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Comparison of Immune Correlates, Age Related Changes and Proteomic Profiling of Healthy Individuals Receiving Influenza Vaccines

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COMPARISON OF IMMUNE CORRELATES, AGE RELATED CHANGES AND PROTEOMIC PROFILING OF HEALTHY INDIVIDUALS RECEIVING INFLUENZA VACCINES

by

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ABSTRACT

COMPARISON OF IMMUNE CORRELATES, AGE RELATED CHANGES AND PROTEOMIC PROFILING OF HEALTHY INDIVIDUALS RECEIVING INFLUENZA VACCINES

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Eastern Virginia Medical School and Old Dominion University, 2009
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The burden of influenza related infections is substantial, both in terms of illness, lives lost and economic impact on society. The degree of impact of influenza related infections is much higher in the elderly population where it is a leading cause of catastrophic disability; greatly affecting the quality of life of elderly persons above 65 years of age. Vaccination is the mainstay for control and prevention of influenza infections. There are two vaccine formulations that are licensed for use at present. The inactivated influenza vaccines (TIIV) which have been used for 60 years in all age groups and the new live attenuated influenza vaccine (LAIV) which is only recommended for use in individuals between 2-49 years of age. The mechanisms by which these two vaccines provide immunity in pre-vaccinated individuals have not been investigated in detail.

In our study we looked at the different immune correlates of vaccine responses and studied the mechanism by which these two vaccines provide immunity. We investigated age related changes in immune response to inactivated influenza vaccines between young and elderly. We also attempted to identify serum specific vaccine and age related immune senescence markers using mass spectrometric approach by using the MALDI-TOF MS technique.

We found contrasting immune responses induced by the two vaccines in different

arms of the immune system. Our results showed that using antibody titer as the only standard to measure vaccine efficacy may lead to a bias towards parentarally administered vaccines. Our study showed significant age related differences in both humoral and cell mediate immune responses in the cohort immunized with inactive influenza vaccine. Elderly showed a decline in IFN-γ secretion as a result of age related decline in the function of influenza specific memory T cells. A positive correlation was observed between Th 1 T cell response and antibody response only in the elderly which suggested an important role of IFN-γ to antibody response in elderly. Our study did not find any relationship in baseline levels of IL-10, IL-6, TNF-α and IL-1β cytokines affecting T cell or antibody response in the elderly which suggests that immune response to vaccination is not affected in the elderly due to a change in these cytokines with age. We also demonstrated that MALDI-TOF MS technique was not feasible in identifying vaccine response or immune senescence markers in healthy vaccinated individuals.

This thesis is dedicated to my family. Without their support this journey would not have been possible.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Influenza Virus

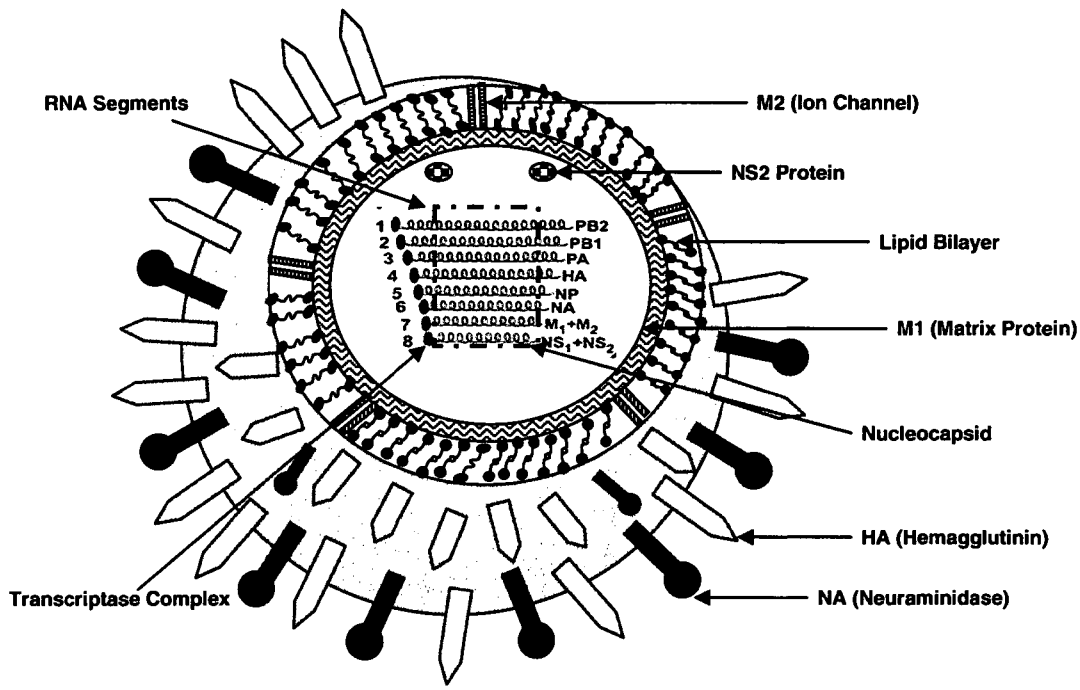
1.1.1. Classification, Structure and Nomenclature

Influenza viruses belong to the family *Orthomyxoviridae* (from the Greek *orthos* meaning 'standard' and *myxa*, meaning 'mucus') [1]. There are three types of influenza viruses categorized as A, B and C based on the antigenic differences of two internal proteins, nucleoprotein (NP) and matrix protein (M) [2]. Influenza virus particles are enveloped and often pleiomorphic, with a diameter of 80 to 120 nm. The host-derived lipid envelope contains spikes, which are integral proteins that project out from the surface of the virus particles (Fig. 1). Influenza viruses contain 7 or 8 negative-sense single stranded RNA (ssRNA) genome segments depending on the type of influenza virus. Influenza type A and B virions are composed of 8 genome segments, which code for ten different viral proteins, while type C has 7 segments, which code for the same number of proteins. The three influenza types not only differ in their genetic composition, but also differ in epidemiology, host range and pathogenicity. Influenza A and B viruses are important human pathogens, while influenza C viruses are rarely associated with infections in humans. Influenza B viruses primarily infect only humans while influenza A viruses are known to infect a number of vertebrates, most importantly aquatic birds [3, 4]. Migrating water fowl are believed to be hosts of all known influenza A sub-types.

The model journal for this dissertation is *Vaccine*

The influenza type A viruses are further divided into subtypes based on the different surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date there have been 16 distinct HA and 9 distinct NA types reported based on sequence analysis and verification of serological reactivity [2, 5]. All influenza strains acquire names based on a standard nomenclature established by the World Health Organization (WHO). For each influenza virus strain the designation comprises the type of influenza virus/abbreviation for animal (if not human species)/place or area of isolation/sequence number at the isolating laboratory/year of isolation (subtype), e.g. *Influenza A/New York/411/2002 (H3N2)* [6, 7].

Fig. 1. Structural diagram of influenza virus



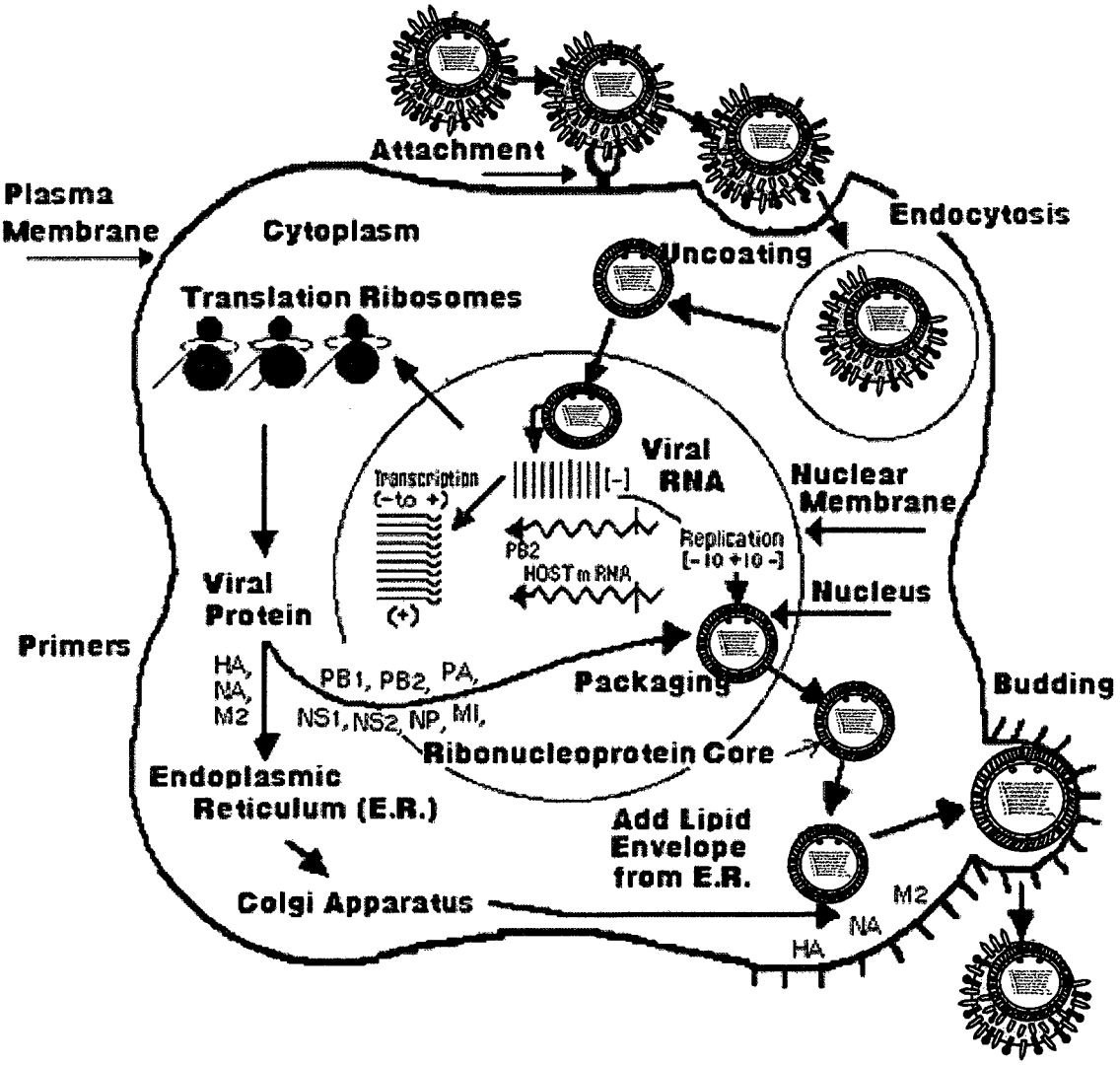
1.1.2 Viral Life Cycle

Influenza virus infects the mucosal epithelial cells of the upper respiratory tract. The viral infection begins when viral HA binds to sialic acid residues on the host cell surface. The removal of sialic acids by the viral NA glycoprotein creates a pathway for the virus through the outer mucus layer to allow cell receptor mediated endocytosis. The virus is engulfed and taken into the cell in a vesicle where it fuses with acidic endosomes. The low pH allows the flow of ions from the endosome into the virion via the viral M2 protein channel resulting in the disassociation of viral ribonucleoproteins (RNPs) from the viral M1 protein. The low pH is also responsible for a conformational change in the HA protein, which results in the fusion of the viral envelope and the endosomal membrane. This step is necessary for virus infectivity [2]. The fusion results in the release of disassociated RNPs into the cytoplasm of the infected cell which are then transported to the nucleus via the NP that has a nuclear localization signal (NLS) [8]. In the nucleus of the infected cells the (-) sense viral RNAs (vRNAs) serve as templates for both transcription of mRNAs and replication of new vRNAs. The viral polymerase requires a 5' RNA primer to initiate transcription of viral mRNAs. This is achieved by a process called "cap-snatching" where the cap specific endonuclease activity of PB2 removes the 5' methylguanosine ends of newly synthesized cellular mRNAs [9]. The elongation of the mRNA chains are mediated by PB1 polymerase which continues up to a point where a stretch of uridine residues is reached where the polymerase complex "stutters" and the poly (A) tail is transcribed. The RNA polymerase is made up of three subunits which are PB1, PB2 and PA which work in conjunction to transcribe the viral genome into a (+) sense mRNA and a (-) sense complimentary RNA (cRNA). While

transcription is carried out by PB1 and PB2 proteins, genome replication requires PB1 and PA subunits of the polymerase complex. The cRNA is used for generating new viral genome and mRNA is used for protein translation. Once the initial proteins are made, then eight complementary positive sense RNA strands are made from the eight (-) sense RNA segments (in influenza A and B). In influenza A and B, ten proteins result from the translation of the eight segments of the genome, including hemagglutinin (HA), neuraminidase (NA), PB1, PB2, nucleoprotein (NP), another RNA polymerase complex, two matrix proteins (M1 and M2) , and two NS proteins (NS1 and NS2). The HA, NA and M2 are translated on the endoplasmic reticulum (ER) while the other proteins are translated in the cytoplasm on free ribosomes. The HA, NA and M2 proteins are transported from the ER to the Golgi apparatus where they undergo further modifications like glycosylation, polymerization, and acylation before being transported to the cell membrane. The RNPs and M1 proteins also migrate to the cell membrane where they interact with the HA and NA proteins. The viral envelope begins to form on the cell membrane by the continuous interaction of M1 and other viral glycoproteins [2]. The viral particles are pinched off via a budding process of the cell membrane by an unknown mechanism. Upon exit, the neuraminidase removes surface sialic acid receptors on the membrane, thus allowing the virus particles to leave the cell. The accumulation of nucleocapsid in the cytoplasm initiates assembly [10, 11]. The production of infectious viral particles is inhibited if the activity of NA is inhibited or HA is not cleaved [12]. How the viral RNA segments are packaged for inclusion in the budding viral particles is not well defined. Most viral particles that bud off from an infected cell are not viable;

only one virion out of 20-50 virion is able to infect a new cell in cell culture [13]. A schematic diagram of the life cycle is described (Fig. 2).

Fig. 2. Schematic diagram of Influenza virus life cycle.
Figure adapted from diagram of virus replication at *www.accessexcellence.org*



1.1.3 Influenza Epidemiology

Influenza viruses are responsible for highly contagious infections and are a major cause of annual epidemics and occasional pandemics. Annual influenza outbreaks usually occur during the winter months in the Northern Hemisphere and between May through September in the Southern Hemisphere. However, year round infections are detected in the tropic and subtropical areas [14]. The reason behind seasonal outbreaks of influenza infections is not clearly known. One possible explanation could be the close proximity of individuals spending more time indoors during winter months, exacerbating person to person transmission. The number of suspected influenza cases in periods of known influenza spread designated as influenza like illness (ILI) is a frequently used measure of epidemiological activity by international and national authorities [15]. The numbers of ILI cases are reported by general practitioners (GP) with patients suffering from typical influenza symptoms and the number of ILI is a good estimate of the magnitude of circulating influenza viruses [16]. The definition of ILI that corresponds best with laboratory confirmed influenza is a sudden onset of fever, cough, myalgia, severe malaise and fatigue [17].

Influenza outbreaks can vary strongly between seasons, however, it is estimated that during annual influenza outbreaks 5-15% of the world's population is infected resulting in one million deaths each year [18]. However, the total number of influenza related deaths worldwide is difficult to estimate, due to a lack of knowledge about influenza epidemics in developing countries [19]. Influenza is a leading cause of catastrophic disability, greatly affecting the quality of life of elderly people above 65 years of age. The degree of impact related to influenza infections is much higher in the

elderly population as opposed to young adults [20, 21]. Most influenza infection related deaths are a result of complications and secondary infections like pneumonia in the elderly and the frail elderly [22]. Along with pneumonia, influenza represents the sixth leading cause of death among individuals 65 years or older in the United States [23]. Influenza infections account for 226,000 hospitalizations and 36,000 deaths annually in the United States, as estimated by the Center for Disease Control and Prevention [20, 24]. Morbidity rates as high as 70% have also been reported in children less than a year old [25]. The burden of influenza related infections is substantial, both in terms of illness, lives lost and economic impact on society [18]. In a recent report it has been estimated that the direct and indirect economic costs of influenza related complications are as high as $71 billion to $166 billion [26]. The major economic impact of influenza is related to loss of productivity, besides the medical costs of hospitalizations as a result of severe disease. Vaccination is the most cost-effective means to prevent seasonal influenza infections and has proven to be effective in prevention and controlling severity of disease [21, 27].

1.1.4 Antigenic Drift

Influenza viruses are unique among the respiratory viruses as they have substantial antigenic variations. The three influenza types continuously undergo antigenic variations leading to changes in the amino acid sequence as a result of mutations through a process called antigenic drift. "New drifted" influenza A variants replace previously circulating strains each year causing seasonal epidemics, whereas influenza B and C accumulate fewer mutations and generally are more antigenically stable [28]. Annual

outbreaks are a result of accumulation of mutations. Antigenic drift is the gradual evolution of viral strains due to frequent mutations and occurs in response to selection pressure to evade human immunity [29, 30]. The process of antigenic drift involves point mutations within antibody-binding sites in the influenza glycoproteins HA and NA, or both, which may potentially occur each time the virus replicates [31]. Influenza viruses like all RNA viruses general have very high frequencies of copy errors during replication as the RNA dependent RNA polymerase does not have the ability to proof read. The average number of mutations in influenza virus per genome per replication cycle is 1.0, compared to 0.0027 for yeast *(Saccharomyces cerevisiae)* [32]. Mutations, especially in the HA which contains the main antigenic determinants allows the virus to escape the host's immunological memory. The annual mutation rate for influenza A envelope glycoproteins HA and NA is 0.4% and 0.7%, respectively [33]. Mutations allow the virus to escape the host's antibody repertoire resulting in new infections. The slower evolution of influenza B and C viruses may be attributed to having a single host to replicate. A hypothesis explaining this has been proposed; both influenza B and C viruses have been coevolving with humans longer and have undergone host specific adaptations, while on the other hand, influenza A has not reached its evolutionary equilibrium with man and is unable to do so as a result of frequent reassortment with viruses from other hosts [4].

1.1.5 Antigenic Shift

Antigenic shift involves major changes in the surface glycoproteins HA and NA of influenza A viruses. It occurs after reassortment of viral genome segments between two different influenza A types. If two different influenza type A viruses co-infect the

same cell and multiply, exchange of genome segments can occur that lead to novel viruses with new combinations of HA and NA surface glycoproteins. If this reassortment generates new subtypes never seen in human populations, the potential for influenza pandemics can occur as these novel viruses spread in humans [34]. It is estimated that the phenomenon of antigenic shift occurs approximately three times every 100 years [35], in fact three pandemics occurred during the 20^{th} century (1918, 1957 and 1968). The most devastating antigenic shift occurred in 1918 and was termed the "Spanish Flu" [36]. There are three main theories to describe the process of antigenic shift: reassortment, recirculation of existing subtypes and a gradual adaptation of animal viruses to human transmission. Reassortment is the most important process that contributes to major shifts in influenza antigenicity and consists of a mixing of genetic material between different viral strains. Genetic reassortment occurs between co-infecting influenza A subtypes from different species for example; reassortment between human and avian virus strains could lead to the production of a highly virulent strain. Co-circulation of different influenza A subtypes is responsible for reassortment [29] and is of particular importance in the evolution of avian flu-associated H3N2 viruses [37]. Once a virus has undergone antigenic shift, it remains susceptible to antigenic drift as occurs with any influenza virus. In most cases the novel viruses are not able to directly infect humans; however, over time the novel viruses can acquire the required mutations, which could enable them to directly infect humans. Direct transfer of highly virulent strain from avian carriers to humans has been confirmed by influenza epidemics in Hong Kong during 1997-1998 [38]. Domestic animals and birds are important reservoirs of influenza viruses. Close proximity to these hosts may lead to higher chances of co-infection and direct transmission of different

influenza types [39]. The highly pathogenic avian influenza A (H5N1) epizootic (animal outbreak) has been reported recently in Asia, Europe, the Near East, and Africa. H5N1 Infections of H5N1 virus have become endemic among domestic poultry in certain parts of the world, which is thought to be responsible for sporadic human infections as a result of direct contact with infected fowl or wild birds who are carriers of the virus. To date, person-to-person transmission of H5N1 infections have been very rare, limited and unsustained. However, this epizootic threat continues to pose a important public health hazard [40]. The World Health Organization (WHO) has reported human cases of avian influenza A (H5N1) in Asia, Africa, the Pacific, Europe and the Near East. The highest numbers of cases to date were reported from Indonesia (141) and Vietnam (109). The total number of deaths reported as a result of H5N1 infections to date is 256 [41]. Overall mortality in reported H5N1 cases is approximately 60%. The majority of cases have occurred among children and adults less than 40 years old. Mortality was highest in patients 10-19 years of age.

1.1.6 Influenza Transmission, Symptoms and Pathogenesis

Influenza viruses are spread in the form of virus-laden droplets from coughing, sneezing or direct person-to-person contact from infected individuals. Human influenza viruses replicate almost exclusively in epithelial cells of the respiratory tract; however, in rare cases infections of the muscle and central nervous system have been documented [1, 42, 43]. Symptoms such as cough, sore throat, myalgia and fever are due to a combination of inflammatory response to viral replication in infected cells and desquamation of the epithelial cell lining. The incubation period is short lasting one to

five days and the onset of illness is abrupt with the initial three days being the period of highest person to person communicability. Clinical symptoms persist for three to four days, although cough and malaise may persist for as long as two weeks. Influenza can cause severe disease and complications in individuals belonging to the high-risk groups. Complications, which include primary viral pneumonia, combined viral-bacterial pneumonia and bacterial pneumonia, are most common in the elderly and frail elderly following influenza infections. Influenza infections cause macrophage-mediated lysis of infected cells, secretion of the inflammatory cytokines tumor necrosis factor-alpha (TNF-α), alpha and beta interferons (IFN-α, IFN-β) and interleukins (IL-1β, IL-6, IL-12), which have been implicated in pathogenesis of influenza infections. For the host, these cytokines, interferons and interleukins help limit production of new viruses by down regulation of intracellular protein synthesis [44, 45].

1.1.7 Immune Responses to Influenza virus

1.1.7.1 Innate Immune Responses

In humans, the first lines of defense against influenza virus infections are multi-component innate immune responses. The main function of the innate immune system is to contain the pathogen until the adaptive immune system is activated and fully functional. The components of the innate immune response consist of mucus, macrophages, IFN-α, IFN-β, the complement system, and natural killer (NK) cells. Most influenza viruses are initially detected and destroyed within hours by non-antigen specific innate immune mechanisms [1, 45]. The complement system is important in recruiting phagocytic cells by acting as an opsonizing and inflammatory initiator. The complement

system together with anti-influenza antibodies present in serum permeabilizes membranes and contributes to the destruction of target cells. Infected monocytes, macrophages and alveolar epithelial cells secrete chemokines that attract other immune cells like neutrophils, macrophages and NK cells to the site of infection. These cells produce additional cytokines, chemokines and anti-viral proteins in response to influenza infections. Macrophages have been shown to secrete IL-1, IL-6, TNF-α, and IL-12 which are known activators of NK cells.

The type 1 interferons, IFN-α and IFN-β, are among the most important cytokines of the innate immune system, as they play a major role in controlling the spread of infection by inhibiting viral replication [1]. Besides recruiting monocytes, the interferons also stimulate increased expression of major histocompatibility complex class I (MHC I) and class II (MHC II) to increase antigen presentation to macrophages and NK cells. Cytokines IL-1, IL-6 and TNF-α induce fever. IL-12 along with TNF-α elicits production of IFN-γ by NK cells. IFN-γ can activate pathways associated with direct antiviral functions [46]. Within 48 hours of the onset of viral infection, NK cells producing IFN-γ are detected which help to limit the spread of virus by perforin mediated cell lysis of infected cells with altered MHC I [45]. It has been shown that NK cells are activated with the help of their activating receptors NKp46 and NKp44 that recognize HA on infected cells and lyse them [47]. Influenza virus has a novel mechanism for inhibiting NK cell lysis activity, thereby enhancing the ability of the virus to spread to other cells [48]. The innate toll-like receptors (TLRs), which are a family of pattern recognition receptors that bind to pathogens and serve as sentinels to induce cytokine production [49], have also been proven to significantly contribute to immune activation in response to influenza

infection. The TLRs are differentially expressed in antigen presenting cells (APCs) and in lymphocytes. The dsRNA intermediate produced by influenza virus during its replication cycle is able to activate TLR 3, TLR 7 and TLR 8 that are constitutively expressed on the APCs or NK cells [50-52].

1.1.7.2 Adaptive Immune Response – Humoral and Cell Mediated

Influenza viruses that are able to evade and escape the initial innate immune response can next be detected and destroyed by components of the adaptive immune system. The adaptive immune response against influenza virus consists of humoral and cell mediated immune responses (CMI). The humoral immune response is elicited by the bone marrow derived lymphocytes called B cells and the cell-mediated immune responses are elicited by a set of thymus-derived lymphocytes called T cells.

The humoral immune system, which includes mucosal and systemic components, plays a major role in immunity against influenza infection and disease. In humans, specific antibodies against all major viral proteins like HA, NA, NP, M1 and M2 are detected after infection [53]. However, antibodies to surface glycoproteins HA and NA have been proven to provide protection and resistance to infection, whereas antibodies to conserved antigens like M and NP proteins are not protective [1]. The antibodies produced against the HA glycoprotein are the most important for neutralizing the virus and preventing virions from binding to host cell receptors, and hence, preventing illness. Antibodies against the NA glycoprotein prevent the release of virus from infected cells and help restrict the infection to the respiratory tract [1]. The specific antibodies generated by the immune system cannot provide lifelong immunity against influenza

viruses due to the ability of the virus to generate new antigenic epitopes as a result of which protection provided by antibodies only lasts up to a few years.

Besides the innate immune system, the mucosal immune system also forms an initial line of defense against infection. Nasal secretions consist of neutralizing antibodies which are primarily secretory immunoglobulin A (S-IgA) [54] and IgM against influenza glycoproteins HA and NA. The mucosal S-IgA prevents entry of virus and can function intracellularly to inhibit viral replication [55]. The S-IgA antibodies are primarily involved in prevention of influenza infections in the upper respiratory tract, whereas serum IgG antibodies predominate in protection of the lower respiratory tract [45]. Subjects who have a local IgA response have been shown to have serum IgA response [55]. It has been observed that IgA is the predominant isotype detected in local secretions after infection and serum IgA responses are detected in serum upon subsequent infection. Serum antibodies play a major role in resistance to or recovery from influenza infections in humans. In humans, levels of serum antibody to surface glycoproteins HA and NA correlate with resistance to illness following challenge with influenza virus under experimental conditions or natural infections [56, 57]. The most commonly measured correlate of protection against influenza are the serum anti-HA antibodies [58]. All three major classes of Ig (IgG, IgA and IgM) are produced by B cells present in the peripheral blood in individuals undergoing influenza infections [59]. The three major Ig classes can be detected within 10-14 days during a primary infection. Peak levels of IgA and IgM are observed after 2 weeks of infection, but then the levels begin to decline, whereas IgG levels peak around 4-6 weeks after infection. The IgG and IgM predominate in primary responses, while IgA and IgG are dominant during secondary immune responses [57].

The serum IgGs are divided into four classes: IgG1, IgG2, IgG3 and IgG4. Most of the serum IgGs belong to the IgG1 sub-group and play an important role in the prevention of influenza infection and provide protection against new infections [60]. In humans, systemic levels of IgG1 and IgG3 are important for complement fixation and antibody-dependent cellular cytotoxicity [61].

1.1.7.3 Cell Mediated Immune Responses

Cell-mediated immune responses play a major role in clearing virus from infected cells, which for influenza infections, aids in recovery from influenza illness and it may also prevent influenza-associated complications. However, its role in preventing influenza infections has not been well defined [1, 55]. The cell-mediated immune component consists of two main cell types, cytotoxic T cells (CD8+ T cells) and helper T cells (CD4+ T cells). The cytotoxic CD8+ T cells (CTLs) are restricted by MHC I antigens. In humans, pre-infection levels of virus specific CTL are associated with accelerated clearance of the virus from the respiratory tract [62]. The CTLs appear in the blood of infected or vaccinated individuals on days 6 to 14 and disappear by day 21 [63]. During a primary infection, the naïve T cells expand and differentiate into cytotoxic effector T cells, which are capable of eliminating virus-infected cells. After viral clearance, the pool of effector T cells contracts and a virus-specific memory T cell persists that can undergo rapid reactivation in the event of future infections. The cytotoxic T cell response has been found to be cross-reactive, providing protection against serologically different influenza A viruses [64]. Internal non-glycosylated proteins like M, NP and PB2 have been shown to be recognized by CTLs [65, 66].

Based on the cytokines produced by helper T cells, they are sub-divided as T helper type 1 or T helper type 2 cells. The type 1 helper cells (Th1) secrete IFN-γ and IL-2 which helps in antibody production and proliferation of MHC I restricted CTLs which primarily drives cellular immunity. The cytokine IL-2 has been shown to be indispensable in stimulating Th1 and CTL proliferation, and differentiation and activation of NK cells [67, 68]. In addition IL-2 contributes to antibody generation by enhancing B and T cell interactions [69]. Another set of CTLs have been found that are CD4+ MHC class II restricted that have peforin mediated cytotoxicity [70]. The cytokine IFN-γ is also an important immune modulator and influences other immune cell functions and antibody subclass switching. It has been demonstrated that IFN-γ is able to upregulate the expression of TLRs and MHC class I and II molecules in macrophages, increasing anti-viral activity [71, 72]. In humans, IFN-γ has also been shown to influence IgG class switching to the IgG1 sub class [73]. The type 2 helper cells (Th2) secrete IL-4, IL-5 and IL-10 which drives humoral immunity by helping antibody producing cells to produce IgA, IgG1 and IgE [74].

1.1.8 Prevention and Prophylaxis of Influenza

There are two major methods to prevent and treat influenza infections, vaccines and antiviral drugs. For vaccines, there are two main types used to prevent annual influenza infections. They are divided into two categories; inactivated influenza vaccines and live virus vaccines.

1.1.8.1 Inactivated Influenza Vaccines

There are three main formulations of the inactivated influenza vaccines that are or have been used clinically. The whole virus vaccine is inactivated using chemical reagents (formalin, β-propiolactone) without destroying the viral envelope [75]. In spite of high immunogenicity, the use of this kind of vaccine formulation was discontinued due to frequent adverse reactions [76, 77]. The other formulation is a split virus vaccine that is produced by chemical agents (ether, tributyl phosphate) by disrupting the viral envelope. The immunogenicity induced by this vaccine is lower compared to the whole virus vaccine; however, there are fewer adverse reactions [76]. The third formulation is a sub-unit vaccine that consists of highly purified surface glycoprotein antigens HA and NA [78]. This formulation is the least immunogenic formulation; however it is one of the safest vaccine formulations and is the most widely used vaccine at present. All inactivated influenza vaccines are administered parenterally, either by an intramuscular or subcutaneous route. The inactivated vaccines are produced in embryonated chicken eggs. All the vaccines are trivalent and are composed of three influenza virus strains to provide protection against the most commonly circulating strains during a season. A normal adult dose of the trivalent influenza vaccine has a concentration of 15μg HA of each influenza strain (H1N1, H3N2 and one B strain). The inactivated vaccines are licensed in the U.S.A. for use in children 6 months or older and all adult age groups. The inactivated vaccines are 60-100% effective in preventing morbidity and mortality and 30-70% effective in prevention of hospitalization in elderly people [79]. The efficacy of inactivated vaccines has been found to be significantly lower in the elderly population when compared to the young [21].

1.1.8.2 Live Attenuated Influenza Vaccines

Live attenuated influenza vaccines (LAIV) have been recently licensed in the U.S.A., but have been used extensively in other countries like Russia [55]. The vaccine consists of a reassorted virus which has the genes encoding the HA and NA glycoproteins from wild-type virus inserted into an attenuated donor virus strain. In the U.S.A. there are two master donor strains: one for influenza A strains, A/Ann Arbor/6/60 (H2N2), and one for influenza B strains, that are used for the production of LAIV. The attenuated donor strains are temperature-sensitive and have limited ability to replicate in the lower respiratory tract [80]. The LAIV vaccines are trivalent and are also produced in embryonated chicken eggs. The LAIV vaccine is administered intranasally (0.25 ml in each nostril) at a dose of approximately 10^7 $TCID_{50}$ of each of three virus stains using a large particle aerosol spray device [81, 82]. The LAIV vaccine has been found to be 78-100% effective at preventing influenza associated illness after an experimental challenge or natural infection [82, 83]. The LAIV vaccines are licensed for use in children 2 years and older and in adults up to the age of 49 years. It has not been approved for use in elderly and immunocompromised adults, who are the most susceptible and high-risk groups that need to be vaccinated. Concerns regarding the possibility of new assortments between live virus present in the vaccine and wild-type influenza virus as a result of simultaneous infections have prevented the use of this vaccine in the high-risk groups.

A comparison of the two licensed vaccine products in the United States of America at present is listed in Table 1.

Table 1. Comparison of different properties of the two currently licensed influenza vaccines in the United States of America.

Category	TIIV (Fluzone®)	LAIV (FluMist®)
Administration	**Intramuscular**	**Intranasal**
Formulation	**Inactivated**	**Live attenuated**
Efficacy in children **Efficacy in adults <50years**	**50%–90%** **70%–90%**	**70%–90%** **70%–90%**
Safety (side effects)	**Sore arm**	**Runny nose**
Growth medium	**Chick embryos**	**Chick cells**
Storage	**Refrigerated**	**Refrigerated**
Age group	**Individuals ≥6 mo**	**2 – 49 yrs* (healthy)**

1.1.8.3 Antiviral Drugs against Influenza

There are three classes of drugs that are used to treat influenza infections. These chemical drugs target different viral proteins to prevent the spread of infection. The M2 channel inhibitors Amantadine and Rimantadine block the M2 ion channel protein to prevent viral uncoating and replication. These drugs only work against influenza A

viruses and have an efficacy rate of 70-90% in healthy adults when used prophylactically. The other class of drugs targets the NA glycoprotein and prevents the release of viral particles from infected cells. These drugs (Oseltamivir [Tamiflu] and Zanamivir [Releza]) act against both Influenza A and B strains with a prophylactic efficacy rate of 80%. The third group of drugs (Ribavirin) targets viral RNA replication [55, 84, 85]. All the chemical drugs help reduce illness within 1-2 days after their administration and reduce the possibility of transmission; however, they do not elicit immunological memory and therefore cannot be used a substitute for vaccination. Another critical issue regarding the use of anti-viral drugs is associated with the development of resistance to antiviral drugs. Studies show that influenza A viruses resistant to amantadine and rimantadine can emerge quickly during treatment, even as early as 2-3 days after initiation of drug therapy [86]. At present, neuraminidase inhibitors are the most widely used antivirals prescribed. A recent report by the CDC demonstrated that a high percentage of circulating influenza A/H1N1 viruses are now resistant to oseltamivir [87]. It is recommended that there should be careful use of antivirals, as monotherapy using a single antiviral agent will lead to further selection of antiviral resistant strains [88].

1.1.9 Aging and Immunosenescence

Aging can be defined as "a process that converts healthy adults into frail ones with diminished reserves in most physiological systems and an exponentially increasing vulnerability to most diseases and to death" [89]. Aging is not a disease but a natural phenomenon, which is genetically controlled and is influenced by environmental factors. Aging is associated with an increase in morbidity and mortality, which has been

attributed to immunosenescence. Immunosenescence is defined as the state of dysregulated immune function that contributes to increased susceptibility of the elderly to infection and possibly to autoimmune diseases and cancer [90, 91]. Immunosenescence results in the decline in the body's ability to fight infection or mount an adequate immune response [92]. Aging-related immunosenescence affects both the innate and adaptive immune systems.

1.1.9.1 Immunosenescence in Innate Immunity

The NK cells are major components of the innate immune system and represent 5-15% of the total human PBMC [93]. These cells are involved primarily in the secretion of IFN-γ and cytotoxicity. Studies have suggested that there is a decline in per cell activity of NK cells in the elderly; however the impact can be compensated as a result of increased number of NK cells in the elderly [94]. The TLRs are also affected by aging. Animal studies have shown that there is reduced expression of TLRs resulting in reduced production of cytokines TNF-α and IL-6 [95]. The APCs, which include macrophages, dendritic cells and monocytes, form an important link between the innate and adaptive immune systems. Animal studies have shown an impaired activation of tissue macrophages as a result of impaired IFN-γ production by macrophage cells [96]. Other studies in mice have also revealed that alveolar macrophages of older animals are less efficient in antigen presentation when compared to the young animals which indicates an age related decline in antigen presentation by APCs [97].

1.1.9.2 Immunosenescence in Adaptive Immunity

The adaptive immune system is composed primarily of T and B lymphocytes, which are responsible for eliciting specific immune responses against pathogens in conjunction with the innate immune system. The T cells are the major source of cytokines in the adaptive system and can be divided into two types based on cell surface expression of CD4 or CD8 molecules. The CD4 T cells are further divided into type 1 (Th1) and type 2 (Th2) cells, each of which produce different profiles of cytokines that are critical in regulating the inflammatory component of the immune response. The Th1 cells typically produce pro-inflammatory cytokines like IFN-γ, TNF-α, IL-2, IL-6 and IL-1 while Th2 cells produce anti-inflammatory cytokines such as IL-10, IL-4 and IL-5 [98, 99]. The production of Th1 and Th2 cytokines usually counteract each other. However, with advanced age and immunosenescence, there is a shift in towards the Th2 cytokine production and a loss in CTL activity [100, 101]. These changes create a pro-inflammatory state that alters immune cell signaling, differentiation, and apoptosis [102]. There is also an increase in memory to naïve ratio of both cytotoxic and helper T cells as a result of thymic involution, which is associated with a decrease in the central production of T cells [103, 104]. The loss in naïve T cells is offset by the expansion of memory T cells in the periphery [105]. The accumulated memory T cells may impair long term T cell activation [106]. An increase in CTL to helper T cells is also observed as a result of immunosenescence due to the apoptosis-resistant nature of CTL [107]. Other changes associated with aging T cells include loss of CD28 surface molecules which significantly reduce the ability of T cells to proliferate [108], calcium deficiency which is critical for cell signaling and other activation functions [109]; inactivation of

transcription factors like NF-KB and AP-1 which results in downregulation of IL-2 which is a key component of T cell proliferation and NK cell activation and differentiation [110].

The changes associated with B cells are less clear; however they appear to have similarities to age-related changes in T cells. B cells from older individuals show impaired activation and proliferation that may also be related to changes in co-stimulatory molecule expression [98, 103]. The age-related dysfunction of T cells in conjunction with the intrinsic impairment of B cells significantly affects the production of antibodies in the elderly [111]. Impaired primary and secondary antibody production after vaccination have been demonstrated; the impairment is greater when helper T cells are involved to drive the antibody production in the elderly. Lower efficacy and specificity of antibodies produced in older individuals when compared to younger individuals has been reported [112].

The field of proteomics, which involves the study of expressed proteins, has made rapid strides in the past two decades. The primary aim of clinical proteomics is to identify biomarkers for the diagnosis of disease by comparing the proteomic profiles of control and disease states from various body fluids like plasma, serum, saliva, cerebrospinal fluid, and urine [194]. Biomarker discovery using ever-advancing mass spectrometry techniques like matrix assisted laser desorption and ionization time of flight (MALDI/TOF), surface-enhanced laser desorption and ionization (SELDI), two dimensional electrophoresis (2D gels), and liquid chromatography-mass spectrometry (LC-MS) have emerged as essential investigative tools for identifying various disease states, especially in the field of early cancer diagnosis [139, 195-199]. I believe that this

technology can be applied to further understand the response to influenza vaccination and infection. Analyzing pre- and post-vaccination serum samples from healthy individuals could provide valuable insights into determining protein biomarker profiles of vaccine responders and non-responders. Attempting to correlate the antibody response and T cell response to proteomic data could facilitate better understanding and establishment of relationships between certain serum biomarkers and T cell and antibody response markers of vaccine response. The use of this technique could also provide specific proteomic profiles to differentiate the two different vaccine compositions.

1.2 Proteomics

1.2.1 Definition of Proteomics

The terms "proteome" and "proteomics" were first coined by Marc Wilkins in 1994 in a conference in Sienna, Italy, and subsequently published in 1995 by an Australian group (Wasinger *et al.*), who defined proteomics as "the study of proteins, how they're modified, when and where they are expressed, how they are involved in metabolic pathways and how they interact with one another" [113, 114].

The word "proteome" is derived from the words "PROTein" and "genOME". Each organism has a set of genes which comprises its genome; similarly, the entire repertoire of proteins expressed in an organism represents its proteome. Although the genome is the source of all the information in an organism, it needs to be decoded (expressed) into proteins that carry out functions in all the different cells of a living organism. The transcription of genes is the first stage of the decoding process, and is followed by translation of the messenger RNA to produce proteins. For most organisms

the genome remains relatively static over time, but the same cannot be said for the proteins being expressed. The proteome of a cell is a highly dynamic entity and undergoes several changes based on the condition, age, function, and intracellular and environmental stimuli. With the successful completion of the Human Genome Project in 2002, it was established that about 30,000 genes code for around 400,000 known proteins and likely several thousand more uncharacterized and undefined proteins [115-117]. The identification and analysis of proteins has turned out to be more complicated than expected, and so far the ambitious goal to identify all proteins expressed by human genome has not been achieved. Hence, proteomics represents a huge, long-term task, which will be more involved and challenging than sequencing the genome.

Proteomics is a combination of several fields of study, which include biochemical techniques, protein separation techniques followed by analytical techniques like mass spectrometry and finally utilizing the power of bioinformatics to identify proteins. Proteomic-based studies are primarily clinical proteomics or functional proteomics. Clinical proteomic based studies are focused essentially on protein expression (profiling) to investigate the differential expression based on a disease state versus a normal state and are dedicated to biomarker identification. Functional proteomic studies are targeted to identify protein-protein interactions and their roles in cell function and signaling pathways.

1.2.2 History of Protein Sequencing Methods

Early protein sequencing techniques were developed in the middle part of the twentieth century. A technique developed by Aberhalden and Brockmann in 1930 was

one of the earliest described protein sequencing methods [118]. The method involved a stepwise degradation procedure that utilized the ability of phenylisocyanate (PIC) to bind to amino acid groups and give rise to an intermediate that is capable of rearrangement under acidic conditions. The modified terminal amino acid from the parent peptide was cleaved in a stepwise process [118]. Nobel laureate Fredrick Sanger, whose work led to the first complete description of a chemical structure of protein "insulin", sequenced the protein by using a novel C-terminal protein labeling method involving the dinitrophenyl (DNP) group. The DNP-labeling method covalently modified the C-terminal amino acids of a peptide. The modification was used as a chemical marker of the peptide that remained bound to the terminal amino acid group after the peptides were hydrolyzed, following which the complete sequence of the peptide could be obtained by aligning all the peptides [117, 119]. In 1949, by using the basic backbone of Sanger's end terminal labeling technique and making modifications by replacing the coupling agent with phenylisothiocyanate (PITC), Pehr Victor Edman, a Swedish biochemist, developed a protein sequencing method that became known as Edman degradation. The method comprises three steps: coupling, cleavage and conversion. The first step entails coupling of PITC to the free N-terminal end of a polypeptide to form a cyclic intermediate phenylthiocarbamyl (PTC). The second step involves the cleavage of the modified PTC amino terminal product from the polypeptide by anhydrous acid. The cleavage results in two parts, the modified amino terminal amino acid and the shortened part of the initial polypeptide. The remaining polypeptide chain can undergo further coupling and cleavage reactions between its reactive N-terminal amino groups with PITC. The final conversion step engages in the removal of the modified amino terminal amino acid from the mixture

containing the shortened polypeptide sequence by a non-polar solvent. The hydrophobicity of the unstable derivative amino acid enables the removal using a non-polar solvent. An aqueous acid is used to convert the unstable derivative amino acid into a more stable phenylthiohydantoin (PTH) product. Following the final conversion step the remaining shortened polypeptide chain is free to undergo several cycles of stepwise degradation steps. The amino acid released in each cycle is identified using analytical liquid chromatography methods [120]. An automated version of the Edman degradation method was introduced in 1967 by Edman and Beg [121]. Despite its reliability and success the Edman degradation method is plagued with several limitations which include requirement of high sample volume, highly purified protein, requirement of an exposed N-terminus of the protein, low throughput and the ability to only sequence polypeptides composed of 50-70 amino acids.

During the period when automated Edman degradation method was introduced, a protein separation technique called two-dimensional gel electrophoresis (2DE) was developed by Klose and O'Farrell in the 1970s to separate and identify complex protein mixtures [122, 123]. The technique offered the separation and fractionation of proteins from complex mixtures by gel electrophoresis in two dimensions. The first dimension is used to separate the proteins based upon isoelectric focusing, which is followed by separation of proteins based on their molecular weight by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins are then visualized using an appropriate stain (silver, coomassie blue or fluorescent). The intensities of the spots after staining reveal the amount of each protein present in the mixture, which can subsequently be compared between two samples to identify

differential expression. The method did provide an effective strategy to separate complex protein mixtures, but it was soon observed that there were several limitations and drawbacks to this technique [114, 117]. Among the major drawbacks were issues regarding reproducibility, co-migration of proteins, the arduous and labor-intensive procedure, low throughput and lack of quantitation of differentially expressed proteins. A number of concerns regarding reproducibility and quantitation were addressed with the introduction of highly sensitive fluorescent dyes with a wide dynamic range. These specially-designed flourophores have been efficiently used in 2D-DIGE (differential gel electrophoresis) [124, 125]. Despite several advances made in the 2DE technique, it still remains a labor-intensive technique requiring a large amount of starting material and suffers from reproducibility concerns. Another concern with 2DE is that it does not resolve very basic proteins or hydrophobic proteins. It is also not suitable for identification of low abundance proteins, as they may not be stained and are hence underrepresented.

1.2.3 Mass Spectrometry

Except for the past 15-20 years, the biological applications of mass spectrometry in proteomic research were limited. Several advances and improvements in instrumentation and ionization techniques have made it possible for researchers to use mass spectrometry as their primary tool for most proteomic studies. There are many different types of mass spectrometers used by researchers involved in proteomic studies. All mass spectrometers consist of three essential components: an ionization source, a mass analyzer and an ion detector. The ionization module confers an electrical charge by

adding protons to the molecules, resulting in the generation of gas phase ions, which pass into the mass analyzer. The mass analyzer component of the mass spectrometer separates the gas phase ions based on electrical and/or magnetic fields. The movement of the ions is dependent on the mass and charge of ion and is measured as mass over charge (m/z) ratio. Commonly used mass analyzers include time of flight (TOF) analyzers, quadrupole, ion traps (typically coupled with quadrupole), fourier transformers (FT) and hybrid instruments (Q-TOF, Q-TRAP). Ions are detected after flowing through the mass analyzer by a device that detects the intensity and m/z values of the ions based on the magnitude of current produced at the detector as a function of time. The data are collected and recorded by a data recorder. The data processor then generates spectra that reflect the m/z values on the x-axis and the intensities on the y-axis [117].

The electron and chemical ionization techniques that were traditionally used for other molecules could not be applied to proteins as they did not ionize easily, owing to their large molecular structure, and they were susceptible to damage as a result of extensive thermal decomposition. The first attempt at ionization of proteins, made in 1981, allowed the analysis of proteins using a mass spectrometer by a method called fast atom bombardment (FAB) [126]. The technique was termed "soft ionization", as it ionized large molecules like proteins efficiently without significant fragmentation. Several advances were made in the development of better "soft ionization" techniques over the next decade and around 1990 two soft ionization methods, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), were made commercially available [117].

1.2.3.1 MALDI Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI)- based mass spectrometry was made possible by the development of the MALDI soft ionization technique in 1988 by Karas and colleagues in Germany and Tanaka *et al.* in Japan [127, 128]. MALDI ionization involves a protein suspended or dissolved in a crystalline structure (matrix) of small, organic, UV-absorbing molecules. The matrix is usually a weak aromatic acid that strongly absorbs energy at the wavelength of an irradiated laser. The analyte is mixed with the matrix material in solution and allowed to dry on a metal target plate. As the analyte-matrix mixture dries, it forms a crystalline coating on the target support. The matrix plays an important role in several different ways: a) it protects the analytes from decomposition by absorbing the excessive energy from the laser, b) the photoionization and photoexcitation of the matrix molecules lead to proton transfer to the analyte molecules, which enhances ion formation, c) sample dilution in matrix prevents association of analyte molecules. There are several different kinds of matrix and the choice of matrix depends on several factors and the interest of the investigator. The selection of matrix varies with kind of laser infrared (IR) or ultraviolet (UV)) lasers that are used to excite the matrix. The most commonly used matrices for MALDI-MS ionization are α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (3, 5- dimethoxy 4-hydroxycinnamic acid) and gentisic acid (3, 5-dihydroxybenzoic acid (DHBA). It has been reported that sinapinic acid provides better signals for higher molecular weight proteins, whereas CHCA is a more suitable choice for smaller proteins and peptides up to 10,000 Daltons. For glycolipid and glycoprotein analysis, DHBA is the ideal choice, as it is completely water soluble [117]. The crystals resulting from the matrix-analyte mixture

are irradiated with laser pulses at wavelengths of maximum matrix spectral absorption. This results in desorption and photoexcitation of the matrix, which leads to the ionization of the analyte through proton transfer from the matrix. The ionization results in the generation of singly charged ions $\{M+H\}^+$ for the most part, however multiply charged and oligomeric forms of the analyte are observed in the case of large molecules. The ionized analyte is then accelerated by an electrostatic field into a mass analyzer (time of flight {TOF} tube).

The TOF mass analyzer is a field-free chamber of high vacuum through which the ions travel and reach the detector. Applying a fixed voltage at the source, the ionized gas phase analyte is accelerated to a fixed kinetic energy and guided into the TOF tube. All ions with the same kinetic energy are separated in the TOF chamber based on their m/z ratios. The ions with low m/z ratios travel faster in the flight tube than those with higher m/z values. The m/z values are determined by measuring the time of flight at the detector since the m/z is proportional to the square of the time of flight. The relative intensities of ions are recorded at the detector, which is at the end of the flight tube. This technique of acquisition of data is termed "linear mode", which is effective for detection of analytes larger than 4000 Daltons and up to 300,000 Daltons. The MALDI-TOF-MS has another variable mode called "reflectron mode" which enables higher resolution and mass accuracy and is used for analyzing smaller peptides with higher precision. The technique incorporates a reflectron, an electrical mirror with an electric potential creating a retarding field at a voltage that is slightly higher than the accelerating voltage. The ions are sequentially slowed down through the reflector until they stop and are reflected back

in the opposite trajectory in a second drift region to a second detector. The reflectron mode provides a longer flight path which results in higher mass accuracy measurements.

The MALDI-TOF-MS is able to provide accurate molecular weights of several proteins and peptides that are part of the analyte. The spectra obtained by MALDI-TOF-MS analysis can be used to determine differentially expressed proteins and peptides between samples for example, disease vs. normal, treated vs. non-treated. With advancements in mass spectrometry techniques, it is now possible on the MALI-TOF platform to determine the protein identities and characterize the differentially expressed proteins using a technique called tandem mass spectrometry, also known as MS/MS. For MALDI-TOF-MS/MS analysis, a parent ion that is to be sequenced is selected from the first MS scan based on its m/z value and is subjected to process called collision-induced dissociation (CID). Only the parent ion is allowed to enter the collision cell, which is filled with a gas (usually helium), using a timed gate to make sure there is only one parent ion of interest that is being fragmented. The CID process, which involves collision of the molecules of the parent ion with the gas molecules, leads to the formation of several smaller cleavage products of the original parent ion. Generally the fragments generated by CID are either b-ions (N-terminal fragments) or y-ions (C-terminal fragment). The fragmentation products are unique for each peptide or protein. A peak map, generated as a result of the fragmentation, is labeled based on the masses and intensities of each of the peaks. The peak intensities and peak masses are comparable to the amino acids that make up the protein. The information generated by fragmentation is used to determine the protein identity by searching appropriate databases to find amino acid combinations that correspond to the MS/MS spectra.

In addition to the CID technique, there is another method of tandem mass spectrometry that can be used on the MALDI-TOF platform. This method uses a tandem MALDI-TOF/TOF technique termed "LIFT", which uses nitrogen laser-induced dissociation (LID) of the parent ion [129]. As in the CID method, the parent ion of interest is selected by the first TOF analysis, which is then filtered out using a precursor ion selector. Once the target parent ion is selected, a low voltage is applied for accelerating the ions. The fragments of the parent ion are generated by laser-induced dissociation. The method LIFT gets its name from the fact that instrument "lifts" the potential energy of the ions to a higher level inside the LIFT cell. The parent ion and the fragments are then directed to the detector where a MS/MS spectrum is generated. The LIFT technique has been proven to be useful for the detection of low mass ions of low abundance. The spectra generated by both CID and LIFT are run through different search engine databases like SEQUEST or MASCOT to ascertain the closest match between the MS/MS spectra and proteins listed in the databases by programmed computer-based algorithm analysis. The databases provide several different options based on the sample preparation and modifications anticipated.

1.2.3.2 Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray Ionization Mass Spectrometry was made possible by the development of the electrospray ionization (ESI) technique by Fenn and his group in 1989 which made ionization of larger complex molecules possible [130]. The ESI technique allows for the ionization of volatile complex protein mixtures from a liquid phase. In the ESI source, a continuous analyte solution is passed through a fine needle,

which leads to the formation of charged droplets due to the high voltage maintained at the end of the needle. In the vacuum chamber, the solvent which surrounds the charged droplet gradually evaporates, and eventually analyte ions are formed and released as gas phase ions. Electrospray results in a continuous production of singly and multiply charged analyte ions. These charged ions are then accelerated in an electric field and are then deflected by a magnetic field. The formation of multiply charged ions from proteins and peptides is dependent on molecular weight and the availability of basic sites like arginine, histidine and lysine. Generally ESI ion sources are coupled with an ion trap or quadrupole mass analyzer. The spectrum generated by ESI is more complex due to the presence of multiply charged ions; however, there are mathematical algorithms that help in deconvoluting the multiply charged ion spectrum in to simple mass spectrum that reveals the molecular weights of the fragments. To obtain protein identification and characterization, the parent ions of interest can be isolated and subjected to further fragmentation using the CID method as described for MALDI mass spectrometry. The major drawback using ESI mass spectrometry is the formation of multiply charged ions that can overlap and hinder the analysis of complex mixtures.

1.2.4 Proteomic Biomarker discovery using Blood: Opportunities and Challenges

Blood has been used a rich source for the identification of biomarkers of disease, treatment and as a diagnostic tool. The blood proteome that includes plasma and serum is a source of proteins secreted from tissues. A change in the blood proteome is indicative of the biochemical and physiological state of an organism. Although serum and plasma are promising clinical fluids for biomarker discovery, there are several challenges that are

associated with them. The wide dynamic range of these protein-rich fluids is the major hurdle, as the protein concentration of serum and plasma spans ten to eleven orders of magnitude [131]. Out of the entire plasma/serum proteome, there are 22 proteins that make up 99% of the entire protein content, with the remaining 1% making up the low abundance proteins [132, 133]. Of the most abundant proteins, albumin constitutes nearly 55% of the blood proteome. The highly abundant proteins are able to mask and prevent the identification of lower abundance proteins. Although the current proteomic-based approaches using mass spectrometers are capable of highly sensitive identification of proteins and peptides, their working range spans only three orders of magnitude of the blood proteome [132, 134]. Hence, major efforts have been directed towards the reduction of these abundant proteins prior to subjecting them to mass spectrometric analysis. Among the most widely used strategies is targeted capture by depleting the most abundant proteins within the serum and plasma samples. This can be achieved by antibody-based albumin and immunoglobulin depletion kits, or by using immunoaffinity kits that can deplete the top 12-20 proteins in the blood proteome [135, 136]. However, the downside of targeted capture by depleting the most abundant proteins is that a number of smaller proteins are bound to these highly abundant carrier proteins and are removed in the process. These less abundant proteins, which are lost as result of fractionation, could be of interest [137]. Another commonly used fractionation method is based on affinity chromatography columns, which are used prior to LC-MS/MS. The drawback of using this technique is that the columns are not suited for automation and are not high throughput. However, the chromatography columns or magnetic particles with higher surface area and binding capacity provide fractionation with less influence from

competition over binding sites [138]. The MALDI-TOF-MS platform utilizes the advantage of higher surface area affinity based magnetic beads as a fractionation measure to reduce the highly abundant proteins from serum and plasma samples prior to MS analysis. There are different types of magnetic affinity beads that are available which can be used in conjunction with automated platforms prior to mass spectrometric analysis using MALDI-TOF [139]. The magnetic beads offer a high throughput, reproducible, automated and efficient method for front-end fractionation of the blood proteome. Despite the technical hurdles, plasma and serum hold great promise for identifying biomarkers, due to their inherent richness of proteins and also the ability to obtain blood relatively easily.

CHAPTER II

SPECIFIC AIMS

The research project was designed to address a number of questions regarding influenza vaccine responses in healthy subjects. We were interested in evaluating the immune correlates of vaccine responses induced by the inactivated trivalent vaccines and live attenuated vaccines. Both vaccines have been proven to be equally efficacious; however, further studies are required to understand the mechanism by which the two vaccines provide immunity. Since live attenuated vaccines have been licensed for use recently, very few studies have focused on investigating different humoral and cellular components of the immune response which are induced in pre-vaccinated individuals. Using the proteomic serum profiling technique to investigate vaccine response biomarkers has never been undertaken. Since this technique has been successful in identifying several cancer and other disease-specific biomarkers, we wanted to utilize this new technique to look for influenza vaccine response markers and immune senescence markers. The study was divided into three parts based on the following aims:

Aim I: Comparison of cellular and humoral immune responses of trivalent inactivated influenza vaccine (TIIV) and live attenuated influenza vaccine (LAIV) in healthy young adults.

Both vaccines are known to have comparable vaccine efficacy, whereas the immune parameters accountable for their perspective vaccine efficacies are not well understood. The goals of this aim were to compare and contrast the humoral and cell-mediated immune responses (CMI) between TIIV and LAIV in healthy young adults, in order to

gain better understanding of immune correlates of vaccine efficacy. We hypothesized that using one gold standard (Hemagglutination inhibition assay {HAI}) to measure vaccine response may not be ideal when evaluating different vaccine compositions and routes of administration. The goals of this aim were achieved by:

A. Evaluation of the humoral antibody response to TIIV and LAIV vaccination. We evaluated the antibody response to vaccination by HAI assay between subjects vaccinated with TIIV and LAIV to determine the difference in humoral response to vaccination.
B. Further characterization of the humoral antibody response was done by evaluating the serum IgG1, IgG2, IgG3 and IgA levels pre- and post- vaccination in subjects vaccinated with TIIV and LAIV.
C. Evaluation of the cellular immune responses was done by comparing the Th1 T cell response, which was defined as IFN-γ secretion by memory T cells pre- and post-vaccination, between the two groups.
D. Further evaluation of the cellular immune responses was done by evaluating the Th2 T cell response by comparing the levels of IL-10 cytokine secretion by PBMCs pre and post-vaccination between the two vaccinated groups.

Aim II: Comparison of age-related changes in immune response to inactivated influenza vaccine response between healthy young and elderly and the role of cytokines in lower vaccine efficacy in elderly.

There is a general consensus that the inactivated influenza vaccine efficacy is much lower in the elderly compared to the young. In an attempt to understand the age-related decline in the immune response to vaccination, we aimed to study different components of the immune response that may have an impact on lower vaccine response rates among the elderly. We hypothesized that age-related change in cytokine levels could lead to a lower antibody response to influenza vaccination in the elderly. The goals of this aim were achieved by:

A. Evaluation of the humoral antibody response by measuring antibody response to TIIV using HAI assay among healthy young and elderly subjects previously vaccinated with inactivated influenza vaccine.
B. Further characterization of the humoral antibody response by measuring the serum IgG1, IgG2, IgG3 and IgA response to influenza vaccination in the young and the elderly.
C. Evaluation of the cellular immune response by measuring the percentage of influenza-specific memory T cells (ISMT) secreting IFN-γ pre- and post-vaccination between the young and elderly.
D. Evaluation of the cellular immune response by measuring the baseline levels of Th1 and Th2 cytokines secreted by PBMCs stimulated with influenza antigen pre- and post-vaccination. Further correlation of the baseline levels of cytokines pre-

vaccination with antibody and T cell responses post-vaccination were performed to evaluate any age-related changes influenza vaccine response.

E. Correlation of humoral antibody and memory T cell responses to establish age-related changes.

Aim III: Proteomic profiling of a longitudinal series of serum samples collected before and after LAIV and TIIV influenza vaccination from healthy subjects to identify biomarkers related to vaccine response and immune senescence.

Proteomic profiling using MALDI-TOF MS has been demonstrated to be a platform of choice for several serum profiling studies to identify biomarkers for various forms of cancer and other disease states. The use of this powerful tool has not been applied to serum-based studies assessing vaccine responses. We achieved this aim by:

A. Serum from a cohort of subjects who had been administered the live attenuated influenza vaccine was evaluated for differentially expressed potential biomarker proteins using the matrix-assisted laser desorption/ionization/time of flight (MALDI-TOF) instrument.

B. Serum from a cohort of inactivated influenza-vaccinated subjects was evaluated for differentially expressed potential biomarker proteins using the matrix-assisted laser desorption/ionization/time of flight (MALDI-TOF) instrument.

CHAPTER III

COMPARISON OF CELLULAR AND HUMORAL IMMUNE RESPONSES OF TRIVALENT INACTIVATED INFLUENZA VACCINE (TIIV) AND LIVE ATTENUATED INFLUENZA VACCINE (LAIV) IN HEALTHY YOUNG ADULTS

Introduction

Influenza viruses are major respiratory pathogens which cause significant morbidity and mortality, resulting in approximately 250,000 - 500,000 deaths annually worldwide [140, 141]. It is estimated that influenza is responsible for 226,000 hospitalizations and 36,000 deaths annually in the United States [20, 24]. Although influenza infections do not usually result in deaths and hospitalizations among healthy young adults, they are responsible for a significant loss of productivity. Based on studies in several industrialized nations, it has been established that annual influenza epidemics are responsible for significant economic burden. Loss of productivity, coupled with the utilization of health care services, is estimated to cost about $1-6 million per 100,000 individuals, based on estimates from France, Germany and the United States [19].

Several antiviral drugs designed to fight influenza infections are available; however, vaccination remains the mainstay among efforts to prevent and control annual influenza outbreaks. The key to protection against subsequent infections and controlling viral replication has been associated with the generation of antibodies against hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins as a result of natural infection or vaccination [6, 7]. The role of cell-mediated immunity, such as T cell responses, in protection against influenza infection is less clear; however, its importance

in clearing influenza virus from infected cells and lowering morbidity and mortality rates is evident [142-144]. Most adults have pre-existing levels of antibodies against influenza A and B viruses as a result of prior exposure or vaccination [145]. Influenza viruses undergo antigenic shift as a result of a series of point mutations in their HA and NA genes during replication in infected cells. The changes in the HA and NA genes render individuals susceptible to these new strains. To ensure optimal vaccine efficacy, it is essential to have a close match between the strains incorporated in the vaccine and the circulating strains. Therefore, influenza vaccines are formulated each year based on recommendations from various international WHO influenza surveillance laboratories.

At present there are two types of licensed influenza vaccines available in the United States. The trivalent inactivated influenza vaccine (TIIV) administered by intramuscular injection, was first used more than 60 years ago for preventing influenza in humans and has been the most widely used vaccine [146]. TIIV is composed of 15 μg of purified HA from each of the three H1N1, H3N2 and influenza B virus strains that are expected to circulate in a season annually. TIIV is licensed for use in children 6 months or older and in adults. In 2003, another influenza vaccine was licensed for use in the United States. The new vaccine is a live attenuated influenza vaccine (LAIV), which is comprised of cold-adapted influenza strains into which HA and NA genes from expected circulating virus strains are inserted by genetic reassortment each year. The genetically modified cold-adaptive live virus strains have reduced ability to replicate in the respiratory tract [147, 148]. The LAIV is administered via an intranasal spray. Although originally licensed to be administered to healthy individuals 5-49 years of age in 2003, in September 2007 it was approved for use in children 2-5 years of age [149]. The two

vaccines differ in their composition and routes of administration but contain similar or identical HA and NA antigens.

Both the TIIV and LAIV vaccines have been shown to have similar efficacy in preventing laboratory-diagnosed influenza (LDI) [79]. It has been shown that TIIV induces significant serum hemagglutination inhibition (HI), IgG, and IgA antibody responses in adults with significant immunological memory to influenza. In contrast, LAIV vaccination studies have shown that, in comparison to TIIV, the magnitude of serum HI and IgG antibody responses is lower in adults [8, 16-18]. In most of the previous studies, the subjects enrolled had mixed influenza vaccination histories. This makes the head-to-head comparison of immune responses elicited by vaccination difficult. Studies involving comparison of parallel cell- mediated immune responses in response to vaccination with TIIV and LAIV have been limited. Studies directly comparing the immunogenicity of the two vaccines in healthy adults previously vaccinated with an influenza vaccine have not been done extensively. The goal of our study was to compare the immune correlates of vaccine response in healthy adults previously vaccinated with influenza vaccine. Several immune correlates including HAI antibody response, serum IgG sub-type (IgG1, IgG2 and IgG3) antibody response, serum IgA antibody response, T cell IFN-γ production and IL-10 response were compared pre- and post-vaccination between TIIV and LAIV recipients.

Materials and Methods

Vaccines

The vaccines used for this study were Fluzone® (Aventis Pasteur), a TIIV vaccine, and FluMist® (MedImmune), a LAIV vaccine. The TIIV vaccines used were from seasons 2005-2006 (T05-06) and 2006-2007 (T06-07), and the LAIV vaccines were from season 2006-2007 (L06-07). The TIIV vaccine of the 2005-2006 season comprised 15 μg each of the HA from A/New Caledonia 20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Jiangsu/10/2003 (B/Shanghai/361/2002-like); the TIIV vaccine of the 2006-2007 season comprised 15 μg each of the HA from A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004. The LAIV vaccine comprised of cold-adapted attenuated influenza virus containing $10^{6.5\text{-}7.5}$ $TCID_{50}$ median tissue culture infectious doses of A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004-like virus strains.

Recruitment of Human Subjects and Vaccination Protocols

Subjects for the study were recruited with written informed consent by the clinical coordinating team at the Glennan Center for Geriatrics and Gerontology, Eastern Virginia Medical School, during the winter influenza seasons of 2005-2006 and 2006-2007. The Institutional Review Board at Eastern Virginia Medical School approved the study protocol and the informed consent form. All study participants were healthy young adults between 18-40 years of age, independently residing in the Hampton Roads area of Virginia. All subjects recruited for the study had been vaccinated with TIIV in the season prior to enrollment to minimize the immunological differences as a result of vaccination.

Subjects with egg allergy, underlying chronic diseases such as diabetes, autoimmune diseases such as lupus erythematosus or rheumatoid arthritis, or congestive heart failure, those on immunosuppressive drugs, and pregnant women were excluded from participating in the study.

There were 27 subjects recruited in season 2005-2006 who were vaccinated with TIIV. During the season 2006-2007 there were two groups of subjects who were randomly divided into two groups. The first group, comprising 22 subjects, was vaccinated with TIIV and the other group, comprising 41 subjects, was vaccinated with LAIV. Blood samples were obtained from each subject on four days: day 0 prior to vaccination, and days 4, 7 and 21 after vaccination.

Hemagglutination inhibition (HAI) assay

Hemagglutination occurs when sialic acid residues, similar to the cellular receptors on erythrocytes, bind to the receptor- binding site present on the tip of the viral HA proteins. Hemagglutination can be inhibited by adding HA-recognizing antibodies before adding erythrocytes in the assay, which is termed hemagglutination inhibition; hence the assay is termed a hemagglutination inhibition assay (HAI). When performing HAI assays, sera from vaccinated or infected subjects are titrated on 96 well plates, to which a known amount of influenza virus and erythrocytes are added. In our study, serum antibody titers specific for each of the three strains present in the vaccine formulations were determined by standard microtiter HAI assay following procedures described previously [150]. Serum HAI antibodies were determined on day 0 (pre-vaccination) and on day 21 (post-vaccination). Non-specific inhibitors in serum were removed by

incubating serum with receptor-destroying enzyme (RDE; Sigma). All influenza H1N1, H3N2 and B antigens (wild strains compatible with the recombinant strains in the vaccine) were grown in 10 day old fertilized chicken eggs. A slight modification was made to the protocol. Guinea pig red blood cells (GPRBCs) were used for agglutination instead of chicken or turkey RBCs. The HAI titer was defined as the reciprocal of the highest dilution of serum that completely inhibited agglutination. Antibody titers of ≥ 40 were considered protective (seroprotection) and a $\geq$ four-fold rise in antibody titer (seroconversion) to one out of three vaccine antigens was considered an adequate response.

Serum IgG1, IgG2 and IgG3 analysis by ELISA

Serum levels of influenza-specific IgG1, IgG2 and IgG3 were determined by ELISA analysis. All the assays were carried out in a similar fashion. For all the above-mentioned variables studied, serum samples obtained at day 0 (prior to vaccination) and at day 21 (post-vaccination) were analyzed. For each of the IgG1, IgG2 and IgG3 analyses, 96-well EIA specific round bottom plates (Immulon 1; Dynatech Laboratories, Chantilly, VA) were used. The 96-well plates were coated with IgG1, IgG2 and IgG3 purified protein as standards (all κ-chains; Sigma Aldrich, MO) and with trivalent influenza vaccine 2005-2006 formula (A/New Caledonia 20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Jiangsu/10/2003; Aventis Pasteur, Swiftwater, PA) or 2006-2007 formula (A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004; Aventis Pasteur, Swiftwater, PA). The plates were incubated at 4°C for 16-18 hours. After overnight incubation, the plates were washed twice with

wash buffer (1X PBS with 0.05% Tween 20, pH 7.4). Following the wash procedure, 200 µl of blocking buffer (10% FBS/1X PBS) was added to each well and incubated at 37°C for 2 hours. The plates were then washed with wash buffer 4 times. Dilutions of serum samples to be analyzed were made for the respective IgG subtype assays. The serum samples were diluted to 1:5000, 1:200 and 1:400 with blocking buffer for IgG1, IgG2 and IgG3 analysis, respectively. The plate was incubated at 37°C for 2 hours. Plates were washed four times with wash buffer. For each IgG subtype, biotin-conjugated anti-IgG subtype antibodies {IgG1 and IgG2 (BD Biosciences)} and IgG3 (Sigma Aldrich) were diluted 1:1000, 1:1000 and 1:4000 with blocking buffer, respectively. Biotin-conjugated anti-IgG specific antibodies were added to the 96-well plate and incubated at 37°C for 1 hour. Plates were washed, 100 µl of avidin-HRP (Sigma Aldrich) diluted 1:400 was added and the plate was incubated at room temperature for 30 minutes. After the final incubation, the plates were washed five times with wash buffer. Peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma Aldrich) was added and color development was allowed to continue for 10 minutes at room temperature in the dark. The color development reaction was stopped by adding 100 µl 1 N sulfuric acid. The plates were read at 450 nm by an ELISA plate reader and analyzed with KC4 (version 3.0) software (PowerWave, Bio-tek Instruments, Winooski, VT). The final analysis was done by normalizing all the readings based on the dilution factors.

Serum IgA analysis by ELISA

The IgA ELISA analysis was done in a similar fashion as the IgG sub-type ELISA. Serum samples obtained at day 0 (prior to vaccination) and at day 21 (post-

vaccination) were analyzed. For IgA analysis 96 well EIA specific round bottom plates (Immulon 1; Dynatech Laboratories, Chantilly, VA) were used. The 96 well plates were coated with IgA (κ-chain; Sigma Aldrich, MO) and with trivalent influenza vaccine 2005-2006 or 2006-2007 formula. The plates were incubated at 4°C for 16-18 hours. After overnight incubation, the plates were washed twice with wash buffer (1 X PBS with 0.05% Tween 20, pH 7.4). Following the wash procedure, 200 µl of blocking buffer (10% FBS/1 X PBS) was added to each well and incubated at 37°C for 2 hours. The plates were then washed with wash buffer 4 times. The serum samples were diluted 1:400 with blocking buffer for IgA and added to the plate. The plate was incubated at 37°C for 2 hours. Plates were washed four times with wash buffer. For IgA, HRP-conjugated anti-IgA antibody (Sigma Aldrich) was diluted 1:4000 with blocking buffer. Diluted HRP-conjugated anti- IgA specific antibody was added to the 96-well plate and incubated at 37°C for 1 hour. Plates were washed four times with wash buffer. After the final incubation, the plates were washed five times with wash buffer. Peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma Aldrich) was added to all the wells and color development was allowed to continue for 10 minutes at room temperature in the dark. The color development reaction was stopped by adding 100 µl 1 N sulfuric acid. The plates were read at 450 nm using an ELISA plate reader and analyzed with KC4 (version 3.0) software (PowerWave, Bio-tek Instruments, Winooski, VT). The final analysis was done by normalizing all the readings based on the dilution factors.

Purification of PBMC

Human peripheral blood mononuclear cells (PBMC) were isolated from blood that was collected in heparinized tubes. Whole blood was first centrifuged at 320 X *g* for 10 minutes. Plasma was aspirated. The buffy coat layer, which is composed of the PBMCs, was carefully aspirated and laid onto a gradient purification Ficoll reagent, Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), followed by centrifugation at 650 X *g* for 30 minutes. The PBMC were collected by aspiration from the interface and washed twice with RPMI 1640 medium (Invitrogen, Carlsbad, CA) at 500 X *g* and 320 X *g* for 10 minutes each. The PBMCs were then counted using an automatic lymphocyte counter (Coulter ACT, Beckman Coulter, Miami, FL) and resuspended in CTL media (RPMI 1640 medium containing 10% FBS, 2 mM of L-Glutamine, 100 U of penicillin, 100 μg/ml of streptomycin and 55 nM of 2-mercaptoethanol). The concentration was calculated and brought up to 1 million PBMCs/ml for the fast immune assay.

Influenza virus used for Fast Immune Assay

All the live influenza virus strains used in the study were propagated in Bio-Safety Level-2 conditions. The seed virus strains were obtained from the Center for Disease Control and Prevention (CDC), Atlanta, GA. All Bio-Safety guidelines detailed in the microbiological and biomedical laboratory manuals published by the CDC and by the Office of Safety at Eastern Virginia Medical School were followed. The live virus strains of influenza used in this study were A/New Caledonia 20/1999 (H1N1), A/New York/55/2004, A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 and B/Shanghai/361/2002. The live viruses were grown in fertilized chicken eggs, using seed

viruses obtained from the CDC, following procedures described previously [151]. Purified allantoic fluids containing live virus were then analyzed to determine the viral titers. After determination of the viral titers, the allantoic fluids were transferred to cryovials and stored at -80°C until required.

Detection of T cell IFN-γ production using Fast Immune Assay

The fast immune procedure to determine T cell IFN-γ secretion was done as described previously [152]. One million PBMC were activated *ex vivo* using a combination of the three strains of virus (10 HA units/ml for each) corresponding to the vaccine strains in 150 µl of complete medium in a 96-well U-bottom tissue culture (TC) plate at 37°C overnight. Brefeldin A (BFA, Sigma-Aldrich, St. Louis, MO) was then added to each well to a final concentration of 5 ug/ml after 17 hours. The culture was incubated at 37°C for 3 more hours before being fixed with 1% paraformaldehyde-PBS and then permeabilized with permeabilization buffer (Becton Dickinson, San Diego, CA) at room temperature for 10 minutes each. The cell culture was stained with the following conjugated antibodies; CD69-PE, CD4-AP, CD8-PerCP and IFN-γ-FITC (BD PharMingen, San Diego, CA). The CD69 is a T cell activation marker used to identify activated T cells, CD4 and CD8 markers are used to identify different T cell sub-types and IFN-γ is a cytokine that is secreted by activated T cells. Intracellular cytokine staining was detected using a flow cytometer (FACSCalibur, CellQuest 3.3 software, BD Biosciences, San Diego, CA).

Cytokine analysis by cytometric bead array (CBA)

Cytometric bead array analysis was used to determine cytokine levels after stimulation of PBMCs for 15 hours with a combination of three strains of influenza viruses present in the vaccines. The supernatants from influenza virus stimulated PBMC were diluted in PBS 1:2 and incubated with CBA beads coated with anti IL-10 antibodies {human inflammatory cytokine kit; BD™ Cytometric Bead Array (CBA)}. The beads were washed and stained with PE-conjugated secondary antibodies. The fluorescent intensity for each cytokine was measured using a flow cytometer (FACSCalibur, BD Biosciences, San Diego, CA). The final concentrations were analyzed and calculated using CBA software (BD Biosciences).

Statistical Analysis

Data analysis of the normally distributed data was performed using Student's t-test. Normal distribution of the data was evaluated using Kolmogorov-Smirnov's test. A modified t-test, Mann-Whitney rank sum test or Wilcoxon signed rank test was applied to determine significant differences between the two groups where data was not normally distributed. Statistical significance was set at $p<0.05$. The correlation coefficient of the two groups was analyzed using Pearson's correlation coefficient test. To determine the statistical relevance of seroprotection and seroconversion rates, Chi Square or Fischer's test were performed ($p<0.05$) based on the size of the group that was being evaluated.

Results

TIIV stimulated higher levels of systemic antibody response than LAIV when measured by hemagglutination inhibition (HAI) assay

The HAI response to vaccination was compared between three groups of subjects who received TIIV (T05-06 and T06-07) and LAIV (06-07) influenza vaccines. We compared HAI responses to each of the three vaccine strains (H3N2, H1N1 and B) independently in each group. We used a cohort vaccinated with TIIV from the 2005-2006 season to observe if there were any season specific variations between the immune responses after TIIV vaccination. Since we did not have a cohort vaccinated with the LAIV for the 05-06 season, we could not evaluate the season specific changes for LAIV. The H3N2 strains T05-06 and T06-07 were able to induce significant increase in HAI antibodies when serum HAI antibody levels at baseline (Day 0, T05-06: 20.86-51.74 and T06-07: 8.56-35.98) were compared with levels on day 21 (post-vaccination) {(62.38-110.08 and 40.5-112.22 ($p = <0.001$)} (Table1). In contrast, LAIV06-07 was able to induce an increase in serum HAI antibody titers to H3N2 (day 0, 14.38-44.82 and day 21 20.33-61.87 ($p = 0.01$). The mean fold of increase (MFI) in serum HAI antibody to H3N2 after LAIV was 1.87 as compared to 5.53 and 7.27 after T05-06 and T06-07, respectively. Although the T06-07 group demonstrated a higher increase in MFI compared to T05-06, it did not reach statistically significant levels.

In the case of H1N1 strain, we observed a similar trend where T05-06 and T06-07 were able to induce significant increase in HAI antibodies when serum HAI levels at baseline (day 0) {81.42-296.98 and 21.63-60.63} were compared with levels on day 21 (post-vaccination) {136.80-382.48 and 61.18-117.08 ($p = 0.001$)}. Again, although

LAIV06-07 was able to induce a significant increase in serum HAI antibodies to H1N1 {day0, 33.28-144.6 and day 21, 32.53-187.99 ($p = 0.01$)}, when compared to T05-06 and T06-07 the magnitude of increase was much lower. The MFI in serum HAI antibody to H1N1 was 3.17, 4.24 and 1.44 in T05-06, T06-07 and LAIV06-07 group, respectively. We did not observe a significant difference in MFI between T06-07 group and the T05-06 group.

For the B strain we observed that the pre-vaccination HAI titers were higher in the T05-06 group compared to T06-07 and LAIV06-07 (Table 2). Regardless of the difference in initial HAI antibody levels, both the T05-06 and T06-07 vaccines were able to induce significant antibody response to the B strains {day 0, 20.57-72.43 and 5.41-7.31; day 21, 42.85-93.45 and 16.9-48.1 ($p = 0.001$)}. Similar to the trend observed for the H3N2 and H1N1 strains, LAIV was able to induce significant HAI antibody response but the magnitude was low compared to T05-06 and T06-07. For the B strains the MFI in serum HAI antibody to vaccination were 2.45, 5.68 and 1.57 for T05-06, T06-07 and LAIV06-07, respectively. We observed a significant difference in the MFI increase for the B strain between T05-06 group and T06-07 group. We expect that this difference was due to a new B strain (B/Malaysia) that was introduced in the T06-07 season as opposed to the T05-06 B/Shanghai strain. It was observed that TIIV and LAIV vaccine recipients who had higher baseline HAI titers showed a lower increase in serum HAI levels compared to individuals with low baseline HAI titers for all the three virus strains (data not shown).

The seroconversion rates were also compared between the three groups. It was observed that seroconversion rates for H3N2, H1N1 and B strains for subjects vaccinated

with T05-06 and T06-07 were significantly higher (χ^2 test, $p<0.05$) than the subjects vaccinated with LAIV (Table 3). A similar trend emerged when seroprotection rates were calculated before and after vaccination. Both T05-06 and T06-07 groups had significantly higher seroconversion rates (χ^2 test, $p<0.05$) for each of the three influenza strains than LAIV06-07 group (Table 3). Taken together, the results show that the two TIIV vaccines were able to induce higher systemic antibody responses compared to the T06-07 LAIV vaccine when measured by HAI assays.

Table 2. Geometric means of HAI Antibody titers against influenza strains after influenza vaccination observed after Hemagglutination Inhibition Assay.

	H3N2 (A/New York or A/Wisconsin) Pre (Day 0)	Post (Day 21)	MFI
Fluzone® (TIIV05-06)	36.30 (95% CI = 20.86-51.74)*	86.23 (95% CI = 62.38-110.08)*a	5.53
Fluzone® (TIIV06-07)	22.27 (95% CI = 8.56-35.98)*	76.36 (95% CI = 40.5-112.22)*,b	7.27
FluMist® (LAIV06-07)	29.60 (95% CI = 14.38-44.82)*	41.10 (95% CI = 20.33-61.87)*,a,b	1.87
	H1N1 (A/New Caledonia)		
	Pre (Day 0)	**Post (Day 21)**	**MFI**
Fluzone® (TIIV05-06)	189.19 (95%CI =81.42-296.98)*	259.65 (95% CI = 136.80-382.48)*,a	3.17
Fluzone® (TIIV06-07)	41.13 (95% CI = 21.63-60.63)*	89.13 (95% CI = 61.18-117.08)*,b	4.24
FluMist® (LAIV06-07)	88.94 (95% CI = 33.28-144.6)*	110.26 (95% CI = 32.53-187.99)*,a,b	1.44
	B (B/Shanghai or B/Malaysia)		
	Pre (Day 0)	**Post (Day 21)**	**MFI**
Fluzone® (TIIV05-06)	46.5 (95% CI = 20.57-72.43)*	68.15 (95% CI = 42.85-93.45)*,a	2.45
Fluzone® (TIIV06-07)	6.36 (95% CI = 5.41-7.31)*,b	32.5 (95% CI = 16.9-48.1)*	5.68
FluMist® (LAIV06-07)	17.5 (95% CI = 12.26-19.74)*,b	22.31 (95% CI = 16.39-28.23)*,a	1.57

* = p= <0.01 comparing Day 0 and Day 21 HAI GMT titers (paired t-Test)
a = p= <0.05 between T05-06 and LAIV06-07 (unpaired t-Test test values)
b = p<0.05 between T06-07 and LAIV06-07 (unpaired t-Test values)

MFI = Mean Fold Increase

n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07

Table 3. Seroprotection and seroconversion rates in response to TIIV and LAIV vaccination.

	% Seroprotection Rates *(n)* (HAI Titers >1:40)					
	H3N2		**H1N1**		**B**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Fluzone® (TIIV 05-06)	37 (10)	74 (20)[a]	63 (17)	85 (23)[a]	37 (10)	63 (17)[a]
Fluzone® (TIIV 06-07)	18 (4)	63 (17)[b]	36 (8)	72 (16)	0 (0)[b]	31 (7)
FluMist® (LAIV 06-07)	23 (9)	31 (12)[a,b]	42 (16)	50 (19)[a]	21 (8)[b]	26 (10)[a]

	% Seroconversion Rates *(n)* (4 Fold or more Increase in HAI Titers)		
	H3N2	**H1N1**	**B**
Fluzone® (TIIV 05-06)	40 (11)[a]	15 (4)	18.5 (5)
Fluzone® (TIIV 06-07)	55 (12)[b]	27 (6)[b]	45 (10)[b]
FluMist® (LAIV 06-07)	10.5 (4)[a,b]	5.2 (2)[b]	5.2 (2)[b]

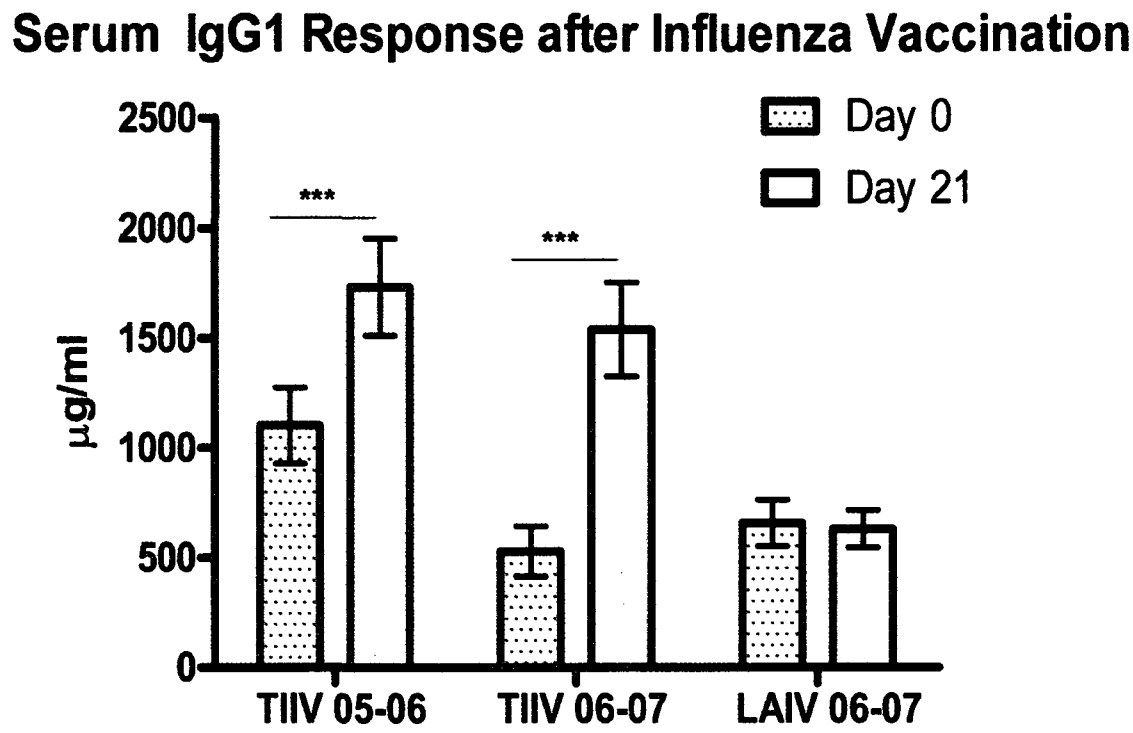
a = p=<0.05 between T05-06 and LAIV06-07 (Chi Square or Fisher's exact test values)
b = p<0.05 between T06-07 and LAIV06-07 (Chi Square or Fisher's exact test values)

n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07)

TIIV, but not LAIV, induced IgG1, IgG2, and IgG3 response after vaccination

The levels of each serum IgG sub-class specific for influenza strains for each vaccine formulation were determined using ELISA assays. Since all the subjects were vaccinated during the previous influenza season with TIIV, they all had pre-vaccination serum antibody IgG subclasses for influenza viruses (H3N2, H1N1 and B strains) (Figs. 3, 4, and 5). The ELISA assay used in our study detected serum antibody IgG1, IgG2 and IgG3 subclass levels against all the three virus strains present in the vaccines. In both the TIIV groups T05-06 and T06-07 there was a significant increase in serum IgG1, IgG2 and IgG3 antibody levels between day 0 (prior to vaccination) and day 21 (post-vaccination) ($p = <0.001$). The increase in serum IgG response among TIIV recipients was dominated by subclass IgG1 which showed a robust increase. The absolute levels of serum IgG1 were about 10 times greater than those of the IgG2 and IgG3 subclasses. However, there was no significant increase observed in serum IgG1, IgG2 or IgG3 antibody levels in subjects vaccinated with LAIV ($p = >0.05$). When serum IgG1 and IgG3 antibody levels of subjects vaccinated with TIIV06-07 and LAIV06-07 were compared there was no significant difference observed at baseline (day 0), however, post-vaccination (day 21) there was a significant difference ($p = <0.001$). Taken together, the results show that TIIV, but not LAIV, was able to induce a robust increase in serum IgG1, IgG2 and IgG3 antibody response after vaccination.

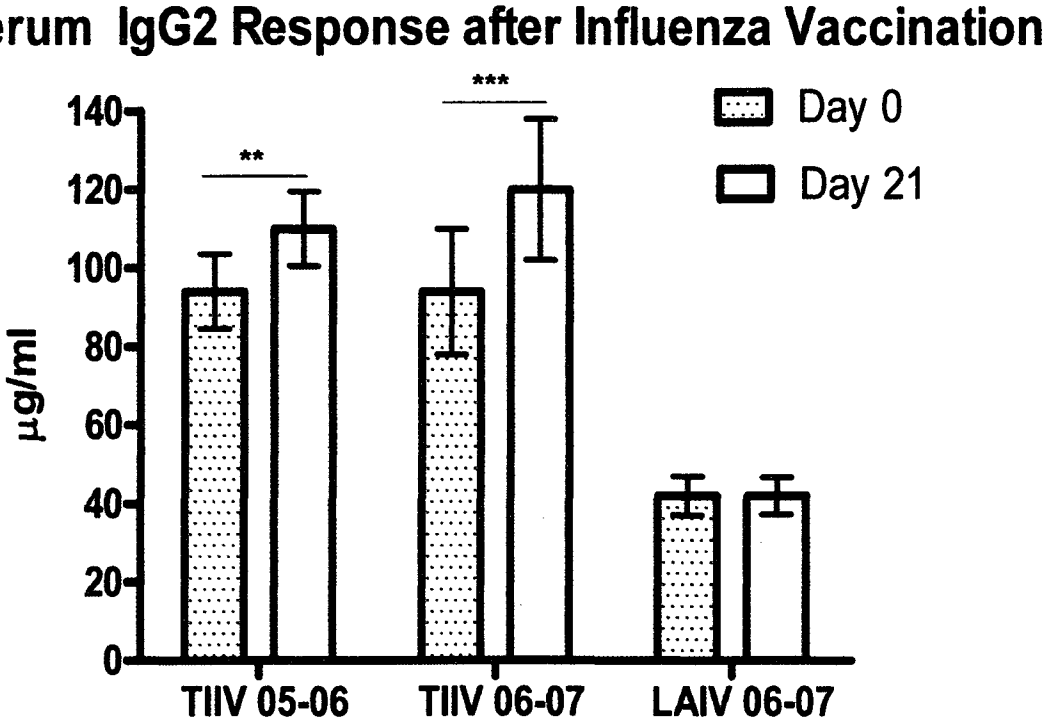
Fig. 3. Serum IgG1 antibody levels before and after TIIV and LAIV influenza vaccination, observed by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean and error bars represent the standard error of mean (S.E.M).



*** = paired t test ($p<0.001$)

n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07)

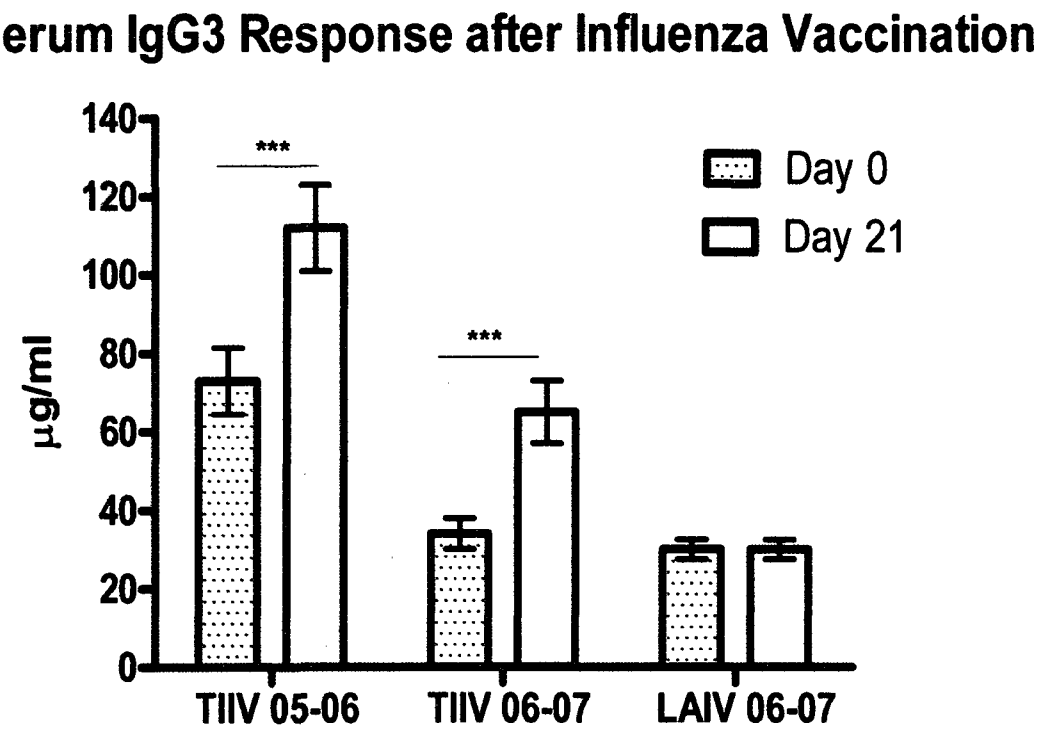
Fig. 4. Serum IgG2 antibody levels before and after TIIV and LAIV influenza vaccination, observed by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean and error bars represent the standard error of mean (S.E.M.).



** = paired t test ($p<0.01$)
*** = paired t test ($p<0.001$)

n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07)

Fig. 5. Serum IgG3 antibody levels before and after TIIV and LAIV influenza vaccination, observed by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean and error bars represent the standard error of mean (S.E.M.).



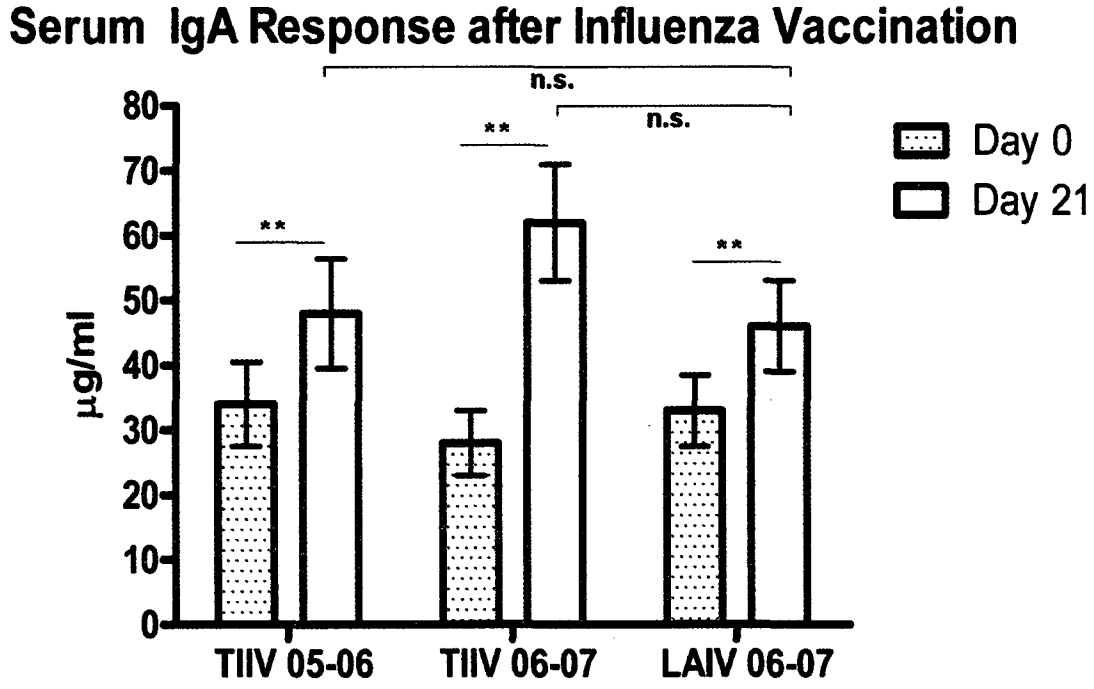
*** = paired t test ($p<0.001$)

n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07)

TIIV was able to induce significant serum IgA antibody response and the magnitude of response was comparable to that of LAIV

Serum IgA antibody response after vaccination against the three influenza strains (H3N2, H1N1 and B) was tested using ELISA. Subjects in all three groups vaccinated with T05-06, T06-07 and LAIV06-07 demonstrated a significant increase in influenza specific serum IgA antibody levels from baseline (pre-vaccination) to day 21 (post-vaccination) ($p = <0.001$) {Fig. 6}. The serum IgA response induced by T06-07 and LAIV06-07 were comparable and did not differ significantly. These results demonstrate that both TIIV and LAIV were able to induce significant and comparable serum IgA antibody response after vaccination.

Fig. 6. Serum IgA antibody levels before and after TIIV and LAIV influenza vaccination, observed by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean and error bars represent the standard error of mean (S.E.M).



** = paired t test ($p<0.01$)

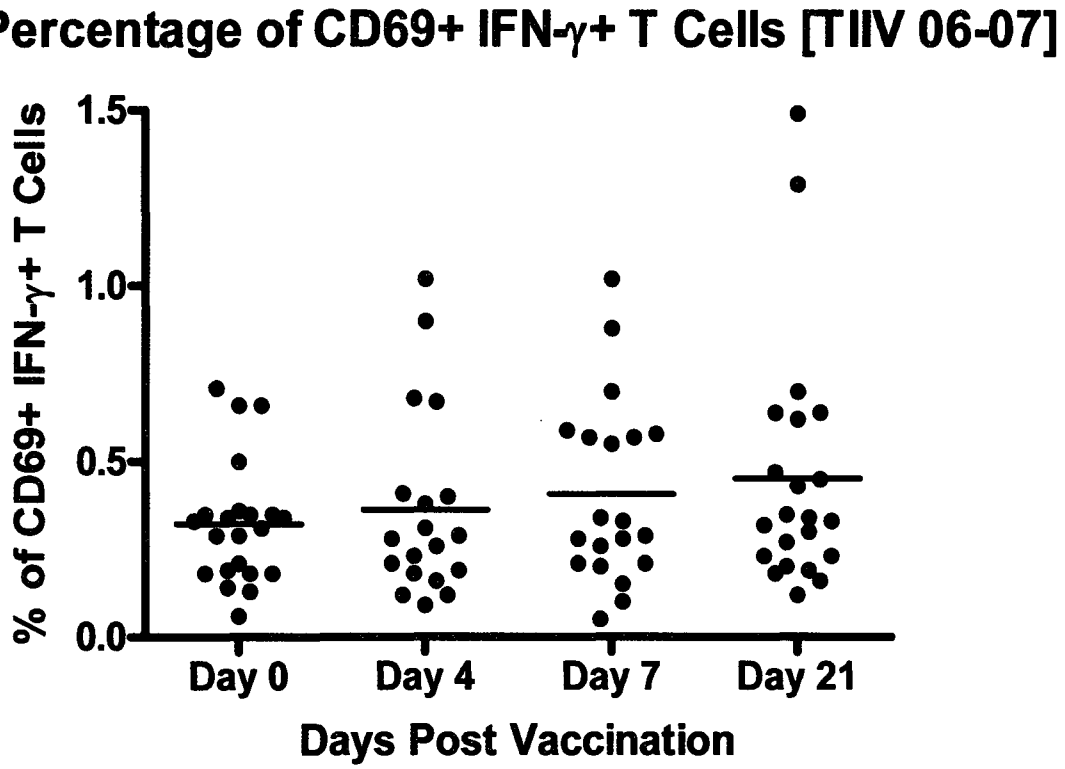
n = 27 (TIIV05-06)
n = 22 (TIIV06-07)
n = 41 (LAIV06-07)

LAIV was able to induce a greater level of type 1 T cell response than TIIV

To monitor the type 1 T cell response elicited by TIIV and LAIV vaccinations, we measured the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells using the Fast Immune assay [152, 153]. We saw that in both the TIIV cohorts T05-06 and T06-07 there was a trend of increase in the percentage of activated ($CD69^+$) T cells secreting IFN-γ from baseline (day 0) to day 4, day 7 and day 21 post-vaccination. The increase however, did not reach statistically significant levels ($p>0.05$) (Figs. 9 and 7).

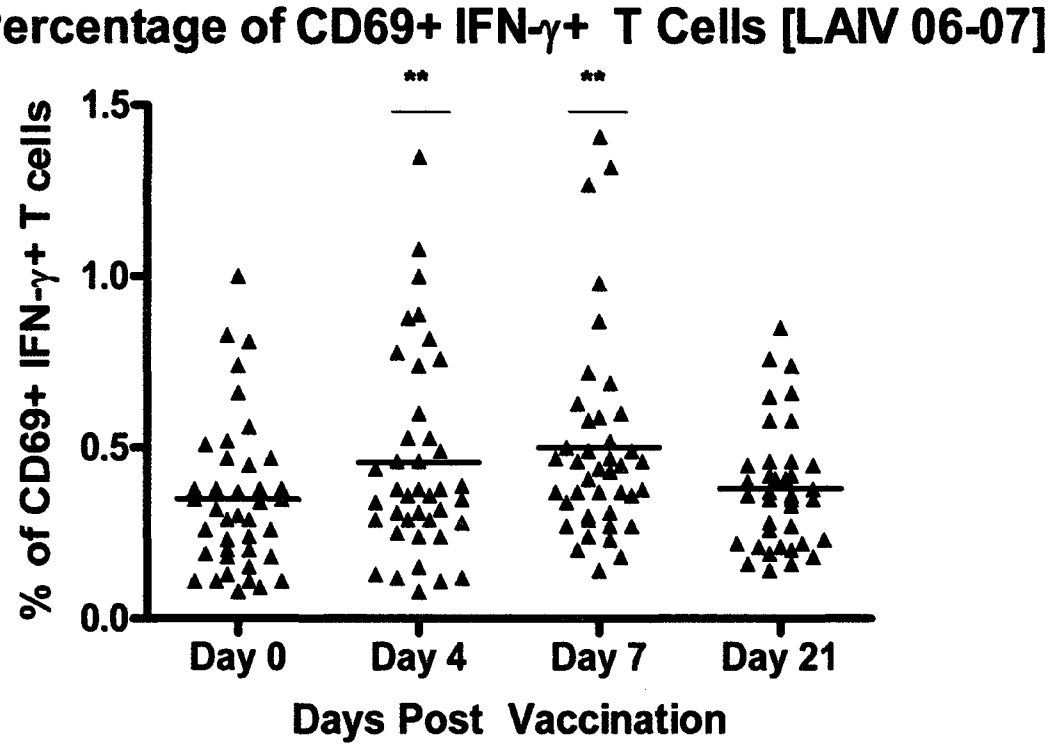
Subjects who were vaccinated with LAIV06-07 showed an increase in the percentage of activated T cells secreting IFN-γ from baseline (day 0) (0.348 ± 0.03 S.E.M.) to day 4 (0.455 ± 0.04 S.E.M.) and day 7 (0.522 ± 0.06 S.E.M.), and this increase reached statistically significant levels ($p = 0.015$ and 0.004, respectively) {Figure 8}. When the frequency of IFN-γ secreting T cells across different days (day 0, day 4, day 7 and day 21) were compared between LAIV06-07 and T06-07 groups, there was no statistical difference observed, although the median levels of IFN-γ secreting T cells remained higher in LAIV06-07 group. These results indicate that there is a difference in the kinetics of vaccine induced IFN-γ secretion by T cells; and LAIV is able to induce a greater increase in IFN-γ secreting T cells following vaccination when compared to TIIV.

Fig. 7. Percentage of $CD69^{+}IFN\text{-}\gamma^{+}$ T cells pre- and post-TIIV06-07 influenza vaccination observed using the Fast Immune Flow Cytometry Assay.
One million PBMCs from each subject vaccinated with TIIV were stimulated with 1 µl of influenza virus antigen in a 96 well plate overnight. Fast Immune assay and flow cytometry was used to determine the frequency of $CD69^{+}IFN\text{-}\gamma^{+}$ T Cells.



n = 22 (TIIV06-07)

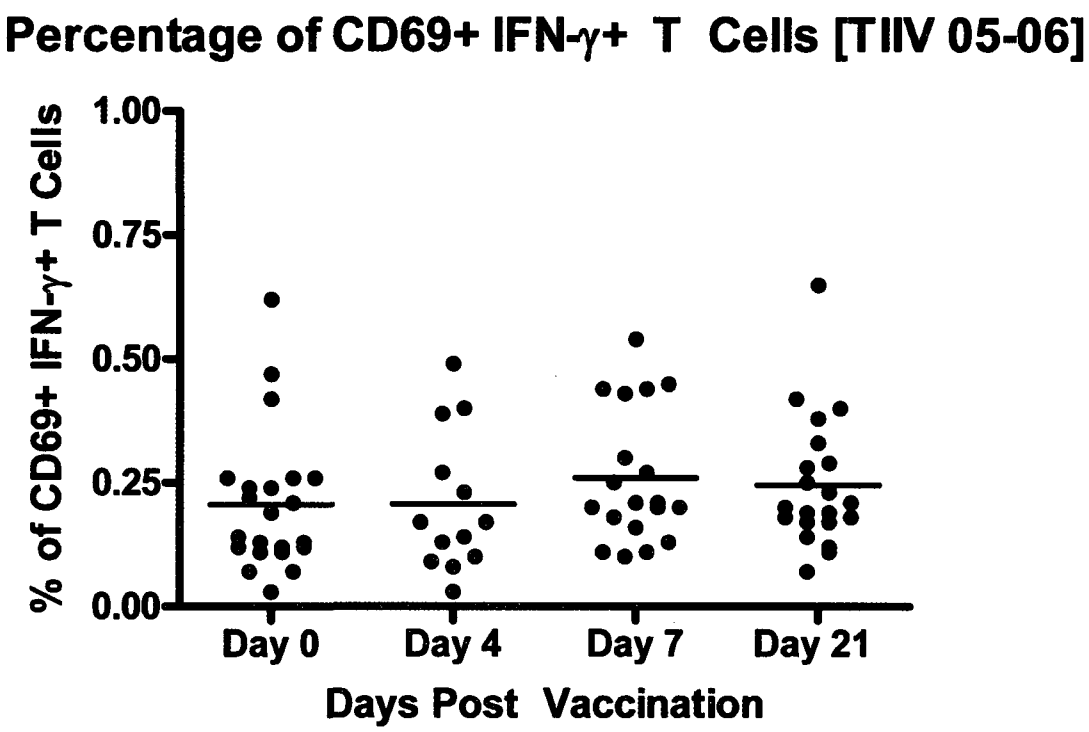
Fig. 8. Percentage of $CD69^{+}IFN\text{-}\gamma^{+}$ T cells pre- and post-LAIV06-07 influenza vaccination observed using the Fast Immune Flow Cytometry Assay.
One million PBMCs from each subject vaccinated with TIIV were stimulated with 1 µl of influenza virus antigen in a 96 well plate overnight. Fast Immune assay and flow cytometry was used to determine the frequency of $CD69^{+}IFN\text{-}\gamma^{+}$ T Cells.



** = $p<0.01$ (paired t-test values Day 0 vs. Day 4, Day 0 vs. Day 7)

n = 41 (LAIV06-07)

Fig. 9. Percentage of $CD69^{+}IFN\text{-}\gamma^{+}$ T cells pre- and post-TIIV05-06 influenza vaccination observed using the Fast Immune Flow Cytometry Assay.
One million PBMCs from each subject vaccinated with TIIV were stimulated with 1 µl of influenza virus antigen in a 96 well plate overnight. Fast Immune assay and flow cytometry was used to determine the frequency of $CD69^{+}IFN\text{-}\gamma^{+}$ T Cells.



n = 27 (TIIV05-06)

Correlation analysis between the T cell and antibody responses to TIIV and LAIV

In addition to the Th1 T cell response to the two vaccines (IFN-γ T cell responses), the Th2 T cell responses to the vaccines were also analyzed using IL-10 as the indicator. The levels of IL-10 in supernatants from influenza-stimulated PBMC from day 0 (pre-vaccination) and at day 7 (post-vaccination) were measured using CBA. We observed a significant increase in IL-10 levels post-vaccination in all groups ($p<0.05$).

To better understand the correlations between the T cell response and antibody response, their correlations were analyzed. We picked IgA and IgG1 as the indicator for antibody response to LAIV and TIIV, respectively and their folds of increase after vaccination were related to the fold of increase in Th1 and Th2 T cell response, respectively. As shown in Table 4, we observed a significant positive correlation between the serum IgA antibody and Th2 T cell (IL-10) response in the LAIV group ($r = 0.56$, Table 4). This positive correlation was only seen in the LAIV vaccinated group but not in the TIIV group ($r = -0.07$, non significant). In addition to correlation between antibody and T cells, we also observed a strong positive correlation between the serum IgG1 and IgA response in TIIV vaccinated group ($r = 0.88$, Table 4), but not in the LAIV vaccinated group ($r = 0.03$). In summary, these data suggest that the Th2 T cell response may play a role in the serum IgA response to LAIV and that the serum IgA response is positively associated with the IgG1 response to TIIV.

Table 4. Correlation analysis between serum IgG1, serum IgA, Th1 and Th2 T cells response in TIIV and LAIV group

	TIIV				LAIV			
	IgA	IgG1	Th1 (IFN-γ)	Th2 (IL-10)	IgA	IgG1	Th1 (IFN-γ)	Th2 (IL-10)
IgA	--				--			
IgG1	0.88	--			n.s	--		
Th1 (IFN-γ)	n.s	n.s	--		n.s.	n.s	--	
Th2 (IL-10)	n.s	n.s.	n.s.	--	0.56	n.s	n.s	--

n.s. = non significant, defined as correlation coefficient r<0.5 or r>-0.5.

Table 5. Correlation analysis between the increase in IgA (LAIV), IgG1 (TIIV) and the increase in HAI antibody titers

	TIIV				LAIV			
	IgG1	IgG2	IgG3	IgA	IgG1	IgG2	IgG3	IgA
HAI titers								
H3/N2	0.06	0.36	0.13	0.05	-0.18	0.14	-0.03	0.42
H1/N1	0.72	0.40	0.29	0.70	0.28	0.29	0.46	0.27
B	0.14	0.25	0.26	0.09	0.67	0.50	0.34	0.55
Over all	0.42	0.56	0.34	0.38	0.34	0.46	0.33	0.66

Discussion

The goals of this study were to compare immune correlates of TIIV and LAIV vaccines administered to previously immunized healthy adults. We examined the humoral immune responses by evaluating the serum HAI, serum IgG sub class, and serum IgA response after vaccination. The cellular immune components evaluated included IFN-γ and IL-10 response after vaccination. Several studies have demonstrated that the efficacies of the two vaccines are essentially comparable and neither approach offers an advantage over another against prevention from influenza virus infection and disease [79, 154-156]. Our study demonstrates that there are important differences in serum antibody responses and cellular immune correlates between the two vaccines when administered to previously vaccinated healthy adults: TIIV induces higher levels of serum antibody response while LAIV induced more robust Th1 T cell response.

The serum HAI antibody titer is used as a surrogate marker for protection after parenteral influenza vaccination. Usually a threshold titer of ≥40 by standard HAI assay is associated with >50% reduction of the risk of contracting an influenza infection or influenza disease [157]. Increases in HAI titer by four-fold, or an HI titer of ≥40, are the standards of measuring influenza vaccine efficacy. In our study, TIIV induced a significantly higher increase in serum HI antibody titers compared to LAIV. The seroconversion and seroprotection rates were also significantly higher in the groups vaccinated with TIIV than LAIV. In our study, LAIV stimulated small increases in HAI titers specific for the H3/N2 and B strain, but no increase in the H1/N1 strain. The increase in serum HAI titers and the seroconversion rates were significantly lower than those by stimulated by TIIV (Table 2 and Table 3). These results are consistent with other

studies which have demonstrated similar observations [11, 16, 17]. LAIV is known to induce a significantly higher mucosal IgA response than TIIV which has been demonstrated in nasal washes from vaccinated individuals [158, 159]. Unfortunately, corresponding nasal washes for IgA testing were not available for our study.

The IgG responses to LAIV have been reported before [60, 160], while in our study, LAIV did not induce any responses in IgG1, IgG1 or IgG3. In our opinion, the explanation for this difference can be attributed to two main factors. First, all subjects involved in our study were vaccinated during the influenza season one year prior to the study. In previous LAIV reports, the study subjects generally did not have any prior immunization history or were immune naïve. A recent study by Sasaki *et al.* [145] which included subjects vaccinated with an influenza vaccine in the year prior to the study showed that that there were significant differences in B cell and antibody responses elicited by TIIV and LAIV vaccines in adults. However that study focused on antibody secreting cells in PBMCs, whereas we investigated the actual level of serum antibody. It was noted that the effector IgG, memory IgG and serum antibody response was much higher in TIIV vaccinated individuals when compared with the LAIV group. Our theory that prior vaccination may contribute to the low IgG response is consistent with the reports that LAIV is not able to induce serum IgG response in elderly subjects when administered alone [160], who typically are vaccinated annually. The second possibility is that the immunogenicity of FluMist for the season of 2006-2007 was particularly low. Thus far, this has not been verified by any other reported studies.

In our study, we also showed that LAIV stimulated a robust serum IgA response. While the exact role of serum IgA in the immunity to influenza is not well understood, we speculate that there may be an increase in serum IgA as a result of the previously reported mucosal IgA response [159]. In addition, we observed a positive correlation between the increases in the serum IgA and HI titers, and these positive correlations are more pronounced than those between IgG1-3 and HI (Table 5). Further studies would be necessary to delineate the relationship between serum and mucosal IgA, as well as the role of IgA in HAI titers.

The cellular immune responses were compared between the TIIV and LAIV vaccinated groups. We observed that individuals vaccinated with LAIV were able to elicit a more robust type 1 T cell response when compared to the TIIV group. Although there was a trend of increase in the frequency of IFN-γ^+T cells after the TIIV vaccination, the increase did not reach statistically significant levels, and this was true for both influenza seasons 05-06 and 06-07 (Fig. 9 and Fig. 7). On the other hand, the Th1 T cell response induced by LAIV was statistically significant (Fig. 8). Of note, the baseline levels of the Th1 T cells were similar between TIIV06-07 and LAIV06-07, while the baseline level for TIIV05-06 was lower. We believe that this difference is contributed by the different levels of the memory Th1 T cells specific for different strains of influenza viruses used in the vaccines during the two different seasons. Regardless of the differences in baseline levels, the kinetics of the T cells responses to TIIV for different influenza seasons were similar (Fig. 9). It also suggests that the difference between the TIIV and LAIV response was not season-specific.

Inactivated or killed virus vaccines induce T cell responses mostly by $CD4^+$ Th1 T cells [152]. On the other hand, LAIV, which is a live virus vaccine capable of replicating in the upper respiratory tract, has the ability to provide $CD8^+$ T cells with viral antigens via dendritic cells and other antigen presenting cells. Hence the LAIV vaccine is able to induce a response from both the $CD4^+$ T cells and the $CD8^+$ T cells resulting in a more pronounced type 1 T cell response to vaccination as opposed to TIIV. We believe this likely explains the more robust Th1 T cell response in LAIV vaccinated subjects compared to TIIV vaccinated to subjects. Further studies are required to examine the different T cell subsets to get a clear picture regarding the immune mechanism of the two vaccines.

In addition to Th1, the Th2 T cell response was also monitored using IL-10 in the PBMC supernatants as the index. We found no differences in the magnitude of IL-10 response between the TIIV and LAIV ($p = 0.72$). It is known that the class and magnitude of antibody responses may be influenced by the types of T cell response (Th1 vs. Th2), which precedes the antibody responses. Here we observed that the antibody response to LAIV correlated positively with the Th2 T cells response ($r = 0.56$, Table 4). This positive correlation was only found in LAIV group. As far as we know, this is the first report on this matter. If confirmed by other independent studies, this information may be useful for enhancing the antibody response to LAIV by modulating IL-10. Conversely, we did not find any significant correlation between the Th1 T cell and the IgG1 antibody response ($r = -0.07$). On the other hand in the same vaccine trial, a positive correlation between the Th1 and IgG1 was observed in a group of healthy elderly (Chapter IV). In addition, this positive correlation is also observed in a group of frail elderly subjects in a

separate vaccine trial done by our group [152]. We reason that as people become older, the Th1 T cells become one of the confounding factors for the serum antibody response to TIIV. These results, together with the results on antibody responses, reiterate that LAIV and TIIV stimulate different arms of the immune system.

Our results also raise the issue of what immune correlates are important for influenza vaccine efficacy. LAIV is less effective in inducing serum IgG response, which has been used as the standard immune correlates for influenza vaccines and other vaccines [60, 161]. One the other hand, LAIV is more effective in stimulating mucosal IgA and Th1 T cell response [79]. The advantage of T cell response is that it can provide cross protection, and studies have shown that LAIV is able to provide protection from antigenically drifted strains in addition to homologous strains [142, 155]. One obvious benefit of mucosal secretory IgA antibodies is their location in the mucosal tract where influenza viruses enter and initiate infection. LAIV and TIIV are known to be similar in their effectiveness in protecting influenza infection. If this holds true for the influenza season 2006-2007, our data supports the notion that IgA and T cell responses should be considered as important immune correlates for influenza vaccine efficacy in addition to serum IgG response.

CHAPTER IV

COMPARISON OF AGE RELATED CHANGES IN IMMUNE RESPONSE TO INACTIVATED INFLUENZA VACCINE RESPONSE BETWEEN HEALTHY YOUNG AND ELDERLY AND THE ROLE OF CYTOKINES IN LOWER VACCINE EFFICACY IN ELDERLY

Introduction

Influenza virus is a very common respiratory pathogen that is responsible for increased annual morbidity and mortality in the elderly population [20]. Annual estimates by the Center for Disease Control and prevention report that influenza is responsible for 226,000 hospitalizations and 36,000 deaths every year [24]. The economic burden of influenza related medical costs among the elderly in the United States alone amounts to greater than 10 billion dollars that are spent on treating severe illness which is prevalent in the elderly populations every influenza season [162, 163]. Current public health measures to reduce influenza related hospitalizations and deaths among elderly call for annual influenza vaccinations among all persons above the age of 65 [164]. Although influenza vaccination is able to substantially reduce severe illness, secondary infections and deaths in the elderly, the efficacy rate of vaccination is 30-50% in this group [21, 162]. Several studies have concluded that the elderly demonstrate a reduction in antibody response after influenza vaccination [165-168]. Besides a reduction in antibody response to vaccination, a decline in cell mediated immune response has also been associated with lower vaccine efficacy in this group [167]. Aging is associated with several changes in different arms of the immune system, however, T cells show the most consistent and

largest alterations with age [101]. Increased susceptibility of the elderly to influenza infections may be related to a lack of development of influenza specific T cell responses after vaccination. Memory T cells mount an immediate vigorous response in response to antigenic challenge and secrete cytokines like IFN-γ and TNF-α. It has been shown that influenza specific memory T cells secreting IFN-γ are crucial for a rapid response to influenza reinfections [169]. Changes in age associated memory T cell response in the frail elderly have been shown to result in an impaired response to influenza vaccination [152]; however, further studies evaluating the effect of memory T cells after influenza vaccination are needed to understand the role of cell mediated immune response in the elderly. Aging has also been associated with dysregulation in cytokine production which play a critical role in modulating immune responses. Murine studies have demonstrated a shift from a Th1 type to a Th2 type cytokine response with increasing age [170]. In humans there have been conflicting reports regarding a shift from a Th1 to a Th2 cytokine profile; some investigators have shown that the capacity of T cells to produce Th1 cytokines is altered while the ability to produce Th2 cytokines is unaffected [171, 172]. However a recent review suggested that there is conflicting data to conclude that there is a shift from a Th1 cytokine profile to a Th2 cytokine profile in the elderly [101]. Ageing has also been associated with an increase in inflammatory cytokine levels, and IL-6 remains the most studied cytokine in the elderly. High levels of IL-6 in elderly have been reported in by some investigators [173, 174]; however, there are contradictory reports with unchanged values as well [175]. A change in cytokine levels as a result of aging could influence antibody response to influenza vaccination. It remains to be evaluated if there is indeed an age related shift in cytokine levels which might influence

vaccine responses. Studies which evaluate both cellular and humoral immune correlates after influenza vaccination in healthy young and the elderly have been limited. In order to understand the relationship between Th1 T cell response and antibody response in the elderly to influenza vaccination it is important to study cellular and humoral immune response to vaccination concurrently.

The current study was conducted to compare different correlates of immune responses among a healthy group of young and elderly individuals who had been previously vaccinated with influenza vaccine. We performed our study over two seasons. Several different humoral and cell mediated immune correlates were evaluated. Our study confirmed previous reports regarding blunted antibody response in elderly. Cell mediated immune response of influenza specific memory T cells (ISMT) demonstrated lower levels of IFN-γ secreting ISMT before and after vaccination in the elderly. We wanted to investigate if there are age related changes in cytokine profiles and if these correlated with the reduced T cell and antibody response to vaccination in the elderly. We also studied aging associated changes in cytokine profiles and the effect of immunization on the cytokine profiles. Our results demonstrate that there is a significant decline in both the humoral and cell mediated immune response in the elderly to influenza vaccination. We did not find any correlation in the cytokine profiles of elderly causing a shift from Th1 to Th2 response before and after vaccination. The pre-vaccination cytokine levels did not have any relation with a reduction in T cell or antibody response in the elderly. A significant observation of our study was the correlation between the Th1 T cell response and the antibody response that was observed only in the elderly which suggests that

reduction in Th1 T cell response affects antibody response to influenza vaccination in the elderly.

Materials and Methods

Vaccines

The vaccines used for this study were Fluzone® (Aventis Pasteur) from seasons 2005-2006 (T05-06) and 2006-2007 (T06-07). The TIIV vaccine of the 2005-2006 season comprised of 15 μg each of the HA from A/New Caledonia 20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Jiangsu/10/2003 (B/Shanghai/361/2002-like) and the TIIV vaccine of the 2006-2007 comprised of each of the HA from A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004.

Recruitment of Human Subjects and Vaccination Protocols

Subjects for the study were recruited with written informed consent by the clinical coordinating team at the Glennan Center for Geriatrics and Gerontology, Eastern Virginia Medical School during the winter influenza seasons of 2005-2006 and 2006-2007. The Institutional Review Board at Eastern Virginia Medical School approved the study protocol and the informed consent form. All study participants were healthy community dwelling young and elderly adults between 21-91 years of age, independently residing in the Hampton Roads area of Virginia. All subjects recruited for the study had been vaccinated with TIIV in the season prior to enrollment to minimize the immunological differences as a result of vaccination. For the T05-06 season the mean age of the study participants was 29 years (S.D. ±5.5) for the young group (n = 27) and 78.5 years (S.D. ±

5.7) for the elderly (n = 33). For the T06-07 season the mean age of the study participants was 27 years (S.D. ± 4) for the young group (n = 22) and 78 years (S.D. ± 7.7) for the elderly (n = 10). Subjects with egg allergy, underlying chronic diseases such as diabetes, autoimmune diseases such as lupus erythematosus or rheumatoid arthritis, or congestive heart failure; those on immunosuppressive drugs; and pregnant women were excluded from participating in the study. There were 60 subjects recruited in season 2005-2006 and 32 subjects recruited for the season 2006-2007 who were vaccinated with TIIV. Blood samples were obtained from each subject on four days: day 0 prior to vaccination and on days 4, 7 and 21 after vaccination.

Hemagglutination inhibition (HAI) assay

Hemagglutination occurs when sialic acid residues, similar to the cellular receptors on erythrocytes bind to the receptor binding site present on the tip of the viral HA proteins. Hemagglutination can be inhibited by adding HA recognizing antibodies before adding erythrocytes in the assay which is termed as hemagglutination inhibition; hence the assay is termed as hemagglutination inhibition assay (HAI). When performing HAI assays sera from vaccinated or infected subjects are titrated on 96 well plates to which a known amount of influenza virus and erythrocytes are added. In our study serum antibody titers specific for each of the three strains present in the vaccine formulations were determined by standard microtiter HAI assay following procedures described previously [150]. Serum HI antibodies were determined on day 0 (pre-vaccination) and on day 21 (post-vaccination). Non-specific inhibitors in serum were removed by incubating serum with receptor destroying enzyme (RDE; Sigma). All influenza H1N1,

H3N2 and B antigens (wild strains compatible with the recombinant strains in the vaccine) were grown in 10 day old fertilized chicken eggs. A slight modification was made to the protocol. Guinea pig red blood cells (GPRBCs) were used for agglutination instead of chicken or turkey RBCs. The HI titer was defined as the reciprocal of the highest dilution of serum that completely inhibited agglutination. Antibody titers of ≥ 40 were considered protective (seroprotection) and a $\geq$ four-fold rise in antibody titer (seroconversion) to one out of three vaccine antigens was considered an adequate response.

Serum IgG1, IgG2 and IgG3 analysis by ELISA

Serum levels of influenza specific IgG1, IgG2 and IgG3 were determined by ELISA analysis. All the assays were carried in a similar fashion. For all the variables serum samples obtained at day 0 (prior to vaccination) and at day 21 (post-vaccination) were analyzed. For each of IgG1, IgG2 and IgG3 analysis 96 well EIA specific round bottom plates (Immulon 1; Dynatech Laboratories, Chantilly, VA) were used. The 96 well plates were coated with IgG1, IgG2 and IgG3 purified protein as standards (all κ-chains; Sigma Aldrich, MO) and with trivalent influenza vaccine 2005-2006 formula (A/New Caledonia 20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Jiangsu/10/2003; Aventis Pasteur, Swiftwater, PA) or 2006-2007 formula (A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004; Aventis Pasteur, Swiftwater, PA). The plates were incubated at 4°C for 16-18 hours. After overnight incubation the plates were washed twice with wash buffer (1 X PBS with 0.05% Tween 20, pH 7.4). Following wash procedure 200 µl of blocking buffer (10%

FBS/1 X PBS) was added to each well and incubated at 37°C for 2 hours. The plates were then washed with wash buffer 4 times. Dilutions of serum samples to be analyzed were made for the respective IgG subtype assays. The serum samples were diluted to 1:5000, 1:200 and 1:400 with blocking buffer for IgG1, IgG2 and IgG3 analysis, respectively. Plates were incubated at 37°C for 2 hours and washed four times with wash buffer. For each IgG subtype, biotin-conjugated anti-IgG subtype antibodies {IgG1 and IgG2 (BD Biosciences) and IgG3 (Sigma Aldrich)} were diluted 1:1000, 1:1000 and 1:4000, respectively with blocking buffer. Biotin conjugated anti IgG specific antibodies were added to the 96 well plate and incubated at 37°C for 1 hour. Plates were washed and 100 µl of avidin-HRP (Sigma Aldrich) diluted 1:400 was added and the plates were incubated at room temperature for 30 minutes. After the final incubation, the plates were washed five times with wash buffer. Peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma Aldrich) was added, and color development was allowed to continue for 10 minutes at room temperature in the dark. The color development reaction was stopped by adding 100 µl per well of 1 N sulfuric acid. The plates were read at 450 nm using an ELISA plate reader and analyzed with KC4 (version 3.0) software (PowerWave, Bio-Tek Instruments, Winooski, VT). The final analysis was done by normalizing all the readings based on the dilution factors.

Serum IgA analysis by ELISA

The IgA ELISA analysis was done in a similar fashion as the IgG sub-type ELISA. Serum samples obtained at day 0 (prior to vaccination) and at day 21 (post-vaccination) were analyzed. For IgA analysis 96 well EIA specific round bottom plates

(Immulon 1; Dynatech Laboratories, Chantilly, VA) were used. The 96 well plates were coated with IgA (κ-chain; Sigma Aldrich, MO) and with trivalent influenza vaccine 2005-2006 or 2006-2007 formula. The plates were incubated at 4°C for 16-18 hours. After overnight incubation, the plates were washed twice with wash buffer (1 X PBS with 0.05% Tween 20, pH 7.4) and 200 µl of blocking buffer (10% FBS/1 X PBS) was added to each well and incubated at 37°C for 2 hours. The plates were then washed with wash buffer 4 times. The serum samples were diluted to 1:400 with blocking buffer for IgA and added to the plate. The plates were incubated at 37°C for 2 hours. Plates were washed four times with wash buffer. For IgA, HRP-conjugated anti-IgA antibody (Sigma Aldrich) was diluted to 1:4000 with blocking buffer. Diluted HRP-conjugated anti-IgA specific antibody was added to the 96-well plate and incubated at 37°C for 1 hour. Plates were washed four times with wash buffer. After the final incubation, the plates were washed five times with wash buffer. Peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma Aldrich) was added to the wells, and color development was allowed to continue for 10 minutes at room temperature in the dark. The reaction was stopped by adding 100 µl 1 N sulfuric acid per well. The plates were read at 450 nm using an ELISA plate reader and analyzed with KC4 (version 3.0) software (PowerWave, Bio-Tek Instruments, Winooski, VT). The final analysis was done by normalizing all the readings based on the dilution factors.

Purification of PBMC

Human peripheral blood mononuclear cells (PBMC) were isolated form blood that was collected in heparinized tubes. Whole blood was first centrifuged at 320 X *g* for

10 minutes. Plasma was aspirated. The buffy coat layer, which contains the PBMCs, was carefully aspirated and laid onto a gradient purification Ficoll reagent Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), followed by centrifugation at 650 X *g* for 30 minutes. The PBMC were collected by aspiration from the interface and washed twice with RPMI 1640 medium (Invitrogen, Carlsbad, CA) at 500 X *g* and 320 X *g* for 10 minutes each. The PBMCs were then counted by an automatic lymphocyte counter (Coulter ACT, Beckman Coulter, Miami, FL) and resuspended in CTL media (RPMI 1640 medium containing 10% FBS, 2 mM of L-Glutamine, 100 U of penicillin, 100 μg/ml of streptomycin and 55 nm of 2-mercaptoethanol). The cell concentration was calculated and brought up to 1 million PBMCs/ml for the fast immune assay.

Influenza virus used for Fast Immune Assay

All the live influenza virus strains used in the study were propagated under Bio-Safety Level-2 conditions. The seed virus strains were obtained from Center for Disease Control (CDC), Atlanta, GA. All Bio-Safety guidelines detailed in the microbiological and biomedical laboratory manuals published by the CDC and by the Office of Safety at Eastern Virginia Medical School were followed. The live virus strains of influenza used in this study were A/New Caledonia 20/1999 (H1N1), A/New York/55/2004, A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 and B/Shanghai/361/2002. The live viruses were grown in fertilized chicken eggs using seed viruses obtained from CDC following procedures described previously [151]. Purified allantoic fluids containing live virus were then analyzed to determine the viral titers. After determination of the viral titers the allantoic fluids were transferred to cryovials and stored at -80°C until required.

Detection of T cell IFN-γ production using Fast Immune Assay

The fast immune procedure to determine T cell IFN-γ secretion was done as described previously [152]. One million PBMC were activated *ex vivo* using a combination of the three strains of virus (10 HA units/ml for each) corresponding with vaccine strains in 150 µl of complete media in 96-well U-bottom tissue culture (TC) plate at 37°C overnight. Brefeldin A (BFA, Sigma-Aldrich, St. Louis, MO) was then added to each well at a final concentration of 5 ug/ml after 17 hours. The culture was incubated at 37°C for 3 more hours before being fixed with 1% paraformaldehyde-PBS and then permeabilized with permeabilization buffer (Becton Dickinson, San Diego, CA) at room temperature for 10 minutes each. The cell culture was stained with the following conjugated antibodies; CD69-PE, CD4-AP, CD8-PerCP and IFN-γ-FITC (BD PharMingen, San Diego, CA). Intracellular cytokine staining was detected using a flow cytometer (FACSCalibur, CellQuest 3.3 software, BD Biosciences, San Diego, CA).

Cytokine analysis by cytometric bead array (CBA)

Cytometric bead array analysis was used to determine the cytokine levels after stimulation of PBMCs for 15 hours with a combination of three strains of influenza viruses present in the vaccines. The supernatants from influenza virus stimulated PBMC were diluted in PBS 1:2 and incubated with CBA beads coated with anti IL-10, IL-6, TNF-α and IL-1β antibodies {human inflammatory cytokine kit; BD™ Cytometric Bead Array (CBA)}. The beads were washed and stained with PE-conjugated secondary antibodies. The fluorescent intensity for each cytokine was measured using a flow

cytometer (FACSCalibur, BD Biosciences, San Diego, CA). The final concentrations were analyzed and calculated using CBA software (BD Biosciences).

Statistical Analysis

Data analysis of the normally distributed data was performed using Student's t-test. Normal distribution of the data was evaluated using Kolmogorov-Smirnov's test. A modified t-test, Mann-Whitney rank sum test or Wilcoxon signed rank test was applied to determine significant differences between the two groups where data was not normally distributed. Statistical significance was set at $p<0.05$. The correlation coefficient of the two groups was analyzed using Pearson's correlation coefficient test. To determine the statistical relevance of seroprotection and seroconversion rates, Chi Square or Fischer's test were performed ($p<0.05$) based on the size of the group that was being evaluated.

Results

Comparison of the Th1 response to TIIV vaccination between young and elderly

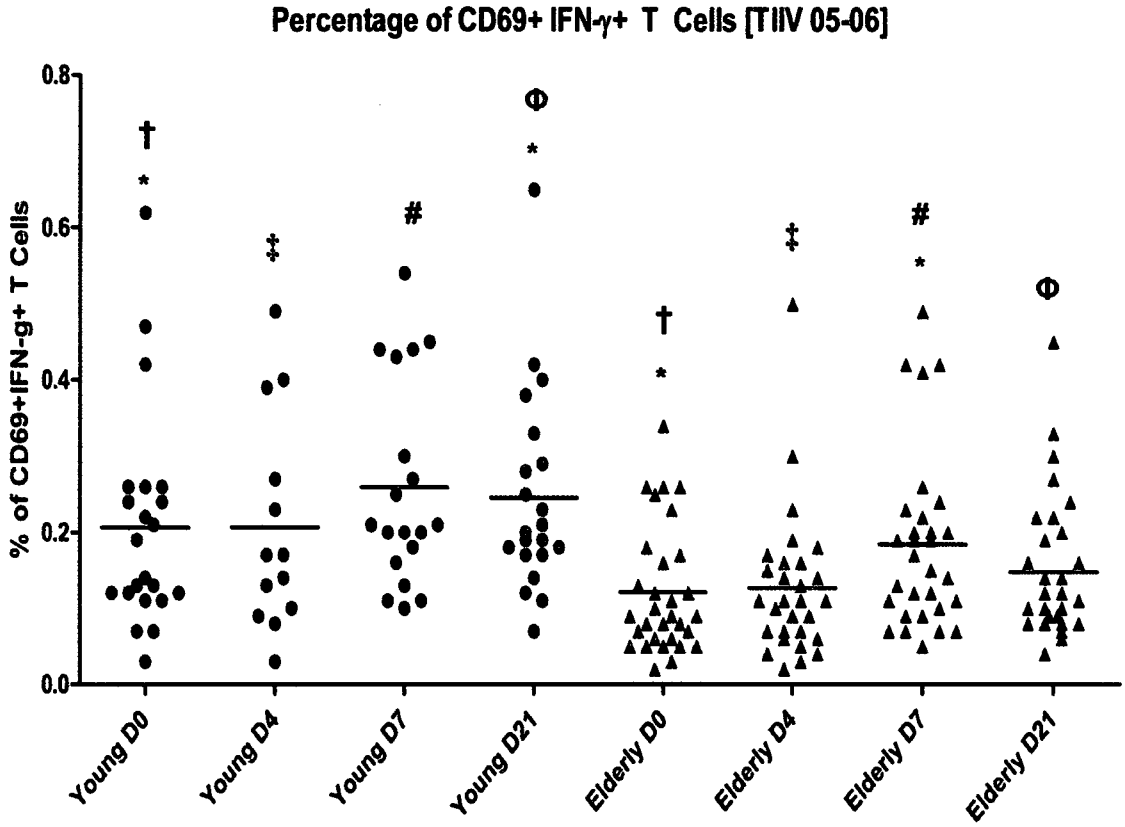
The type 1 T cell response was measured between the young and the elderly after TIIV vaccination by monitoring the change in the percentage of IFN-γ secreting ISMT cells ($CD8^+$ and $CD4^+$ memory T cells) using the FastImmune Assay. We observed that there was a trend in increase in the percentage of IFN-γ secreting T cells after TIIV vaccination in the elderly and young group. Observations were made at day 0 (prior to vaccination), day 4, day 7 and day 21 post-vaccination. Although there was an increase in the percentage of IFN-γ secreting T cells in both the young and the elderly groups there was a difference in the kinetics that was observed. In the elderly group there was a

significant increase in the percentage of IFN-γ secreting T cells between day 0 and day 7 (T05-06 day 0 0.118 s.e.m ± 0.01; T05-06 day 7 0.215 S.E.M. ± 0.04, p = 0.02) (T06-07 day 0 0.161 S.E.M. ± 0.02; T06-07 day 7 0.223 S.E.M. ± 0.03, p = 0.03), however the increase was transient and went back to baseline levels by day 21 (Fig. 10 and Fig. 11). The trend was observed for both the T05-06 and T06-07 season. On the other hand in the young group there was a trend in increase in the number of IFN-γ producing T cells between day 0, day 4, day 7 and day 21. The increase in the young group was not transient and significantly higher levels in the percentage of IFN-γ secreting T cells were observed on day 7 in the young group (T05-06 day 0 0.203 S.E.M. ± 0.03; T05-06 day 7 0.257 S.E.M. ± 0.02, p = 0.003) (T06-07 day 0 0.320 S.E.M. ± 0.03; T06-07 day 7 0.404 S.E.M. ± 0.05, p = 0.05) (Fig.10 and Fig. 11). The trend was consistent for both the T05-06 and T06-07 group. The percentage of T cells secreting IFN-γ did not go down to baseline levels by day 21 in the young group and there was sustained increase in Th1 type T cell response. These results indicate that although there is a trend of increase in the Th1 type T cell response in both the elderly and the young after TIIV vaccination, there is a major difference in the ability to induce a sustained increase in the levels of IFN-γ secreting T cells.

We also observed that there was a significant difference in the percentage of IFN-γ secreting T cells between the young and the elderly for each time point that was measured. When the levels were compared across day 0, day 4, day 7 and day 21 we found that there were significantly higher levels of IFN-γ secreting T cells in the young group as opposed to the elderly group. (Elderly T05-06 day 0 0.118 S.E.M. ± 0.01; Young T05-06 day 0 0.203 S.E.M. ± 0.01, p = 0.007) (Elderly T05-06 day 4 0.125

S.E.M. ± 0.01; Young T05-06 day 4 0.205 ± 0.01, p = 0.05) (Elderly T05-06 day 7 0.215 S.E.M. ± 0.04; Young T05-06 day 7 0.257 ± 0.01, p = 0.02) (Elderly T05-06 day 21 0.145 S.E.M. ± 0.01; Young T05-06 day 21 0.243 ± 0.02, p = 0.002) (Elderly T06-07 day 0 0.161 S.E.M. ± 0.02; Young T06-07 day 0 0.320 S.E.M. ± 0.03, p = 0.006) (Elderly T06-07 day 4 0.195 S.E.M. ± 0.02; Young T05-06 day 4 0.360 ± 0.06, p = 0.112) (Elderly T06-07 day 7 0.223 S.E.M. ± 0.03; Young T06-07 day 7 0.404 ± 0.05, p = 0.05) (Elderly T06-07 day 21 0.173 S.E.M. ± 0.02; Young T06-07 day 21 0.449 ± 0.07, p = 0.003) (Fig. 10, Fig. 11). These results show that with age there is decline in the baseline levels of T cells that secrete IFN-γ specific for influenza viruses. Influenza vaccination does induce an increase in the percentage of IFN-γ specific T cells; however, the response is blunted in the elderly compared to the young.

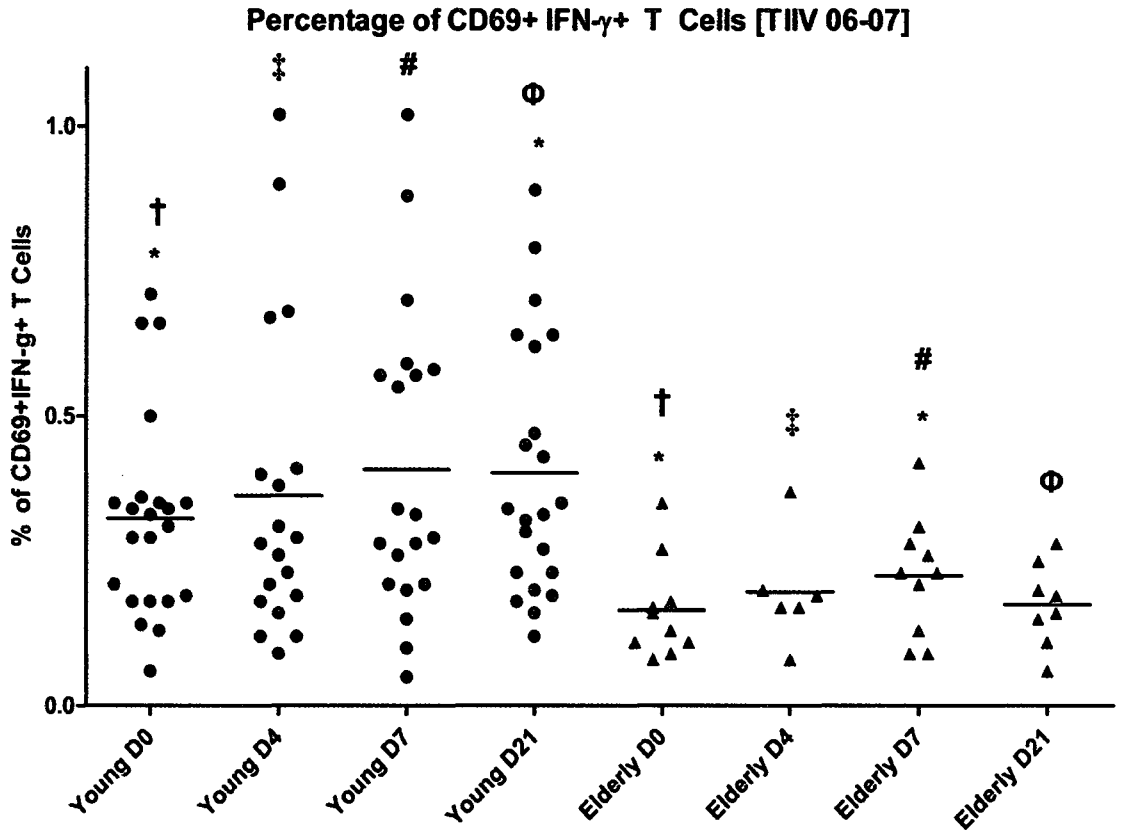
Fig. 10. Percentage of $CD69^{+}IFN\text{-}\gamma^{+}$ T cells pre- and post-TIIV05-06 influenza vaccination observed in young and elderly subjects using the Fast Immune Flow Cytometry Assay.
One million PBMCs from each subject vaccinated with TIIV were stimulated with 1 µl of influenza virus antigen in a 96-well plate overnight. The Fast Immune assay and flow cytometry were used to determine the frequency of $CD69^{+}IFN\text{-}\gamma^{+}$ T Cells.



* = significant difference between two time points (paired t test $p<0.05$)
† = significant difference between two groups Day 0 (unpaired t test $p<0.05$)
‡ = significant difference between two groups Day 4 (unpaired t test $p<0.05$)
# = significant difference between two groups Day 7 (unpaired t test $p<0.05$)
Φ = significant difference between two groups Day 21 (unpaired t test $p<0.05$)

Young (n) = 27
Elderly (n) = 33

Fig. 11. Percentage of $CD69^{+}IFN\text{-}\gamma^{+}$ T cells pre- and post-TIIV06-07 influenza vaccination observed in young and elderly subjects using the Fast Immune Flow Cytometry Assay.
One million PBMCs from each subject vaccinated with TIIV were stimulated with 1 µl of influenza virus antigen in a 96-well plate overnight. The Fast Immune assay and flow cytometry were used to determine the frequency of $CD69^{+}IFN\text{-}\gamma^{+}$ T Cells.



* = significant difference between two time points (paired t test $p<0.05$)
† = significant difference between two groups Day 0 (unpaired t test $p<0.05$)
‡ = significant difference between two groups Day 4 (unpaired t test $p<0.05$)
# = significant difference between two groups Day 7 (unpaired t test $p<0.05$)
Φ = significant difference between two groups Day 21 (unpaired t test $p<0.05$)

Young (n) = 22
Elderly (n) = 10

Comparison of antibody response to TIIV vaccination between young and elderly

The antibody responses to TIIV vaccination were measured by several different assays in the two groups. We measured the HAI antibody response to each influenza strain present in the vaccine by standard hemagglutination inhibition assay. We also measured the serum IgG1, IgG2, IgG3 and IgA levels by ELISA pre- and post-vaccination. There was a significant increase ($p = 0.001$) in HAI antibody titers after TIIV vaccination in both the elderly and young group for each of the three influenza vaccine strains (Table 6) except for B/Malaysia strain for the T06-07 season in the elderly group. Although there was no difference between the young and the elderly population in pre-vaccination antibody titers except for H1N1 strain in the T05-06 season, the elderly showed significantly lower antibody titers to all the three strains compared to young after vaccination except for H3N2 strain in the T05-06 season (Table 6) (H1N1 A/New Caledonia $p<0.05$, B/Shanghai $p<0.05$ for the T05-06 season, H3N2 A/Wisconsin $p<0.05$, A/New Caledonia $p<0.05$, B/Malaysia $p<0.01$ for the T06-07 season). The mean fold increase in HAI antibody titers was low for the elderly in both the seasons when compared to young (Table 6).

We also observed that the incidence of pre-vaccination antibody titers equal to the seroprotection rate defined by HAI titers ≥ 40 was comparable between the young and the elderly; however, post-vaccination levels of antibody seroprotection rates were much higher in the young group compared to the elderly (Table 7). For H3N2 strains (A/New York and A/Wisconsin), 77% young had post-vaccination HAI antibody titers of ≥ 40 for season 05-06 and 06-07 compared to 54% and 30% in the elderly group, respectively (χ^2; $p = 0.05$). Similarly for the H1N1 strain (A/New Caledonia), only 15% and 30% of the

elderly had HAI titers ≥40 for the season 05-06 and 06-07, respectively, compared to 88% and 72% in the young group for the respective seasons (χ^2; p = 0.05). We observed a similar trend with regard to the B strains (B/Shanghai and B/Malaysia) where 65% and 31% of the young had HAI titers ≥40 for the season 05-06 and 06-07, respectively and in the elderly the rates were 21% and 10% for the respective seasons. Overall, although there was an increase in HAI antibody titers in the elderly after vaccination, the response was much lower compared to the young. Seroconversion (4-fold rise in antibody titers) rates between young and old were comparable, but the seroprotection rates of the elderly were lower post-vaccination (Table 7). These results reflect that although there was an increase in HAI antibody titers in the elderly, it was not able to reach protective levels.

We observed that the baseline levels of HAI antibody levels were an important factor in the level of HAI antibody response post-vaccination. In both the young and the elderly groups, we found that subjects with higher baseline HAI antibody levels had a lower mean fold increase in antibody titers post-vaccination. On the other hand, subjects with low pre-vaccination HAI antibody titers showed a much higher mean fold increase in HAI antibody response post-vaccination (data not shown).

Table 6. Geometric means of HAI antibody titers against influenza strains after influenza vaccination observed after Hemagglutination Inhibition Assay.

	H3N2 (A/New York or A/Wisconsin)		
	Pre	**Post**	**MFI**
TIIV (T05-06)			
Young	36.30 (95% CI = 20.86-51.74)*	86.23 (95% CI = 62.38-110.08)*	5.53
Elderly	19.75 (95% CI= 11.42-28.08)*	79.72 (95% CI = 43.88-115.56)*	4.27
TIIV (T06-07)			
Young	22.27 (95% CI= 8.56--35.98)*	76.36 (95% CI= 40.5-112.22)*,b	7.27
Elderly	15.5 (95% CI= 7.06-23.94)*	27.6 (95% CI= 21.95-33.25)*,b	2.66
	H1N1 (A/New Caledonia)		
	Pre	**Post**	**MFI**
TIIV (T05-06)			
Young	189.19 (95% CI= 81.42-296.98)*,a	259.65 (95% CI = 136.80-382.48)*,a	3.17
Elderly	11.42 (95% CI= 8.14-14.7)*,a	26.8 (95% CI= 8.48-45.19)*,a	1.94
TIIV (T06-07)			
Young	41.13 (95% CI= 21.63--60.63)*	89.13 (95% CI = 61.18-117.08)*,b	4.24
Elderly	16.5 (95% CI=10.3-22.7)*	27.7 (95% CI= 14.3-41.5)*,b	1.62
	B (B/Shanghai or B/Malaysia)		
	Pre	**Post**	**MFI**
TIIV (T05-06)			
Young	46.5 (95% CI = 20.57-72.43)*	68.15 (95% CI = 42.85-93.45)*,a	2.45
Elderly	13.96 (95% CI= 9.24-18.68)*	33.27(95% CI= 21.92-44.62)*,a	1.9
TIIV (T06-07)			
Young	6.36 (95% CI = 5.41--7.31)*	32.5 (95% CI = 16.9--48.1)*,b	5.68
Elderly	8.2 (95% CI= 6.72-9.68)	13 (95% CI= 6.6-19.4)b	1.54

* = Significant increase in HAI antibody titers between Day 0 and Day 21, p = <0.05 (paired t test)
a = Significant difference between HAI antibody titers between young (T05-06) and elderly (T05-06), p = <0.05 (unpaired t test)
b = Significant difference between HAI antibody titers between young (T06-07) and elderly (T06-07), p = <0.05 (unpaired t test)
MFI= Mean fold increase

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

Table 7. Seroprotection and seroconversion rates between the young and elderly after TIIV vaccination.

	% Seroprotection Rates *(n)* (HAI Titers >1:40)					
	H3N2		**H1N1**		**B**	
TIIV (T05-06)	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Young	38 (10)	77 (20)	65 (17)[a]	88 (23)[a]	38 (10)[a]	65 (17)[a]
Elderly	18 (6)	54 (18)	3 (1)[a]	15 (5)[a]	6 (2)[a]	21 (7)[a]
TIIV (T06-07)						
Young	17 (4)	77 (18)[a]	36 (8)	72 (16)[a]	0 (0)	31 (7)
Elderly	20 (2)	30 (3)[a]	10 (1)	30 (3)[a]	0 (0)	10 (1)

	% Seroconversion Rates *(n)* (4 Fold or more Increase in HAI Titers)		
	H3N2	**H1N1**	**B**
TIIV (T05-06)			
Young	42 (11)	15 (4)	23 (6)
Elderly	54 (18)	15 (5)	15 (4)
TIIV (T06-07)			
Young	55 (12)	27 (6)	41 (9)
Elderly	40 (4)	0 (0)	10 (1)

a = Significant difference between seroprotection rates between young and elderly (Chi square test p = 0.05)

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

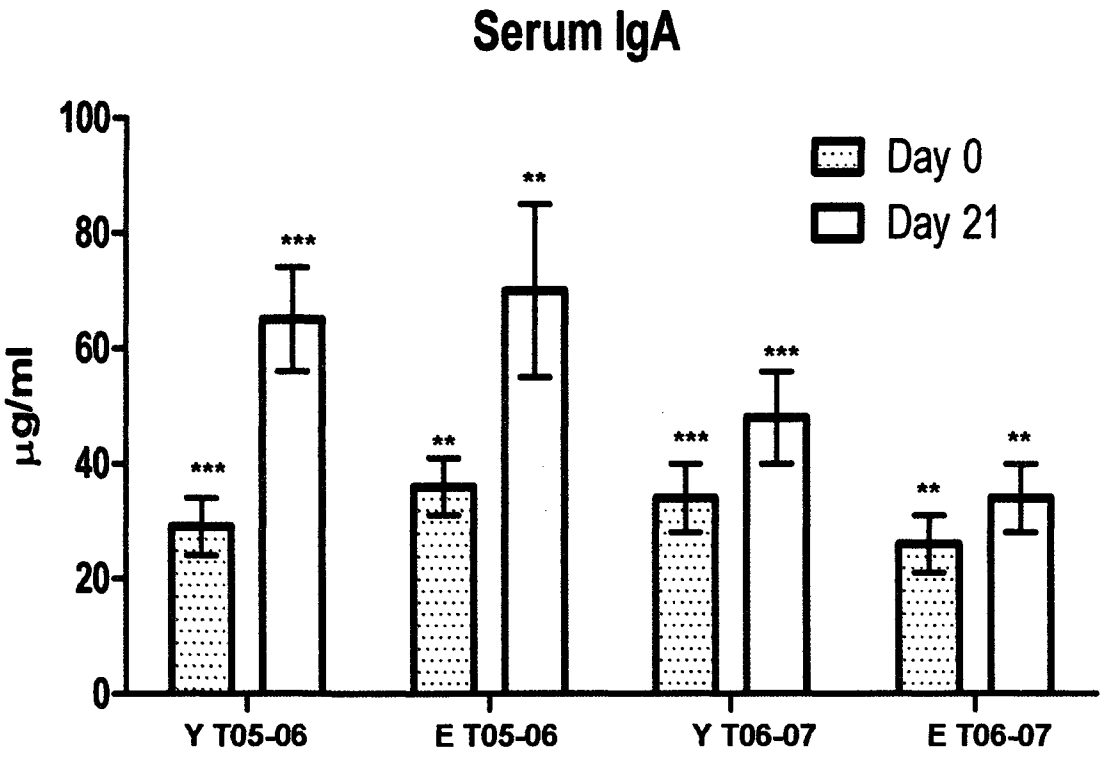
The levels of serum IgA increased after vaccination in both the young and the elderly group. The levels of serum IgA pre and post-vaccination were comparable between the young and the elderly group (T05-06Young day 0, 29.1 μg/ml; S.E.M. ± 5, T05-06Elderly day 0, 36 μg/ml; S.E.M. ± 4.8; T05-06Young day 21, 64.6 μg/ml; S.E.M. ± 9.1, T05-06Elderly day 21, 69.9 μg/ml; S.E.M. ± 15.3) (T06-07Young day 0, 34.1 μg/ml; S.E.M. ± 6.4, T06-07Elderly day 0, 25.9 μg/ml; S.E.M. ± 5.2; T06-07Young day 21, 47.9 μg/ml; S.E.M. ± 8.3, T06-07Elderly day 21, 33.7 μg/ml; S.E.M. ± 5.9) (Fig. 12). This shows that there is no change as a result of age in the ability to induce a serum IgA response by TIIV vaccination.

The serum IgG sub-class specific for influenza strains for both the T05-06 and T06-07 vaccines were determined using ELISA. All the subjects in our study had been vaccinated during the previous influenza season with TIIV and had pre-vaccination serum antibody IgG subclasses for influenza viruses (H3N2, H1N1 and B strains) (Fig. 13, Fig. 14 and Fig. 15). The ELISA assay used in our study detected serum antibody IgG1, IgG2 and IgG3 subclass levels against all the three virus strains present in the vaccines. We observed that IgG1 had the greatest increase in levels following vaccination in both the young and the elderly groups. In the case of serum IgG1 we observed that the levels of pre-vaccination serum IgG1 levels were comparable in both the young and the elderly group for both the 05-06 and 06-07 seasons (Fig. 13). There was a significant increase in serum IgG1 levels in both the young and the elderly groups ($p = <0.001$) (Fig. 13). However, the magnitude of increase in the levels of IgG1 was much lower in the elderly group (T05-06 50%, T06-07 26%) compared to the young group (T05-06 190%, T06-07 74%) (Fig. 13). These results show that there is marked decline in the elderly

group's ability to mount a serum IgG1 response after vaccination. When serum IgG2 and IgG3 levels were compared, there was a significant increase in both IgG2 and IgG3 levels after vaccination in both the elderly and the young groups ($p = <0.001$) (Fig. 14 and Fig. 15). The elderly had higher levels of serum IgG2 when compared to the young group, both pre- and post-vaccination ($p = 0.05$) (Fig. 14 and Fig. 15).

After evaluating the antibody responses, we looked at the correlation of the HAI antibody response to serum IgA, IgG1, IgG2 and IgG3 among the elderly and young groups for both the T05-06 and T06-07 seasons combined. There was a positive correlation between the HAI antibody response and serum IgA ($r = 0.62$), IgG1 ($r = 0.71$) and serum IgG2 ($r = 0.40$) in the elderly, and there was a weak correlation between HAI antibody response and serum IgG1 ($r = 0.45$) and IgA ($r = 0.43$).

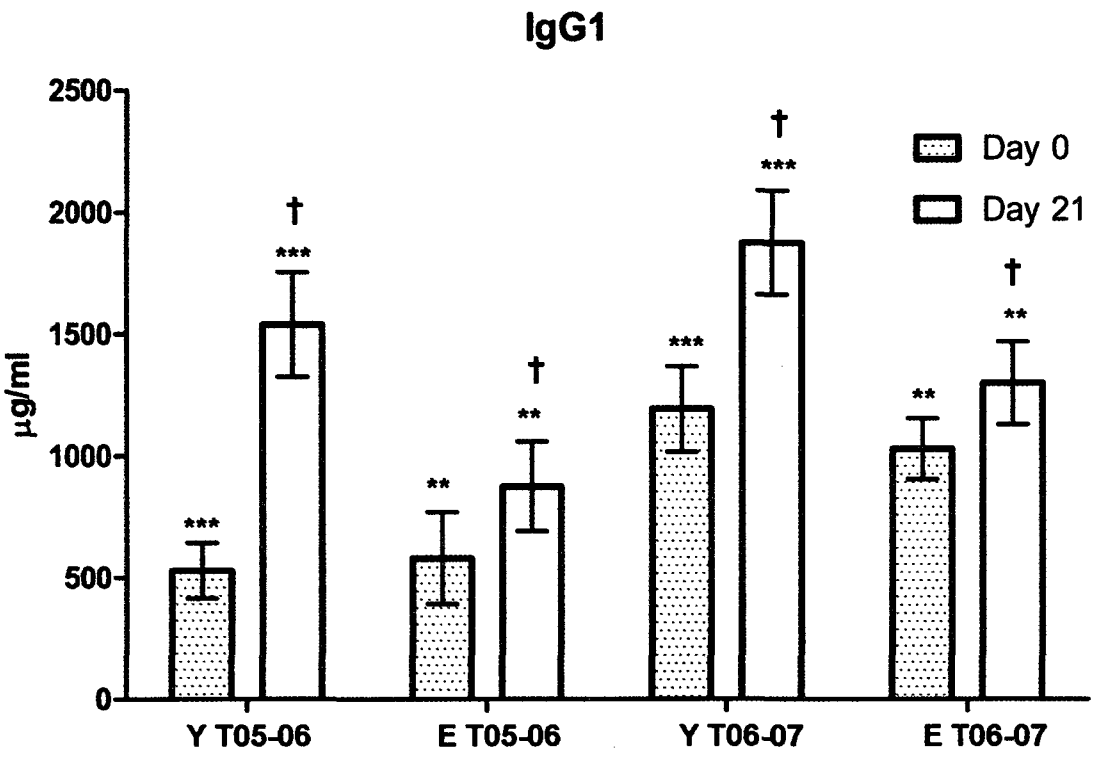
Fig. 12. Serum IgA antibody levels before and after TIIV influenza vaccination observed in the young and the elderly by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean, and error bars represent the standard error of the mean (S.E.M.).



*** = significant difference between Day 0 and Day 21 among young and old group paired t test ($p<0.001$)
** = significant difference between Day 0 and Day 21 among young and old group paired t test paired t test ($p<0.01$)

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

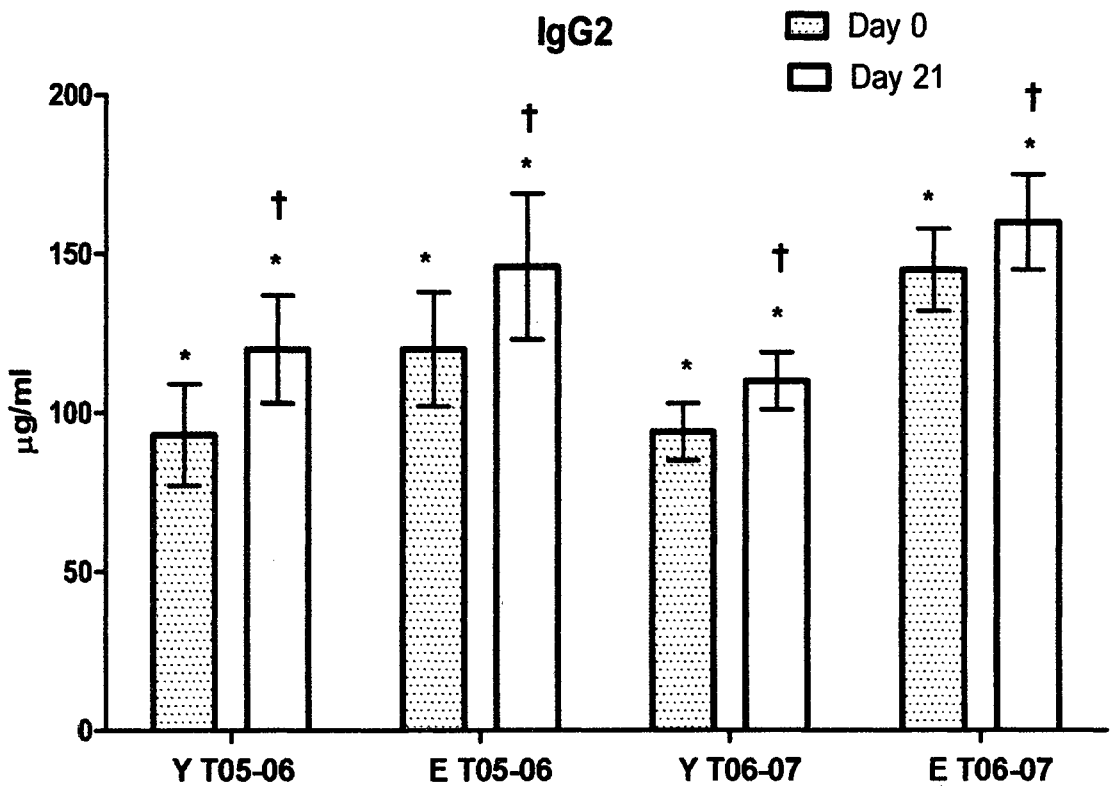
Fig. 13. Serum IgG1 antibody levels before and after TIIV influenza vaccination observed in young and elderly by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean, and error bars represent the standard error of mean (S.E.M).



*** = significant difference between Day 0 and Day 21 among young and old group paired t test ($p<0.001$)
** = significant difference between Day 0 and Day 21 among young and old group paired t test paired t test ($p<0.01$)
† = significant difference between young and old at Day 0 and Day 21 unpaired t test ($p<0.05$)

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

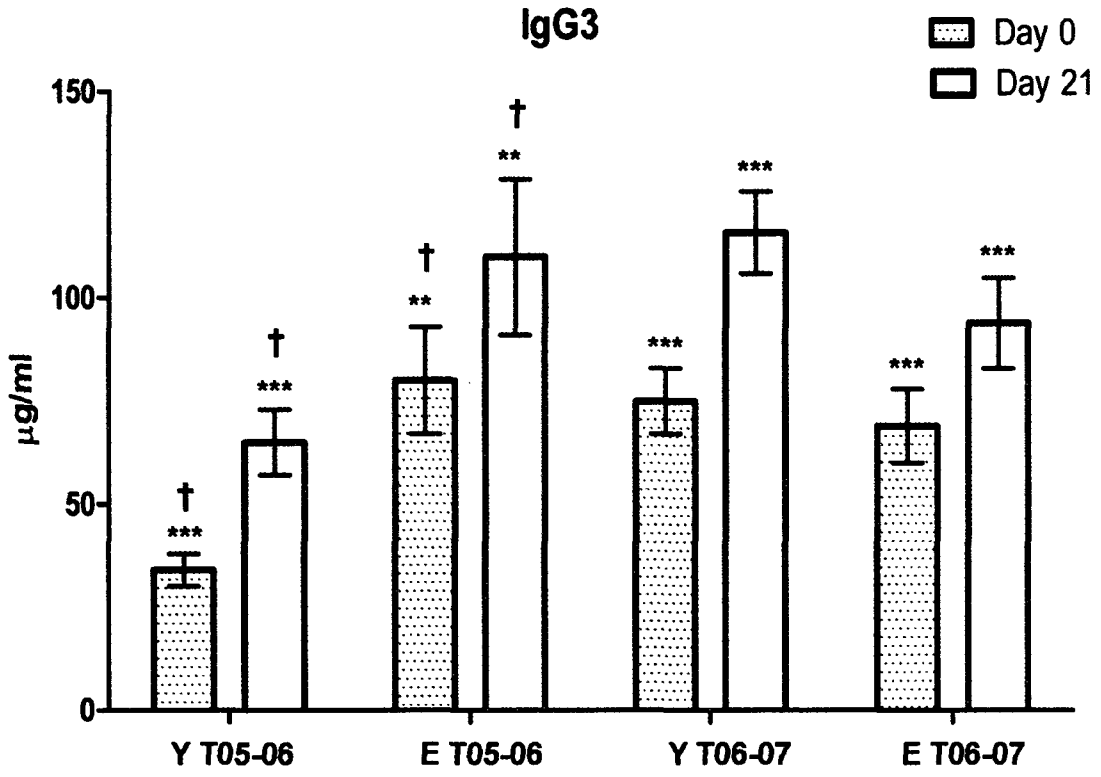
Fig. 14. Serum IgG2 antibody levels before and after TIIV influenza vaccination observed in young and elderly by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated by ELISA. Vertical bars represent the mean, and error bars represent the standard error of mean.



* = significant difference between Day 0 and Day 21 among young and old group paired t test paired t test ($p<0.05$)
† = significant difference between young and old at Day 0 and Day 21 unpaired t test ($p<0.05$)

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

Fig. 15. Serum IgG3 antibody levels before and after TIIV influenza vaccination observed in young and elderly by ELISA.
Serum samples were collected on day 0 (pre-vaccination) and on day 21 (post-vaccination). Serum antibody response was evaluated using ELISA assay. Vertical bars represent the mean, and error bars represent the standard error of mean (S.E.M).



*** = significant difference between Day 0 and Day 21 among young and old group paired t test ($p<0.001$)
** = significant difference between Day 0 and Day 21 among young and old group paired t test paired t test ($p<0.01$)
† = significant difference between young and old at Day 0 and Day 21 unpaired t test ($p<0.05$)

Young (n) = 27 (TIIV05-06) : 22 (TIIV06-07)
Elderly (n) = 33 (TIIV05-06) : 10 (TIIV06-07)

Th1 T cell response correlated positively with the antibody response in the elderly but not young subjects

The antibody response to influenza vaccination and its correlation with type 1 T cell response was also analyzed between the young and the elderly groups. It was observed that the elderly group had a strong positive correlation between antibody response and type 1 T cell response. There was a positive correlation between Th1 response and serum IgA ($r = 0.74$), IgG1 ($r = 0.64$), IgG2 ($r = 0.51$) and IgG3 ($r = 0.43$). There was no correlation observed between type 1 T cell response and antibody response in the young group. These results indicate that the type 1 T cell response is associated with IgA, IgG1, IgG2 and IgG3 antibody response to influenza vaccination in the elderly.

Cytokine response to influenza vaccination in the elderly and the young

In an attempt to understand the underlying mechanism of age-related decline in Th1 and antibody response to inactivated influenza vaccination, the inflammatory and Th2 cytokine levels prior to vaccination, as well as how they responded to vaccination, were measured in young and elderly subjects. The inflammatory cytokines tested were TNF-α, IL-1β and IL-6, and the Th2 cytokine tested was IL-10. The cytokine levels from PBMC supernatants before and after influenza virus stimulation were measured using a multibead cytokine assay (CBA). Among all the cytokines evaluated, IL-6 was the most abundant. Although the mean levels of IL-6 were greater in the young group, we did not observe any statistically significant ($p>0.05$) difference in levels between the young and the elderly groups. The IL-10 levels were comparable in the young and the elderly groups pre- and post-vaccination. There was an increase in IL-10 levels in both the young and

the elderly groups after vaccination, but it was not significant. In the case of TNF-α, we observed that the young group had significantly higher levels both pre- and post-vaccination compared to the elderly group. However, there was no significant increase in TNF-α levels post-vaccination in either group. Similarly for IL-1β, the levels were higher in the young group when compared to the elderly both pre- and post-vaccination, and did not increase significantly post-vaccination. We did not observe an enhanced Th2 cytokine response in the elderly group before and after influenza vaccination.

These results suggest that the blunted type 1 T cell response in the elderly was not due to the increase in Th2 cytokines. We also evaluated the correlation between baseline levels of all the Th1 and Th2 cytokines and the antibody response in both elderly and young adults, in an effort to investigate the relationship between cytokine levels and their effect on the T cell and antibody response to vaccination. We did not find any correlations between the T cell response or antibody response and baseline cytokine levels (data not shown), which suggests that cytokines do not influence the humoral antibody outcome after vaccination in the elderly or the young. We did not find any age-related changes in cytokine profile before vaccination that had any influence on the T cell or antibody response to vaccination.

Discussion

It has been well established that influenza is a serious public health hazard, and the elderly are a highly vulnerable population affected by influenza-related illness and other secondary infections caused as a result of influenza infections [164, 176]. Although vaccination has been recommended to reduce influenza-related morbidity and mortality,

it has been found to be less efficacious in this population compared to the young [165, 168]. One of the most crucial factors when evaluating age-related changes in immune response to vaccination is the state of health of the subjects. It has been shown in various studies that the health criteria of subjects can influence the results of a study [168]. In order for a study to be reflective of immune response elicited by a general healthy elderly population, we excluded any elderly subjects with immunodeficiency diseases, malignancies or those who were taking medications known to affect the immune system. In our study, we evaluated the humoral and cell-mediated immune responses simultaneously from a group of healthy and young individuals over a two year period to determine the effects of age on immune response to influenza vaccination. Most studies in the past have focused on either humoral or cell-mediated immune responses separately. Very few studies have looked into the age-associated changes in both arms of the immune system in parallel to delineate the age-related immune dysfunction to influenza vaccination [152, 166, 167, 177, 178]. We performed a comprehensive study looking at various humoral and cell mediated immune components pre- and post- influenza vaccination in a cohort of healthy community-dwelling young and elderly individuals to investigate the age-related decline in vaccine response.

We demonstrated that the humoral immune response is compromised in the elderly compared to the young. Our results are in agreement with previous reports which have demonstrated an age-related decline in humoral antibody response to influenza vaccination [165, 168]. Although we did not observe any significant difference in the levels of four-fold rise after vaccination between young and elderly, it was evident that the baseline levels were much lower in the elderly compared to the young, since post-

vaccination seroprotection levels in the elderly were significantly lower than in the young. Inactivated influenza vaccination was able to induce an increase in HAI antibody titers to each of the three strains, but was not able to provide an increase equivalent to seroprotective levels defined by >1:40 (HAI titer standard for seroprotection). We observed a trend of lower humoral immune response for both influenza seasons T05-06 and T06-07 in the elderly. Another interesting trend observed in both the young and the elderly groups was that subjects with lower baseline HAI antibody levels tended to have a higher increase in antibody response as opposed to the individuals who had high levels of pre- existing HAI antibodies for any strain. Our data confirms reports from prior studies that have demonstrated inadequate humoral immune response to influenza vaccination in the healthy elderly compared to healthy young individuals.

We further characterized the humoral immune response by determining serum IgG subclass and serum IgA levels pre- and post-vaccination. We investigated serum levels of IgG1, IgG2 and IgG3, which are subclasses of total serum IgG. Influenza immunization has been shown to induce serum IgG and IgA responses and is correlated with resistance to illness [56]. Total human serum IgG is divided into four subclasses: IgG1, IgG2, IgG3 and IgG4; their distribution is approximately 65%, 23%, 8% and 4% of the total serum IgG, respectively [179]. In humans, IgG1 and IgG3 are the most important IgG subclasses in complement-fixation reactions and antibody cellular cytotoxicity [180]. The greatest increase in vaccination-induced titers was observed in IgG1 levels, whose concentrations were significantly higher than IgG2, IgG3 or IgA levels. We observed an age-related decline in IgG1 levels in the elderly group. Since HAI antibody response correlates with IgG1 titers more than with any other isotype [181],

these results suggest that the difference in IgG1 antibody levels could reflect the overall blunted humoral antibody response to vaccination in healthy elderly adults. Another study reported similar results where they observed an age-associated decline in IgG1 antibody response to vaccination [182]. We also observed an age-related difference in serum IgG2 levels. Previous studies have observed that there is an age-related increase in levels of influenza specific serum IgG2 antibodies [161]. It has also been shown that only 3-4% of anti-influenza antibodies in convalescent sera bear the IgG2 isotype [183]. Hence, although serum IgG2 subclass antibodies increase with age, the role of the IgG2 subclass in immunity to influenza may be limited and may not be able to compensate for the age-related decline in serum IgG1. We observed a significant positive response to serum IgG3 levels post-vaccination in the elderly and the young. There were no dramatic differences observed in serum IgG2 and IgG3 levels as opposed to serum IgG1 levels between the two groups. Other investigators have also observed that inactivated influenza vaccines stimulate moderate IgG2 and IgG3 responses [60, 182]. In the case of serum IgA levels, we observed that the pre- and post-vaccination levels in the young and the elderly were comparable and did not show any age-related decline in vaccine-induced immune response. While the biological role of IgA antibodies is evident at the mucosal surfaces, their function in systemic immunity with regard to influenza is still not clear [179].

We investigated the difference in the type 1 T cell response to influenza vaccination in the young and the elderly by studying IFN-γ secretion from ISMT (CD4 and CD8 T cells). Memory T cells have been shown to mount a vigorous immune response to challenge previously encountered antigens. We observed a significant decline

in the percentage of IFN-γ secreting T cells in the elderly for both seasons T05-06 and T06-07. Other studies have also observed an age-related decline of IFN-γ secretion after vaccination in the elderly [152, 166, 184]. An interesting observation in our study was that the decline was across longitudinal time points between the two groups. In a previous report, an impaired T cell response was found in the frail elderly who showed a blunted response to influenza vaccination [152]. We observed a similar trend in our study among the healthy elderly. Another interesting observation was that although there was an increase in the percentage of IFN-γ secreting ISMT cells after vaccination in both groups, the young were able to maintain increased levels 21 days after vaccination as opposed to the elderly, where the levels went down to baseline after day 7 post-vaccination. Our data were different from those of a previous study [152], in which the levels of IFN-γ-secreting ISMT cells went down in the young population after 7 days. One of the reasons could have been the small sample size in that study (n = 7) as opposed to our study {T05-06 (n = 27) and T06-07 (n = 22)}. An observation similar to that in our study in the young group was made in another study, in which young subjects demonstrated a sustained increase in IFN-γ production after vaccination [106]. The study by Deng *et al.* showed that there is no change in the number of ISMT cells between the young and the elderly. Hence, aging is not associated with a decline in the number of ISMT cells, but their ability to secrete IFN-γ is compromised. Based on our data and previous findings, we can conclude that there is a functional difference between the ISMT of the elderly and the young, and that influenza vaccination is not able to induce significant and long- lasting IFN-γ secretion from the ISMT cells in the elderly. Studies aimed to decipher the mechanism of functional changes that result in the reduced IFN-γ

response of the ISMT cells in the elderly will help elucidate the reasons for immune senescence in memory T cells in the elderly. We also observed a difference in the scale of the IFN-γ response between the two vaccine seasons. This could be due to the composition of the vaccine; however, it was important to note that the trends between the young and the elderly were similar in both seasons which demonstrates that the dysfunction of the ISMT cells is not specific for certain strains of the virus. The importance of IFN-γ, a classical Th1 cytokine promoting the Th1 response and suppressing the Th2 response, has been well documented [169]. It has also been demonstrated that IFN-γ secreting influenza-specific ISMT cells are critical for a rapid response to influenza vaccination [169]. Investigators have emphasized the importance of IFN-γ in the defense against viruses and intracellular pathogens. We show in our study that there is a decline in IFN-γ secreting ISMT cells *ex vivo* when stimulated with influenza antigen. Age-related defects in the ability of the ISMT cells to secrete IFN-γ at the same levels as in the young may be responsible for lower vaccine efficacy rates and increased susceptibility to influenza infections in the elderly. The decline in the CTL response in the elderly could also be related to a decline the IFN-γ levels which promote activation and proliferation of influenza-specific CD8 memory T cells. These results could also explain the reduction in the Th1-driven antibody response in the elderly compared to the young.

We next investigated the cytokine profiles from PBMCs stimulated with influenza antigens *ex vivo* in the young and the elderly to study the effects of aging on cytokine responses to influenza vaccination. Cytokines play a critical role in regulating communication among cells of the immune system and in effector activity during an

immune response. Investigators have been interested in studying the age-related changes in cytokine profiles as a result of aging and its impact on the immune response. While most of the murine studies support an age-related shift from a Th1 response to a Th2 response, in humans the data remains inconclusive [101]. Experimental considerations are important when studying cytokine profiles. Variable results have been documented as a result of a change in stimulating agent, types of cells studied and health status of the subjects [101]. Using influenza as a stimulating agent as opposed to other mitogens yields contradictory results when measuring cytokine secretions from PBMCs [167, 185]. Using mitogens to evaluate the immune response may not reflect an immune response specific for a specific pathogen. By keeping the experimental conditions consistent, our study evaluated the both the Th1 and Th2 cytokine profiles from the same lymphocyte preparation (PBMCs) using influenza as a stimulating antigen in a group of healthy young and an elderly population. We observed that IL-6 was the most abundant cytokine secreted by the lymphocytes in both groups. There was considerable heterogeneity in both groups, and we did not see an age-related change in IL-6 levels secreted by the elderly pre- or post-influenza vaccination. Studies using influenza as a stimulating antigen have observed a similar trend when there was no significant change in the IL-6 response to vaccination [152, 167, 186]. The IL-10 levels remained unchanged or there was a moderate increase in IL-10 levels post-vaccination. We did not find a correlation of IL-10 and IL-6 with the IFN-γ T cell response; thus, there was no shift in cytokine profiles of the elderly from a Th1 response to a Th2 response. The proinflammatory cytokines TNF-α and IL-1β both were found to be higher in the young group when compared to the elderly and increased post-vaccination. However, the increase did not

reach statistically significant levels. In order to assess the role of baseline cytokine levels prior to vaccination, which might be responsible for lower T cell or antibody responses in the elderly, we measured cytokines secreted by PBMCs before and after vaccination. We did not observe any correlation between the baseline levels of IL-6, TNF-α, IL-1β or IL-10 and T cell or antibody responses, which points out the lack of age-associated changes having an impact on the T cell or antibody responses to influenza vaccination. We did not observe an age-related shift in the cytokine response from a predominantly Th1 response to a Th2 response. It is important to note that our study group comprised healthy elderly and young individuals who reflect the majority of the population to which influenza vaccination is administered. We excluded any individuals with underlying inflammatory diseases such as arthritis, which have been shown to influence the results in previous studies. We also evaluated the Th1 and Th2 cytokines concurrently from the same lymphocyte preparation to eliminate any assay-related bias. No relationship between baseline changes in Th1 and Th2 cytokine levels with respect to antibody response were observed, which demonstrated that the age-related changes in IL-6, IL-10, IL-1β or TNF-α do not affect T cell or antibody responses to influenza vaccination.

Finally, we looked at the correlation between the Th1 T cell response and the antibody response among the elderly and young adults. We found that in the elderly group, there was a positive correlation between the Th1 T cell response and the IgG1, IgG2, IgG3 and IgA antibody responses. Similar results have been reported in previous studies in healthy and frail elderly [152, 166]. We did not find a correlation between antibody and Th1 T cell responses among the young group. In this regard, there have been previous studies with conflicting data. In one study, a positive correlation was

observed between antibody and T cell responses in the young [152], however the sample size was relatively small (n = 7). On the other hand, results similar to those in our study (n = 27 for T05-06 and n = 22 for T06-07) were reported in a cohort of young individuals (n = 30) vaccinated with inactivated influenza vaccine [185]. These results show that the Th1 T cell response is an important component of the immune response in the elderly compared to the young, who show discordance between antibody and T cell responses. Results from our study indicate that in the elderly the Th1 T cell response (IFN-γ secretion) becomes critical as it drives the antibody response to vaccination. Vaccines that enhance both cellular and humoral immunity in the elderly are likely to offer optimal protection. Further studies need to be done to decipher the relationship between ISMT cells and antibody responses induced by vaccination, which will help in designing a more effective influenza vaccine for the elderly.

In summary, we studied a comprehensive set of immune correlates to influenza vaccine response between young and elderly over a two year period. The data yielded several important conclusions. Clearly, the elderly have a blunted immune response to vaccination in both cellular and humoral arms of the immune system. We show that there is a significant decline in the humoral antibody response to all three vaccine strains in the elderly when compared to the young. The dramatic decline in IgG1 in the elderly, which forms an important component of the HAI antibody response, is responsible for the overall decline in stimulation of the HAI antibody response. Changes in B cell repertoire with age may be responsible for the reduced IgG1 response in the elderly. We also found that there was significant change in the ability of ISMT cells to secrete IFN-γ in the elderly. Vaccination was able to induce a moderate increase in IFN-γ response in the

elderly. However, there was a difference in the kinetics of the IFN-γ response which was observed between the young and elderly; the young had a sustained increase in IFN-γ response post-vaccination as opposed to the elderly. Since IFN-γ is an important component of the cell-mediated and antibody responses, the overall change in ISMT function which results in a decline in IFN-γ production could be a major factor in the blunted response to vaccination in the elderly. We also found that T cell responses correlated positively with antibody responses only in the elderly, which could mean that the IFN-γ response becomes critical in the elderly.

The antibody levels which offer protection in the young may not adequately protect the elderly. The underlying mechanisms of functional changes in ISMT cells need to be investigated; these will provide insights for the development of better influenza vaccines for the elderly. Another important observation of our study was that there was no shift in cytokine profiles from a Th1 T response to a Th2 T cell response in the elderly. There was no correlation between baseline levels of cytokines that influenced the antibody responses in the elderly. The most significant observation of our study was the correlation between T cell response and antibody response in the elderly. Since the elderly showed reduced vaccine efficacy, one of the reasons for the reduced immune response could be due to a lower T cell response which influences the antibody response. Our results point out that the T cell response to influenza vaccination becomes more critical in the elderly compared to the young. Cellular immune responses targeted towards more conserved internal proteins of the influenza virus offer cross-reactive protection against different strains. Improving the induction of cellular immunity towards these proteins offers promise for developing better vaccines for the elderly, which will

help in boosting not only the T cell response but the antibody response to vaccination as well.

CHAPTER V

PROTEOMIC PROFILING OF A LONGITUDINAL SERIES OF SERUM SAMPLES COLLECTED BEFORE AND AFTER LAIV AND TIIV INFLUENZA VACCINATION FROM HEALTHY SUBJECTS TO IDENTIFY BIOMARKERS RELATED TO VACCINE RESPONSE AND IMMUNE SENESCENCE

Introduction

Influenza infections account for 40,000 deaths in the United States annually. The burden of influenza related infections is substantial, both in terms of illness, lives lost and economic impact on society [18, 20]. The impact of influenza-related infections is much higher in the elderly population. Influenza is a leading cause of catastrophic disability; greatly affecting the quality of life of elderly persons above 65 years of age [20, 21, 187]. Vaccination is the most cost-effective means to prevent severe influenza infections, especially for individuals who have a risk of morbidity and mortality. These include elderly individuals, infants, immunocompromised individuals, health care workers, and diabetic patients. Unlike other types of vaccines, the composition of the most common vaccines available in the market changes from year to year depending on the virus strain prevalent. At present there are two types of vaccines that are licensed in the U.S. market; a trivalent split inactivated influenza vaccine (TIIV) and a live virus vaccine, FluMist® (LAIV). The TIIV has been used for the past 60 years. Despite vaccination efforts, studies have shown that the vaccines are only 30-40% effective in the elderly population, in contrast to 70-90% in the young population [20, 166, 188, 189]. The inadequate vaccine response is largely attributable to immunosenescence. The underlying mechanism of immunosenescence still remains poorly understood. Continued and

focused research efforts are needed to understand the underlying mechanisms of immunosenescence; they will eventually translate into better vaccines or age group-specific vaccines [22, 27]. Various studies have demonstrated a relationship between influenza vaccine response in the elderly and immunosenescence. Most of the data, however, are focused on T cells [152, 190]. Other aspects of immunity are most probably also affected by immunosenescence. There is a lack of evidence on other immune senescence markers; they might also be affected as a result of aging, which leads to a decline in the efficacy of influenza vaccine response. The most established method to determine vaccine efficacy is the hemagglutination inhibition (HAI) antibody titer increase post-vaccination. This technique does not help in identifying immune response markers as a result of vaccination over time. The HAI assay is helpful only in determining increases in serum antibody levels after vaccination. Other techniques involving measuring T cell activity, NK cell activity and other cell surface markers have also been used to demonstrate immune responses to vaccination [166, 191-193]; however, they do not give us a complete picture of immunological changes taking place after administering the vaccine. The field of proteomics, which involves the study of expressed proteins, has made rapid strides in the past two decades. The primary aim of clinical proteomics is to identify biomarkers for the diagnosis of disease by comparing the proteomic profiles in control and disease states from body fluids like plasma, serum, saliva, cerebrospinal fluid, and urine [194]. Biomarker discovery uses ever advancing mass spectrometry techniques like matrix-assisted laser desorption and ionization time of flight (MALDI/TOF), surface-enhanced laser desorption and ionization (SELDI), two dimensional electrophoresis (2D gels), and liquid chromatography mass spectrometry

(LC-MS), which have emerged as essential investigative tools for identifying various disease states, especially in the field of early cancer diagnosis [139, 195-199]. Studies have successfully utilized mass spectrometry based proteomic techniques to identify blood-based biomarkers from human plasma and serum for cancer diagnostic, asthma, Alzheimer's disease, and rheumatoid arthritis [194]. To date, no data are available on the use of mass spectrometry-based proteomic techniques to assess the efficacy of influenza vaccines. We wanted to determine if we could identify any influenza vaccine-related biomarkers using mass spectrometry-based proteomic technology. We used mass spectrometry-based profiling techniques to observe longitudinally collected serum samples from subjects vaccinated with the TIIV and LAIV influenza vaccines to quantify the immune responses to influenza vaccination and identify vaccine response biomarkers. In this study, we used the MALDI-TOF MS in conjunction with chemical affinity beads for proteomic profiling of a longitudinal series of serum samples obtained from two different vaccinated cohorts. The first cohort comprised young and elderly healthy volunteers who were immunized with TIIV and the second cohort comprised healthy young volunteers vaccinated with LAIV. Unfortunately, to our surprise, the study did not reveal any candidate immune response markers in the LAIV group, due to high levels of variability of peptide/protein peaks observed in the vaccinated subjects. The same case applied to the TIIV group, in which we did not observe any host response biomarkers to influenza vaccination. Our comparisons between the young and the elderly groups also did not reveal any age-related immune senescence markers. To our knowledge, this was the first effort to use proteomic profiling (MALDI-TOF MS) technology to evaluate vaccine response markers. Our exploratory efforts using proteomic profiling approaches

did not succeed in identifying serum biomarkers of influenza vaccine immune response. Although the use of proteomic technology appeared to be a novel and unique tool to investigate influenza vaccine responses, the use of serum as a source to identify vaccine response biomarkers seems to be more complicated than anticipated at this time.

Materials and Methods

Vaccines

The vaccines used for this study were Fluzone® (Aventis Pasteur), a TIIV vaccine and FluMist® (MedImmune) a LAIV vaccine. The TIIV vaccines used were from seasons 2005-2006 (T05-06) and 2006-2007 (T06-07) and the LAIV vaccines were from season 2006-2007 (L06-07). The TIIV vaccine of the 2005-2006 season comprised 15 μg each of the HA from A/New Caledonia 20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Jiangsu/10/2003 (B/Shanghai/361/2002-like), and the TIIV vaccine of the 2006-2007 season comprised 15 μg each of the HA from A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. The LAIV vaccine comprised cold adapted attenuated influenza virus containing $10^{6.5\text{-}7.5}$ $TCID_{50}$ median tissue culture infectious doses of A/New Caledonia 20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004-like virus strains.

Study Population

Subjects for the study were recruited by the clinical coordinating team at the Glennan Center for Geriatrics and Gerontology, Eastern Virginia Medical School during the winter influenza season of 2005-2006 and 2006-2007 through written informed

consent. The Institutional Review Board at Eastern Virginia Medical School approved the study, protocol and the informed consent form. The study group comprised healthy independently living individuals divided into two groups. One group received Fluzone, an intramuscular inactivated split virus vaccine, and the other group received FluMist, an intranasal live attenuated virus vaccine. The subjects that received the Fluzone vaccine were further divided in to two groups and two categories. The two groups were divided into young subjects and elderly subjects, and the two categories were divided into 2005-2006 influenza season and 2006-2007 influenza season groups. Subjects who received the FluMist vaccine were enrolled in the 2006-2007 influenza season. For the Fluzone study, a total of 92 subjects were recruited, out of which 49 were young (ages 28 ± 5 S.D. years) and 43 were elderly (ages 78 ± 6 S.D. years) elderly. For the FluMist study, a total of 41 (ages 31 ± 9 S.D. years) subjects were recruited. Subjects allergic to eggs, suffering from underlying chronic diseases such as diabetes, autoimmune diseases like systemic lupus erythematosus or rheumatoid arthritis, or congestive heart failure, pregnant women and those on immunosuppressive drugs were excluded from the study. In order to reduce any differences in immunological background against influenza, only those individuals were included in the study who had been vaccinated with the influenza vaccine during the previous influenza season (2004-2005 or 2005-2006). Serum samples were collected on day 0 (before vaccination) and on day 4, day 7 and day 21 post-vaccination. All the samples were frozen at -80°C after collection until further analysis.

Hemagglutination inhibition (HAI) assay

Hemagglutination occurs when sialic acid residues, similar to the cellular receptors on erythrocytes bind to the receptor binding site present on the tip of the viral HA proteins. Hemagglutination can be inhibited by adding HA recognizing antibodies before adding erythrocytes in the assay which is termed as hemagglutination inhibition; hence the assay is termed as hemagglutination inhibition assay (HAI). When performing HAI assays sera from vaccinated or infected subjects are titrated on 96 well plates to which a known amount of influenza virus and erythrocytes are added. In our study serum antibody titers specific for each of the three strains present in the vaccine formulations were determined by standard microtiter HAI assay following procedures described previously [150]. Serum HI antibodies were determined on day 0 (pre-vaccination) and on day 21 (post-vaccination). Non-specific inhibitors in serum were removed by incubating serum with receptor destroying enzyme (RDE; Sigma). All influenza H1N1, H3N2 and B antigens (wild strains compatible with the recombinant strains in the vaccine) were grown in 10 day old fertilized chicken eggs. A slight modification was made to the protocol. Guinea pig red blood cells (GPRBCs) were used for agglutination instead of chicken or turkey RBCs. The HI titer was defined as the reciprocal of the highest dilution of serum that completely inhibited agglutination. Antibody titers of $\geq$40 were considered protective (seroprotection) and a $\geq$ four-fold rise in antibody titer (seroconversion) to one out of three vaccine antigens was considered an adequate response.

MALDI/TOF of Serum Samples

Serum samples from all subjects vaccinated with either TIIV (n = 92) or LAIV (n = 41) collected at different time points pre and post-vaccination were processed in duplicate. For each sample, 20 µl of serum was subjected to fractionation with 10 µl Magnetic Weak Cation exchange beads (MB-WCX) on the ClinProt robotic platform as per manufacturer's instruction (Bruker Daltonics, Germany). The samples were washed twice with binding buffer and unbound proteins were discarded. Proteins bound to the beads were eluted, mixed with alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix 1:1 in an acetone and ethanol mixture of 1:2. For each sample 1 µl was robotically spotted in duplicate onto a pre-structured sample support (600 µm AnchorChip™ target, Bruker Daltonics) and allowed to air dry at room temperature. The samples were analyzed using an UltraFlex 1 matrix assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics) equipped with a pulsed ion extraction ion source. The samples were assayed randomly and blinded to the operator. Ionization was achieved by irradiation with a nitrogen laser (337 nm) operating at 15 Hz. Each spectrum was detected in linear positive mode and was externally calibrated using a mixture of peptide standards. Profile spectra were acquired from an average of 400 laser shots. The serum profiles were processed using ClinPro Tools 2.0 Software (Bruker Daltonics, Germany). The Clin Pro Tools software was used to facilitate the processing and comparison of multiple spectra by automatic normalization, base line subtraction, peak definition and recalibration. The peak mass-to-charge ratio (m/z) values and intensities in the range of 1000 Da to 10,000 Da were analyzed with a signal to noise ratio of >3. A mass window of 0.5% was used to align the spectra. The processed spectra

were used under the quick classifier method to classify differentially expressed peaks between the groups that were being investigated. A k-nearest neighbor genetic algorithm as a part of the software was used to generate statistically significant differences in protein peaks in the groups analyzed. These identified ions represent candidate protein/peptide biomarkers of influenza vaccine response. The relative peak intensities of significant peaks were calculated among the different groups that were compared and a student t test was performed to determine the statistically significant peaks.

Results

Serum MALDI-TOF MS profiling of subjects vaccinated with LAIV

We first evaluated the serum profiles from subjects vaccinated with LAIV vaccine. A four-fold rise in antibody response to any one of the three strains in the vaccine which is used as a standard measure of vaccine response was not used to classify subjects as responders and non-responders since the humoral response to vaccination was low in all subjects as mentioned in Chapter III. In order to evaluate influenza vaccine immune response we categorized serum samples collected from all subjects based on days collected pre and post-vaccination in the form of a longitudinal data set. Serum samples collected at day 0, day 4, day 7 and day 21 were fractionated using WCX affinity based magnetic beads on the ClinProt robotic platform and subjected to MALDI-TOF MS serum profiling as described in the methods section. We observed 138 peaks in the 1000 to 10000 m/z range when evaluated with the ClinPro Tools software. Statistically evaluation did not yield any peaks which were differentially expressed between day 0 (pre- vaccination) and subsequent post-vaccination days (day 4, day 7 and day 21). We

performed student's t-test for the most intense peaks across the groups and did not find any peaks of interest which showed differential expression as a result of vaccination. We observed a number of the peptide peaks which have been reported in other cancer biomarker discovery studies by our group and others [195, 200] which are listed in Table 8. We compared the expression of some of these most commonly identified peptide peaks and found that there was no difference in expression levels over time (Fig. 16, Fig. 17 and Fig. 18). We also observed a high level of subject to subject variation. The majority of the outliers which were observed in our analysis had high or low levels of expression throughout the longitudinal time points that were evaluated.

There were three peaks which did show some amount of differential expression hence we looked at each of those individual peaks and arranged the data from the individual subjects to determine if we could find any correlation between differential expression of these peaks and antibody or T cell responses. We arranged the data based on the following three peaks 1979 m/z, 2861 m/z and 3059 m/z. A representation of differential expression of these peaks over different time points pre- and post-vaccination is depicted in Fig. 19. Keeping the change in the intensity of the peaks at day 4 and day 7 relative to the baseline (pre-vaccination) levels as the variables of interest we divided vaccinated subjects into two groups. The first group (G1) showed an increase in the intensity of three peaks on day 4 and day 7 post-vaccination and the second group (G2) did not show any change in intensity at day 4 and day 7 post-vaccination. Once the two groups were established, we then compared the antibody response (based on HAI response) and the T cell response (based on IFN-γ response) of the vaccinated subjects that fell in each group G1 and G2 (Table 9). When all the variables were compared we

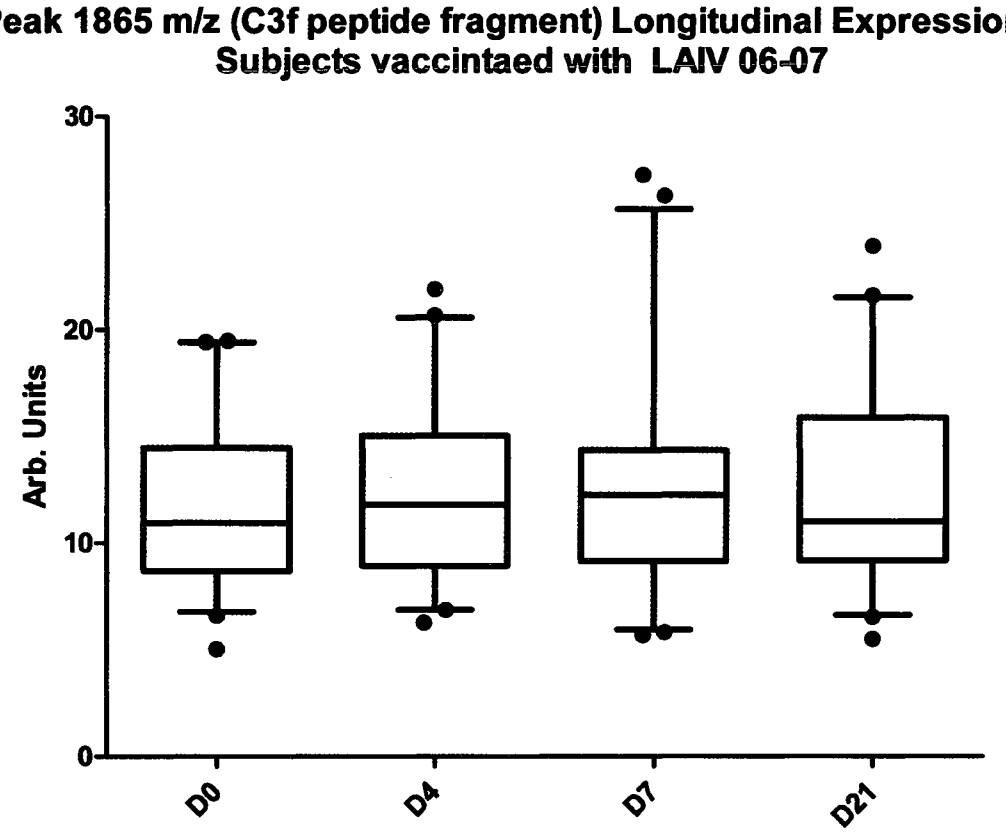
were not able to establish any correlation between change in the peak intensities with either the antibody response or the T cell response. There were no meaningful statistical relationships that could be recognized. No trend emerged which could justify the change in peak intensities were a result of vaccination as they did not correlate with any other immune response markers. Based on these data we concluded that the variations in the peak intensities were most likely attributed to subject to subject variation and were not a reflection of a change in protein profiles as a result of influenza vaccination.

We had anticipated observing differences in serum profiles which would have been correlated with other immune correlates evaluated in Chapter III. However, such correlation studies were not possible due to lack of identification of any differentially expressed peaks.

Table 8. List of peaks observed in our study which have been observed by other groups involving cancer biomarker studies [195, 200].

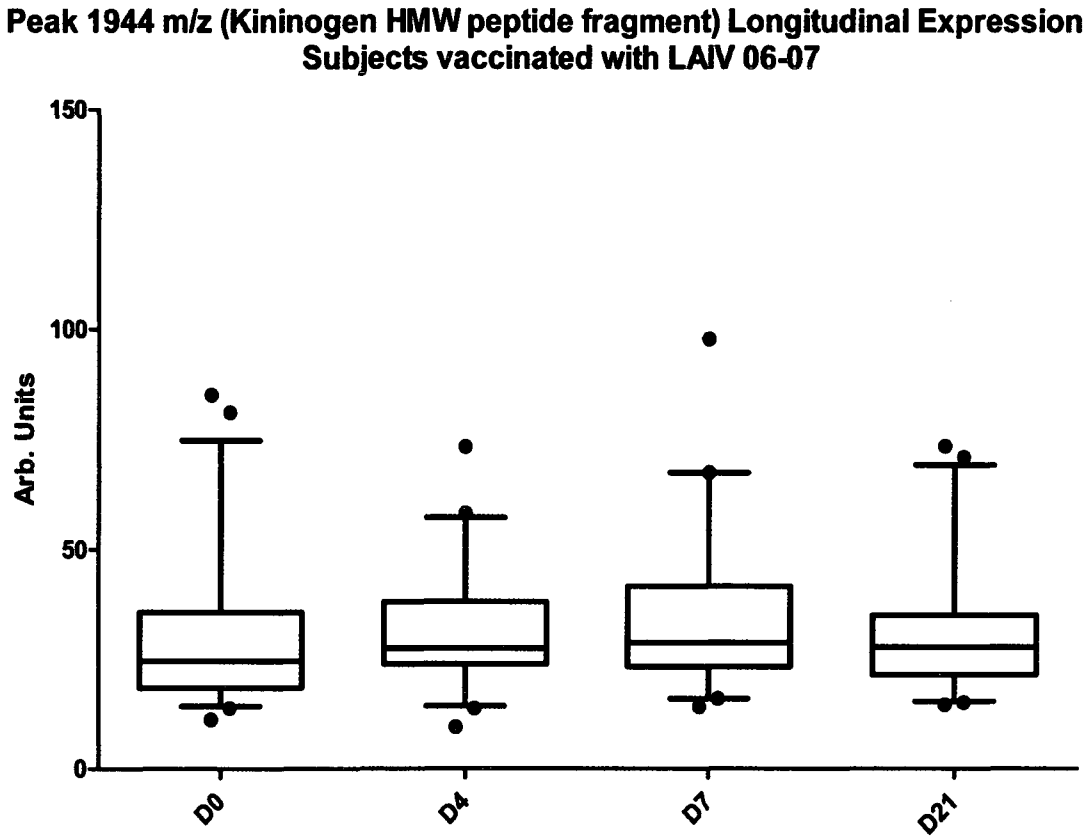
Peak (m/z)	Peptide fragment ID
3261	Fibrinogen alpha
3190	Fibrinogen alpha
2931	Fibrinogen alpha
2768	Fibrinogen alpha
2553	Fibrinogen alpha
2816	Fibrinogen alpha
3239	Fibrinogen alpha
2659	Fibrinogen alpha
2021	Fibrinogen alpha
2861	Fibrinogen alpha
3240	Fibrinogen alpha
1465	Fibrinogen alpha
2021	Complement C3f
1865	Complement C3f
1895	Complement C4a
1740	Complement C4a
2704	Complement C4a
2183	ITIH4
3970	ITIH4
2271	ITIH4
3156	ITIH4
2755	apoA-IV
1944	Kininogen HMW
2081	Kininogen HMW
2209	Kininogen HMW

Fig. 16. Box and whiskers plot of the distribution of relative intensities of peak 1865 m/z in the LAIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



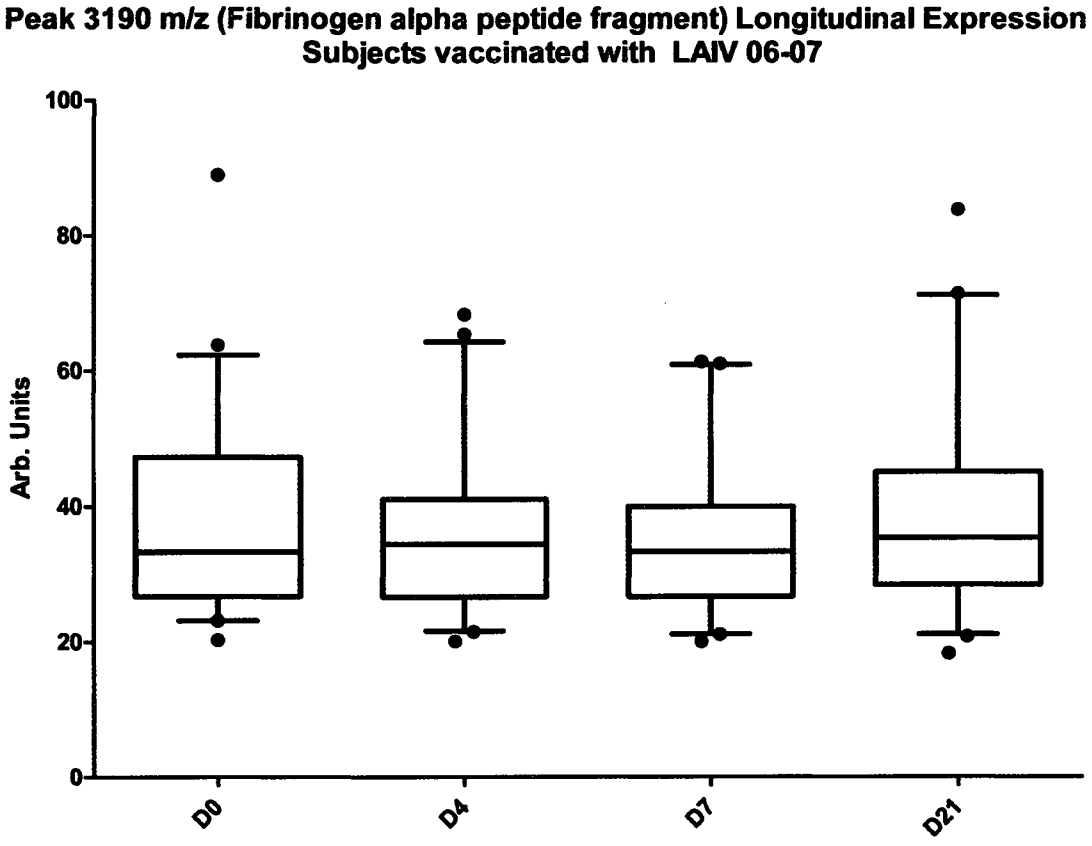
n = 41

Fig. 17. Box and whiskers plot of the distribution of relative intensities of peak 1944 m/z in the LAIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



n = 41

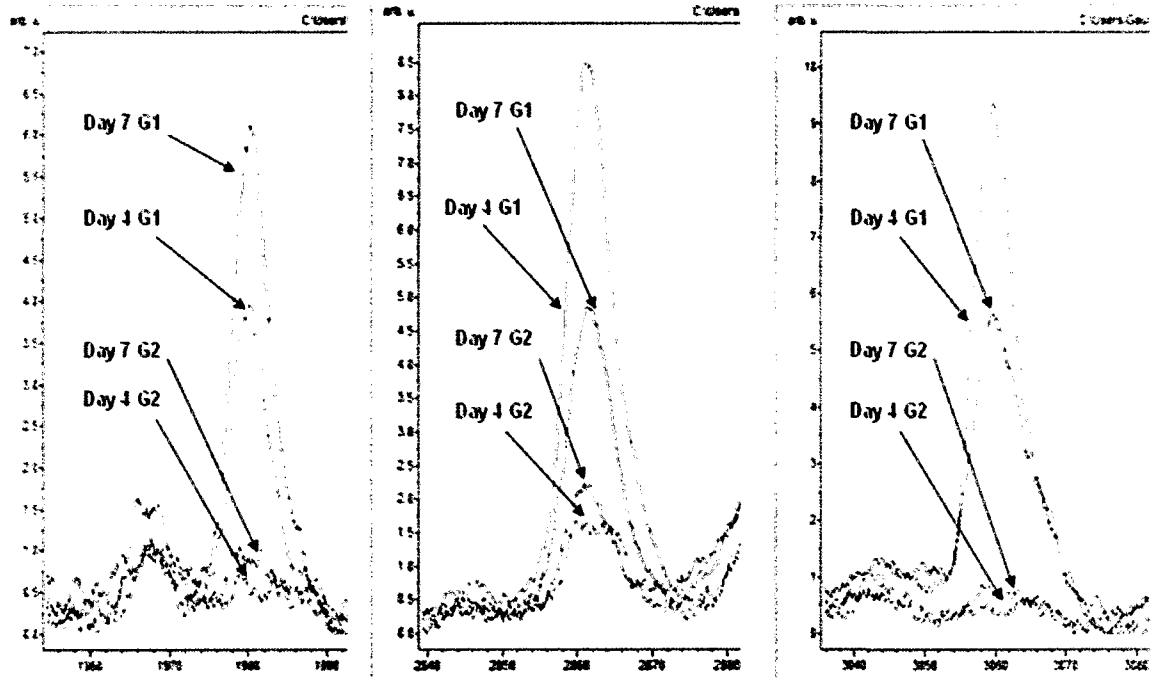
Fig. 18. Box and whiskers plot of the distribution of relative intensities of peak 3190 m/z in the LAIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



n = 41

Fig. 19. Example of three possible differential peaks 1979 m/z, 2861 m/z and 3059 m/z observed in LAIV vaccinated subjects.

Three possible differentially expressed peaks that were observed in LAIV vaccinated subjects based on differential expression at day 4 and day 7 post-vaccination. First group (G1) demonstrated increase in peak intensity on day 4 and day 7 post-vaccination and second group (G2) did not show any increase in peak intensity at day 4 and day 7 post-vaccination.



Peak 1979 m/z Peak 2861 m/z Peak 3059 m/z

Table 9. Comparison of subjects vaccinated with LAIV with an increase in peaks 1979 m/z, 2861 m/z and 3059 m/z to subjects with no significant change in respective peaks with relation to T cell and antibody response.

A

Subjects with an increase in peak intensities on Day 4, Day 7 after vaccination

Subject # G1	Fold Increase IFN-γ	HAI Ab Response
311	3.68	NR
314	2.8	WR
317	0.9	NR
319	0.66	NR
324	1.14	WR
326	1.98	R
329	3.48	WR
331	1.1	NR
333	1	WR

B

Subjects with no increase in peak intensities on Day 4, Day 7 after vaccination

Subject # G2	Fold Increase IFN-γ	HAI Ab Response
305	3.27	WR
318	1.35	NR
320	0.41	NR
328	1.16	WR
334	0.54	WR
336	1.4	NR
341	1.33	NR

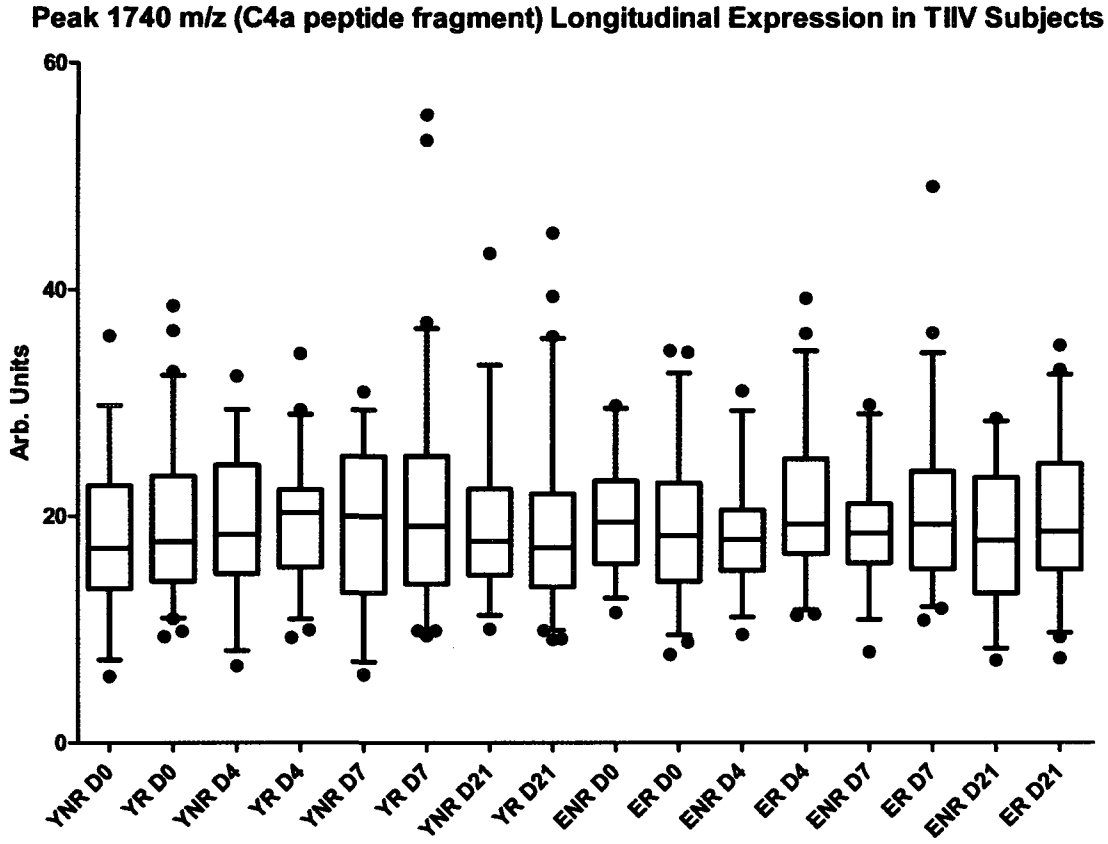
The numbers (311, 314 etc.) denote each subject vaccinated with influenza vaccine.
R = Responder (based on four-fold increase of HAI antibody titers to all three strains of influenza virus present in the vaccine)
WR = Weak responder (based on four-fold increase of HAI antibody titers to one of the three strains of influenza present in the vaccine)
NR = Non-responder (no four-fold increase in HAI antibody titers to any of the three strains of influenza virus present in the vaccine)

Serum MALDI-TOF MS profiling of subjects vaccinated with TIIV

We next evaluated the serum profiles from subjects who were vaccinated with TIIV. Since subjects vaccinated with TIIV demonstrated a strong humoral antibody response as opposed to LAIV (Chapter III), we divided this cohort further into sub-groups based on four-fold increase in HAI antibody titers to any of the three strains present in the vaccine. Subjects who demonstrated a four-fold increase in HAI antibody titers were classified as responders. The different sub-groups were as follows: young responder, young non-responder, old responder, and old non-responder. We divided the cohorts in this manner in an attempt to look for serum biomarkers of vaccine response as well as immune senescence markers. It has been described in detail in Chapter IV how the immune response to TIIV vaccination is markedly reduced in elderly compared to young. We attempted to identify candidate markers in the serum which are differentially expressed in the young and elderly as a result of vaccine response. All serum samples were subjected to fractionation using WCX affinity magnetic beads on the ClinProt robotic platform as and subjected to MALDI-TOF MS as described in the methods section. Overall we observed 122 peaks in the 1000 to 10000 m/z range when evaluated with the ClinPro Tools software. We performed statistical analysis on the most intense peaks that were observed. To our surprise we did not find any statistically significant peaks that were observed among any of the groups that were evaluated. We failed to identify any vaccine response specific peaks; we also failed in our attempt to identify aging associated differentially expressed peaks between the young and the elderly groups. The classification based on antibody response did not yield any statistically significant differentially expressed peaks. Again we were able to observe the most abundant

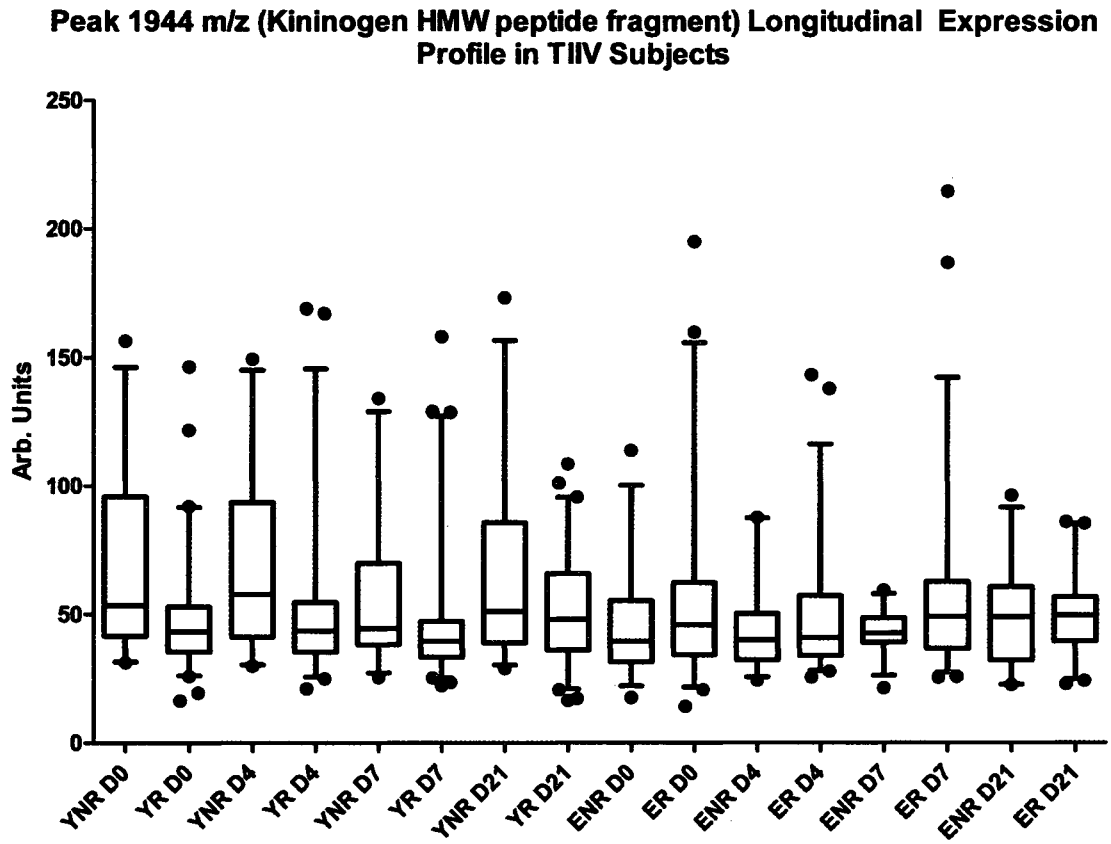
peptide/protein peaks that are identified in most cancer biomarker related studies [195, 200] which are listed in Table 8. The identified peaks were similar to the ones observed in the LAIV cohort. We compared the expression of some of these peaks across all different groups as we did for the LAIV cohort and found no statistically different peaks similar to the LAIV cohort (Fig. 20, Fig. 21 and Fig. 22). A trend was observed similar to LAIV group where the majority of the outliers in analysis had high or low levels of expression throughout the longitudinal time points that were evaluated. The failure of our endeavor in identifying vaccine response or aging related biomarkers was not due to experimental errors in the assay per se since in both the LAIV and TIIV cohorts we were able to observe the most abundant peptide/protein peaks that have been observed in other studies using this technology. These results show that the technology is not able to identify influenza vaccine response markers or aging related immune senescence markers in healthy subjects.

Fig. 20. Box and whiskers plot of the distribution of relative intensities of peak 1740 m/z in the TIIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



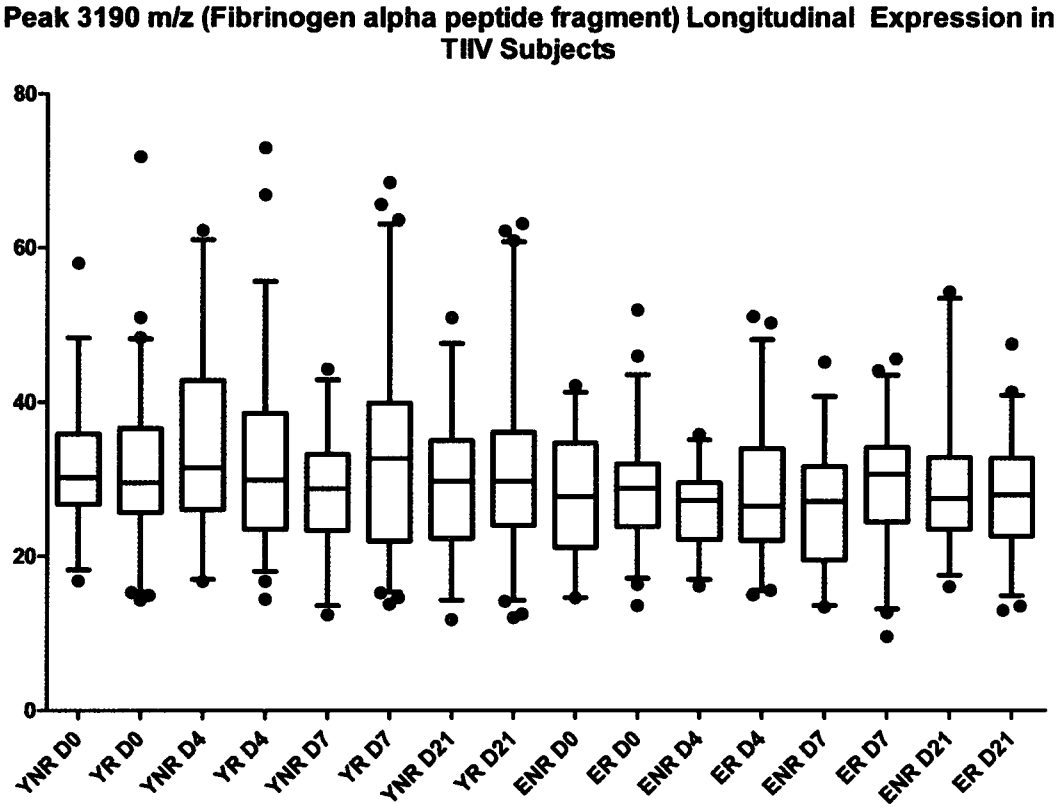
Young (n) = 49
Elderly (n) = 43

Fig. 21. Box and whiskers plot of the distribution of relative intensities of peak 1944 m/z in the TIIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



Young (n) = 49
Elderly (n) = 43

Fig. 22. Box and whiskers plot of the distribution of relative intensities of peak 3190 m/z in the TIIV cohort.
The box denotes where the intensities of majority of the samples lie and the whiskers of the box plot demonstrate the range of intensities of all samples that are not considered outliers. The outliers are shown as individual points. The median of the group is denoted by a solid line in the respective boxes. There were no statistically significant comparisons observed between groups.



Young (n) = 49
Elderly (n) = 43

Discussion

Our studies showed that MALDI-TOF serum profiling using affinity capture magnetic beads was not able to identify differential peptide/protein expression to influenza vaccination and aging associated immune senescence markers among healthy individuals. We used the technique to identify differential expression of peptides longitudinally in two separate vaccine cohorts. In both the TIIV and LAIV cohort we did not find any statistically significant differentially expressed serum peptides/proteins. There could be a number of reasons for the failure of our study to identify vaccine response markers and age related immune senescence markers.

Previous studies involving detection of cancer biomarkers have been successful in identifying signature biomarkers in the form of differentially expressed peptides/proteins in serum samples using the MALDI-TOF platform. The majority of the differentially expressed peptides are derived from the most abundant proteins that are secreted or otherwise released from tissues into the bloodstream [132]. Most cancers involve transformation and proliferation of altered cell types that produce high levels of specific proteins and enzymes like proteases [201, 202]. This results in the modification of the existing serum proteins (serum proteome) along with their metabolic products serum peptidome [132]. In addition there is complex interplay between tumor tissue and the surrounding microenvironment, resulting in an alteration of the serum protein profile [203]. A recent study showed that a large portion of the human serum peptidome that is detected by MALDI-TOF MS is produced *ex vivo* by degradation of endogenous substrates by endogenous proteases [204]. Another study also provided evidence of exoprotease activity activities superimposed on the *ex vivo* coagulation and complement

degradation pathways contributed not only to cancer specific but also cancer type specific serum peptides [195]. The MALDI-TOF MS profiling detection method is suitable for such studies since proteases and exoproteases are well established components of cancer progression and invasiveness [205-207]. In our study we were attempting to identify differentially expressed peaks corresponding to vaccine response related changes in healthy subjects. Since influenza vaccination does not involve any transformation or proliferation of cell types that produce high levels of proteases or exoproteases, it may have impacted the experimental outcome and led to our inability to identify any peptides/proteins of vaccine response in the window of 1000 to 10000 m/z that we evaluated. In our study we observed a number of peptide peaks that corresponded to peptide fragments that have been reported to be differentially expressed in previous cancer studies which rules out possibility of any assay related errors. When these peptide fragments were studied longitudinally there was no significant difference that was observed over time after vaccination. It has also been observed that the sensitivity to identify distinct peptides from subjects with later stages of cancer is higher than from the ones with benign tumors or in initial stages of tumor progression using the MALDI-TOF MS profiling method. Our results imply that there are no dramatic systemic changes in the entire proteome as a result of vaccination as opposed to cancer, which is why this technique failed to identify any signature biomarkers.

Expression based profiling studies employing the MALDI-TOF platform mostly examine endogenous low molecular mass peptides/proteins (1-20 kDa) of serum and plasma [138]. Due to the large size of most proteins captured by magnetic affinity beads (e.g. IgGs and IgAs) are not effectively resolved in the MALDI-TOF instrument. Unless

the larger proteins are broken down into smaller fragments within the detection range of the instruments they will not be detected. In our study we only investigated the peptides/proteins in the 1-10 kDa range; hence it is likely that a number of proteins that were captured by WCX fractionation were not detected due to the absence of the peptide fragments representative of the larger proteins that were beyond the detection range of the instrument. The WCX affinity capture beads are based on cation exchange principle where a weak acid (carboxymethyl functional group) is bound to the bead which enables the beads to only bind positively charged proteins present in the serum due to its negative polarity. The entire serum proteome is too complicated and it is impossible to study all the proteins present in the serum at one time. Different fractionation methods are employed to capture particular classes of peptides/proteins. It is unlikely that WCX fractionation alone could account for the enrichment all the low abundant serum proteins. Other fractionation techniques using other affinity bead types for example WAX (weak anionic exchange); HIC C8 (based on hydrophobic interactions) and IMAC Cu (based on metal ion affinity) can be used to investigate other serum fractions to identify different groups of proteins which may demonstrate differential expression. Another approach that could be employed is to digest each sample (e.g., using trypsin) after front end fractionation and then evaluating the complex protein mixtures. However, this method will require multiple MS runs to detect differentially expressed peptides which is not feasible for large sample sizes and is labor intensive.

Another explanation for our results could be sensitivity, ion suppression and mass resolution issues that are associated with the MALDI-TOF MS profiling technology. For example, prostate specific antigen (PSA) which is a universally accepted marker for

prostate cancer is secreted in the serum of prostate cancer patients. However, MALDI-TOF MS technology currently available has not been able to detect PSA from serum. It is possible that the changes in serum proteins that occur after vaccination are not detectable using MALDI-TOF MS platform due to their low concentrations in the serum. It has been reported that concentrations of cytokines are so low that these proteins are beyond the detection range of the instrument [208]. There is a general consensus that only 1% of the entire serum protein content is made up of proteins that are considered low abundance which are of primary interest from a biomarker discovery point of view [133]. However, it has been observed during the analysis of complex mixtures like serum using MALDI-TOF MS that complex components of highest molar abundance generally dominate the spectrum and tend to suppress detection of lower abundance proteins [138]. A number of reports have suggested that there are difficulties in determining the protein identities of potential biomarker peaks, and there is a concern that the sensitivity and dynamic range of prevalent proteins in serum or plasma prohibits identification of proteins which continues to hamper expression profiling approaches [209, 210]. At the moment there are no clear answers about the interrelationship between changes of peptide protein profiles obtained from serum and pathological changes in an organism.

In conclusion, our results, although negative, shed some light into the use of MALDI-TOF MS technology to identify vaccine response markers. This is the first report to use this technology for the identification of vaccine response markers. It is often the case that a quest of discovery using a new technology meets with failures. Future studies involving vaccine response should be undertaken with caution and careful consideration should be given to the kind of systemic changes that take place after vaccination which

could yield any significant biomarkers in the serum. The field of serum profiling using MALDI-TOF MS technology currently is and will remain a very active field in the search for cancer biomarkers. Its use for vaccine response markers however, remains limited to date and needs further investigation.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions Aim I: Comparison of cellular and humoral immune responses of trivalent inactivated influenza vaccine (TIIV) and live attenuated influenza vaccine (LAIV) in healthy young adults

A. Subjects vaccinated with TIIV had a significantly higher increase in serum antibody titers (HAI) compared to the LAIV group.
B. The seroconversion and seroprotection rates were also found to be significantly higher in the groups vaccinated with TIIV than LAIV.
C. Comparable serum IgA responses were observed between the TIIV and LAIV groups, however, serum IgA correlated positively with HAI antibody response and IL-10 cytokine response only in LAIV group.
D. Significant increase in serum IgG1, IgG2 and IgG3 responses after vaccination were only observed in the TIIV group which correlated positively with the serum HAI antibody response. There was no significant increase in serum IgG1, IgG2 and IgG3 response in the LAIV group.
E. We observed that subjects vaccinated with LAIV were able to elicit a more robust type 1 T cell response (increase in percentage of IFN-γ producing T cells) compared to the TIIV group.

Future Directions for Aim I

The efficacy of both TIIV and LAIV vaccines have been shown to be comparable in several studies. Studies have shown that LAIV predominantly induces local secretion of polymeric secretory IgA antibodies on the mucosal surface [79] and that polymeric secretory IgA antibodies can neutralize influenza virus efficiently independently of serum IgGs [211]. Our study along with other reports has clearly demonstrated a limited humoral antibody response after LAIV vaccination as opposed to TIIV. Keeping these considerations in mind some investigators have proposed that the levels of IgA in nasal secretions which are significantly higher after LAIV to TIIV immunization should be used to account for vaccine efficacy for LAIV [79]. The exclusive consideration of HAI as a serological response marker will result in a bias in favor of parenteral vaccine and an underestimation of the efficacy of LAIV.

The difference in the immune response to TIIV and LAIV can be explained by the different routes of immunizations employed. Systemic immunizations are generally inclined to induce a greater systemic antibody response as opposed to mucosal immunization especially in previously immunized individuals who have been primed. The antibody responses induced after LAIV immunization could be predominantly taking place locally in the nasal mucosa and the upper respiratory tract and only a limited proportion of the local antibody responses are actually reflected in systemic circulation. Previous studies have demonstrated the ability to induce local response after mucosal immunizations [158, 212, 213]. On the other hand parenteral influenza immunization did

not result in an increase in antibody responses in the nasal mucosa but showed an increase in the blood and tonsils [214].

Future studies should be designed to investigate nasal washes for mucosal IgA, and IgGs pre- and post-vaccination from a group of pre-vaccinated healthy young individuals vaccinated with the TIIV and LAIV vaccine. Such studies will provide us with better data to interpret the differences in immune responses of the two vaccines. Simultaneous assessment of serum IgA and IgGs should also be conducted and pre and post-vaccination values should be correlated to provide us with a better understanding of the relationship between mucosal and systemic antibody responses.

We speculate that LAIV which is capable of replicating in the upper respiratory tract, has the ability to provide $CD8^+$ T cells with viral antigens via dendritic cells and other antigen presenting cells. As a result LAIV vaccine is able to induce a response from both the $CD4^+$ T cells and the $CD8^+$ T cells resulting in a more pronounced type 1 T cell response to vaccination. Further studies should be targeted to examine immune response to vaccination from different T cell subsets to get a clear picture regarding the immune mechanism of the two vaccines. We believe this will help explaining a more robust Th1 T cell response in LAIV group compared to TIIV group. Another observation made in our study which requires further investigation is the correlation of IL-10 cytokine and antibody response in LAIV group. Further studies need to be done to verify this observation and studies to modulate IL-10 response to induce antibody response in LAIV vaccination should be designed.

Aim II: Comparison of age related changes in immune response to inactivated influenza vaccine response between healthy young and elderly and the role of cytokines in lower vaccine efficacy in elderly.

A. The humoral antibody (HAI) immune response to inactivated influenza vaccination is compromised in the elderly compared to the young.
B. Subjects with lower baseline HAI antibody levels had a higher increase in antibody response as opposed to those who had high levels of pre existing HAI antibodies for any strain of the influenza virus present in the vaccines.
C. There was a dramatic decline in serum IgG1 response to influenza vaccination in the elderly which forms an important component of the HAI antibody that reflects the overall decline in of the HAI antibody response.
D. The overall ability of ISMT cells to secrete IFN-γ pre- and post-vaccination was lower in the elderly compared to the young across longitudinal time points.
E. There was a difference in the kinetics of the IFN-γ response observed between young and elderly where young had a sustained increase in IFN-γ response post-vaccination as opposed to the elderly.
F. Overall change in ISMT function which results in a decline in IFN-γ production could be a major factor in the blunted response to vaccination in the elderly.
G. Influenza specific T cell responses correlated positively with antibody response only in the elderly which reflects that IFN-γ response becomes critical in elderly.
H. No correlation between baseline levels of IL-10, IL-6, TNF-α and IL-1β cytokines was observed in relation to the antibody and T cell responses to vaccination in the

elderly which demonstrated that these cytokines are not related to a reduced immune response in elderly.

Future Directions of Aim II

The results from our study demonstrated a marked decline in humoral and cell mediated Th1 T cell immune responses in the elderly compared to the young after inactivated influenza vaccination. Our study clearly demonstrated that there is decline in the IFN-γ secretion levels from ISMT cells in elderly and vaccination was not able to compensate for this decline. We also observed a correlation between Th1 T cell response and antibody response only in the elderly which suggest the importance of Th1 T cell response to antibody production in the elderly. It will be useful to investigate the mechanism of decreased IFN-γ production as a result of the functional decline of ISMT cells which would help in developing approaches to compensate for the decreased influenza vaccine efficacy in elderly. Future studies using proteomic techniques could be employed to determine difference in proteins between isolated T cells from young and elderly. These isolated pure cell populations could be digested using proteolytic enzymes and labeled with isotope labeled tags (iTRAQ) and differential expression of proteins present in these cells can be evaluated using LC-MALDI MS or LTQ. Such studies could provide us with insights into the mechanism of functional decline in T cells. It will also be useful in identifying other cell types associated with IFN-γ production in response to influenza vaccination which will allow for modulation of those specific cell types for antibody generation. Efforts should be made to develop vaccines that can stimulate the Th1 T cell response as it appears to be the most effective way to increase the efficacy of

influenza vaccines in elderly. The use of IFN-γ as an adjuvant to influenza vaccines could also be investigated. An alternative approach would be the use of adjuvants to stimulate IFN-γ response in elderly. The role of other cytokines like IL-7 which has an important role in thymic functioning should be investigated since modulation of these cytokines may offer a target for increasing the response to vaccination in the elderly.

Conclusions Aim III: Proteomic profiling of a longitudinal series of serum samples collected before and after LAIV and TIIV influenza vaccination from healthy subjects to identify biomarkers related to vaccine response and immune senescence.

A. Although we observed a number of peptide/protein peaks using MALDI-TOF MS serum profiling, we were unable to identify any vaccine response specific peaks in the LAIV group after vaccination.
B. The TIIV group also did not reveal any influenza vaccine response specific peaks or any ageing related immune senescence markers using the MALDI-TOF MS technique.

Future Directions of Aim III

Our study was a first study which utilized this technology for the identification of vaccine response markers. Our results bring out some very important points in regard to study design for the use of MALDI-TOF MS technology to identify vaccine response markers. It is clear from our study that there is high level of heterogeneity in peptide/peak response in healthy individuals to vaccination. These individuals have been exposed to various strains of influenza along with several other pathogens over the years. The varied

immunological background and susceptibility to other medical conditions of the subjects makes it challenging to search for vaccine response markers using MALDI-TOF MS. Cancer and other systemic diseases which are characterized by dynamic changes in protein expression and function are ideal candidates for the use of this technology. Future studies involving vaccine response should be undertaken with careful considerations to the kind of population in which the study is being conducted and systemic changes that take place after vaccination which could yield any significant biomarkers in the serum. Other fractionation techniques and tryptic digestion methods can be employed to mine deeper into the serum for identification of vaccine response or immune senescence markers. Future studies could be carried in out in immune naïve populations, for example in children who have relatively similar immunological background, which would reduce the complexity and heterogeneity in immune response. Studies evaluating other vaccines besides influenza vaccines could also help us better understand if the field of serum profiling can be applied for vaccine response markers. At this point though the use of MALDI-TOF MS technique for identification of vaccine response markers remains limited and needs further investigation.

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PUBLICATIONS

Basu, G., Rossouw., J., Sebunya, T.K., Gashe, B.A., deBeer, M., Dewar, J.B., Steele, A.D., "Prevalence of Rotavirus, Adenovirus and Astrovirus infection in young children with gastroenteritis in Gaborone, Botswana"; East African Medical Journal 2003; 80(12): 652-5

HONORS AND AWARDS

2007 Selected for Health Professions Scholars Program, EVMS, Norfolk, VA
2006 1st Place, Graduate Student Poster Presentation, Annual Research Exposition Old Dominion University, Norfolk, VA
2005 1st Place, Graduate Student Oral Presentation, American Society for Microbiology Virginia Chapter, Norfolk, VA
2004 1st Place, Graduate Student Poster Session (Travel Award), Research Day, EVMS, Norfolk, VA
2002 Selected to represent University of Botswana at the WHO African Rotavirus Workshop, Medical University of South Africa, Pretoria, South Africa
1999 Academic Honor Award, Department of Chemistry, University of Lucknow, Lucknow, India