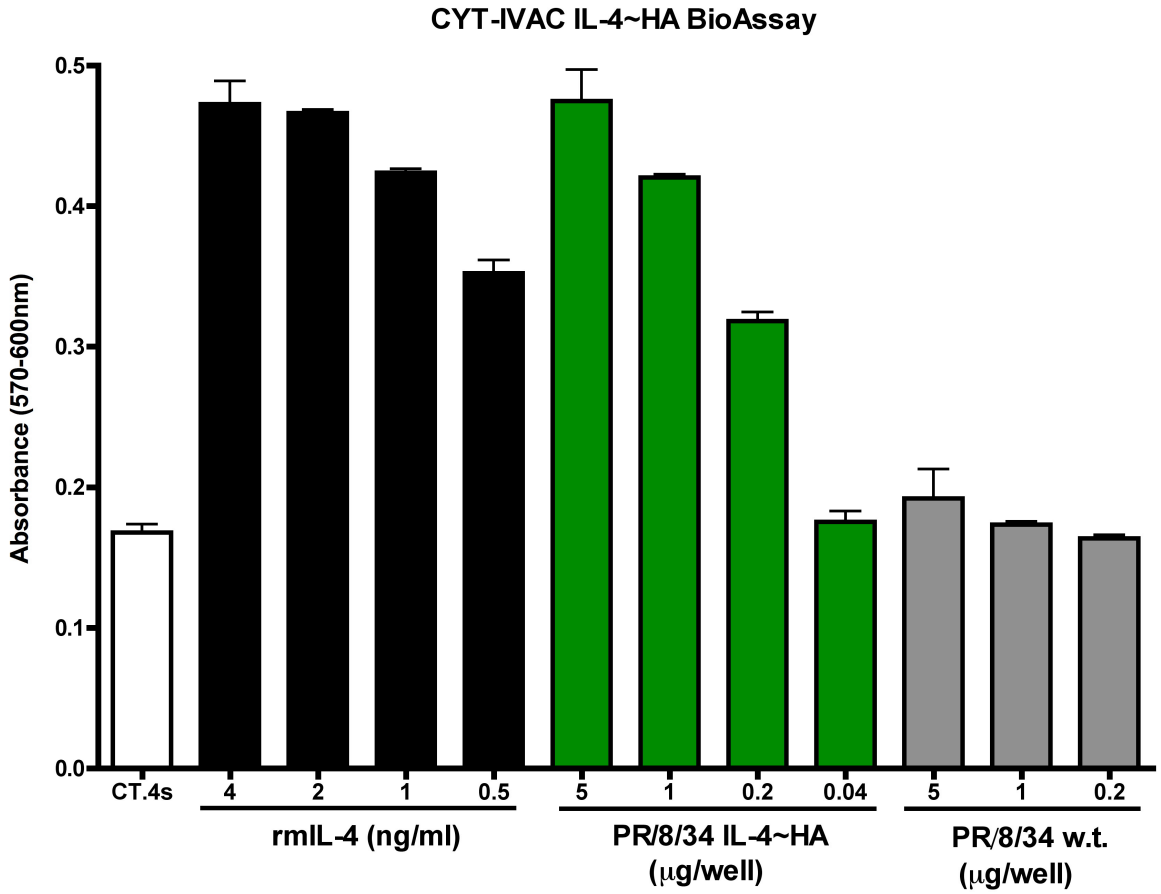


Figure 10. Membrane-bound immunomodulators retain bioactivity following viral inactivation. CT.4s cells, as IL-4 specific indicator cells, were incubated with decreasing concentrations of ß-propiolactone inactivated wild-type vaccine or IL-4 CYT-IVAC. Proliferation was determined by Alamar Blue® reduction. Recombinant murine IL-4 was used as the positive control.

antibodies in our evaluation. The IL-2 and IL-4 bioassays were shown to be specific for their respective cytokines as the IL-4 CYT-IVAC failed to induce significant proliferation of IL-2 dependent CTLL-2 cells (Figure 11 A) and similarly, the IL-2 CYT-IVAC failed to induce the proliferation of IL-4 dependent CT.4s cells (Figure 11 B). Furthermore, the addition of neutralizing anti-IL-2 antibodies to the culture media reduced proliferation of IL-2 CYT-IVAC stimulated CTLL-2 cells in a dose dependent manner (Figure 12). Several inactivation methods were considered when deciding how to inactivate CYT-IVACs and still maintain bioactivity. While all inactivation methods tested (heat, UV, formalin, ß-propiolactone) preserved GM-CSF bioactivity, this was not the case for all membrane-bound cytokines (Figure 13). ß-propiolactone consistently preserved

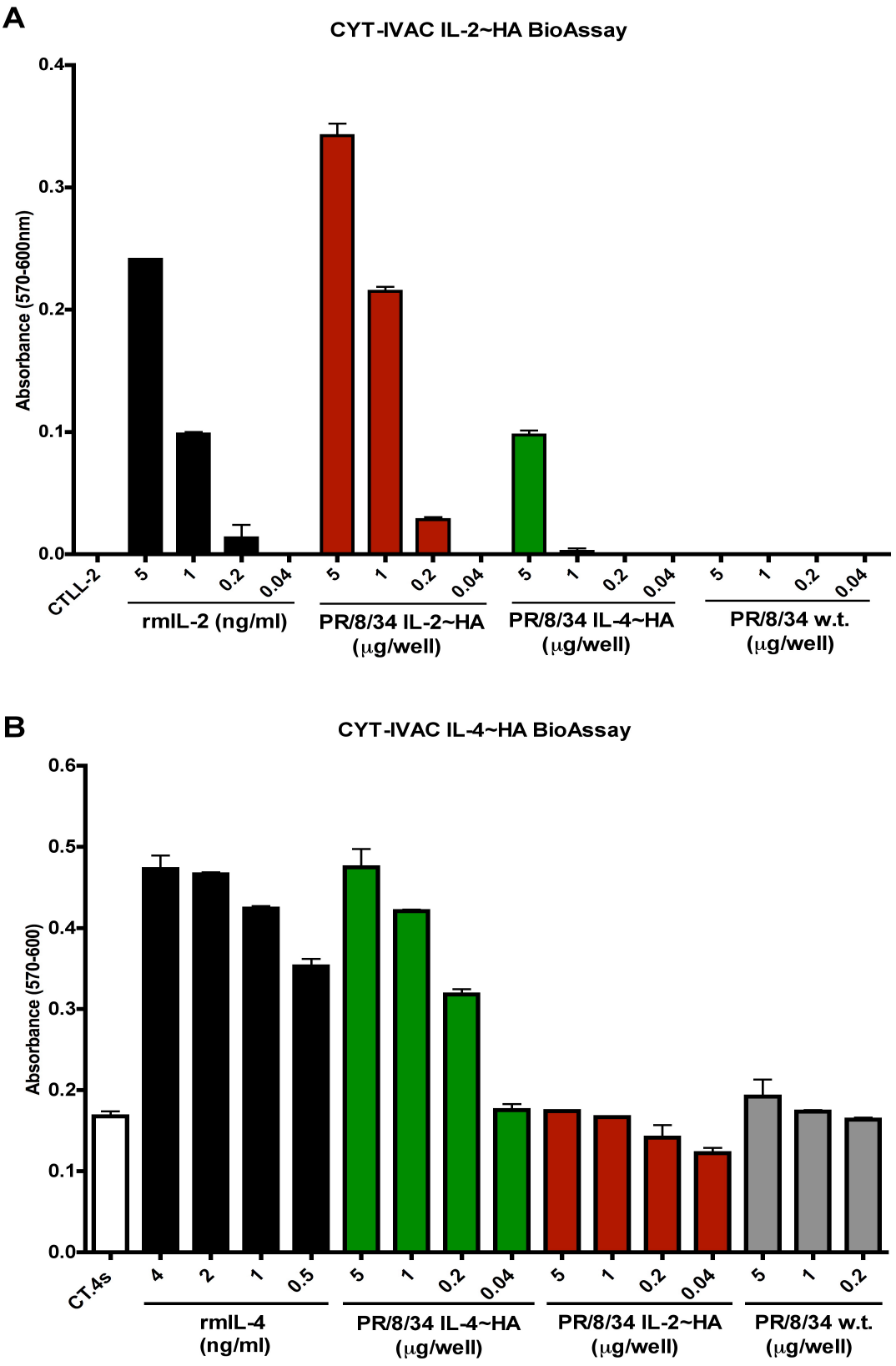


Figure 11. Proliferation induced by CYT-IVACs is specific and dependent on respective membrane-bound cytokine. Proliferation of cytokine responsive cell lines CTLL-2 (A) and CT. 4s (B) was measured following incubation with ß-propiolactone inactivated wild-type PR/8/34 or mIL-2 or mIL-4 bearing CYT-IVACs. Proliferation was determined by Alamar Blue® reduction. Recombinant protein was used as a positive control.

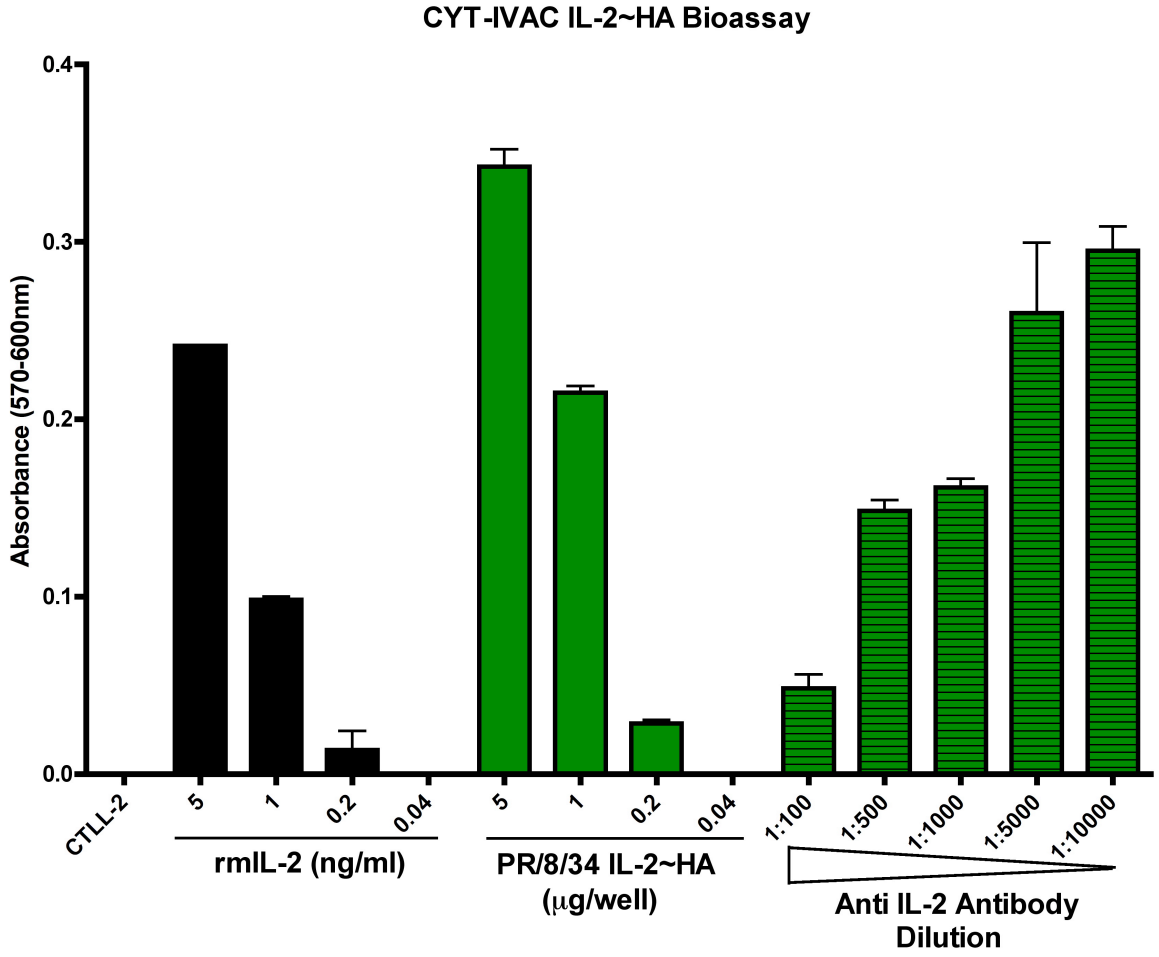


Figure 12. Proliferation induced by CYT-IVACs is specific and dependent on respective membrane-bound cytokine. Proliferation of CTLL-2 cells was measured following incubation with ß-propiolactone inactivated mIL-2 bearing CYT-IVACs with or without anti-mIL-2 neutralizing antibodies, at decreasing concentrations. Proliferation was determined by Alamar Blue® reduction. Recombinant protein was used as a positive control.

cytokine bioactivity for all constructs tested, therefore, it was selected as the inactivation method of choice for subsequent vaccine studies. CYT-IVACs can be stored long term (> 1 year) at 4°C without significant loss of bioactivity and furthermore, membrane-bound cytokines retain bioactive function following storage at -80°C (Figure 14) demonstrating there preserved functionality after freezing.

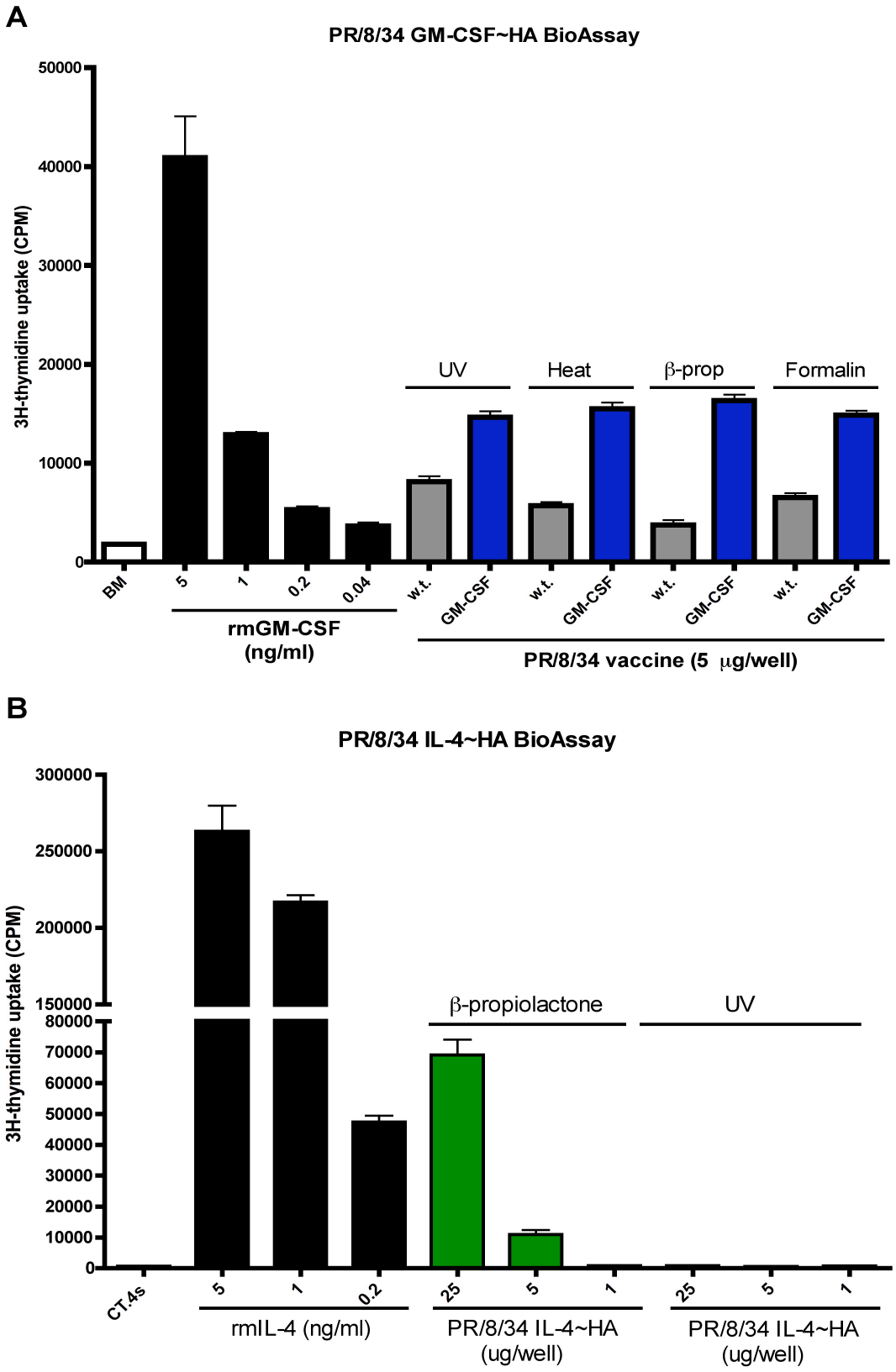


Figure 13. Inactivation methods for preserving membrane-bound cytokine bioactivity. Cytokine responsive cell lines were incubated with wild-type vaccine and mGM-CSF CYT-IVAC (A) or mIL-4 CYT-IVAC (B) inactivated using UV, heat, ß-propiolactone, and formalin (A) or ß-propiolactone and UV (B) inactivation methods. Proliferation of cell lines was evaluated using ^{3}H-thymidine incorporation. Recombinant protein was used as a positive control.

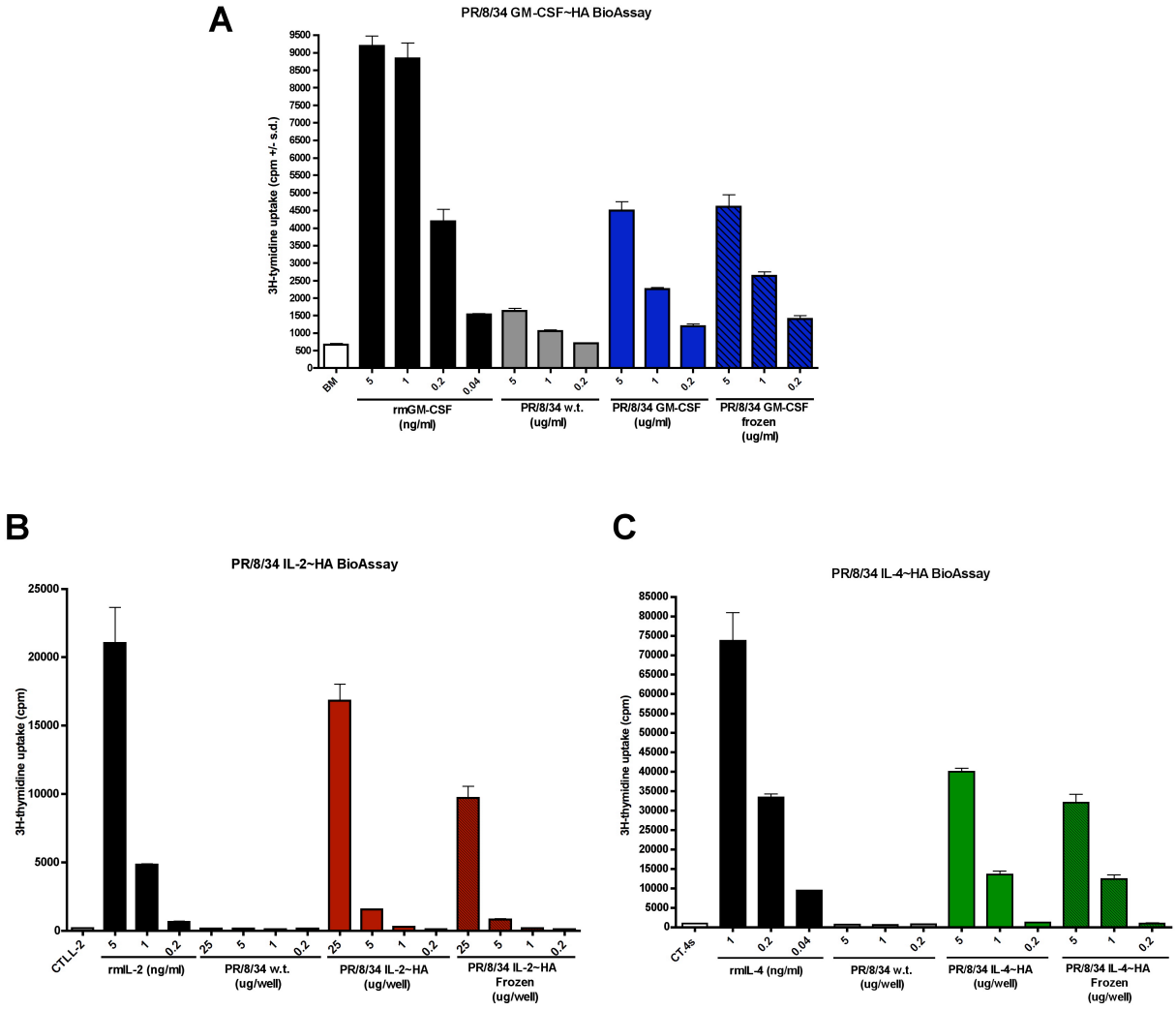


Figure 14. Membrane-bound cytokine bioactivity is preserved following storage at -80°C. Cytokine responsive cell lines were incubated with ß-propiolactone inactivated wild-type vaccine, mGM-CSF CYT-IVAC (A) mIL-2 CYT-IVAC (B) or mIL-4 CYT-IVAC (C) with or without freezing at -80°C. Cellular proliferation was evaluated using ^{3}H-thymidine incorporation. Recombinant protein was used as a positive control.

Aim 2: Evaluation of CYT-IVACs ability to enhance protection against challenge and induce humoral and cellular immunity.

After demonstrating the feasibility of producing CYT-IVACs it was necessary to determine if cytokines in their membrane-bound form offered any beneficial adjuvant properties for vaccination against influenza. We considered several methodologies to evaluate the adjuvant potential of membrane-bound

immunomodulators and decided to evaluate protection against lethal challenge. We chose to vaccinate with a single dose, either intranasally or subcutaneously, to determine if membrane-bound cytokines offered protection following a single vaccination without the need for a booster vaccine. We also chose to vaccinate with a suboptimal dose that offered protection to only 20 percent of wild-type vaccinated mice so as to illustrate even minute adjuvant effects afforded by CYT-IVACs. As stated earlier, cellular mediated immunity is important for fighting off influenza infections and induction of cellular mediated immunity is a desirable characteristic for influenza vaccine candidates. Classically, inactivated vaccines fail to induce cellular mediated immunity and we were optimistic that CYT-IVACs my generate some form of influenza specific cellar immunity by skewing the immune response toward a T_h1-type immune response as opposed to a T_h2-type response typically seen with conventional inactivated influenza vaccines. To strengthen any argument that CYT-IVACs skew the immune response to a T_h1-type response we chose to use the Balb/c model of influenza vaccination and infection because this strain of mouse is known to generate T_h2 mediated immunity when vaccinated with inactivated influenza vaccines [148-155]. This method of evaluating vaccine efficacy would allow us to rapidly screen many CYT-IVACs and identify those immunomodulators that afford superior protection compared to conventional inactivated whole-virus influenza vaccine.

3.7 Establishment of Minimal Protective Dose 20 for wild-type influenza vaccine.

To illustrate the adjuvant potential of membrane-bound cytokines it was necessary to define a suboptimal dose for vaccination in order to highlight minute enhancements in vaccine efficacy afforded by membrane-bound cytokines. Importantly, we chose not to include a boosting dose so that we could determine whether single dose vaccination with CYT-IVACs offered more protection than wild-type vaccine. We established the MPD_{20} for both crude vaccine preparations (single gradient purified vaccine) and ultra-purified vaccine preparations (double gradient purified vaccine). Groups of female, Balb/c mice were vaccinated with a

single dose of differing amounts of inactivated, non-adjuvanted wild-type influenza vaccine either intranasally or subcutaneously. Mice were challenged with a 100 LD_{50} of mouse-adapted influenza A/PR/8/34 on day 35 post-vaccination. Weight loss and survival were monitored until completion of the study. As with all studies evaluating survival, a mouse succumbed to infection, to the point of death, if a mouse experienced 25% weight loss for two consecutive days and was thereafter euthanized.

To establish the MPD_{20} for crude vaccine preparations (single-gradient purified) mice were vaccinated either intranasally or subcutaneously with 10, 5, 2.5 or 1 μg of single gradient purified wild-type vaccine. Fifty percent of mice vaccinated intranasally with 5 μg of vaccine survived lethal challenge and none of the mice vaccinated intranasally with 2.5 μg were protected (Figure 15 A). The MPD_{20} for intranasal vaccination using crude vaccine preparations was determined to be 3.75 μg and this dose was used for preliminary studies evaluating CYT-IVACs. Surprisingly, all mice vaccinated subcutaneously, regardless of the dose, survived lethal challenge (Figure 15 B). Based on these preliminary findings, the MPD_{20} for crude vaccine preparations was known to be less than 1 μg and we estimated the MPD20 for subcutaneous vaccination with single gradient purified vaccine to be 0.2 μg. This dose was used for preliminary studies to evaluate CYT-IVACs.

To determine the MPD_{20} of ultra-purified vaccine (double-gradient purified), mice were vaccinated intranasally with 3, 2, 1 or 0.5 μg or subcutaneously with 0.5, 0.25, 0.125 or .06125 μg of double gradient purified wild-type vaccine. Mice vaccinated intranasally with 3, 2, or 1 μg of vaccine demonstrated 100, 90 and 60 percent protection, respectively (Figure 15 C). Only 20% of mice vaccinated intranasally with 0.5 μg survived. Because the two surviving mice in the 0.5 μg group and two of the surviving mice in the 1 μg group did not show signs of illness it is possible that they received a sub-lethal dose of mouse adapted virus, therefore, the MPD_{20} for intranasal vaccination likely lies between 0.5 and 1 μg. Upon subsequent studies using 0.8 μg of ultra-purified wild-type vaccine, we

learned that none of the mice were protected against lethal challenge. Therefore, we calculated the MPD_{20} for intranasal vaccination with ultra-purified vaccine to be 1 µg and this dose was used for subsequent vaccine studies. Only 40% of mice vaccinated subcutaneously with 0.5 µg of wild-type vaccine survived lethal challenge whereas 10% of mice vaccinated with 0.25 µg survived (Figure 15 D). The MPD_{20} for subcutaneous vaccination with ultra-purified vaccine was determined to be 0.375 µg and this dose was used for subcutaneous vaccination in subsequent studies.

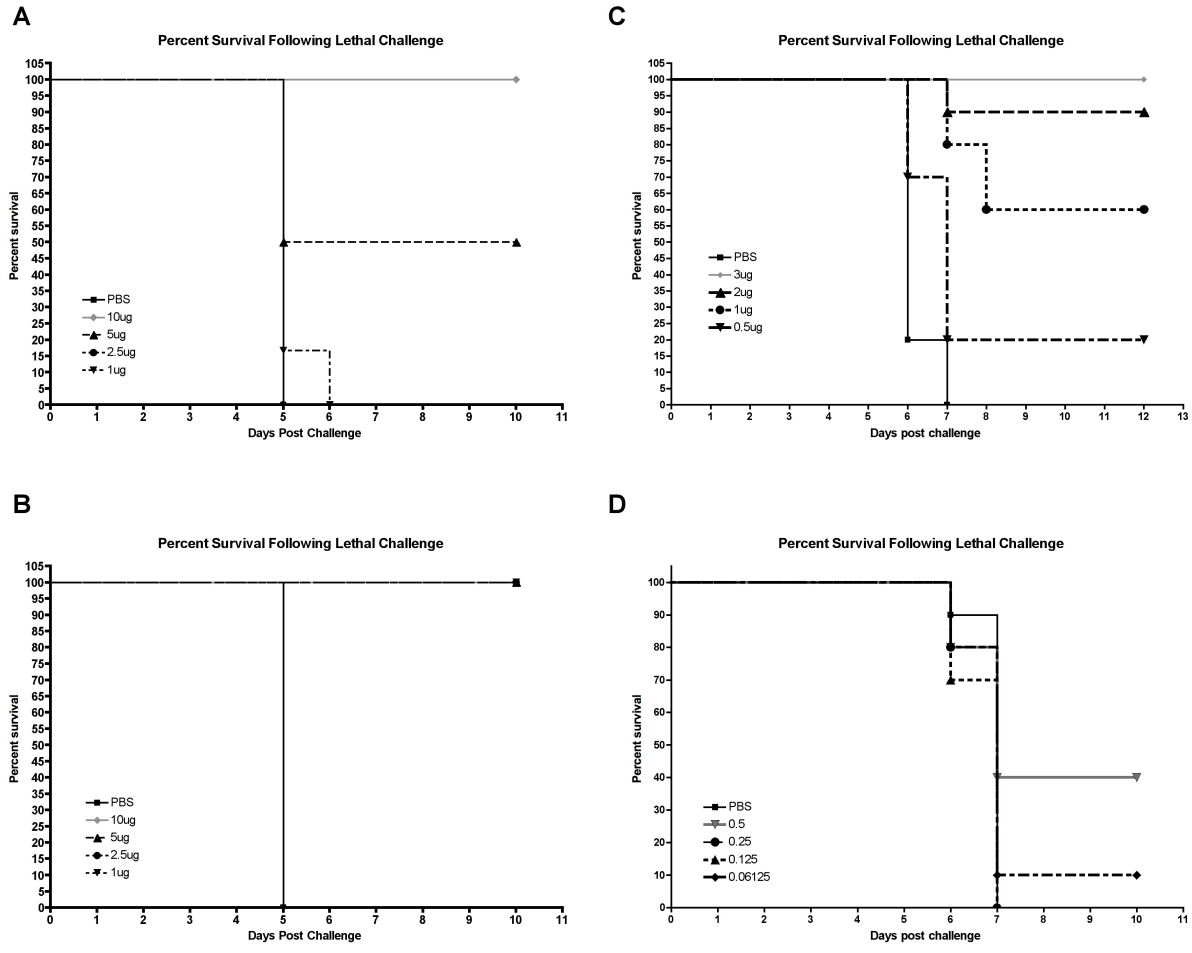


Figure 15. Minimal Protective Dose 20 (MPD20) for wild-type influenza vaccine that protects 20% of vaccinated mice from lethal challenge. Groups of Balb/c mice (n=10) were vaccinated intranasally (A,C) or subcutaneously (B,D) with decreasing doses of non-adjuvanted, inactivated wild-type PR/8/34 vaccine, crude vaccine preparations (A,B) or ultra-purified vaccine preparations (C,D). PBS served as negative vehicle control. Mice were challenge day 35 post-vaccination with 100 LD_{50} of mouse-adapted A/PR/8/34 and survival was monitored over time

3.8 CYT-IVACs enhance serum anti-viral antibodies and skew immune response toward T_h1.

To evaluate the adjuvant potential of our CYT-IVACs, we vaccinated groups of Balb/c mice (8-12 weeks) with a MPD_{20} dose (previously described) of CYT-IVACs or wild-type vaccine, administered either intranasally (i.n.) or subcutaneously (s.c.). A representative vaccine protocol is provided in Appendix F. It should be noted that no adjuvant, other than the particulate matter of the vaccine itself or the incorporated cytokine, was administered. Blood was collected from mice at day 21 post-vaccination and serum was evaluated by ELISA against whole viral antigens to determine elicited anti-viral antibody titers. Early studies using single gradient purified vaccine (Figure 16 A) demonstrated that intranasal vaccination with mGM-CSF and mIL-4 bearing CYT-IVACs significantly enhanced influenza specific total serum IgG antiviral antibody end-titers compared to wild-type vaccination. An increase in antibody end-titer was also seen in mice vaccinated intranasally with mIL-2 bearing CYT-IVAC compared to the wild-type vaccine group, albeit not significant ($p > 0.05$). Following subcutaneous vaccination with single gradient purified vaccine, we did not detect any discernable differences in influenza specific antibody titers between vaccine groups (Figure 16 B). To further characterize the immune response elicited by CYT-IVACs we determined antibody titers for influenza specific IgG_1 to IgG_{2a} by ELISA. It is well established that a high IgG_{2a} titer relative to IgG_1 is indicative of a T_h1 mediated immune response whereas a high IgG_1 titer relative to IgG2a is indicative of a predominately T_h2-type response. In early experiments using single gradient purified vaccine preps, intranasal vaccination with wild-type vaccine resulted in a high IgG_1 titer which is the typical immune response elicited by inactivated influenza vaccines in Balb/c mice, which are skewed towards T_h2-type responses (Figure 16). In contrast, intranasal vaccination with mIL-2 bearing CYT-IVAC resulted in elevated IgG_{2a} titers indicative of isotype class switching and T_h1 mediated immunity (Figure 16 A). No significant differences in IgG_1 or IgG_{2a} titers were observed between mice vaccinated subcutaneously with wild-type vaccine or

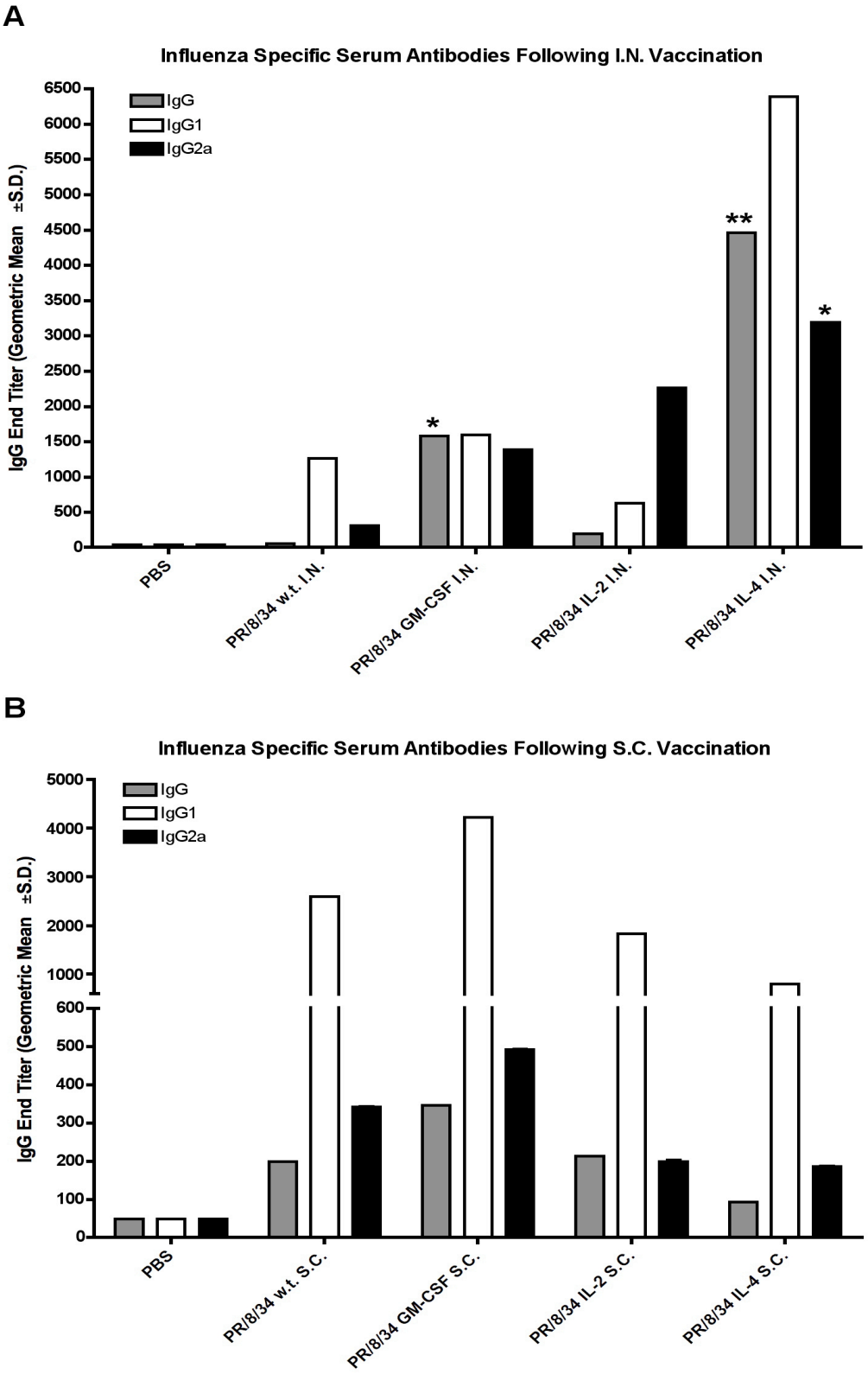


Figure 16. Inactivated influenza vaccines bearing membrane-bound immunomodulators enhance serum anti-viral antibody titers. Balb/c mice were vaccinated intranasally (A) or subcutaneously (B) with 3.5 or 0.2 μg, respectively, of crude preparations of A/PR/8/34 wild-type (n=10) or A/PR/8/34 bearing membrane-bound GM-CSF (n=10), IL-2 (n=10), and IL-4 (n=10). PBS served as negative vehicle control. Serum was collected on day 21 post-vaccination and antibody titers for influenza virus specific IgG and isotypes IgG1 (Th2) and IgG2a (Th1) were determined by ELISA. Data is displayed as the geometric mean end titer for each group. (* $p < 0.05$ compared to PR/8/34 w.t., ** $p < 0.01$ compared to PR/8/34 w.t.)

CYT-IVACs and the IgG_1 isotype was the dominant isotype detected across all vaccine groups.

We postulated that ultra-purified vaccine preparations would better demonstrate the adjuvant properties of CYT-IVACs by further removing any contaminates that could adversely affect the outcome of the vaccine efficacy studies through introducing unknown and uncontrollable variables. Furthermore, by removing contaminates we could be sure that the wild-type vaccine preparations contained only the particulate matter of the influenza virus itself and that the only adjuvant present in the CYT-IVAC preparations was the membrane-bound cytokine. Subsequent studies using ultra purified vaccine partially confirmed previous findings in that intranasal vaccination with mIL-4 bearing vaccine resulted in elevated, albeit not significant, influenza specific total serum IgG antiviral antibody levels compared to wild-type vaccine (Figure 17 A). Subcutaneous vaccination with double gradient purified vaccine resulted in significantly higher titers ($p < 0.01$) of influenza specific serum IgG for mice vaccinated with the mIL-2 CYT-IVAC compared to wild-type vaccinated mice (Figure 17 B). Evaluation of influenza specific IgG_1 and IgG_{2a} following intranasal vaccination demonstrated that all vaccine groups had low IgG_1 to IgG_{2a} ratios indicating a skewing towards a T_h1 type humoral response. This is unlike earlier studies in which only the IL-2 CYT-IVAC mice were skewed towards T_h1 mediated immunity (Figure 16 A). Mice vaccinated subcutaneously with either the mIL-2 CYT-IVAC or the mIL-4 CYT-IVAC had significantly higher IgG_{2a} titers compared to wild-type vaccinated mice (Figure 17 B). Although significantly higher IgG_1 titers were detected in IL-2 CYT-IVAC vaccinated mice compared to wild-type vaccinated mice, the IgG_{2a} isotype remained the predominate influenza specific isotype detected in serum samples collected from mIL-2 or mIL-4 CYT-IVAC vaccinated mice, indicating a skewing towards a T_h1 immune response.

It is important to note that there was no direct correlation between elevated antibody titers and protection when evaluated on a mouse-by-mouse basis. That is, mice with high influenza specific antibody titers were not necessarily protected following lethal challenge and several mice from the IL-2 and IL-4 CYT-IVAC

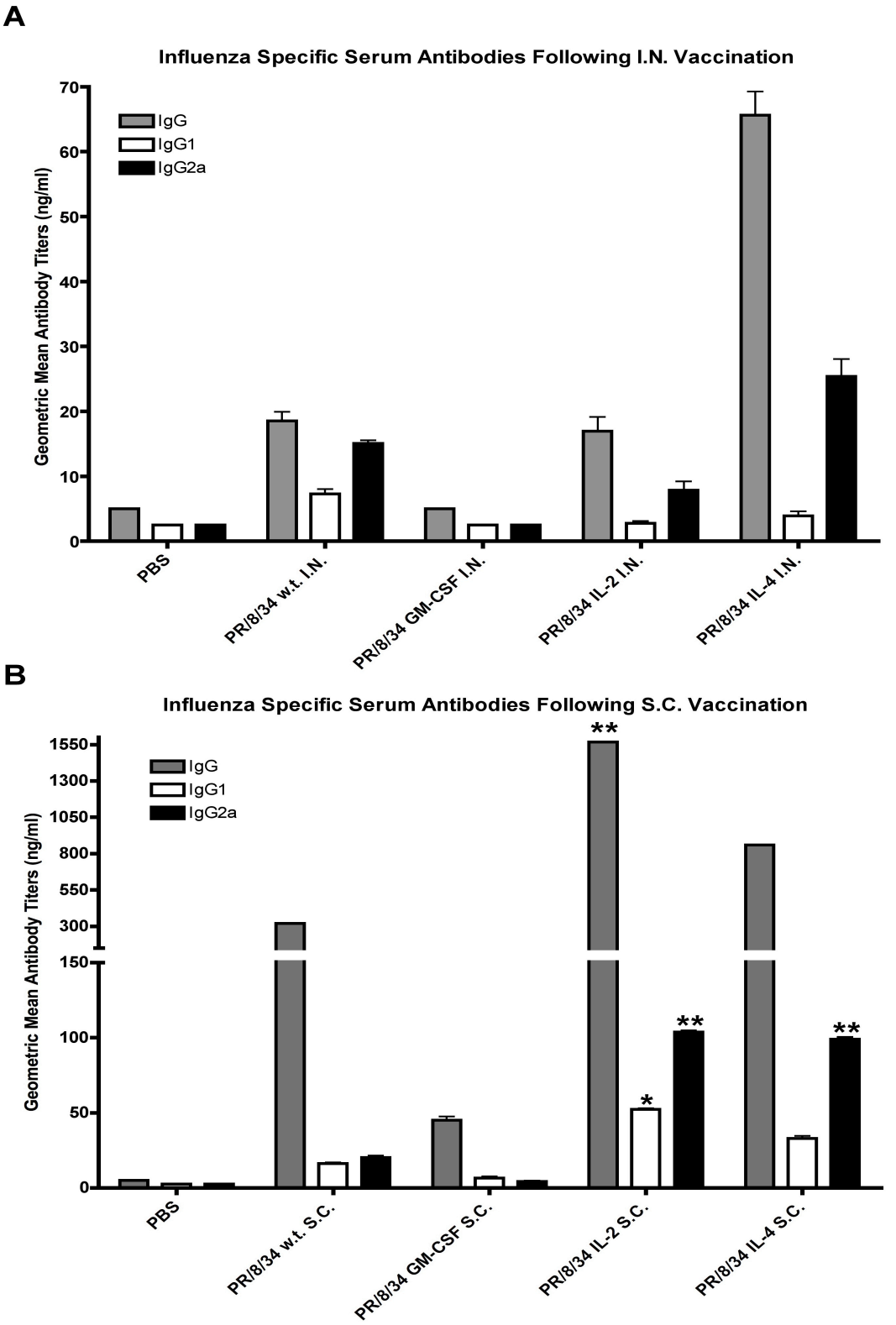


Figure 17. Inactivated influenza vaccines bearing membrane-bound immunomodulators enhance serum anti-viral antibody titers. Balb/c mice were vaccinated intranasally (A) or subcutaneously (B) with 1 or 0.375 μg, respectively, of double-gradient purified A/PR/8/34 wild-type (n=20) or A/PR/8/34 bearing membrane-bound GM-CSF (n=10), IL-2 (n=19), and IL-4 (n=20). PBS served as negative vehicle control. Serum was collected on day 21 post-vaccination and antibody titers for influenza virus specific IgG and isotypes IgG1 (Th2) and IgG2a (Th1) were determined by ELISA. Data is displayed as the geometric mean titer in ng/ml of serum for each group. (* $p < 0.05$ compared to PR/8/34 w.t., ** $p < 0.01$ compared to PR/8/34 w.t.)

groups, which displayed low seroconversion titers survived lethal challenge. We were unable to detect neutralizing antibodies in any of the serum samples, however, neutralizing immune responses were clearly evoked upon challenge as viral loads were significantly reduced in the IL-2 and IL-4 CYT-IVAC vaccinated animals at day 4 post-challenge (see Figure 20).

3.9 Vaccination with CYT-IVACs results in enhanced protection against lethal influenza virus challenge.

The most compelling evidence supporting the immunostimulatory or immunomodulatory properties of CYT-IVACs was the protection against lethal challenge. Here, mice vaccinated with a single MPD_{20} dose were challenged on day 35 post-vaccination with a lethal dose of homotypic mouse-adapted influenza A/PR/8/34 (100 LD_{50}). Weight loss and survival were monitored following challenge. Early studies using crude preparations demonstrated that mice vaccinated intranasally (3.75 μg) with mIL-2 or mIL-4 bearing-CYT-IVACs exhibited less weight loss following lethal challenge compared to mice vaccinated with wild-type, non-cytokine bearing vaccine (Figure 18 A). These groups of mice also exhibited higher survival rates than wild-type vaccinated mice (Fig 18 B). Only 50 percent of mice vaccinated with wild-type vaccine survived past day 5, whereas 83 percent of mice receiving mIL-2 or mIL-4 bearing CYT-IVACs survived challenge. Despite inducing significantly higher influenza specific IgG titers, vaccination with the mGM-CSF-bearing CYT-IVAC did not afford better protected against lethal challenge compared to wild-type (Figure 18 B). Weight loss in mice vaccinated subcutaneously (0.2 μg) with the mIL-2 or mIL-4-bearing CYT-IVAC was delayed and reduced compared to wild-type vaccinated mice (Figure 18 C). All mice vaccinated subcutaneously with wild-type vaccine succumbed to infection by day 5, but 50 percent of mice vaccinated with mIL-2 bearing CYT-IVAC survived lethal challenge (Figure 18 D). No significant increase in protection from lethal challenge was observed for those mice vaccinated with the mGM-CSF or mIL-4 bearing CYT-IVACs (Figure 18 D) in these early studies using crude preparations of inactivated virus vaccine.

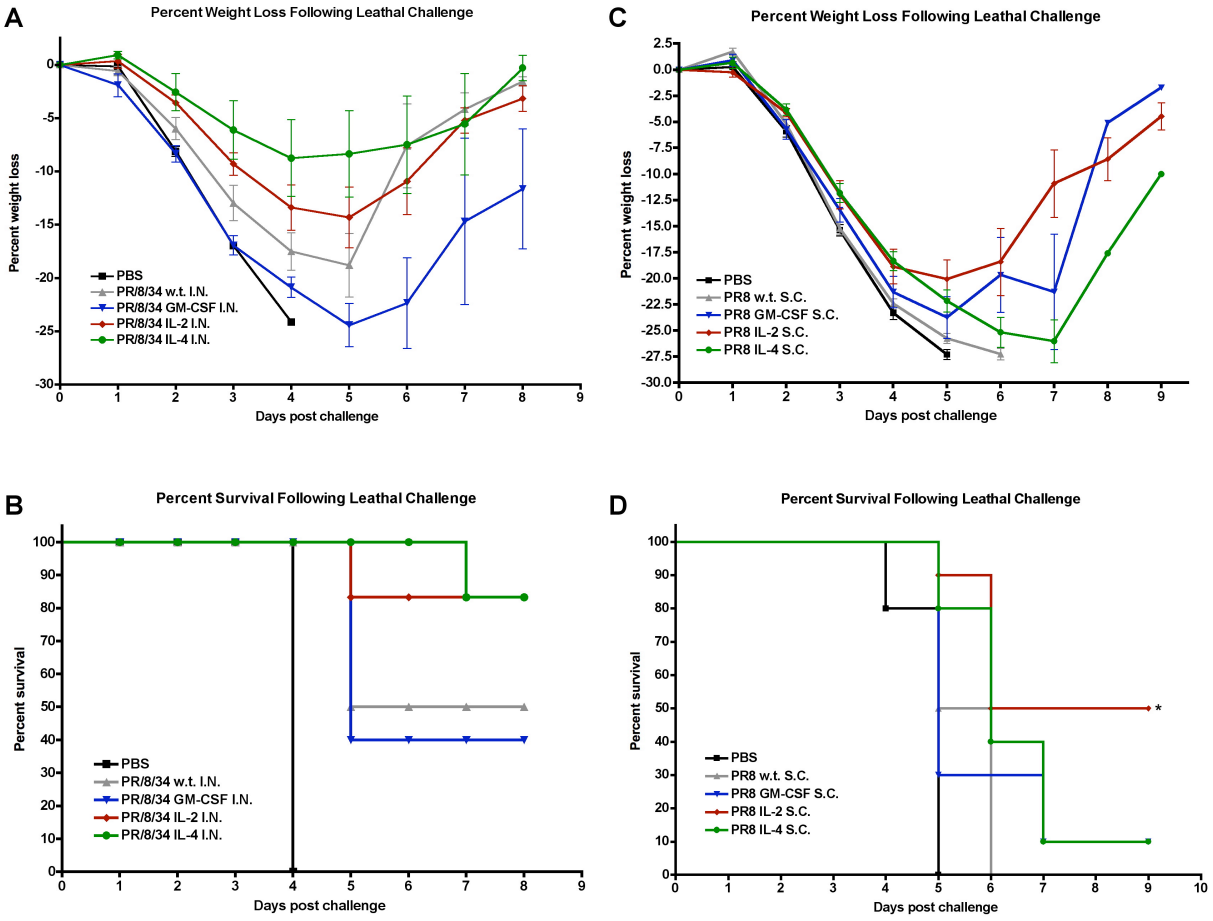


Figure 18. Inactivated influenza vaccines bearing membrane-bound immunomodulators protects mice against lethal challenge. Balb/c mice (N=10) were challenged with 100 LD_{50} of mouse-adapted A/PR/8/34 on day 35 post vaccination. Mice were vaccinated intranasally with 3.5 μg (A, B) or subcutaneously with 0.2 μg (C, D) of inactivated wild type vaccine or CYT-IVACs bearing membrane-bound GM-CSF, IL-2, or IL-4 using crude vaccine preparations. PBS served as negative vehicle control. Percent weight change (A, C) and survival (B, D) was monitored over time.

Later studies evaluating ultra-purified vaccine preparations provided clearer support for the adjuvant properties of CYT-IVACs. Mice were vaccinated intranasally (1 μg) or subcutaneously (0.375 μg) with ultra-purified vaccine preparations (double gradient purified) and subsequently challenged 35 days later with 100 LD_{50}. Mice vaccinated intranasally with the mIL-4 CYT-IVAC (n=10) experienced less weight loss compared to wild-type (n=10) vaccinated mice (Figure 19 A) and 20 percent of mIL-4 CYT-IVAC vaccinated mice survived lethal challenge while all wild-type vaccinated mice succumbed to infection (Figure 19 B). Weight loss in mice vaccinated subcutaneously with wild-type vaccine or mGM-

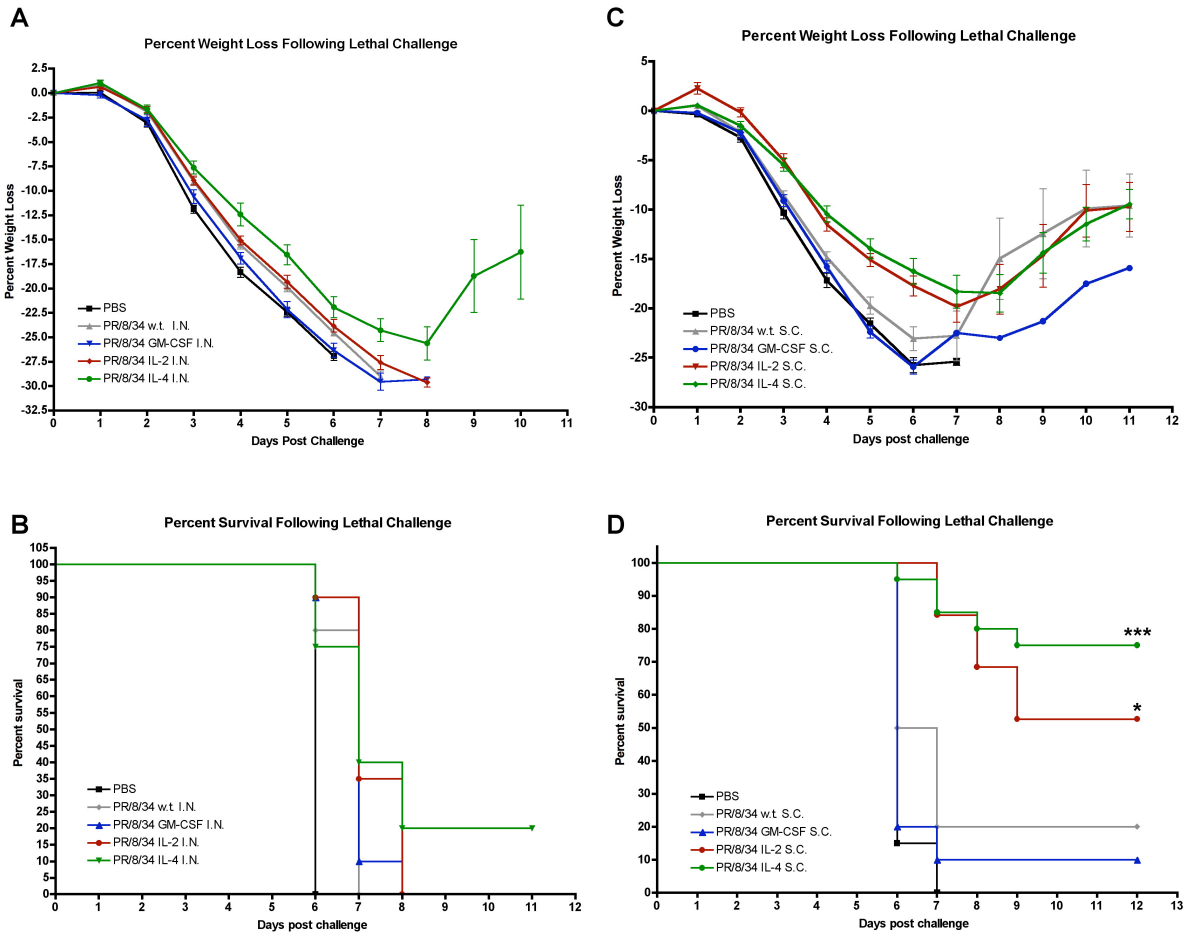


Figure 19. Inactivated influenza vaccines bearing membrane-bound immunomodulators protects mice against lethal challenge. Balb/c mice were challenged with 100 LD_{50} of mouse-adapted A/PR/8/34 on day 35 post vaccination. Mice were vaccinated intranasally with 1 µg (A, B) or subcutaneously with 0.375 µg (C, D) of inactivated wild type vaccine (n=20) or CYT-IVACs bearing membrane-bound GM-CSF (n=10), IL-2 (n=20), or IL-4 (n=20) using ultra-purified vaccine preparations. PBS served as negative vehicle control. Percent weight change (A, C) and survival (B, D) was monitored over time.

CSF bearing CYT-IVAC closely mimicked that of PBS (sham) inoculated mice (Figure 19 C). Sudden increases in weight for these groups between days 6 and 8 can be explained by a combination of recovering weight of remaining mice and loss of mice due to death; albeit mostly due to the latter. Only 20 percent of mice vaccinated subcutaneously with wild-type (n=20) vaccine and 10 percent of mGM-CSF CYT-IVAC (n=10) vaccinated mice were protected against lethal homotypic challenge (Figure 19 D). Mice vaccinated subcutaneously with mIL-2 (n=19) or mIL-4 (n=20) bearing CYT-IVAC exhibited both reduced and delayed weight loss compared to mice vaccinated with wild-type vaccine (Figure 19 C). Over 50

percent ($p<0.05$) of mice vaccinated with mIL-2 bearing CYT-IVAC and 75 percent ($p<0.001$) of mIL-4 CYT-IVAC vaccinated mice survived lethal challenge (Figure 19 D) and those mice that succumbed to infection took considerably longer to do so.

3.10 CYT-IVAC vaccination resulted in reduced viral loads in lungs of infected mice.

As another correlate of vaccine induced protection, we assessed the viral loads in lungs of vaccinated mice at day 4 following challenge. Mice vaccinated with ultra-purified (or double gradient purified) vaccine preparations, intranasally (1 μg) or subcutaneously (0.375 μg), were challenged on day 35 post-vaccination. Lungs were harvested from 3 mice per vaccine group on day 4 post-challenge and viral loads of lung homogenates were determined for each mouse. We chose to omit the mGM-CSF CYT-IVAC from this study because previously recorded results indicated no adjuvant effect for this CYT-IVAC. Intranasal vaccination with wild-type vaccines reduced viral loads marginally compared to unvaccinated mice but only mIL-4 CYT-IVAC vaccinated mice had significantly lower viral titers compared to wild-type vaccinated mice (Figure 20 A). Viral titers in the lungs of mice vaccinated subcutaneously with either the mIL-2 or mIL-4 CYT-IVAC were approximately a full log lower compared to mice vaccinated with the wild-type vaccine resulting in a percent reduction of 85.3 and 88.9, respectively (Figure 20 B).

Aim 3: Characterization of immunostimulatory properties of CYT-IVACs.

After demonstrating the adjuvant properties of the mIL-2 and mIL-4 CYT-IVACs, it was necessary to begin evaluating how these CYT-IVACs were eliciting their adjuvant effect. This has proven to be a challenging task due to the complexity and vastness of the immune response mounted during influenza vaccination. The intricate series of immunological events that occur following vaccination provide numerous pathways that could potentially be affected by the

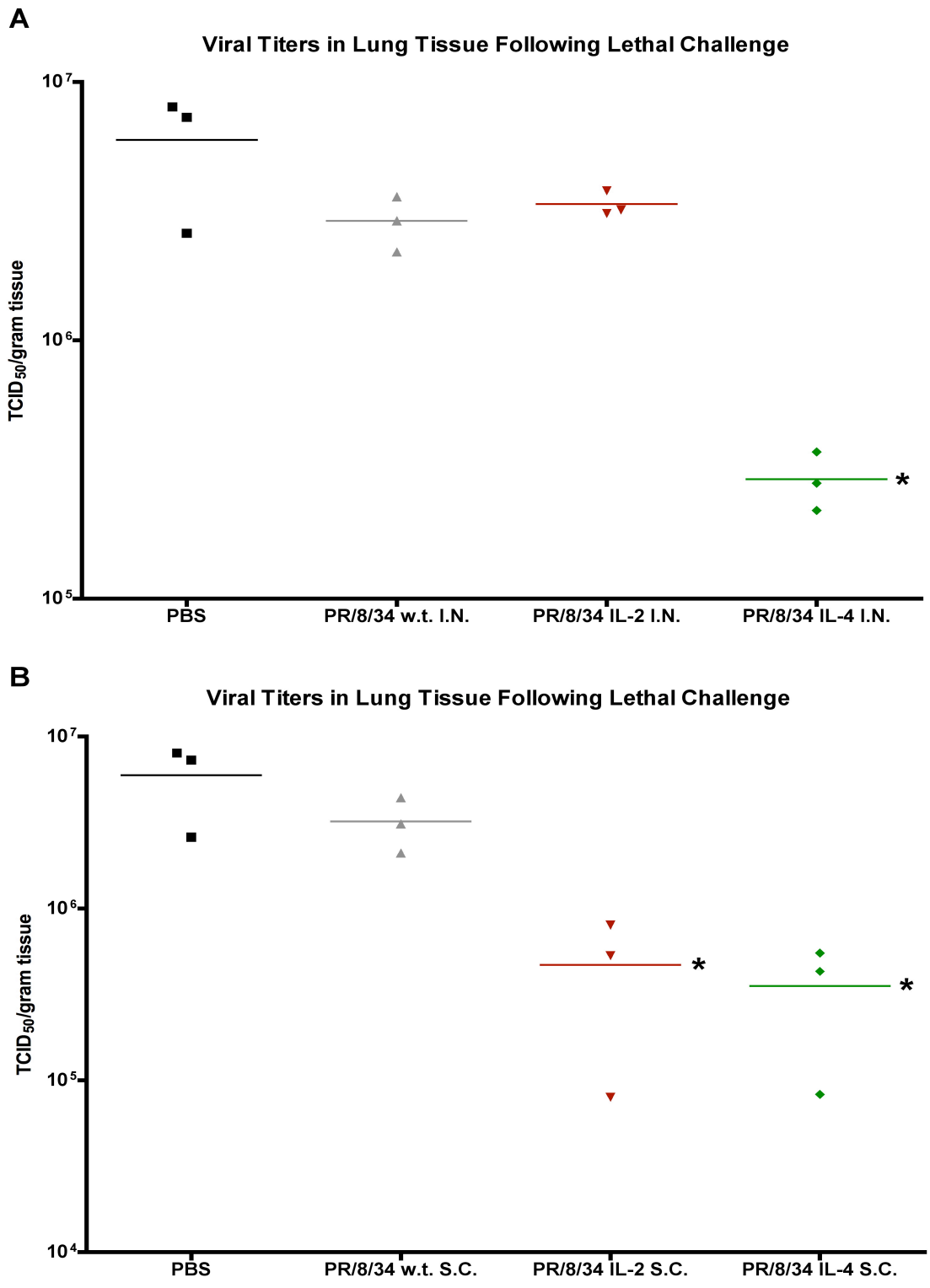


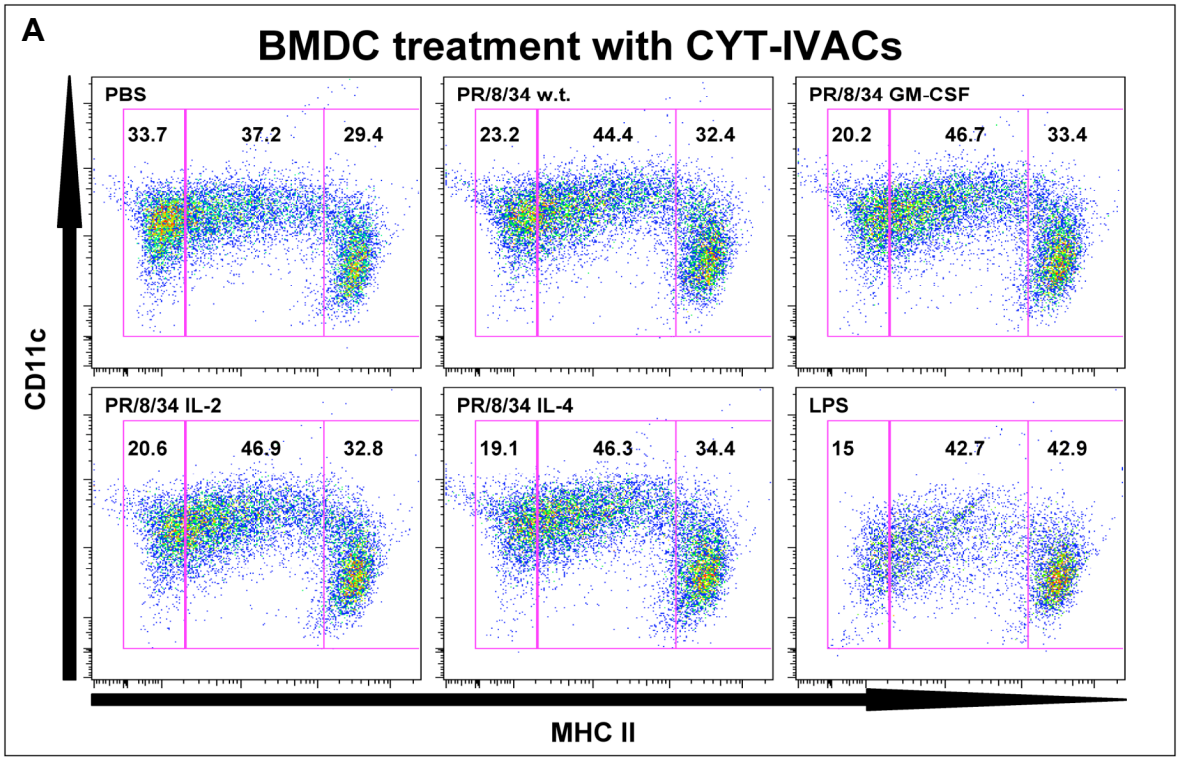
Figure 20. CYT-IVAC vaccination significantly reduces viral loads in lung tissue following lethal challenge. Mice vaccinated intranasally (A) or subcutaneously (B) with either wild-type vaccine or CYT-IVACs were challenged on day 35 post-vaccination with 100 LD_{50} of mouse-adapted A/PR/8/34. Mice were sacrificed on day 4 post-challenge and viral loads from homogenized lung tissue (n=3) were determined by tissue culture infectious dose assay. Data is expressed as $TCID_{50}$ per gram of lung tissue. (* $p < 0.05$ compared to PR/8/34 w.t.)

presence of membrane-bound immunomodulators. We first chose to investigate the affect CYT-IVACs had on early innate immunological events by examining the response of antigen presenting cells (APCs) to CYT-IVAC stimulation. APC's are likely responsible for initiating immune responses against CYT-IVACs and because these cells express receptors for many of the immunomodulators, it is possible that the adjuvant effects afforded by CYT-IVACs could be directed at APCs. Because earlier antibody data (Figure 16, 17) suggested the possibility of influenza specific cellular mediated immunity being generated during CYT-IVAC vaccination, we wanted to determine if in fact membrane-bound mIL-2 or mIL-4 was shifting the immune response from the classical T_h2 mediated or antibody driven immunity toward a T_h1 or cellular mediated immune response.

3.11 CYT-IVAC effect on Antigen Presenting Cells (APC).

To begin to understand the mechanism by which CYT-IVACs enhance immunity to influenza infection, we evaluated the role APCs may play in augmenting the immunogenicity of CYT-IVACs compared to wild-type vaccine. Dendritic cells (Bone marrow derived dendritic cells (BMDCs) or macrophages (murine alveolar macrophage-like cells (MH-S)) were incubated with wild-type vaccine or CYT-IVACs and FLOW cytometric analysis was used to evaluate expression of maturation markers following stimulation. CYT-IVAC treatment of BMDCs did not significantly enhance MHC II expression compared to wild-type vaccine treated cells, although MHC II expression did increase with vaccine treatment compared to untreated cells (Figure 21 A). Expression of co-stimulatory molecules CD40, CD80, CD86, CD70, CD274 and CCR7 were elevated in vaccine treated cells compared to untreated, however, there was no significant increase in expression with CYT-IVAC treatment compared to wild-type vaccine treatment (Figure 21 B).

As depicted in Figure 22 A, vaccine stimulation of MH-S macrophages did not increase expression of MHC II molecules regardless of the presence of membrane-bound cytokines. LPS stimulation, however, did upregulate expression of MHC II molecules on the surface of treated MH-S cells. Evaluation of



B

Treatment	CD40	CD80	CD86	CD70	CCR7	CD274
PBS	21.1	17.1	25.9	3.68	4.97	67.5
PR/8/34 w.t.	29.9	19.7	31.5	3.41	4.52	67.2
PR/8/34 GM-CSF	33.2	20.6	33.6	ND	ND	ND
PR/8/34 IL-2	29.8	20.6	32.1	2.85	3.61	62.2
PR/8/34 IL-4	34.6	22.6	33.9	3.25	4.12	65.5
LPS	87.5	30	61.4	6.52	4.24	86.3

Figure 21. Evaluation of CYT-IVAC induced maturation of BMDCs. BMDCs were treated with ß-propiolactone inactivated wild-type vaccine, mGM-CSF CYT-IVAC, mIL-2 CYT-IVAC or mIL-4 CYT-IVAC (1 µg) for 48 hours. BMDCs were collected and their state of maturation was determined by FLOW cytometric analysis evaluating MHC II (A), CD40, CD80, CD86, CD70, CCR7 and CD274 (B) expression on the surface of $CD11c^+$ cells. LPS (1 µg/ml) or media alone were used as positive and negative controls, respectively. A. From left to right, MHC II percentages represent negative, low and high expressing populations. B. Data is expressed as the percentage of $CD11c^+$ cells that are positive for the respective co-stimulatory molecule.

costimulatory molecule expression yielded similar results in that treatment of MH-S cells with wild-type vaccine or CYT-IVACs failed to increase expression of CD40, CD80, or CD86 (Figure 22 B). Again, LPS stimulation resulted in enhanced expression of all three costimulatory molecules evaluated proving that MH-S cells are capable or upregulation of CD40, CD80, and CD86 given the proper stimulus.

A

Alveolar Macrophage (MH-S) Maturation					
	PR/8/34 w.t.	PR/8/34 GM-CSF	PR/8/34 IL-2	PR/8/34 IL-4	LPS
MHC II Low	95.5	94.5	94.9	94.9	87.7
MHC II High	4.49	5.51	5.12	4.98	12.3

B

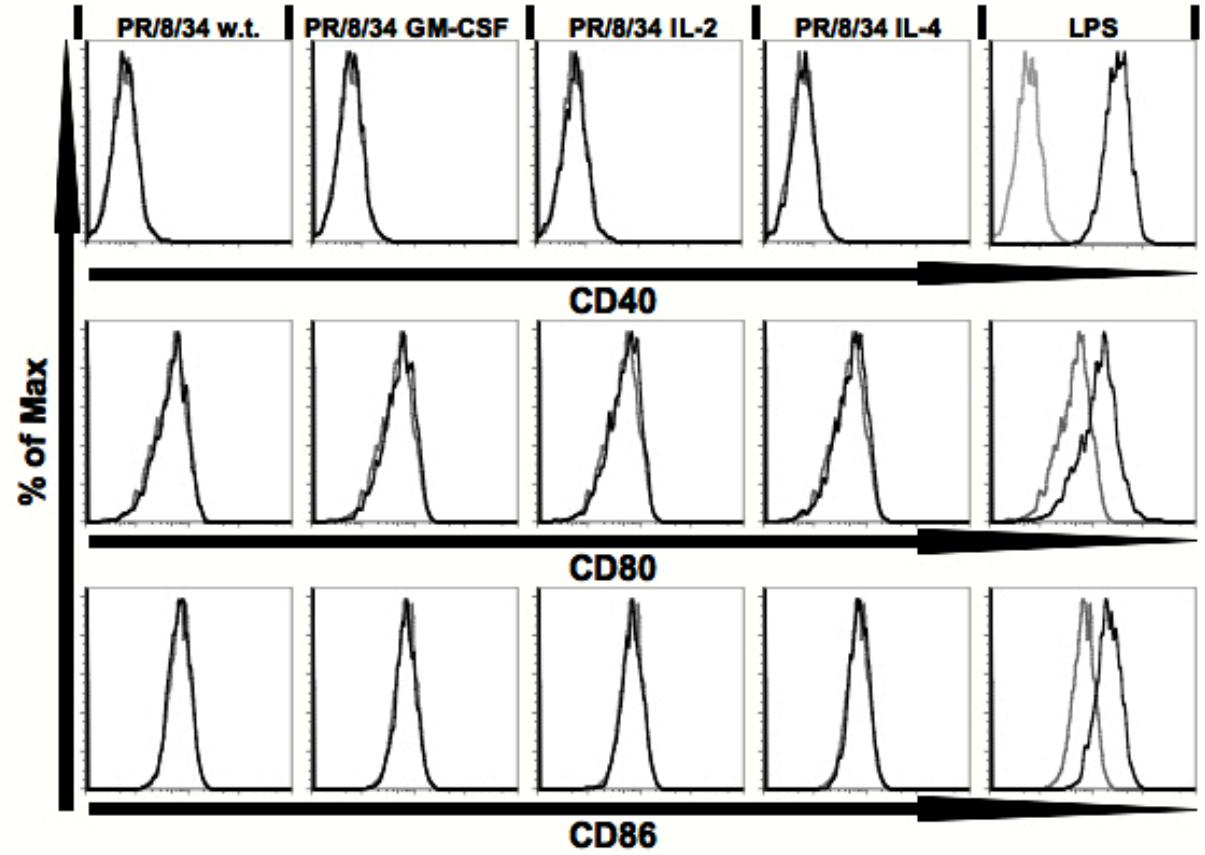


Figure 22. Evaluation of CYT-IVAC induced maturation of Alveolar Macrophage-like MH-S cells. MH-S cells were treated with ß-propiolactone inactivated wild-type vaccine, mGM-CSF CYT-IVAC, mIL-2 CYT-IVAC or mIL-4 CYT-IVAC (1 µg) for 24 hours. MH-S cells were collected and their state of maturation was determined by FLOW cytometric analysis evaluating MHC II (A), CD40, CD80 and CD86 (B) expression on the surface of $CD11c^+$ cells. LPS (1 µg/ml) or media alone were used as positive and negative controls, respectively. A. Percentage of MHC II expressing $CD11c^+$ cells following treatment by indicated vaccine or LPS. B. Histogram of wild-type vaccine (lane 1), mGM-CSF CYT-IVAC (lane 2), mIL-2 CYT-IVAC (lane 3), mIL-4 CYT-IVAC (lane 4) or LPS (lane 5) treated MH-S cells (black line) compared to negative control (gray dashed line).

3.12 Vaccination for the assessment of cellular immunity induced by CYT-IVACs.

As previously stated, stimulation of cellular mediated immunity (CMI) is important for fighting off influenza infection and is a desirable characteristic of an effective influenza vaccine. To evaluate potential CMI induced by CYT-IVACs, it

was necessary to increase the vaccine dose so that CMI induced by wild-type or CYT-IVAC vaccination could be readily detectable as detection of CMI usually requires multiple vaccinations with large doses. We chose to continue with the single vaccination regimen in order to evaluate subtle differences in cellular mediated immune responses that might otherwise be lost by using multiple vaccinations with high doses of vaccine. Further, multiple high doses of vaccine may mask the effects of responses induced by individual cytokines. Using multiple high doses of vaccine may make it difficult to identify cellular immunity stimulated by membrane-bound immunomodulators and cellular immunity induced simply by overloading the immune system with antigen. The opposite is also true in that a single dose vaccination event may result in cellular immunity that is below detection and for this reason, we chose to assess CMI post-challenge to allow for the clonal expansion of memory T cells generated during vaccination but prior to expansion of influenza specific T cells stimulated by infection [156-159]. We chose not to include the mGM-CSF-bearing CYT-IVAC in these studies due to poor performance in previous studies and a lack of any detectable adjuvant effect. To explore cellular immune responses induced by wild-type vaccine and CYT-IVACs, mice received a single high dose of CYT-IVAC or wild-type vaccine, intranasally (5 μg) or subcutaneously (1 μg). Serum was collected on day 21 post-vaccination for serological analysis and mice were challenged on day 28 post-vaccination. On day 4 post-challenge mice were euthanized and serum, nasal washes, lungs and spleens were collected (Appendix G). Differences in antibody titers were not as distinct using a large dose of vaccine as they were in the MPD_{20} studies (Figure 23 A, B). However, intranasal vaccination with mIL-4 CYT-IVAC did result in significantly higher influenza specific total IgG and IgG_1 titers and IgG_{2a} titers were higher, albeit not significant, in mIL-2 and mIL-4 CYT-IVAC vaccinated mice, compared to wild-type vaccinated mice. While there was no significant difference between antibody titers following subcutaneous vaccination, trends in antibody titers resembled those seen in earlier MPD_{20} vaccine studies. We did not see any difference in weight loss or viral loads in the lungs following intranasal vaccination when using a high dose of vaccine (Figures 24 A, 25 A). We did see a delay in

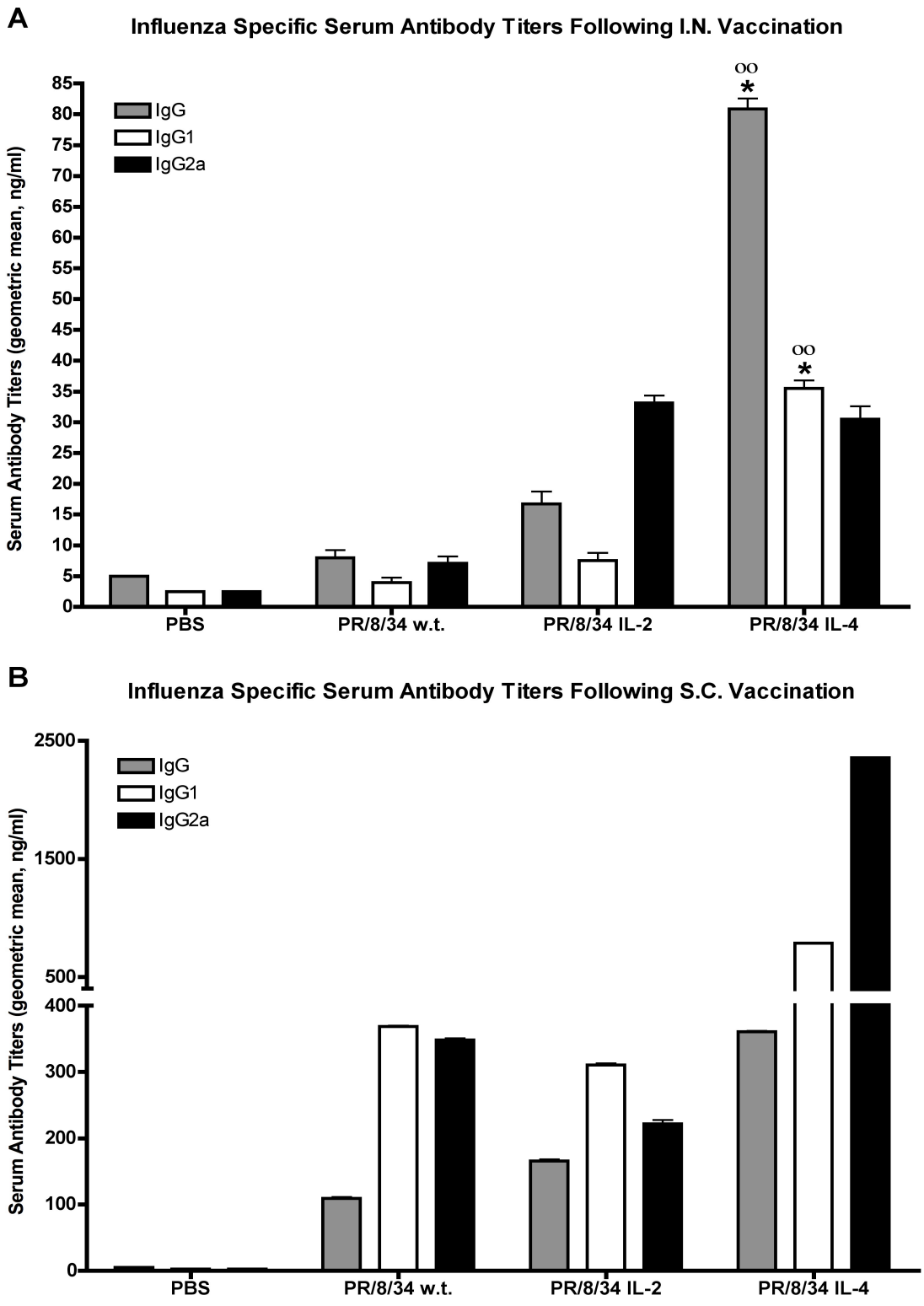


Figure 23. Vaccination with high dose of vaccine for determination of cellular mediated immunity. Mice were vaccinated intranasally (A) or subcutaneously (B) with 5 or 1 μg of either wild-type vaccine, mIL-2 CYT-IVAC, or mIL-4 CYT-IVAC. Serum was collected on day 21 post-vaccination and antibody titers for influenza virus specific IgG and isotypes IgG1 (Th2) and IgG2a (Th1) were determined by ELISA. Data is displayed as the geometric mean titer in ng/ml of serum for each group. (* $p < 0.05$ compared to PR/8/34 w.t., ^{oo} $p < 0.01$ compared to PBS)

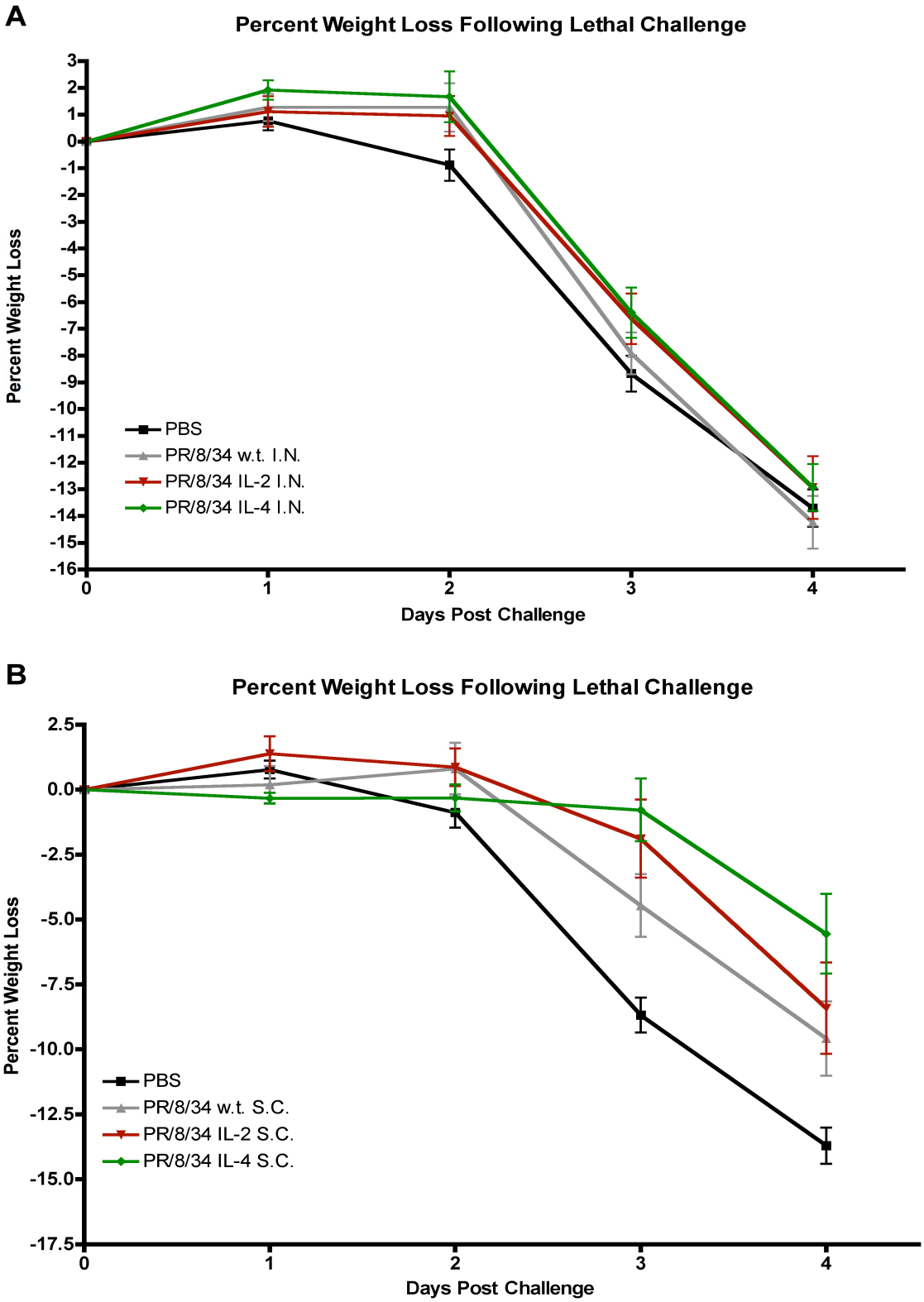


Figure 24. Vaccination with high dose of vaccine for determination of cellular mediated immunity. Mice were vaccinated intranasally (A) or subcutaneously (B) with 5 or 1 μg of either wild-type vaccine, mIL-2 CYT-IVAC, or mIL-4 CYT-IVAC. Mice (n=6) were challenged with 100 LD_{50} of mouse-adapted A/PR/8/34 on day 28 post-vaccination and weight loss was monitored over time.

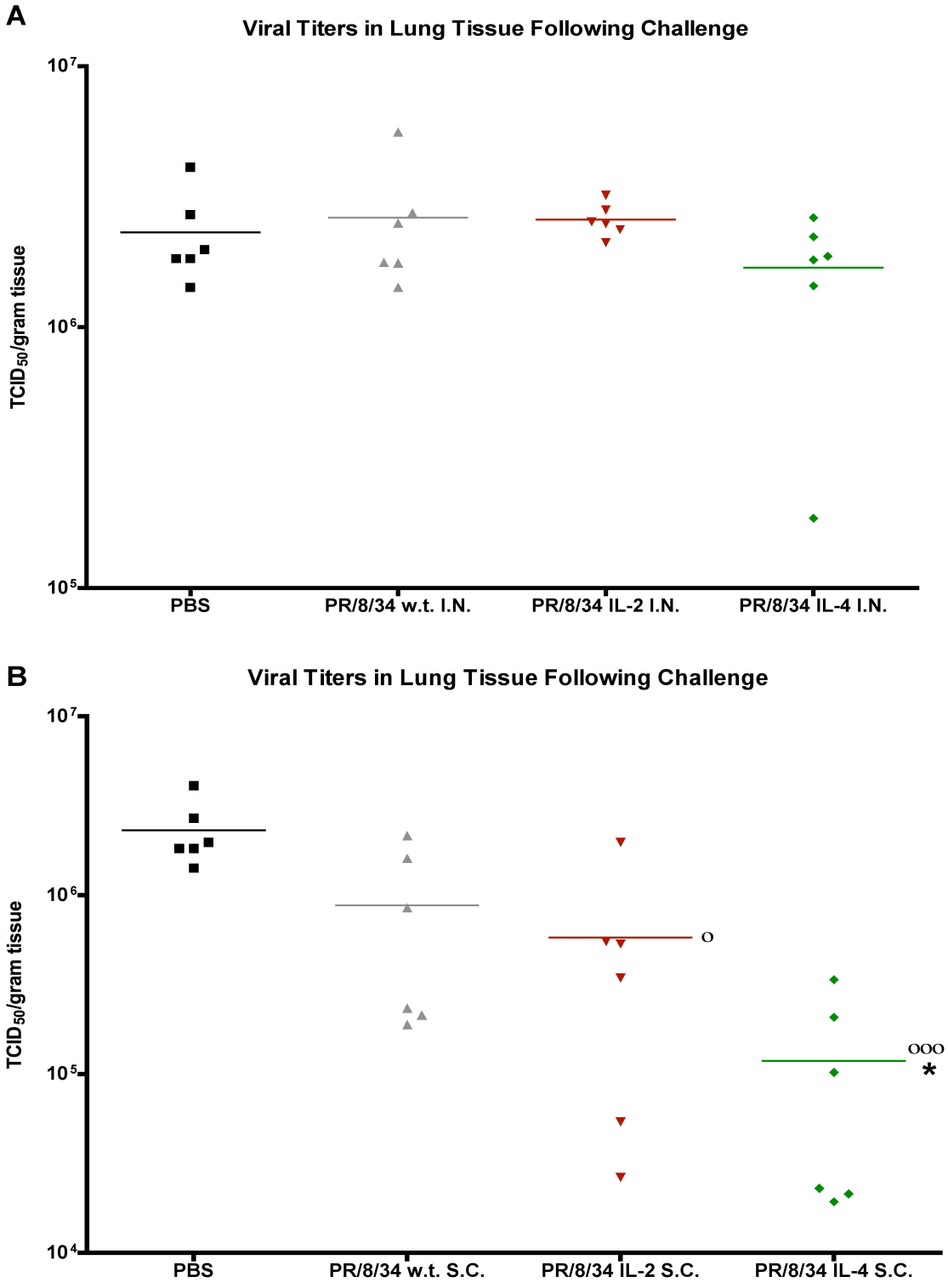


Figure 25. Vaccination with high dose of vaccine for determination of cellular mediated immunity. Mice (n=6) were vaccinated intranasally (A) or subcutaneously (B) with 5 or 1 μg of either wild-type vaccine, mIL-2 CYT-IVAC, or mIL-4 CYT-IVAC and challenged with 100 LD_{50} of mouse-adapted A/PR/8/34 on day 28 post-vaccination. Mice were sacrificed on day 4 post-challenge and viral loads from homogenized lung tissue were determined by tissue culture infectious dose assay (E, F). Data is expressed as $TCID_{50}$ per gram of lung tissue. (* $p < 0.05$ compared to PR/8/34 w.t., ^{o} $p < 0.05$ compared to PBS, ^{ooo} $p < 0.001$ compared to PBS)

weight loss for mice vaccinated subcutaneously with the mIL-2 and mIL-4 CYT-IVACs compared to wild-type vaccinated mice (Figure 24 B). We also observed a reduction in overall weight loss and viral titers in the lungs of mice vaccinated with the mIL-4 CYT-IVAC compared to wild-type vaccinated mice (Figures 24 B, 25 B)

3.14 Splenocyte proliferation assay for evaluation of CYT-IVAC induced influenza specific T cells.

Splenocyte proliferation assays were conducted using splenocytes isolated from vaccinated mice 4 days post challenge. Cells were stimulated with inactivated wild-type vaccine, MHC I, or MHCII restricted influenza peptides (Appendix E) to stimulate proliferation of influenza specific T cells. Proliferation was detected by reduction of Alamar Blue® and compared to background proliferation with media alone (negative control) to determine significance. We were unable to detect any significant influenza specific proliferation for splenocytes isolated from intranasally vaccinated mice regardless of the vaccine used (Figure 26 A). However, influenza specific proliferation was detected in splenocytes isolated from mice vaccinated subcutaneously with the mIL-4 CYT-IVAC ($p<0.01$) when splenocytes were stimulated with inactivated influenza vaccine but not with MHC peptides (Figure 26 B). We were unable to detect any significant influenza specific proliferation above background in the remaining groups following subcutaneous vaccination.

3.15 Characterization of T cell mediated immunity induced by CYT-IVAC vaccination.

To better understand the breadth of T cell immunity induced during vaccination with wild-type vaccine and CYT-IVACs we evaluated the maturation ($CD69^+$) and cytokine secretion (IL-4, IFN-gamma, and IL-17a) of splenocytes isolated from vaccinated mice. Splenocytes were harvested on day 4 post-challenge from mice vaccinated intranasally (5 μg) or subcutaneously (1 μg) with a high dose of vaccine. Splenocytes were stimulated in vitro with inactivated wild-type vaccine and T cell maturation and cytokine secretion was analyzed by FACS analysis. We did not detect any significant differences in the activation status of

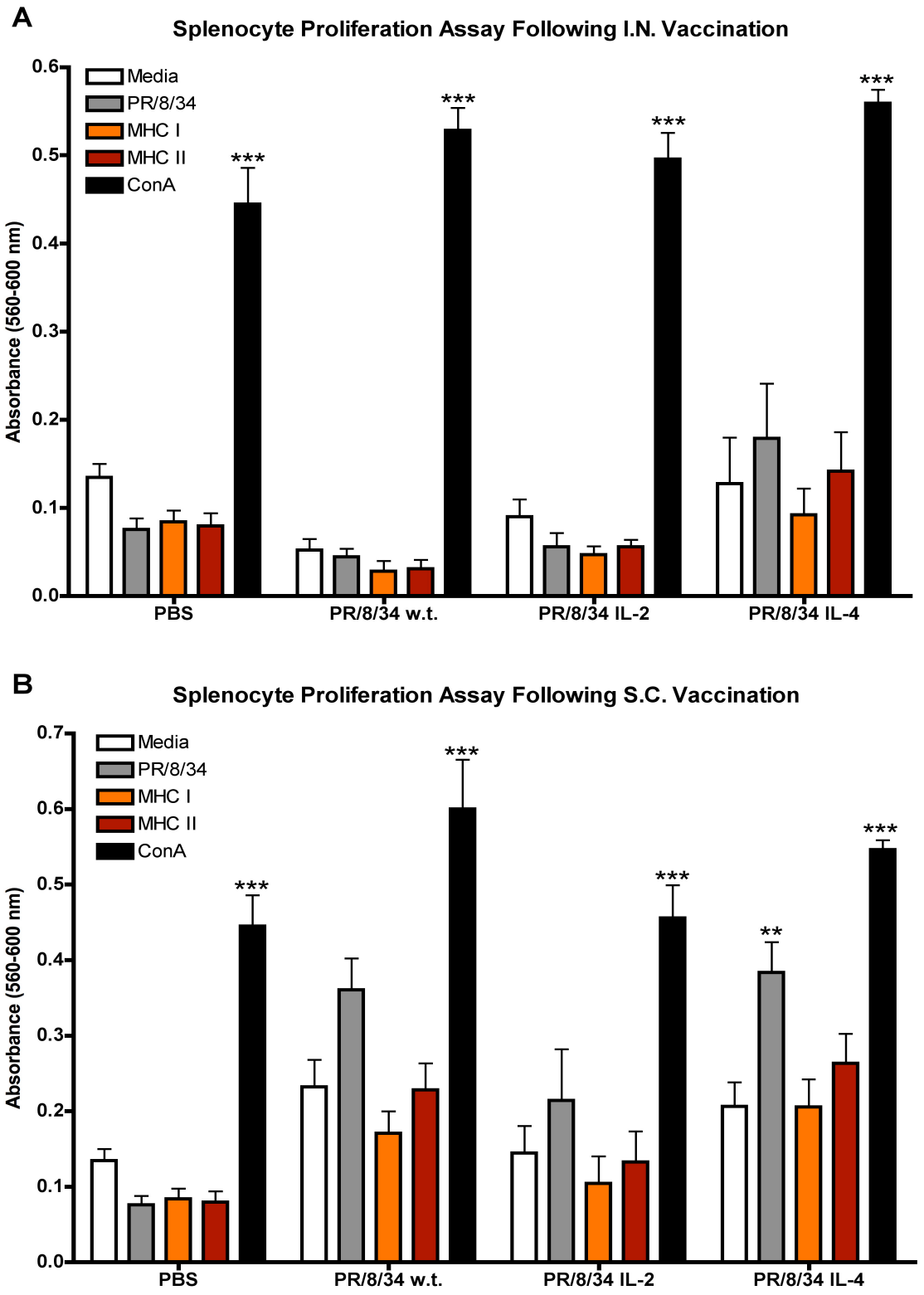
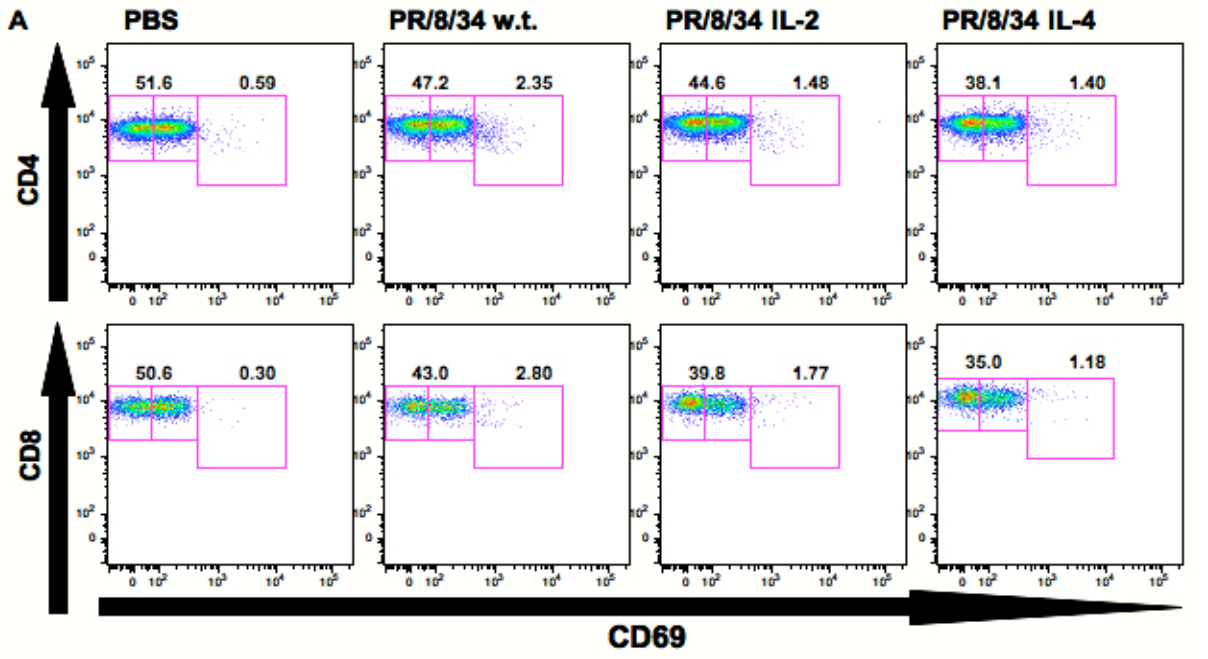


Figure 26. Splenocyte proliferation assay for evaluation of influenza specific cellular responses. Mice (n=6) were vaccinated intranasally (A) or subcutaneously (B) with 5 or 1 μg, respectively. Splenocytes were isolated on day 4 post-challenge and stimulated with media alone, inactivated PR/8/34, MHC I or MHC II influenza peptides, or LPS. Proliferation was measured by Alamar Blue® reduction. Media alone for each vaccine group served as background control and concanavalin A served as positive control. (** $p < 0.01$ compared to negative control, *** $p < 0.001$ compared to negative control)

CD4+ or CD8+ T cells isolated from mice vaccinated intranasally with wild-type vaccine compared to mice vaccinated with mIL-2 or mIL-4 CYT-IVACs, although the percentage of T cells expressing CD69 from vaccinated mice were elevated compared to unvaccinated mice (Figure 27 A). Intracellular cytokine production by CD4+ and CD8+ T cells isolated from mice vaccinated intranasally did not differ significantly between vaccine groups but again the percentage of cytokine secreting T cells were slightly higher in vaccinated mice versus unvaccinated mice

A

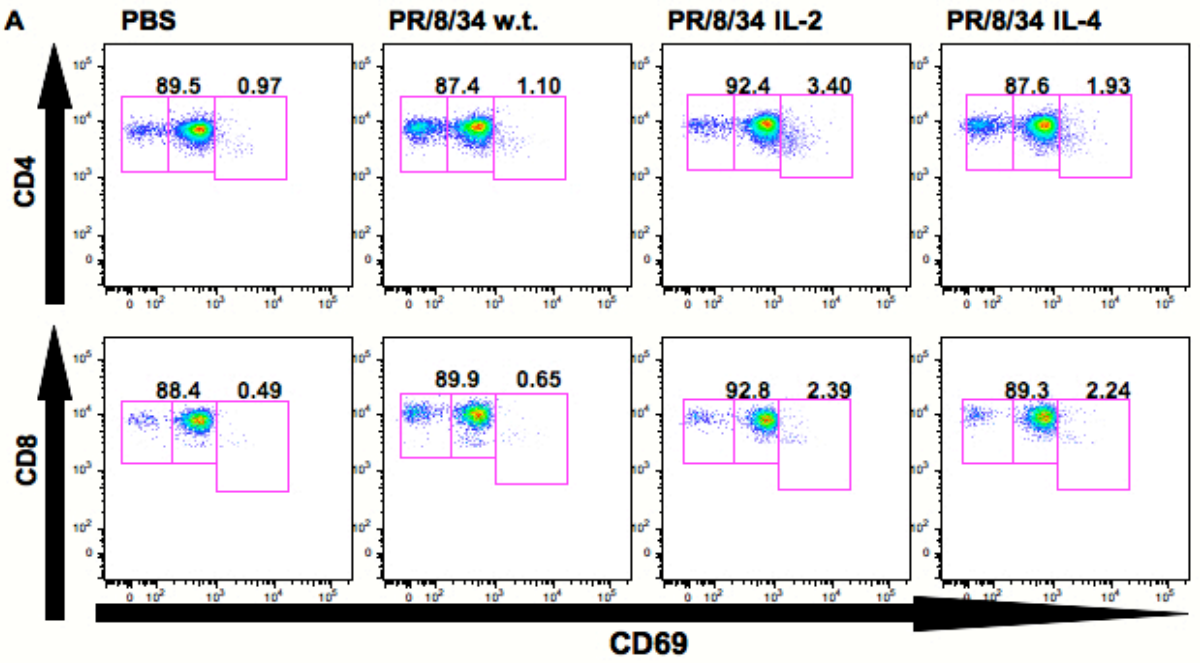


B

	CD4+			CD8+
Vaccine Group	**IL-4+**	**IFN-g+**	**IL-17a+**	**IFN-g+**
PBS	0.21±0.04	0.21±0.04	0.27±0.02	0.29±0.25
PR/8/34 w.t.	0.86±0.40	1.19±0.68	2.26±2.02	1.92±0.94
PR/8/34 IL-2	0.51±0.06	0.53±0.04	0.56±0.09	0.73±0.13
PR/8/34 IL-4	0.69±0.35	0.88±0.40	0.63±0.30	1.06±0.69

Figure 27. T cell activation and cytokine secretion of splenocytes from vaccinated mice following in vitro stimulation with wild-type vaccine. Mice (n=6) were vaccinated intranasally with 5 µg of wild-type vaccine, mIL-2 CYT-IVAC, or mIL-4 CYT-IVAC. PBS served as the negative control. Splenocytes were harvested day 4 post-challenge and stimulated in vitro with inactivated PR/8/34. Activation (A) and cytokine secretion (B) for CD4+ and CD8+ T cells was analyzed by FLOW cytometry. A. Average percentage of CD69 low expressing (middle gate) and CD69 high expressing (right gate) CD4+ and CD8+ T cells calculated from replicates for each vaccine group. B. Average percentage of IL-4, IFN-gamma, and IL-17a secreting CD4+ and IFN-gamma secreting CD8+ T cells calculated from replicates for each vaccine group.

(Figure 27 B). Evaluation of T cell activation and cytokine secretion from mice vaccinated subcutaneously did uncover some subtle differences between vaccine groups. A detectable increase in the percentage of $CD69^+$ T helper cells was observed in splenocytes isolated from the mIL-2 CYT-IVAC (3.4%) vaccinated group compared to unvaccinated (0.97%), wild-type (1.10%) vaccinated, or mIL-4 CYT-IVAC (1.93%) vaccinated mice (Figure 28 A). We also detected a sizable increase in the percentage of activated $CD8^+$ T cells in splenocytes isolated from



B

	CD4+			CD8+
Vaccine Group	**IL-4+**	**IFN-g+**	**IL-17a+**	**IFN-g+**
PBS	0.12±0.0	0.11±0.04	0.10±0.04	0.19±0.14
PR/8/34 w.t.	0.33±0.11	0.46±0.14	0.20±0.08	0.91±0.55
PR/8/34 IL-2	0.42±0.06	0.66±0.31	0.51±0.14	0.59±0.15
PR/8/34 IL-4	0.62±0.15	0.81±0.04	0.51±0.22	1.49±0.26

Figure 28. T cell activation and cytokine secretion of splenocytes from vaccinated mice following in vitro stimulation with wild-type vaccine. Mice (n=6) were vaccinated subcutaneously with 1 μg of wild-type vaccine, mIL-2 CYT-IVAC, or mIL-4 CYT-IVAC. PBS served as the negative control. Splenocytes were harvested day 4 post-challenge and stimulated in vitro with inactivated PR/8/34. Activation (A) and cytokine secretion (B) for $CD4^+$ and $CD8^+$ T cells was analyzed by FLOW cytometry. A. Average percentage of CD69 low expressing (middle gate) and CD69 high expressing (right gate) $CD4^+$ and $CD8^+$ T cells calculated from replicates for each vaccine group. B. Average percentage of IL-4, IFN-gamma, and IL-17a secreting $CD4^+$ and IFN-gamma secreting $CD8^+$ T cells calculated from replicates for each vaccine group.

the mIL-2 (2.39%) and mIL-4 CYT-IVAC (2.24%) vaccinated mice compared to unvaccinated (0.49%) or wild-type (0.65%) vaccinated mice (Figure 28 A). Marginal increases in IL-4, IFN-gamma, and IL-17a secreting $CD4^+$ T cells were noted for splenocytes isolated from subcutaneously vaccinated mice compared to unvaccinated mice, however, no significant differences were observed between vaccine groups (Figure 28 B). Upon analysis of IFN-gamma secreting $CD8^+$ T cells, splenocytes from vaccinated mice had higher percentages of IFN-gamma secreting CTL cells compared to unvaccinated mice and furthermore, mice vaccinated with the mIL-4 CYT-IVAC had the highest percentage of IFN-gamma producing $CD8^+$ T cells among the vaccine groups (Figure 28 B).

4. Discussion

In the present study we described a novel approach to immunopotentiate the anti-viral, protective response induced by whole virus inactivated influenza vaccines without the need for additional adjuvants or boosting doses of vaccine. Not only were our cytokine-bearing influenza vaccines (CYT-IVACs) more efficacious than non-adjuvanted whole virus vaccine, they also skewed the elicited humoral response towards a T_h1 mediated humoral immune response. Previously, we demonstrated feasibility of this platform for production of avian influenza vaccines bearing a membrane-bound form of chicken-derived IL-2 and GM-CSF [131]. CYT-IVAC-bearing chIL-2 significantly boosted antiviral antibody titers in vaccinated chicks compared to non-adjuvanted vaccine. Here, we have extended those studies and were able to successfully develop a platform upon which membrane-bound forms of mammalian-derived immunomodulatory proteins such as mouse IL-2, IL-4, or GM-CSF can efficiently be incorporated into budding virus particles. Importantly, we confirmed that bioactivity was retained following inactivation of the virus with either formaldehyde or ß-propiolactone, two virus inactivating agents commonly used during the formulation of current influenza vaccines [81]. It should be noted that not all inactivation methods allowed for retention of bioactivity for every CYT-IVAC. Specifically, ultraviolet and heat inactivation abrogated IL-4 bioactivity whereas they had no adverse effect on GM-CSF bioactivity (Figure 6). Further, we were able to demonstrate that the intrinsic proliferative-inducing activity associated with each individual CYT-IVACs was specific for the incorporated membrane-bound cytokine (Figure 5). This suggested that it was not simply the inclusion of the fusion protein itself that conveyed immune stimulating properties, but the demonstrated bioactivity of the incorporated cytokine. It should also be noted that long-term storage (> 12 months at 4°C) did not result in any detectable loss of cytokine specific bioactivity associated with the inactivated CYT-IVACs. Furthermore, in our hands, CYT-IVACs were stable and remained bioactive even following freeze/thaw when stored at -80°C (Figure 7). The stability of CYT-IVACs may allow for long-term storage of vaccine stockpiles,

generated potentially months in advance, with no deleterious affects on vaccine efficacy better preparing us should a pandemic arise.

Viral incorporation of membrane-bound cytokines was achieved through interactions between the viral matrix protein and cytoplasmic tail domains of the cytokine fusion construct during the budding process; this is the same interaction used to incorporate native full-length viral hemagglutinin. One caveat is that following infection of CYT-IVAC producer cells, there was the potential for decreased full-length viral HA incorporation during virus assembly and release due to the presence of membrane-bound fusion constructs. Although we were unable to determine exact full-length HA protein levels, for lack of a purified standard, optical density measurements were highly similar among CYT-IVACs using HA1 (H1) specific antibodies in slot blot assays. This suggests that total HA levels were not markedly reduced in the CYT-IVACs compared to wild-type vaccine (Table 2). In addition, hemagglutination units (HAU/μg total viral protein) of CYT-IVAC and wild-type vaccines did not differ significantly (Table 2). Clearly, our *in vivo* efficacy studies demonstrated that adequate antiviral humoral immune responses were elicited by our CYT-IVACs, indicating sufficient hemagglutinin content to stimulate a protective immune response. Precise quantitation of virus-incorporated cytokine was essential for standardizing adjuvant dose and we employed two methodologies for quantitating membrane-bound cytokines (Table 2). While quantitation of virus-incorporated cytokine is important, quantitation of bioactive membrane-bound cytokine may be more indicative of dosage, as we do not fully understand how anchoring the cytokine to the virus particle may affect its full biological capacity. Differences observed following quantitation of incorporated cytokine and quantitation of biologically active cytokine illustrate this point. For example, membrane-bound GM-CSF was incorporated at relatively high levels yet was poorly bioactive. Both IL-2 and IL-4 CYT-IVACs exhibited similar cytokine specific bioactivity, yet had variable amounts of incorporated cytokines. There was a clear discrepancy between these two methodologies in terms of the amount of cytokine measured, however, the biologically active measure is likely more indicative of the true adjuvant dose. Of note, membrane-bound cytokine

incorporation was relatively consistent across several independent vaccine preparations based on associated bioactivity per μg of viral protein (data not shown). This suggested that the observed variation in incorporation was specific for a given fusion construct and not due to variation in growth propagation of the virus in cell culture. The observed variability may partially explain why the GM-CSF CYT-IVAC, with low associated bioactive GM-CSF, did not provide better protection than the wild-type vaccine. Future formulations in which the GM-CSF molecule is extended further out from the virus particle may help enhance its bioactivity. Clearly, the amount of incorporated cytokine necessary to achieve an immunopotentiating effect will likely be cytokine specific and will require additional testing to optimize *in vivo* immunomodulatory effective dose.

Results reported here illustrate the feasibility of generating cell culture derived influenza vaccines containing membrane-bound adjuvants that are readily incorporated into newly budding virion particles during propagation of virus for vaccine preparation. Retention of adjuvant effect following inactivation of viral preparations eliminates the need for the addition of adjuvants post vaccine production further streamlining the vaccine production process. This approach also addressed a federal mandate issued in 2008 designed to encourage investigations into cell-based vaccine technologies for the production of influenza vaccines with emphasis on pandemic influenza preparedness [160]. Interest in cell-based vaccine production has become a priority, in part, because of recent shortages of yearly flu vaccines. Shortages often arise due to inadequacies in current egg-based vaccine technologies, that include long lag periods for vaccine production of up to 12 months, and the large number of special pathogen free eggs required to produce adequate numbers of vaccine doses [161-163]. Furthermore, propagation of wild-type, highly pathogenic avian influenza (H5N1) to high titers in eggs is challenging because these strains are highly lethal to chicken embryos [164]. We remain vulnerable to pandemic threats associated with HPAI strains until more efficient production methods for HPAI vaccines are realized. Recent advancements in microcarrier technology have allowed for large-scale production of MDCK based cell-culture derived influenza vaccine stocks, for both epidemic human strains and

HPAI, in amounts that are equivocal to egg-based vaccine production methods [164-166]. Concerns regarding the immunogenicity and safety of cell-based vaccines have limited their use and FDA approval, but studies are beginning to address these issues. Several studies alluded to the fact that cell-based and egg-based influenza vaccines have comparable antigenic properties and in some cases, cell-based vaccine preparations elicited superior antibody responses [167-169]. In fact, influenza vaccines propagated in eggs often accumulate genetic mutations in the receptor-binding region of hemagglutinin altering the amino acid sequence and host-dependent glycosolation patterns of hemagglutinin leading antigenic variation [170-173]. This, however, has yet to be documented in cell-based vaccines. Numerous studies evaluating the sequence similarity and antigenic likeness of clinical isolates and those same clinical isolates passaged in MDCK cells demonstrated identical HA1 amino acid sequences as well as antigenic similarity and homogeneity [174-178]. Several clinical trials evaluating the safety and tolerance of cell-based influenza vaccines demonstrated no remarkable differences or adverse reactions following vaccination and overall, cell-based vaccines were well tolerated in children, healthy adults, and elderly participants [162, 179-181]. Use of immortalized cell lines for vaccine production raises concerns of tumor induction and the presence of adventitious pathogens, however, due to their origin, the risk of MDCK cell induced tumors in humans is low. In addition, MDCK cell lines have been shown to be free of harmful pathogens and the risk of serum associated diseases from serum based cell cultures can be eliminated through the use of serum free cell culture technology for the propagation of vaccine preparations (CYT-IVAC producer cells tolerate serum free culture mediums) [181-184]. CYT-IVAC producer cell lines can be stored long term and rapidly scaled up for vaccine production in a short amount of time reducing the lag period typically associated with egg-based vaccine production. In addition, CYT-IVAC vaccine production does not rely on a stable poultry industry for a steady supply of special pathogen free eggs; a poultry industry that could be in jeopardy, due to widespread culling, should an outbreak of HPAI occur. CYT-IVACs and other cell-based vaccines also provide a potential vaccine for individuals with

allergies to egg products who are left vulnerable to influenza infection due to the absence of a non-egg derived influenza vaccine alternative. Application of CYT-IVAC adjuvant technology with microcarrier technology has the potential to generate large amounts of vaccine in a relatively short period of time by lowering the effective dose required to protect vaccinated individuals and by increasing production capacity, better preparing us to effectively address a pandemic event.

Protective immunity to influenza is best developed during the course of an active mild infection as it induces both humoral and cellular immunity that provides homotypic protection against re-challenge by the same or similar influenza strains [81]. Humoral immunity induced by influenza infection is characterized primarily by influenza specific mucosal IgA and serum IgG and to some extent IgM [81, 185]. Antibodies specific for hemagglutinin and neuraminidase are primarily responsible for subtype-specific protection against infection [186, 187]. Hemagglutinin and neuraminidase subtype-specific IgA in the mucosal lining has been directly correlated to protection against homotypic challenge as well as challenge from drift viruses within the same subtype, especially early on, whereas serum antibodies and CTLs in the respiratory tract play a limited role in early protection [188-190]. While comparatively low to HA and NA antibodies in term of protective capability, M2 antibodies provide additive protection against infection and because of its conserved nature across influenza A viruses, M2 antibodies provide some cross-protection against subtype viruses [188, 191, 192]. It should be noted that homotypic protection afforded by antibody-mediated immunity is often short lived due to antigenic shift leaving us vulnerable to infection by antigenically distinct sub-types [193]. While cellular immunity is not directly involved in preventing infection, it is important for clearance of virally infected cells [81]. Both $CD4^+$ and $CD8^+$ T lymphocytes are important for recognition and clearance of the virus and are generally thought to be important for establishment of heterotypic protection as they also recognize epitopes derived from the internal viral proteins, including NP, M1, PA, and PB1 epitopes, that are more conserved across subtypes [194, 195]. Memory CTLs developed during the course of infection play a role in clearance of influenza upon re-infection and there is a direct correlation between the number of

memory CTLs and the rate of viral clearance [196]. T cell memory responses are generally longer-lived and more promiscuous than antibody-mediated immunity and aid in cross-protection [197-199]. Due to the synergistic effect of both antibody and cellular-mediated immunity, induction of a balanced humoral and cellular immune response to influenza affords optimal protection and is the prime objective for effective influenza vaccines.

Immunity induced by Live Attenuated Influenza Vaccine (LAIV) is comparable to immunity induced by a natural course of influenza infection in that both humoral and cellular immunity is achieved [81, 200]. While LAIV induces influenza specific cellular immunity as well as serum antibody responses, it may be the influenza specific mucosal IgA response developed during vaccination that makes this vaccine approach so effective [201]. Neutralizing antibodies (IgA and IgG) in the upper respiratory mucosa in addition to serum antibodies and CTLs have been implicated in cross-protection against drift variants and heterotypic influenza strains [202, 203]. Safety concerns over adverse reactions to the live vaccine and the potential for the loss of attenuation of the vaccine due to reassortment with existing active influenza infections lessens its allure as an ideal vaccine candidate [204]. As a result of these safety concerns, the LAIV is only licensed for use in the United States for those individuals between the ages of two and 49 omitting the two most vulnerable groups, infants and the elderly [188]. Future studies using recombinant influenza virus modified to express membrane-bound forms of immunostimmulatory molecules may allow us to further enhance efficacy of LAIV. The expression of the membrane-bound immunomodulator by a recombinant LAIV should allow for immune stimulation in a highly localized immune microenvironment, minimizing systemic immune stimulation and associated toxic side effects that can occur when soluble cytokine is co-delivered or expressed.

Trivalent inactivated influenza vaccines (TIVs) are more commonly used and they are available in three formulations, whole virus, split, or subunit vaccines with whole virus vaccines being the most immunogenic, yet are not used in the United States [188, 205-207]. TIVs are generally well tolerated with few, if any,

adverse reactions reported [204]. Adverse reactions have been reported in children vaccinated with whole virus formulations and they are generally administered split or subunit vaccines [81, 208]; however, CYT-IVACs might reduce side effects of whole virus formulations if they permit the use of lower antigenic doses. Immunity induced by TIVs is dominated by humoral (T_h2) immunity, predominantly influenza specific serum IgG_1 [78-80, 82]. TIVs are effective (60-100%) at preventing morbidity and mortality from antigenically similar influenza strains in healthy individuals, however they are largely ineffective at preventing infection when the circulating strain of influenza is antigenically distinct from the vaccine strains as the humoral response induced by TIV vaccination provides poor cross-protection [188, 204]. Efficacy of TIVs in elderly and immunocompromised individuals is poor (30-70%) due in part to decreased immune function in these individuals that results in lower antibody titers following vaccination [81]. The inability of TIVs to effectively protect the elderly and to induce cross-protection has led to investigations into adjuvants that aid in enhancing the immune response to inactivated influenza vaccines. Microfluidized Emulsion 59 (MF59) as an adjuvant of inactivated influenza vaccines is effective at boosting antibody responses mediated by T_h2 type responses [209]. Aluminum based adjuvants have also proven effective as an adjuvant for influenza vaccines by inducing protective immunity with lower antigen doses [210]. Toxin type adjuvants [cholera toxin B (CTB) and E. coli heat-labile toxin B (LTB)] targeting innate immune responses induced cross protective antibody responses in the upper respiratory mucosa mediated primarily by secretory IgA [211-216]. Safety concerns regarding the use of CTB and LTB as adjuvants limit their application as CTB and LTB delivered intranasally have been linked to incidents of facial paralysis [217, 218]. FLU-ISCOMs not only induced significantly higher HI titers compared to the conventional influenza vaccine but also provided cross protection [92]. Cross protection conferred by FLU-ISCOMs is dependent on generating influenza specific cytotoxic T lymphocytes and reiterates the need to stimulate cellular immunity during vaccination [93].

Our approach of anchoring immunostimulators directly to the virus particle was designed to elicit a more robust immune response that boosts both humoral

and cellular immunity. Results from vaccine efficacy studies using highly purified vaccine preparations will be discussed as these results best represent the true adjuvant capability of membrane-bound immunomodulators. Ultra-purified vaccine preparations were required to maintain only the particulate matter of the virus itself, with or without membrane-bound cytokines, and helped to avoid making any false claim about adjuvant effects that may have been attributed to unknown contaminates within crude vaccine preparations. Specific CYT-IVACs induced a more T_h1 mediated immune response as evident by the higher levels of antiviral IgG_{2a} antibodies compared to wild-type non-adjuvanted virus vaccine. Isotype switching from IgG_1 to IgG_{2a} is known to be stimulated during T_h1-type immune responses, and has been further implicated in increased clearance of influenza infections following influenza vaccination [148-155]. Influenza vaccination in Balb/c mice conventionally leads to the production of influenza specific IgG_1 antibodies however the ratio of IgG_1:IgG_{2a} antibodies can vary depending on the formulation of the vaccine as well as site of administration. It has been well documented that whole viral vaccine formulations induce lower IgG_1:IgG_{2a} ratios compared to split or subunit vaccine formulations [150, 219] and while we did not directly compare different vaccine formulations, our results correlated well with previous reports in that non-adjuvanted whole virus vaccine did induce low IgG_1:IgG_{2a} antibody ratios. Following subcutaneous vaccination, murine IL-2 was effective at eliciting significantly higher IgG_{2a} antibody responses compared to non-adjuvanted vaccine when incorporated in a membrane-bound form on CYT-IVACs. Our finding correlated well with previous studies demonstrating that IL-2 served to ameliorate antigen specific IgG_{2a} titers and in fact, skewed the humoral response toward a IgG_{2a} dominate isotype [117, 220-222]. These findings are also supported by reports that characterize IL-2 as both an enabler and product of T_h1 mediated immunity that, as stated above, is associated with elevated IgG_{2a} titers [119, 223, 224]. The critical role IL-4 plays in stimulating T_h2 type immune effectors and suppressing T_h1 mediated immunity is well documented. Contrary to our hypothesis, the IL-4 bearing CYT-IVAC also induced elevated IgG_{2a} antibody titers compared to wild-type vaccination, suggesting that membrane-bound IL-4

polarizes immune effectors in a different manner than that described for soluble IL-4 [225-227]. Interestingly, other groups have reported that IL-4 in a membrane-bound form and in a highly localized environment can induce IL-12 production, a potent T_h1 inducer, by APCs [228, 229]. Results obtained with the GM-CSF bearing CYT-IVAC were less conclusive. Failure of GM-CSF bearing CYT-IVAC to enhance protection from lethal challenge when administered subcutaneously may be due in part to the amount of membrane-bound GM-CSF incorporated into virus particles. Large doses of GM-CSF can have an inhibitory effect on effector T cell function [120, 230]. Our GM-CSF bearing CYT-IVAC may present excessive GM-CSF leading to suppression of immunological functions by activation and expansion of myeloid suppressor cells [120]. This will require further clarification and additional studies to fully understand why the GM-CSF CYT-IVAC, despite bioactive function, failed to enhance immunity. Due to the low vaccine doses evaluated, significant differences in serum antibody titers between vaccine groups were not detected following intranasal vaccination, which is not all that surprising given the route of vaccination where one would expect higher mucosal antibody titers compared to serum antibodies (Figure 10 A). We were hopeful that differences in influenza specific IgA titers in the respiratory tract of vaccinated mice would be detected. We evaluated IgA titers in nasal washes collected from vaccinated mice on day 4 post-challenge by ELISA but were unable to detect any measurable influenza specific IgA antibodies at the lowest dilution (1:1) tested (data not shown). This was likely do to the large volume of PBS used to collect the nasal wash that may have diluted the sample making detection unattainable. Lessening the nasal wash volume may alleviate this problem allowing for the detection of influenza specific IgA and will be considered in future studies.

As previously stated, we observed no direct correlation between elevated antibody titers and protection when evaluated on a mouse-by-mouse basis in efficacy studies using the MPD_{20} dose of vaccine. Interestingly, in studies using a high dose of vaccine, there was a direct correlation between reduced viral loads in the lungs and elevated IgG_{2a} antibody titers detected in serum samples collected post vaccination. In fact, those mice for which influenza specific IgG_{2a} was the

dominate isotype had significantly lower viral loads in the lungs post challenge compared to those mice for which IgG_1 was the dominate influenza specific antibody isotype. Neutralizing antibody titers are often used as correlates of protection; though, recent studies suggest that this may not be a valid correlate in human studies. As with serum collected from MPD_{20} studies, we were unable to detect any neutralizing antibody titers. It is therefore possible that our microneutralization assay was not sensitive enough to detect the low levels of neutralizing antibody induced by the single low dose of vaccine administered. Alternatively, we were likely using too little vaccine to induce detectable neutralizing antibodies as neutralizing antibodies are typically detectable following repeated vaccinations or with a single large dose of vaccine [150].

The adjuvant properties of membrane-bound immunomodulators, specifically murine derived IL-2 and IL-4, have been clearly demonstrated following subcutaneous vaccination illustrated by enhanced protection from lethal challenge and reduced viral loads (see Figures 11-13). However, the adjuvant potential following intranasal vaccination has yet to be fully demonstrated. The intranasal vaccination route has proven to be difficult to reproduce as evident by the inability to accurately predict the MPD_{20} dose for wild-type vaccination. This is potentially due to the multitude of variables involved with intranasal vaccination. With subcutaneous vaccine delivery, the full dose is delivered in a single location that is relatively easy to reproduce across many vaccination events and vaccine delivery is easily monitored by visual conformation. With our method of intranasal vaccination, mice are first anesthetized followed by delivery of the vaccine at the nasal passage opening. It is then up to the mouse to aspirate the vaccine deep into the lungs to achieve full respiratory tract delivery. This vaccination method allows for the introduction of several variables that may differ from mouse to mouse. Aspiration volume, depth of unconsciousness, amount of vaccine lost to ingestion can all influence the actual dose of vaccine any one mouse receives and potentially lead to inaccurate dosing. Additionally, by altering the delivery volume it is also possible to target vaccine delivery to different areas of the respiratory mucosa, some of which have very different immunological environments [231-233].

One must consider these variables when interpreting the results for studies involving intranasal vaccination. A more accurate method of intranasal delivery, such as aerosolization, may better illustrate the overall adjuvant properties of membrane-bound immunomodulators for vaccines that intend to protect against mucosal associated infections [234, 235]; although this again has numerous variables associated with it, especially quantitating initial dose. The immunological environments unique to the respiratory mucosa versus subcutaneous injection sites are quite diverse and may also contribute to variations in vaccine efficacy [231, 232, 236]. A better mechanistic understanding of how CYT-IVACs enhance immunity is needed to fully appreciate the significance of the immunological environment. Still, intranasal vaccination with the IL-4 CYT-IVAC yielded reduced viral loads in the lungs and marginally enhance protection compared to non-adjuvanted wild-type vaccine. Yet, further studies are clearly needed to confirm that membrane-bound IL-4 enhances the efficacy of intranasally delivered influenza vaccine. It is to be expected that not all membrane-bound immunomodulators will act as adjuvants and those that do may not behave comparably for every vaccine delivery method. It will require in-depth analysis of each immunomodulator for each vaccination strategy to identify the optimal vaccination method paired with the best possible immunomodulator(s) to provide superlative protection against infection.

Understanding the mechanisms by which IL-2 and IL-4 augment the immunogenicity of whole virus influenza vaccine is essential to maximizing the potential of CYT-IVAC technology. This understanding is multifactorial in that we must 1) identify the correlates of immunity afforded by CYT-IVACs that are not induced by wild-type vaccination and 2) develop an understanding of how membrane-bound IL-2 and IL-4 function to reprogram the natural immune response to inactivated flu vaccine. The classical biological functions of IL-2 and IL-4 are defined as being quite different in that IL-2 promotes the differentiation and proliferation of T helper cells and CTL responses and IL-4 promotes the development of T_h2 mediated immunity [237-239]. Based on these classical functions, one would expect the adjuvant effects elicited by IL-2 and IL-4 to be quite different and indeed they may be. However, our preliminary evidence

suggests that these cytokines, in their membrane-bound forms, may be functioning in a similar manner to boost immunity to influenza infection. IL-2 and IL-4 bearing CYT-IVACs both enhanced influenza specific IgG_{2a} antibody titers, reduced weight loss and viral loads, and increased protection from lethal challenge in similar fashion. We also observed increases in the percentage of activated influenza specific $CD4^+$ and $CD8^+$ T cells isolated from the spleens of IL-2 and IL-4 CYT-IVAC vaccinated mice compared to wild-type vaccinated mice. These results suggest enhancement in vaccine efficacy could be attributed to the development of influenza specific T_h1 cells and possibly cytotoxic T lymphocytes, although further conformational analysis is required. Correlates between induction of influenza specific T_h1 and CTL responses and reduced viral loads, along with increased survival, have long been recognized [240-244]. Protection from subsequent re-infection by heterosubtypic influenza A viruses is also associated with the induction of T_h1 and CTL mediated immunity [245, 246]. In addition, the mutual importance of both antibody and cellular mediated immunity for the clearance of highly pathogenic influenza viruses has been demonstrated [247, 248]. In this regard, current inactivated vaccines leave us vulnerable to infection with highly pathogenic influenza as they only induce antibody driven immunity. Evidence collected by our laboratory supports the hypothesis that membrane bound IL-2 and IL-4 may be skewing the immune response toward T_h1 mediated immunity, immunity that is characterized by a dominate IgG_{2a} antibody isotype as well as influenza specific T_h1 cells and cytotoxic T lymphocytes, which lead to the rapid clearance of influenza virus and virally infected cells lessening morbidity and mortality of infected mice.

The manner in which IL-2 and IL-4 bearing CYT-IVACs coordinate a predominantly T_h1 mediated immune response has yet to be fully understood. We have focused our efforts on defining the relationship between CYT-IVACs and antigen presenting cells (APCs), as this is the first, and most likely immunological synapse to be altered by membrane-bound immunomodulators [114]. APCs are potentially one of the first cell types to engage CYT-IVACs (or any foreign antigen) following vaccination and are responsible for bridging the gap between innate and

adaptive immunity [249, 250]. For this reason, APCs, specifically dendritic cells, are of particular interest because of their critical role in educating naïve T helper cells and because they are known to express functional receptors for both IL-2 and IL-4 [251-253]. IL-2 and IL-4 receptor stimulation on APCs may lead to enhanced antigen processing and co-stimulatory molecule expression important for T cell priming [251, 254, 255], however our initial evaluation of BMDC (Figure 14) and MH-S (Figure 15) cells following treatment with CYT-IVACs showed no enhanced activation of APCs. It is more likely that IL-2 and IL-4 receptor stimulation could alter the cytokine profile of APCs. Specifically, studies have identified IL-4 as a potent stimulator of IL-12 production by dendritic cells [229, 251, 256, 257]. This challenges conventional wisdom regarding the immunological functions of IL-4 and provides a potential explanation for how IL-4 CYT-IVAC may skew the immune response toward T_h1 driven immunity. Interestingly, IL-4-dependent IL-12 secretion by dendritic cells is dependent on the context and timing for which the IL-4 stimulation is given. When IL-4 is delivered to dendritic cells at the time of antigen stimulation, i.e. maturation, IL-4 then becomes a potent initiator of bioactive IL-12 (IL-12p70 heterdimer) production and in fact inhibits the production of non-bioactive IL-12 (IL-12p40 homodimer) and IL-10 providing an optimal cytokine environment for priming T_h1-type $CD4^+$ T cells [229, 257-259]. The inherent conformation of our CYT-IVAC provides optimal opportunity for IL-4 to be present at the onset of dendritic cell stimulation potentially leading to IL-12 secretion and T_h1 priming during T cell education. Initiating a T_h1 priming environment during influenza vaccination has proven beneficial for enhancing protection against infection [153] and this could be the mechanism by which the IL-4-bearing CYT-IVAC elicits superior protection. This speculation is supported by the elevated IgG_{2a} titers, reduced viral loads, and greater percentage of activated $CD4^+$ and $CD8^+$ T cells detected in IL-4 CYT-IVAC vaccinated mice which could all be attributed to T_h1 mediated immunity. However, further in depth studies are needed to confirm or deny this theory. The role of IL-2 receptors on dendritic cells is less defined, although the importance of IL-2 for the development of effective cross talk between dendritic cells and naive T cells during T cell education has been

illustrated [260]. IL-2 produced by dendritic cells, in response to microbial stimulus, is required at the immunological synapse between mature dendritic cells and naïve T cells to facilitate proliferation of both $CD8^+$ and $CD4^+$ T cells and to promote maximal immunity [255, 260, 261]. IL-2 stimulation has also been shown to augment IL-12-dependent interferon-γ (IFN-γ) production by dendritic cells [253]. Natural IL-2 enhancement of dendritic cell derived IFN-γ is apt to occur during normal T cell priming events in the regional lymph node with the source of IL-2 being newly primed naïve T cells, ultimately leading to T_h1 skewing. The IL-2 bearing CYT-IVAC may provoke a similar response by providing the IL-2 stimulus needed to enhance IL-12-dependent IFN-γ production ensuring matured dendritic cells are primed for T_h1 skewing when they arrive at the draining lymph nodes. Ongoing studies will undoubtedly provide us with a better understanding of how IL-2 and IL-4 bearing CYT-IVACs manipulate conventional immunological progression resulting in more robust immunity.

It is important to acknowledge the potential for adverse side-effects that could be associated with the use of immunomodulatory adjuvants. Some immunomodulatory proteins are capable of stimulating the expansion and activation of regulatory T cells (T regs), T cells responsible for maintaining immunological homeostasis and suppressing immune functions to promote “self tolerance” [262, 263]. If during vaccination with CYT-IVACs T regs are activated, it is theoretically possible that, through suppressive functions of regulatory T cells, the CYT-IVAC could be mistaken for a self antigen and immunological tolerance to the vaccine would be established, thus providing no protections against subsequent infection. While this is clearly not the case for the IL-2 and IL-4-bearing CYT-IVACs, given the immunostimulatory properties documented, T reg activation offers a possible explanation for the shortcomings of the GM-CSF-bearing CYT-IVAC and should be considered when evaluating future CYT-IVACs. The opposite is also true in that induction of autoimmunity against the immunomodulators, mediated by auto-reactive antibodies or auto-reactive T cells, is a real possibility. Because immunomodulators are anchored directly to foreign antigens, it is

possible that the immune system may mistake these self-antigens as foreign to generate an immune response against the immunomodulators themselves, a phenomenon termed the “bystander effect” [264-266]. Careful consideration and evaluation of possible side-effects are required to address safety concerns regarding the use and application of CYT-IVAC technology.

A wide range of applications exist for our cytokine-bearing viral vaccine technology. This vaccine approach is adaptable to a variety of species including avian, swine, canine, and equine by simply introducing species-specific immunomodulators. Likewise, using human-specific immunomodulators we can tailor this platform to the production of human influenza vaccines bearing membrane-bound immunomodulators. Importantly, depending on the location of the bioactive domains, immunomodulators can be presented either as type I or type II membrane-bound molecules on the virus particle. This also serves to overcome potential steric hindrances that may occur during cytokine folding and/or presentation. In our laboratory, we have been able to incorporate these membrane-bound immunomodulators in H3N2, H1N1 as well as H6N2 (data not presented) influenza virus strains using the same CYT-IVAC producer cell line. Thus, vaccines against newly emerging influenza strains can be readily produced using our CYT-IVAC producer cell lines given that the emerging strains grow efficiently in cell culture. It should also be noted, that this approach is amenable to virtually any enveloped virus, requiring only virus specific adaptation of the membrane-anchoring domain to ensure incorporation during the budding process. It has also recently been demonstrated that this approach is amenable for inclusion into virus-like particles. Wang et al (2008) demonstrated the feasibility of influenza VLP incorporation of a membrane-bound form of flagellin using the baculovirus expression system and furthermore, demonstrated the enhanced immunogenicity of flagellin containing VLPs compared to unadjuvanted VLPs [267]. This study provides independent, corroborating evidence supporting the versatility and practicality of membrane-bound immunomodulators as effective vaccine adjuvants.

5. Conclusions and Scientific Contributions

We have demonstrated both the feasibility of viral incorporation of membrane-bound immunomodulators by influenza viruses and the enhanced efficacy of our CYT-IVACs compared to conventional, non-adjuvanted influenza virus vaccines. Superior immunogenicity of CYT-IVACs was manifested as elevated influenza specific antibodies, particularly IgG_{2a} isotypes implicating T_h1 mediated immunity. Enhanced protection from infection was also demonstrated for IL-2 and IL-4 CYT-IVAC vaccinated mice further illustrating the adjuvant effect of membrane-bound IL-2 and IL-4. The adjuvant or immune stimulating properties of CYT-IVACs makes them attractive candidates for inducing a more robust and protective immune response in the elderly and immunocompromised individuals where immune responses are waning or compromised. Further, the membrane-bound immunomodulators may be helpful in either augmenting the immunogenicity of influenza vaccines that require large antigen doses to confer protection or in reducing the dose required for protection. This could significantly increase vaccine availability targeting low immunogenic strains such as H5N1. Current studies in our lab, encompassing additional immunostimulatory molecules, the intranasal route of vaccine delivery and other virus platforms, will help define the utility and efficacy of the CYT-IVAC approach. While this body of work has helped to define the adjuvant potential of membrane-bound immunomodulators as it applies to inactivated influenza vaccines, it also lends support for extending membrane-bound adjuvant technology to other enveloped viruses for which more effective vaccines are need.

6. Future Directions

As is often the case at the end of a dissertation project, we are left with many unanswered questions. The most pressing question, and arguably the most difficult to answer, involves identifying the mechanism by which IL-2 and IL-4-bearing CYT-IVACs augment the immune response. Cellular mediated immunity has been proposed to play a critical role in protecting IL-2 and IL-4 CYT-IVAC vaccinated mice but further evidence is required. Adaptive transfer experiments, in which CD4 and/or CD8 T cells from wild-type or CYT-IVAC vaccinated mice are delivered to naïve mice prior to challenge, would help to determine both the quality and relevance of cellular mediated immunity induced by CYT-IVAC vaccination. Passive transfer of serum from wild-type or CYT-IVAC vaccinated mice to naive mice prior to challenge would help to define the importance of humoral immunity in protecting CYT-IVAC vaccinated mice and provide further insight into the role of cellular mediated immunity. In addition to adaptive transfer experiments, evaluation of heterotypic protection afforded by CYT-IVACs would not only offer auxiliary data regarding CYT-IVAC induced cellular immunity, it would also aid in defining the overall utility of IL-2 and IL-4-bearing CYT-IVACs. To further define the overall aptitude of IL-2 and IL-4-bearing CYT-IVACs, long-term protection studies would be valuable. These studies would help to characterize the longevity of immunity afforded by CYT-IVAC vaccination compared to non-adjuvanted vaccine. In addition, because the elderly population is left vulnerable to influenza infection by the currently available vaccines, a more robust vaccine that provides protection for the elderly is in dire need. Vaccine efficacy studies using aged mice would divulge useful information regarding the effectiveness of CYT-IVACs for the elderly population. Finally, the need to fully characterize the response of APCs to CYT-IVAC stimulation remains. Evaluation of dendritic cell cytokine profiles, by either real-time PCR or protein quantitation methodologies, induced by CYT-IVAC stimulation compared to non-aduvanted vaccine could facilitate a better understanding of how CYT-IVACs alter the bridge between innate and adaptive immunity as well as offer support for theories proposed in the discussion.

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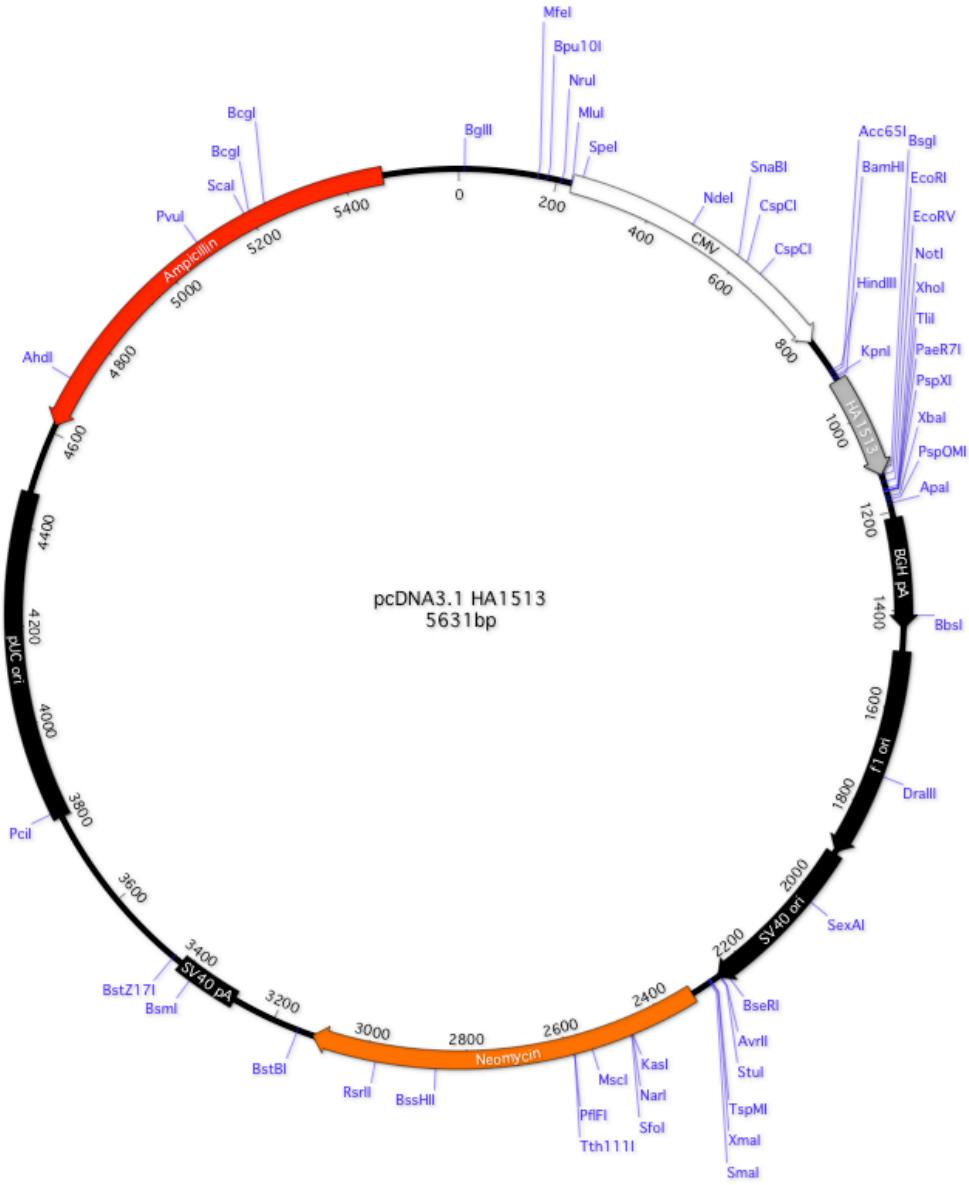
Appendix A.

HA1513 Expression Vector



HA1513 Gene Sequence

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GGGAAAAGATAGATGGAGTGAAATTGGAATCAATGGGGGTGTATCAGATTCTG
GCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGG

CAATCAGTTTCTGGATGTGTTCTAATGGGTCTTTGCAGTGCAGAATATGCATCT
GA***GAATTC***

Restriction Sites:

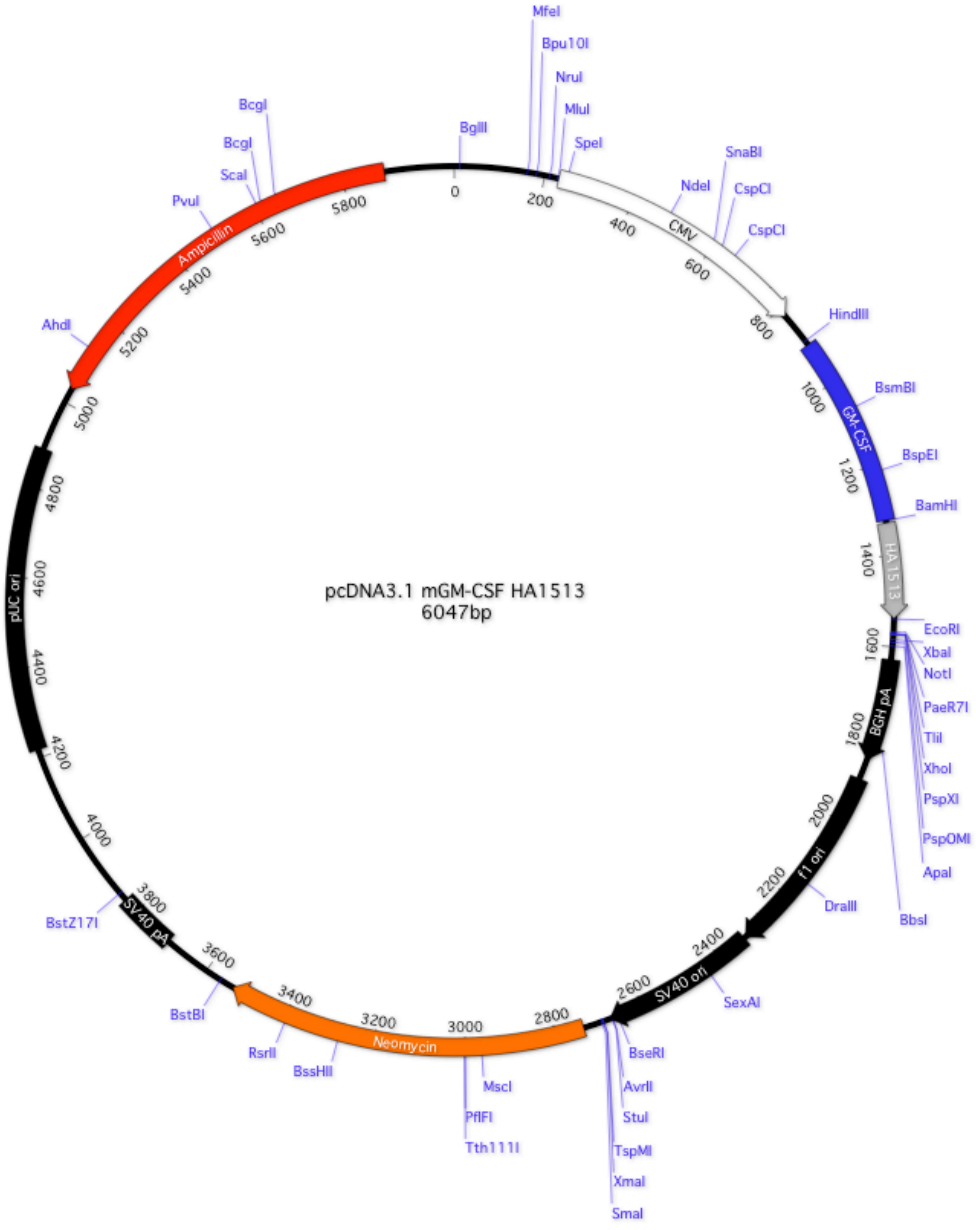
Acc65I	895	DraIII	1730	PvuI	5078
AhdI	4708	EcoRI	1123	RsrII	2980
ApaI	1178	EcoRV	1135	ScaI	5188
AvrII	2254	HindIII	889	SexAI	2021
BamHI	907	KasI	2463	SfoI	2465
BbsI	1416	KpnI	899	SmaI	2277
BcgI	5247	MfeI	161	SnaBI	590
BcgI	5213	MluI	228	SpeI	249
BglII	12	MscI	2546	StuI	2253
Bpu10I	180	NarI	2464	TliI	1156
BseRI	2250	NdeI	484	TspMI	2275
BsgI	1125	NotI	1150	Tth111I	2582
BsmI	3384	NruI	208	XbaI	1168
BssHII	2861	PaeR7I	1156	XhoI	1156
BstBI	3146	PciI	3815	XmaI	2275
BstZ17I	3436	PflFI	2582		
CspCI	661	PspOMI	1174		
CspCI	626	PspXI	1156		

HA1513 Amino Acid Sequence

GS**NGTYDYPKYSEESKLNREKIDGVKLE**SMGVYQILAIYSTVASSLVLLVSLGAISF
WMCSNGSLQCRICI*

Appendix B.

Murine GM-CSF~HA1513 Expression Vector



murine GM-CSF HA1513 Construct Gene Sequence

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AGCCTCTCAGCACCCACCCGCTCACCCATCACTGTCACCCGGCCTTGGAAGC
ATGTAGAGGCCATCAAAGAAGCCCTGAACCTCCTGGATGACATGCCTGTCACA
TTGAATGAAGAGGTAGAAGTCGTCTCTAACGAGTTCTCCTTCAAGAAGCTAAC
ATGTGTGCAGACCCGCCTGAAGATATTCGAGCAGGGTCTACGGGGCAATTTCA
CCAAACTCAAGGGCGCCTTGAACATGACAGCCAGCTACTACCAGACATACTGC
CCCCCAACTCCGGAAACGGACTGTGAAACACAAGTTACCACCTATGCGGATTT
CATAGACAGCCTTAAAACCTTTCTGACTGATATCCCCTTTGAATGCAAAAAACC
AGTCCAAAAA***GGATCC***AATGGGACTTATGATTATCCAAAATATTCAGAAGAATCA
AAGTTGAACAGGGAAAAGATAGATGGAGTGAAATTGGAATCAATGGGGGTGTA
TCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTTTGGTCT
CCCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGGTCTTTGCAGTGCAGA
ATATGCATCTGA***GAATTC***

Restriction Sites:

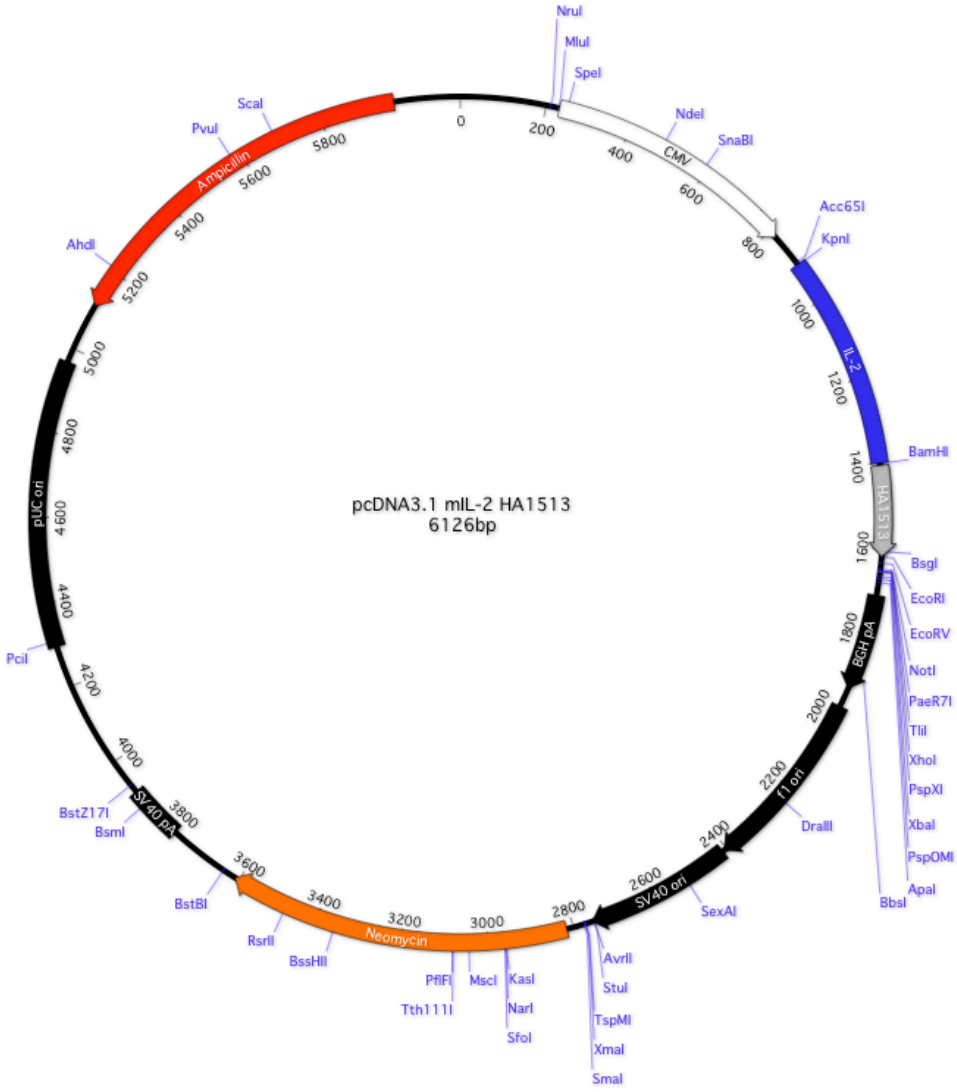
AhdI	5124	CspCI	626	ScaI	5604
ApaI	1594	DraIII	2146	SexAI	2437
AvrII	2670	EcoRI	1539	SmaI	2693
BamHI	1323	HindIII	889	SnaBI	590
BbsI	1832	MfeI	161	SpeI	249
BcgI	5663	MluI	228	StuI	2669
BcgI	5629	MscI	2962	TliI	1572
BglII	12	NdeI	484	TspMI	2691
Bpu10I	180	NotI	1566	Tth111I	2998
BseRI	2666	NruI	208	XbaI	1584
BsmBI	1073	PaeR7I	1572	XhoI	1572
BspEI	1214	PflFI	2998	XmaI	2691
BssHII	3277	PspOMI	1590		
BstBI	3562	PspXI	1572		
BstZ17I	3852	PvuI	5494		
CspCI	661	RsrII	3396		

murine GM-CSF HA1513 Construct Amino Acid Sequence

MWLQNLLFLGIVVYSLSAPTRSPITVTRPWKHVEAIKEALNLLDDMPVTLNEEVEV
VSNEFSFKKLTCVQTRLKIFEQGLRGNFTKLKGALNMTASYYQTYCPPTPETDCE
*TQVTTYADFIDSLKTFLTDIPFECKKPVQK*GS**NGTYDYPKYSEESKLNREKIDGVK**
LESMGVYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI*

Appendix C.

Murine Interleukin-2~HA1513 Expression Vector



murine IL-2 HA1513 Construct Gene Sequence

GGTACCAGCATGCAGCTCGCATCCTGTGTCACATTGACACTTGTGCTCCTTGT
CAACAGCGCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCACAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGGAGCAGCTGTTG
ATGGACCTACAGGAGCTCCTGAGCAGGATGGAGAATTACAGGAACCTGAAACT
CCCCAGGATGCTCACCTTCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGA

AAGATCTTCAGTGCCTAGAAGATGAACTTGGACCTCTGCGGCATGTTCTGGAT
TTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCATCAGCAAT
ATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGACAACACATTTGAGTGCCAA
TTCGATGATGAGTCAGCAACTGTGGTGGACTTTCTGAGGAGATGGATAGCCTT
CTGTCAAAGCATCATCTCAACAAGCCCTCAA***GGATCC***AATGGGACTTATGATTA
TCCAAAATATTCAGAAGAATCAAAGTTGAACAGGGAAAAGATAGATGGAGTGAA
ATTGGAATCAATGGGGGTGTATCAGATTCTGGCGATCTACTCAACTGTCGCCA
GTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCT
AATGGGTCTTTGCAGTGCAGAATATGCATCTGA***GAATTC***

Restriction Sites:

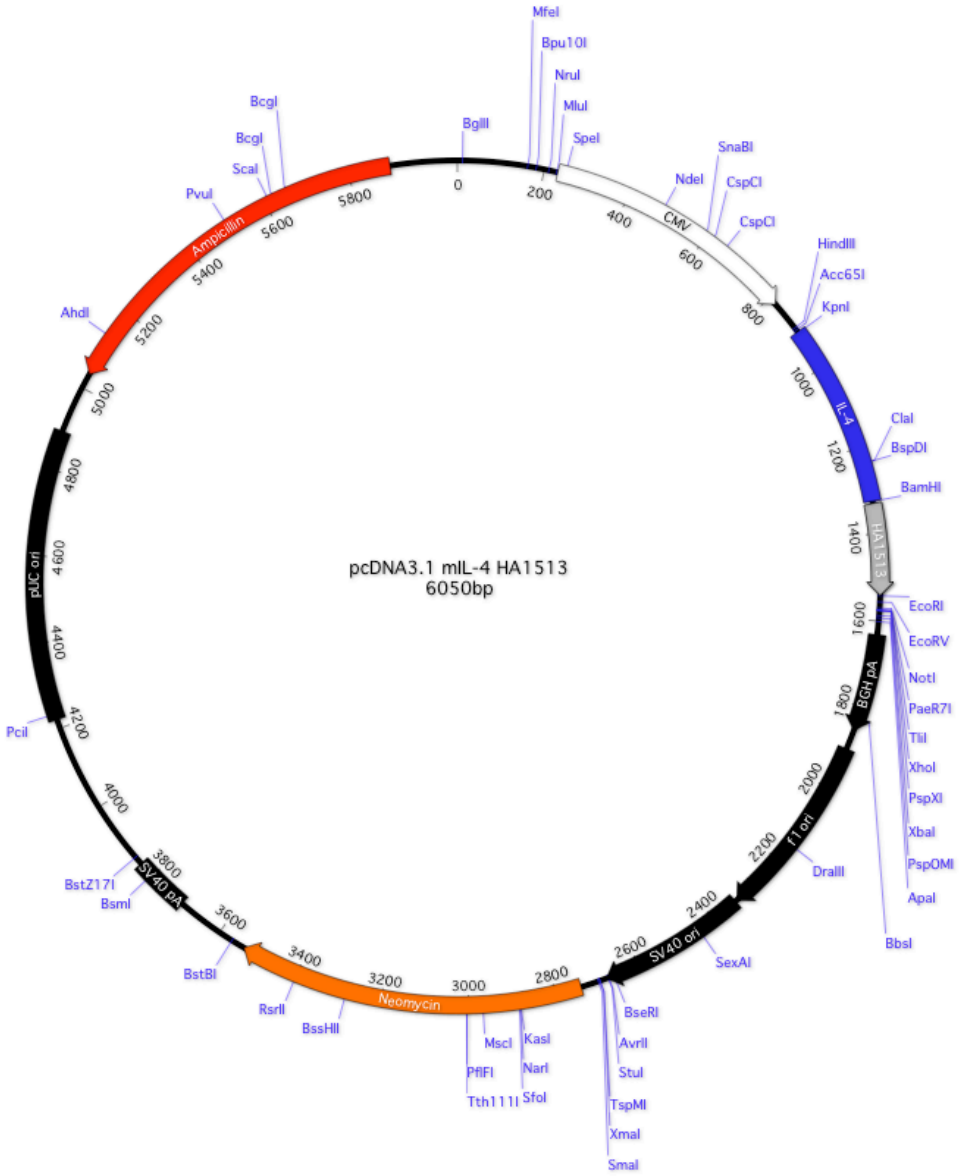
Acc65I	895	KasI	2958	RsrII	3475
AhdI	5203	KpnI	899	ScaI	5683
ApaI	1673	MluI	228	SexAI	2516
AvrII	2749	MscI	3041	SfoI	2960
BamHI	1402	NarI	2959	SmaI	2772
BbsI	1911	NdeI	484	SnaBI	590
BsgI	1620	NotI	1645	SpeI	249
BsmI	3879	NruI	208	StuI	2748
BssHII	3356	PaeR7I	1651	TliI	1651
BstBI	3641	PciI	4310	TspMI	2770
BstZ17I	3931	PflFI	3077	Tth111I	3077
DraIII	2225	PspOMI	1669	XbaI	1663
EcoRI	1618	PspXI	1651	XhoI	1651
EcoRV	1630	PvuI	5573	XmaI	2770

murine IL-2 HA1513 Construct Amino Acid Sequence

MQLASCVTLTLVLLVNSAPTSSSTSSSTAEAQQQQQQQQQQQQHLEQLLMDLQE
LLSRMENYRNLKLPRMLTFKFYLPKQATELKDLQCLEDELGPLRHVLDLTQSKSF
QLEDAENFISNIRVTVVKLKGSDNTFECQFDDESATVVDFLRRWIAFCQSIISTSPQ
GS**NGTYDYPKYSEESKLNREKIDGVKLE**SMGVYQILAIYSTVASSLVLLVSLGAISF
WMCSNGSLQCRICI*

Appendix D.

Murine Interleukin-4~HA1513 Expression Vector



murine IL-4 HA1513 Construct Gene Sequence

GGTACCGCACCATGGGTCTCAACCCCCAGCTAGTTGTCATCCTGCTCTTCTTT
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GATCATCGGCATTTTGAACGAGGTCACAGGAGAAGGGACGCCATGCACGGAG

ATGGATGTGCCAAACGTCCTCACAGCAACGAAGAACACCACAGAGAGTGAGC
TCGTCTGTAGGGCTTCCAAGGTGCTTCGCATATTTTATTTAAAACATGGGAAAA
CTCCATGCTTGAAGAAGAACTCTAGTGTTCTCATGGAGCTGCAGAGACTCTTT
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GTCCACATCACTGAAAGACTTCCTGGAAAGCCTAAAGAGCATCATGCAAATGG
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AGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTTTGGTCTCC
CTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGGTCTTTGCAGTGCAGAAT
ATGCATCTGA***GAATTC***

Restriction Sites:

Acc65I	895	CspCI	626	PspXI	1575
AhdI	5127	DraIII	2149	PvuI	5497
ApaI	1597	EcoRI	1542	RsrII	3399
AvrII	2673	EcoRV	1554	ScaI	5607
BamHI	1326	HindIII	889	SexAI	2440
BbsI	1835	KasI	2882	SfoI	2884
BcgI	5666	KpnI	899	SmaI	2696
BcgI	5632	MfeI	161	SnaBI	590
BglII	12	MluI	228	SpeI	249
Bpu10I	180	MscI	2965	StuI	2672
BseRI	2669	NarI	2883	TliI	1575
BsmI	3803	NdeI	484	TspMI	2694
BspDI	1236	NotI	1569	Tth111I	3001
BssHII	3280	NruI	208	XbaI	1587
BstBI	3565	PaeR7I	1575	XhoI	1575
BstZ17I	3855	PciI	4234	XmaI	2694
ClaI	1236	PflFI	3001		
CspCI	661	PspOMI	1593		

murine IL-4 HA1513 Construct Amino Acid Sequence

MGLNPQLVVILLFFLECTRSHIHGCDKNHLREIIGILNEVTGEGTPCTEMDVPNVLT
ATKNTTESELVCRASKVLRIFYLKHGKTPCLKKNSSVLMELQRLFRAFRCLDSSIS
*CTMNESKSTSLKDFLESLKSIMQMDYS*GS**NGTYDYPKYSEESKLNREKIDGVKL**
ESMGVYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI*

Appendix E.

Influenza A MHC I and MHC II Peptides

Manufactured by: GeneScript Corporation
120 Centennial Ave.
Piscataway, NJ 08854
Tel: (732) 885-9188
Fax: (732) 210-0262
E-mail: order@genscript.com

MHC I Peptides

Peptide 1: Influenza A NP 147-155
Sequence: TYQRTRALV — Purity: 97.2%
Molecular Weight: 1107.27 — Quantity: 14mg

Peptide 2: Influenza A/PR/8/34 HA
Sequence: IYSTVASSL — Purity: 96.2%
Molecular Weight: 940.05 — Quantity: 14mg

Peptide 3: Influenza A/PR/8/34 HA
Sequence: LYEKVKSQL — Purity: 94.0%
Molecular Weight: 1107.31 — Quantity: 14mg

MHC II Peptides

Peptide 4: Influenza A NP 55-69
Sequence: RLIQNSLTIERMVLS — Purity: 84.9%
Molecular Weight: 1773.11 — Quantity: 14mg

Peptide 5: Influenza A/PR/8/34 HA
Sequence: SFERFEIFPKE — Purity: 98.3%
Molecular Weight: 1428.59 — Quantity: 14mg

Peptide 6: Influenza A/PR/8/34 HA
Sequence: HNTNGVTAACSH — Purity: 75.6%
Molecular Weight: 1211.27 — Quantity: 14mg

Peptide 7: Influenza A/PR/8/34 HA
Sequence: CPKYVRSAKLRM — Purity: 88.0%
Molecular Weight: 1451.81 — Quantity: 14mg

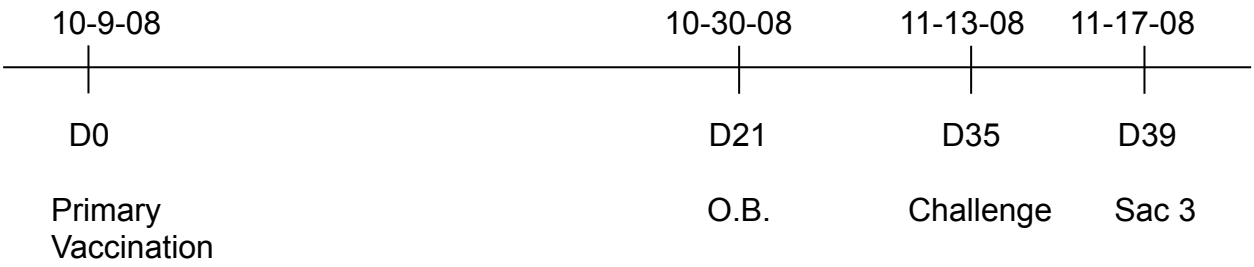
Peptide 8: Influenza A/PR/8/34 HA
Sequence: KLKNSYVNKKGK — Purity: 98.1%
Molecular Weight: 1406.68 — Quantity: 14mg

Peptide 9: Influenza A/PR/8/34 HA
Sequence: NAYVSVVTSNYNRRF — Purity: 90.2%
Molecular Weight: 1789.95 — Quantity: 12mg

Appendix F.

Vaccination Protocol for Evaluation of CYT-IVAC Efficacy.

Vaccination Group	# of mice per group	ug of viral protein	Total viral protein needed (ug)	# of mice challenged D35 post-vaccination
PBS	**13**	**0**	**0**	**13**
PR8 wt I.N.	**13**	**1**	**13**	**13**
PR8 wt S.C.	**13**	**0.375**	**4.875**	**13**
PR8 IL-2 I.N.	**13**	**1**	**13**	**13**
PR8 IL-2 S.C.	**13**	**0.375**	**4.875**	**13**
PR8 IL-4 I.N.	**13**	**1**	**13**	**13**
PR8 IL-4 S.C.	**13**	**0.375**	**4.875**	**13**



Sac 3 mice per group at D39 (day 4 post challenge)
Collect Lungs, N.W., spleens for:

- -N.W. IgA
- -Serum IgG, IgG subtypes
- -Lung viral loads post challenge
- -Splenocyte Proliferation Assay
- -AFC from spleens

Appendix G.

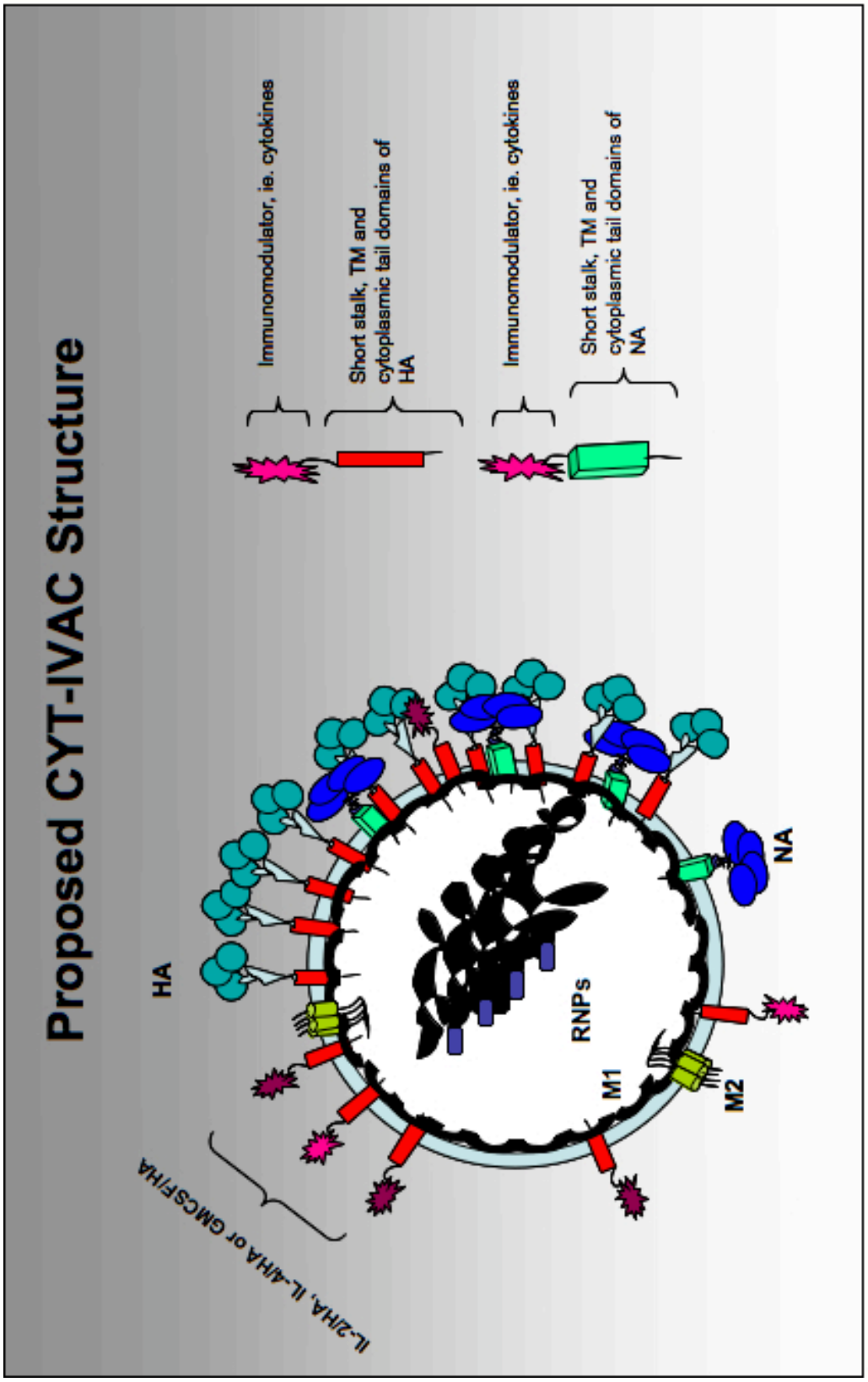
Vaccination Protocol for Evaluation of Cellular Immunity Induced by CYT-IVACs.

Vaccination Group	# of mice per group	ug of viral protein	Total viral protein needed (ug)	# of mice challenged D35 post-vaccination
PBS	**6**	**0**	**0**	**6**
PR8 wt I.N.	**6**	**5**	**30**	**6**
PR8 wt S.C.	**6**	**1**	**6**	**6**
PR8 IL-2 I.N.	**6**	**5**	**30**	**6**
PR8 IL-2 S.C.	**6**	**1**	**6**	**6**
PR8 IL-4 I.N.	**6**	**5**	**30**	**6**
PR8 IL-4 S.C.	**6**	**1**	**6**	**6**

Sac 6 mice per group at D32 (day 4 post challenge)
Collect serum, lungs, N.W., spleens for:

- -N.W. IgA
- -Serum IgG, IgG subtypes
- -Lung viral loads post challenge
- -Splenocyte Proliferation Assay
- -AFC from spleens
- -Intracellular Cytokine Staining of Splenocytes

Appendix H.


Proposed CYT-IVAC Structure
Immunomodulator, ie. cytokines
Short stalk, TM and cytoplasmic tail domains of HA
Immunomodulator, ie. cytokines
Short stalk, TM and cytoplasmic tail domains of NA
HA
RNPs
M1
M2
NA
IL-2/HA, IL-4/HA or GMCSF/HA