Characterizing immunogenetic factors associated with Influenza cross-reactive responses and disease severity

By

Dr. Yoav Keynan M.D

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Department of Medical Microbiology

University of Manitoba

Winnipeg

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ABSTRACT

Influenza is a common and potentially life threatening infection. The constant evolution of the virus poses challenges on the cross-reactive response of the immune system, and emergence of new strains renders the antibody-mediated protection insufficient. Cell-mediated immunity (CMI) may attenuate the severity of illness and provide better hetero-subtypic coverage. A myriad of underlying comorbidities affect the outcomes of influenza infection; however, such known risk factors fail to explain a significant proportion of severe influenza infections.

To investigate cross reactive antibody and cell-mediated responses and predictors of disease severity we employed several projects and distinct cohorts- after natural infection; live- attenuated vaccine and inactivated vaccine. The main contributions of this project were the development of assays to measure antibody responses to multiple influenza strains, using a microbead based assay and application of phenotypic and functional assays to the study of influenza specific responses. Using these methods in healthy volunteers it was shown that repeated vaccination using a recurring strain failed to elicit increased antibody or cytotoxic T cell (CTL) responses. The administration of live attenuated influenza vaccine (LAIV) resulted in generation of measurable cross-reactive antibody responses. The study showed that even in a vaccine naïve adult population, LAIV resulted in limited generation of CD4 or CD8 responses. Furthermore, the microbead assay was applied to the study of prevalence rates of 2009 H1N1 pandemic during the first wave, demonstrating acceptable specificity with increased sensitivity along with the added benefits of high throughput and ability to simultaneously study responses to multiple strains of influenza. The study of severe influenza infection during

the 2009 pandemic was able to characterize the profile of several pro-inflammatory cytokines and chemokines that trended towards higher concentrations in those individuals that succumbed to pandemic H1N1 infection. This adds to the accumulating evidence suggesting that a cytokine storm together with inability to contain it are involved in determining the outcome of pandemic H1N1 infection. This may potentially aid in early identification of patients with poor prognosis and provide targets for tailored anti-inflammatory interventions. In addition the study identified, for the first time, the association between CCR5 deletion and pandemic influenza severity, illustrating the importance of this polymorphism beyond HIV and flaviviral infections.

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List of Abbreviations

Haemagglutinin- HA
Neuraminidase- NA
Influenza A viruses- IAV
A/H1N1/California/2009 virus- pdmH1N1
Intensive care unit- ICU
Acute respiratory distress syndrome- ARDS
Matrix 2 ion channel protein- M2
Nucleoprotein- NP
Acid polymerase- PA
Basic polymerase- PB
Cluster determinant 4- CD4
Cluster determinant 8- CD8
Electroporation- EP
Hemagglutination inhibition- HAI
Neuraminidase inhibitors- NAIs
Matrix protein- M1
Ribonucleoprotein-RNP
Ribonucleoprotein complex-RNPC
Non-structural protein- NS
Nuclear export protein- NEP
RNA polymerase proteins- PB2, PB1 and PA
Interferon- IFN
Viral ribonucleoprotein- vRNP
Killer immunoglobulin-like receptor –KIR
Interferon inducible transmembrane 3- IFTIM3
Toll-like receptors- TLR
B cell receptors- BCRs
Interleukin- IL
Immunglobulins- Ig (eg. A,G,M,D,E)
Major histocompatibility complex- MHC
T cell receptor- TCR
Central memory T cells- TCM
Effector memory T cells- TEM
Nuclear factor kappa-light-chain-enhancer of activated B cells NFκB
RIG-I like receptor- RLR
Nod like receptor – NLR
Natural Killer- NK cells
Natural Killer T cells-NKT
NK cytotoxic receptor 1- NCR1
Tumor necrosis factor- TNF
Trivalent Inactivated influenza vaccine- TIV
Live-attenuated influenza vaccine- LAIV
Phosphate buffered saline- PBS
Paraformaldehyde- PFA
fetal calf serum- FCS

A/New Caledonia/20/1999 (H1N1)- NC
A/Wisconsin/67/2005 (H3N2)- WISC
B/Malaysia/2506/2004- BMAL
A/Brisbane/59/2007 (H1N1)- H1 BRIS
A/Brisbane/10/2007 (H3N2)- H3 BRIS
B/Brisbane/60/2008- B BRIS
Pandemic strain- A/Mex/04/2009- MEX
A/California/04/2009- pdmH1N1
A/Solomon Island/03/06- SI
A/Beijing/262/95- BJ
A/Taiwan/1/86- TA
A/New York/55/04- NY
A/Wyoming/3/03- WYO
B/Florida/04/06- BFLO
B/Victoria/504/00-BVIC
Carboxyfluorescein diacetate succimidyl ester- CFSE
Monocyte chemotactic protein- MCP
Macrophage inflammatory protein- MIP
Peripheral blood mononucleocytes- PBMCs
Mean Fluorescent Intensity- MFI
Interferon induced protein- IP-10
Intracellular cytokine staining- ICS

Chapter 1. Introduction

1.1 Influenza epidemiology

Influenza viruses, members of the orthomyxoviridae family, are significant respiratory pathogens that cause both seasonal epidemics as well as periodic, unpredictable pandemics [1, 2]. Influenza infections are among the most common causes of respiratory infections affecting individuals of all age groups with a global distribution and [1, 3] these infections are associated with significant morbidity and mortality [3-5]. The disease associated with an influenza infection can run the gamut of severity, from a mild, self-limited disease, typically characterized by the brisk onset of coryza, sore throat, cough, high fever, headache, muscle ache, malaise and inflammation of the upper respiratory tree and trachea [1, 3-6]. At the severe end of the spectrum, progressive respiratory distress, pneumonitis and respiratory failure may ensue. It is estimated that influenza viruses infect roughly 24 million Americans annually, and cause approximately 40,000 deaths. The estimated global annual death toll is around 500,000, with the brunt of morbidity and mortality during seasonal influenza involving patients with chronic diseases, compromised immune systems and at the extremes of age, generally infants and the elderly (>59 years). Although the attack rates are highest among children, risks for serious complication, hospitalization and death from seasonal influenza are highest among the elderly. These complications may be directly caused by the influenza virus affecting the lower respiratory tract or by mucosal injury and possibly immune dysregulation leading to secondary bacterial infections [7-10].

1.1.1 Seasonal influenza

Influenza infections follow a seasonal pattern in temperate climates, with the peak of incidence occurring in winter months. The staggering burden of disease annually is estimated at 5–10% of the world's population. New strains causing epidemic influenza A, arise every 1 to 2 years as a result of selected point mutations within two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). The mutations enable the newly arising strains to evade human host pre-existing protective, neutralizing immunity provided by either natural infection or after vaccination. These small changes in the antigenicity of influenza A viruses are termed antigenic drift, and are the fundamental process that underlies the regular occurrence of influenza epidemics. In addition to the mutagenesis, the simultaneous presence of multiple viral lineages of the same virus subtype leads to ongoing process of re-assortment, whereby co-circulating strains of the same viral subtype exchange segments of the genome to create new strains, with unique antigenic composition and at times with novel virulence attributes. These changes involve significant viral segments and are termed antigenic shift. These changes are of epidemiological significance and antigenic shift may result in the emergence of new strains with the potential to affect hosts that are rendered susceptible by the change in antigenic pattern [11]. The success of the new strain in causing human clinical illness is determined by the strain's adaptation to binding human respiratory tract receptors, its ability to replicate in the host and by the virulence of the arising strain. The severity of disease in an individual during an influenza outbreak is variable. It is estimated that about a half of infections lead to no clinical symptoms or signs. Among the symptomatic individuals, clinical presentation varies from mild respiratory symptoms without systemic disease, mimicking the common cold. Others develop a febrile illness ranging in severity

from mild to severe, and the infection may not be limited to the respiratory tract, with involvement of the heart, brain, liver, kidneys, and muscles [12, 13]. The clinical course is determined by a complex interaction of the properties of the unique viral strain, the patient's age, the degree of pre-existing immunity, presence of co-morbidities, immunosuppression, and pregnancy [13]. Most fatalities occur as a consequence of primary viral pneumonia or of secondary respiratory bacterial infections, and individuals over the age of 65 or under 2 and those with co-morbidities (especially chronic cardiac and respiratory conditions) are disproportionately affected by seasonal influenza epidemics, with hospital admission rates of 25.3 to 64.0/100,00 for the elderly, ages ≥65 years in 2009-2012 seasons [14].

1.1.2 Pandemic influenza

Pandemic influenza occurs upon the emergence of a novel strain, where the preexisting antibodies directed primarily against the surface hemagglutinins fail to provide protection. The major natural hosts of influenza A viruses (IAV) are thought to be a myriad of avian species with phylogenetic evidence linking the major H1N1 pandemic strain that caused the worst pandemic of the 20th century with an avian source [15-17] . IAV's adaptation and stable replication in a variety of animals, including avian and mammalian species, has been associated with the occurrence of pandemics. The avian strains generally possess limited ability to attach to human respiratory lining cells. Attachment of influenza A virions is achieved through mature trimerised viral HA glycoproteins. A variety of different sialyloligosaccharides are restricted to tissue and species origin in the disparate hosts of influenza. Distinct terminal sialic acid species (N-

acetyl- or N-glycolylneuraminic acid), the type of glycosidic linkage to penultimate galactose (α2-3 or α2-6) and the composition of further inner fragments of sialyloligosaccharides present at the cell surface of different tissues in the various animal hosts, determine the host and tissue tropism of influenza viruses [18, 19]. In order to move from animal species to a human host, adaptation in both the viral HA and the NA glycoprotein to the human receptor types are necessary, and these evolutionary steps form a prerequisite for efficient replication [20-24]. The ability to predict likelihood of an influenza strain to traverse the species barrier and become a human pathogen is more complex and only part insight is gleaned with the use of structural studies [25]. This intricate selection process acts as a sieve, limiting the number of influenza strains that can transcend the species barrier. Since 1510, there have been approximately fourteen IAV pandemics; in the past 120 years there were 6 pandemics in 1889, 1918, 1957, 1968, 1977 and 2009 [1]. The worst pandemic on record occurred in 1918, resulting in approximately 675,000 total deaths in the United States and an estimated global mortality of up to 50 million people [3]. The 1957 and 1968 pandemics caused approximately 70,000 and 34,000 excess deaths in the United States, respectively, and this H2N2 subtype influenza (Asian flu) resulted in approximately 1 million deaths globally. The crystal structures of 1957 H2 HAs have been determined, and the structural basis for their ability to traverse the binding barrier, from avian to human have been elucidated [26]. Up to 50% of the population can be infected in a single pandemic year, and this upswing in human cases, along with limited pre-existing protection, can be associated with a dramatic increase in number of severe infections and deaths, over burdening the health-care systems that are working at close to full capacity even prior to a pandemic onset. In contrast to the

seasonal influenza epidemics where the elderly and individuals with weakened immune systems are most severely affected, during some of the pandemics (eg. 1918), young, healthy individuals were more likely to succumb to the infection. This observation may be explained at least in part by the presence of cross protective immunity against certain viral epitopes among older adults or by the role that an exuberant inflammatory response plays in pathogenesis of severe influenza (see 1.4.2). Despite the incredible diversity of zoonotic influenza viruses, the rare emergence of pandemic strains suggests that host switch and production of stable IAV in humans entails a complex and incompletely understood process that makes the ability to predict future pandemics difficult.

H1N1 influenza viruses emerged from presumed or documented multiple steps of re-assortment of segments from viruses of zoonotic origin with human adapted influenza viruses. The resulting viruses were fit for replication and managed to propagate, eventually causing pandemic spread in 1918 and in 2009 [2]. The 2009 appearance of a swine origin re-assortant virus led to the first pandemic of the 21st century. The pandemic caused by the 2009 A/H1N1 virus (pdmH1N1) was associated with increased admission rates and mortality with some distinguishing features compared to seasonal influenza. The mean incubation period of pdmH1N1 was approximately 4 days, with an average of 7 day duration of symptoms [27]. The latent period is estimated to be 2.6 days, and duration of infectiousness of 2.5-3.4 days, which may be slightly longer than typically observed with seasonal influenza, but not dramatically different [28, 29]. The severe infections were not limited to the elderly and patients with the usual risk factors. The median age of hospitalized adults was significantly younger (38–46 years) and one-quarter to one-half of patients had no evidence of co-morbid medical conditions

(diabetes; heart failure; chronic lung disease; immunosuppression that are traditionally associated with severe influenza disease and complications). In Canada, only 30–48% of severe infections occurred in individuals with the aforementioned comorbidities, with similar results from additional studies in North America and Europe [30-33]. Notably, pregnancy appeared as an important risk factor; among hospital admissions, pregnancy accounted for close to a third of female cases aged 20–39 years old [30, 34, 35]. Pandemic A/H1N1 infection was associated with a higher than expected rates of hospitalization and clinical deterioration among pregnant woman, accounting for 7–10% of hospitalized patients, 6–9% of intensive care unit (ICU) patients and 6–10% of fatalities [31, 34, 36]. The same risk factors were mirrored in infections culminating in fatality. In addition, some novel risk factors were evident including morbid obesity and certain indigenous ethnic groups. The clinical manifestations of the 2009 pandemic influenza were similar to seasonal influenza. Among individuals admitted to healthcare facilities, upper respiratory symptoms were frequently absent. Chest radiographs reflected diffuse involvement with multifocal patchy or diffuse ground-glass infiltrates or consolidations; these features at times were similar to those of pulmonary edema and acute respiratory distress syndrome (ARDS) [37-39]. The clinical course among admitted patients was often rapidly progressive, resulting in refractory hypoxemia and the requirement of intubation and mechanical ventilation. In patients admitted to ICU, ARDS and multi-organ failure developed frequently and were associated with a prolonged and complicated course resulting in high fatality rates. Similar to seasonal influenza, pandemic H1N1 influenza was associated with secondary bacterial infections, being present in approximately 5–15% of all hospitalized cases and in a larger proportion of

individuals admitted to ICUs (up to 25–30% in ICU cases) and among children [40]. Varying rates were reported in the context of the different influenza pandemics, with a staggering 70-80% reported during the 1957 Asian flu [41, 42] and much lower rates during the pdmH1N1 pandemic [43-45]. The most common infecting bacterial pathogens include *Streptococcus pneumoniae, beta-hemolytic streptococcus* and *Staphylococcus aureus* [46] and these secondary bacterial infections contribute significantly to morbidity and mortality. The underlying mechanisms for increased susceptibility to post-influenza bacterial pneumonia are multiple and only partially understood [47, 48].

During past pandemics, it was clear that certain individuals or populations appear to be more susceptible to severe disease but the ability to conduct studies in order to understand the immune mechanisms that underlay the increased propensity for complications was limited. The pdmH1N1 pandemic was accompanied by improved surveillance and laboratory testing capacity allowing for more accurate epidemiological characterization of the outbreak. In addition better estimation of disease severity and better methods to examine the immune mechanisms behind complicated disease led to improved understanding of the mechanisms behind differential susceptibility [32, 34, 49-53]. These will be discussed in greater detail in chapters focusing on the immune response to influenza.

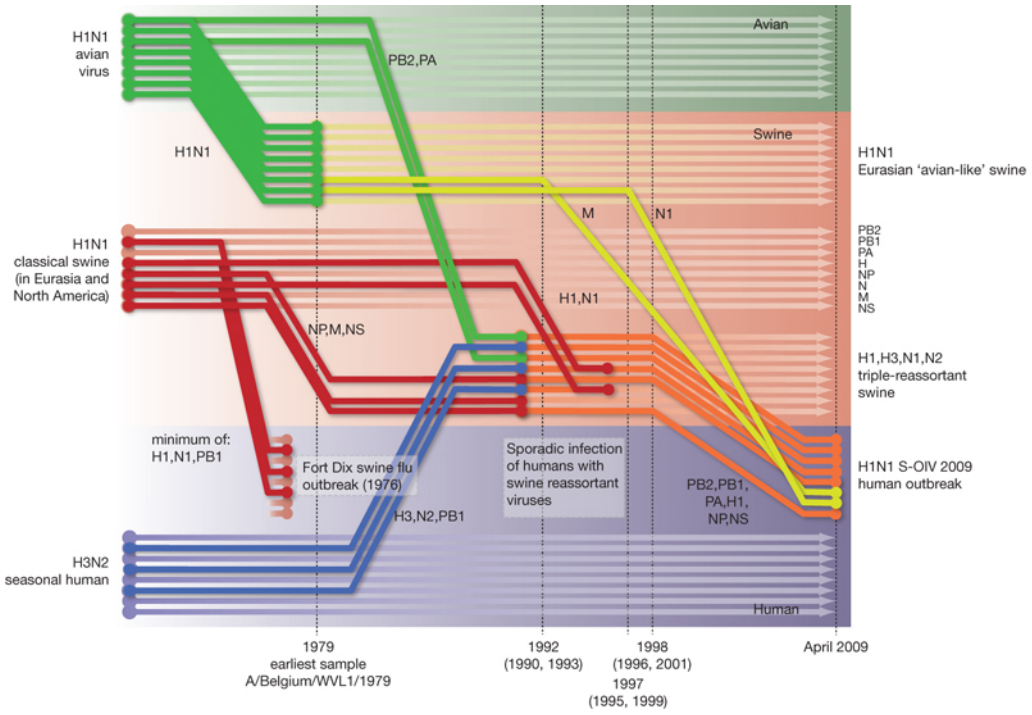


Figure 1.1. Reconstruction of the sequence of reassortment events leading to the emergence of swine-origin influenza. **From: GJD Smith et al. Nature 459, 1122-1125 (2009) doi:10.1038/nature08182[54]**.

URL: http://www.nature.com.proxy2.lib.umanitoba.ca/nature/journal/v459/n7250/full/nature08182.html (as per NPG publishing author license policy)

1.1.3 Evolution of influenza viruses in human and non-human host

Influenza viruses have a capacity for rapid evolution driven by selection pressures applied by the environment and by host defense mechanisms. The production of novel viral genotypes through re-assortment following mixed infections, coupled with the ability of IAV to stably adapt to new avian and mammalian species, has tremendous implications for the control as well as the ability to forecast influenza virus outbreaks [15, 55]. The first isolation of Mammalian influenza virus in 1931 by Shope was followed shortly by the isolation of the first human influenza in 1933 [56]. In 2005, after a long collaborative effort, the 1918 pandemic influenza viral genome sequencing was completed, and this in turn led to reconstruction of the virus using plasmid-based reverse genetics, allowing for detailed investigation of the pathogenicity in mice [57, 58]. Review of the complex processes for every influenza type and subtype is beyond the scope of this introduction, therefore it will focus on an example, namely the 2009 A/H1N1 pandemic strain (pdmH1N1 2009). The 2009 pandemic influenza A virus had been circulating undetected in swine and through incompletely understood sequence of events, entered the human population. Several events in the process of viral evolution and adaptation to host were required to make this an efficient human pathogen. Unlike most swine influenza infections of humans, this virus was able to establish sustained human-to-human transmission with rapid spread leading to a global pandemic. The pdmH1N1 2009 virus is the result of multiple re-assortment events that led to incorporation of genomic segments from several host species and disparate geographic origins: classical H1N1 swine influenza virus, human seasonal H3N2 influenza virus, North American avian influenza virus, and Eurasian avian-origin swine influenza viruses [59] . The result of the complex

re-assortment led to a H1N1 strain that possesses a number of unusual features: the RNA-dependent RNA polymerase, PB2 from pdmH1N1 2009 possesses some mutations, particularly a basic residue at position 591 and a mutation to S at position 590 that are thought to contribute to efficient human infection [60-62]. In addition, the pdmH1N1 PB1-F2 is truncated due to the presence of premature stop codons [59] potentially linked to increased virulence [63]. Unlike prior pandemic viruses, the 2009 pdmH1N1 does not encode for the virulence factor PB1-F2, and generation of viruses with restored productive PB1-F2 had minimal effect on the virulence of pdmH1N1in animal models [64]. NS1 is a multifunctional protein that is thought to play a role in disruption of the innate immune recognition of viral RNA and/or activation of inflammatory pathways [65-67]. In pdmH1N1, NS1 is truncated at the C terminus and contains a mutation (K217E) that prevents it from binding to the Crk/CrkL signaling adapters [59]. Modifying pdmH1N1 NS1 to more closely resemble NS1 of seasonal strains of influenza results in rapid viral clearance and reduced the virulence of pdmH1N1, despite the fact that the modified protein was able to more effectively antagonize innate immunity. The NS1 protein is a potent interferon antagonist, but in contrast to other human H1N1 influenza viruses, NS1 from pdmH1N1 is unable to block general host gene expression in humans. Even restoration of binding by mutagenesis resulting in increased binding of NS1 to the cellular pre-mRNA, and increased capacity to antagonize host innate immune responses in human epithelial cells had no effect on the viral replication in tissue culture [68]. This is a non-comprehensive compilation of examples taken from the most recent pandemic influenza strains, and the purpose of including it here is to underscore the myriad of factors that may affect the virus-host interactions. In summary, it is clear that a complex

process of acquisition of virulence and host adaptation mutations is necessary to allow for establishment of the novel H1N1, its ability to replicate and to be transmitted efficiently from human to human. The complex interplay between the segments acquired from the different animal host viruses and host adaptation makes it difficult to predict the virulence of emerging influenza strains. For further description of the structure and function of influenza viruses see section 1.3.1; 1.3.2.

1.2 Influenza Prevention and Treatment

1.2.1 Influenza Vaccines

Apart from social distancing, isolation of infected patients and cough etiquette, vaccines provide the best means for influenza prevention. The approved vaccines for broad usage can be divided into inactivated and live-attenuated preparations. The current practice is to offer vaccine to anyone 6 months and older and to repeat vaccination annually. This broad recommendation appeared after the onset of the 2009 influenza pandemic and was aimed to remove the need to determine the specific indication for vaccination for a given individual, and protect as many people as possible against influenza. Influenza vaccination is a proven safe preventive health measure with potential benefits across all age groups, and vaccine is to be repeated on an annual basis, even if the vaccine composition doesn't change from previous season. Although the recommendation is general, the vaccine is especially important in order to protect individuals who are at high risk of having serious complications or because they live with, or care for people at high risk for developing such severe complications. These

recommendations are summarized by the CDC influenza, health professionals resource, (http://www.cdc.gov/flu/professionals/vaccination/vax-summary.htm):

- Children aged 6 months--4 years (59 months);
- People 50 years and older;
- People with chronic pulmonary (including asthma), cardiovascular (except hypertension), renal, hepatic, neurologic, hematologic, or metabolic disorders (including diabetes mellitus);
- People who are immunosuppressed (including immunosuppression caused by medications or by human immunodeficiency virus);
- Women who are or will be pregnant during the influenza season;
- Children aged 6 months to 18 years receiving long-term aspirin therapy and who therefore might be at risk for experiencing Reye syndrome after influenza virus infection;
- Residents of nursing homes and other chronic-care facilities;
- American Indians/Alaska Natives;
- People who are morbidly obese (body-mass index is 40 or greater);
- Health-care personnel;
- Household contacts and caregivers of children aged younger than 5 years and adults aged 50 years and older, with particular emphasis on vaccinating contacts of children aged younger than 6 months; and
- Household contacts and caregivers of persons with medical conditions that put them at higher risk for severe complications from influenza.

The WHO estimates that influenza vaccines can prevent 70-90% of influenza-specific illness among healthy adults and up to 80% of deaths in the elderly, and for this reason the United States and Canada have implemented a policy of universal immunization that recommends that all individuals over 6 month of age should be immunized [69]. Despite the prospects of influenza prevention, current influenza vaccines are hampered by the requirement for the identification of specific seasonally circulating strains on a yearly basis for vaccine production, requiring an educated guess to select the antigens for the next season's vaccine, based on surveillance data from 130 sites across 101 countries in which the trends are monitored throughout the year. Each year, the seasonal influenza vaccine contains three influenza viruses — one influenza A (H3N2) virus, one seasonal influenza A (H1N1) virus, and one influenza B virus. The selection of the constituent influenza viruses to be included in the seasonal flu is derived from surveillance-based forecasts, leading to a prediction of the influenza strains that are expected to cause disease during the upcoming influenza season. Numerous observational studies have been able to document substantial benefits of influenza vaccination for the prevention of serious outcomes such as all-cause mortality or hospitalizations for pneumonia or influenza. Most of the literature is based on measuring influenza-like illnesses, a clinically relevant, but not very specific outcome, as it may be caused by numerous viruses in addition to influenza virus. The credibility of some of the evidence has been compromised by reductions that cannot be explained by the vaccination, selection bias and lack of laboratory confirmation of influenza infections as the measure of vaccine success [70-74]. A recent study used nucleic acid based testing for laboratory confirmation, among community-dwelling elderly adults aged >65 years in Ontario,

Canada, during the 2010-2011 winter. The authors demonstrated that seasonal influenza vaccination was associated with a 42% reduction in laboratory-confirmed influenza hospitalizations [74]. Despite years of research, only one randomized control trial of vaccine efficacy in the elderly has been published [75] and this study was not able to detect differences in admissions or mortality. These guidelines, and especially the focus on older individuals, are based on the observations of reductions in hospitalizations and deaths among the vaccinated elderly, derived primarily from retrospective data supporting the effectiveness of the vaccines, with studies afflicted by a long list of limitations including the "healthy user bias" (whereby healthier individuals are more likely to receive the vaccine) [69].

The anticipated effectiveness of influenza vaccines has been correlated with a rise in serum antibody titres, as long as the virus strain used in the vaccine matches the strain circulating in the community. However, high risk individuals, including the elderly and immunocompromised patients frequently fail to mount an adequate antibody response. Moreover, the reliance upon the humoral immune response, that is generated primarily against the most variable surface glycoproteins, means that the protection offered is narrow and relatively short lived [76]. The limited efficacy, inability to induce local mucosal immunity, lack of significant cellular response and the short- lived protection afforded by inactivated vaccines sparked research into ways of improving upon these shortcomings. During wild-type influenza infection the immunologic memory to the whole replicating virus results in long-lived resistance to re-infection with homologous virus. Topical nasal administration results in induction of mucosal immune response. Taken together these advantages, along with the hope for generating some degree of

cross-protection within a subtype of influenza have been the drivers behind the development of Live Attenuated Influenza Vaccines (LAIV). The vaccine is trivalent, containing the same strains recommended for seasonal vaccination. The strains are cold-adapted, able to replicate at 25^0, but temperature-sensitive, rendering them incapable to replicate at $37^{0\,[77]}$. The LAIV nasally-administered vaccine was shown to be efficacious, potentially superior to inactivated vaccines in children, resulting in serum IgG as well as mucosal IgA [78-81]. In addition, some studies claimed that induction of cell-mediated immunity may provide some additional protection against clinical disease [82]. The effectiveness was lower among adults and the vaccine is not approved for individuals over the age of 50 [79, 83, 84]. Another approach to bolster the immune response to influenza vaccines has been adjuvant usage. Adjuvants have been used to augment the immune response to vaccine antigens for several decades, however, the ability to activate innate immune responses underlies the benefits as well as toxicities associated with them [85, 86]. In addition, adjuvants permit antigen-sparing which is of particular importance in the setting of epidemics, where manufacturing capacity is overwhelmed by the needs. The squalene-containing oil-in-water emulsions currently used as adjuvants for licensed inactivated influenza vaccines include MF59 and AS03. MF-59 induces a local influx of antigen-presenting cell (APC) types into the site of injection, resulting in generation of larger pools of antigen specific and cross-reacting B cells that can be boosted years later by a mismatched MF59-adjuvanted vaccine to induce robust neutralizing antibody responses [87]. AS03 enhances the antigen-specific adaptive immune response by promotion of cytokine production and by inducing monocytes as the principal APCs [88,

89]. Alternative approaches, whereby conserved regions of influenza antigens are the targets, are being investigated with the prospect of a broader cross-reactivity.

Some of the explored conserved epitopes of the viral proteins include the extracellular domain of matrix 2 ion channel protein (M2) and NP as protective antigens, or conserved regions within the HA stem [90, 91]. The advantages of DNA vaccines include the ease of manufacturing and lower associated costs, easy formulation of multivalent vaccines, absence of pre-existing immunity to vector components, relative safety compared to vector based approaches. However, these advantages were traditionally undermined by the fact that DNA-based vaccine platforms have produced low cytotoxic T lymphocyte (CTL) levels and extremely weak or nonexistent antibody responses in humans [92, 93]. More recently improvements to this technology in the form of enhancements in plasmid antigen design, including codon optimization, RNA stabilization, cytokine based enhancement, enhanced leader sequence utilization, plasmid production at high concentrations and electroporation (EP) as a method of plasmid delivery, have significantly enhanced the ability to induce measurable CD4, CD8 and humoral responses. [94-101]. Encouraging results have been attained by combining several consensus influenza antigens and inoculating them using electroporation, leading to generation of protective cellular and humoral immune responses in mice, ferrets and non-human primates [101]. A more recent double-blind, placebo-controlled phase 1 study involved the administration of adjuvanted plasmid DNA vaccine or placebo to 103 healthy adults, 21 days apart. The study showed good tolerability and no serious adverse events, as well as adequate rises in antibody titres measured by hemagglutination inhibition (HAI). A study conducted by Inovio Pharmaceuticals Inc, is an ongoing phase

1 clinical trial evaluating safety and immunogenicity of the two consensus H1 DNA constructs. An open-label (non-blind), parallel-design study to evaluate the safety, tolerability, and immunogenicity of influenza virus hemagglutinin 1 DNA used as a prime in healthy elderly individuals prior to administration of the seasonal influenza vaccine is ongoing in Manitoba.

Further detailed review of the immune response to influenza vaccines is included in the chapter of influenza immune response.

1.2.2 Antiviral Therapy

Antivirals that possess anti-influenza activity belong to three groups: the neuraminidase inhibitors (NAIs; laninamivir, oseltamivir, peramivir and zanamivir), adamantanes (amantadine and rimantadine) and ribavirin. Adamantanes were the first available anti-influenza drugs and have been used for several decades. The mechanism of antiviral activity relies on their ability to inhibit an early step of the influenza A virus replication cycle, by interfering with the function of the viral M2 protein. M2 is an ion channel that allows hydrogen ions into the viral particle, leading to the dissociation of the matrix protein (M1) from the ribonucleoprotein (RNP) complex, allowing the entry of RNP into the nucleus thereby initiating replication [102-104]. These agents are able to prevent about 70 to 90% of illnesses when used as prophylaxis. The therapeutic benefit of adamantanes leads to an average 1-day reduction in the duration of symptoms when the drugs are administered within 48 hours of the onset of symptoms. The factors limiting the use of adamantanes are the lack of effect against influenza B and the associated central nervous system side effects, reported more frequently with amantadine compared to

rimantidine [105-107]. NAIs interfere with the activity of viral neuraminidase, a key protease required for the cleavage of HA from the cellular receptor and release of progeny viral particles from infected host cells. The two licensed agents approved for both treatment and prophylaxis are inhaled zanamivir and oral oseltamivir. Oral admistration of oseltamivir and delivery of zanamivir via respiratory route are the approved modes of administration. Alternative delivery methods and new antivirals, particularly the parenteral agents (for example, intravenous oseltamivir, intravenous peramivir and intravenous zanamivir) are being investigated specifically for hospitalized patients. Both oseltamivir and zanamivir are pregnancy category C medications, however, limited clinical experiences have suggested their tolerability without evident teratogenicity. Therefore, pregnancy should not be considered a contraindication for NAI treatment as available data suggest the benefit may outweigh the potential risks. The pharmacological properties of these agents and indications are beyond the scope of this review. The effectiveness of these agents is hampered by emergence of resistance. Resistance to NAIs arise as a result of mutations in the active site of the NA enzyme or as a consequence of alteration in the amino acid sequence of HA, affecting the area near the receptor binding site rendering the virus less dependent on NA activity [108-110]. Several mutations, namely R292K and E119V are the main changes demonstrated in oseltamivir-resistant H3N2 viruses. The H275Y mutation is the most common mutation leading to oseltamivir resistance in H1N1 strains [111, 112]. Peramavir, an additional NAI agent with antiviral effects that were thought to be useful for treatment of severe cases of pdmH1N1, received FDA approval emergency use authoirization, for intravenous administration to hospitalized patients, in cases where patients are unable to

take inhaled zanamivir or resistance to oseltamivir exists. The emergency approval expired in the summer of 2010, during the window of approval the drug was used for at least 1274 patients, however the clinical efficacy has not been determined conclusively [113]. The use of combination antiviral therapy may have a role in severe cases, when the effectiveness of either class of antivirals cannot be empirically predicted.

1.3 Influenza Virology

1.3.1 Influenza classification and structure

Influenza virus is a member of the family *Orthomyxoviridae* encompassing negative sense, single strand RNA viruses. The name *orthomyxoviridae* is based on the two Greek words: *orthos* (standard or correct) and *myxa* (mucus) pointing to the salient features of these viruses, namely the ability to bind, and infect through the mucus membrane lining the respiratory tract [114, 115]. This family of RNA viruses consists of five genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus* and *Isavirus* [116]. The first three genera each have one species or type: Influenza A virus, Influenza B virus and Influenza C virus respectively. These three types can be distinguished based on the antigenic differences in their internal proteins, NP and M protein. IAV is the most important human pathogen and this may, in part, be attributed to its broad host range (including avian and mammalian species- swine, dogs, horses, whales, and mink). In contrast influenza B virus has a much narrower host range, infecting humans and seals, while influenza C virus is uncommon, restricted primarily to humans and swine, and capable of causing a milder disease compared to types A and B . Influenza host-range restriction is polygenic and most of the viral genes play a role in influenza virus adaptation to a specific host. IAV is the predominant pathogen

responsible for the seasonal, highly contagious, acute respiratory illness in humans and other mammals. The genome consists of a single strand of (−) RNA segmented into eight fragments that code for 10 proteins. The RNA is protected by the close proximity of the associated NP forming a helical structure around it called the nucleocapsid. The NP is a type-specific antigen and occurs in one of three types, and it is the variant of NP that is the basis for the classification of human influenza viruses into A, B and C [116].

The outer layer of the influenza viruses is composed of a lipid bilayer acquired as the virus buds through the host cell's cytoplasmic membrane. Two important glycoproteins are inserted into the membrane giving the virus a spiky appearance under the electron microscope, these two major surface glycoproteins –HA and NA form the basis for sub-division of influenza A viruses into various sub-types. Seventeen variants of HA and 10 NA variants combine to give the diversity of viral subtypes [117].

Wild aquatic birds are thought to harbor influenza viruses without apparent symptoms and are considered the natural reservoir for all subtypes of influenza A virus. This natural reservoir maintains and accounts for the diversity as well as the occasional emergence of influenza A viruses that are able to cross the boundaries of host restriction to other avian and mammalian species and to establish clinical diseases. Subtypes of HA can be grouped based on phylogenetic analysis of the eight genes: Group 1 HAs (subtypes H1, 2, 5, 6, 8, 9, 11, 12, 13, and 16) and Group 2 HAs (H3, 4, 7, 10, 14, and 15) [118]. They can be further divided to four clades: H1, H2, H5, H6, H11 and H13; H8, H9 and H12; H3, H4 and H14; and H7, H10 and H15 [119].

Table 1.1 describes the distribution of influenza subtypes among animal and human hosts.

Table 1.1.includes known influenza subtypes and their respective host species.

HA Subtype	Predominant host/s	NA Subtype	Predominant host/s
H1	Human, pig, birds	N1	Human, pig, birds
H2	Human, pig, birds	N2	Human, pig, birds
H3	Birds, human, pig, horse, dog	N3	Birds
H4	Birds	N4	Birds
H5 Birds, (human)	Birds, (rarely human)	N5	Birds
H6	Birds	N6	Birds
H7	Birds, horse, (human)	N7	Horse, birds
H8	Birds	N8	Horse, birds, dog
H9	Birds, (human)	N9	Birds
H10	Birds	N10	Bats
H11	Birds		
H12	Birds		
H13	Birds		
H14	Birds		
H15	Birds		
H16	Birds		
H17	Bats		

Structure of the virion: Influenza A viruses are small, negative sense, RNA viruses. The spherical virions range from 80 to 120 nm in diameter. Fresh isolates obtained from tissues are generally filamentous, and can measure up to several micrometers in length [120]. The envelope consists of a host-derived lipid bi-layer with the two abundant surface glycoproteins, HA, and NA embedded within. M2 ion channels also project from this envelope [121, 122]. Within the lipid envelope M1 matrix proteins form the inner shell which encompasses the viral RNA genome. Multiple NPs surround each viral RNA segment in a helical symmetry and together they form the RNP complex. Three RNA dependent RNA polymerase proteins (PB2, PB1 and PA) remain attached to the end of each viral RNA segment of the RNP complex [120, 122].

1.3.2 Viral genome segments and the proteins they encode

The genome of Influenza A virus (about 13.6 kb) consists of 8 single strand negative sense RNA segments which altogether encode for at least eleven major viral proteins.

Hemagglutinin-HA

The fourth largest gene (1778 nucleotides) in the influenza genome encodes the most abundant membrane glycoprotein (MW 61.5 kDa monomer), hemagglutinin or HA [122]. This glycoprotein accounts for roughly 95% of surface antigen and plays a key role in mediating receptor binding and membrane fusion during viral entry into the cell. During the attachment process, multiple HAs bind to sialic acids on the carbohydrate side chains of cell-surface glycoproteins and glycolipids [123, 124]. In addition, the HAs function as membrane fusion glycoproteins. After the virus binding to receptor and uptake into the endosomes, acidification of the endosome results in conformational changes leading to HA activation and fusion of the virus to the endosomal membranes

[125]. Each HA polypeptide can be divided into three functional sub-domains: receptor binding, vestigial esterase and fusion domain, the first two contained within HA1 and the latter function residing within the HA2 [125-127]. Mutations throughout HA, have been documented to have important functional effects on the pH sensitivity of the conformational changes as well as membrane fusion [128-132]. In order to become activated the HA undergoes three posttranslational modifications: (a) cleavage of the amino terminal signal (b) glycosylation and palmitoylation; (c) proteolytic cleavage into two disulfide-linked subunits, HA1 and HA2 [133].

Sialyloligosacharides on cell membranes serve as the docking targets for all HA, however, the host specificity is thought to depend on the terminal sialic acid residue linkage to galactose . The α-2,3 link being favored by avian influenza viruses while α-2,6 links are associated with human viruses preferential binding [134], with similar affinities shown for all 3 common human pathogenic influenza, namely H1, H2 and H3 [135]. A relatively minor, two amino acid substitution is sufficient to change the α-2,3 affinity into α-2,6 affinity [136]. Studies of swine lower respiratory tract cells conducted by Ito et al. illustrated that pig trachea co-express both types of receptors, and this dual expression makes them an ideal “mixing vessel”, where reassortement of avian-human viruses can occur [137]. Moreover, this environment is conducive to the replication, and avian viruses are directed towards a shift in receptor specificity to NeuAcα2,6Gal linkages exclusively, thus becoming the “kindle” for generation of novel influenza viruses with pandemic potential [137-139]. HA serves a dual function, after HA binds to its receptors and the virions are endocytosed into intracellular endosomes, HA undergoes conformational changes. The changes are driven by the acidity within the endosome and

lead to fusion with the endoplasmic membrane with ensuing release of viral RNP into the cytoplasm [140]. During the 2009 H1N1 pandemic, high- resolution structural analysis coupled with structural and glycan microarray analyses allowed determination of HA receptor affinity. Study of thousands of 2009 pandemic H1 HA protein sequences demonstrated that most of them possess Asp190 and Asp225 [141, 142]. These viruses, thus, have receptor specificity for α2-6-linked glycans, confirming the correlation between human receptor-binding specificity and efficient transmission in humans. Rare variants with minor substitutions have functional implications that may translate into significant clinical impact. One such substitution- HA D225G mutants has been associated with severe disease and fatality in Norway [143]. Structural analysis of this variant suggests that it affords the increased affinity to α2-3 sialylated glycans, at the expense of loss of α2-6 avidity and hence potentially less transmission [144]. Similar changes in the 1918 H1N1 had a much greater impact on receptor binding, explained by sequence variation in adjacent positions [145]. These studies underpin the added complexity, where even minor changes can lead to dramatic changes in host receptor tropism, transmissibility and disease severity.

Neuraminidase- NA

The NA gene encodes for neuraminidase, the second most abundant surface glycoprotein, constituting the remaining 5% of surface glycoprotein of influenza virus [122]. The NA is composed of four identical subunits forming a homotetramer of around 240 kDa that binds to sialic acid. It has 4 catalytic sites residing within the head region, involved in cleavage and release of newly formed virions from host cell membrane receptors, while the N-terminus is important in the docking of the protein to the viral

envelope [146, 147]. The NA is the most common target for drug therapy, and mutations in the protein lead to resistance, albeit with associated fitness cost to the virus [148]. Next-generation sequencing of NA genes from seasonal and pandemic influenza, with screening for mutations capable of regenerating the fitness lost in the NAI resistant H1N1 influenza viruses led to the identification of compensatory mutations. The identified mutations either partially or completely restored replication capacity of the viruses [149]. Indeed, the success and global distribution of the influenza A/Brisbane/59/2007-like (H1N1) viruses carrying the H274Y NA mutation, in the absence of NAI therapy was seen during the 2007–2009 seasons, supporting the limited fitness costs and ability to overcome those with compensatory mutations [150-152]. Additional functions that have been ascribed to the NA include the prevention of viral aggregation by removal of sialic acid residues from viral membrane glycoproteins, breakdown of viscous mucins of the respiratory tract and enhancement of membrane binding of the M1 protein. This latter effect can be attenuated by mutations in the cytoplasmic tail of the NA [153].

Matrix- M

The M gene encodes two viral proteins: the matrix protein M1 (MW 27.8 kDa) and ion channel protein M2 (MW 11 kDa). M1 is the most abundant viral protein that underlies the lipid envelope and provides structural rigidity, as well as tethering of the cytoplasmic tails of HA and NA [153]. The C-terminal domain of M1 binds to RNPs and is associated with their nuclear-cytoplasmic trafficking, a process requiring phosphorylation [154, 155]. It also plays a role in amalgamating viral and host derived components to facilitate budding of the virus [156]. The M2, is a product of spliced mRNA derived from M RNA segment. It functions as a proton channel, controlling

influx from the endosome, thus providing the acidic environment that is critical for the conformational changes necessary for uncoating of the RNP complex and replication. The M protein is a membrane protein composed of 3 domains: an ectodomain, a transmembrane domain and a cytoplasmic tail [157, 158]. Recent studies during the 2009 pdmH1N1 epidemic revealed specific M1 residues that may result in release of spherical progeny, potentially contributing to the transmissibility of the pandemic virus [159]. The extra-cellular domain of the M2 is highly conserved between influenza A virus strains compared to hemagglutinin and neuraminidase, and for this reason it has been studied as a potential target for broadly protective vaccine, despite limited immunogenicity and short lived responses in wild-type infection [160, 161].

Polymerase

Influenza virus polymerase is a heterotrimeric complex consisting of polymerase A (PA), polymerase B1 (PB1) and polymerase B2 (PB2). Together with the viral NP they form the ribonucleoprotein complex (RNPC) composed of single polymerase bound to the complementary viral RNA and multiple copies of the viral NP that run along the entire length of each of the eight single-stranded viral genome segments [162]. The complex possesses multiple enzymatic and ligand binding capabilities that are responsible for the synthesis of capped, poly-adenylated mRNAs during viral transcription [163]. The only acidic subunit (MW 84.2 kDa) of the viral polymerase complex is encoded by the **PA** gene which has a length of about 2233 nucleotides [122]. It forms a part of the polymerase complex and contains a 25 kD N-terminal domain, exhibiting endonuclease activity that is thought to play a role in mRNA de-capping and a 55 kD C-terminal domain that is involved in the interaction with PB [164-167]. The PA

contains two nuclear localization domains [168], and plays a role in priming the replication activities, although not all functions are clearly understood [162]. The **PB1** gene of influenza A virus is similar length as PB2 and it encodes one of two basic subunits (MW 96.5 kDa) of the viral polymerase complex. It serves as an anchor that can bind to the other two polymerases as well as nucleoprotein (18, 19). PB1 is the catalytically active RNA dependent RNA polymerase which is involved in transcription initiation and elongation of messenger RNA (mRNA) and viral RNA [169, 170]. In addition to the polymerase activity, PB1 also performs an endonuclease cleavage of cellular mRNA to generate capped RNA primers. This endonuclease is comprised of two active sites, one required for cap binding and the other for endonuclease cleavage [171-173]. A novel protein PB1-F2 has been identified to be expressed from an alternate open reading frame of PB1 gene. This viral protein localizes to the mitochondria and is probably an important player in influenza virus pathogenesis through its ability to induce apoptosis, and pro-inflammatory cytokine production [174]. PB1F2 has variable sizes with truncations either at the C- or N-terminal ends. The 2009 pandemic H1N1 viral genome contains three stop codons preventing PB1-F2 expression. Generation of mutants expressing PB1-F2 virus led to altered levels of pro-inflammatory cytokines, with a minimal effect on virulence in animal models [64, 175]. Although greater virulence of the 1918 H1N1 pandemic strain has been attributed, at least in part, to the presence of a functional PB1-F2 [176, 177].

The PB2 gene of influenza A virus is 2341 nucleotide long and encodes for a polypeptide that constitutes one of the components of the heterotrimeric polymerase complex. The PB2 subunit plays a pivotal role in viral mRNA transcription. It binds to the host mRNA

to produce capped primers, required for the initiation of transcription, in a process called "cap snatching" [122, 178, 179]. PB2 locus within the C-terminus is thought to be a key virulence determinant, as it interacts with a mammalian host factor, capable of blocking its NP binding site. Avian viruses without favorable mutations in the domain demonstrate inhibited activity attributed to an unidentified host factor, leading to host restriction [180-182].

Nucleoprotein- NP

The NP gene (1565 nucleotides) encodes a highly basic, single-strand RNA binding protein called nucleoprotein, forming the key component of the RNPC [122]. NP forms helical filament structures in an unclear way [183]. It serves multiple functions, encapsidating viral RNA, forms homo-oligomer important for the maintenance of the RNP structural integrity, and mediates interactions with host proteins serving as adaptor molecules [184-186]. The NP is an helical protein consisting of a head domain, body domain and tail, the latter mediating oligomerization [187]. This polypeptide contains at least two nuclear localization signals that together with localization sites on the PB are essential for intracellular trafficking of RNP in and out of the nucleus [188, 189]. NP also interacts with several viral proteins including the M protein and non-structural protein.

Non-structural proteins- NS

The smallest gene, NS, encodes for two viral proteins: NS1 (MW 26.8 kDa) and nuclear export protein NEP (formerly NS2, MW 14.2 kDa). Although NS1 is not incorporated as a structural component into the virion, it is a highly conserved protein of 230–237 amino acids, abundantly synthesized during virus replication. NS1 is

polyfunctional, involved in viral replication as well as protection of the virus against host anti-viral responses [190, 191]. The NS1 interacts with a myriad of cellular proteins, with strain-specific differences for some of these interactions. Among the key pathways that NS1 affects are: inhibition of the host interferon (IFN) system mediated by multiple mechanisms, regulation of viral RNA and protein synthesis and viral mRNA splicing, and activation of the PI3K pathway [66, 191-193]. The expanding understanding of the role played by this viral protein along with its conserved structure make it a topic of intense study with the potential for therapeutic targets.

The second protein, NEP, accumulates preferentially in the nuclei of infected host cells. The original designation as NS2 was changed to NEP after elucidation of its main function as an adaptor, serving to transport viral ribonucleoproteins from the nucleus to the cytoplasm in conjunction to the host transporter CRM1 [194, 195]. In addition the protein is thought to play a role in viral replication [196, 197]. More recent research illuminated M1 and NEP cooperation that has viral polymerase inhibitory capacity, prompting attempts to mutate NEP and attenuate viruses for use in vaccine design [198].

1.3.3 Influenza Life Cycle

See Figure 1.2

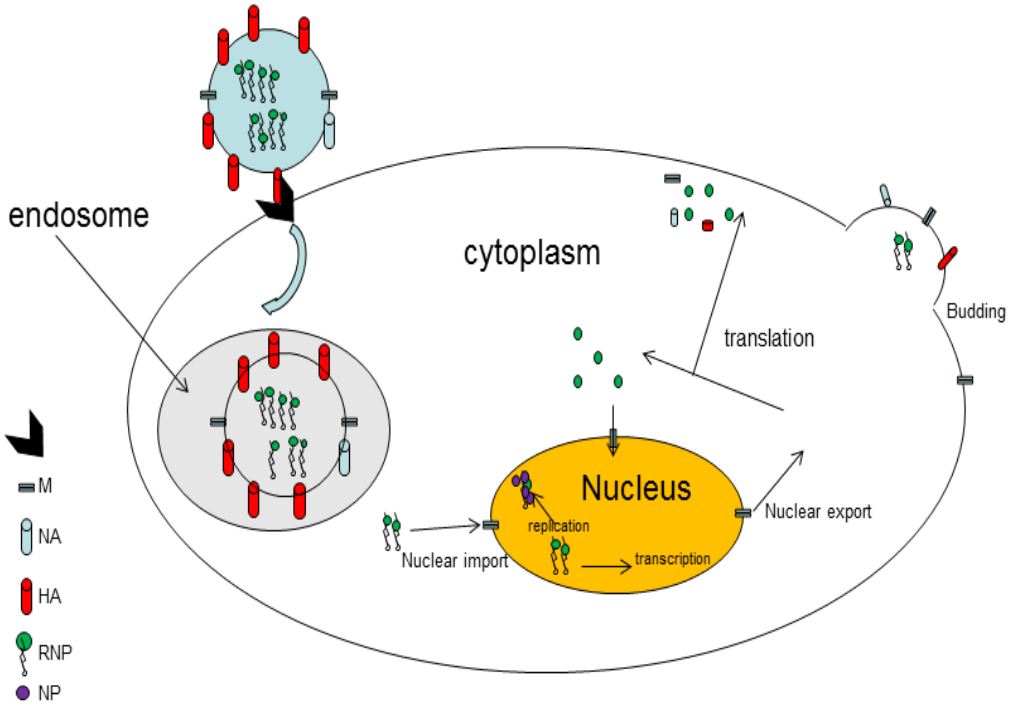


Figure 1. influenza A virus life cycle. The RNPs are represented by green hairpins, with the polymerase subunits (PA/PB are included in the greenand NP (purple). In the nucleus, the viral transcription and replication processes are depicted, followed by export, translation, with re-entry of viral protein necessary for replicative machinery. Also depicted is the viral budding.

The initial event leading to viral replication is binding of virus particles to receptors on the cell surface. Binding is mediated by interaction of the surface HA protein with sialyloligosaccharides on proteins and lipids on the host cell membranes. Following the binding the virus enters the cell through receptor-mediated endocytosis. The low pH

within the endosomes triggers a conformation change in HA, resulting in fusion of the viral and endosomal membranes. After fusion occurs, the viral ribonucleoprotein (vRNP) complexes, containing the viral genetic material and replicative machinery, are released into the cytoplasm. After their import into the nucleus, replication and transcription ensue, leading generation of vRNAs and the transcription of mRNAs for viral protein synthesis. Newly produced vRNPs are subsequently exported to the cytoplasm, aided by M1 and NEP proteins (reviewed in previous section). These vRNPs are packaged together with viral proteins at the plasma membrane, and this process is followed by budding and release of influenza virions from the cell membrane [122, 162].

1.4 Influenza Pathogenesis

1.4.1 Influenza Transmission

Influenza viruses are highly communicable, transmitted through the airborne route, through coughing and sneezing, with dispersion of large particle droplets (>5 microns) [199, 200]. The virus can also be transmitted by direct contact with mucous. The transmission is very efficient and is potentiated by close social contact in the workplace or in instances of overcrowding [201]. Influenza viruses infect additional mammalian and avian hosts, and the close association between humans and animals especially in domestic environment has been implicated in the initiation of new epidemics [202-204]. During seasonal influenza outbreaks, most commonly occurring in the winter in temperate climates, up to 10–20% of the population are affected with predilection for higher attack rates in high-risk groups (these are reviewed in section 1.2.1) and both extremes of age. Such annual epidemics are associated with high burden

of respiratory illness, and tremendous economic impact, the latter could be decreased by broader coverage with vaccines [201, 205, 206]. In addition, influenza may appear as pandemics i.e large, global scale epidemics caused by emergence of novel influenza viruses harboring HA proteins not previously seen by the population. These emerging strains, against which no preexisting immune memory exists may result in high disease burden within a short period of time. Such pandemics occurred in 1918-1919, 1957, 1968 and most recently in 2009 with the appearance of novel influenza virus in Mexico. The latest H1N1 subtype spread globally with reproductive numbers (defined as the average number of secondary cases caused by one index, denoted *R*) estimated around a median value of 1.6 with a range between 0.5 and 3.3 [29, 207-209].

1.4.2 Disease Severity

After an incubation period ranging from 1-5 days, with a mean of 2 days [210], an acute respiratory illness ensues. The disease begins abruptly with fever, sore throat, headache, chills, coryza and myalgia. The clinical spectrum runs the gamut from symptomless or barely symptomatic infections to life-threatening disease, accompanied by respiratory and systemic complication that may have fatal outcome. The clinical outcome is a complex interplay of a myriad of intrinsic properties of the virus, social determinants of health, underlying co-morbidities and health of the individual and preexisting immunity [211]. Medical conditions that play an important role in predisposing to severe disease include heart or lung disease, immunological disorders, renal failure, immunosuppressive therapy, organ transplant and smoking. In addition pregnancy increases the risk of severe disease, an effect that was notable during the last pandemic [31, 35, 49]. During the 2009 pandemic several studies documented disparities

in social conditions that were associated with higher risk of severe disease, requiring hospital admission. For example, a Spanish study reported non-Caucasian ethnicity, overcrowding, and the lack of previous preventive information, as risk factors for severe disease while secondary or higher education was found to be protective [212]. Gene segments are exchanged between influenza viruses during the process of re-assortment. The role of specific segments in determining severity has been studied in the context of highly pathogenic influenza viruses. Examples include the role of NS1 gene or gene product in evasion of host interferon response which in-turn contributes to virulence. In the context of Avian influenza A (H5N1/97) NS1 has been shown to mediate the observed elevated levels of cytokines associated with the systemic inflammatory response that is thought to be an important contributor to disease severity [191, 213, 214]. Additional sequence variations in viral segments have been implicated in determining disease severity [215-217]. In addition to traditional host related risk factors and viral sequence variations, a growing list of host genetic polymorphisms are being identified as contributors to the disease outcome. These polymorphisms in innate and adaptive immune genes help to shed light on the pathogenesis of severe influenza and the complications associated with it.

Table 1.2. Genetic polymorphisms associated with H1N1 susceptibility and severity. Modified from [218].

Gene	Polymorphism	Functional Significance	
KIR	2DL2/L3	Increased allele frequency among Canadian H1N1 ICU cases	[219]
IFTIM 3	rs12252 altered splice acceptor	Increased among hospitalized H1N1from England and Scotland	[220, 221]
FcγRIIa, IGHG2	IGHG2 *n/*-n FcγRIIa-R131H	IgG2 Subclass deficiency reported in association with severe H1N1. Polymorphisms linked to IgG2 deficiency were not confirmed in H1N1 patients	[222]

1.4.3 Post-influenza bacterial infections

Several days after resolution of the viral illness, some individuals will go on to develop a second peak of illness with recurrence of fever accompanied by a productive cough and shortness of breath. This apparent “relapse” frequently represents the development of a superimposed bacterial infection. Although estimating the exact contribution of bacterial pneumonia is difficult, it is clear that bacterial pneumonia is an important cause of morbidity and mortality [10, 223, 224]. The most frequently isolated organisms are *Streptococcus pneumonia, Staphylococcus aureus, and Haemophilus influenzae*. The increased susceptibility to bacterial infection post-influenza is brought about by a complex interplay of viral, bacterial and host factors [225]. The host predisposition is mediated through changes in epithelial defenses and changes in epithelial cell wall, caused by the cytolytic effects of the virus, leading to increased bacterial adherence and ability to invade. Immune changes involve several alterations of innate mechanisms affecting several aspects of function, including the ability of the innate cells to recognize the pathogen, impaired migration of neutrophils, or inability of macrophages and other immune cells to clear the invading organism [226-228]. Of particular interest is the role of toll-like receptors (TLR). Desensitization of airway macrophages to bacterial TLR agonists as well as impaired neutrophil recruitment in response to TLR2, TLR4, and TLR5 agonists, decreased TLR-induced cytokine production have been shown to persist for up to 6 weeks following influenza infection [226, 229]. The magnitude of the risk is difficult to assess and complicated by reporting bias, variable effects of particular strains of influenza virus and the degree of local

epithelial damage and immune dysregulation the viral infection induces as well as the virulence of the secondary infecting bacterial organism [225].

1.5 Basic Immunology

1.5.1 Innate Immune System

Innate immunity to infection is an oversimplified term used to describe immune responses that are constitutively expressed without the need for prior contact with a pathogen, in contrast to the adaptive responses that require an encounter with a pathogen. The main characteristics of system include the immediacy and non-specificity of the response. This system is made of numerous components that can be classified into:

1. Physical barriers- skin, epithelial and mucous membrane surfaces, mucous secretions
2. Cells- Epithelial cells, phagocytic cells (neutrophils, monocytes, macrophages), platelets, natural killer cells, antigen presenting cells (dendritic cells and monocytes)
3. Antimicrobial proteins- defensins, cathelicidin, lysozyme
4. Inflammatory proteins- complement, C-reactive protein, lectins,
5. Cellular receptors that recognize microbial patterns- Toll-like receptors, nucleotide-binding oligomerization domain (NOD), RIG-1-like receptors
6. Cells active through the release of inflammatory mediators- macrophages, mast cells, natural-killer cells

The early engagement of the innate immune system protects against microorganisms. In case of microbial invasion, it aids in detecting the infection and providing the first line of

defense. In addition to antimicrobial response it acts as a bridge involved in activation and shaping of the ensuing adaptive immune responses [230].

1.5.2 Adaptive Immune System

1.5.2.1 B cells and humoral immunity

B cells are produced in the bone marrow. They mature into circulating lymphocytes, present in blood and lymphoid organs. B cells express surface receptors termed the B cell receptors (BCRs). The same globular proteins are secreted into the bloodstream and form the immunoglobulin antibodies. These cells offer a tremendous diversity and specificity that is controlled and maintained by recombination and mutation processes. The great diversity of antibody specificities is present in low abundance, with exposure to cognate antigen through binding to the receptor, leading to clonal expansion and production of increasing amount of immune globulins [231]. B cell differentiation occurs in an extrafollicular pathway, resulting in early antibody production, and simultaneously through a germinal center pathway- the process that leads to germinal center formation, immunological memory, and the production of plasma cell. The B cells that become activated by antigenic exposure migrate to T cell-rich zones in the lymph nodes and spleen. The outcome of the response will depend on the successful cooperation with T cells and the presence of a ”supportive environment”- a milieu of signals that include co-stimulatory molecules and cytokines such as IL-2, IL-4, IL-6, IL-10 and growth factors. The expanded clones contract upon elimination of the antigenic challenge, only to remain as memory cells, offering greater abundance compared to the pre-exposure stage, and these memory cells are capable of mounting a larger scale and faster response when faced with second encounter with the cognate antigen [230, 232].

Five classes of immunglobulins have been identified: IgG, IgA, IgM, IgD and IgE, based on structure and function. Naïve cells produce IgM with the other immunoglobulins being produced following stimulation and subsequent affinity maturation. The latter process leads to changing of the constant region [233]. The process of antibody recombination and affinity maturation provide an important mechanism of antibody diversity, however they are beyond the scope of this text. The most common immunoglobulins are IgG in the circulatory system and IgA on mucosal surfaces. These antibodies carry a tremendous repertoire, may provide protective immunity against repeated exposure and form the basis of protection afforded by many available vaccines (HBV for example).

1.5.2.2 T cells

1.5.2.2.1 T cell memory

T cells are generated and undergo differentiation and conditioning within the Thymus. Progenitor cells become progressively committed to T cell lineages that are marked by surface expression of adhesion molecules [230, 234]. T cells recognize antigens when they are presented, as short peptides, after breakdown within antigen-presenting host cells. The short peptides are presented on the major histocompatibility complex (MHC) molecules. A T cell interacts with the MHC-antigen complex through a T cell receptor (TCR) and the diversity of the receptor accounts for the specificity of the response. T cell clones produce different TCRs through a process that is similar to the B cells recombination that is responsible for antibody diversity. Unlike B cells, no affinity maturation occurs in T cells and the TCR is determined by the T cell lineage. MHC class I molecules bind the CD8 subset of T cells, these cells become activated upon exposure to the 8-10 amino acid epitope, and function as cytotoxic T lymphocytes (CTLs)

equipped to destroy the host cell. MHC class II molecules present a 13-17 amino acid peptide to CD4 T cells [235] resulting in activation and production of cytokines and chemokines that are critical to support and stimulate an antibody or CTL response [230]. After an expansion of the cognate lymphocytes in response to microbial pathogen, once the antigenic stimuli is removed, most responding cells undergo apoptosis, a process that is important in the prevention of excessive tissue damage, an inevitable consequence of immune activation. The remaining long-lived memory cells include B, CD4 and CD8 T cells, and these are able to survive for many years following infection and to mount rapid as well as stronger response upon re-challenge. The concept of immune memory was evident since ancient times and forms the basis of vaccination. The duration of persistence of immunological memory depends on the infectious organism or vaccine and on the cell subset [236].

1.5.2.2.2 CD8 memory and subsets

The major driver of characterization of T cell subsets has been the development of multiparametric flow cytometry. The use of multiple cell surface and intracellular markers simultaneously allowed for classification of phenotypic and functional CD8 T cell subsets [237, 238]. The topic is a moving target with application of numerous markers and constant changes in the nomenclature and further differentiation into subsets and sub-subsets [239]. Moreover, the subset differentiation depends on the type of infectious agent the cells are responding to [240]. Naïve T cells are characterized by the expression of CD45RA, CCR7, the high expression of CD27 and to lesser degree CD28, and the lack of expression of cytolytic molecules. Central memory T cells (TCM), reside most frequently in lymphoid organs, lose CD45RA expression and have decreased CD27

on the cell surface. TCM cells are characterized by robust proliferative capacity and secretion of high levels of IL-2. Effector memory T cells (TEM) are more abundant in the periphery, do not express CD45RA or CCR7 and may be sub-classified based on surface expression of CD27/CD28. TEM cells produce IFN-γ secretion and possess cytotoxicity, however their proliferative capacity is decreased compared to TCM. Another subset termed terminally differentiated TEM, regain expression of CD45RA but are devoid of surface CCR7, CD27, and CD28 expression. These cells are strong effectors with no IL-2 production and very limited ability to proliferate [241, 242]. Table 1.3 characterizes the phenotype and functional attributes of each of the memory subsets, as identified in the literature.

Table 1.3. Some of the surface and functional markers that are frequently used in varying combinations to classify CD8 T cell subsets.

Subset	**Phenotype**	**Effector expression/proliferation**
Naïve	CD8+CD45RA+, CD62L+, CCR7+, CD127+ CD27+++,CD28+	None/ proliferation+++
Central Memory	CD8+CD45RA-, CD62L^{+}, CCR7^{+}, CD127^{+}, CD27++,CD28++	IL-2/ proliferation+++
Effector memory	CD8+CD45RA-CCR7- CD27/CD28+/- (SUBSETS)	IFNG, granzyme/proliferation+
Effector	CD8+CD45RA+,CCR7-,CD27/CD28+/- (SUBSETS)	Increasing IFNG, granzyme, perforin

Footnote: - indicates no expression; + expression of the marker; +++highest level of surface expression/function

1.5.3 Mucosal Immunology

1.5.3.1 Influenza in the respiratory tract mucosa

The respiratory tract is the initial point of contact with influenza virus. The mucosal surface provides a combination of non-specific and specific barriers to infection. The respiratory epithelial barrier, along with respiratory secretions, provides the first line of defense against viral invasion. The mucosal barriers are imbedded with cell (dendritic cells, macrophages) and molecules with non-specific anti-viral activities. Sensing of viral products by both airway epithelial cells and resident immune cells through activation of pattern recognition receptors leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription with ensuing outpouring of interferons. In addition NFκB leads to early production of proinflammatory cytokines, chemokines and growth factors, that initiate homing of additional immune cells to the site of invasion [243, 244]. The adaptive immune response that ensues, brings antibodies, transuding IgG as well as mucosal IgA, to the site of infection. Secretory IgA, if present as a result of prior exposure, may prevent viral invasion and lead to immune exclusion [245, 246]. Because such IgA response is of low inflammatory potential compared to an IgG, due to the inability of IgA to fix complement it has generated research interest with the hope of providing protection without inflammatory response in the context of highly pathogenic influenza viruses [247]. An additional attractive property of the IgA response is the potential for providing cross protection, with mouse studies documenting that transfer of

S-IgA from respiratory tract secretions of immunized to naïve animals resulted in good protection against homologous and to a lesser extent heterologous strain challenges [248, 249]. The potential of nasally administered influenza vaccine- with suggested induction of superior secretory IgA as well as proposed CD8 T cell responses, resulted in disappointing non-superiority compared to the traditional inactivated vaccine [250]. The reasons are not clear, but preexisting immunity limiting the viral replication and hence CD8 T cell responses may underlie this observation and is supported by the fact that the vaccines performed better in studies of pediatric populations.

1.6 Influenza Immunology

1.6.1 Innate response to influenza infection

Upon mucosal attachment, influenza viruses are recognized by receptors present on epithelial cells and immune cells. Among these receptors are TLR (TLR3 TLR7 are key responders to influenza), RIG-I like receptor (RLR), Nod like receptor (NLR) family and C-type lectin receptor family [251]. Both RIG-I and TLR7 pathways recognize 5′-triphosphates on genomic single stranded RNA and activate a cascade that culminates in induction of type I interferons (IFNs) and additional inflammatory cytokines with antiviral activity. Recent research highlighted the role of triggering of inflammasome activation by TLR and RIG signaling as a key anti-influenza effector mechanism [252]. Viral induced production of interleukin (IL)-1β and IL-18 in macrophages is the result of caspase-1 activation. Several elegant studies illustrated that the ability to achieve early viral clearance is dependent on NLRP3 and the presence of inflammasomes, and animals deficient in NLRP3 fail to clear influenza infection [253-255].

Dendritic cells that reside in airways and lung tissues also express sensors that contribute to identification of influenza viruses and induction of IFN as well as bridging with the adaptive response [256, 257]. Myeloid DCs mainly sense influenza viruses through RIG-I and TLR3 [258, 259], while plasmacytoid DCs express high levels of TLR7, which recognizes influenza virus ssRNA in the endosomes resulting in high and early induction of type I IFN [260, 261]. In the lungs, alveolar macrophages express TLR3, TLR4, TLR5, and TLR6 and are similarly capable of interferon and proinflammatory mediator induction [262, 263]. Interestingly, binding specificity to sialic acid, results in more robust proinflammatory response to influenza viruses of the "avian" α2,3 affinity, potentially contributing to the hypercytokinemia associated with highly pathogenic influenza strains [264].

1.6.2 Natural Killer (NK) cells and Natural Killer T cells (NKT) in response to influenza

The role of natural killer cells (NK) in recognition of influenza infected cells is gaining better understanding. The function of NK cells is determined by an intricate balance between stimulation of inhibitory and activating receptors. The former include (but not limited to) the killer immune globulin-like receptors KIR2DL1, KIR2DL2 and CD94/NKG2A, while the latter include KIR2DS2 and DNAM/CD96 [265].When the balance leans towards the former, cells are identified as "self" and are not targeted. When a net state of activation occurs, NK cells become cytotoxic and carry out direct killing of infected cells. During influenza infection, NK cells migrate into infected lung tissue. HA is thought to be the major influenza virus target recognized by NK cells, through their sialic acid residues containing cytotoxic receptors NKp44 and NKp46. The interaction

with HA expressed on the infected cells results in NKp44/NKp46-mediated killing [266-269]. The importance of this mechanism of protection is supported by experiments with NK cytotoxic receptor 1 (NCR1) knockout mice (the murine equivalent of NKp46), in which infection with influenza strains proved fatal [270]. A recent study demonstrated that influenza NA protein is capable of removing sialic acid residues from NKp46, suggesting this viral protein mediates protection against NK recognition of HA. Blocking of NA activity using monoclonal antibody or the anti-viral agent oseltamivir, resulted in salvage of the NK mediated killing [271].

Natural killer T cells (NKT) represent a subset of T lymphocytes that express NK cell surface markers, and recent evidence is emerging to support a role they play in bolstering innate responses to influenza, decreasing viral titres in infected tissues and decreasing mortality [272, 273]. Most attention has been drawn to the effect of the ligand αGalCer used in conjunction with influenza vaccine. This adjuvant induced high levels of systemic IgG and mucosal s-IgA Abs, high levels of IFN-γ and IL-4 both locally and systemically, as well as Ag-specific CD8 responses, that resulted in protection against influenza challenge [274, 275]. These results spark enthusiasm with the potential of improving the adaptive responses by utilizing NKT stimulating adjuvants, and perhaps generating a greater degree of cross-protective immunity [276].

1.6.3 Influenza-specific antibody response

Protective immunity against influenza is correlated with the titres of anti-HA antibodies. These are thought to achieve protection through blockage of the initial viral attachment to host cells. Preexisiting immunity, mediated by IgG or local IgA, is the only

mechanism capable of fully neutralizing viral growth, leading to aborted infection [277, 278]. Virus specific IgG can persist in serum for years, and long-lived plasma cells in the bone marrow can be generated by replicating, wild-type viral infection [279] . The serum HAI titer is considered the gold standard and a titre of 1:40 or 4 fold increase is the correlate of anti-influenza immunity induced by vaccines [4, 280]. The limitation of HA antibody responses generated by inactivated vaccines lays in the fact that the antigenic regions of HA are selectively pressured to undergo perpetual modifications, rendering it ineffective when new viral strains emerge. An additional shortcoming is the fact that the most vulnerable (elderly, immunocompromised) are less likely to mount a protective anti-HA antibody response [281, 282]. Antibodies to neuraminidase do not prevent infection, but do lessen the severity of disease by limiting viral release from infected host cells. The desire to achieve broader cross-protective immunity led to exploration of conserved antigenic targets such as the HA stalk domain, consensus sequences of HA, extracellular domain of M2 and NA [283]. An attractive and conserved target is the M2. Antibodies bind to M2 extra cellular component on the surface of influenza infected cells and allow for antibody-dependent cytotoxicity to destroy these cells. However, although this mechanism may attenuate disease it is unable to prevent initial infection [284, 285].

1.6.4 Influenza-specific T cells

Antibody titres are the traditional correlate of influenza protection and vaccine efficacy, however, despite the ability of anti-HA antibodies to provide complete protection against re-challenge the protection is limited for the reasons reviewed in the previous section. In contrast to the anti-HA antibody response, CD8+ CTLs recognize epitopes of HA, NA or internal proteins M, NP, or PB2 presented in the context of HLA

class I molecules [76]. This CTL-mediated immunity is not protective, however, the infection that ensues is attenuated by the presence of CTLs. Moreover, the presence of cross-reactive CTL may be crucial in determining the outcome of influenza infection caused by a novel, emerging strain, where the antibody response is non-existent [286]. This phenomenon has been observed during the recent 2009 pandemic [287]. CTLs are subtype-specific. However, since the targets are relatively conserved, directed at internal antigens rather than surface glycoproteins, there may be a degree of cross-reactivity among influenza A strains. Most convincing evidence for this was initially provided by studies of murine models, where a very limited number of immunodominant epitopes generate the majority of the CD8 response [288-290]. The extensive cross-protection brought about by a limited number of epitopes has been held as the "holy grail" of influenza vaccine. The mechanisms by which CTLs clear influenza infected cells are likely multiple. Cytokine responses may contribute to the elimination of influenza-infected cells, but it seems likely that the main mechanism by which CD8+ effector T cells eradicate infected cells is through a CD69/ CD95 (Fas)-mediated or perforin- and granzyme-mediated cytotoxicity [291, 292]. Intracellular cytokine staining indicates that influenza epitope–specific CD8+ T cells produce variable amounts of IFN-γ, tumor necrosis factor (TNF) and IL-2, and that the exact composition of the cytokine output differs depending on the epitope [293]. Cytokine storm is now a well-documented driver of severe disease caused by highly pathogenic strains of H5N1 and H1N1 viruses, however the role of cytokines released from responding CD8 T cells in exacerbating immunopathology and causation of the cytokine storm that occurs in the course of highly pathogenic influenza infections, remains to be determined [213, 294-296]. Measures of

the cellular immune response to influenza arguably provide a better correlate of protection against influenza in the presence of limited antibody response among the elderly [281, 297]. The importance of preexisting CD8 responses has been suggested based on retrospective epidemiological study of the Cleveland Family cohort. In this cohort, reported by Epstein, 5.6% of the adults who had prior symptomatic influenza A infection developed influenza during the pandemic, in contrast to 55.2% of the children [298]. Although the mechanism was not studied, a role for repeated heterotypic exposure has been suggested. Similarly, mining of epitope databases conducted during the 2009 H1N1 pandemic identified a limited overlap in B-cell epitopes between previously circulating H1N1 strains and the pdmH1N1 strain. In contrast, more than 2/3 of the CD8 epitopes were identical. The same authors also showed that the existing CD8+ T cell memory in the adult was similar in magnitude to that found against seasonal H1N1 influenza [299]. A study using overlapping peptides, examined the cross-reactivity of CD4 and CD8 memory induced by H5N1 and H3N2 viruses. Most participants exhibited cross-reactive response, mainly directed against viral NP and M1conserved epitopes. This study of individuals residing in the UK who were not exposed to H5N1 virus, provides encouraging evidence of the potential for heterotypic protection [300] . After initial viral exposure, CD8 memory T cells require minimal co-stimulatory signals in comparison to naïve T cells and respond with a quicker more robust proliferation and cytokine output following antigenic re-stimulation. Memory CD8 T cells are also present locally, providing more immediate reactivity [301-303]. Coupling the cross-subtypic and, to lesser extent, heterotypic reaction, the conserved epitopes targeted and the accelerated

response upon re-exposure it is easy to envision the potential benefits of a vaccine strategy that will be able to harness these elements.

1.7 Vaccine induced protection

1.7.1 Inactivated influenza vaccine

The goal of inactivated influenza vaccines is to induce Abs against the globular head domain of HA, thereby blocking viral attachment and neutralizing infectivity. Vaccines are trivalent and contain two influenza A (H3N2, H1N1) and one influenza B strain (TIV). They are produced in chicken eggs causing delay in availability when reformulation is required in the context of emergence of a new strain. They contain residual amounts of egg protein resulting in risk for inducing hypersensitivity reactions in susceptible individuals. The vaccines contain a standardized amount of 15 μg of hemagglutinin (HA) protein for each included strain, however, the content of NA protein included is not standardized. The selection of strains for each season is typically made in February and vaccine is administered in the fall [122, 304]. In addition to the limitations already mentioned in the preceding chapters, vaccine coverage among vulnerable population is insufficient, with an estimated average cost of influenza-associated hospitalizations in elderly patients of $372 million per year, based on US Medicare data collected over several influenza season, suggesting that finding strategies that obviate the need for annual vaccine administration is a desirable goal [305-307].

1.7.1.1 Antibody Correlates of Protection

Inactivated influenza vaccine is the most common vaccine used, with an efficacy of 70–90% for preventing infection with influenza virus, and with the correlate of protection being a rise in serum antibody titre to >1:40 or 4 fold increase. This level is achievable after vaccination in young healthy adults, however, levels are typically lower among elderly individuals with underlying medical conditions as well as in the context of vaccine mismatch-when the circulating viruses do not match the strains included in the vaccine [72, 308, 309]. The study by Skowronski, based on the 2005-2006 influenza season in Canada, found that three-quarters of influenza A and all B isolates were mismatched with the vaccine strains [309]. Among individuals above the age of 65, hospitalization and pneumonia rates during influenza seasons are increased, and TIV decreased these rates. One prospective cohort study documented similar immunogenecity among hospitalized persons ≥65 years or with chronic medical conditions compared with outpatients [69, 310]. Immunogenicity of higher dose preparation administered to individuals ≥65 years of age was improved compared to standard dosing in three studies [311-313]. The ability to protect individuals over the age of 60 in community was addressed by a single randomized controlled trial showing a vaccine efficacy of 58%, with similar rates of protection among participants >70 years old, although the size of the latter group was limited [75, 314]. Influenza vaccine effectiveness in preventing respiratory illness among elderly confined to nursing homes had conflicting results with wide range of estimates from as low as 20%-40% up to 80% for prevention of influenza-related deaths [315-320]. A second group of individuals that are at increased risk for severe consequences of influenza, that is relevant for this thesis, are HIV infected

patients. Among individuals with advanced HIV disease and immunosuppression, with low CD4 T cell counts of less than 200 per µl, administration of TIV does not consistently lead to protective antibody titres [321, 322]. Boosting with a second dose of vaccine does not result in increased protection [322, 323]. A Canadian study tested different modes of inactivated vaccine administration, including a double-dose given twice with a 4 week interval. In the end this approach was not deemed cost-effective [324]. The efficacy of vaccine among individuals receiving highly active antiretroviral therapy and in whom CD4 counts are >200 per µl, has been equivalent to uninfected individuals [325]. During the 2009 pandemic, administration of the TIV with an adjuvant increased antibody titres compared to historic controls [326].

1.7.1.2 Cell-Mediated Correlates of Cross-Protection

The role of antibodies and the drawbacks of the protection they offer have been reviewed in previous chapters. Cell-mediated response to influenza is important in clearance of the virus and tissue recovery. Transgenic mice lacking CD8 responses succumb to H3N2 infection at higher rates than intact counterparts, and survival upon re-challenge is possible even in the absence of antibodies [327, 328]. The exposure to influenza virus leads to clonal expansion and this activation of effector CTLs is followed by a contraction of the responding population with remaining generation of long-lived, specific, memory T cells. The CD8 T cell specificity has been shown to be directed to a large extent at the relatively conserved internal proteins. These long-lasting memory CD8 T cells have the potential to respond rapidly by proliferation and release of cytokines when the cognate antigen is encountered. The presence of such memory has been extensively documented in mice and humans [329-335]. The epitopes against which the

CD8 responses are mounted are predominantly within NP, PA, M, HA, NS. Results from mouse model demonstrate immunodominance for NP366-374 and PA224-233 accounting for 2/3 of responding CD8 T cells. The magnitude of these responding clones is similar during primary influenza infections and they contract approximately 10 fold and remain as stable memory T cells. After secondary exposure the NP366-374 increases 5-10 fold more than the PA224-233 specific CD8 T cells, and this can be manipulated by mutations and changes in antigen dose [336, 337]. The picture of immunodominance is obviously more complicated among humans, with great HLA diversity, the fact that virtually any exposure to influenza after infancy is to some degree “secondary”. In the context of the 2009 pdmH1N1, screening epitopes revealed over 2/3 CD8 epitope conservation relative to previously circulating strains of H1N1 [299]. A similar study using slightly different screening methodology, arrived at similar conclusions: greater than 50% agreement in CD4 and CTL epitopes of HA, with lower concordance rates for NA [338]. Harnessing of the potential advantages of CD8-specific responses may provide a mechanism of attenuating influenza illness.

1.7.2 Live Attenuated influenza vaccine

Hemagglutination inhibition following inactivated influenza vaccine, attain such associated protection in less than 60% of adults [250]. This, coupled with underutilized mucosal antibody responses, ineffective stimulation of CTL response and the need for injection, clearly demonstrate that alternative vaccination approaches are important. Live-Attenuated, cold adapted, influenza vaccine (LAIV) for intra-nasal administration has been FDA approved. The vaccine is produced by reassortment between a stable, attenuated, cold-adapted donor virus and the projected epidemic wild-type strain,

resulting in a live virus that maintains the backbone of genetic segments that render it attenuated, temperature sensitive and thus incapable of replication in the lower respiratory tract. The wild-type strains donate the HA and NA and allow for induction of antibody responses against them [339-341]. The inoculation with mucosal live attenuated virus is theoretically expected to yield improved mucosal antibody, systemic CTL response and potentially longer lasting protection. In contrast to the promise, comparative analysis of the efficacy of LAIV and TIV failed to consistently show these benefits. Although some reports documented positive attributes, meta-analysis of studies removed significant overall differences in the immunogenicity or efficacy of the two vaccine types. It also failed to induce better protection against modified, drifted strains [342]. In contrast, a few studies and meta-analyses of studies focused on pediatric population were able to show higher efficacy as well as longer lasting protection conferred by LAIV, especially upon administration of two consecutive years of vaccination [343-346]. The apparent differences between adult and pediatric studies receive some support and plausible mechanistic explanation from studies in US military. In two studies LAIV was more efficacious than trivalent inactivated TIV among recruits without prior influenza immunization history, compared to a greater efficacy associated with TIV in those who had been subjected to previous annual immunization [347-349].

1.7.2.1 T cell responses

Numerous animal studies provide clear support to the notion that T-cell immunity may provide heterosubtypic protection. It is therefore expected that a replication competent attenuated virus will induce some degree of CD8 response aimed at the conserved influenza epitopes and will be associated with better protection against

"drifted" influenza strains. Much less evidence has been generated in humans. A pediatric study compared prime/boost approaches involving different combinations of TIV and LAIV. They were able to show that only regimens containing LAIV induced influenza-specific CD4 and CD8 T cells. Cytokine production and proliferation in response to conserved NP and M1/M2 influenza peptides was seen exclusively in those who received at least one dose of LAIV [350]. Differential efficacy, with LAIV providing superior protection in children [351, 352], and evidence suggesting that TIV may be more protective among adults [308], may in part be explained by differences in induction of T cell responses. This discrepancy in responses may be the result of prior antibody effect, decreasing the amount of antigen available for CD8 induction [331, 353]. This concept of preexisiting immunity preventing the generation of CTL, is supported by the finding of lack of boosting of influenza A virus-reactive IFNg producing T cells and NK cells among adults given either LAIV or TIV [354]. Moreover, the same group found that in young children between 6 months to 4 years of age, who were vaccine naive and received 2 doses of TIV, a significant increase in the percentage of influenza-specific tetramer positive, IFNγ+CD8+ T cells was observed along with increased perforin and reduced CD27 expression- compatible with an effector phenotype. In older children aged 5 to 9 years, LAIV, but not TIV, resulted in CD8 effector induction. In contrast, in adults, neither vaccine resulted in detectable increase in CD8 effector expression, in agreement with a previous study [354]; they did notice a decrease in CD27 expression after LAIV, with opposite effect after TIV administration [355] , indicating that both vaccines were able to induce some degree of CD8 response, albeit of different phenotype. The authors speculate that the locally replicating LAIV infects antigen-presenting cells and thus viral

proteins are presented through a proteasome and MHC-I related mechanism, leading to the phenotype that is consistent with a memory phenotype.

1.7.2.2 Humoral Immunity – IgG, IgA

LAIV induces similar systemic antibody responses in young children, however, a somewhat decreased rate of seroconversion was seen in older children and adults [356]. In terms of protective efficacy, a wide range of results have been reported- from superior to inferior protection rates in the major trials, respectively[345, 357]. Taken together, the rates of seroconversion following LAIV are lower than TIV- with a range of responses measured by HAI: 23 to 70% to H1N1 component; 18-30% to the H3N2 component and 0-30% to the B component [358-360], however, protection rates were similar overall and slightly higher among those without pre-exisiting antibody responses [361]. The high rates of protection, despite lower seroconersion, have been attributed, with some evidence, to superior ability to induce CTL by LAIV [82, 362]. Another mechanism for the disparity between low systemic antibody response and high protection rate has been attributed to mounting of greater mucosal IgA response, especially in seropositive children [362]. LAIV has not been evaluated methodically for the ability to provide cross protection against antigenic drift influenza strains. Studies in porcine model suggest that LAIV may provide that coverage, with meta-analysis of 34 human RCTs supporting a degree of cross protection [363, 364].

1.7.3 Influenza cross-protection

1.7.3.1 Cross protection induced by virus exposure

As reviewed in previous sections, induction of antibody response against HA, using TIV results in protection that is limited to the strains included in the vaccine. Heterosubtypic immunity is defined as the induction of immunity by a given influenza subtype that mediates some degree of protection against challenge with a different subtype. This concept was first supported almost half a century ago when it was shown that infecting mice with one strain resulted in reduced pathology and viral load in the respiratory tract when exposed to a distinct subtype of influenza [365]. Although not fully protective, the ability of these cross-reactive responses to attenuate the severity of influenza infection, led to research in attempt to identify the correlates of cross-responses. Antibodies may provide cross protection. In mice, maternal immunization generated heterosubtypic protection in the offspring [366]. The generation of mucosal IgA response has not been consistently shown to correlate with cross protection, and such protection was induced in the absence of IgA [328, 367, 368]. Antibodies directed at conserved HA stalk, NA, and M2 have been promoted as promising targets for induction of cross protection, however, these have not been reproducibly shown after influenza infection in humans. The ectodomain of M2 is weakly immunogenic, and research is ongoing, with use of adjuvants, aimed at improving the ability to induce responses against this conserved influenza protein [160, 369]. NP is another viral protein that remains highly conserved across influenza strains, is present in large quantities and is strongly immunogenic [370], however, evidence for its role in heterosubtypic protection is lacking.

More convincing evidence for cross reactive T cell response is available. Most of the dominant CTL responses target the conserved cytoplasmic or nuclear viral proteins. Since

the internal proteins share high degree of homology across subtypes, T cells are thought to account for the majority of heterosubtypic immunity induced by exposure to live influenza viruses. These cross reactive T cell responses do not prevent infection, but can provide a rapid response, facilitating expedient viral clearance and decreasing the resulting pathology. Numerous studies have documented protection afforded by prior exposure to influenza, both against human strains and strains of zoonotic origin [298, 371, 372]. Although the vast majority of convincing evidence has been derived from studies in mice, it appears that CTL responses are playing a role in influenza heterosubtypic protection among humans. During the 2009 H1N1 pandemic, it was shown through examination of longitudinal samples from a single donor, that pre-existing cross-reactive memory CD8 T cells were able to expand robustly and rapidly 8-10 days following infection during the second wave of the pandemic [373]. T-cell responses against immunodominant epitopes have been shown to lead to cross reactivity to heterosubtypic challenge. The assessment is complicated by the diverse HLA alleles and hence repertoire of epitopes. Among a Caucasian population, the immunodominant epitope GILGFVFTL derived from the M1, amino acid 58–66, is capable of producing a strong, long-lasting CTL response among individuals bearing the HLA-A2 allele [286, 374-376]. An attempt to elucidate the hierarchy of influenza epitopes in humans was undertaken by Liu et al. [377]. They synthesized immunodominant epitopes encompassing the entire proteome of 2009 pdmH1N1 virus and seasonal influenza A and characterized the CD8 T cell responses. The study again recapitulates the dominant role of the immunodominant M1 epitope among an Asian population. In addition it provides a

comprehensive list of potential cross reactive targets within NS, PA, PB, NP, NA as well as M1.

From the preceding review, it becomes clear that CTLs are major contributors to cross-protection and that variation in the ability to mount CTL responses to influenza, due to co-morbidities or immunogenetic factors may underlie some of the differences in disease severity when a novel strain of influenza emerges.

1.8 Gaps in Knowledge and Project rationale

The projects included in this thesis were undertaken to try and address several gaps in knowledge:

1. Antibodies play a role in the protection and termination of influenza infection but a gap in our knowledge is that we do not know if they provide significant cross protective responses, in the context of drifted or shifted strains. Some of the limitations in the ability to study cross-reactive antibody responses stems from the reliance on HAI. This method requires production of HA and use of live virus neutralization, making it cumbersome for use in the context of a pandemic, when biosafety requirements are upgraded. In addition, it measures the response to a single HA and cannot determine the breadth of the response. We sought to develop and utilize a microbead- based assay, in order to increase the throughput and ability to interrogate the response to multiple influenza strains simultaneously. The use of protein coupled to the beads which obviates the need for live viruses.

2. CTL responses to influenza do not provide complete protection, however they attenuate the severity of illness and may provide hetero-subtypic and heterotypic coverage. They are therefore an attractive focus of research, with the hope of finding a

"universal influenza vaccine". A significant knowledge gap is the ability of TIV and LAIV to induce cross reactive CTL, so this was investigated as one of the components of this project. In addition, repeated exposure to a recurring circulating influenza strain offered an opportunity to examine the effect of such repeated encounters with the same strain to induce cell-mediated responses. During the 2009 H1N1 pandemic, the emergence of a novel influenza strain allowed us to assess the presence of cross reactive CTL and antibody response that are generated in response to circulating influenza viruses, inactivated vaccine and live-attenuated influenza vaccine.

3. When this project was initiated, it was evident that influenza infection leads to a wide range of disease severity. A significant gap in our knowledge was that the variation could not be explained by traditional risk factors and co-morbidities. During the spring of 2009 an epidemic caused by swine origin reassortant H1N1 spread from Mexico, and by June 11, 2009, the WHO declared the novel H1N1 influenza virus to be the cause of a pandemic. As of August 24, 2009, Manitoba had 886 laboratory confirmed cases of H1N1 flu and seven H1N1 related deaths. Of the 886 confirmed cases in Manitoba, 327 were First Nations or Metis people- suggesting an over-representation of FN in severe disease. This information combined with records from Hudson Bay company, indicating that during the 1918 pandemic Norway House experienced the highest rates- with mortality of approximately 20% of the community sparked our interest in exploring the role of immunogenetic factors in disease susceptibility.The 2009 H1N1 pandemic, provided a unique opportunity to study novel immunogenetic factors that may be involved in determining the outcome of influenza infection.

1.8.1 Global Hypotheses

- **The strength of humoral and cellular responses to influenza can be predicted on the basis of previous exposure and level of immunocompetence.**
- **Cross reactive cellular influenza-specific responses correlate with immunogenetic factors and may affect disease severity.**

Specific hypotheses:

- **Cross reactive antibody responses can be measured after 1) natural infection 2) Live attenuated vaccine 3) inactivated vaccine**
- **Cross reactive CTL responses are strongest after natural infection followed by Live attenuated vaccine, with inactivated vaccine giving the weakest responses**
- **Decreased cross reactive antibody and cell mediated responses correlate with severity of illness caused by novel natural influenza infection**
- **Novel immunogenetic factors contribute to the disparate disease susceptibility during H1N1 influenza infection**

1.8.2 Projects

To try and address the gaps in knowledge and hypotheses put forward, we employed several projects:

- Administration of inactivated trivalent seasonal influenza vaccine- to examine the ability of repeated exposure to recurring influenza strain (circulating as wild-type and included as seasonal vaccine component for several years prior to the study), to boost antibody and CTL against the strain.
- Flumist- seasonal, live-attenuated, cold adapted influenza virus administered via nasal mucosa. We used this model of exposure through the mucosa, to a live attenuated virus, to assess the antibody and CTL responses to vaccine components as well as the ability to induce cross reactive responses against pandemic H1N1.
- Study of serum specimens collected for routine prenatal screening before and after the onset of the first wave of the 2009 H1N1 pandemic. We compared the prevalence of antibodies against the pandemic strain as well as cross-reactive antibodies against additional strains.
- H1N1- severe respiratory illness- individuals admitted to intensive care units during the 2009 H1N1 pandemic- this cohort was used to determine whether antibody or CTL responses are correlated with disease severity and outcome. In addition, we explored immunogenetic candidates that may affect the likelihood of requiring admission to the intensive care unit.

Using these projects we attempt to meet the following objectives:

- **To measure cross reactive antibody responses generated by vaccines and circulating strains of influenza**
- **To measure cross reactive cellular responses generated by vaccines and circulating strains of influenza**
- **To characterize immunogenetic determinants of cross reactive immunity and disease severity**
- **To characterize the cross reactive humoral and cellular responses based on pre-existing degree of immunocompetence**

1.8.3 Project outline and section-specific hypotheses

Chapter 3. Antibody responses induced by influenza infections and immunization: Cross reactive antibody responses can be measured after 1) natural infection 2) Live attenuated vaccine 3) inactivated vaccine

Chapter 4. Cell-mediated immunity induced by influenza infections and vaccines: Inactivated influenza vaccine is capable of limited induction of CTL responses, especially in the context of prior exposure to a recurring influenza strain; and cross reactive CTL responses are strongest after natural infection followed by Live attenuated vaccine and lowest after inactivated vaccine

Chapter 5. Systemic and mucosal cytokine response induced by pandemic influenza infection and administration of LAIV: LAIV administered intra-nasally results in increased levels of proinflammatory cytokines and chemokines systemically; LAIV

administered intra-nasally results in increased levels of proinflammatory cytokines measured in cervicovaginal lavage; Pandemic H1N1 influenza infection results in cytokine "storm" and the levels of cytokines correlate with clinical outcomes

Chapter 6. Immunogenetic factors that influence pandemic H1N1 disease severity: Immunogenetic factors that influence innate and adaptive immune response affect disease severity caused by pandemic H1N1 influenza infection.

Chapter 7. Influenza vaccine strategies for HIV infected individuals: Increasing the dose and adding a booster dose of seasonal influenza vaccine results in improved responses among HIV infected individuals

Chapter 2. Materials and Methods

2.1 General Reagents

2.1.1 Solutions

a. Phosphate buffered saline (PBS): 48.5g of PBS powder: 137.93mM NaCl, 2.67mM KCl, 8.1mM Na_2HPO_4, 1.47mM KH_2PO_4 (Gibco), dissolved in 1L of ddH_2O.

b. 10% paraformaldehyde (PFA): 43ml of ddH_2O, 2ml of 5M NaCl and 5gr of PFA. The solution was heated for 1 minute followed by addition of 20ul of NaOH. The PFA was made fresh on weekly basis.

c. FACS Wash: PBS with addition of 2% fetal calf serum, heat inactivated for 1 hr in 56^0C (FCS, Gibco).

d. Cell culture media, R10: RPMI-1640, L-glutamine 2.05 mM (Thermo Scientific), with 10% heat inactivated FCS and 1% streptomycin/penicillin.

e. Freezing Media: 10% DMSO (Sigma) and 90% cell culture media.

2.1.2 Antigens used for humoral assays

Multiplex suspension bead array technology is based on 5.5 micron, polysterene microspheres that contain a distinct ratio of two luminescent dyes creating 100 bead sets with unique, identifying spectral addresses. This allows for the simultaneous assay of up to 100 analytes within a single sample as small as 50µL. Antigens of interest are coupled to their own specific bead set and mixed with the sample to allow complimentary antibodies to bind. Analysis using a dual laser, flow-cytometry allows quantitative and qualitative determination of antibodies against multiple influenza strains simultaneously. We used an in-house assay, where recombinant HA from influenza strains were coupled

to naked beads, according to bead coupling protocol. In brief: unconjugated, carboxylated microspheres were dispersed with sonication and vortexed for 60 s, and 5×10^6 microspheres (400 μL) were dispensed into a 1.5 mL centrifuge tube and spun down at 14,000 x g for 4 min. The pellet was resuspended in 80 μL of activation buffer, and sonicated until homogeneous. Ten microlitres of Sulfo-NHS solution (10 mg Sulfo-NHS per 2 mL activation buffer) was added to the microsphere suspension and vortexed, and 10 μL of EDC solution (10 mg EDC per 2 mL activation buffer) was added to the solution. The beads were incubated for 20 min in the dark at room temperature. The activated microspheres were centrifuged at 14,000 x g for 4 min and the supernatant aspirated. The pellet was resuspended in 250 μL of coupling buffer, centrifuged and supernatant aspirated. Each specific bead set was resuspended with 250 μL (100 μg/mL) of its assigned antigen preparation (see table 2.1 for the list of coupled antigens). The solutions were incubated at room temperature for 1 h to mix and then washed twice. After the second wash cycle, 250 μL of phosphate-buffered saline of pH 7.4, 1% bovine serum albumin and 0.05% sodium azide (PBSBN) was added. The tube was incubated for 30 min in the dark at room temperature, after the pellet was resuspended in 100 μL of PBSBN. The conjugated beads in each set were enumerated by a hemocytometer. The bead sets were maintained at 4°C in the dark until needed (Coupled beads could be used up to one year from the original date of coupling).

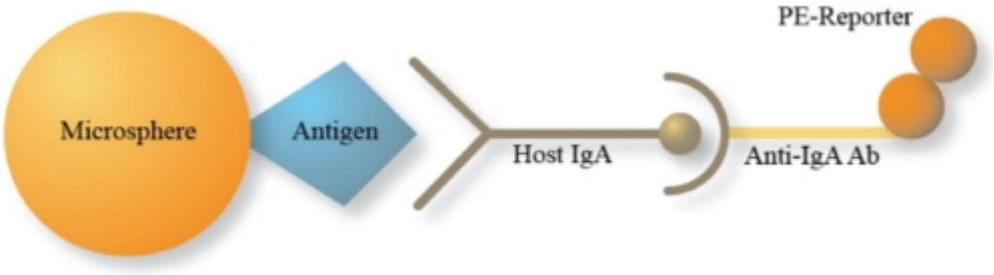


Figure 2.1. Schematic representation, showing antigen coupled to polystyrene bead. For the purpose of our studies, HA from various influenza viruses of interest were coupled. Secondary anti-IgA or anti-IgG antibodies are added, followed by the addition of a reporter- allowing for quantification of the emanating fluorescence.

Table 2.1. List of influenza HA coupled to beads for the detection of antibody response in the various projects. The recombinant proteins were purchased from Prospec. (http://www.prospecbio.com/)

A/H1N1	A/H3N2	Influenza B
A/California (pdmH1N1)/04/2009	A/Brisbane/10/07	B/Florida/04/06
A/Solomon Island/03/06	A/New York/55/04	B/Victoria/504/00
A/Beijing/262/95	A/Wisconsin/67/05	B/Malaysia/2506/04
A/Taiwan/1/86	A/Wyoming/3/03	
A/New Caledonia/20/99		

2.1.3 Antigens and viruses used for stimulation

PBMCs from each donor were incubated in the presence of media (negative control), staphylococcal enterotoxin B 10µg/ml (SEB; Sigma), phytohaemagglutinin at 5µg/ml (positive controls), and the following influenza strains: three influenza vaccine strains namely: A/New Caledonia/20/1999 (H1N1) (NC), A/Wisconsin/67/2005 (H3N2) (WISC) and B/Malaysia/2506/2004 (MAL). For the pandemic cross-reactivity projects: A/Brisbane/59/2007 (H1N1); A/Brisbane/10/2007; (H3N2); B/Brisbane/60/2008 (Courtesy of Dr. Yan Li, NML) and pandemic strain- A/Mex/04/2009 (Courtesy of Dr. Kobinger, NML)

2.2 General Methods

2.2.1 Cohorts and studies

2.2.1.1 Local healthy donors- Inactivated vaccine study

Study subjects included 14 healthy adult donors. The mean age of participants was 37.5 years (range 23–60 years). Seven of the volunteers had received influenza vaccine in preceding years and seven had not. The volunteers attended a vaccination clinic at the University of Manitoba, Winnipeg, Canada. All subjects provided their informed consent, and the study was approved by the ethics review committee of the University of Manitoba. Participants were immunized by intramuscular injection with inactivated trivalent influenza vaccine according to the Northern Hemisphere recommendation for 2006–2007: A/New Caledonia/20/1999 (H1N1)-like, A/Wisconsin/67/2005 (H3N2)-like and B/Malaysia/2506/2004-like antigens [378].

Blood samples were collected at baseline and repeated sampling was obtained on days 7 and 30 post-immunization. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation at 1400 rpm for 25 min, using Ficoll-hypaque (Bio-Lynx, Brockville, Canada) and washed twice in RPMI media (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS (Gibco, Carlsbad, CA, USA).

2.2.1.2 Antibody responses among pregnant women and among marginalized inner city populations in Manitoba

We tested a random sample of 296 serum specimens collected from women who were screened as part of the routine Manitoba Maternal Serum Screening Program. This is a random sampling from a province-wide prenatal screening program offered to all pregnant women between 15 and 22 weeks' gestation. The screening was done on 252 samples obtained before the first wave of pdmH1N1 (March 2009) and these were compared to samples that were obtained at the end of the first wave (August 2009). We used a hemagglutination inhibition assay to detect IgG antibodies against the pandemic strain of the virus and microbead array assay to assess the response to additional influenza viruses, according to protocol outlined in section 2.2.3 [379]. For the second project, a convenience sample of adults presenting to three inner city community clinics in Winnipeg from October 2009 to December 2009 were recruited as study participants (n=458). During the initial phase of the participant enrollment, only adults who had not received the pdmH1N1 vaccine were eligible to participate in the study. Starting on November 13, 2009, this restriction on eligibility was removed due to the rollout of the

pdmH1N1 mass vaccination campaign. We performed HAI and MBA on all samples and analyzed the performance of the two assays.

2.2.1.3 Flumist- Majengo Commercial Sex Worker (CSW) Cohort

This study was conducted in Nairobi, Kenya, at the Majengo clinic. This cohort of commercial sex workers has been followed by a collaborative effort of University of Manitoba and University of Nairobi, for over 30 years [380]. For the purpose of this study, we conducted participant perception assessment prior to study. The study was approved by institutional ethics review committee at the University of Manitoba and University of Nairobi; all participants signed informed consent. In this study we enrolled sixty individuals- 30 highly exposed seronegative (HESN), that have been enrolled in the cohort for over 7 years (we used the strict epidemiological definition). The participants were age matched to controls and screened for participation. The control group consisted of 30 new negatives (NN)- enrolled in the cohort for less than 3 years without evidence of HIV infection based on serology and HIV seronegativity was confirmed using PCR. Baseline samples were collected prior to intranasal LAIV Flumist administration, after 1 day, 7 days, 1 month and at the 4-6~month- last collection was completed in April 2010. Samples included PBMC's, plasma, CVL and nasal aspirates. The samples were stored in -80 and subsequently shipped for further studies in Winnipeg, Manitoba.

2.2.1.4 Severe H1N1 Influenza- intensive care unit (ICU)

In response to the outbreak of pandemic (H1N1) 2009 in Mexico, we collaborated with a multi-centre observational study of critically ill patients, admitted to ICUs, and requiring mechanical ventilation support, due to pandemic influenza infection in Canada.

The research was approved by the local research ethics board. The study protocol is described in detail [30]. We examined blood samples from 20 patients with laboratory-confirmed pdmH1N1 for the presence of genetic polymorphisms. We obtained PBMCs for flow cytometry and plasma for measurement of cytokines and antibodies using microbead array. For cytokine determination the samples included: 34 individuals, 22 women, 6 fatalities overall, average age was 48.45 (range-22-73). Clinical data included ventilation requirements, oxygen requirement, hemodynamic measures, evidence for secondary infections, antimicrobial therapy and outcomes (discharge, transfer to non-ICU bed, mortality). All study participants were coded and data analysis was non-nominal.

2.2.1.5 Influenza vaccine in HIV infected individuals

We obtained serum samples from a multi-centre study. The study was aimed to evaluate the immune response of three different seasonal influenza (Fluviral®) vaccine dosing strategies in HIV infected adults. Study participants were randomized into one of three groups: Group A: single standard dose injection of Fluviral; Group B: standard dose injection of Fluviral followed 28 days later by a booster standard dose of Fluviral; Group C: a double dose of Fluviral followed 28 days later by a second double dose of Fluviral. The study is described in detail in [381]. We received serum samples and were blinded to the group each participant belonged to.

2.2.2 Samples

2.2.2.1 Blood sample collection and processing

Blood was obtained by venipuncture applying sterile technique. Peripheral blood mononucleocytes (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-hypaque (Bio-Lynx, Brockville, Canada), whole blood was layered onto ficoll and centrifuged at 1400 rpm for 25 minutes. The PBMC layer was extracted, diluted with FACS wash and washed twice in RPMI media (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS (Gibco, Carlsbad, CA, USA). Cell were resuspended in media and counted using trypan blue.

Plasma was cryopreserved in cryovials and kept at -80 for use in antibody assays and to determine cytokine concentrations.

2.2.3 Multiplex suspension bead array antibody assays

Coupled beads were stored at 4°C in the dark until needed. A master mix of the pre-coupled beads was prepared according to the list of antigens of interest for each project. Plate preparation followed the manufacturer's instructions. In Brief: 100 microlitres of master bead mix were added to each well followed by vacuuming and washing with 100 µL of Bio-Plex Wash Buffer (Bio-Rad Laboratories). The serum samples were diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} concentration with PBSBN, for most experiments in this thesis we used a dilution of 10^{-3} or 10^{-4} for optimized detection (based on optimization showing that these dilutions represent the steep portion of the titration curve). After incubation with sample, 100 µL at a concentration of 0.5 mg/mL of detection antibody (Mouse Anti-Human IgG1, IgG2 or IgA_1-Biot) (1:400 PBSBN) were added to each well. The plate was incubated in the dark at room temperature for 30 min. Following incubation, the wells were vacuum dried and washed three times with Bio-Plex Wash Buffer and 100 µL of Bio-Plex Streptavidin-PE (Bio-Rad Laboratories), 1 mg/mL

(1:500 PBSBN), were added to each well. The plate was covered and incubated on a shaker at room temperature for 10 min. The last step included repeated washing and the addition of 125 μL of Bio-Plex Assay Buffer for resuspension. Before each plate was assessed, calibration and validation beads (Bio-Rad Laboratories) were used to ensure quality control. We used known positive samples as well as PBS and uncoupled beads as positive and negative controls for each plate that was evaluated. All samples were run in duplicates. The flat-bottom 96-well plate was run on the Bio-Plex Protein Array System (Bio-Rad Laboratories) using Luminex xMap technology. The system was managed with Bio-Plex Manager 4.0 software (Bio-Rad Laboratories). The results are presented as fluorescence-background.

2.2.4 Flow Cytometry

2.2.4.1 Surface staining

Following PBMC isolation, cells were suspended in $1x10^6$ /ml of media, and aliquoted into 5ml snap-capped FACS tubes or 96 v-bottomed plates and incubated in the presence of media (negative control), staphylococcal enterotoxin B (SEB; Sigma), phytohaemagglutinin (PHA) (positive controls), and live influenza strains, according to the specific experiment. Frequently used strains included: NC, WISC, MAL. For stimulation using the pandemic strains MEX, the stimulations were carried out inside a level 3 laboratory with additional application of N95 mask. The antigens were added at a final concentration of 10 haemagglutinin units/mL. After stimulation, the cells were stained using a panel of fluorochrome-conjugated mAb. The panels used varied according to the specific project. The commonly used surface markers included: CD4-AmCyan,CD3-Alexa Fluor 700, CD8-Pacific Blue, CD62L-PE (for fresh samples only),

CCR7-PE-Cy7, HLA DR-APC-Cy7 and CD38-APC (all from BD Biosciences, San Diego, CA, USA) and CD45RA-ECD (Beckman Coulter, Fullerton, CA, USA). Cells were incubated with the antibodies at 4^0C in the dark for 30 minutes. Cells were then washed in Dulbecco's phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) (Gibco), fixation was accomplished with the use of 1% PFA in cases where intracellular staining was not done. The fixed samples were resuspended in PBS. The staining was carried out in 96 well, V shaped bottom-plates, and prior to specimen acquisition the samples were transferred back to 5 ml FACS tubes. At least one hundred thousand events per sample were acquired using an LSRII flow cytometer and analysis was performed with FACSDiva software (BD Biosciences).

After 1,3,6 days of *in vitro* culture, depending on the experiment and staining panel, cells were incubated for a further 6 h in the presence of 1µl/ml GolgiPlug containing brefeldin A (BD Biosciences) or 1µl/ml of GolgiStop containing momensin (BD Biosciences) or both (depending on the specific cytokines assessed) to prevent secretion of cytokines, followed by staining for flow cytometric analysis. Table 2.2 lists the commonly used surface markers and fluorochromes.

Table 2.2. Commonly used surface markers and fluorochromes. Calibration and optimization of each antibody was done prior to usage in a staining panel.

Antibody	**Fluorochrome**	**Antibody**	**Fluorochrome**
CD3	AmCyan, V500	CCR5	PE

CD4	Alexa 700	CD38	PE, PECY5
CD8	Pacific Blue, V450	CD69	FITC, PECY7
CD45RA	ECD		
CCR7	PECy7		
HLA DR	APCCy7		
CD62L	PECY5		

2.2.4.2 Intracellular cytokine staining (ICS)

After surface staining, the cells were washed with 200µL FACS wash, subsequently, incubated in 100µL of fixation/permeabilization solution for 30 minutes (this step was extended from 20 to 30 minutes to achieve inactivation of the influenza viruses used for stimulation), in the dark (BD cytofix/cytoperm fixation/permeabilization kit, BD Biosciences). Cells were then washed with 150µL of the perm/wash solution included in the BD fixation/permeabilization kit. The combination of the solutions in the kit enables the antibodies to gain access into the cells. After the cells were permeabilized, staining with a mix of antibodies against cytokines were prepared according to concentrations that were determined in calibration experiments. The cells were incubated in the dark, at 4^0 for 30 minutes, followed by washing. The cells were resuspended in PBS for flow cytometry acquisition.

2.2.4.3 Carboxyfluorescein succinimidyl ester proliferation assay

Proliferation assays were performed with the use of CFSE (carboxyfluorescein diacetate succimidyl ester) dye dilution. The principles behind the assay lay in the ability of CFSE to enter cells thanks to the acetate side chains. Inside cells, the acetate groups are cleaved by intracellular esterases and the carboxyfluorescein exits from cells at a much slower rate, allowing for time for the CFSE to covalently couple to intracellular amines. After the cells are stained the amount of fluorescent intensity decreases as each

cell divides and the intensity is halved with each cell division [382]. The protocol used: CFSE (Invitrogen) was reconstituted in DMSO (Sigma-Aldrich). Prior to staining the stock solution was thawed and diluted to 1μL CFSE/ml of PBS. The cells were washed to remove traces of FCS and resuspended at $1x10^7$cells/ml in PBS. An equal volume of diluted CFSE was added to the cells and incubated in the dark for 8 minutes (at 37^0). After incubation, the reaction was quenched by addition of cold FCS for 1 minute. This step was followed by washing with FACS wash, twice. Cells were then stimulated according to the specific experimental design, resuspended in media, and kept in a tissue culture incubator, at 37^oC, for 6 days. Following incubation, the cells were stained with surface and intracellular stains as described in previous sections. CFSE was detected by flow cytometry using the channel reserved for FITC detection on the blue laser.

2.2.4.4 Compensations

The compensation procedure entails the measurement of the fluorescence of each fluorophore dye included in the multiparametric analysis, individually and in every other channel that was used for detection. After the measurement of each individual constituent along with a negative and positive control, a computer algorithm is used to remove spectral overlaps, by the use of complex matrix algebra allowing for simultaneous solution of the equations for the contributions of the spectral overlaps
of each of the colors into every detector. The use of computer generated compensation overcomes the difficulties caused by multiple spectral overlaps, obviating the need for manual compensation (impossible in complex multi-parametric experiments). The compensations were accomplished using commercial microsphere beads (CompBeads, BD Biosciences), coupled to an antibody specific for the Kappa light chain of Ig, from

mouse and rat. The staining of CompBeads was performed according to manufacturer instructions. In brief: the beads were vortexed and resuspended in PBS. Each antibody in the staining panel was represented by a single FACS tube (along with a negative control CompBead), 1μL of the corresponding antibody was added to each tube followed by 20-30 minutes of incubation. For CFSE experiments, a tube of unstimulated cells was used.

2.2.4.5 Data acquisition and analysis

Stained samples were acquired using LSRII flow cytometer (BD Biosciences). Fixed samples were resuspended in at least 200 μL of FACS buffer. The number of events collected varied between experiments. For surface staining at least 50,000 events within the PBMC gate were acquired. For the intracellular staining 100,000-500,000 were acquired in an attempt to ensure sufficient events for each cytokine. The flow cytometry data were analyzed using FACS Diva verion 6 (BD Biosciences).

2.2.5 Stimulations

PBMCs from each donor were aliquoted into either 5ml FACS tubes (BD Falcon), at 10^6/ml in media, and incubated in the absence of additional stimuli (negative control), or in the presence of staphylococcal enterotoxin B (SEB; Sigma), phytohaemagglutinin (positive controls), and the following influenza strains: three influenza vaccine strains namely: NC, WISC and MAL. For the pandemic cross-reactivity projects: H1 BRIS; H3 BRIS; B BRIS (Courtesy of Dr. Yan Li, NML) and pandemic strain- MEX (Courtesy of Dr. Kobinger, NML). For the ICS assays, golgi transport inhibitors were added (1 μL GolgiPlug containing Brefeldin A and/ or GolgiStop containing monensin, BD

Biosciences) to prevent the secretion of cytokines. These inhibitors were added 8-14 hours prior to staining.

2.2.6 Cytokine and chemokine bead arrays

Chemokine and cytokine levels were determined using the Milliplex MAP multiplex kit (Human Cytokine/Chemokine I, II from Millipore, Billerica, MA) and analyzed on the BioPlex-200 (Bio-Rad, Mississauga, ON, Canada). We used the same assay for detection of cytokines/chemokines in plasma and cervicovaginal lavages, in the following studies: ICU pandemic H1N1 study and flumist LAIV study among commercial sex workers. The assays were performed in accordance to protocol provided by the manufacturer. The assay principle: 96 well plate was custom tailored to include the chemokine/cytokines of interest. The protocol follows these steps: capture antibody–coupled beads are incubated with antigen standards or samples followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin conjugate. Following washes for removal of excess SA-PE, the beads were subjected to flow cytometry, using the Bio-Plex array reader (Bio-Plex 200 system), which measures the fluorescence of the bead and of the bound SA-PE. The standard curve was used to convert mean fluorescence intensity into corresponding concentrations (BioPlex software, Bio-Rad, Mississauga, ON, Canada). We used a panel of the following 19 cytokines/chemokines for our assays: interferon (IFN) alpha2 (IFNα2); IFNγ; interleukin (IL) IL-1ra (receptor agonist); IL-1a; IL-1b; IL-2; soluble IL-2 receptor sIL-2rα; IL-6; IL-10; IL-12(p40); IL-12(p70); IL-15; IL-17; IFNγ induced protein IP-10; monocyte

chemotactic protein (MCP) MCP-1; MCP-3; macrophage inflammatory protein (MIP) MIP-1a; MIP-1b; tumor necrosis factor (TNF) TNFa.

2.3 Section Specific Methods

2.3.1 Local healthy donors – *ex vivo* T cell phenotyping

Study subjects included 14 healthy adult donors. The mean age of participants was 37.5 years (range 23–60 years). Seven of the volunteers had received influenza vaccine in preceding years and seven had not. Participants were immunized with inactivated trivalent influenza vaccine according to the Northern Hemisphere recommendation for 2006–2007. Blood was obtained prior to vaccination and after 7 and 30 days. Peripheral blood mononucleocytes (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-hypaque as described in section 2.2.1.1. PBMCs were enumerated using hemocytometer, stimulated with media, SEB and PHA (positive controls). Viral strains included in the seasonal influenza vaccine were used: NC, WISC and MAL. The cells were incubated at 37^0 until staining.

2.3.1.1. Flow cytometry panels for *ex vivo* T cell phenotyping and functional assays

We used the following antibody panels for surface staining: CD4-AmCyan, CD3-Alexa Fluor 700, CD8-Pacific Blue (BD Pharmingen, San Diego, CA, USA)- for T cell subset classification, CD62L-PE, CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA, USA) and CD45RA-ECD (Beckman Coulter) for memory subset classification. We stained with HLA DR-APC-Cy7 and CD38 as markers of immune activation (BD Pharmingen, San Diego, CA, USA. After surface staining, intracellular cytokine staining for IFN-γ-fluorescein isothiocyanate and IL-2-allophycocyanin (BD Biosciences) was performed, as

described by Betts et al [383]. Cells were incubated in the presence of stimuli for 3 and 6 days.

2.3.1.2 Flow cytometry gating strategies

We gated on lymphocytes and, subsequently, on CD3+ and CD8+ cells. Within the CD8+ cells, we gated on IFN-γ and IL-2 and recorded the proportions of CD3+CD8+ lymphocytes producing each cytokine, upon each stimulation condition. In parallel gating, we separated CD8 into memory subsets based on surface expression of CD45RA and CCR7, defining CD8+CD45RA+CCR7+ as Naïve; CD8+CD45RA-CCR7+ as central memory (CM); and CD8+CD45RA-CCR7- as effector memory (EM).

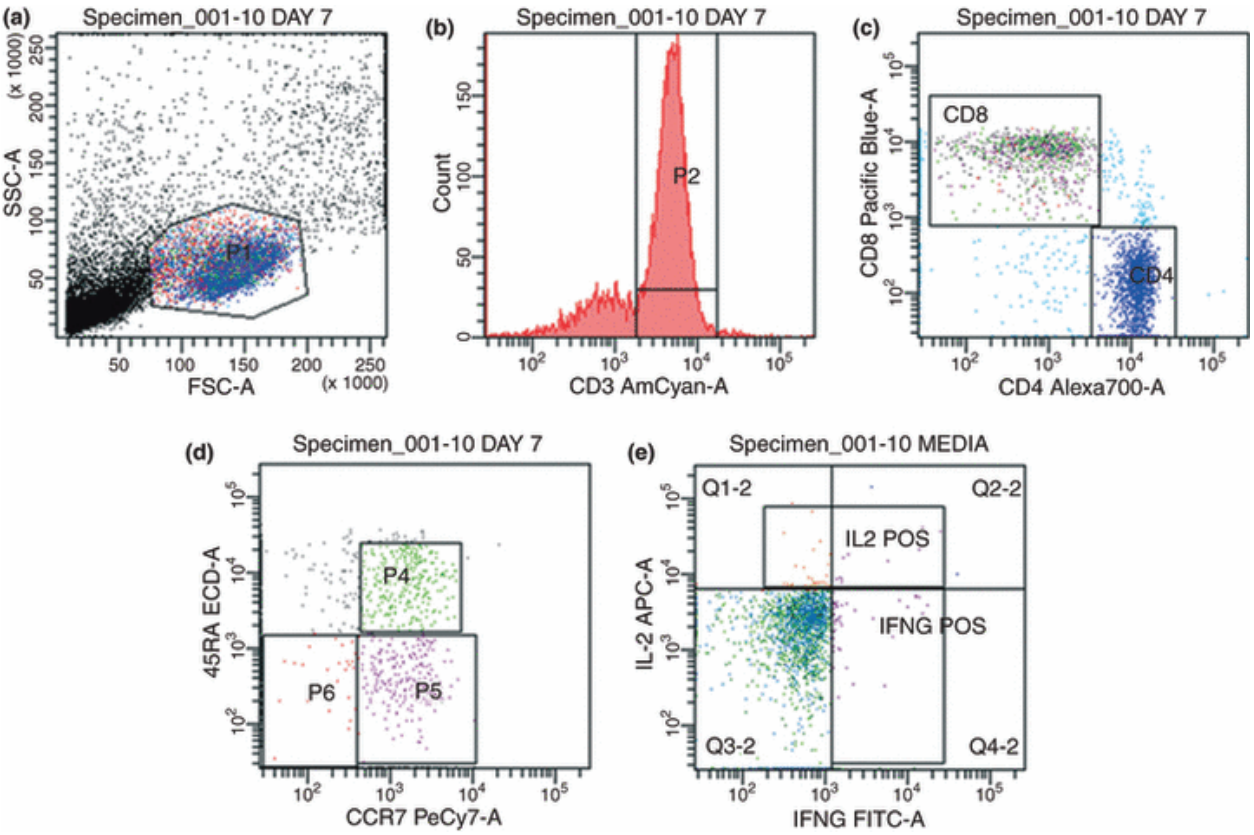


Figure 2.2. Depiction of the gating strategy. The gating strategy employed was to first gate on lymphocytes (a) followed by CD3+ cells (b) and finally T cell subsets (CD4 or CD8) (c). Then memory subsets, defined by CD45RA and CCR7, were gated on (d). Functional responses on either T cell subsets (CD4/CD8) or memory subsets (naïve, T_{CM}, T_{EM}) were assessed by determining interleukin (IL)-2 and interferon (IFN)-γ responses (e) using the described intracellular staining protocol [378].

Similarly we performed memory subset classification by gating on CD8, and dividing the CD8+ T cells into naïve – CD8+ CD45RA+, central memory (T_{CM}) – CD8+ CD45RA− CCR7+ and effector memory (T_{EM}) cells – CD8+ CD45RA− CCR7−. Memory subsets were determined before, 7 days and 30 days after vaccination with the seasonal TIV. Immune activation, as assessed by HLA-DR and CD38 expression, was measured for the CD8+ T cells as well as for each memory subset.

2.3.1.3 Statistical analysis of *ex vivo* T cell phenotyping study

To determine the phenotype and function of influenza CD8+ T cells in response to repeated vaccination, we compared the activation, memory subset distribution and IFN-γ and IL-2 production in response to stimulation with 'old' (NC, the stain circulating for the 5-6 seasons prior to vaccine) and 'new' vaccine components, prior to and after vaccination with TIV. Mean values (percent cells expressing a particular phenotype) were compared between groups using the nonparametric Mann–Whitney U-test; $p \leq 0.05$ was considered statistically significant. The Kruskal–Wallis nonparametric test was used for the calculation of trend between the three time points.

2.3.2 Flumist-Majengo CSW cohort

2.3.2.1 IgG Antibody response- antigens

Antibody levels against a panel of influenza HA antigens: H1N1: A/California (pdmH1N1)/04/2009; A/Solomon Island/03/06 (SI); A/Beijing/262/95 (BJ); A/Taiwan/1/86 (TA); H1N1: NC; H3N2: H3 BRIS; A/New York/55/04 (NY); WISC; A/Wyoming/3/03 (WYO); B: B/Florida/04/06 (BFLO); B/Victoria/504/00 (BVIC); B/Malaysia/2506/04.

Where antibodies reactive with HA antigen were present at baseline (MFI $\geq$ 250), increases of >500MFI and to a value >1000 MFI were considered to indicate positive humoral responses to the vaccine. Without pre-existing antibody responses against increases in MFI to $\geq$ 500 were considered to indicate positive humoral responses to the vaccine.

2.3.2.2 Flow cytometry panels for CMI study

Cryopreserved cells were maintained in aliquots at -135^{0}. Prior to incubation and stimulations, cells were thawed, viability of PBMCs-based crystal violet staining and microscopy was determined and experiment proceeded when the viability rate was >80%. Three antibody panels were used for this project: 1. A 24 hour stimulation, focusing on measurement of NK cytokine response; a 3 day intracellular panel- characterizing subsets, activation, memory and cytokine production and a 6 day stimulation, designed to measure proliferation among subsets in response to the vaccine. The panels that were used are depicted in Table 2.3 (A, B, C respectively).

Table 2.3. Antibody panels used for measuring CMI in response to LAIV.

A Day 1 NK Panel

Antibody	Fluorochrome
CD3	AmCyan
CD16	ApcCy7
CD56	Alexa 700
IFN gamma	APC
Perforin BD48	PE
CD 107a	PeCy5
Nkp46	FITC
CD69	Pac Blue
NKG2D	PECY7

B Day 3 ICS panel

Antibody	Fluorochrome
CD3	AmCyan
CD4	Alexa 700
CD8	Pac Blue
CD45RA	ECD
CCR7	PECy7
HLA DR	APCCy7
IL2	APC
IFN gamma	FITC
CCR5	PE
CD38	PECy5

C Day 6 Proliferation panel

Antibody	Fluorochrome
CD3	AmCyan
CD4	Alexa 700
CD8	Pac Blue
CD45RA	ECD
CCR7	PECy7
HLA DR	APCCy7
IL2	APC
CFSE	
CCR5	PE
CD38	PECy5

2.3.2.3 Statistical Analysis of T cell function study

To determine the phenotype and function of influenza CD4+ and CD8+ T cells in response to repeated vaccination, we compared the activation, cytokine production (IFN-γ and IL-2) and proliferation in response to stimulation with H1N1 component of the seasonal LAIV prior to and after vaccination with Flumist. Mean values (percent cells expressing a particular phenotype) were compared between groups using the nonparametric Mann–Whitney U-test; $p \leq 0.05$ was considered statistically significant. The Kruskal–Wallis nonparametric test was used for the calculation of trend between the three time points. In addition, we compared the baseline and post vaccine responses to pandemic H1N1 stimulation in order to assess for cross-reactive responses to a strain that was not included in the vaccine formulation. GraphPad Prism Software version 6.0, GraphPad Software, Inc. La Jolla, CA,USA was used for statistical tests.

2.3.4 Severe H1N1 influenza – ICU study

This project was done during the 2009 pandemic of novel H1N1 influenza. We obtained PBMC and plasma samples of individuals admitted to ICUs with laboratory confirmed (culture or PCR) pandemic H1N1 influenza infection, as part of a collaborative project, involving health care facilities in Canada, as described in [30]. Thirty three individuals who had multiple samples obtained during their stay in the ICU, including an early sample, within the first day of admission, were included in the analysis. Of them, 6 succumbed to influenza or its complications and 27 survived for at least 28 days after admission.

2.3.4.1 IgG Antibody- antigen panel

Micro-bead array assay was used as previously described. The influenza HA antigens coupled, included: pdmH1N1; SI; BJ; TA; NC, H3 BRIS; NY; WISC; WYO; BFLO; BVIC; BMAL. Mean Fluorescent Intensity (MFI) was measured at the time of admission, 5-7 days later and after 10-14 days. The change in antibody response to the pandemic H1N1- California was measured as well as to heterosubtypic and heterotypic viruses included in the panel.

2.3.4.2 Cytokine levels

The same panel of 19 cytokines/chemokines (section 2.2.5 on page 76) was used. The panel included: IFN alpha2 (IFNα2); IFNγ; IL: IL-1ra (receptor agonist); IL-1a; IL-1b; IL-2; soluble IL-2 receptor sIL-2rα; IL-6; IL-10; IL-12(p40); IL-12(p70); IL-15; IL-17; IFNγ induced protein (IP-10); MCP: MCP-1; MCP-3; MIPs: MIP-1a; MIP-1b; TNFa. The clinical features and outcomes were correlated with the cytokine levels.

2.3.4.3 Flow Cytometry panels for phenotyping

PBMC from whole blood were obtained from the individuals enrolled in the ICU study. Due to the limited quantity of whole blood for each collection, we were not able to perform an elaborate functional characterization as such assays require large numbers of PBMCs. Instead the focus was on activation of CD4+ and CD8+ T cells, based on surface staining, requiring less (50,000-100,000 events to be recorded). Activation markers may provide some surrogate information about the antigen specific responses and have been shown to expand and contract in a way that mirrors the antigen specific responses in the context of HIV infection and vaccinia virus administration [384, 385]. A rudimentary

panel composed of subset classification surface markers (CD3; CD4; CD8) and activation markers (CD38; CCR5; HLA-DR) was used.

2.3.4.4 Genetic polymorphisms

DNA was extracted from stored PBMCs. The samples were thawed and resuspended in 200 uL phosphate-buffered saline. Genomic DNA was extracted by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions .This analysis included samples from 20 patients suffering from severe respiratory illness caused by confirmed H1N1 pandemic influenza. The average age was 40.3, 10 individuals were of Aboriginal descent, 9 Caucasians and one unknown ethnicity. The presence of the CCR5delta32 allele was determined by PCR amplification of the delta32 locus and the size of the resulting PCR product. DNA was amplified using previously reported primers surrounding the 32bp deletion in the CCR5 gene: 5' primer: TCATTACACCTGCAGCTCTC; 3' primer: TGGTGAAGATAAGCCTCAC [386, 387].

Wild-type CCR5 DNA results in a 197bp product, compared to the 165bp product of the Δ32 allele. Comparison was made to a known heterozygote as a control sample. Odds ratio (OR) and 95% confidence limits were calculated using a recessive genetic model comparing proportions of wild-type CCR5 and CCR5Δ32 heterozygotes between this study and previously reported rates among Caucasian population [388]. Significance was determined by Chi-square test using a two-sided P value (with the threshold of significance set at $p<0.05$) and Yates' continuity correction using Prism statistics program version 6 (GraphPad Software).

2.3.5.1 Antibody responses among pregnant women Manitoba

Micro-bead array assay was used as previously described. The influenza HA antigens coupled, included: A/California (pdmH1N1)/04/2009; A/Solomon Island/03/06; A/Beijing/262/95; A/Taiwan/1/86; A/New Caledonia/20/99 H3N2: A/Brisbane/10/07; A/New York/55/04; A/Wisconsin/67/05; A/Wyoming/3/03; B: B/Florida/04/06; B/Victoria/504/00; B/Malaysia/2506/. The incidence of pandemic H1N1 influenza was determined among two panels of stored frozen serum specimens collected for routine prenatal screening. The samples were randomly selected for testing before (March 2009, n = 252) and after (August 2009, n = 296) the first wave of the pandemic [379]. A standard HAI assay was used to detect the presence of IgG antibodies against the pandemic (H1N1) 2009 virus. The cumulative incidence of pandemic (H1N1) influenza was calculated as the difference between the point prevalence rates in the first and second panels. The results of HAI were compared to those obtained with the in-house bead based assay.

2.3.6 Influenza vaccine response among HIV infected individuals

2.3.6.1 Antibody response-antigen panel

This study (Canadian HIV Trials Network- CTN 237) was designed to evaluate the immune response of three different seasonal influenza (Fluviral®) vaccine dosing strategies in HIV infected adults. Study participants were randomized into one of three groups: A. single standard dose injection of Fluviral; B. standard dose injection of Fluviral followed 28 days later by a booster standard dose of Fluviral; C. a double dose of Fluviral followed 28 days later by a second double dose of Fluviral. We sought to

determine the antibody responses to the vaccine strain of influenza, to evaluate the ability to generate cross reactive antibody responses and to compare the performance of HAI and micro-bead array based measurement of IgG1 responses. A panel of influenza HAs was used: H1N1: pdmH1N1; SI; BJ; TA; NC; H3N2:H3 BRIS; NY; WISC; WYO B: BFLO; BVIC; B/MAL. In addition P24 and GP120 were included to assess whether influenza vaccination had an effect on HIV specific antibody responses.

2.3.6.2 Statistical analysis of cytokine and antibody response

Correlation analysis between age and CD4 T cell count were performed. A comparison of the 3 vaccination strategies, and the ability to induce a rise in IgG1 titres, was assessed using non-parametric, Wilcoxon matched-pairs signed rank test. In order to assess overall trend in antibody response the Friedman repeated measure ANOVA was applied. $P \leq 0.05$ were considered statistically significant.

Chapter 3. Influenza specific antibody responses

3.1 Rationale

As reviewed in previous chapters, antibodies play a role in the protection and termination of influenza infection but do not provide significant cross-protective responses, in the context of drifted or shifted strains. The stalwart hemagglutination inhibition assay (HAI) is based on binding of hemagglutinin protein on the surface of influenza to sialic acid receptors on the cell surface of erythrocytes, causing agglutination. If antibodies to a specific influenza strain are present, they will occupy the antigenic sites on the HA protein inhibiting viral binding and thus inhibit agglutination. Although rising titres and HAI of >1:40 are hailed as the gold-standard for vaccine induced protection, delay for antibody levels to rise, the requirement for acute and convalescent sera for diagnostic use of this assay, make it largely a tool for confirming epidemic cases, if respiratory specimens are not available.

Some additional limitations include an inability to study cross-reactive antibody responses simultaneously, the prerequisite of production of HA and use of live virus neutralization. This makes it restrictive, especially in the context of highly pathogenic influenza strains, necessitating high containment laboratories that are scarce. In addition, it measures the response to a single HA, and cannot determine the breadth of the response. In regions were surveillance is limited, using a assay that will be able to detect the serological evidence of prior circulating strains is of tremendous value. In order to try and increase the throughput and ability to interrogate the response to multiple influenza strains simultaneously we sought to develop and utilize a microbead based assay. In addition to the higher throughput, using protein coupled to the beads obviates the need for live viruses.

3.2 Hypotheses

- **Cross reactive antibody responses can be measured in decreasing magnitude after natural infection> Live attenuated vaccine> inactivated vaccine**

3.3 Objectives

A. **To determine the IgG responses induced by vaccination with seasonal Trivalent influenza vaccine (TIV)**
B. **To compare the ability of TIV to increase antibody responses in the context of repeated exposure to the recurring influenza strain**
C. **To measure antibody response to LAIV among vaccine naïve, healthy commercial sex workers in Nairobi, Kenya**
D. **To measure IgG responses induced by circulating seasonal and pandemic influenza strains in Manitoba**
E. **To compare the assay performance of HAI and micro-bead array in detection of IgG responses induced by circulating seasonal and pandemic influenza strains in Manitoba**
F. **To measure the antibody responses induced by the 2009 pandemic influenza in severely ill individuals admitted to ICU**
G. **To measure the IgG antibody response induced by different influenza vaccination strategies in HIV infected individuals**
H. **To compare the cross-reactive antibody responses induced by different influenza vaccination strategies in HIV infected individuals**

3.4 Results

3.4.1 Study populations

- Inactivated trivalent seasonal influenza vaccine was administered to local healthy volunteers, to address objectives A and B.
- Flumist- seasonal, live-attenuated, cold adapted influenza virus administered via nasal mucosa was used to address objective C, in a population of healthy, HIV negative commercial sex workers in Nairobi, Kenya.
- Study prenatal samples pre and after the onset of the first wave of the 2009 H1N1 pandemic. This study was used to address objectives D and E.
- H1N1- severe respiratory illness- individuals admitted to intensive care units during the 2009 H1N1 pandemic- this cohort was used to address objective F.
- A collaboration with Dr. Cooper and CTN study 237 (CIHR- Canadian HIV trials network sponsored study assessing the immunogenicity and efficacy s influenza vaccine strategies in HIV infected Adults. The study entailed 3 seasonal influenza TIV vaccination strategies was used to explore objectives G and H.

3.4.2 IgG antibody response induced by seasonal influenza vaccine in healthy individuals

This study involved 14 healthy adult donors from Winnipeg. The mean age of participants was 37.5 years (range 23–60 years). Seven of the volunteers had received influenza vaccine in preceding years and seven had not. Samples were collected before vaccination and then 7 and 30 days afterwards. The HAI was performed on all samples for each of the three vaccine components. Mean pre-vaccine titres were 52 (95% CI 95 0–

130.7), 18.86 (95% CI 95 −6.45 to 31.26) and 4.857 (95% CI 95 3.005–6.709) for H1N1New Caledonia/20/1999, A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004, respectively. HAI titres on day 0 (before vaccination) and days 7 and 30 (after vaccination) for each of the strains contained in the vaccine are shown in figure 3.1. Over this time period, HAI titres for NC did not change, but statistically significant increases in antibody titres for WISC and MAL were noted (Kruskal–Wallis p values for trend were: NC, 0.16; WISC, 0.0064 and MAL, 0.0019) [378].

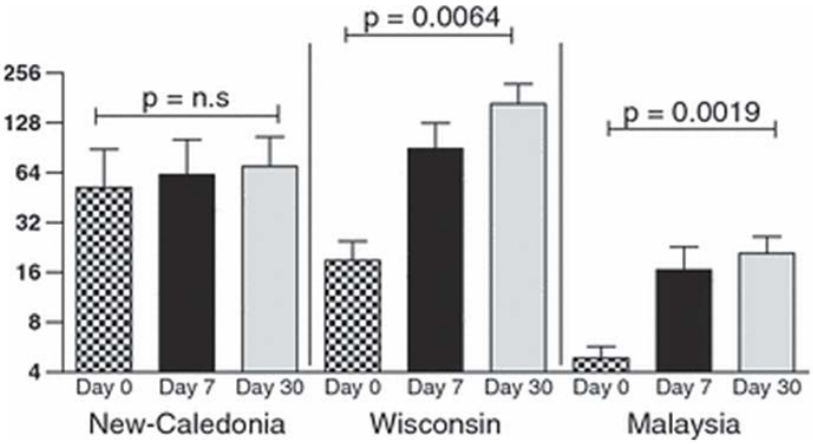


Figure 3.1.HAI titres for each of the three vaccine components. The recurring strain New-Caledonia 20/99 failed to induce a rise in antibody titres while the two other components resulted in significant increases of antibody titres.

3.4.3 IgG antibody response induced by seasonal LAIV in healthy commercial sex workers

In this project we administered a seasonal Flumist, LAIV to influenza vaccine naïve, HIV negative, commercial sex workers in Nairobi, Kenya. The vaccine was given to 60 participants. We used our micro-bead based (MBA) assay to assess antibody response to vaccine and non-vaccine strains. The antibody titres for H1N1 Brisbane 59/2007, reported as mean fluorescent intensity were high at baseline, with MFI in the range of 345-3430, mean-1998, with no further increase at 7 or 30 days post-vaccination (Figure 3.2 A). At the outset, prior to vaccine administration 92% had preexisting IgG1 antibodies against H1N1 Brisbane. The baseline titres for H1N1 Solomon Island, a previously circulating strain that was not included in the vaccine preparation of 2009, were higher, at a mean of 3340, with no increase following vaccination. The antibody titres against the pandemic H1N1 California were lower at baseline, without a significant increase after immunization. Of note is the finding that >40% of the study population had titres >1000 MFI, a value that was used as the cutoff in other studies we conducted. This suggests that by the fall of 2009, approximately 6 month after the onset of the 2009 H1N1 pandemic, a significant proportion of the study population was exposed to the pandemic strain.

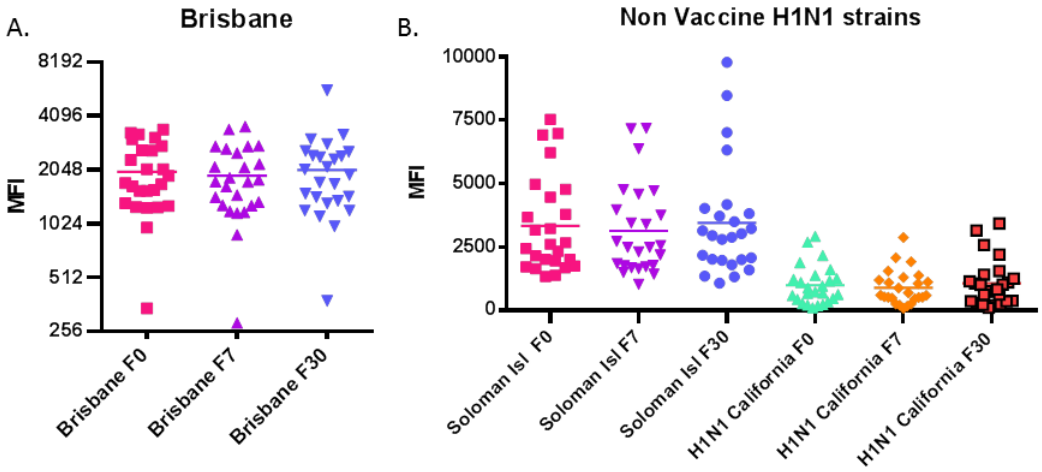


Figure 3.2. IgG1 titres against influenza A/H1N1 strains. Panel A. depicts the response to H1N1, A/ Brisbane 59/2007, the H1N1 component of the Flumist vaccine administered to study participants. Panel B depicts the IgG1 titres to non-vaccine strains of H1N1-A/Solomon Island/03/06, a seasonal strain that was circulating in previous years and A/California (pdmH1N1)/04/2009; the pandemic strain that emerged in the spring, several month prior to this study.

3.4.4 IgG antibody response induced by circulating influenza virus in Manitoba

This study used archived serum samples collected during antenatal screening. The first set of samples were obtained from before the pandemic H1N1 first wave (March 2009) and these were compared to samples that were obtained at the end of the first wave (August 2009). A hemagglutination inhibition assay was used to detect IgG antibodies against the pandemic strain of the virus and microbead array assay to assess the response to additional influenza viruses. The median participant age was similar in the two sampling periods: 28 (range 16–40) years for the women whose samples were collected in March and 27 (range 16–43) years for those whose samples were collected in August. The seroprevalence rates, for pandemic H1N1 virus, measured with HAI were: 7.1% of the serum specimens collected in March and 15.7% of those collected in August (Table 3.1). The calculated cumulative incidence (the point prevalence rate in August 2009-point prevalence rate in March 2009) was 8.6% (95% CI 3.2%–13.7%). The cumulative incidence was higher among younger women (age 14–27 years; 17.2%, 95% CI 8.6%–25.4%) than among older women (3.6%, 95% CI 0.0%–10.6%).

Table 3.1. The point prevalence and cumulative incidence of pandemic H1N1 in March and August 2009 [379].

Cut-off point for positivity; age group, yr	No.*	Point prevalence, %		Cumulative incidence,† % (95% CI)
		In March 2009 *n = 252*	In August 2009 *n = 296*	
HIA titre ≥ 1:20				
14–27	150	23.9	36.0	12.1 (1.1–23.3)
28–44	146	19.3	23.3	4.0 (0.0–13.1)
All	296	21.4	27.3	5.9 (0.0–12.6)
HIA titre ≥ 1:40				
14–27	150	6.8	24.0	17.2 (8.6–25.4)
28–44	146	7.4	11.0	3.6 (0.0–10.6)
All	296	7.1	15.7	8.6 (3.2–13.7)
HIA titre ≥ 1:80				
14–27	150	2.6	14.0	11.4 (5.5–18.0)
28–44	146	1.5	6.8	5.4 (1.2–10.1)
All	296	2.0	10.0	8.0 (4.4–12.2)

Note: CI = confidence interval, HIA = hemagglutination inhibition assay.
*The number of specimens tested in August.
†Calculated as the difference between the point prevalence estimates in March and August 2009.

Footnote: Table taken from CMAJ. 2010 Oct 5;182(14):1522-4. doi: 0.1503/cmaj.100488

In addition, we compared the performance of the HAI with micro-bead array. We used different thresholds, based on our previous experience with assay [389]. We applied a comparison based on MFI thresholds of 500 and 1000 (Table 3.2 A. and B. respectively). The cumulative incidence was 22.9% and >40% for the entire province and in Northern Manitoba, respectively. Increasing the positivity threshold to 1000 MFI led to a decrease in baseline rate to 0.7% while the cumulative incidence remained similar at 23.7% with good agreement in the province sub-regions. In addition, we tried to ascertain the correlation between the two assays. For that purpose, cutoffs of 20, 40 and 80 were used for the HAI and MFI of 200, 500, 1000 for the bead based assay cutoff. The concordance of the assays was determined for pre-pandemic and post- pandemic samples, and representative results are presented in Table 3.3. The correlation between the results of HAI titres and the bead array MFI measurements was stronger for the post-epidemic panel (r=0.63) than for the pre-epidemic panel (r=0.52). However, agreement between the two assays in terms of classifying specimen into seropositive or seronegative (Table 3.3) was much stronger, and was highest when HAI titres of 1:40 or 1:80 were used and ranged from 75% to 98%. The pattern of disagreement between the two tests was dependent on the panel. In the pre-epidemic panel, specimens were likely to test positive in the HAI and negative in the micro-bead array regardless of the cutoff point used (data not shown). In the post-epidemic panel, specimens were likely to test positive according to bead array results but negative in the HAI. As a result, estimates of period prevalence based on the results of the MBA were generally higher than those based on the HAI. For instance, the period seroprevalence (based on MFI $\geq$ 500) was 21.2% (13.0-29.5) for the

whole province. Seroprevalence in Northern Manitoba was 42.1% (18.3-65.0), a rate that was much higher than in the south, although with overlapping confidence intervals due to the smaller number of specimens tested using the MBA (270 total tested using MBA, 135 from March 2009 and 135 from August 09). The period prevalence estimates based on different cutoff points for the MBA did not change significantly.

Another study was conducted during the 2009 pandemic influenza emergence, and it had two aims: to assess whether marginalized inner city populations are at higher risk of acquiring pandemic H1N1 as well as vaccine uptake. The second aim was to compare the performance of HAI and MBA. We found the positivity rate in the pre-vaccine phase (before November 13, 2009) subsample was 5.7%. On the November 13, 2009 samples, the positivity rate in the non-immunized individuals increased to 16% [390]. Positivity was more likely among female, Aboriginal participants, lower level of education and lower income. Since this study entailed testing of equal numbers of men and women, the higher positivity among women cannot be the result of higher testing rates. For the assay comparison, we used the cutoff derived from the previous study as described above. A positive HAI result was defined as titres ≥1:40 and a positive MBA result was a mean fluorescence intensity (MFI) ≥500. Using these positivity cutoffs, higher rates of seropositivity were detected with MBA compared to HAI across age groups, ethnicities as well as socioeconomic strata. When applying the same criteria to measurement of vaccine response, the rate of increase in HAI positivity against pandemic H1N1 is higher (20% increase versus 5% for the MBA), despite the fact that this strain was not included in the vaccine formulation. After administration of the pandemic H1N1 vaccine, the rates of positivity for MBA were 12% higher than for HAI (Table 3.4). In

addition, we examined the cross-reactive IgG1 responses to non-vaccine strains. The strongest cross-reactive responses among individuals that received the pandemic H1N1 vaccine is seen against A/Solomon Island/03/06; this is the closest phylogenetic relative with some degree of similarity to the emerging pandemic strain.

Table 3.2. Cumulative incidence of pandemic H1N1 infection, Manitoba, 2009. A. Using the Micro bead assay lower threshold of 500 MFI, the cumulative incidence was 22.9% with >40% cumulative incidence in Northern Manitoba. B. using the higher threshold of 1000 MFI, the baseline rates decreased significantly to 0.7% while the cumulative incidence remained similar art 23.7% with good agreement in the province sub-regions. WRHA denotes Winnipeg Regional Health Authority.

A. Bead Array At MIF=500				B. Bead Array At MIF=1000			
Region	% +ve in March	% +ve in August	Aug minus March	Region	% +ve in March	% +ve in August	Aug minus March
Total	4.5	27.4	22.9 (15.2-31.3)	Total	0.7	23.7	23.0 (16.8-30.9)
WRHA	7.7	27.4	19.7 (7.8-31.3)	WRHA	1.5	21.9	20.4 (10.7-30.9)
North	5.6	47.6	42.1 (16.7-65.9)	North	0	42.9	42.9 (22.7-63.6)
Other	0	17.1	17.1 (7.3-30.6)	Other	0	17.1	17.1 (7.3-30.6)

Table 3.3. Agreement between assays, for the various HAI and MFI thresholds.

Mean Fluorescence Intensity Bead Array	**HAI titre**	**Concordance rate**	**Discordance rate**
Prepandemic samples March 2009			
MFI > 500	40	89.60%	10.40%
MFI > 1000	40	90.30%	9.70%
MFI > 500	80	94%	6%
MFI > 1000	80	97.80%	2.20%
Aug samples			
MFI > 500	40	85.90%	14.10%
MFI > 1000	40	85.20%	14.80%
MFI > 500	80	83.00%	17.00%
MFI > 1000	80	85.20%	14.80%

Table 3.4. Comparison of HAI and MBA, among marginalized inner city population. MBA positivity was higher across the age groups, ethnicities, education and income. Notably, the positivity rates for HAI increased more (27.3 to 47.1%) following the seasonal immunization, that did not contain the pandemic H1N1strain. Higher MBA positivity rates for pandemic H1N1 following the introduction of the corresponding vaccine (82.5% vs 70.2% 15 days after immunization).

Variable	Category	N	Positive by HIA at = 1:40 (%)	P-value	Positive by SBA (%)	P-value
Total		458	96(21.0)		224(48.9)	
Age	18-29	120	27(22.5)	0.220	53(44.2)	0.089
	30-39	97	13(13.4)		41(42.3)	
	40-49	147	33(22.4)		75(51.0)	
	50+	94	23(24.5)		55(58.5)	
Ethnicity	Aboriginal	296	72(24.3)	0.040	173(58.4)	0.000
	Caucasian	127	17(13.4)		31(24.4)	
	Other	35	7(20.0)		20(57.1)	
Education	Not High School Graduate	236	53(22.5)	0.043	123(52.1)	0.117
	High School Graduate	191	32(16.8)		83(43.5)	
	Unknown	31	11(35.5)		18(58.1)	
Annual Household Income						
	<$10,000	242	58(24.0)	0.077	117(48.3)	0.584
	$10,000-29,999	126	27(21.4)		66(52.4)	
	$30k+	51	4(7.8)		21(41.2)	
	Unknown	39	7(17.9)		20(51.3)	
# Days Between Seasonal Vaccination and Study Enrollment						
	0-14	22	6(27.3)	0.000	16(72.7)	0.000
	15+	85	40(47.1)		66(77.6)	
	Not Vaccinated	328	48(14.6)		135(41.2)	
	Unsure	23	2(8.7)		7(30.4)	
# Days Between H1N1 Vaccination and Study Enrollment						
	0-14	21	14(66.7)	0.000	16(76.2)	0.000
	15+	57	40(70.2)		47(82.5)	
	Not Vaccinated	359	40(11.1)		156(43.5)	
	Unsure	21	2(9.5)		5(23.8)	

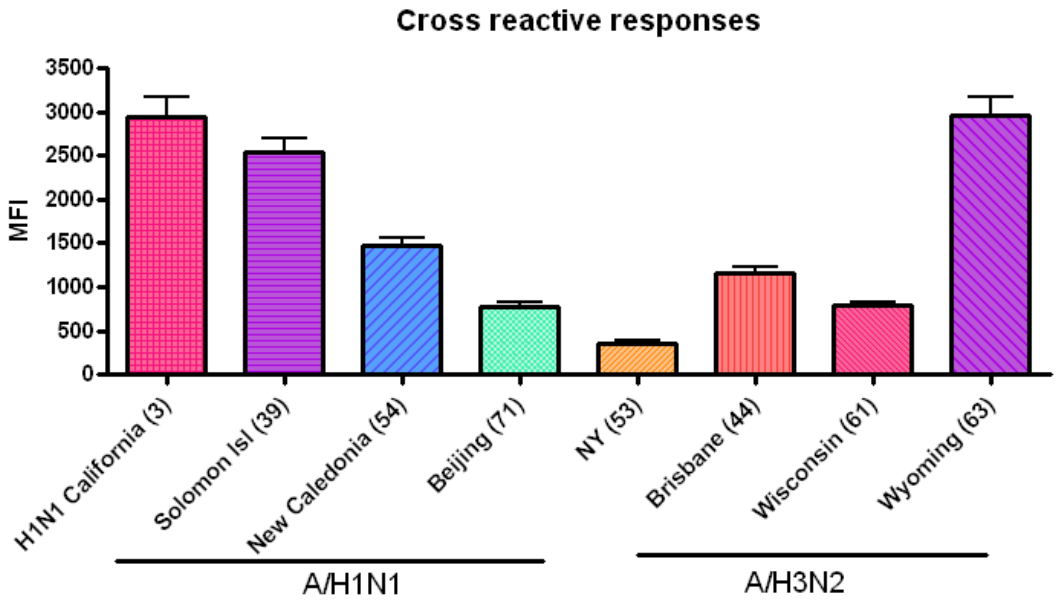


Figure 3.3. Cross reactive responses induced by natural pandemic H1N1infection, against selected related H1N1 strains as well as heterotypic responses against H3N2. Of note is the high rate of antibody positivity to H1N1/Solomon Island. The results represent individuals enrolled in the prenatal seroprevalence study conducted in Manitoba.

3.4.5 IgG antibody response induced by pandemic influenza virus in severely ill individuals

Here, we were interested in addressing the question, whether individuals admitted to ICU with severe pandemic H1N1 infection that necessitates ventilator support, are predisposed to severe disease due to inability to mount an IgG antibody response and are less likely to have cross reactivity to other H1N1. Using the same MBA panel described earlier in this section, we evaluated samples that were collected early, intermediate and late during the ICU stay (day 1; day 5-7 and day 10-14 respectively). All cases had confirmed pandemic H1N1/04/2009 infection, and indeed, we found an increase in IgG1 titres against pandemic A/California (pdmH1N1)/04/2009, with high titres achieved by day 5-7 (Figure 3.4panel A). In addition, a boosting of cross-reactive IgG1 directed against A/Solomon Island/03/06, a previously circulating strain, was detected, albeit, with approximately ten-fold lower titres (Table 3.4, panel B). Similarly, significant increases were noted for A/H1N1 Brisbane, the component of seasonal H1N1 vaccine for 2009, in the absence of exposure, indicating cross-reactive response. Such cross-reactivity was limited to heterosubtypes but wasn't demonstrated for heterotypic strains (Figure 3.4, panel C).

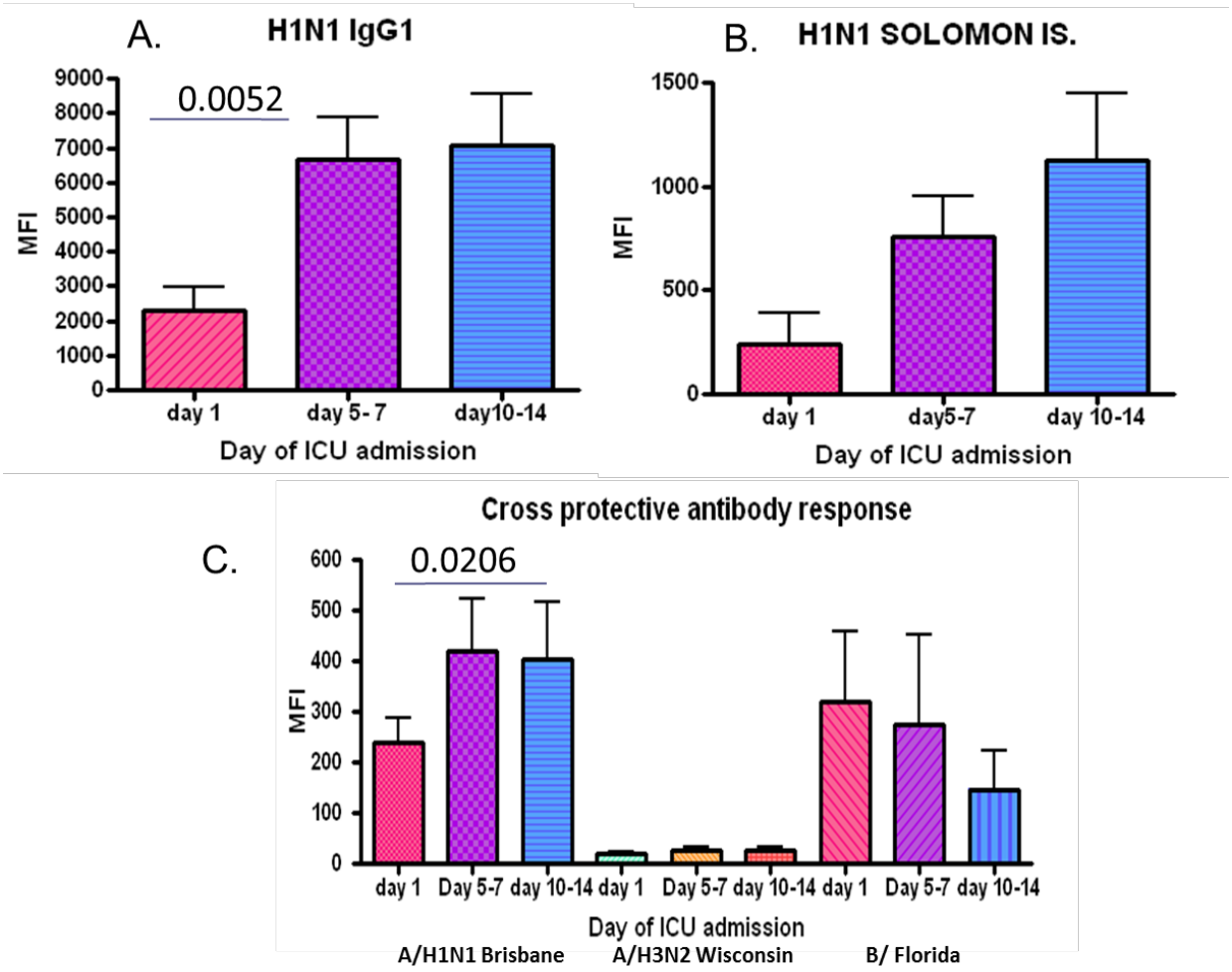


Figure 3.4. IgG1 antibody responses among individuals admitted to ICU with documented pandemic H1N1 infection, 2009/California. The figure depicts IgG1 titres against the infecting H1N1 pandemic strain (A.) measured by MBA. Panel B and C show cross-reactivity with additional strains, the A/H1N1 Solomon Island and A/H1N1 Brisbane. Error bars indicate mean and SEM.

3.5 Summary

We measured antibody responses against vaccine and circulating strains of influenza in diverse cohorts, with three main purposes: to validate the in-house MBA assay for measuring IgG1 responses to influenza HA; to determine the presence of cross-reacting antibodies and their induction upon vaccination or influenza infection and to evaluate the correlation between the ability to mount antibody response and disease severity in critically ill patients infected with pandemic H1N1 influenza during the 2009 early pandemic phase**.** We were able to show comparable sensitivity and potentially a better specificity of the MBA assay using the seroepidmiological studies in Manitobans. We also demonstrated the cross-reactive responses in all studies.

Chapter 4. Influenza-specific T cell function

4.1 Rationale

A CTL response to influenza has been long hailed as the “holy grail” of vaccine development. Although it does not provide complete protection, several features make it attractive: the ability to attenuate the severity of illness; the coincidence of viral clearance with the peak of effector CD8 response [293]; the persistence of antigen-specific memory cells at the site of infection [391]; the ability to provide hetero-subtypic and heterotypic coverage as a result of conserved epitopes; the ability to induce long-lived immunological memory [76, 337, 392, 393]. The ability of TIV and LAIV to induce cross reactive CTL was investigated as one of the components of this project. The repeated exposure to a recurring circulating influenza strain, represented by A/New Caledonia/20/99 offered an opportunity to examine the effect of such repeated encounters with the same strain to induce cell-mediated responses, and the emergence of pandemic H1N1 during the spring of 2009 provided a unique opportunity to study the cross-reactivity of the specific CTLs.

4.2 Hypotheses

- **Inactivated influenza vaccine is capable of limited induction of CTL responses, especially in the context of prior exposure to a recurring influenza strain**
- **Cross reactive CTL responses are strongest after natural infection followed by Live attenuated vaccine and minimal after inactivated vaccine**

4.3 Objectives

- **4.3.1 To measure vaccine strain-specific CTL response in individuals receiving annual inactivated and live attenuated influenza vaccine**

- **4.3.2 To measure cross-reactive CTL responses in individuals receiving live attenuated influenza vaccine**
- **4.3.3 To correlate cross-reactive CTL responses with severity of illness caused by influenza**
- **4.3.4 To characterize the influenza-specific memory subset composition and function after inactivated and live-attenuated influenza vaccines**

4.4 Results

4.4.1 Study Populations

Local seasonal influenza vaccine recipients, detailed in 4.4.2

Flumist seasonal influenza vaccine in healthy commercial sex workers, detailed in 4.4.3

ICU-pandemic influenza virus in severely ill individuals, the study is detailed in 4.4.4.

4.4.2 Local study seasonal influenza vaccine in healthy individuals

In order to address objective 4.3.1, we conducted a study of the antibody and T cell response to seasonal influenza vaccination. Prior to the study, flow cytometry panels were developed and protocols for *in-vitro* stimulation of PBMCs with live influenza viruses were adapted and calibrated. After ethics approval and informed consent, we enrolled 14 healthy adult donors. The mean age of participants was 37.5 years (range 23–60 years). Seven of the volunteers had received influenza vaccine in preceding years and seven had not, Heparinized blood was collected before vaccination and then 7 and 30 days afterwards. Participants received a dose of TIV in the fall, when seasonal vaccines became available. The composition of the TIV seasonal influenza vaccine included the

A/New Caledonia/20/1999 (NC) (H1N1); A/Wisconsin/67/2005 (H3N2) (WISC) and B/Malaysia/2506/2004 (MAL). New Caledonia has been circulating for 6-7 years prior to this study and was a component of TIV for the preceding 6 years. We studied the antigen-specific responses at baseline and following vaccination with each strain**.** To determine the function of CD8+ T cells in response to vaccine components, we used the antibody panels for phenotyping and function (cytokine staining as outlined in ICS staining) as well as proliferation, after 3 days and 6 days of incubation, respectively. The PBMCs were separately incubated with each of the three vaccine strains as previously described. We gated on lymphocytes followed by positive gating on CD3+ and CD8+ cells. Within the CD8+ cells, we gated on cytokine producing cells with IFN-γ and IL-2 as the measured cytokine output. The response of each participant to the newly included vaccine components was compared to the same participant's response to NC, the recurring strain of influenza. We characterized the cellular phenotype and level of immune activation, as well as classified memory subsets of the responding cells. Memory subsets were defined by gating on CD8, and dividing the CD8+ T cells into naïve – CD8+ CD45RA+, central memory (TCM) – CD8+ CD45RA− CCR7+ and effector memory (TEM) cells – CD8+ CD45RA− CCR7−. Memory subsets were determined prior to vaccination and 7 days and 30 days after vaccination with TIV. The distribution of memory subsets within the entire CD8 T-cell population did not change significantly after vaccine administration. The level of immune activation was estimated based on the surface CD38 expression, and the level of activation was measured on each of the memory subsets (naïve, TCM and TEM). The proportions of activated CD8+ T cells were increased significantly after immunization with inactivated influenza virus in the naïve subpopulation only (Figure

4.1). We then compared the IFN-γ production by the three memory subsets pre-vaccination and post- vaccination. We pooled the combined results for all three influenza stimulations (corresponding to the vaccine strains). We found a significant increase in IFN-γ production by CD8+ TEM cells after immunization, whereas the proportion of CD8+ TCM cells that produce IFN-γ showed a significantly decreased response (Figure 4.2). This likely represents the differentiation of central memory T cells into effector cells and effector memory cells.

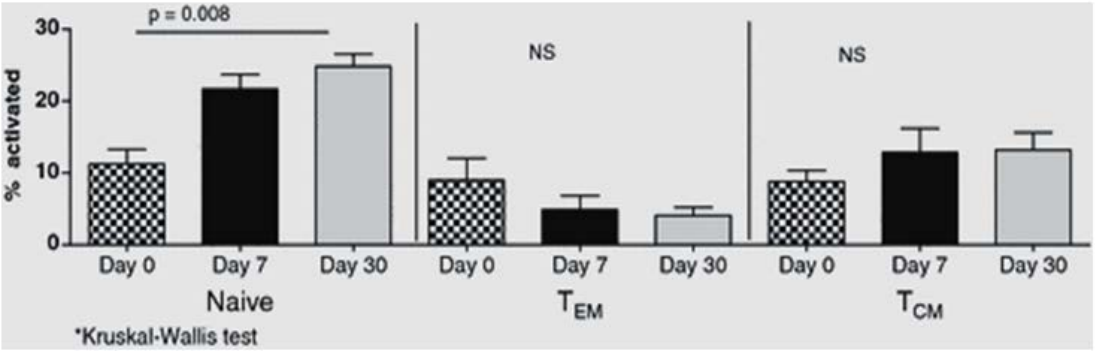


Figure 4.1 [378]. Total level of cellular activation within CD8+ memory subsets. The activation state is expressed as the percentage of CD8+ T cells within the population expressing the activation surface marker CD38; p values represent the trend between three time points using Kruskal–Wallis analysis of variance. NS- not significant. Increase in activation is only observed on naïve CD8+ T cells.

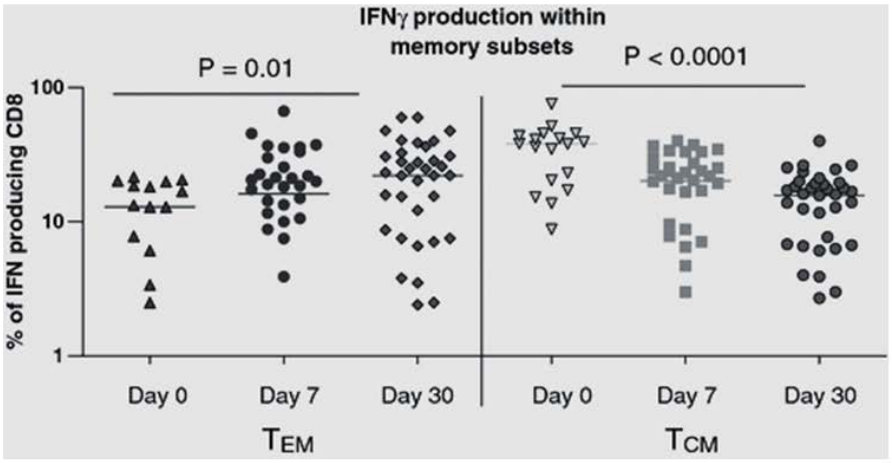


Figure 4.2 [378]. Intracellular staining for Interferon (IFN)-γ response within the CD8+ memory subsets. The responses pre- and post-vaccination to all three vaccine components are plotted together; p values represent the trend between three time points using Kruskal–Wallis analysis of variance.

We then went on to compare the cytokine production by CD8+ T cells in response to the recurring NC component of the seasonal TIV. IFN-γ and IL-2 production were measured after 3 days of incubation with the corresponding NC strain (labeled “old”) and the non-recurring strains (A/Wisconsin and B/Malaysia), marked as ‘new’. Again, pre-vaccination and post-vaccination were compared as depicted in figure 4.3. Gated on IL-2 producing cells, the memory subset of responding T cells was determined according to the classification scheme outlines earlier. The IL-2 produced by TEM in response to ‘new’ components increased significantly ($p=0.0071$) after immunization, whereas IL-2 produced by TCM decreased significantly ($p=0.0006$). Administration of the recurring NC strain did not result in increased IL-2 production within either subset in response to *in-vitro* corresponding strain stimulation. A similar observation was recapitulated when IFN-γ production was measured, again showing significant rise in the production among TEM (Figure 4.3 panel B). This project examined the cytokine production within memory subsets, in response to immunization. The ability to boost the functional capacity, measured as IL-2 and IFN-γ production, was limited to the CD8+ effector memory subset, with a corresponding decrease within the central memory compartment. This increased functional capacity was only induced by strains of influenza that were relatively new, and included in the TIV formulation for the first time. Commensurate increases were not induced by the recurring strain that was circulating and included in previous year’s vaccines.

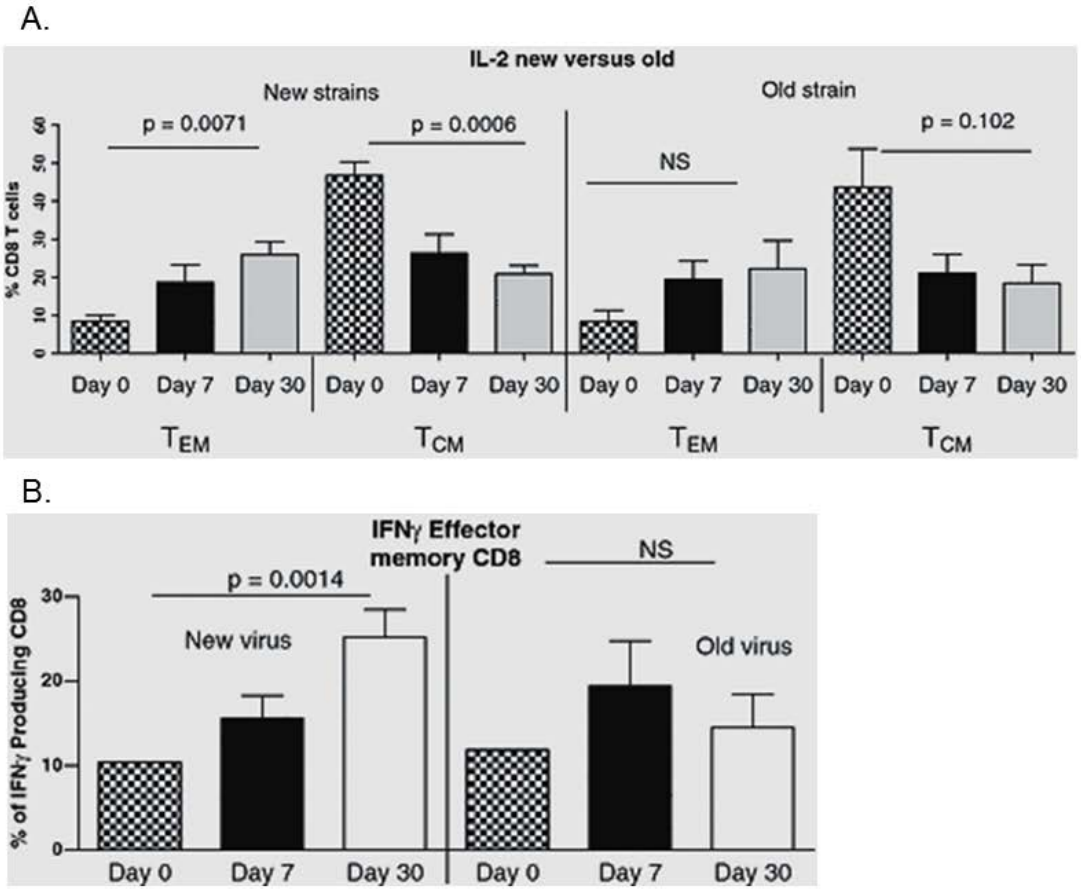


Figure 4.3 [378]. IL-2 and IFN-γ antigen specific CD8 responses. A. Interleukin (IL)-2 responses within memory subsets upon stimulation with the 'new' strains (A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004) compared to the 'old' strain (A/New Caledonia/20/1999). The responses are expressed as the percentage of CD8+ T cells of that memory class (either TEM or TCM); B. IFN-γ antigen-specific response of TEM is measured following stimulation of PBMCs with the old and new strains. The responses are expressed as the percentage of memory CD8+ T cells. p values represent the trend between three time points using Kruskal–Wallis analysis of variance.

4.4.3 Flumist study-seasonal LAIV in healthy commercial sex workers

In order to address the second component of objective 4.3.1, and 4.3.2, we utilized a study that was conducted in Nairobi, Kenya. The study took place in Majengo Clinic, in Nairobi, in a cohort of commercial sex workers. Prior to the study, community and participant's acceptance were assessed. After feasibility was favourably assessed, adaptation of laboratory and clinical specimen collection methods and ethics approval, the study was launched in the fall of 2009. Based on the phenotype and immunogenetic correlates of protection identified during >15 years of intensive research in a cohort of highly exposed commercial sex workers (CSW) that remained HIV uninfected (highly exposed seronegative-HESN), the study was designed to examine the innate and adaptive response of these women to an unrelated viral pathogen administered mucosally. Since the chosen antigen was Flumist- an FDA approved, safe, live-attenuated influenza virus. It offered an opportunity to characterize the early mucosal and systemic responses to live influenza virus. In addition, the onset of the 2009 influenza pandemic, prompted us to try and measure the cross protective responses to this emerging H1N1 strain, not included in the seasonal vaccine that was administered. After giving concent, we enrolled 60 CSWs. Baseline samples were obtained followed by intranasal LAIV administration. The vaccine was well tolerated. Analysis of the questionnaire completed by study participants at the time of each of the sample collection disclosed 4 reports of runny nose; 4 nasal congestion; 37 reports of headache (all visits included)- these events were not temporally related to vaccine. Three individuals reported cough; runny nose beginning within 3 days of vaccine administration, presumed to be vaccine related.

We preformed flow cytometry on cryopreserved PBMCs using 3 panels as outlined in table 2.3 of the Methods section. We sought to determine the phenotype, memory subset, cytokine production, activation and proliferation of T cells after vaccination, following stimulation with the vaccine strain of H1N1 (A/Brisbane/10/2007) and with the pandemic strain A/Mex/04/2009 to determine cross-reactive responses. All experiments involving the pandemic strain took place in a level 3 laboratory. Figure 4.4 depicts the gating strategy used to determine the activation and cytokine production induced by *in-vitro* stimulation with the aforementioned influenza strains.

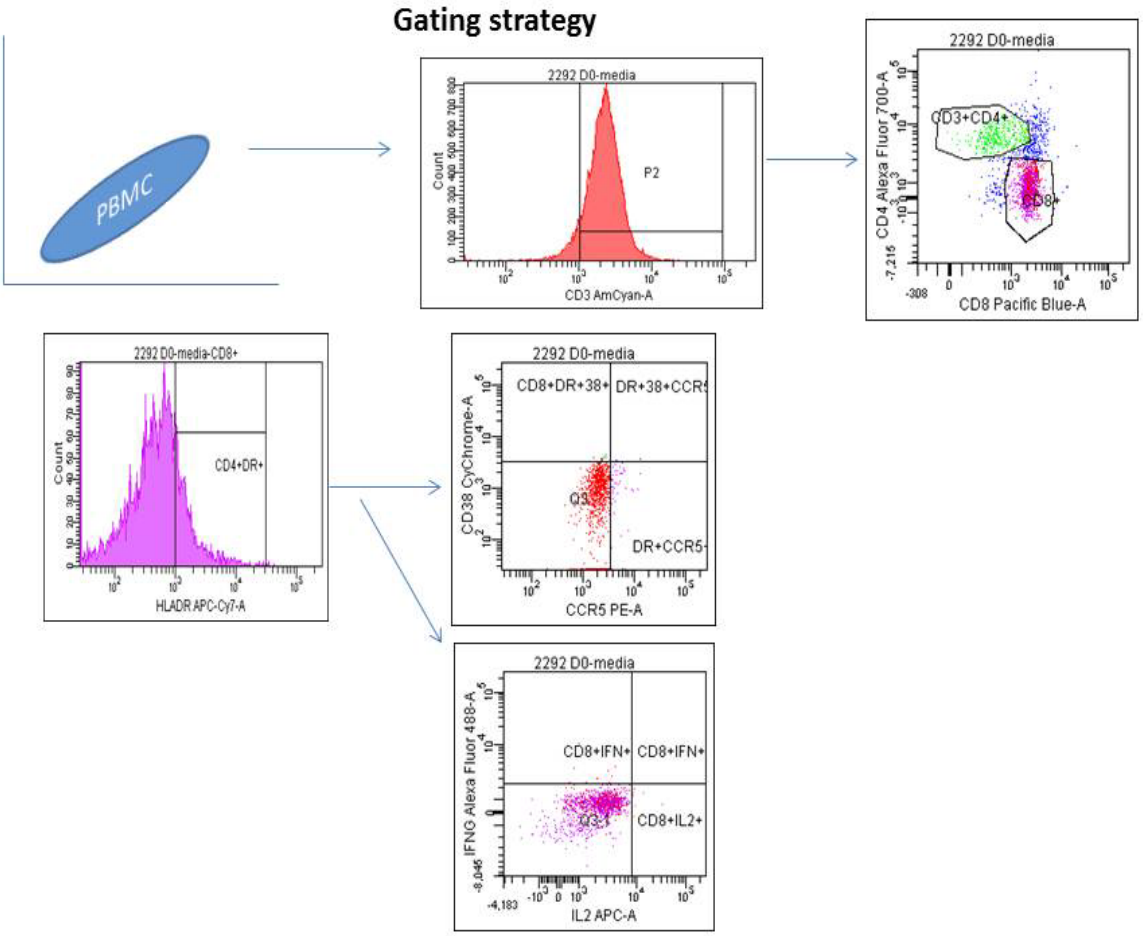


Figure 4.4. Flumist vaccine recipient gating strategy for the day 3 stimulation experiments. Gating started on PBMCs, followed by CD3+. The CD3+ T cells were further characterized by CD4/CD8 surface expression. Within the CD4/CD8 subsets activation was determined based on surface expression of HLA-DR, CD38 and CCR5, individually or in combination. In addition, IFNγ and IL-2 intracellular staining positive cells were determined within the CD4/CD8 populations.

Activation of the CD4 and CD8 T cells was performed according to the gating strategy depicted in Figure 4.4. We compared the surface expression of the activations markers CD38, HLA-DR and CCR5, individually or in combination, on both CD4 and CD8 cells before and after administration of the LAIV, Flumist. Increase in accumulation of activation markers is thought to represent the expansion phase of antigen-specific response [384, 385], as noted earlier in this chapter.

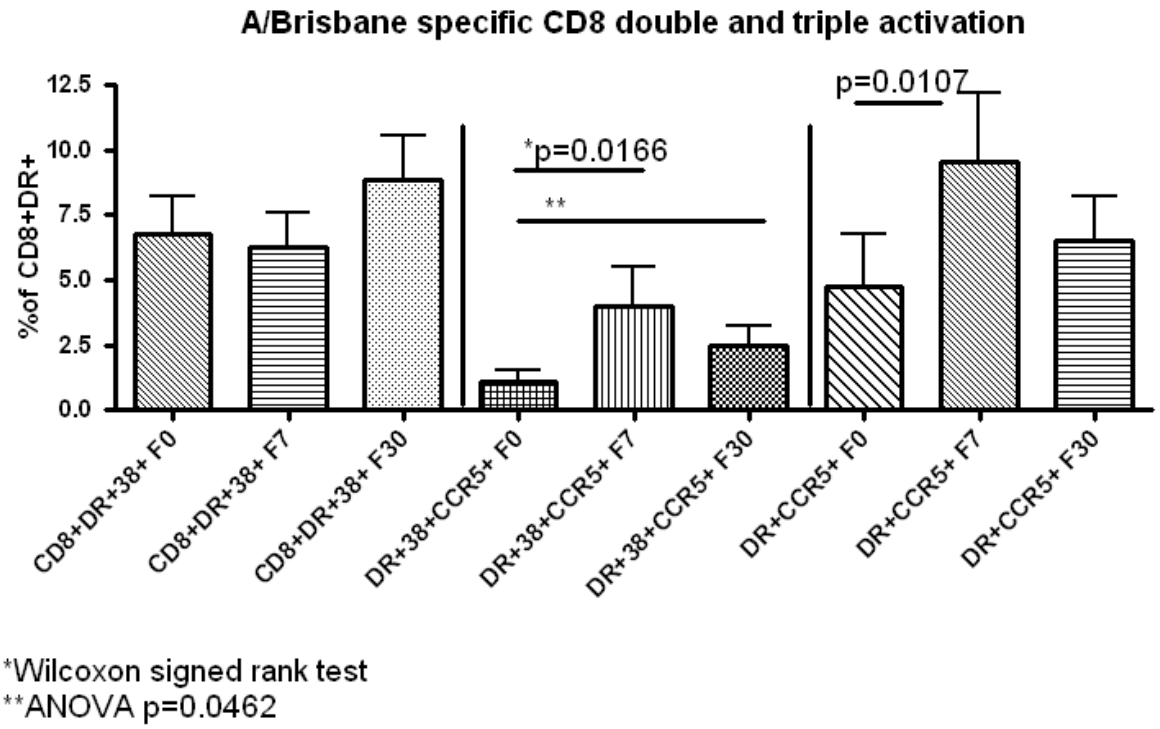


Figure 4.5. The expression of multiple activation markers on CD8+ T cells. The left third of this graph depicts the co-expression of HLA-DR and CD38 on CD8 T cells following stimulation with A/Brisbane- the H1N1 component of the LAIV administered. No significant increase could be demonstrated.

The middle graphs illustrate the triple expression of activation markers, namely: DR/CD38/CCR5 on the surface of CD8+ T cells. There is a significant increase in the number of of cells expressing the triple activation markers by day 7 after LAIV administration, with a non-significant decrease by day 30 after vaccine administration (p=0.0166, paired, non-parametric, Wilcoxon's matched pair signed rank test). The right third of the graph shows the increase in percent of CD8+ cells that co-express HLA-DR and CCR5 7 days after vaccination (p=0.0107, paired, non-parametric, Wilcoxon's matched pair signed rank test).

F0-before vaccine administration; **F7**- seven days after LAIV; **F30**- 30 days after LAIV.

We next sought to determine the cross reactive T cell responses that are induced by seasonal live attenuated influenza vaccine. We measured baseline (pre administration of Flumist) T cell responses to the 2009 pandemic H1N1, to control for exposure or pre-existing cross-reactivity that might have been present (the study was conducted in the fall of 2009, several month after the declared 2009 pandemic). We applied the same flow cytometry protocols and antibody panels, and stimulated *ex-vivo*, using live influenza A/Mex/04/2009, a strain isolated during the early pandemic in Mexico. Figure 4.6 shows the antigen specific responses, measured after stimulation with the pandemic strain, not included in the LAIV that was administered.

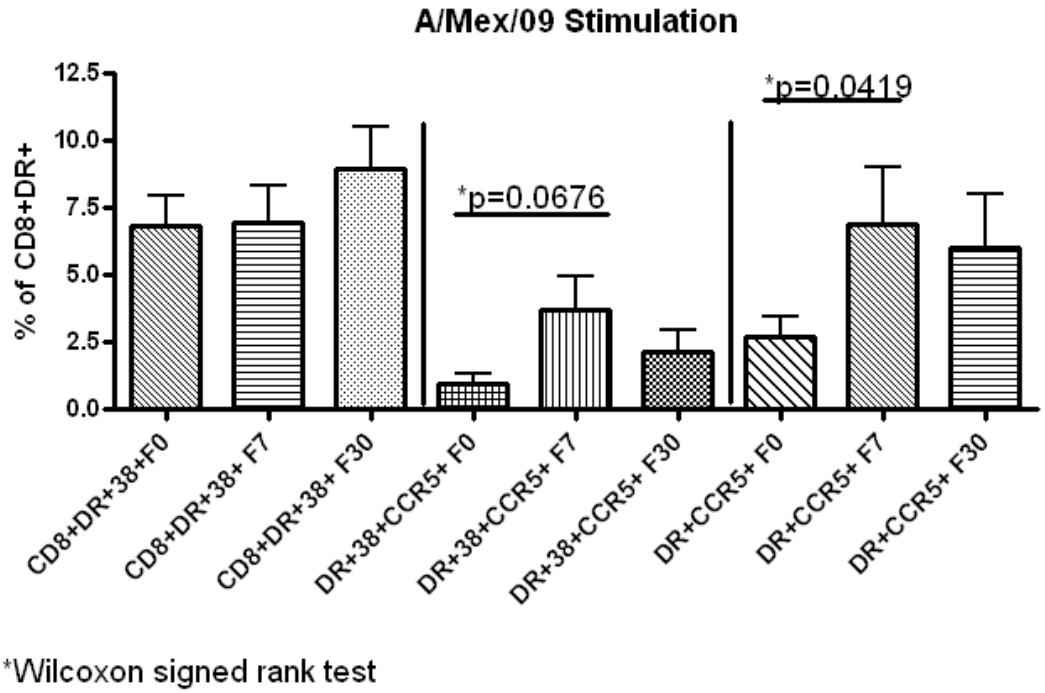


Figure 4.6. The expression of activation markers on CD8+ T cells before and after incubation with A/Mex/04/09. The left third of this graph, depicts the co-expression of HLA-DR and CD38 on CD8 T cells following stimulation with A/Mex/04/09- the H1N1 pandemic strain not included in the LAIV administered. No increase in CD8+HLA-DR+CD38+ could be measured after vaccination. In contrast, the middle bars, show a trend towards increase in co-expression of the three activation markers together, namely, DR/CCR5/CD38. The bars on the right hand side of the figure illustrate a significant increase in the co-expression of HLA-DR and CCR5 on the surface of CD8 T cells, 7 days after Flumist was administered.

In addition, we measured the production of intracellular cytokine after ex-vivo stimulation with the vaccine H1N1 strain or pandemic H1N1. We compared the ratio of perforin, IL-2 and IFNγ producing CD4 and CD8 T cells after *ex-vivo* stimulation, before and after immunization. Figure 4.7 shows the measured cytokines pre-LAIV and post-LAIV administration. Panels A-C show the perforin and IFNγ staining on CD8 T cells at day 0, 7, and 30 when the antigen used was the A/Brisbane/10/2007, a component of the LAIV used for this study. Panels D-F show the production of the same cytokines, after stimulation with the pandemic A/Mex/04/09 strain, that was not included in the vaccine formulation. The results from all individuals that had sufficient viable cells, and in which positive control (PHA/SEB) stimulation had cytokine responses, were included in the analysis. We were not able to find measurable increase in IL-2, IFNγ or perforin production after stimulation with either the vaccine strain or pandemic H1N1. The summary of the results of flow-cytometry experiments, including the phenotyping, ICS and proliferation panels is shown in Table 4.1.

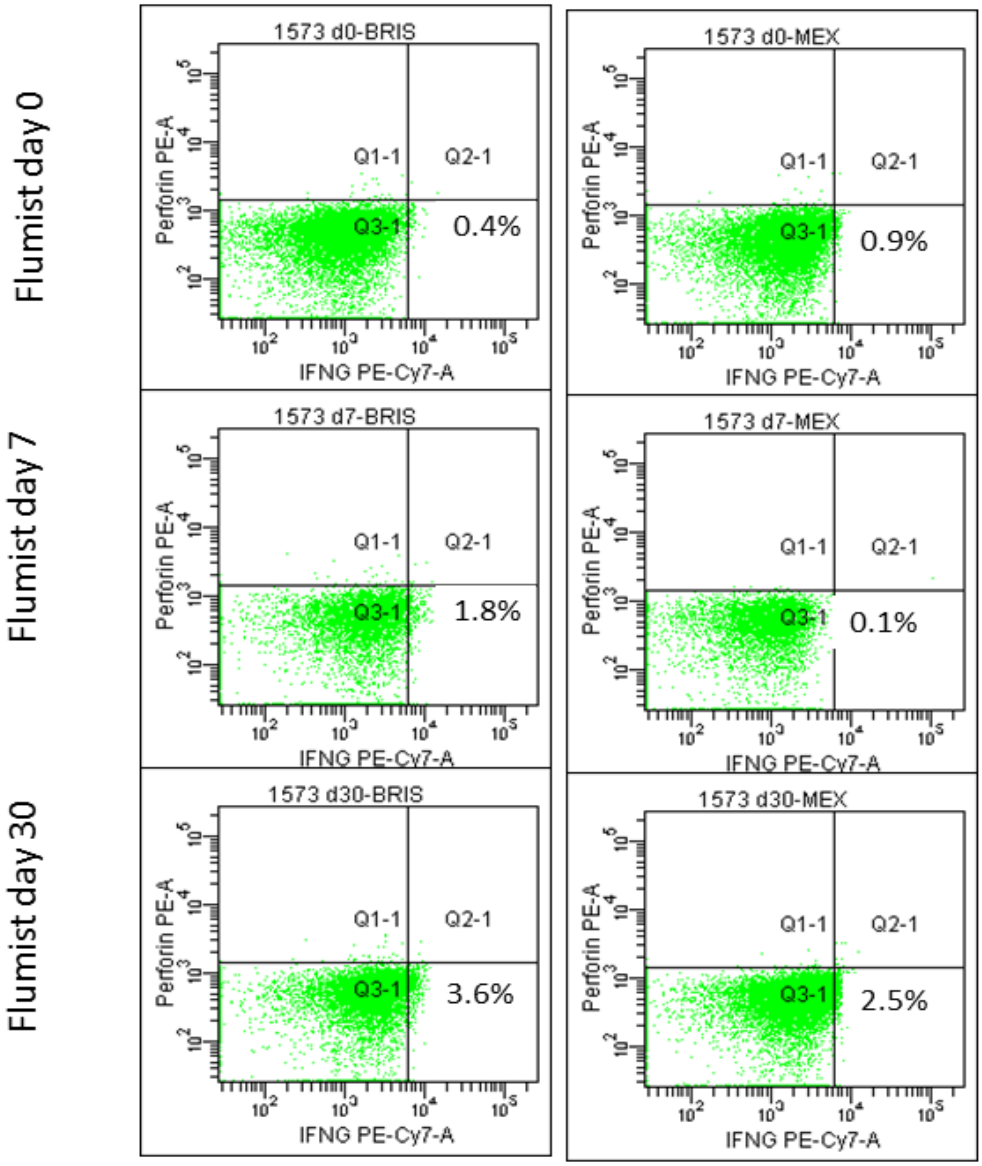


Figure 4.7. Measured cytokines using ICS pre and post LAIV administration. Panels A-C show the perforin and IFNγ staining on CD8 T cells at day 0, 7, and 30 when the antigen used was the A/Brisbane/10/2007, a component of the LAIV used for this study. Panels D-F, show the production of the same cytokines, after stimulation with the pandemic A/Mex/04/09 strain on days 0, 7 and 30.

Table 4.1. Summary of the results of flow-cytometry experiments, including the phenotyping, ICS and proliferation.

Measure-systemic	Influenza response-Vaccine strain A/Brisbane/10/2007	Non-vaccine strain (cross reactive)-A/Mex/04/2009
Activation	• increase in antigen specific triple activated CD8 • Significant antigen specific increase in DR+CCR5+CD8 in response to A/Brisbane	• Significant antigen specific increase in DR+CCR5+CD8 in response to A/Mex/04/09 • Trend towards increase in antigen specific triple activated CD8
ICS for cytokines	• Limited measurable effect of vaccine	No difference
Proliferation	No difference	No difference

In addition to the phenotype and ICS panels we attempted to ascertain the functional response of NK cells. In brief, we gated on CD3- PMBCs, and within this gate, went on to gate on CD56+ and CD16 bright and dim populations. We used CD69 as an activation marker, CD107 as marker of degranulation and perforin as the cytokine output of functioning cells. We had limited recovery, and in this analysis, only 13 participants had sufficient NK population for the purpose of the study. We applied the same gating and a batch analysis and compared the perforin, CD69 and CD107 expression at baseline and after LAIV administration.

There were non-significant differences in the levels of activation, perforin production or degranulation marker expression on the CD16 bright NK subpopulation. The surface expression of CD107, a degranulation marker, on CD3-CD56+CD16 dim subset of NK cells, after *ex-vivo*, overnight stimulation with influenza A/Brisbane/10/2007, was increased 7 days after the administration of LAIV.

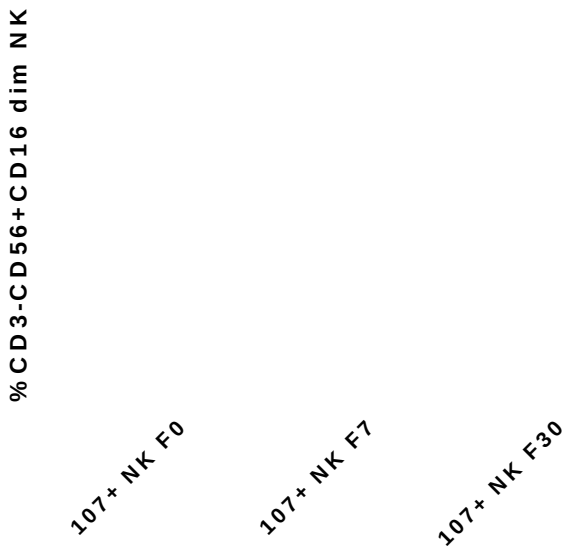


Figure 4.8. CD107+ CD56+CD16 dim NK. Seven days after LAIV, stimulation with the cognate vaccine H1N1 strain, led to increase in surface expression of degranulation marker. p=0.0186, non-parametric, Wilcoxon matched-pairs signed rank test.

4.4.4 ICU-pandemic influenza virus in severely ill individuals

This study was embedded in a multi-centre study aimed at characterizing the clinical features of the outbreak of pandemic (H1N1) 2009 in Canada. We obtained whole blood from critically ill individuals admitted to intensive care units in Winnipeg, enrolled in the observational study. The research was approved by the local research ethics board. We were able to obtain blood samples from patients with laboratory-confirmed pandemic (H1N1) 2009, however, PBMC recovery was variable and we elected to perform a study focused on surface staining for phenotypic analysis. The limited PBMC recovery precluded the performance of intracellular staining. The samples included: 34 individuals, 22 women, 6 fatalities overall, average age was 48.5 (range-22-73). BMI- 36.31, and Ethnicities consisted of: Caucasian-22; First nations-9; Asian- 2; other 1. Six of the individuals succumbed to the infection. Age, BMI and ethnicities were not predictive of the likelihood of fatal outcome in our substudy. The markers of T cell activation CD38/CCR5 and HLA-DR on CD4 and CD8 T cells were used as surrogate markers for the expansion of contraction phases of the cell-mediated response [384, 385]. We gated on PBMCs, followed by gating on CD3+. The CD3+ T cells were further characterized by CD4/CD8 surface expression. Activation within the CD4/CD8 was determined based on surface expression of CD38 and CCR5, individually or in combination. Figure 4.9 and 4.10 depict the activation on CD4 T cells and CD8 T cells, respectively. The surface expression of CD38 on CD4 T cells did not increase significantly, nor did the percentage of CD4+ T cells expressing the activation marker CCR5. A trend ($p=0.06$) to increased surface co-expression of CD38 and CCR5 was seen

on the CD4 T cells, when the earlier time point (first day of admission) was compared to the second sample obtained on day 3-5 (Figure 4.9).

The surface expression of CD38 on CD8+ T cells, did not significantly increase. In contrast the percentage of CD8+ cells expressing CCR5 increased significantly upon re-sampling 3-5 days into the ICU admission (Figure 4.10).

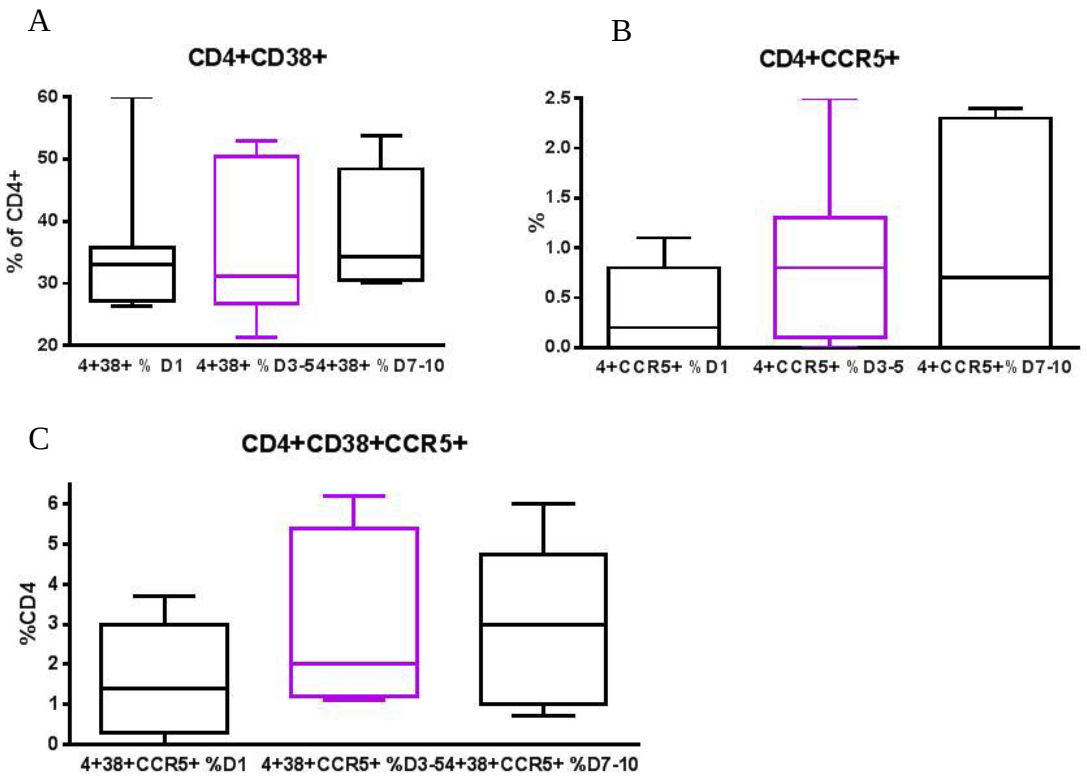


Figure 4.9. Activation of CD4 T lymphocytes among individuals admitted to ICU with pdmH1N1. Clockwise from upper left: A.CD4+ T lymphocytes expressing CD38 are not significantly increased 3-5 days into admission. B. CD4+ T cells expressing surface activation marker, CCR5 appear to increase over the first 3-5 days of ICU admission, however the increase was not statistically significant. C. CD4+ T cell co-expressing CD38 and CCR5 show a trend towards increase by day 3-5 of admission (p=0.06, paired, non-parametric Wilcoxon's matched pair signed rank test).

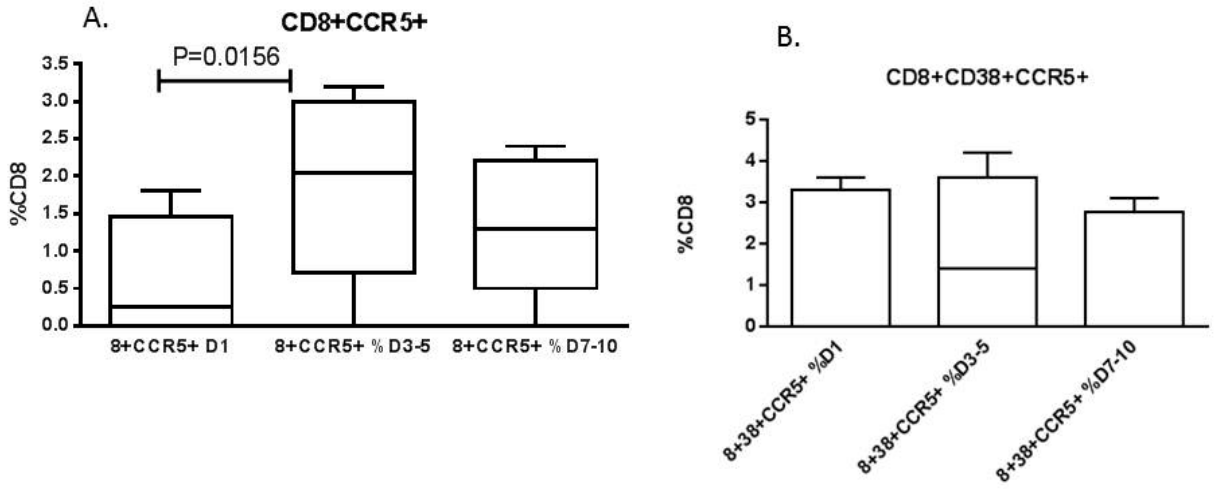


Figure 4.10. Activation of CD8 T lymphocytes among individuals admitted to ICU with pdmH1N1. Panel A. CD8+ T lymphocytes expressing CCR5 are significantly increased 3-5 days into admission, with no further increase by day 5-7 (p=0.0156, paired, non-parametric Wilcoxon's matched pair signed rank test). B. CD8+ T lymphocytes co-expressing CD38 and CCR5 represent a smaller proportion of CD8+ cells and did not increase significantly over time.

4.5 Summary

We were able to prove the first hypothesis of this project, stating that inactivated influenza vaccine is capable of limited induction of CTL responses, especially in the context of prior exposure to a recurring influenza strain. Using the study in healthy volunteers, we were able to measure an increase in IL-2 and IFNγ production in response to new vaccine strains. In contrast, the strain that was previously circulating and included in vaccine preparations for several years, did not induce a measurable boosting of the cell mediated responses.

In the Flumist project, we were able to show that administration of LAIV resulted in expansion of activated T cell populations, with significant increases in the surface markers on CD8 T cell 7 days after LAIV was administered. The increased activation was demonstrated using ex-vivo stimulation with the corresponding H1N1 contained in the vaccine formulation. In addition, we sought to determine whether LAIV results in cross-reactivity when the ex-vivo stimulus was the 2009 pandemic H1N1, not included in the vaccine. We demonstrate the expansion of CD8 activated cell population, by day 7 after vaccine administration. The measurable increase in triple expression of HLA-DR/CD38/CCR5 and the significant increase of CD8 T cells co-expressing HLADR/CCR5 are consistent with the timing of expansion seen in other studies with flaviviral, and pox virus models or influenza [385, 394, 395].

ICU- This aspect of the project was limited by the specimens we were able to obtain. The use of blood specimens that were “leftover” decreased the quantity of viable

PBMC's and rendered the ability to conduct functional analysis, suboptimal. However, we were able to document the expansion phase of CMI, as manifested by up-regulation of the surface expression of surface markers. Despite limited sample size, we were able to demonstrate an increase in CCR5, chemokine receptor and activation marker on CD8+ T cells by the 3-5 days of ICU admission and a trend towards increased double positivity (CD38/CCR5 co-expression) on CD4+ T cells at the same time point. However, we were not able to correlate the CTL response with the severity of illness caused by the pandemic H1N1 strain.

Chapter 5. Systemic Cytokine responses induced by influenza infection and vaccine

5.1 Rationale

To complement the previous objectives, we sought to measure cytokine levels induced by natural influenza infection and influenza vaccine. In addition we were interested in measuring the cytokine response in systemic and mucosal compartments following the administration of LAIV, delivered intra-nasally. We also examined the antigen specific response by measuring the cytokine levels in supernatant of PBMCs that were stimulated with influenza viruses, from samples obtained prior to and after vaccination.

5.2 Hypotheses

- **LAIV administered intra-nasally results in increased levels of proinflammatory cytokines and chemokines systemically**
- **LAIV administered intra-nasally results in increased levels of proinflammatory cytokines measured in cervicovaginal lavage**
- **Pandemic H1N1 influenza infection results in cytokine "storm" and the levels of cytokines correlate with clinical outcomes**

5.3 Objectives

- **5.3.1 To measure systemic and mucosal cytokines/chemokines induced by LAIV**
- **5.3.2 To measure vaccine strain specific cytokine/chemokine production after LAIV**

- **5.3.3 To measure the cytokine/chemokine production after stimulation with hetero-subtypic influenza virus stimulation among vaccine recipients**
- **5.3.4 To measure the kinetics of cytokine responses in severe pandemic H1N1 infected individuals admitted to ICU.**
- **5.3.5 To correlate cytokine responses with the clinical outcomes in severe pandemic H1N1 infected individuals admitted to ICU.**

5.4 Results

5.4.1 Study populations

5.4.2 Concentrations of cytokines and chemokines induced by LAIV

In order to address the second component of objective 5.3.1-5.3.3 we used the study that was conducted in Nairobi, Kenya as previously described. For this aim, we included the 60 CSWs included in the study. We obtained blood and cervicovaginal lavage prior to vaccine administration and measured cytokine and chemokine concentrations using the panel of 19 cytokines/chemokines (section 2.2.5a).

We measured plasma cytokines before and after vaccination. The cytokine/chemokine levels in unstimulated plasma samples were compared to pre-vaccine levels using ANOVA to detect a trend between day 0, 7 and 30 and with non-parametric repeated measures Wilcoxon signed-rank test to compare 2 time-points. Cytokine levels that were low or undetectable in plasma for more than a 1/3 of samples were not further analyzed. These included IL-1ra, IL-12 (p40), IL-12 (p70), sIL-2ra, IL-15 and IL-17. Selected cytokines/chemokines are represented in Figure 5.1, no significant increases could be detected in un-stimulated samples.

In addition, we collected supernatents from *in-vitro* stimulation experiments that are described in detail in section 4.4.3. We measured the concentrations of the same panel of 19 cytokines/chemokines before and after vaccine administration and in response to stimulation with the matching A/H1N1 strain (A/Brisbane/10/07) and the non-vaccine, pandemic A/H1N1/Mex/04/09. Figure 5.2 shows the results of selected cytokines and chemokines. Most cytokine concentrations were not increased following the stimulation with the matching vaccine strain, A/Brisbane/59/07.

In addition, cervico-vaginal lavage (CVL) was collected prior and after vaccine administration. We measured the same 19 cytokines using the same cytokine/chemokine platform. The cytokine concentrations were highly variable and we could detect no significant changes following the administration of LAIV (Figure 5.3).

Figure 5.1. Unstimulated plasma cytokines before and after influenza seasonal LAIV. Panel A depicts the cytokines IL-6 and IL-10, and the chemokines MIP 1 alpha and beta. Although levels were measurable in the majority of participants, measurable increases in cytokines/chemokines follwing vaccine administration could be detected in plasma (Bar from left to right- at baseline, 7 days and 30 days after LAIV). B. represents the pro-inflammatory cytokines IFNγ and TNF alpha; again, no significant rise in concentration could be detected following LAIV administration.

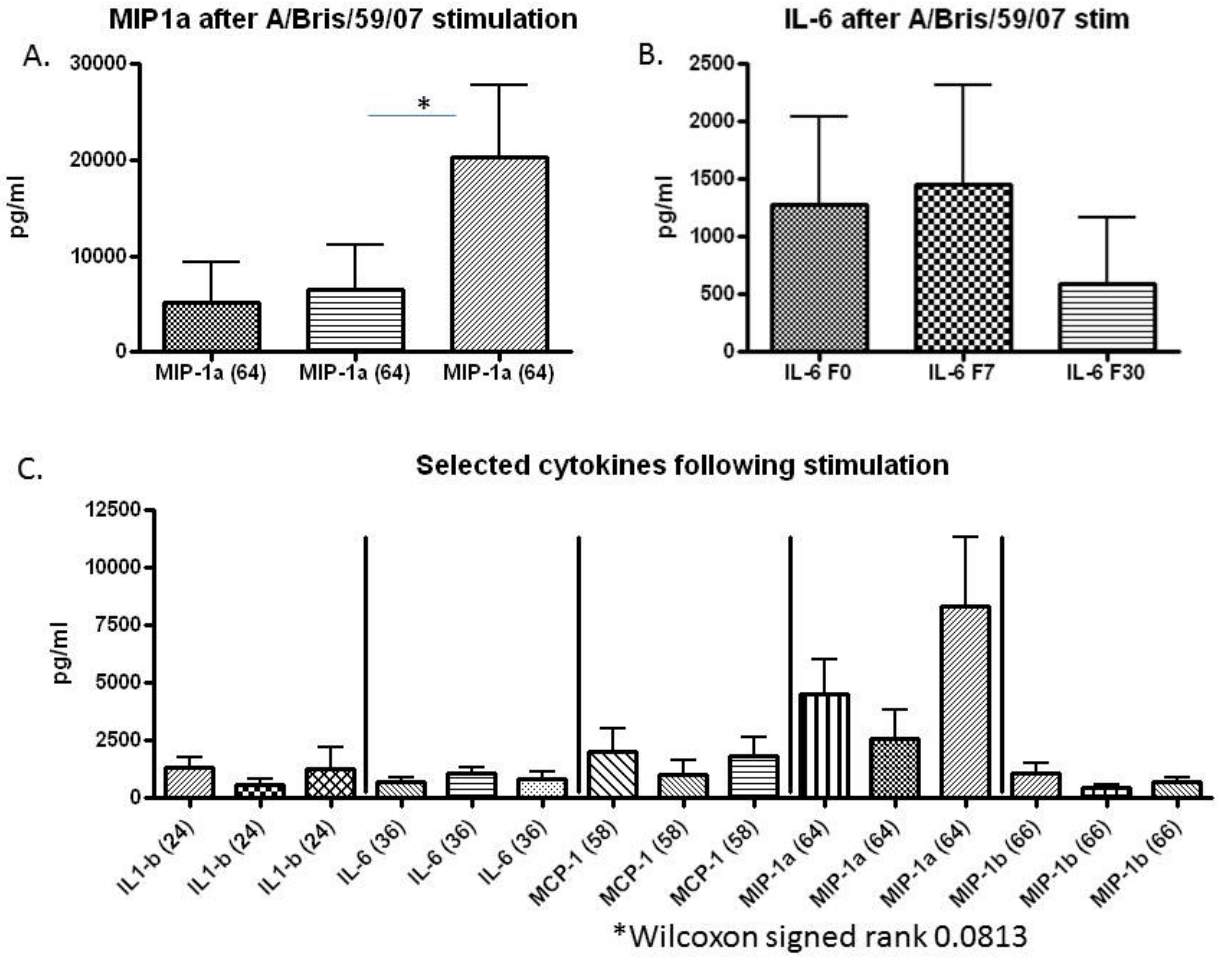


Figure 5.2. Stimulated plasma cytokines, before and after influenza seasonal LAIV. Panel A depicts the cytokine MIP 1 alpha concentrations after stimulation with the vaccine strain A/Bris/59/07. A trend towards increase MIP 1 alpha is seen (p=0.08). Figure B. shows IL-6 concentrations when PBMCs were stimulated with the vaccine strain, no significant increase could be measured. Concentrations of IL1 beta, MCP1, MIP 1 beta, were not altered significantly after in vitro stimulation with the vaccine strain.

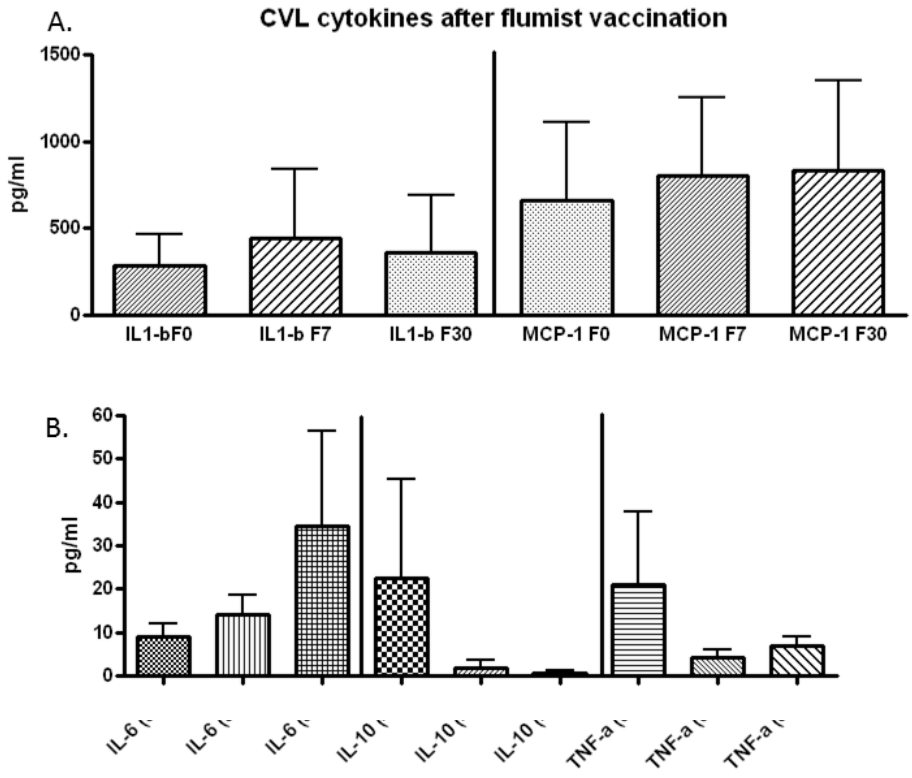


Figure 5.3. Selected concentration of cytokines in the CVL, prior to and 7 and 30 days after administration of intranasal LAIV. Cytokine concentrations were highly variable and no significant effect of vaccine could be measured in terms of CVL cytokines/chemokines.

5.4.3 Concentrations of cytokines and chemokines in severely ill individuals

To address the objectives in 5.3.4 and 5.3.5, we obtained plasma from individuals enrolled in the multi-centre collaboration of intensive care units, aimed to characterize individuals requiring ICU admission due to severe pandemic H1N1 influenza during the 2009 outbreak. The total number of individuals included in this analysis was 34. The inclusion criteria for this analysis were: the availability of plasma samples on the first day of ICU admission, the availability of repeated sample on day 2 and day 4. We used the multiplex cytokine assay described earlier, and excluded cytokines where the concentrations were low or undetectable in plasma for more than a 1/3 of samples. These included IL-1ra, IL-12 (p40), IL-12 (p70), sIL-2ra, IL-15 and IL-17.

We performed ANOVA tests for trend in cytokine concentrations across the three time points as well as two-tailed, Wilcoxon matched-pairs signed rank test to compare cytokine/chemokine concentrations between 2 points during the ICU admission. Results for selected cytokines are depicted in Figure 5.3. Moderate increases in IL-6, MCP-1, soluble CD40 ligand and IP-10 are seen between the first and second days of ICU admission, however the differences do not reach statistical significance. The fact that not all cytokines were detected in every individual and each time point, precluded performing repeated measure analysis. For interferon 2α and interferonγ the peak cytokine concentration was seen on the second day into the ICU course with subsequent trend to declining plasma concentrations (5.4 E,F)

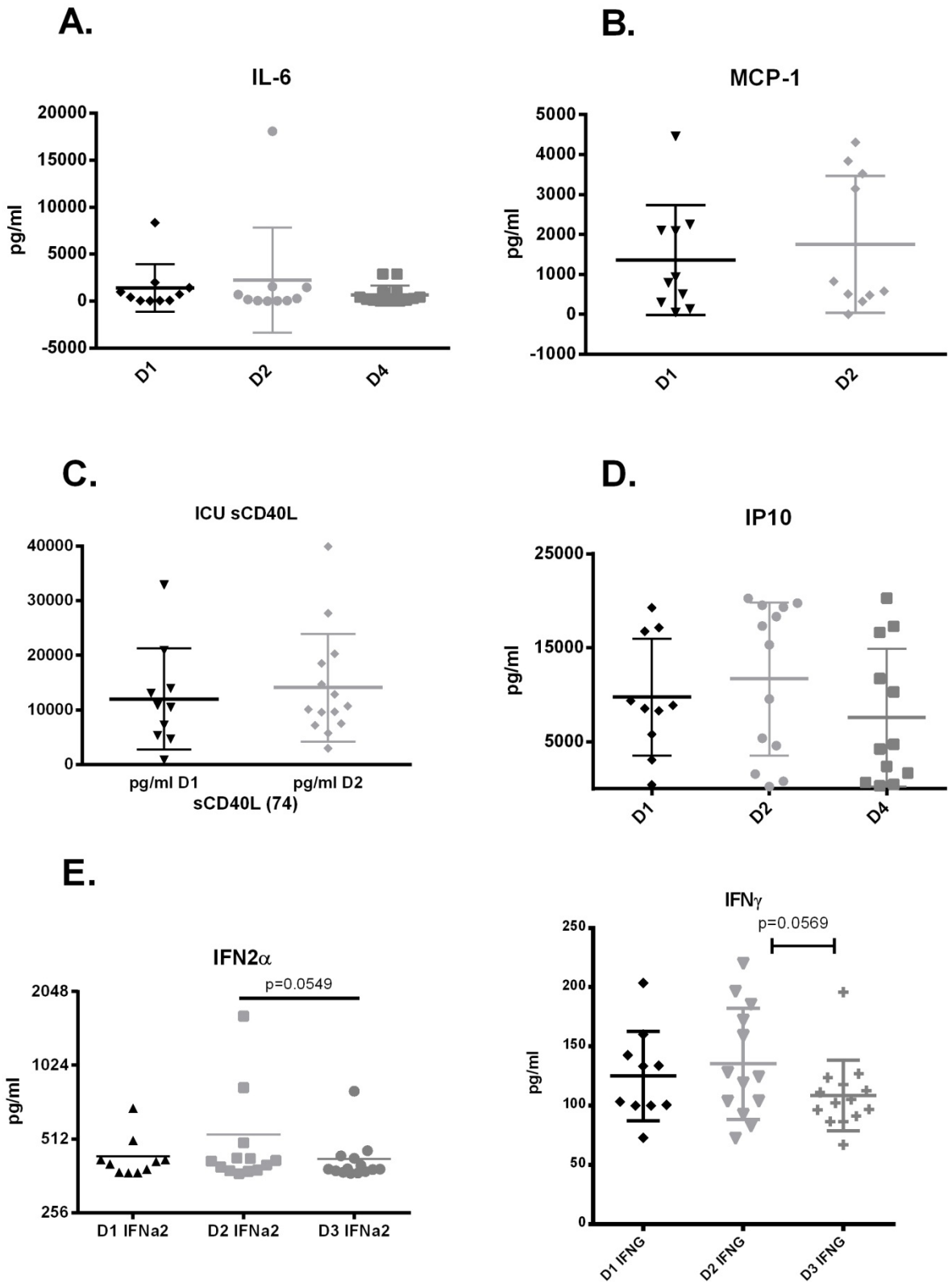

A.
IL-6
pg/ml
20000
15000
10000
5000
0
-5000
D1
D2
D4
B.
MCP-1
pg/ml
5000
4000
3000
2000
1000
0
-1000
D1
D2
C.
ICU sCD40L
40000
30000
20000
10000
0
pg/ml D1
pg/ml D2
sCD40L (74)
D.
IP10
pg/ml
25000
15000
5000
D1
D2
D4
E.
IFN2α
p=0.0549
pg/ml
2048
1024
512
256
D1 IFNa2
D2 IFNa2
D3 IFNa2
IFNγ
p=0.0569
pg/ml
250
200
150
100
50
0
D1 IFNG
D2 IFNG
D3 IFNG

Figure 5.4. Plasma concentrations of selected cytokines and chemokines among individuals with laboratory confirmed pdmH1N1 infection necessitating ICU admission. IL-6 concentrations on day 1, 2 and 4 in Panel A. MCP-1, soluble CD40 ligand and IP-10 are represented by panels B-D, respectively. An increase in all 4 chemokines/cytokines is seen between the first and second days of ICU admission, however the differences do not reach statistical significance. Concentrations of interferon 2α and interferonγ on day 1-3 into the course in ICU illustrate that the peak cytokine concentration was seen on the second day into the ICU course with subsequent trend to declining plasma concentrations (panels E and F, respectively).

We next sought to determine if the cytokine and chemokine concentrations were correlated with the presence of well-established risk factors for influenza disease severity and with the clinical outcomes. Twenty eight of the individuals recovered from the infection and survived beyond the first month of the study while 6 succumbed to the infection.

Table 5.1 captures the demographic parameters, comparing individuals with favorable outcome to those that did not survive the ICU admission. We attempted to examine predictors of adverse outcome by correlating the demographic and clinical information as well as the cytokine/chemokine concentrations with the endpoint of fatality or survival. The comparison was limited but the good outcomes experienced by 28/34 individuals. Again we used the multiplex cytokine assay described earlier, and excluded cytokines for which concentrations were low or undetectable in plasma in more than a 1/3 of samples. We performed non-parametric, t-tests to compare cytokine/chemokine concentrations between each time point for fatal versus non-fatal cases. Insufficient numbers of participants had detectable concentrations of IL-12 (p40), IL-12 (p70), sIL-2ra, IL-15 and IL-17, and these were omitted from further analysis. No differences in plasma concentrations of TNF, MCP1, MCP3, IFNα, IFNγ could be found between fatal cases and individuals that recovered. Figure 5.5 depicts selected cytokines and chemokines. Of note is the finding of significantly higher plasma concentrations of s IL-ra among fatal outcomes, on the day of ICU admission and at day 3 into the stay in ICU.

Table 5.1. Age and BMI of fatal cases compared with survivors. There was no significant difference in mean age or BMI.

Fatal Outcome	**Number of Observations**	**Mean age**	**Min age**	**Max age**	**Mean BMI**
Yes	28	47.55	22	73	36.42
No	6	52.5	40	73	35.79

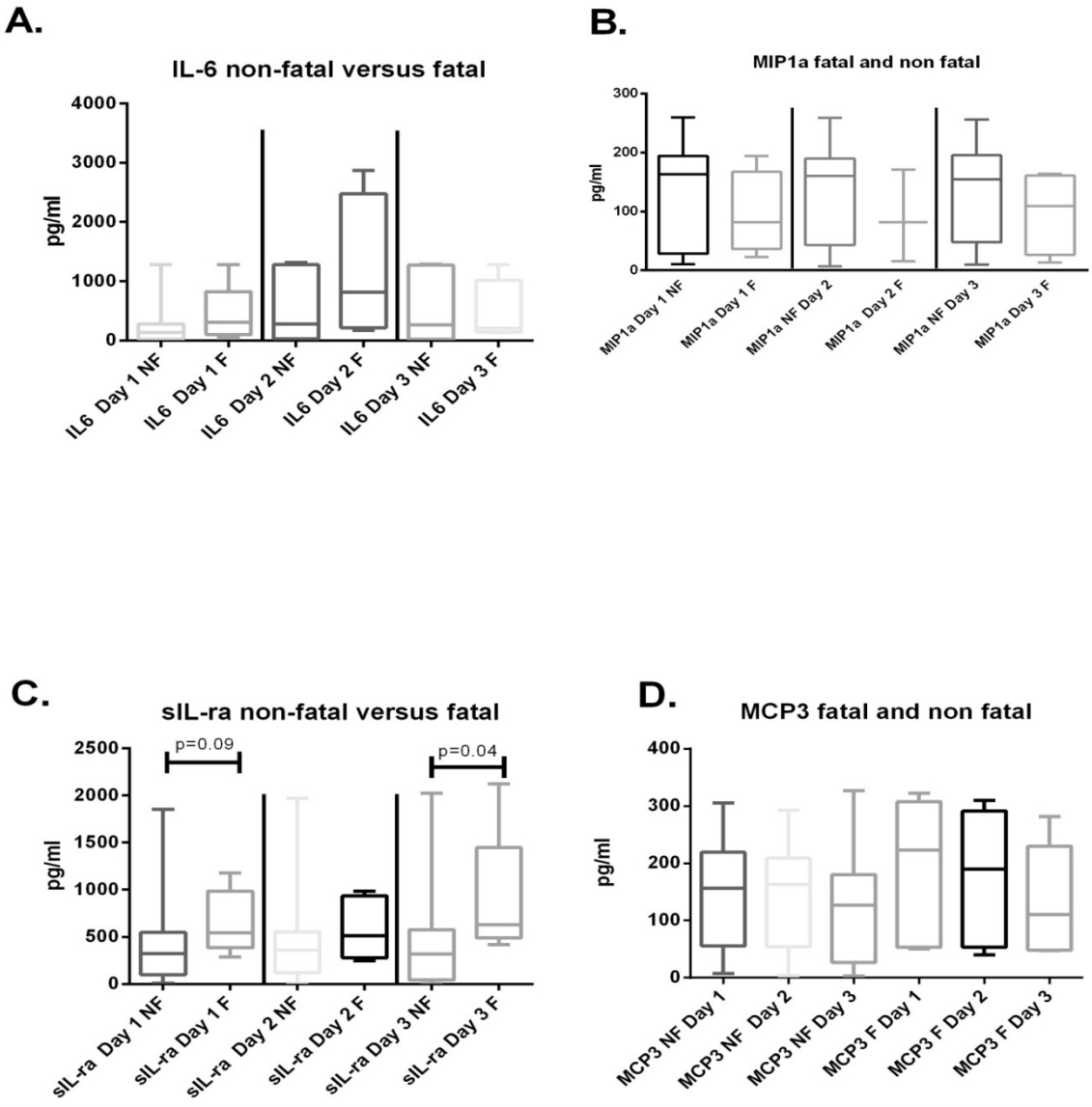


Figure 5.5. Comparison of cytokine and chemokine concentrations among survivors and fatal outcomes. Panel A depicts IL-6 concentrations on the first day of admission and at days 2 and 3. Similar time points for MIP1 alpha are shown in panel B. Panel C shows sIL-1-ra concentrations, a trend towards higher levels are seen on day 1 in individuals that eventually succumbed to the infection on day 1, and a significantly higher concentration is seen on day 3 (Two-Tailed, non-parametric, Mann-Whitney test).

The limited number of individuals with fatal outcomes precluded meaningful statistical comparisons, however, several trends are worth mentioning. As shown in Table 5.2, individuals with a favorable outcome had lower minimum and mean IL-6 concentration on the day of admission and at day 2. In addition, MCP-1 concentrations trended towards significance, with higher levels among individuals that did not survive the infection. Table 5.3 shows the comparison of MCP-1 concentration on the first day of ICU admission, among those that survived and those that succumbed to laboratory confirmed pandemic H1N1 infection. Individuals that survived the ICU admission had lower MCP-1 concentrations on the first day. However, this trend did not reach statistical significance.

In addition, we wanted to understand the kinetics of cytokines/chemokines during the course of ICU admission for severe H1N1 infection during the 2009 pandemic. We reviewed the area under the curve- in an attempt to determine the timing of peak cytokine/chemokine response. The goal was to determine the main cytokines and their production kinetics early in the course of ICU admission for the purpose of devising a tool to predict the severity and outcome of illness. In this vein we examined all of the cytokines/chemokines measured, excluding those that had no measurable concentrations in more than a third of participants. Figure 5.6 depicts the cytokines and chemokines that were measurable in most individuals enrolled in this cohort (>75%) and that had demonstrable changes in concentration during the admission.

Table 5.2. IL-6 concentrations among survivors and non-survivors. Higher minimum and mean IL-6 concentrations are seen early during the stay in the ICU, among those that did not survive the admission.

Column stats	IL6 Day 1 Survivors	IL6 Day 2 Survivors	IL6 Day 1 Fatality	IL6 Day 2 Fatality
Minimum	13.23	3.82	45.87	168.7
25% Percentile	17.64	30.82	102.6	214.1
Median	134.4	280.1	309.3	817.4
75% Percentile	280.9	1279	826.5	2476
Maximum	1280	1315	1282	2873
Mean	**299.4**	**508.7**	**433.5**	**1169**
Std. Deviation	447.9	531.6	491.3	1237
Std. Error of Mean	102.8	122	219.7	618.3
Lower 95% CI of mean	83.5	252.5	-176.6	-798.6
Upper 95% CI of mean	515.3	765	1043	3137

Table 5.3. Monocyte chemoattractant factor 1 (MCP-1) concentration on the first day of ICU admission.

A trend towards higher concentrations of MCP1 among individuals with unfavorable outcomes could be seen. p=0.1009, non-parametric, two way analysis of variance using Kruskal-Wallis analysis.

Death	No. observations	Mean	Median	Minimum	Maximum	St Dev
Yes	6	1742.83	1820.1	794.95	11379.81	911.7
No	28	1624.2	698.55	50.99	2613.44	2590.83

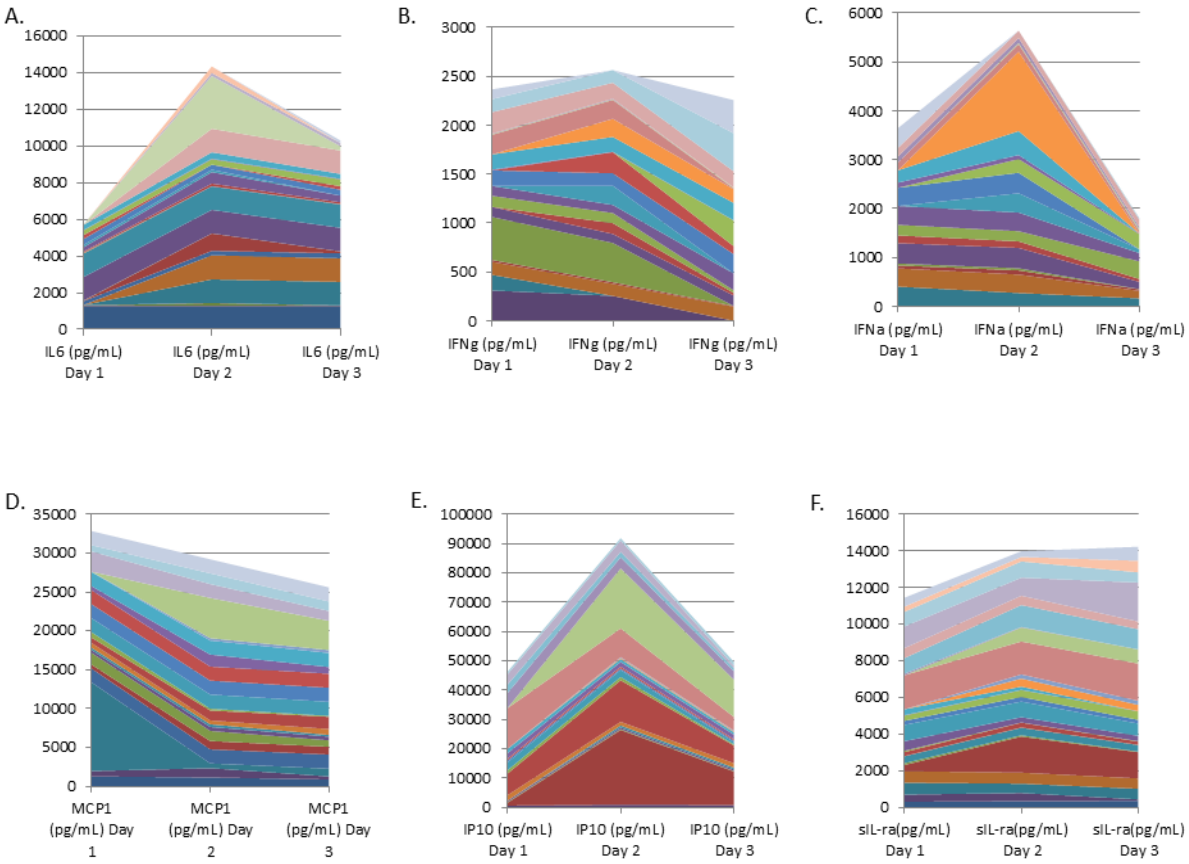


Figure 5.6. Plasma concentration of selected cytokines/chemokines, represented as area under the curve. This representation highlights the peak concentrations seen of A) IL-6, B) IFNγ, C) IFNα and E) IP-10 on the second day of ICU admission. These figures include all individuals, demonstrating the peak of most cytokines occurs within the first 2 days of ICU admission.

5.4 Summary

In this project we attempted to measure cytokine and chemokine concentration, with the hypothesis that high pro-inflammatory cytokines or cytokine dysregulation are correlated with adverse outcomes. We sought to determine the kinetics of the exuberant pro-inflammatory phase, in order to better understand what may predict disease outcome during pandemic H1N1 infection and what would constitute the optimal timing of anti-inflammatory strategies, if those are to be applied. We found that the peak of cytokine levels was on the first to second day of ICU admission, and found a set of cytokine that had measurable peaks, namely, IL-6; IFNγ; IFNα; IP-10 and s IL-ra. We found a trend to higher levels of IL-6 and MCP-1 among patients with a severe course that culminated in fatal outcome. Our analysis was limited by the small number of individuals that had fatal outcomes. It is possible to speculate that having a composite score that includes the concentrations of the aforementioned cytokines, may enable healthcare providers to predict the severity of illness and potentially guide the use of anti-inflammatory treatment modalities for the selected subset of patients with projected bad outcomes.

Chapter 6. Genetic predisposition to severe pandemic H1N1 infection

6.1 Rationale

During influenza pandemics caused by the emergence of novel strains, variable degrees of individual susceptibility have been observed. Studies during previous influenza pandemics pointed to high rates of mortality among young adults, in contrast to the propensity for adverse outcomes among older adults during seasonal influenza. Well established risk factors reviewed in section 1.4.2 only partially explained disease severity and in most studies, between 25%-50% of patients with severe pdmH1N1 infection had underlying health conditions. In Canada, 30%-48% of infections presented in persons with comorbidities; diabetes, heart disease and immunosuppression were associated with the highest risk of severe infection, while lung diseases and obesity were among the most common underlying conditions [30-32]. Ethnicity was another risk factor identified during pandemics caused by H1N1 in studies from several populations in North America and Australasia [30]. An over-representation of individuals of aboriginal descent presenting with severe pdmH1N1 infection was seen in the 1918 H1N1 Spanish influenza pandemic during which mortality in aboriginal communities in North America (3%-9% of Aboriginal populations) was significantly higher than among non-aboriginal communities [3, 396]. During the 2009 H1N1 pandemic a plethora of data from North America and Australasia document an predisposition to severe disease among individuals belonging to indigenous populations. Pacific Islanders account for 2.5% of the Australian population but made up 9.7% of patients admitted to Australian ICUs with confirmed pdmH1N1. Maori represent 13.6% of the New Zealand population, but accounted for

25% of ICU admissions in the ANZIC study [397]. In Manitoba, Kumar et al. [30] also reported 25.6% of the individuals admitted to ICUs in Canada belonged to First Nations, Inuit or Metis ethnicities. Similarly, two U.S. states (Arizona and New Mexico) observed a disproportionate number of deaths related to pdmH1N1 among American Indian/Alaska Natives, with subsequent validation of the observations in 12 additional state health departments. The results indicated that pdmH1N1 mortality rates among American Indian/Alaska Natives were four times higher than in persons of all other ethnic populations combined [398]. Comorbidities failed to entirely account for the discrepancies in disease severity. As a novel strain of influenza emerges, the pre-existing antibody responses directed at the surface glycoproteins are ineffective. In this context, the innate and CD8 T cell responses are thought to play an instrumental role. This led us to explore whether immune dysfunction caused by underlying genetic polymorphisms may lead to impaired responses. One of the factors we focused our project around was chemokine receptor 5 (CCR5) because of its known impact in a number of viral infections. The CCR5 chemokine receptor is expressed primarily on T cells, macrophages and dendritic cells and plays a pivotal role in mediating leukocyte chemotaxis in response to its ligands (RANTES, MIP-1a and MIP-1b). CCR5 is believed to be important in mucosal homing of several immune cell subsets, including regulatory T cells and Th17 cells, to sites of infection. Individuals who are homozygous for the CCR5Δ32 allele, a condition in which a 32bp deletion in the CCR5 gene prevents its expression on the cell surface, have been shown to have a reduced susceptibility to HIV infection, but more recently the role of CCR5 in other viral infections has been realized. The CCR5Δ32 allele is now known to correlate with increased risk of symptomatic and fatal West Nile virus

(WNV) infection [387, 399, 400], severe adverse reaction to the live yellow fever virus vaccine as well as a link with severe tickborne encephalitis symptoms [401, 402]. This purported expanded role of CCR5 along with reports based on animal models, suggest that CCR5 may have an impact on the response to influenza [403, 404] therefore formed the basic rationale for this line of investigation. In addition we explored the presence of SNPs in IL-6 and IP-10, both of which result in increased cytokine production and thus may play a role in disease severity [405-408].

6.2 Hypotheses

- **Immunogenetic factors that influence innate and adaptive immune response affect disease severity caused by pandemic H1N1 influenza infection.**

6.3 Objectives

- **6.3.1 To characterize immunogenetic determinants of pandemic H1N1 disease severity**
- **6.3.2 To compare rates of selected single nucleotide polymorphisms including CCR5delta32 among individuals with severe pandemic H1N1 infection**

6.4 Results

6.4.1 Study populations

In brief, an observational study conducted in Manitoba in response to the spring/summer pdmH1N1 outbreak.

The study was approved by local ethics committee. DNA was extracted from lymphocytes obtained from 20 patients suffering from laboratory confirmed severe

respiratory illness caused by H1N1 pandemic influenza. The average age was 40.4, 10 individuals were of Aboriginal descent, 9 Caucasians and one unknown ethnicity.

6.4.2 Genetic polymorphisms

DNA was extracted using QIAmp DNA mini kits (Qiagen) and used as a template for PCR amplification of the CCR5Δ32 locus. CCR5 genotype was determined by visual inspection of the size of the resulting PCR product: the wild-type allele produces a band of 197bp, while the polymorphic allele produces a band of 165bp. In addition, we performed genotyping of polymorphisms using sequence-based analysis. PCR products containing SNPs of interest in the IL-6 and IP-10 genes were purified, sequenced and genotyped.

Table 6.1 summarizes the three genetic polymorphisms. The frequency of the IL6 -174G allele, an allele that leads to increased IL-6 production, was higher than expected among the Caucasian population inflicted with severe pandemic H1N1. The IP-10 -1596T allele, known to result in increased transcription and production of the interferon dependent IP-10 was more frequent than expected among non-Caucasian pdmH1N1 patients.

Table 6.1. Genetic variants among individuals admitted to ICU with severe pandemic H1N1 infection, compared to historical population allele frequencies. The allele frequencies are derived from historical population based samples.

Ethnicity	Gene	Observed Allele Frequency	Expected Allele Frequency	Observed C allele Heterozygote Frequency	Expected Heterozygote Frequency
Caucasian	CCR5	27.8%	7-9%	55.5%	10-15%
Non-Caucasian		0	0	0	0
Caucasian	IL-6 G-174C (G allele frequency)	66.6%	47-50%	44.4%	45-50%
Non-Caucasian	IP-10 C-1596 T (T allele frequency)	31.2%	8-12%	62.5%*	16-21%

*IP-10 T allele frequency compared to non-Caucasian historical heterozygote frequency, OR 4.514, CI-1.254 to 16.25, p=0.02, Two-sided Fisher's exact test.

6.4.3 The role of CCR5 in severe disease

We took several lines of investigation in order to explore the potential association of this truncation in CCR5 with influenza susceptibility and disease severity. Figure 6.1 depicts the results of visual examination of the PCR product, where wild-type CCR5 DNA results in a 197-bp product, and the Δ32 allele results in a 165-bp product. We compared the allele frequency among individuals of Caucasian ethnicities that were admitted to ICU with confirmed 2009 pandemic H1N1 infection to historical data based on publications involving Caucasian blood donors. We performed Chi-Square analysis with Yate's correction, to compare the prevalence and found an odds ratio of 6.033, with a 95% confidence interval of 1.613 to 22.57, and a significant P value of 0.0094, indicating that the observed frequency is significantly higher than expected.

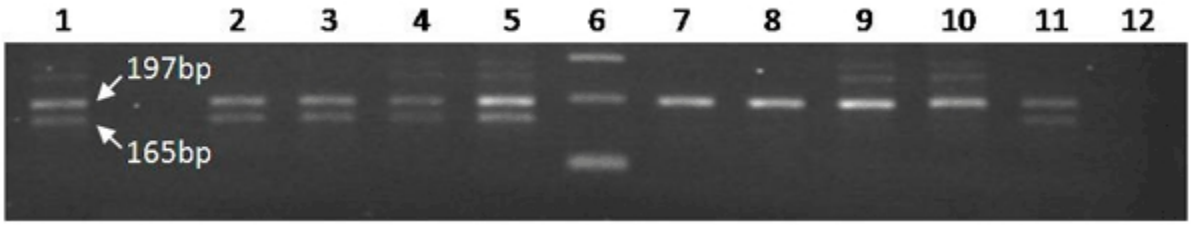


Figure 6.1. Amplification of the chemokine receptor 5 (CCR5) Δ32 locus in Caucasian patients. Lane 1, heterozygous positive control; lanes 2–5 and 7–11, patient's samples; lane 6, 100-bp ladder; lane 12, negative control. CCR5Δ32 heterozygosity is observed in samples 2, 3, 4, 5, and 11. Adapted from [409].

CCR5 is the first host protein to be targeted by HIV therapy. Maraviroc is an orally administered, noncompetitive inhibitor of CCR5 with potent *in-vitro* activity, binding to the trans-membrane co-receptor cavity with ensuing conformational changes [410, 411]. Its use received FDA approval in 2007 for the therapy of advanced HIV. This approval was based primarily on the MOTIVATE 1 and MOTIVATE 2 clinical trials in which the drug demonstrated superior antiviral and immunological efficacy compared to an optimized backbone regimen with placebo in HIV infected patients [412-414]. After our observation on the association of CCR5 delta 32 and severe H1N1 infection, we sought to determine the occurrence of respiratory infections with specific attention to viral Upper and Lower Respiratory Tract Infections (URTI and LRTI) in the aforementioned randomized controlled Maraviroc trials. We obtained the information regarding upper and lower respiratory from the two landmark trials as well as from the FDA approval documents. The results of this analysis are shown in Table 6.2. We found lower respiratory tract infections occur more frequently in the Maraviroc treatment arms- OR 10.32, CI 1.375 to 77.46, p=0.01 (Two-sided Fisher's xact test) [415, 416]. In addition, the cases of Pneumocystis jirovecii pneumonia (PJP or PCP) were limited to the treatment arms in all 3 trails. It is unclear whether this is related to different reporting pattern, and because of the small numbers, it is difficult to ascertain whether an association between the use of Maraviroc and the occurrence of PJP is significant.

Table 6.2. Maraviroc large RCT's- comparison of respiratory infections among treatment and control arms. Data extracted from the 3 trials. Modified from [416].

Publication	**N=; Drug**	**Trial Name**	**LRTI among CCR5 antagonist**	**LRTI Control arm**	**LRTI**
Cooper JID 2010 [412]	721; maraviroc	MERIT: Maraviroc in treatment Naïve	2 Lobar Pneumonia; 1PJP	0	
Saag JID 2009 [413]	186 (OBT versus OD and BID maraviroc)	Maraviroc in treatment experienced	5 Bronchitis; PJP 4	0 bronchitis PCP-0 Pneumonia-1	
Gulick NEJM 2008 [414]	1049; maraviroc	MOTIVATE 1+2: (601+474) PLACEBO-209	2 PJP; Pneumonia 4		URTI 16+20 (Placebo =1)
			11 LRTI; 7 PJP	**1 LRTI; 0 PJP**	

6.5 Summary

It was clear at the outset of this study that the known risk factors associated with severity of pandemic influenza account for some, but not all, of the discrepancy in disease severity. We focused our search for immunogenetic factors associated with disease severity on CCR5- a chemokine receptor that plays a role in trafficking of CD8 cells to mucosal sites, with well described role in HIV pathogenesis, and an emerging role in flaviviral diseases. We found it to be over-represented among Caucasians suffering from confirmed 2009 pandemic H1N1 requiring ventilator support and ICU admission. We also speculated that medical blockage of CCR5 with Maraviroc would result in increased respiratory viral infection. Indeed, review of the key clinical trials that resulted in the registration of this competitive CCR5 inhibitor revealed high rates of lower respiratory tract infections. The significance of these is likely minor but highlights the purported importance of CCR5 in viral immunity (beyond the well-established role in HIV susceptibility). In addition, we investigated the role of SNPs in the IL-6 and IP-10 genes that are result in increased cytokine production, as it is known that high levels of cytokines and the so called “cytokine storm” contribute to the adverse outcomes of severe disease. The frequency of the IL6 -174G allele was higher than expected in the Caucasian population, and this allele is known to result in increased IL-6 production. Similarly, the IP-10 -1596T allele was more frequent than expected among non-Caucasian pdmH1N1 patients. This allele has also been shown to increase transcription and production of IP-10 and may contribute to the heightened inflammatory response and potentially to the adverse outcomes among individuals of non-Caucasian ethnicities. Taken together, the results of this study add to the knowledge of genetic determinants of disease severity and

one can speculate that high levels of systemic inflammatory response coupled with impaired capacity of activated cells to migrate to the mucosal site of infection, may contribute to development of severe pandemic influenza illness.

Chapter 7. Influenza vaccine strategies in HIV infected individuals

7.1 Rationale

Immunosuppressed individuals are at higher risk of contracting influenza viruses and also for experiencing complications after influenza infections [417]. Among HIV infected individuals higher incidence of influenza illness is thought to occur, with several studies documenting higher hospitalization rates, longer duration of illness and increased mortality [69, 418-422].

In healthy adults, the inactivated seasonal influenza vaccine provides an estimated protective efficacy of 70% to 90% (15). As reviewed in previous chapters, this protection is correlated with antibody titres against viral hemagglutinin, as measured by the serum HAI, with titers of >=1:40 corresponding to relative protection from the influenza virus [105, 423, 424]. Several studies documented inferior antibody responses after seasonal influenza immunization in HIV-infected persons compared to non-infected population, while others failed to document the same disparity, showing restored capacity to mount antibody responses among individuals on effective HAART [323, 325, 425-428]. Protection levels were very low among individuals with advanced HIV disease, and lower than among uninfected even among those with asymptomatic HIV disease [429]. We sought to evaluate the use of different vaccination strategies on serological responses using two methods to measure antibody titres.

7.2 Hypotheses

- **Increasing the dose and adding a booster dose of seasonal influenza vaccine results in improved responses among HIV infected individuals**

7.3 Objectives

- To evaluate the immune response of three different seasonal influenza (Fluviral®) vaccine dosing strategies in HIV infected adults.
- To compare the performance of HAI and MBA assays for detection of vaccine responses

7.4 Results

7.4.1 Study participants

Study participants (n=297) were HIV infected individuals, that were randomized into one of three groups: Group A: single standard dose injection of Fluviral;Group B: standard dose injection of Fluviral followed 28 days later by a booster standard dose of Fluviral; Group C: a double dose of Fluviral followed 28 days later by a second double dose of Fluviral (A/Brisbane/59/2007 (H1N1)-like, A/Brisbane/10/2007 (H3N2)-like, and B/Florida/4/2006).

7.4.2 Antibody response and cross-reactive responses

Serum hemagglutinin inhibition (HAI) activity among 297 participants receiving at least one injection was measured. Ninety percent were male; 89% on HAART; the median CD4 was 470 cells/mm_3 and 76% undetectable HIV viral loads. The primary outcome was a doubling of antibodies relative to baseline (start of study) at week 8, comparing groups A to C and B to C. Over the 20-week period of assessment, 32 influenza-like illnesses (ILI) were reported. Six influenza infections were confirmed. ILI and positive infection results were evenly distributed between groups A, B and C. All dosing strategies, included the doubling of dose and administration of booster were well tolerated.

Using the HAI titres, the observed protection rates 8 weeks after vaccination were poor- ranging from 19% to 58%. The overall immunogenicity of influenza vaccine at the 3 time points and across the influenza strains assessed was poor (Range HAI ≥ 40 = 31-58%). Doubling the dose and providing a booster only slightly increased the measured response rate for A/Brisbane and B/Victoria at weeks 4, 8 and 20 compared to standard vaccine dose [430]. Using MBA assay, we applied the restrictive threshold of MFI >1000 as constituting a response. We found that the rate of vaccine response was 64.5%, higher than the rates reported for HAI. Figure 7.1 illustrates the ability to detect increases in IgG1 titres after vaccine administration. Responses to the vaccine components increased, however, there was a third of patients with undetectable antibody titres at all the time points and across all vaccine strains and those were not included in figure 7.1.

Table 7.1 shows the increase in titres against pandemic H1N1. The baseline MBA titres were low and the increase after vaccination was modest, albeit statistically significant ($p=0.0093$ comparing baseline titres to 4 weeks after the second dose, Two-tailed, non-parametric Mann-Whitney test). Figure 7.2 shows the effect of age and CD4 counts on the MBA IgG1 titres. The antibody titres do not correlate with either age or CD4 count in this study. We further sought to compare the effect of administration of a single dose versus the administration of booster on the antibody titres. We found no differences between the different vaccine strategies with respect to ability to induce a rise in titres measured by MBA or HAI. Figure 7.3 shows the comparison of single dose to a boosting with a second dose. Pre-vaccination, T2-representing the MBA titres measured after the second dose and T3 is the measurement of MBA titres expressed as MFI 4 weeks after the second dose boosting (8 weeks after the initial dose of vaccine. The left bar of each

pair represents the single dose and the right bar the boosted vaccine. Comparison of titres for A/Solomon Island 04/2006; A/Brisbane/10/07; A/California/04/09 are presented for each time-point.

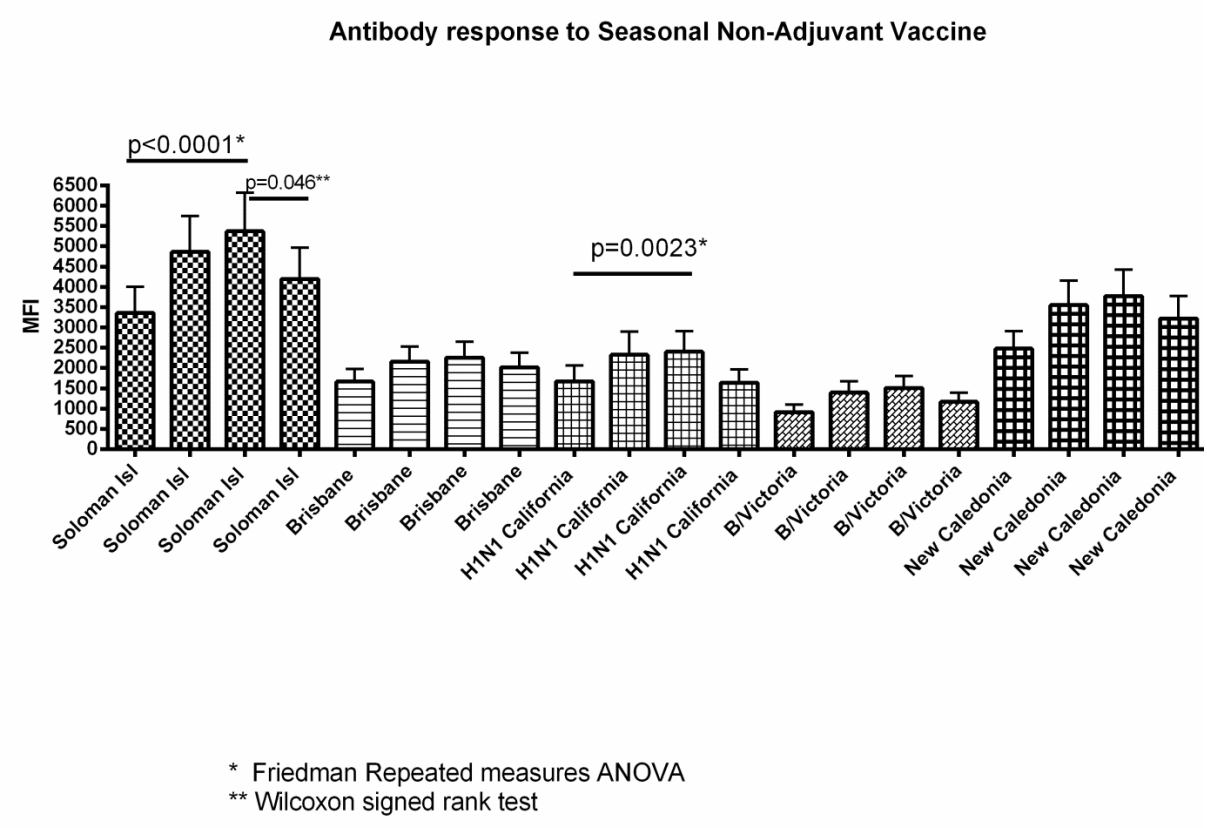
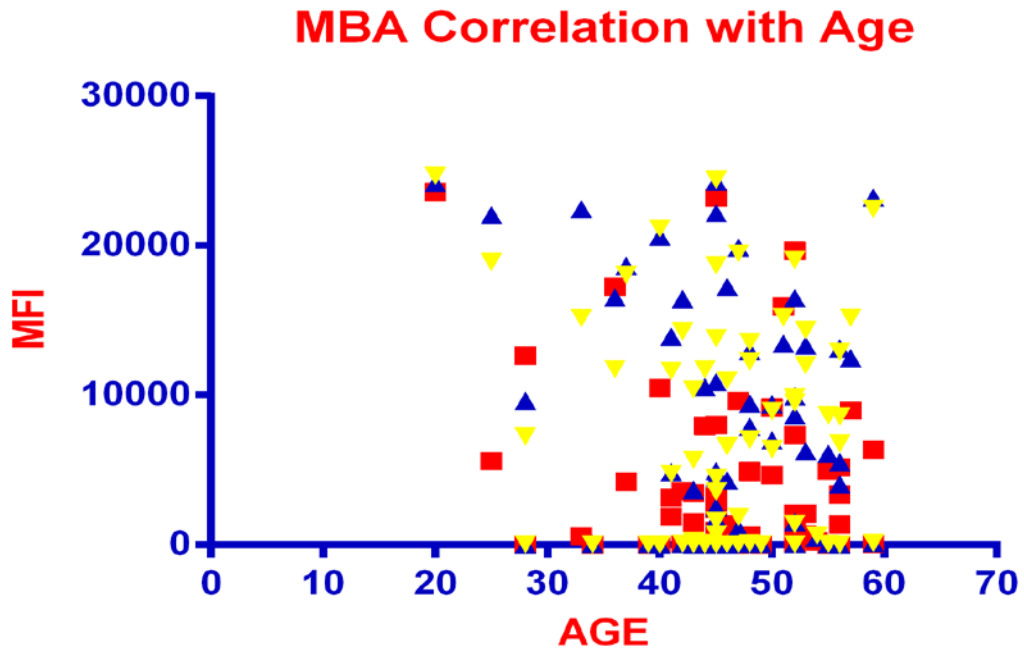


Figure 7.1. Antibody response to seasonal TIV measured by MBA. Vaccine responders, MBA IgG1 titres increased for vaccine strains H1N1/Brisbane and B/Victoria, as well as for H1N1/Solomon Island/3/2006 that was not included in the vaccine formulation. In addition, increased titres albeit at much lower absolute levels were seen for the pandemic H1N1/California/04/2009. For each set of graphs, from left to right- pre vaccine, 4 weeks, 8 weeks, 20 weeks. The IgG1 titres reach a peak for all strains, at 8 weeks with subsequent decline by week 20.

Table 7.1 summarizes the increases in MBA titres, against the non-vaccine pandemic H1N1 strain. The baseline titres were low and increased after vaccine administration.

	H1N1 California Baseline	H1N1 California T1	H1N1 California T2
Minimum	6.5	6.5	6.5
25% Percentile	7	7.5	7.75
Median	10.5	25	28
75% Percentile	25	66	76
Maximum	233	211.5	621
Mean	28.43	42.81	79.01
Lower 95% CI of mean	16.82	25.84	31.40
Upper 95% CI of mean	40.03	59.78	126.6



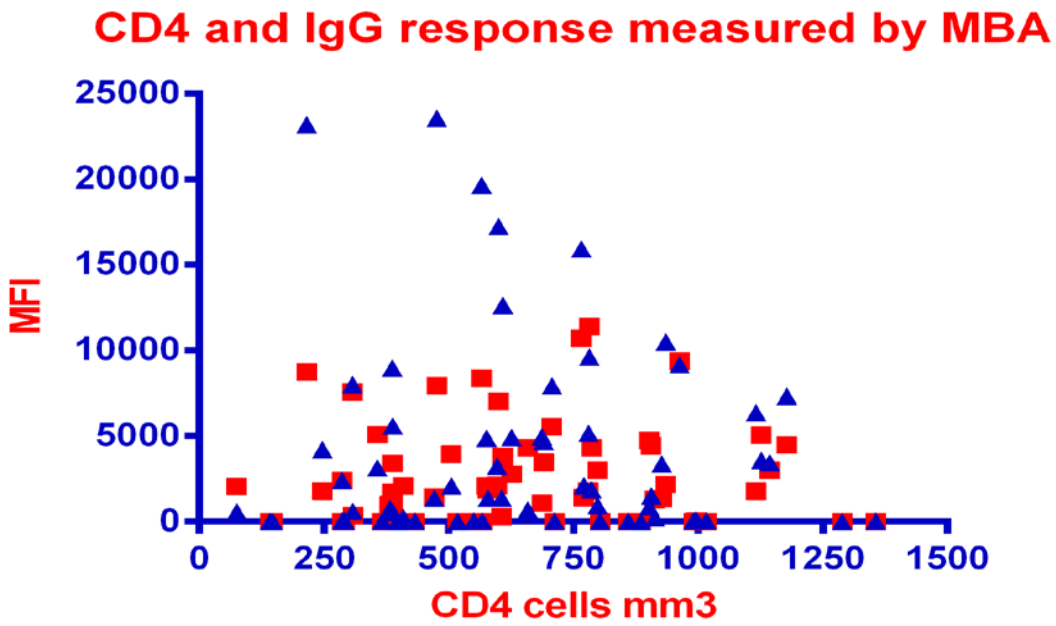


Figure 7.2 shows the effect of age and CD4 count on IgG1 antibody titres. No correlation between age or CD4 count could be detected using nonparametric Spearman correlation test.

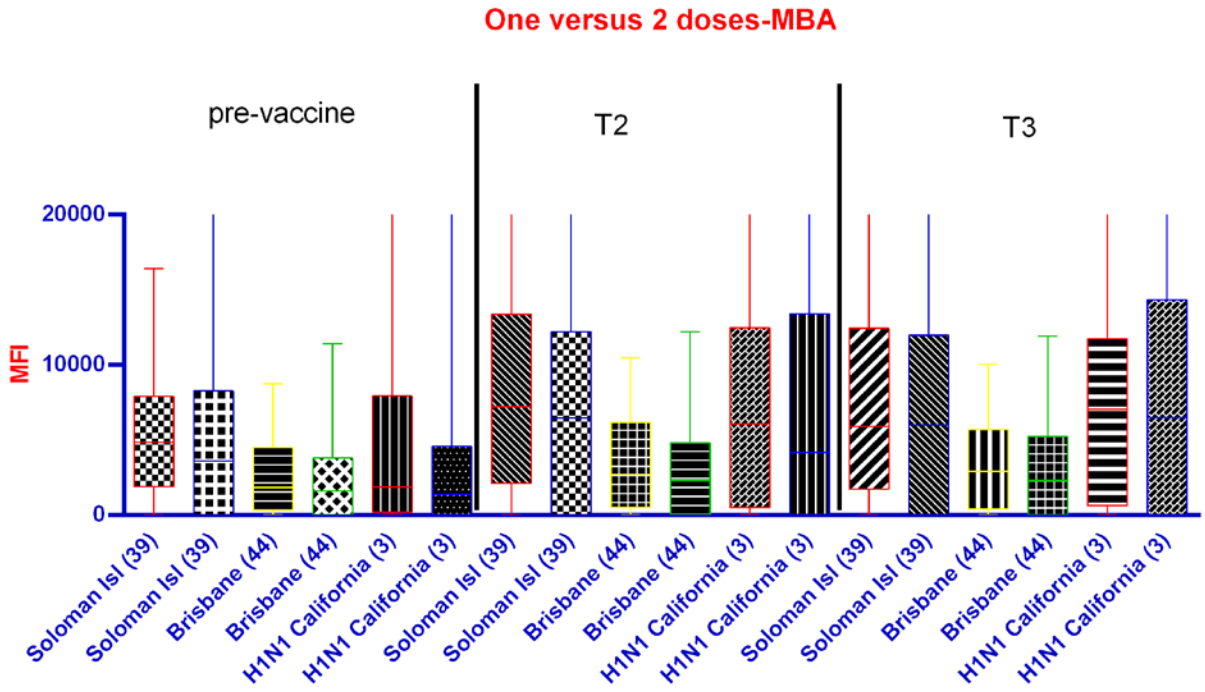


Figure 7.3 Comparison of single vaccine dose and boosting using a second dose. The antibody titres are measured using micro-bead array, and are expressed as mean fluorescence intensity. The two vaccination strategies resulted in similar antibody titres against vaccine strain (A/Brisbane/10/07) and the non-vaccine H1N1 strains, A/Solomon Island 04/2006 and the pandemic A/California/04/09, measured at the time of the second dose and 4 weeks after the second dose (T2 and T3, respectively).

7.5 Summary

In this component of the project we wanted to evaluate the immune response induced by different seasonal influenza (Fluviral®) vaccine dosing strategies in HIV infected adults and to compare the performance of HAI and MBA assays for detection of vaccine responses.

The results from HAI among 297 participants receiving at least one injection indicated the safety and tolerability of repeated vaccine administration, but the results indicated poor overall protection rates for all vaccine components. Administration of a booster dose of Fluviral only marginally increased the seroprotection offered by the vaccine [430]. Using the in-house MBA we were able to detect antibodies in a larger proportion of vaccinated individuals. For the vaccine component A/Brisbane/10/07, positive responses were detected in 64.5% even when using the restrictive threshold of 1000 MFI. We also show that administration of the booster dose of seasonal vaccine, 28 days after the first dose, did not result in improved responses, confirming the observations based on the HAI titres. Moreover, we found no correlation between the CD4 count and the vaccine response, but this lack of association may stem from the fact the patient population enrolled in the study had relatively preserved immune system, with a median CD4 of 470 cells/mm^3; the majority were on effective HAART and had undetectable viral loads. We found that administration of the seasonal vaccine resulted in increase in IgG1 titres against non-vaccine strains (A/Solomon Island and A/California), representing cross-reaction that could be measured using MBA. The increase in anti A/California IgG titres was statistically significant, albeit low. This observation points to the utility of the assay in investigating the ability to generate cross-reactive responses.

Chapter 8. Discussion

8.1 Cross-reactive antibodies induced by infection and vaccination

We hypothesized that cross reactive antibody responses can be measured after 1) natural infection 2) Live attenuated vaccine 3) inactivated vaccine. Microbead assay was employed to measure cross reactive responses in the various studies and cohorts described.

The correlate of protection against influenza is the presence of IgG anti-HA antibody titre of >1:40 or a 4 fold increase compared to baseline. The limitation of this protective response lays in its ephemeral nature and the lack of cross protection against the ever changing antigenic structure of influenza surface proteins. When facing a novel, or antigenically shifted influenza virus, the question of heterosubtypic and homosubtypic immunity invariably arises. Survival after challenge with emerging strains of influenza has been correlated with the presence of cross protection against drift viruses within a subtype or against diverse subtype [431-434]. Further evidence for the importance of cross reactive antibodies is offered by the fact that during emergence of novel pandemic influenza strains, healthy young adults experience disproportionate morbidity and mortality [435]. This is in contrast to morbidity and mortality associated with archetypal influenza strains among patients with weakened immune systems, generally the young (<5 years) and the elderly (>59 years). Our aim was to measure cross reactive responses. In order to achieve this aim we developed a micro-bead based assay that will be able to measure the antibody responses (both IgG and IgA) against multiple influenza subtypes, simultaneously. This method provides several advantages over the stalwart HAI. The

latter is plagued by the requirement for culturing the virus and the ability to measure titers for a single strain while micro-bead array provides a high throughput tool enabling the rapid gathering of information on multiple viral isolates that may assist public health resources allocation. The second aim was to determine the cross reactive responses induced by circulating influenza strains, live attenuated influenza vaccine and inactivated influenza vaccines.

We utilized the MBA assay for several projects. We were able to show the benefits of the assay compared to HAI for the detection of anti-influenza IgA1 and IgG1. We showed that this high throughput, sample sparing method could be effectively used to survey the seroepidemiology of influenza in the absence of culture based surveillance by conducting a comparative study in Canada and Kenya [389]. In further studies using the same assay, we examined the IgG1 response to TIV in healthy adults. The first study conducted in Winnipeg showed that the HAI and MBA titres against the 3 strains of influenza included in the seasonal vaccine preparation induced different responses. Two strains that were used for the first time (A/Wisconsin and B/Malaysia), and were relatively newly introduced to the environment resulted in measurable increase in antibody titres. The third vaccine component, A/New Caledonia/20/1999, was a strain that was circulating for the 6-7 years prior to this study, and that half of the participants had already received in previous years vaccine preparation [436] (figure 3.1). The A/New Caledonia component failed to induce an increase in antibody titres, a fact that may be related to the higher pre-immunization titres against this recurring strain. This higher baseline titre against A/New Caledonia/20/1999 strain was seen even in the group that did not receive the vaccine in previous years, suggesting that the circulating virus may have contributed to the high

antibody titres at baseline, and this may have blocked the induction of additional response to the vaccine. An alternative explanation may be the so-called “original antigenic sin”, when exposure to viruses with some antigenic relatedness, especially after long exposure intervals leads to clonal selection removing less avid antibody clones. This phenomena, popular in older literature, results in epitope competition between naïve and antigen-specific B cells which in-turn leads to inability to generate a response to closely related strains [437-441].

We applied the MBA method to study archived serum samples collected during antenatal screening. In this project there were two notable findings: measurement of the cumulative incidence of 8.6% between March and August 2009 among pregnant women in Manitoba and the comparison of assay performance. We found that using two thresholds resulted in better specificity for the more restrictive definition (MFI>=1000), without jeopardizing the sensitivity. The cumulative incidence was around 23% using either MFI of 500 or 1000 threshold, and the agreement of the MBA with the HAI ranged from 75% to 98% (Table 3.3). The disagreement between the two tests was higher in the pre-epidemic period, suggesting that the HAI may have detected non-specific responses. In the post-pandemic period rates of positivity were higher using MBA compared to HAI. The second study conducted among marginalized inner city population showed that among vulnerable populations the sero-positivity was higher among female, Aboriginal participants, and correlated with lower level of education and lower income, using HAI (Table 3.4). Again, using the same assay cutoffs, higher rates of sero-positivity were detected with MBA compared to HAI across the study population. The better specificity

of MBA is further supported by this study because the rate of increase in HAI positivity against pandemic H1N1 was higher (20% increase versus 5% for the MBA), even though this strain was not included in the seasonal vaccine preparation (Table 3.3). In addition, the studies illustrated that TIV induced cross-reactive IgG1 responses to non-vaccine strains, with the most closely related strain A/Solomon Island/03/06 showing the greatest cross reactivity.

We used the MBA assay to determine if individuals admitted to ICU with severe pandemic H1N1 infection produce IgG antibody response and whether they mount cross reactive responses to other H1N1 (Fig 3.4). We found an increase in IgG1 titres against pandemic A/California (pdmH1N1)/04/2009 among individuals admitted to ICU, with high titres achieved by day 5-7, similar to the expected peak of antibody response in less severely affected individuals [442]. In addition, we showed that infection with the pandemic H1N1 strain resulted in a boosting of cross-reactive IgG1 directed against A/Solomon Island/03/06, at ten-fold lower titres, similar to the observations in the Manitoba sero-survey studies. The consistent ability to detect better cross-reactive responses with a gradient based on the antigenic relatedness is encouraging for this tool and suggests it could be useful for predicting the breadth of protection that is induced by various immunization strategies, making it an interesting research method [389, 443]. Finally, we used a MBA assay to assess antibody response to LAIV among 60 individuals from a vaccine naïve, commercial sex workers cohort in Nairobi (Figure 3.2). We documented high baseline titres for H1N1 Brisbane 59/2007, with no further increase at 7 or 30 days post-vaccination. This lack of increase in titres may be attributed to the presence of blocking antibodies at the level of the respiratory mucosa, limiting the

availability of antigen. This is in keeping with the fact that, in contrast to experimental animal models which entail the application of antigens to influenza-naive hosts, all adult human vaccine recipients have had prior exposures [444-446]. Prior exposure has been suggested as one of the explanations for LAIV's better efficacy in early childhood compared to studies in adults [348, 447]. Thus, although vaccine naïve, this population was previously exposed to closely related strains as is evidenced by high titres of antibody at baseline. The assay showed high levels of antibodies against A/Solomon Island and although the antibody titres against the pandemic H1N1 California were lower at baseline, they did not increase after immunization. This finding suggests that by the fall of 2009, approximately 6 months after the onset of the 2009 H1N1 pandemic, a significant proportion of the study population was exposed to the pandemic strain.
The MBA based method, has clearly shown some merit- being high throughput, sample sparing, able to measure simultaneous titres against multiple strains, not requiring bio-safety cabinet for performing the assay. This optimistic outlook needs to be balanced by the fact it is an in-house assay, with reproducibility that depends on the antigens available and coupling reaction. An additional limitation lays in the fact that the method has not been correlated with clinical protection and the measured antibody titres observed in cross- reactivity studies, may represent an in-vitro phenomena rather than a true contribution to immunity.

8.2 CD 8 T cell responses to influenza

The cell mediated response to influenza has been understudied in humans in part because antibodies are pivotal in protecting people from influenza and as such vaccine

manufacturers are required to demonstrate the ability of new products to elicit HAI titres > 1:40. Cell-mediated immunity (CMI) as standard measure of influenza immunity is not used outside of the research laboratory, correlates of protection are not available, and study of CMI is not required by the licensing agencies, and thus less frequently assessed. Despite the inability to confer sterilizing immunity, CMI responses to wild-type influenza virus infection are known to play a principle role in the protection of humans against drifted or shifted strains [448]. Cross-reactive T-cell responses targeting conserved epitopes may reduce virus replication and improve clinical outcomes [88, 449]. We sought to investigate the ability of seasonal, inactivated trivalent influenza vaccine (TIV) to induce CTL responses, especially in the context of prior exposure to a recurring influenza strain. For this purpose we studied healthy adults immunized by seasonal TIV. We characterized the phenotype and level of immune activation and also elucidated the effector function of T cells. We found little change in memory subsets within the entire CD8 T-cell population after vaccine administration, but were able to demonstrate an increased level of CD8 T cell activation, measured on naïve subset (Figure 4.1). Moreover, we found increased IFNγ ICS staining on the effector memory subset of CD8+ T cells, with a corresponding, reciprocal decrease in central memory CD8+ production of interferon (Figure 4.2). Taken together, an antigen-driven increase in activation and differentiation of central memory T cells into effector and effector memory cells, representing a subtle CD8+ vaccine response is suggested [436]. When cytokine production by CD8+ T cells in response to the recurring A/New Caledonia/99 component of the seasonal TIV was investigated, we found no measurable effect, with no demonstrable increase in IL-2 or IFNγ (Figure 4.3). The limited ability to generate an

increase in CD8 response by a TIV is not surprising. The inability to boost the functional capacity by the recurring strain that was circulating and included in previous years vaccines, suggests the futility of the approach of re-vaccination with the same strain. One can put forward a suggestion that, if a similar situation, of repeated circulating strain, reoccurs; the use of a divergent strain may be beneficial in the place of the recurring strain. We went on to study the effect of LAIV, on the cell mediated responses to influenza. We showed activation of the CD4 and CD8 T cells peaking a week after vaccine administration, representing the expansion phase of the antigen specific response [384, 385]. We speculated that a live, replicating virus, despite its localization to the upper respiratory tract, will have better ability to produce cell-mediated responses and that such responses will possess some degree of heterosubtypic capacity against the 2009 pandemic H1N1 [363, 364, 450-452] . We were not able to find measurable increase in IL-2, IFNγ or perforin production after stimulation with either the vaccine strain or pandemic H1N1, suggesting that even in vaccine naïve adult population the ability to induce CTL responses is limited (Table 4.1). This again, may be explained by the fact that adults are previously exposed to a myriad of influenza strains and that the presence of pre-exisiting mucosal responses, accounting for the disparity of LAIV efficacy, with best results in children aged 6 month-7 years [250, 364, 444, 446, 447]. It can be postulated although not proven, that such topical immune responses prohibit the persistence of antigen in the quantity and duration that is required for generation of CMI. In addition, we examined the CMI of critically ill individuals admitted to intensive care units in Winnipeg, with the aim of correlating the response with severe disease. We were able to show trend towards an increased surface co-expression of CD38 and CCR5 on the

CD4 T cells, but not of other activation markers or on CD8 T cells (Figure 4.6). This project was hampered by the limited availability of viable cells and the lack of an adequate control group, as individuals with 2009 pandemic H1N1 infection either required ICU admission or were managed as outpatients (we were not able to enroll mildly ill or asymptomatic individuals).

Overall, we were able to adapt the phenotypic and functional assays to the study of responses to vaccines, and were able to show slight degree of CD8 response to TIV, the lack of significant systemic CMI responses to LAIV.

This component of the project has several limitations: the assays were performed on both fresh and frozen PBMC's and the use of frozen samples may have contributed to the limited CMI measured in the LAIV and ICU studies. The fact that mucosal responses were not elucidated and that a comparison of the TIV and LAIV modes of vaccination was not carried out in the same populations, limits the ability to draw conclusions from this segment of the project.

8.3 Cytokine levels as a correlate of cross-reactive response and disease severity in influenza

Given the limitation of cell recovery in the LAIV and ICU project, we investigated the systemic and mucosal concentrations of cytokines induced by LAIV as a surrogate of the innate and adaptive vaccine response. We also set out to determine the magnitude of cytokine "storm" caused by pandemic H1N1 influenza infection and whether it correlates with clinical outcomes.

In the LAIV, Flumist study, plasma cytokines and chemokines were measured before and after vaccination and after in-vitro stimulation using vaccine and non-vaccine strains. There were no demonstrable increases in un-stimulated cytokine/chemokine levels, after vaccination. After the PBMCs of individuals that received the vaccine were exposed to the matching A/H1N1 strain (A/Brisbane/10/07) a trend towards increase MIP 1 alpha was seen. The stimulation with pandemic H1N1 not included in the LAIV, did not lead to measurable increases in cytokine levels. These results are in keeping with the minimal observed effect of LAIV on CMI discussed in the previous section.

For the ICU study, we measured plasma cytokines in order to document the kinetics of cytokine concentrations and to try to correlate those with clinical outcomes. We observed moderate increases in IL-6, MCP-1, soluble CD40 ligand and IP-10 between the first and second days of ICU admission, and for most cytokines/chemokines with measurable concentrations, the peak was on the 1-2 days of ICU admission. The limited number of individuals with fatal outcomes precluded meaningful statistical comparisons. We have, however, noted that individuals with a favorable outcome had lower minimum and mean IL-6 concentration on the early days of admission, similarly the MCP-1 concentrations were higher (trending but not significant) among individuals that succumbed to the infection. It is conceivable that pooling the results of selected cytokines/chemokines, during the first day of ICU admission may provide a “severity score” that can predict the eventual outcome of admission and which of the individuals may potentially benefit from the administration of adjunctive anti-inflammatory agents. This concept is supported by the finding of substantially elevated concentrations of 7 cytokines/chemokines (among them IL-6, MIP1-β and MCP1) in lung tissues of patients

that perished during the 2009 pdmH1N1 [453]. Similar results with emphasis on the potential role of IL-6 as a severity marker are supported by several studies of systemic cytokines [454-458]. This study, and others that examined the role of cytokines in disease severity, are limited by availability of samples, the lack of milder cases affected by pandemic H1N1 as controls, by the presence of multiple concurrent conditions and interventions in the severely ill individual that may account for increase in plasma cytokines. The findings that are corroborated by these publications from diverse studies, implicate the MCP-1 (CCL2) and IL-6 at least as potential markers of the immune dyregulation that occurs during severe pandemic H1N1 infection. Early identification of those with projected unfavorable outcomes, may provide a window of opportunity to attempt targeting this dysfunctional chemokine/cytokine activation, with monoclonal antibodies, such as those developed for oncologic indications [459].

8.4 Genetic susceptibility to severe influenza

This component of the work was aimed at identifying novel risk factors that contribute to the variable individual susceptibility. During influenza pandemics, in the absence of protective antibody responses, due to unique antigenic properties of the emerging influenza strains, it is presumed that innate and T cell broadly responsive mechanisms are critical for attenuation of the illness. The traditional comorbidities that are associated with influenza severity are present in 25-50% of patients with severe disease; moreover, ethnicity was associated with disease severity during the 1918 H1N1 Spanish influenza pandemic, with overrepresentation of aboriginal communities in North America. Similar trends were observed during the 2009 H1N1 pandemic with high rates

of severe disease among individuals of aboriginal ethnicities in divergent studies from distinct geographic regions. In Manitoba, more than a quarter of the individuals admitted to ICUs were First Nations, Inuit or Metis [30]. We characterized immunogenetic determinants of pandemic H1N1 disease severity, focusing on CCR5. CCR5 deletion has a well-established protective effect in the context of HIV. A recent large meta-analysis of over 13,000 cases and controls confirmed this association with protection while dispelling the association with a wide array of previously implicated single-nucleotide polymorphisms [460]. CCR5 delta 32 deletion was further associated with the pathogenesis of several flaviviral diseases, and at least in animal models with impaired response to influenza [404, 461]. We found a high proportion of the CCR5 deletion among individuals of Caucasian ethnicities that were admitted to ICU with confirmed 2009 pandemic H1N1 infection. The CCR5 delta 32 heterozygocity rate was 55.5% in this patient population, compared to 10-15% in historical population based studies among Caucasians [409]. Following this observation, we investigated the hypothesis that CCR5 blockage using Maraviroc, a FDA approved HIV treatment, will increase the occurrence of respiratory infections with specific attention to viral Upper and Lower Respiratory Tract Infections (URTI and LRTI). Analysis of the landmark randomized controlled trials, we confirmed this hypothesis by finding significantly more lower respiratory tract infections in the Maraviroc treatment arms- OR 10.32, CI 1.375 to 77.46 [416]. This analysis is limited by the fact that culture-based diagnosis of LRTIs in the studies was not uniform. Despite this reservation, it does suggest that a more detailed examination of the impact of targeting an important host factor for treatment of HIV is warranted.

These observations are interesting as they suggest that impaired migration of immune cells to the mucosal site of influenza infection may lead to higher viral burden and potentially result in more severe disease. Recently one unpublished study (in review) from Spain, found more severe disease and worse outcomes among individuals bearing the CCR5 deletion. In addition, a recently reported fatal case caused by pandemic H1N1, occurred in a CCR5 delta 32 homozygous individual [462]. Although the mechanism behind this association is unclear, most efforts focused on T cells proliferation and migration. Another potential explanation received some support from a recent study of NK cells. This group of investigators found reduced amounts of RANTES in the liver of CCR5-deficient mice, with interrupted proliferation and migration of NK cells upon influenza virus stimulation [463].

In addition to CCR5, the frequency of the IL6 -174G allele, an allele that leads to increased IL-6 production was higher among severe pandemic H1N1, than expected in the Caucasian population. This observation together with the previously mentioned association of elevated IL-6 concentration with poor outcomes, suggest that immune dysfunction that underlies the cytokine storm in severe pandemic H1N1 infections, could be at least in part, explained by host genetic polymorphism. In order to establish the association of this genetic polymorphism to the disease outcome, measuring concurrent levels of mRNA or IL-6 would be required.

Our observation in this project adds to the growing list of genetic polymorphisms that may predispose to severe pandemic H1N1. The development of newer tools, including massively parallel sequencing platforms capable of simultaneously measuring gene expression levels as well as genetic polymorphisms will undoubtedly result in the

discovery of some genetic predictors of morbidity and mortality caused by pdmH1N1. Establishing the role of each of the host genes implicated by additional measurement of cytokine levels based on pathways identified as a result of the genomics approach will help to deepen the understanding of the role of specific signaling pathways. These may bestow researchers with a new stockpile of potential targets for therapeutic interventions [218].

8.5 Influenza specific response in HIV infected individuals

Multiple observations, mostly in advanced HIV disease documented inferior antibody responses to seasonal influenza immunization compared to HIV uninfected. Some evidence is indicating that the capacity to mount antibody responses in HIV infected individuals is effectively restored by HAART. Although doubling of dose or providing an additional booster dose has minor impact among healthy adults it was speculated that among HIV infected individuals it may improve the suboptimal responses seen with standard vaccination strategy. We collaborated with an ongoing study, assessing the effects of various fortified vaccination strategies on the antibody responses among HIV infected individuals. In addition, the study provided the opportunity to compare the performance of HAI and the in-house MBA, as previously described. The results of the HAI following vaccination showed poor protection rates 8 weeks after vaccination, ranging from 19% to 58%. The poor immunogenicity was seen across the vaccination strategies with doubling the dose and providing a booster resulting in only marginal increments in rate of sero-protection. We applied a restrictive threshold (MFI >1000), based on prior studies, for the comparison of MBA. We found that the rate of

vaccine response was 64.5%, higher than the rates reported for HAI. However, a third of the participants had undetectable antibody titres at all the time points, and across all vaccine strains. The antibody titres did not correlate with either age or CD4 count in this study, nor did they correlate with the vaccination strategy (single dose versus the administration of booster). The study was able to establish the safety and tolerability of repeated vaccine administration, but the results indicated poor overall protection rates for all vaccine components. Administration of a booster dose of Fluviral only marginally increased the seroprotection offered by the vaccine. Using the in-house MBA, we were able to detect antibodies in a larger proportion of vaccinated individuals for the vaccine component A/Brisbane/10/07 and confirmed the observations based on the HAI titres (Figure 7.1). The lack of finding the expected correlation between the CD4 count and the vaccine response may be attributed to the inclusion of individuals with relatively preserved immune system (median CD4 of 470 cells/mm^3), with approximately 90% on effective HAART and with undetectable viral loads (Figure 7.2). The addition of MBA to the study also demonstrated that administration of the seasonal vaccine resulted in increase in IgG1 titres against non-vaccine strains (A/Solomon Island and A/California).

8.6 Summary of observations

At the outset of this research endeavor we hypothesized that: **a**. The strength of humoral and cellular responses to influenza can be predicted on the basis of previous exposure and level of immunocompetence and **b**.that cross reactive cellular influenza-specific responses correlate with immunogenetic factors and may affect disease severity. We used several projects and distinct cohorts to develop the methods and address these hypotheses.

- Cross reactive antibody and cell mediated responses can be measured after natural infection, Live attenuated vaccine and inactivated vaccine- The main contribution of this project was the development of assays to measure antibody responses to multiple influenza strains, using a sample sparing, high-throughput, microbead based assay and application of phenotypic and functional assays to the study of influenza specific responses. These resulted in the following publications [378, 464]. We were able to document the antibody and CTL responses to TIV and LAIV. The key finding from the TIV study in healthy volunteers was that repeated vaccination using a recurring strain failed to result in increased antibody or CTL responses. The administration of LAIV resulted in generation of cross-reactive antibody responses. The study showed that even in a vaccine naïve adult population, LAIV resulted in limited generation of CD4 or CD8 responses.
  The use of MBA assay in local studies, contributed to characterization of the prevalence rates of 2009 H1N1 pandemic during the first wave, and the

performance of the assay suggests acceptable specificity with increased sensitivity along with the added benefits of high throughput and ability to simultaneously study responses to multiple strains of influenza [379, 465] and an additional manuscript in preparation..

- Decreased cross reactive responses correlate with severity of illness caused by novel influenza- We were able to document CTL responses, as measured by activation of T cells in severely ill patients, admitted to ICU. We found that several pro-inflammatory cytokines and chemokines trended towards higher concentrations in those individuals that succumbed to pandemic H1N1 infection. This adds to accumulating evidence that a cytokine storm with elevated concentrations of IL-6 and MCP-1 is one of the predictors of adverse outcomes, and may eventually help in identifying patients with poor prognosis, early in the course of illness providing the grounds for attempting to modulate the hypercytokinemia. These studies resulted in one manuscript in preparation.
- Novel immunogenetic factors contribute to the disparate disease susceptibility during H1N1 influenza infection- We were the first to identify the association between CCR5 deletion and pandemic influenza severity. This observation is receiving some support from additional studies and adds to the ongoing efforts to understand the factors that explain the discrepancies in disease severity that are not accounted for by traditional risk. These observations led to several publications [218, 409, 415, 416].
- Flumist- seasonal, live-attenuated, cold adapted influenza virus administered via nasal mucosa. We used this model of exposure through the mucosa, to a live

attenuated virus, to assess the antibody and CTL responses to vaccine components as well as the ability to induce cross reactive responses against pandemic H1N1. The results of this study are still being analyzed and 2 manuscripts are in preparation.

8.7 Pandemic Influenza susceptibility model

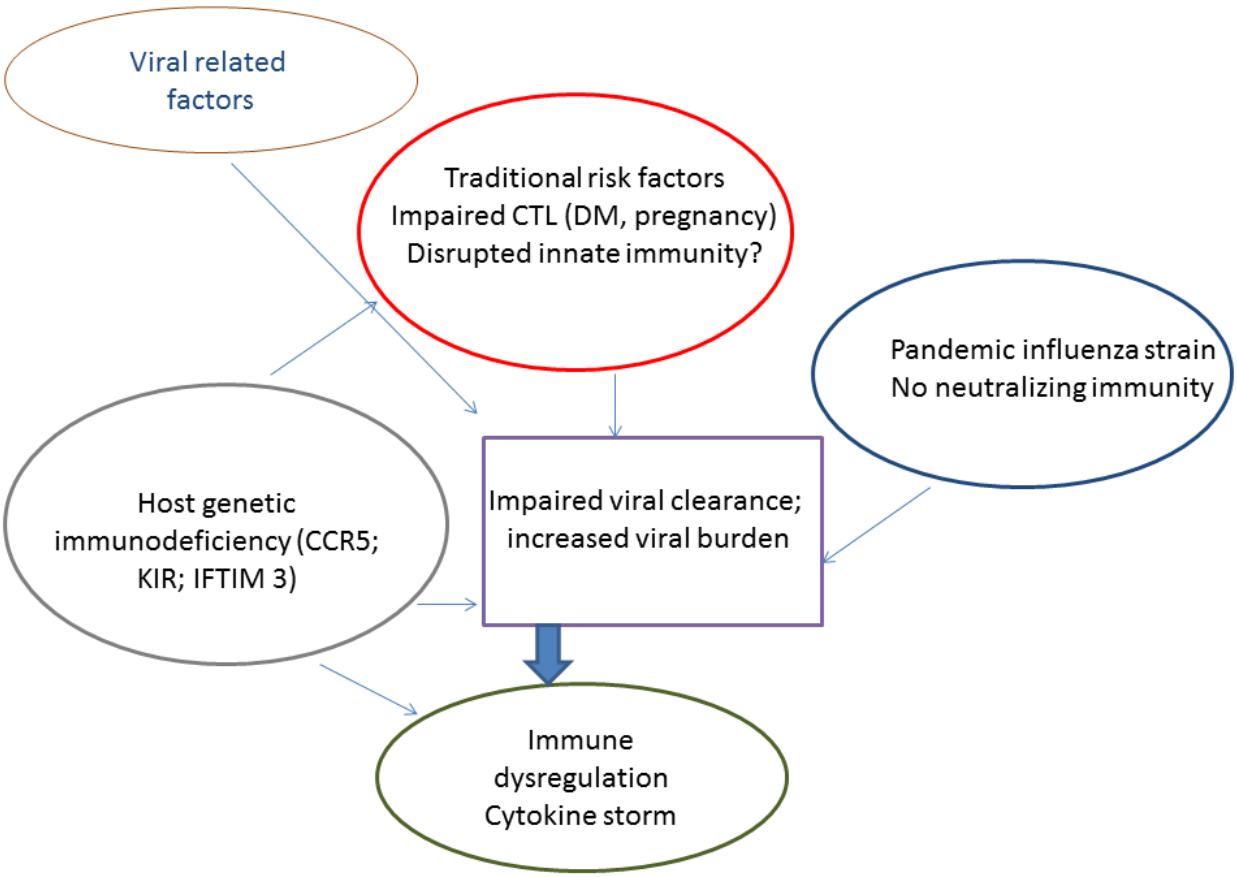


Figure 8.1 Pandemic influenza susceptibility model. In the absence of neutralizing antibodies, due to emergence of novel influenza, the fate of the host relies on the cell-mediated and cross protective innate mechanisms. If those are impaired by co-morbidities or genetic subtle deficiencies, viral burden is increased. These, along with a myriad of virulence factors of the emerging strain, determine the risk of immune dysregulation and

cytokine storm. The magnitude of the resulting cytokine storm is a chief influence on disease outcome.

The model in Figure 8.1 proposes the following interactions to explain the pathogenesis of severe pandemic H1N1 infection: viral factors associated with increased virulence and ability to evade the immune response, act in concert with risk factors that result in impaired innate or cell-mediated immunity. Individual's susceptibility is also influenced by a growing list of host genetic polymorphisms that alter the innate and cell-mediated response. The appearance of a novel, pandemic strain of influenza, poses a unique challenge, since antibody mediated protection is usually absent, exposing the underlying innate or cell-mediated dysfunction. In this scenario, the result is an increased viral burden and ensuing uncontrolled cytokine production, leading to respiratory failure and potentially to spiraling the multi-organ dysfunction frequently seen in individuals succumbing to severe pandemic H1N1 influenza.

8.8 Future directions and recommendations

The main avenues for continued investigation involve the ongoing examination of the proteomics in response to LAIV. In this project we collected cervical, nasal and plasma samples and subjected them to mass spectrometry, measuring protein abundance patterns before and after the administration of seasonal LAIV. We identified significant increases in the relative of abundance of numerous proteins, and these could be clustered to several main pathways using Ingenuity Pathway Analysis (IPA) software. The main findings are of increased expression of proteins involved in acute phase responses, complement activation and IL-17 signaling. The patterns of expression differed among

the two groups of study participants (HESN and new negative commercial sex workers) and current analysis of the entire cohort is ongoing. The objectives are to further characterize the compartmental distribution of pathway activation and to identify key pathway that are involved in the innate and adaptive responses to the vaccine. Conducting a comparative study applying these techniques to TIV, LAIV and other novel influenza vaccines is of great interest.

To further characterize the immunogenetic factors, namely CCR5, IL-6 and IP-10 in severe influenza, additional studies from diverse cohorts, including larger sample size are required. In addition, measuring and correlating the gene expression using RNA sequencing techniques, with the concentration of cytokine will be required as steps to establish a causative role for the polymorphisms. The availability of CCR5 knock-out mouse model, provides compelling avenues to investigate the effect of this deficiency of the magnitude of cytokine response to pandemic H1N1 strains.

In terms of recommendations, three results of the study can potentially be translated to workable proposals:

a. Use of recurring influenza strain in vaccine formulation may have limited efficacy in inducing boosting of either the humoral or cell mediated immunity. Incorporating a divergent strain, instead of the repeated strain, may be beneficial.
b. Individuals with the highest concentrations of IL-6, MCP-1 and MIP1 alpha early in the course of pandemic H1N1 infection, may be at increased risk of poor outcome and should be targeted for specific, focused immunomodulatory interventions, as part of multi-centre clinical trials.

c. The importance of CCR5 in immune response to an expanding array of viral agents is noted. It is imperative that more detailed virological investigations are added to future studies that entail blockage of this chemokine receptor.

8.9 Publications that are the result of the work included in this thesis:

- Keynan, Y., S. Malik, and K.R. Fowke, *The role of polymorphisms in host immune genes in determining the severity of respiratory illness caused by pandemic H1N1 influenza.* Public Health Genomics, **2013**. 16(1-2): p. 9-16.
- Keynan, Y., et al., *Toll-Like Receptors Dysregulation after Influenza Virus Infection: Insights into Pathogenesis of Subsequent Bacterial Pneumonia.* ISRN Pulmonology, 2011. **2011**: p. 6.
- Juno, J.A., Y. Keynan, and K.R. Fowke, *Invariant NKT cells: regulation and function during viral infection.* PLoS Pathog, **2012**. 8(8): p. e1002838.
- Keynan, Y., et al., *Cellular immune responses to recurring influenza strains have limited boosting ability and limited cross-reactivity to other strains.* Clin Microbiol Infect, **2010**. 16(8): p. 1179-86.
- Mahmud, S.M., et al., *Estimated cumulative incidence of pandemic (H1N1) influenza among pregnant women during the first wave of the 2009 pandemic.* CMAJ, **2010**. 182(14): p. 1522-4.
- Keynan, Y., et al., *Evaluation of influenza-specific humoral response by microbead array analysis.* Can J Infect Dis Med Microbiol, **2011**. 22(1): p. 25-9.
- Thompson, L.H., et al., *Serological survey of the novel influenza A H1N1 in inner city Winnipeg, Manitoba, 2009.* Can J Infect Dis Med Microbiol, **2012**. 23(2): p. 65-70.
- Keynan, Y., et al., *Chemokine receptor 5 delta 32 allele in patients with severe pandemic (H1N1) 2009.* Emerg Infect Dis, **2010**. 16(10): p. 1621-2.
- Keynan, Y., et al., *Targeting the chemokine receptor CCR5: good for HIV, what about other viruses?* J Infect Dis, **2011**. 203(2): p. 292; author reply 293.
- Keynan, Y et al., *Maraviroc Induced CCR5 Blockage for HIV Infected Individuals is Associated with Increased Rates of Respiratory Tract Infections.* Virology & Mycology, **2012.**

9. References:

1. Taubenberger, J.K. and D.M. Morens, *Pandemic influenza--including a risk assessment of H5N1.* Rev Sci Tech, 2009. **28**(1): p. 187-202.
2. Morens, D.M., J.K. Taubenberger, and A.S. Fauci, *The persistent legacy of the 1918 influenza virus.* N Engl J Med, 2009. **361**(3): p. 225-9.
3. Johnson, N.P. and J. Mueller, *Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic.* Bull Hist Med, 2002. **76**(1): p. 105-15.
4. Thompson, W.W., et al., *Mortality associated with influenza and respiratory syncytial virus in the United States.* JAMA, 2003. **289**(2): p. 179-86.
5. Thompson, W.W., et al., *Estimating influenza-associated deaths in the United States.* Am J Public Health, 2009. **99 Suppl 2**: p. S225-30.
6. Morens, D.M., et al., *An historical antecedent of modern guidelines for community pandemic influenza mitigation.* Public Health Rep, 2009. **124**(1): p. 22-5.
7. Ballinger, M.N. and T.J. Standiford, *Postinfluenza bacterial pneumonia: host defenses gone awry.* J Interferon Cytokine Res, 2010. **30**(9): p. 643-52.
8. Gupta, R.K., R. George, and J.S. Nguyen-Van-Tam, *Bacterial pneumonia and pandemic influenza planning.* Emerg Infect Dis, 2008. **14**(8): p. 1187-92.
9. Hussell, T., E. Wissinger, and J. Goulding, *Bacterial complications during pandemic influenza infection.* Future Microbiol, 2009. **4**(3): p. 269-72.
10. Morens, D.M., J.K. Taubenberger, and A.S. Fauci, *Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness.* J Infect Dis, 2008. **198**(7): p. 962-70.
11. Holmes, E.C., et al., *Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses.* PLoS Biol, 2005. **3**(9): p. e300.
12. Nicholson, K.G., et al., *Influenza-related hospitalizations among young children in Leicestershire.* Pediatr Infect Dis J, 2003. **22**(10 Suppl): p. S228-30.
13. Nicholson, K.G., J.M. Wood, and M. Zambon, *Influenza.* Lancet, 2003. **362**(9397): p. 1733-45.
14. *Update: Influenza activity - United States, September 30, 2012-February 9, 2013.* MMWR Morb Mortal Wkly Rep, 2013. **62**(7): p. 124-30.
15. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.
16. Gorman, O.T., et al., *Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses.* J Virol, 1991. **65**(7): p. 3704-14.
17. Shu, L.P., et al., *Genetic reassortment in pandemic and interpandemic influenza viruses. A study of 122 viruses infecting humans.* Eur J Epidemiol, 1996. **12**(1): p. 63-70.
18. Herrler, G., H.J. Gross, and R. Brossmer, *A synthetic sialic acid analog that is resistant to the receptor-destroying enzyme can be used by influenza C virus as a receptor determinant for infection of cells.* Biochem Biophys Res Commun, 1995. **216**(3): p. 821-7.

19. Gambaryan, A., et al., *Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain.* Virology, 2005. **334**(2): p. 276-83.
20. Ito, T., et al., *Receptor specificity of influenza A viruses from sea mammals correlates with lung sialyloligosaccharides in these animals.* J Vet Med Sci, 1999. **61**(8): p. 955-8.
21. Banks, J., et al., *Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy.* Arch Virol, 2001. **146**(5): p. 963-73.
22. Matrosovich, M., et al., *The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties.* J Virol, 1999. **73**(2): p. 1146-55.
23. Suzuki, Y., et al., *Sialic acid species as a determinant of the host range of influenza A viruses.* J Virol, 2000. **74**(24): p. 11825-31.
24. Suzuki, Y. and M. Nei, *Origin and evolution of influenza virus hemagglutinin genes.* Mol Biol Evol, 2002. **19**(4): p. 501-9.
25. Matrosovich, M.N., S. Krauss, and R.G. Webster, *H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity.* Virology, 2001. **281**(2): p. 156-62.
26. Xu, R., et al., *Structure, receptor binding, and antigenicity of influenza virus hemagglutinins from the 1957 H2N2 pandemic.* J Virol, 2010. **84**(4): p. 1715-21.
27. Tuite, A.R., et al., *Estimated epidemiologic parameters and morbidity associated with pandemic H1N1 influenza.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2010. **182**(2): p. 131-136.
28. Balcan, D., et al., *Seasonal transmission potential and activity peaks of the new influenza A(H1N1): a Monte Carlo likelihood analysis based on human mobility.* BMC medicine, 2009. **7**: p. 45.
29. Lessler, J., et al., *Outbreak of 2009 pandemic influenza A (H1N1) at a New York City school.* N Engl J Med, 2009. **361**(27): p. 2628-36.
30. Kumar, A., et al., *Critically ill patients with 2009 influenza A(H1N1) infection in Canada.* JAMA : the journal of the American Medical Association, 2009. **302**(17): p. 1872-1879.
31. Zarychanski, R., et al., *Correlates of severe disease in patients with 2009 pandemic influenza (H1N1) virus infection.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2010. **182**(3): p. 257-264.
32. Campbell, A., et al., *Risk of severe outcomes among patients admitted to hospital with pandemic (H1N1) influenza.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2010. **182**(4): p. 349-355.
33. Hanslik, T., P.Y. Boelle, and A. Flahault, *Preliminary estimation of risk factors for admission to intensive care units and for death in patients infected with A(H1N1)2009 influenza virus, France, 2009-2010.* PLoS currents.Influenza, 2010: p. RRN1150.
34. Vaillant, L., et al., *Epidemiology of fatal cases associated with pandemic H1N1 influenza 2009.* Euro surveillance : bulletin europeen sur les maladies

transmissibles = European communicable disease bulletin, 2009. **14**(33): p. 19309.
35. Creanga, A.A., et al., *Severity of 2009 pandemic influenza A (H1N1) virus infection in pregnant women.* Obstetrics and gynecology, 2010. **115**(4): p. 717-726.
36. Archer, B., et al., *Interim report on pandemic H1N1 influenza virus infections in South Africa, April to October 2009: epidemiology and factors associated with fatal cases.* Euro Surveill, 2009. **14**(42).
37. Ramsey, C. and A. Kumar, *H1N1: viral pneumonia as a cause of acute respiratory distress syndrome.* Curr Opin Crit Care, 2011. **17**(1): p. 64-71.
38. Agarwal, P.P., S. Cinti, and E.A. Kazerooni, *Chest radiographic and CT findings in novel swine-origin influenza A (H1N1) virus (S-OIV) infection.* AJR Am J Roentgenol, 2009. **193**(6): p. 1488-93.
39. Jartti, A., et al., *Chest imaging findings in hospitalized patients with H1N1 influenza.* Acta Radiol, 2011. **52**(3): p. 297-304.
40. Rothberg, M.B. and S.D. Haessler, *Complications of seasonal and pandemic influenza.* Crit Care Med, 2010. **38**(4 Suppl): p. e91-7.
41. Hers, J.F., N. Masurel, and J. Mulder, *Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza.* Lancet, 1958. **2**(7057): p. 1141-3.
42. Louria, D.B., et al., *Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza.* J Clin Invest, 1959. **38**(1 Part 2): p. 213-65.
43. Louie, J.K., et al., *Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California.* JAMA, 2009. **302**(17): p. 1896-902.
44. *Intensive-care patients with severe novel influenza A (H1N1) virus infection - Michigan, June 2009.* MMWR Morb Mortal Wkly Rep, 2009. **58**(27): p. 749-52.
45. Webb, S.A., et al., *Critical care services and 2009 H1N1 influenza in Australia and New Zealand.* N Engl J Med, 2009. **361**(20): p. 1925-34.
46. Rice, T.W., et al., *Critical illness from 2009 pandemic influenza A virus and bacterial coinfection in the United States.* Crit Care Med, 2012. **40**(5): p. 1487-98.
47. Peltola, V.T. and J.A. McCullers, *Respiratory viruses predisposing to bacterial infections: role of neuraminidase.* Pediatr Infect Dis J, 2004. **23**(1 Suppl): p. S87-97.
48. Metersky, M.L., et al., *Epidemiology, microbiology, and treatment considerations for bacterial pneumonia complicating influenza.* Int J Infect Dis, 2012. **16**(5): p. e321-31.
49. Presanis, A.M., et al., *The severity of pandemic H1N1 influenza in the United States, from April to July 2009: a Bayesian analysis.* PLoS medicine, 2009. **6**(12): p. e1000207.
50. Presanis, A.M., et al., *The severity of pandemic H1N1 influenza in the United States, April -- July 2009.* PLoS currents, 2009. **1**: p. RRN1042.
51. Archer, B., et al., *Interim report on pandemic H1N1 influenza virus infections in South Africa, April to October 2009: epidemiology and factors associated with*

fatal cases. Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin, 2009. **14**(42): p. 19369.
52. Girard, M.P., et al., *The 2009 A (H1N1) influenza virus pandemic: A review.* Vaccine, 2010. **28**(31): p. 4895-4902.
53. Louie, J.K., et al., *Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California.* JAMA : the journal of the American Medical Association, 2009. **302**(17): p. 1896-1902.
54. Smith, G.J.D., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009. **459**(7250): p. 1122-1125.
55. Taubenberger, J.K. and J.C. Kash, *Influenza virus evolution, host adaptation, and pandemic formation.* Cell Host Microbe, 2010. **7**(6): p. 440-51.
56. Taubenberger, J.K. and J.C. Kash, *Insights on influenza pathogenesis from the grave.* Virus Res, 2011. **162**(1-2): p. 2-7.
57. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(7060): p. 889-93.
58. Tumpey, T.M., et al., *Characterization of the reconstructed 1918 Spanish influenza pandemic virus.* Science, 2005. **310**(5745): p. 77-80.
59. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science (New York, N.Y.), 2009. **325**(5937): p. 197-201.
60. Mehle, A. and J.A. Doudna, *Adaptive strategies of the influenza virus polymerase for replication in humans.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21312-6.
61. Mehle, A., et al., *Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers.* J Virol, 2012. **86**(3): p. 1750-7.
62. Yamada, S., et al., *Biological and structural characterization of a host-adapting amino acid in influenza virus.* PLoS Pathog, 2010. **6**(8): p. e1001034.
63. Conenello, G.M., et al., *A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence.* PLoS Pathog, 2007. **3**(10): p. 1414-21.
64. Hai, R., et al., *PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models.* J Virol, 2010. **84**(9): p. 4442-50.
65. Ehrhardt, C., T. Wolff, and S. Ludwig, *Activation of phosphatidylinositol 3-kinase signaling by the nonstructural NS1 protein is not conserved among type A and B influenza viruses.* J Virol, 2007. **81**(21): p. 12097-100.
66. Ehrhardt, C., et al., *Interplay between influenza A virus and the innate immune signaling.* Microbes Infect, 2010. **12**(1): p. 81-7.
67. Hale, B.G., et al., *The multifunctional NS1 protein of influenza A viruses.* J Gen Virol, 2008. **89**(Pt 10): p. 2359-76.
68. Hale, B.G., et al., *Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein.* J Virol, 2010. **84**(14): p. 6909-22.
69. Fiore, A.E., et al., *Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010.* MMWR Recomm Rep, 2010. **59**(RR-8): p. 1-62.

70. Jefferson, T., et al., *Vaccines for preventing influenza in the elderly.* Cochrane Database Syst Rev, 2010(2): p. CD004876.
71. Simonsen, L., et al., *Impact of influenza vaccination on seasonal mortality in the US elderly population.* Arch Intern Med, 2005. **165**(3): p. 265-72.
72. Jackson, L.A., et al., *Evidence of bias in estimates of influenza vaccine effectiveness in seniors.* Int J Epidemiol, 2006. **35**(2): p. 337-44.
73. Campitelli, M.A., et al., *Influenza vaccination and all-cause mortality in community-dwelling elderly in Ontario, Canada, a cohort study.* Vaccine, 2010. **29**(2): p. 240-6.
74. Kwong, J.C., et al., *Vaccine effectiveness against laboratory-confirmed influenza hospitalizations among elderly adults during the 2010-2011 season.* Clin Infect Dis, 2013.
75. Govaert, T.M., et al., *The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial.* JAMA, 1994. **272**(21): p. 1661-5.
76. Doherty, P.C., et al., *Influenza and the challenge for immunology.* Nat Immunol, 2006. **7**(5): p. 449-455.
77. Murphy, B.R. and K. Coelingh, *Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines.* Viral Immunol, 2002. **15**(2): p. 295-323.
78. Belshe, R.B., et al., *Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine.* The Journal of pediatrics, 2000. **136**(2): p. 168-175.
79. Belshe, R.B., C.S. Ambrose, and T. Yi, *Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age.* Vaccine, 2008. **26 Suppl 4**: p. D10-6.
80. Belshe, R., et al., *Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine.* Expert review of vaccines, 2004. **3**(6): p. 643-654.
81. Harper, S.A., et al., *Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP).* MMWR Recomm Rep, 2004. **53**(RR-6): p. 1-40.
82. Forrest, B.D., et al., *Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children.* Clin Vaccine Immunol, 2008. **15**(7): p. 1042-53.
83. Block, S.L., et al., *Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5-49 years of age.* Vaccine, 2008. **26**(38): p. 4940-6.
84. Belshe, R.B., *An introduction to influenza: lessons from the past in epidemiology, prevention, and treatment.* Manag Care, 2008. **17**(10 Suppl 10): p. 2-7.
85. Leroux-Roels, G., *Unmet needs in modern vaccinology: adjuvants to improve the immune response.* Vaccine, 2010. **28 Suppl 3**: p. C25-36.
86. Gupta, R.K., et al., *Adjuvants--a balance between toxicity and adjuvanticity.* Vaccine, 1993. **11**(3): p. 293-306.
87. O'Hagan, D.T., et al., *MF59 adjuvant: the best insurance against influenza strain diversity.* Expert Rev Vaccines, 2011. **10**(4): p. 447-62.

88. Ellebedy, A.H. and R.J. Webby, *Influenza vaccines.* Vaccine, 2009. **27 Suppl 4**: p. D65-8.
89. Valkenburg, S.A., et al., *Immunity to seasonal and pandemic influenza A viruses.* Microbes Infect, 2011. **13**(5): p. 489-501.
90. Rao, S.S., et al., *Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret.* PLoS One, 2010. **5**(3): p. e9812.
91. Nabel, G.J. and A.S. Fauci, *Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine.* Nat Med, 2010. **16**(12): p. 1389-91.
92. Choi, S.Y., et al., *Enhancement of DNA Vaccine-induced Immune Responses by Influenza Virus NP Gene.* Immune Netw, 2009. **9**(5): p. 169-78.
93. Kutzler, M.A. and D.B. Weiner, *DNA vaccines: ready for prime time?* Nat Rev Genet, 2008. **9**(10): p. 776-88.
94. Donnelly, J.J., et al., *Further protection against antigenic drift of influenza virus in a ferret model by DNA vaccination.* Vaccine, 1997. **15**(8): p. 865-868.
95. Tao, P., et al., *Enhanced protective immunity against H5N1 influenza virus challenge by vaccination with DNA expressing a chimeric hemagglutinin in combination with an MHC class I-restricted epitope of nucleoprotein in mice.* Antiviral Res, 2009. **81**(3): p. 253-60.
96. Park, K.S., et al., *Complete protection against a H5N2 avian influenza virus by a DNA vaccine expressing a fusion protein of H1N1 HA and M2e.* Vaccine, 2011. **29**(33): p. 5481-7.
97. Ulmer, J.B., et al., *Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA.* J Virol, 1998. **72**(7): p. 5648-53.
98. Fu, T.M., et al., *Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge.* J Immunol, 1999. **162**(7): p. 4163-70.
99. Saha, S., et al., *A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus.* Virology, 2006. **354**(1): p. 48-57.
100. Lim, K.L., et al., *Co-administration of avian influenza virus H5 plasmid DNA with chicken IL-15 and IL-18 enhanced chickens immune responses.* BMC Vet Res, 2012. **8**: p. 132.
101. Laddy, D.J., et al., *Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens.* PLoS One, 2008. **3**(6): p. e2517.
102. Monto, A.S. and N.H. Arden, *Implications of viral resistance to amantadine in control of influenza A.* Clin Infect Dis, 1992. **15**(2): p. 362-7; discussion 368-9.
103. Monto, A.S., et al., *Safety and efficacy of long-term use of rimantadine for prophylaxis of type A influenza in nursing homes.* Antimicrob Agents Chemother, 1995. **39**(10): p. 2224-8.

104. Martin, K. and A. Helenius, *Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import.* Cell, 1991. **67**(1): p. 117-30.
105. Couch, R.B., *Prevention and treatment of influenza.* N Engl J Med, 2000. **343**(24): p. 1778-87.
106. Kawai, N., et al., *Factors influencing the effectiveness of oseltamivir and amantadine for the treatment of influenza: a multicenter study from Japan of the 2002-2003 influenza season.* Clin Infect Dis, 2005. **40**(9): p. 1309-16.
107. Keyser, L.A., et al., *Comparison of central nervous system adverse effects of amantadine and rimantadine used as sequential prophylaxis of influenza A in elderly nursing home patients.* Arch Intern Med, 2000. **160**(10): p. 1485-8.
108. Abed, Y., et al., *Characterization of 2 influenza A(H3N2) clinical isolates with reduced susceptibility to neuraminidase inhibitors due to mutations in the hemagglutinin gene.* J Infect Dis, 2002. **186**(8): p. 1074-80.
109. McKimm-Breschkin, J.L., *Resistance of influenza viruses to neuraminidase inhibitors--a review.* Antiviral Res, 2000. **47**(1): p. 1-17.
110. McKimm-Breschkin, J.L., *Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance.* Influenza Other Respi Viruses, 2013. **7 Suppl 1**: p. 25-36.
111. Kiso, M., et al., *Resistant influenza A viruses in children treated with oseltamivir: descriptive study.* Lancet, 2004. **364**(9436): p. 759-65.
112. Ives, J.A., et al., *The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo.* Antiviral Res, 2002. **55**(2): p. 307-17.
113. Yu, Y., et al., *Peramivir use for treatment of hospitalized patients with influenza A(H1N1)pdm09 under emergency use authorization, October 2009-June 2010.* Clin Infect Dis, 2012. **55**(1): p. 8-15.
114. McCauley, J.W. and B.W. Mahy, *Structure and function of the influenza virus genome.* Biochem J, 1983. **211**(2): p. 281-94.
115. Skehel, J.J. and D.C. Wiley, *Influenza viruses and cell membranes.* Am J Respir Crit Care Med, 1995. **152**(4 Pt 2): p. S13-5.
116. Wright, P.F. and R.G. Webster, *Orthomyxoviruses.* Fields virology, 2001. **1**: p. 1533-1579.
117. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.
118. Air, G.M., *Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus.* Proceedings of the National Academy of Sciences of the United States of America, 1981. **78**(12 II): p. 7639-7643.
119. Nobusawa, E., et al., *Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses.* Virology, 1991. **182**(2): p. 475-85.
120. Choppin, P.W., J.S. Murphy, and I. Tamm, *Studies of two kinds of virus particles which comprise influenza A2 virus strains. III. Morphological characteristics:*

independence to morphological and functional traits. J Exp Med, 1960. **112**: p. 945-52.
121. Hoyle, L., R.W. Horne, and A.P. Waterson, *The structure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether.* Virology, 1961. **13**: p. 448-59.
122. Nicholson, K.G., R.G. Webster, and A.J. Hay, *Textbook of influenza*. 1998: Blackwell Science Ltd.
123. Sauter, N.K., et al., *Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: a 500-MHz proton nuclear magnetic resonance study.* Biochemistry, 1989. **28**(21): p. 8388-8396.
124. Takemoto, D.K., J.J. Skehel, and D.C. Wiley, *A Surface Plasmon Resonance Assay for the Binding of Influenza Virus Hemagglutinin to Its Sialic Acid Receptor.* Virology, 1996. **217**(2): p. 452-458.
125. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.
126. Gamblin, S.J., et al., *The structure and receptor binding properties of the 1918 influenza hemagglutinin.* Science, 2004. **303**(5665): p. 1838-42.
127. Russell, R.J., et al., *H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes.* Virology, 2004. **325**(2): p. 287-96.
128. Bullough, P.A., et al., *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. **371**(6492): p. 37-43.
129. Korte, T., et al., *Conformational intermediates and fusion activity of influenza virus hemagglutinin.* J Virol, 1999. **73**(6): p. 4567-74.
130. Chen, J., J.J. Skehel, and D.C. Wiley, *N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil.* Proc Natl Acad Sci U S A, 1999. **96**(16): p. 8967-72.
131. Daniels, R.S., et al., *Fusion mutants of the influenza virus hemagglutinin glycoprotein.* Cell, 1985. **40**(2): p. 431-9.
132. Lin, Y.P., et al., *Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion.* Virology, 1997. **233**(2): p. 402-10.
133. Klenk, H.D., et al., *Activation of influenza A viruses by trypsin treatment.* Virology, 1975. **68**(2): p. 426-39.
134. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin.* Virology, 1983. **127**(2): p. 361-73.
135. Rogers, G.N. and B.L. D'Souza, *Receptor binding properties of human and animal H1 influenza virus isolates.* Virology, 1989. **173**(1): p. 317-22.
136. Connor, R.J., et al., *Receptor Specificity in Human, Avian, and Equine H2 and H3 Influenza Virus Isolates.* Virology, 1994. **205**(1): p. 17-23.
137. Ito, T., *Interspecies transmission and receptor recognition of influenza A viruses.* Microbiol Immunol, 2000. **44**(6): p. 423-30.

138. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.
139. Bateman, A.C., et al., *Glycan analysis and influenza A virus infection of primary swine respiratory epithelial cells: the importance of NeuAc{alpha}2-6 glycans.* J Biol Chem, 2010. **285**(44): p. 34016-26.
140. Skehel, J.J., et al., *Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion.* Proc Natl Acad Sci U S A, 1982. **79**(4): p. 968-72.
141. Bao, Y., et al., *The influenza virus resource at the National Center for Biotechnology Information.* J Virol, 2008. **82**(2): p. 596-601.
142. Chutinimitkul, S., et al., *Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding.* J Virol, 2010. **84**(22): p. 11802-13.
143. Kilander, A., et al., *Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010.* Euro Surveill, 2010. **15**(9).
144. Xu, R., et al., *Structural characterization of the hemagglutinin receptor specificity from the 2009 H1N1 influenza pandemic.* J Virol, 2012. **86**(2): p. 982-90.
145. Yang, H., P. Carney, and J. Stevens, *Structure and Receptor binding properties of a pandemic H1N1 virus hemagglutinin.* PLoS Curr, 2010. **2**: p. RRN1152.
146. Colman, P.M. and C.W. Ward, *Structure and diversity of influenza virus neuraminidase.* Curr Top Microbiol Immunol, 1985. **114**: p. 177-255.
147. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase.* Virology, 1974. **61**(2): p. 397-410.
148. Renaud, C., J. Kuypers, and J.A. Englund, *Emerging oseltamivir resistance in seasonal and pandemic influenza A/H1N1.* Journal of Clinical Virology, 2011. **52**(2): p. 70-78.
149. Wu, N.C., et al., *Systematic identification of H274Y compensatory mutations in influenza A virus neuraminidase by high-throughput screening.* J Virol, 2013. **87**(2): p. 1193-9.
150. Dharan Nj, G.L.V.M.J.J. and et al., *INfections with oseltamivir-resistant influenza a(h1n1) virus in the united states.* JAMA, 2009. **301**(10): p. 1034-1041.
151. Bloom, J.D., L.I. Gong, and D. Baltimore, *Permissive secondary mutations enable the evolution of influenza oseltamivir resistance.* Science, 2010. **328**(5983): p. 1272-5.
152. Govorkova, E.A., *Consequences of resistance: in vitro fitness, in vivo infectivity, and transmissibility of oseltamivir-resistant influenza A viruses.* Influenza Other Respi Viruses, 2013. **7 Suppl 1**: p. 50-7.
153. Enami, M. and K. Enami, *Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein.* J Virol, 1996. **70**(10): p. 6653-7.
154. Ye, Z.P., et al., *Functional and antigenic domains of the matrix (M1) protein of influenza A virus.* J Virol, 1987. **61**(2): p. 239-46.

155. Bui, M., et al., *Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins.* J Virol, 2000. **74**(4): p. 1781-6.
156. Gomez-Puertas, P., et al., *Influenza virus matrix protein is the major driving force in virus budding.* J Virol, 2000. **74**(24): p. 11538-47.
157. Lamb, R.A., S.L. Zebedee, and C.D. Richardson, *Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface.* Cell, 1985. **40**(3): p. 627-33.
158. Sugrue, R.J. and A.J. Hay, *Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel.* Virology, 1991. **180**(2): p. 617-24.
159. Bialas, K.M., E.A. Desmet, and T. Takimoto, *Specific residues in the 2009 H1N1 swine-origin influenza matrix protein influence virion morphology and efficiency of viral spread in vitro.* PLoS One, 2012. **7**(11): p. e50595.
160. Feng, J., et al., *Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2.* Virol J, 2006. **3**: p. 102.
161. Gabbard, J., et al., *A humanized anti-M2 scFv shows protective in vitro activity against influenza.* Protein Engineering, Design and Selection, 2009. **22**(3): p. 189-198.
162. Fields, B., D. Knipe, and P. Howley, *Fields virology. 5th*, 2007, Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.
163. Lamb, R.A. and R.M. Krug, 2001: p. 1487-1531.
164. Dias, A., et al., *The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit.* Nature, 2009. **458**(7240): p. 914-918.
165. Yuan, P., et al., *Crystal structure of an avian influenza polymerase PA N reveals an endonuclease active site.* Nature, 2009. **458**(7240): p. 909-913.
166. Hara, K., et al., *Amino acid residues in the N-terminal region of the PA subunit of influenza a virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding.* Journal of Virology, 2006. **80**(16): p. 7789-7798.
167. Guu, T.S.Y., et al., *Mapping the domain structure of the influenza A virus polymerase acidic protein (PA) and its interaction with the basic protein 1 (PB1) subunit.* Virology, 2008. **379**(1): p. 135-142.
168. Nieto, A., et al., *Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit.* Journal of General Virology, 1994. **75**(1): p. 29-36.
169. Biswas, S.K., P.L. Boutz, and D.P. Nayak, *Influenza virus nucleoprotein interacts with influenza virus polymerase proteins.* J Virol, 1998. **72**(7): p. 5493-501.
170. Nakagawa, Y., K. Oda, and S. Nakada, *The PB1 subunit alone can catalyze cRNA synthesis, and the PA subunit in addition to the PB1 subunit is required for viral RNA synthesis in replication of the influenza virus genome.* J Virol, 1996. **70**(9): p. 6390-4.
171. Li, M.L., P. Rao, and R.M. Krug, *The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits.* EMBO J, 2001. **20**(8): p. 2078-86.

172. Manz, B., M. Schwemmle, and L. Brunotte, *Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier.* J Virol, 2013. **87**(13): p. 7200-9.
173. Paterson, D. and E. Fodor, *Emerging roles for the influenza A virus nuclear export protein (NEP).* PLoS Pathog, 2012. **8**(12): p. e1003019.
174. Chen, W., et al., *A novel influenza A virus mitochondrial protein that induces cell death.* Nat Med, 2001. **7**(12): p. 1306-12.
175. Pena, L., et al., *Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine.* J Virol, 2012. **86**(10): p. 5523-32.
176. Chakrabarti, A.K. and G. Pasricha, *An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses.* Virology, 2013. **440**(2): p. 97-104.
177. Schnitzler, S.U. and P. Schnitzler, *An update on swine-origin influenza virus A/H1N1: A review.* Virus Genes, 2009. **39**(3): p. 279-292.
178. Ulmanen, I., B.A. Broni, and R.M. Krug, *Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7355-9.
179. Moeller, A., et al., *Organization of the influenza virus replication machinery.* Science, 2012. **338**(6114): p. 1631-4.
180. Tarendeau, F., et al., *Host determinant residue lysine 627 lies on the surface of a discrete, folded domain of influenza virus polymerase PB2 subunit.* PLoS Pathog, 2008. **4**(8): p. e1000136.
181. Labadie, K., et al., *Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells.* Virology, 2007. **362**(2): p. 271-82.
182. Mehle, A. and J.A. Doudna, *An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase.* Cell Host Microbe, 2008. **4**(2): p. 111-22.
183. Compans, R.W., J. Content, and P.H. Duesberg, *Structure of the ribonucleoprotein of influenza virus.* J Virol, 1972. **10**(4): p. 795-800.
184. Kobayashi, M., et al., *Molecular dissection of influenza virus nucleoprotein: deletion mapping of the RNA binding domain.* J Virol, 1994. **68**(12): p. 8433-6.
185. Prokudina-Kantorovich, E.N. and N.P. Semenova, *Intracellular oligomerization of influenza virus nucleoprotein.* Virology, 1996. **223**(1): p. 51-6.
186. Portela, A. and P. Digard, *The influenza virus nucleoprotein: A multifunctional RNA-binding protein pivotal to virus replication.* Journal of General Virology, 2002. **83**(4): p. 723-734.
187. Ye, Q., R.M. Krug, and Y.J. Tao, *The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA.* Nature, 2006. **444**(7122): p. 1078-82.
188. Wang, P., P. Palese, and R.E. O'Neill, *The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal.* J Virol, 1997. **71**(3): p. 1850-6.

189. Weber, F., et al., *A classical bipartite nuclear localization signal on Thogoto and influenza A virus nucleoproteins.* Virology, 1998. **250**(1): p. 9-18.
190. Das, K., et al., *Structures of influenza A proteins and insights into antiviral drug targets.* Nature Structural and Molecular Biology, 2010. **17**(5): p. 530-538.
191. García-Sastre, A., *Induction and evasion of type I interferon responses by influenza viruses.* Virus Research, 2011. **162**(1-2): p. 12-18.
192. Ayllon, J., A. García-Sastre, and B.G. Hale, *Influenza A viruses and PI3K: are there time, place and manner restrictions?* Virulence, 2012. **3**(4): p. 411-414.
193. Ehrhardt, C. and S. Ludwig, *A new player in a deadly game: Influenza viruses and the PI3K/Akt signalling pathway.* Cellular Microbiology, 2009. **11**(6): p. 863-871.
194. Iwatsuki-Horimoto, K., et al., *Generation of influenza A virus NS2 (NEP) mutants with an altered nuclear export signal sequence.* Journal of Virology, 2004. **78**(18): p. 10149-10155.
195. O'Neill, R.E., J. Talon, and P. Palese, *The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins.* EMBO J, 1998. **17**(1): p. 288-96.
196. Bullido, R., et al., *Influenza A virus NEP (NS2 protein) downregulates RNA synthesis of model template RNAs.* Journal of Virology, 2001. **75**(10): p. 4912-4917.
197. Robb, N.C., et al., *NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome.* Journal of General Virology, 2009. **90**(6): p. 1398-1407.
198. Akarsu, H., et al., *Structure-based design of NS2 mutants for attenuated influenza A virus vaccines.* Virus Research, 2011. **155**(1): p. 240-248.
199. Brankston, G., et al., *Transmission of influenza A in human beings.* Lancet Infect Dis, 2007. **7**(4): p. 257-65.
200. Mubareka, S., et al., *Transmission of influenza virus via aerosols and fomites in the guinea pig model.* J Infect Dis, 2009. **199**(6): p. 858-65.
201. Kawaoka, Y. and G. Neumann, *Influenza Viruses: An Introduction #*, in *T Influenza Virus*. 2012. p. 1-9.
202. Yang, Y., et al., *Detecting human-to-human transmission of avian influenza A (H5N1).* Emerg Infect Dis, 2007. **13**(9): p. 1348-53.
203. Aditama, T.Y., et al., *Risk factors for cluster outbreaks of avian influenza A H5N1 infection, Indonesia.* Clin Infect Dis, 2011. **53**(12): p. 1237-44.
204. Aditama, T.Y., et al., *Avian influenza H5N1 transmission in households, Indonesia.* PLoS One, 2012. **7**(1): p. e29971.
205. Gasparini, R., et al., *Influenza epidemiology in Italy two years after the 2009-2010 pandemic: Need to improve vaccination coverage.* Hum Vaccin Immunother, 2013. **9**(3).
206. Baguelin, M., et al., *Health and economic impact of the seasonal influenza vaccination programme in England.* Vaccine, 2012. **30**(23): p. 3459-62.
207. Hahne, S., et al., *Epidemiology and control of influenza A(H1N1)v in the Netherlands: the first 115 cases.* Euro Surveill, 2009. **14**(27).

208. Hens, N., et al., *Estimating the effective reproduction number for pandemic influenza from notification data made publicly available in real time: a multi-country analysis for influenza A/H1N1v 2009.* Vaccine, 2011. **29**(5): p. 896-904.
209. Boelle, P.Y., et al., *Transmission parameters of the A/H1N1 (2009) influenza virus pandemic: a review.* Influenza Other Respi Viruses, 2011. **5**(5): p. 306-16.
210. Cox, N.J. and K. Subbarao, *Influenza.* Lancet, 1999. **354**(9186): p. 1277-82.
211. Juno, J., K.R. Fowke, and Y. Keynan, *Immunogenetic factors associated with severe respiratory illness caused by zoonotic H1N1 and H5N1 influenza viruses.* Clin Dev Immunol, 2012. **2012**: p. 797180.
212. Mayoral, J.M., et al., *Social factors related to the clinical severity of influenza cases in Spain during the A (H1N1) 2009 virus pandemic.* BMC Public Health, 2013. **13**: p. 118.
213. Cheung, C.Y., et al., *Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease?* The Lancet, 2002. **360**(9348): p. 1831-1837.
214. García-Sastre, A., *Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses.* Virology, 2001. **279**(2): p. 375-384.
215. Kilander, A., et al., *Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010.* Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin, 2010. **15**(9): p. 19498.
216. Rykkvin, R., et al., *Within-patient emergence of the influenza A(H1N1)pdm09 HA1 222G variant and clear association with severe disease, Norway.* Euro Surveill, 2013. **18**(3).
217. Balraj, P., et al., *Molecular analysis of 2009 pandemic influenza A(H1N1) in Malaysia associated with mild and severe infections.* Malays J Pathol, 2011. **33**(1): p. 7-12.
218. Keynan, Y., S. Malik, and K.R. Fowke, *The Role of Polymorphisms in Host Immune Genes in Determining the Severity of Respiratory Illness Caused by Pandemic H1N1 Influenza.* Public Health Genomics, 2013. **16**(1-2): p. 9-16.
219. La, D., et al., *Enrichment of variations in KIR3DL1/S1 and KIR2DL2/L3 among H1N1/09 ICU patients: an exploratory study.* PLoS One, 2011. **6**(12): p. e29200.
220. Everitt, A.R., et al., *IFITM3 restricts the morbidity and mortality associated with influenza.* Nature, 2012. **484**(7395): p. 519-23.
221. John, S.P., et al., *The CD225 Domain of IFITM3 Is Required for both IFITM Protein Association and Inhibition of Influenza A Virus and Dengue Virus Replication.* J Virol, 2013. **87**(14): p. 7837-52.
222. Zuniga, J., et al., *Genetic variants associated with severe pneumonia in A/H1N1 influenza infection.* Eur Respir J, 2012. **39**(3): p. 604-10.
223. Ballinger, M.N. and T.J. Standiford, *Postinfluenza bacterial pneumonia: host defenses gone awry.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 2010. **30**(9): p. 643-652.

224. Gupta, R.K., R. George, and J.S. Nguyen-Van-Tam, *Bacterial pneumonia and pandemic influenza planning.* Emerging infectious diseases, 2008. **14**(8): p. 1187-1192.
225. Keynan, Y., et al., *Toll-Like Receptors Dysregulation after Influenza Virus Infection: Insights into Pathogenesis of Subsequent Bacterial Pneumonia.* ISRN Pulmonology, 2011. **2011**: p. 6.
226. Didierlaurent, A., J. Goulding, and T. Hussell, *The impact of successive infections on the lung microenvironment.* Immunology, 2007. **122**(4): p. 457-465.
227. Mao, H., et al., *Inhibition of human natural killer cell activity by influenza virions and hemagglutinin.* Journal of virology, 2010. **84**(9): p. 4148-4157.
228. Meunier, I., et al., *Influenza pathogenesis: lessons learned from animal studies with H5N1, H1N1 Spanish, and pandemic H1N1 2009 influenza.* Critical care medicine, 2010. **38**(4 Suppl): p. e21-9.
229. Heltzer, M.L., et al., *Immune dysregulation in severe influenza.* Journal of leukocyte biology, 2009. **85**(6): p. 1036-1043.
230. Delves, P.J., S.J. Martin, and D.R. Burton, *Essentials : Roitt's Essential Immunology (12th Edition)*. 2011, Hoboken, NJ, USA: Wiley-Blackwell.
231. Linton, P.-J., *B Lymphocyte Repertoire*, in *Encyclopedia of Immunology (Second Edition)*, J.D. Editor-in-Chief: Peter, Editor. 1998, Elsevier: Oxford. p. 359-362.
232. Kehrl, J.H., *B Lymphocyte Differentiation*, in *Encyclopedia of Immunology (Second Edition)*, J.D. Editor-in-Chief: Peter, Editor. 1998, Elsevier: Oxford. p. 355-359.
233. Rajewsky, K., *Affinity Maturation*, in *Encyclopedia of Immunology (Second Edition)*, J.D. Editor-in-Chief: Peter, Editor. 1998, Elsevier: Oxford. p. 52-54.
234. Kruisbeek, A.M., *T Lymphocyte Differentiation*, in *Encyclopedia of Immunology (Second Edition)*, J.D. Editor-in-Chief: Peter, Editor. 1998, Elsevier: Oxford. p. 2334-2341.
235. Doyle, C. and J.L. Strominger, *Interaction between CD4 and class II MHC molecules mediates cell adhesion.* Nature, 1987. **330**(6145): p. 256-9.
236. Hammarlund, E., et al., *Duration of antiviral immunity after smallpox vaccination.* Nat Med, 2003. **9**(9): p. 1131-7.
237. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
238. Sallusto, F., J. Geginat, and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance.* Annu Rev Immunol, 2004. **22**: p. 745-63.
239. Rocha, B. and C. Tanchot, *The Tower of Babel of CD8+ T-cell memory: known facts, deserted roads, muddy waters, and possible dead ends.* Immunol Rev, 2006. **211**: p. 182-96.
240. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. **8**(4): p. 379-85.
241. Romero, P., et al., *Four Functionally Distinct Populations of Human Effector-Memory CD8+ T Lymphocytes.* The Journal of Immunology, 2007. **178**(7): p. 4112-4119.

242. Halwani, R., et al., *Generation and maintenance of human memory cells during viral infection.* Springer Seminars in Immunopathology, 2006. **28**(3): p. 197-208.
243. Piqueras, B., et al., *Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors.* Blood, 2006. **107**(7): p. 2613-8.
244. Schmitz, N., et al., *Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection.* J Virol, 2005. **79**(10): p. 6441-8.
245. Renegar, K.B., et al., *Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract.* Journal of Immunology, 2004. **173**(3): p. 1978-1986.
246. Stokes, C.R., J.F. Soothill, and M.W. Turner, *Immune exclusion is a function of IgA.* Nature, 1975. **255**(5511): p. 745-746.
247. van Riet, E., et al., *Mucosal IgA responses in influenza virus infections; thoughts for vaccine design.* Vaccine, 2012. **30**(40): p. 5893-5900.
248. Asahi-Ozaki, Y., et al., *Secretory IgA antibodies provide cross-protection against infection with different strains of influenza B virus.* Journal of Medical Virology, 2004. **74**(2): p. 328-335.
249. Tamura, S., et al., *Cross-protection against influenza A virus infection by passively transferred respiratory tract IgA antibodies to different hemagglutinin molecules.* European Journal of Immunology, 1991. **21**(6): p. 1337-1344.
250. Osterholm, M.T., et al., *Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis.* Lancet Infect Dis, 2012. **12**(1): p. 36-44.
251. Pang, I.K. and A. Iwasaki, *Inflammasomes as mediators of immunity against influenza virus.* Trends in Immunology, 2011. **32**(1): p. 34-41.
252. Pothlichet, J., et al., *Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells.* PLoS Pathog, 2013. **9**(4): p. e1003256.
253. Ichinohe, T., et al., *Inflammasome recognition of influenza virus is essential for adaptive immune responses.* Journal of Experimental Medicine, 2009. **206**(1): p. 79-87.
254. Allen, I.C., et al., *The NLRP3 Inflammasome Mediates In Vivo Innate Immunity to Influenza A Virus through Recognition of Viral RNA.* Immunity, 2009. **30**(4): p. 556-565.
255. Thomas, P.G., et al., *The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1.* Immunity, 2009. **30**(4): p. 566-75.
256. Demedts, I.K., et al., *Identification and characterization of human pulmonary dendritic cells.* Am J Respir Cell Mol Biol, 2005. **32**(3): p. 177-84.
257. van Haarst, J.M., et al., *Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung.* Am J Respir Cell Mol Biol, 1994. **10**(5): p. 487-92.

258. Perrot, I., et al., *TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA.* J Immunol, 2010. **185**(4): p. 2080-8.
259. Matsumoto, M., et al., *Subcellular localization of Toll-like receptor 3 in human dendritic cells.* J Immunol, 2003. **171**(6): p. 3154-62.
260. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.* Science, 2004. **303**(5663): p. 1529-31.
261. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5598-603.
262. Maris, N.A., et al., *Toll-like receptor mRNA levels in alveolar macrophages after inhalation of endotoxin.* Eur Respir J, 2006. **28**(3): p. 622-6.
263. Wang, J., et al., *Innate immune response of human alveolar macrophages during influenza A infection.* PLoS One, 2012. **7**(3): p. e29879.
264. Ramos, I., et al., *Effects of receptor binding specificity of avian influenza virus on the human innate immune response.* J Virol, 2011. **85**(9): p. 4421-31.
265. Stanietsky, N. and O. Mandelboim, *Paired NK cell receptors controlling NK cytotoxicity.* FEBS Lett, 2010. **584**(24): p. 4895-900.
266. Achdout, H., et al., *Enhanced recognition of human NK receptors after influenza virus infection.* Journal of immunology (Baltimore, Md.: 1950), 2003. **171**(2): p. 915-923.
267. Achdout, H., I. Manaster, and O. Mandelboim, *Influenza virus infection augments NK cell inhibition through reorganization of major histocompatibility complex class I proteins.* Journal of virology, 2008. **82**(16): p. 8030-8037.
268. Achdout, H., et al., *Killing of avian and swine influenza virus by natural killer cells.* Journal of Virology, 2010. **84**(8): p. 3993-4001.
269. Mandelboim, O., et al., *Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells.* Nature, 2001. **409**(6823): p. 1055-1060.
270. Gazit, R., et al., *Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1.* Nature Immunology, 2006. **7**(5): p. 517-523.
271. Bar-On, Y., et al., *Neuraminidase-mediated, NKp46-dependent immune-evasion mechanism of influenza viruses.* Cell Rep, 2013. **3**(4): p. 1044-50.
272. De Santo, C., et al., *Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans.* The Journal of clinical investigation, 2008. **118**(12): p. 4036-4048.
273. Ho, L.P., et al., *Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection.* European journal of immunology, 2008. **38**(7): p. 1913-1922.
274. Ko, S.Y., et al., *alpha-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor.* Journal of immunology (Baltimore, Md.: 1950), 2005. **175**(5): p. 3309-3317.
275. Youn, H.J., et al., *A single intranasal immunization with inactivated influenza virus and alpha-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system.* Vaccine, 2007. **25**(28): p. 5189-5198.

276. Juno, J.A., Y. Keynan, and K.R. Fowke, *Invariant NKT cells: regulation and function during viral infection.* PLoS Pathog, 2012. **8**(8): p. e1002838.
277. Gerhard, W., *The role of the antibody response in influenza virus infection.* Curr Top Microbiol Immunol, 2001. **260**: p. 171-90.
278. Riberdy, J.M., et al., *Protection against a lethal avian influenza A virus in a mammalian system.* J Virol, 1999. **73**(2): p. 1453-9.
279. Coleclough, C., et al., *Respiratory vaccination of mice against influenza virus: dissection of T- and B-cell priming functions.* Scand J Immunol, 2005. **62 Suppl 1**: p. 73-83.
280. Food, U., *Drug Administration: Guidance for Industry. Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines*, 2010.
281. McElhaney, J.E., et al., *T cell responses are better correlates of vaccine protection in the elderly.* Journal of immunology (Baltimore, Md.: 1950), 2006. **176**(10): p. 6333-6339.
282. Govaert, T.M.E., et al., *The efficacy of influenza vaccination in elderly individuals: A randomized double-blind placebo-controlled trial.* Journal of the American Medical Association, 1994. **272**(21): p. 1661-1665.
283. Kang, S.M., J.M. Song, and R.W. Compans, *Novel vaccines against influenza viruses.* Virus Res, 2011. **162**(1-2): p. 31-8.
284. Jegerlehner, A., et al., *Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity.* J Immunol, 2004. **172**(9): p. 5598-605.
285. El Bakkouri, K., et al., *Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection.* J Immunol, 2011. **186**(2): p. 1022-31.
286. Tu, W., et al., *Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus.* Journal of virology, 2010. **84**(13): p. 6527-6535.
287. Scheible, K., et al., *CD8+ T cell immunity to 2009 pandemic and seasonal H1N1 influenza viruses.* Vaccine, 2011. **29**(11): p. 2159-2168.
288. Belz, G.T., P.G. Stevenson, and P.C. Doherty, *Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses.* J Immunol, 2000. **165**(5): p. 2404-9.
289. Effros, R.B., et al., *Generation of both cross reactive and virus specific T cell populations after immunization with serologically distinct influenza A viruses.* Journal of Experimental Medicine, 1977. **145**(3): p. 557-568.
290. Jenkins, M.R., et al., *Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting.* J Immunol, 2006. **177**(5): p. 2917-25.
291. Topham, D.J., R.A. Tripp, and P.C. Doherty, *CD8+ T cells clear influenza virus by perforin or Fas-dependent processes.* J Immunol, 1997. **159**(11): p. 5197-200.
292. Marshall, D.R., et al., *Effector CD8+ T cells recovered from an influenza pneumonia differentiate to a state of focused gene expression.* Proc Natl Acad Sci U S A, 2005. **102**(17): p. 6074-9.

293. La Gruta, N.L., S.J. Turner, and P.C. Doherty, *Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity.* J Immunol, 2004. **172**(9): p. 5553-60.
294. de Jong, M.D., et al., *Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia.* Nature medicine, 2006. **12**(10): p. 1203-1207.
295. Woo, P.C., et al., *Cytokine profiles induced by the novel swine-origin influenza A/H1N1 virus: implications for treatment strategies.* J Infect Dis, 2010. **201**(3): p. 346-53.
296. Kobasa, D., et al., *Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus.* Nature, 2007. **445**(7125): p. 319-23.
297. McElhaney, J.E. and R.B. Effros, *Immunosenescence: what does it mean to health outcomes in older adults?* Curr Opin Immunol, 2009. **21**(4): p. 418-24.
298. Epstein, S.L., *Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature.* J Infect Dis, 2006. **193**(1): p. 49-53.
299. Greenbaum, J.A., et al., *Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population.* Proc Natl Acad Sci U S A, 2009. **106**(48): p. 20365-70.
300. Lee, L.Y., et al., *Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals.* J Clin Invest, 2008. **118**(10): p. 3478-90.
301. Ely, K.H., et al., *Memory T cell populations in the lung airways are maintained by continual recruitment.* J Immunol, 2006. **176**(1): p. 537-43.
302. Woodland, D.L., *Cell-mediated immunity to respiratory virus infections.* Curr Opin Immunol, 2003. **15**(4): p. 430-5.
303. Woodland, D.L. and I. Scott, *T cell memory in the lung airways.* Proc Am Thorac Soc, 2005. **2**(2): p. 126-31.
304. Orr, P., *An Advisory Committee Statement (ACS). National Advisory Committee on Immunization (NACI). Statement on influenza vaccination for the 2004-2005 season.* Can Commun Dis Rep, 2004. **30**: p. 1-32.
305. *Interim results: state-specific influenza vaccination coverage--United States, August 2010-February 2011.* MMWR Morb Mortal Wkly Rep, 2011. **60**(22): p. 737-43.
306. *Influenza vaccination coverage levels in selected sites--United States, 1989.* MMWR Morb Mortal Wkly Rep, 1990. **39**(10): p. 159-60, 165-7.
307. McBean, A.M. and P.L. Hebert, *New estimates of influenza-related pneumonia and influenza hospitalizations among the elderly.* Int J Infect Dis, 2004. **8**(4): p. 227-35.
308. Ohmit, S.E., et al., *Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines.* N Engl J Med, 2006. **355**(24): p. 2513-22.
309. Skowronski, D.M., et al., *Estimating vaccine effectiveness against laboratory-confirmed influenza using a sentinel physician network: results from the 2005-*

2006 season of dual A and B vaccine mismatch in Canada. Vaccine, 2007. **25**(15): p. 2842-51.
310. Berry, B.B., et al., *Influenza vaccination is safe and immunogenic when administered to hospitalized patients.* Vaccine, 2001. **19**(25-26): p. 3493-8.
311. Couch, R.B., et al., *Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects.* Vaccine, 2007. **25**(44): p. 7656-63.
312. Falsey, A.R., et al., *Randomized, double-blind controlled phase 3 trial comparing the immunogenicity of high-dose and standard-dose influenza vaccine in adults 65 years of age and older.* J Infect Dis, 2009. **200**(2): p. 172-80.
313. Keitel, W.A., et al., *Safety of high doses of influenza vaccine and effect on antibody responses in elderly persons.* Arch Intern Med, 2006. **166**(10): p. 1121-7.
314. Thijs, C., et al., *Mortality benefits of influenza vaccination in elderly people.* Lancet Infect Dis, 2008. **8**(8): p. 460-1; author reply 463-5.
315. Monto, A.S., K. Hornbuckle, and S.E. Ohmit, *Influenza vaccine effectiveness among elderly nursing home residents: A cohort study.* American Journal of Epidemiology, 2001. **154**(2): p. 155-160.
316. Ohmit, S.E., N.H. Arden, and A.S. Monto, *Effectiveness of inactivated influenza vaccine among nursing home residents during an influenza type A (H3N2) epidemic.* J Am Geriatr Soc, 1999. **47**(2): p. 165-71.
317. Coles, F.B., G.J. Balzano, and D.L. Morse, *An outbreak of influenza A (H3N2) in a well immunized nursing home population.* J Am Geriatr Soc, 1992. **40**(6): p. 589-92.
318. Libow, L.S., et al., *Sequential outbreak of influenza A and B in a nursing home: efficacy of vaccine and amantadine.* J Am Geriatr Soc, 1996. **44**(10): p. 1153-7.
319. Jefferson, T., et al., *Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review.* Lancet, 2005. **366**(9492): p. 1165-74.
320. Patriarca, P.A., et al., *Efficacy of influenza vaccine in nursing homes. Reduction in illness and complications during an influenza A (H3N2) epidemic.* JAMA, 1985. **253**(8): p. 1136-9.
321. Staprans, S.I., et al., *Activation of virus replication after vaccination of HIV-1-infected individuals.* J Exp Med, 1995. **182**(6): p. 1727-37.
322. Kroon, F.P., et al., *Antibody response after influenza vaccination in HIV-infected individuals: a consecutive 3-year study.* Vaccine, 2000. **18**(26): p. 3040-9.
323. Miotti, P.G., et al., *The influence of HIV infection on antibody responses to a two-dose regimen of influenza vaccine.* JAMA, 1989. **262**(6): p. 779-83.
324. Nosyk, B., et al., *The cost-effectiveness and value of information of three influenza vaccination dosing strategies for individuals with human immunodeficiency virus.* PLoS One, 2011. **6**(12): p. e27059.
325. Zanetti, A.R., et al., *Safety and immunogenicity of influenza vaccination in individuals infected with HIV.* Vaccine, 2002. **20 Suppl 5**: p. B29-32.
326. Cooper, C.L., *Pandemic H1N12009 influenza and HIV: a review of natural history, management and vaccine immunogenicity.* Curr Opin Infect Dis, 2012. **25**(1): p. 26-35.

327. Bender, B.S., et al., *Transgenic mice lacking class I major histocompatibility complex- restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge.* Journal of Experimental Medicine, 1992. **175**(4): p. 1143-1145.
328. Epstein, S.L., et al., *Mechanism of protective immunity against influenza virus infection in mice without antibodies.* Journal of Immunology, 1998. **160**(1): p. 322-327.
329. Roberts, A.D., K.H. Ely, and D.L. Woodland, *Differential contributions of central and effector memory T cells to recall responses.* J Exp Med, 2005. **202**(1): p. 123-33.
330. Kohlmeier, J.E., et al., *Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge.* Immunity, 2010. **33**(1): p. 96-105.
331. McMichael, A.J., et al., *Cytotoxic T-cell immunity to influenza.* N Engl J Med, 1983. **309**(1): p. 13-7.
332. McMichael, A.J., et al., *Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes.* Journal of General Virology, 1986. **67**(4): p. 719-726.
333. McMichael, A.J., F.M. Gotch, and J. Rothbard, *HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes.* Journal of Experimental Medicine, 1986. **164**(5): p. 1397-1406.
334. Gotch, F., et al., *Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes.* Journal of Experimental Medicine, 1987. **165**(2): p. 408-416.
335. Townsend, A.R.M., et al., *The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides.* Cell, 1986. **44**(6): p. 959-968.
336. Kedzierska, K., S.J. Turner, and P.C. Doherty, *Conserved T cell receptor usage in primary and recall responses to an immunodominant influenza virus nucleoprotein epitope.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 4942-7.
337. Kedzierska, K., et al., *Establishment and recall of CD8+ T-cell memory in a model of localized transient infection.* Immunological Reviews, 2006. **211**(1): p. 133-145.
338. De Groot, A.S., et al., *Immunoinformatic comparison of T-cell epitopes contained in novel swine-origin influenza A (H1N1) virus with epitopes in 2008-2009 conventional influenza vaccine.* Vaccine, 2009. **27**(42): p. 5740-5747.
339. Gomez Lorenzo, M.M. and M.J. Fenton, *Immunobiology of influenza vaccines.* Chest, 2013. **143**(2): p. 502-10.
340. Gasparini, R., et al., *Live attenuated influenza vaccine--a review.* J Prev Med Hyg, 2011. **52**(3): p. 95-101.
341. Belshe, R., et al., *Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine.* Expert Rev Vaccines, 2004. **3**(6): p. 643-54.
342. Beyer, W.E., et al., *Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis.* Vaccine, 2002. **20**(9-10): p. 1340-1353.

343. Rhorer, J., et al., *Efficacy of live attenuated influenza vaccine in children: A meta-analysis of nine randomized clinical trials.* Vaccine, 2009. **27**(7): p. 1101-1110.
344. Ambrose, C.S., X. Wu, and R.B. Belshe, *The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination.* Pediatr Infect Dis J, 2010. **29**(9): p. 806-11.
345. Belshe, R.B., et al., *Live attenuated versus inactivated influenza vaccine in infants and young children.* The New England journal of medicine, 2007. **356**(7): p. 685-696.
346. Zangwill, K.M. and R.B. Belshe, *Safety and efficacy of trivalent inactivated influenza vaccine in young children: a summary for the new era of routine vaccination.* Pediatr Infect Dis J, 2004. **23**(3): p. 189-97.
347. Eick, A.A., et al., *Comparison of the trivalent live attenuated vs. inactivated influenza vaccines among U.S. military service members.* Vaccine, 2009. **27**(27): p. 3568-75.
348. Wang, Z., et al., *Live attenuated or inactivated influenza vaccines and medical encounters for respiratory illnesses among US military personnel.* JAMA, 2009. **301**(9): p. 945-53.
349. Dormitzer, P.R., et al., *Influenza vaccine immunology.* Immunol Rev, 2011. **239**(1): p. 167-77.
350. Hoft, D.F., et al., *Live and Inactivated Influenza Vaccines Induce Similar Humoral Responses, but Only Live Vaccines Induce Diverse T-Cell Responses in Young Children.* Journal of Infectious Diseases, 2011. **204**(6): p. 845-853.
351. Ashkenazi, S., et al., *Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections.* Pediatr Infect Dis J, 2006. **25**(10): p. 870-9.
352. Belshe, R.B., et al., *Live attenuated versus inactivated influenza vaccine in infants and young children.* N Engl J Med, 2007. **356**(7): p. 685-96.
353. McMichael, A.J., et al., *Influenza virus-specific cytotoxic T lymphocytes recognize HLA-molecules. Blocking by monoclonal anti-HLA antibodies.* J Exp Med, 1980. **152**(2 Pt 2): p. 195s-203s.
354. He, X.S., et al., *Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines.* J Virol, 2006. **80**(23): p. 11756-66.
355. He, X.S., et al., *Phenotypic changes in influenza-specific CD8+ T cells after immunization of children and adults with influenza vaccines.* J Infect Dis, 2008. **197**(6): p. 803-11.
356. Block, S.L., et al., *Comparative immunogenicities of frozen and refrigerated formulations of live attenuated influenza vaccine in healthy subjects.* Antimicrob Agents Chemother, 2007. **51**(11): p. 4001-8.
357. Monto, A.S., et al., *Comparative efficacy of inactivated and live attenuated influenza vaccines.* N Engl J Med, 2009. **361**(13): p. 1260-7.
358. Keitel, W.A., et al., *Trivalent attenuated cold-adapted influenza virus vaccine: Reduced viral shedding and serum antibody responses in susceptible adults.* Journal of Infectious Diseases, 1993. **167**(2): p. 305-311.

359. Atmar, R.L., et al., *Comparison of trivalent cold-adapted recombinant (CR) influenza virus vaccine with monovalent CR vaccines in healthy unselected adults.* Journal of Infectious Diseases, 1995. **172**(1): p. 253-257.
360. Treanor, J.J., et al., *Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses.* Vaccine, 1999. **18**(9-10): p. 899-906.
361. Treanor, J.J., et al., *Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses.* Vaccine, 1999. **18**(9–10): p. 899-906.
362. Belshe, R.B., et al., *Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine.* The Journal of infectious diseases, 2000. **181**(3): p. 1133-1137.
363. Vincent, A.L., et al., *Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease.* J Virol, 2012. **86**(19): p. 10597-605.
364. Tricco, A.C., et al., *Comparing influenza vaccine efficacy against mismatched and matched strains: a systematic review and meta-analysis.* BMC Med, 2013. **11**(1): p. 153.
365. Schulman, J.L. and E.D. Kilbourne, *INDUCTION OF PARTIAL SPECIFIC HETEROTYPIC IMMUNITY IN MICE BY A SINGLE.* Journal of bacteriology, 1965. **89**: p. 170-174.
366. Mbawuike, I.N., et al., *Vaccination with inactivated influenza A virus during pregnancy protects neonatal mice against lethal challenge by influenza A viruses representing three subtypes.* Journal of Virology, 1990. **64**(3): p. 1370-1374.
367. Epstein, S.L., et al., *Mechanisms of Heterosubtypic Immunity to Lethal Influenza A Virus Infection in Fully Immunocompetent, T Cell-Depleted, β2-Microglobulin-Deficient, and J Chain-Deficient Mice.* Journal of Immunology, 1997. **158**(3): p. 1222-1230.
368. Benton, K.A., et al., *Heterosubtypic immunity to influenza a virus in mice lacking IgA, all Ig, NKT cells, or γδ T cells.* Journal of Immunology, 2001. **166**(12): p. 7437-7445.
369. Kang, S.M., M.C. Kim, and R.W. Compans, *Virus-like particles as universal influenza vaccines.* Expert Rev Vaccines, 2012. **11**(8): p. 995-1007.
370. Chen, W., et al., *Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses.* Immunity, 2000. **12**(1): p. 83-93.
371. Slepushkin, A.N., *The effect of a previous attack of A1 influenza on susceptibility to A2 virus during the 1957 outbreak.* Bulletin of the World Health Organization, 1959. **20**(2-3): p. 297-301.
372. Sonoguchi, T., et al., *Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses.* Journal of Infectious Diseases, 1985. **151**(1): p. 81-88.

373. Wagar, L.E., et al., *Humoral and cell-mediated immunity to pandemic H1N1 influenza in a Canadian cohort one year post-pandemic: implications for vaccination.* PLoS One, 2011. **6**(11): p. e28063.
374. Wu, C., et al., *Systematic identification of immunodominant CD8+ T-cell responses to influenza A virus in HLA-A2 individuals.* Proceedings of the National Academy of Sciences, 2011. **108**(22): p. 9178-9183.
375. Boon, A.C., et al., *The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype.* Journal of virology, 2002. **76**(2): p. 582-590.
376. Bednarek, M.A., et al., *The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2.* J Immunol, 1991. **147**(12): p. 4047-53.
377. Liu, J., et al., *Conserved epitopes dominate cross-CD8+T-cell responses against influenza A H1N1 virus among Asian populations.* European Journal of Immunology, 2013: p. n/a-n/a.
378. Keynan, Y., et al., *Cellular immune responses to recurring influenza strains have limited boosting ability and limited cross-reactivity to other strains.* Clin Microbiol Infect, 2010. **16**(8): p. 1179-86.
379. Mahmud, S.M., et al., *Estimated cumulative incidence of pandemic (H1N1) influenza among pregnant women during the first wave of the 2009 pandemic.* CMAJ, 2010. **182**(14): p. 1522-4.
380. Bandewar, S.V., J. Kimani, and J.V. Lavery, *The origins of a research community in the Majengo Observational Cohort Study, Nairobi, Kenya.* BMC Public Health, 2010. **10**: p. 630.
381. Cooper, C., et al., *Vitamin D Supplementation Does Not Increase Immunogenicity of Seasonal Influenza Vaccine in HIV-Infected Adults.* HIV Clinical Trials, 2011. **12**(5): p. 275-276.
382. Weston, S.A. and C.R. Parish, *New fluorescent dyes for lymphocyte migration studies: Analysis by flow cytometry and fluorescence microscopy.* Journal of Immunological Methods, 1990. **133**(1): p. 87-97.
383. Betts, M.R., et al., *Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation.* Journal of Immunological Methods, 2003. **281**(1–2): p. 65-78.
384. Zaunders, J.J., et al., *Early proliferation of CCR5(+) CD38(+++) antigen-specific CD4(+) Th1 effector cells during primary HIV-1 infection.* Blood, 2005. **106**(5): p. 1660-1667.
385. Zaunders, J.J., et al., *CD127+CCR5+CD38+++ CD4+ Th1 effector cells are an early component of the primary immune response to vaccinia virus and precede development of interleukin-2+ memory CD4+ T cells.* Journal of virology, 2006. **80**(20): p. 10151-10161.
386. Abdi, R., et al., *Chemokine Receptor Polymorphism and Risk of Acute Rejection in Human Renal Transplantation.* Journal of the American Society of Nephrology, 2002. **13**(3): p. 754-758.

387. Glass, W.G., et al., *CCR5 deficiency increases risk of symptomatic West Nile virus infection.* J Exp Med, 2006. **203**(1): p. 35-40.
388. Promrat, K., et al., *Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C.* Gastroenterology, 2003. **124**(2): p. 352-60.
389. Keynan, Y., et al., *Evaluation of influenza-specific humoral response by microbead array analysis.* Can J Infect Dis Med Microbiol, 2011. **22**(1): p. 25-9.
390. Thompson, L.H., et al., *Serological survey of the novel influenza A H1N1 in inner city Winnipeg, Manitoba, 2009.* Can J Infect Dis Med Microbiol, 2012. **23**(2): p. 65-70.
391. Wiley, J.A., et al., *Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection.* J Immunol, 2001. **167**(6): p. 3293-9.
392. Stambas, J., P.C. Doherty, and S.J. Turner, *An in vivo cytotoxicity threshold for influenza A virus-specific effector and memory CD8(+) T cells.* J Immunol, 2007. **178**(3): p. 1285-92.
393. Kedzierska, K., et al., *Quantification of repertoire diversity of influenza-specific epitopes with predominant public or private TCR usage.* J Immunol, 2006. **177**(10): p. 6705-12.
394. Miller, J.D., et al., *Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines.* Immunity, 2008. **28**(5): p. 710-22.
395. Fox, A., et al., *Severe pandemic H1N1 2009 infection is associated with transient NK and T deficiency and aberrant CD8 responses.* PLoS One, 2012. **7**(2): p. e31535.
396. Graham-Cumming, G., *Health of the original Canadians, 1867-1967.* Med Serv J Can, 1967. **23**(2): p. 115-66.
397. Investigators, A.I., et al., *Critical care services and 2009 H1N1 influenza in Australia and New Zealand.* The New England journal of medicine, 2009. **361**(20): p. 1925-1934.
398. *Deaths related to 2009 pandemic influenza A (H1N1) among American Indian/Alaska Natives - 12 states, 2009.* MMWR Morb Mortal Wkly Rep, 2009. **58**(48): p. 1341-4.
399. Lim, J.K., et al., *Genetic deficiency of chemokine receptor CCR5 is a strong risk factor for symptomatic West Nile virus infection: a meta-analysis of 4 cohorts in the US epidemic.* J Infect Dis, 2008. **197**(2): p. 262-5.
400. Glass, W.G., et al., *Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection.* J Exp Med, 2005. **202**(8): p. 1087-98.
401. Pulendran, B., et al., *Case of yellow fever vaccine--associated viscerotropic disease with prolonged viremia, robust adaptive immune responses, and polymorphisms in CCR5 and RANTES genes.* J Infect Dis, 2008. **198**(4): p. 500-7.
402. Kindberg, E., et al., *A deletion in the chemokine receptor 5 (CCR5) gene is associated with tickborne encephalitis.* J Infect Dis, 2008. **197**(2): p. 266-9.

403. Kohlmeier, J.E., et al., *The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections.* Immunity, 2008. **29**(1): p. 101-113.
404. Dawson, T.C., et al., *Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus.* The American journal of pathology, 2000. **156**(6): p. 1951-1959.
405. Falleti, E., et al., *Genetic polymorphisms of interleukin-6 modulate fibrosis progression in mild chronic hepatitis C.* Hum Immunol, 2010. **71**(10): p. 999-1004.
406. Smith, A.J., et al., *Association of serum interleukin-6 concentration with a functional IL6 -6331T>C polymorphism.* Clin Chem, 2008. **54**(5): p. 841-50.
407. Tang, N.L.S., et al., *Early enhanced expression of interferon-inducible protein-10 (CXCL-10) and other chemokines predicts adverse outcome in severe acute respiratory syndrome.* Clinical Chemistry, 2005. **51**(12): p. 2333-2340.
408. Tang, N.L., et al., *Genetic association between a chemokine gene CXCL-10 (IP-10, interferon gamma inducible protein 10) and susceptibility to tuberculosis.* Clin Chim Acta, 2009. **406**(1-2): p. 98-102.
409. Keynan, Y., et al., *Chemokine receptor 5 big up tri, open32 allele in patients with severe pandemic (H1N1) 2009.* Emerg Infect Dis, 2010. **16**(10): p. 1621-2.
410. Baba, M., et al., *A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5698-703.
411. Castonguay, L.A., et al., *Binding of 2-aryl-4-(piperidin-1-yl)butanamines and 1,3,4-trisubstituted pyrrolidines to human CCR5: a molecular modeling-guided mutagenesis study of the binding pocket.* Biochemistry, 2003. **42**(6): p. 1544-50.
412. Cooper, D.A., et al., *Maraviroc versus efavirenz, both in combination with zidovudine-lamivudine, for the treatment of antiretroviral-naive subjects with CCR5-tropic HIV-1 infection.* J Infect Dis, 2010. **201**(6): p. 803-13.
413. Saag, M., et al., *A double-blind, placebo-controlled trial of maraviroc in treatment-experienced patients infected with non-R5 HIV-1.* J Infect Dis, 2009. **199**(11): p. 1638-47.
414. Gulick, R.M., et al., *Maraviroc for previously treated patients with R5 HIV-1 infection.* N Engl J Med, 2008. **359**(14): p. 1429-41.
415. Keynan, Y., et al., *Targeting the chemokine receptor CCR5: good for HIV, what about other viruses?* J Infect Dis, 2011. **203**(2): p. 292; author reply 293.
416. Yoav, K., *Maraviroc Induced CCR5 Blockage for HIV Infected Individuals is Associated with Increased Rates of Respiratory Tract Infections.* Virology & Mycology, 2012.
417. Kunisaki, K.M. and E.N. Janoff, *Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses.* The Lancet Infectious Diseases, 2009. **9**(8): p. 493-504.
418. Fine, A.D., et al., *Influenza A among patients with human immunodeficiency virus: an outbreak of infection at a residential facility in New York City.* Clin Infect Dis, 2001. **32**(12): p. 1784-91.

419. Neuzil, K.M., et al., *Cardiopulmonary hospitalizations during influenza season in adults and adolescents with advanced HIV infection.* J Acquir Immune Defic Syndr, 2003. **34**(3): p. 304-7.
420. Radwan, H.M., et al., *Influenza in human immunodeficiency virus-infected patients during the 1997-1998 influenza season.* Clin Infect Dis, 2000. **31**(2): p. 604-6.
421. Safrin, S., J.D. Rush, and J. Mills, *Influenza in patients with human immunodeficiency virus infection.* Chest, 1990. **98**(1): p. 33-7.
422. Lin, J.C. and K.L. Nichol, *Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome.* Arch Intern Med, 2001. **161**(3): p. 441-6.
423. Couch, R.B. and J.A. Kasel, *Immunity to influenza in man.* Annu Rev Microbiol, 1983. **37**: p. 529-49.
424. Ennis, F.A., et al., *Correlation of laboratory studies with clinical responses to A/New Jersey influenza vaccines.* J Infect Dis, 1977. **136 Suppl**: p. S397-406.
425. Brydak, L., et al., *Humoral Response to Influenza Vaccination in HIV-Infected Patients.* Clinical Drug Investigation, 1999. **17**(6): p. 441-449.
426. Durando, P., et al., *Safety and immunogenicity of two influenza virus subunit vaccines, with or without MF59 adjuvant, administered to human immunodeficiency virus type 1-seropositive and -seronegative adults.* Clin Vaccine Immunol, 2008. **15**(2): p. 253-9.
427. Kroon, F.P., et al., *Restored humoral immune response to influenza vaccination in HIV-infected adults treated with highly active antiretroviral therapy.* AIDS, 1998. **12**(17): p. F217-23.
428. Malaspina, A., et al., *Compromised B Cell Responses to Influenza Vaccination in HIV-Infected Individuals.* Journal of Infectious Diseases, 2005. **191**(9): p. 1442-1450.
429. Nelson, K.E., et al., *The influence of human immunodeficiency virus (HIV) infection on antibody responses to influenza vaccines.* Ann Intern Med, 1988. **109**(5): p. 383-8.
430. Cooper, C., et al., *Immunogenicity is not improved by increased antigen dose or booster dosing of seasonal influenza vaccine in a randomized trial of HIV infected adults.* PLoS One, 2011. **6**(3): p. e17758.
431. Liew, F.Y., et al., *Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity.* Eur J Immunol, 1984. **14**(4): p. 350-6.
432. Yoshikawa, T., et al., *Total viral genome copies and virus-Ig complexes after infection with influenza virus in the nasal secretions of immunized mice.* J Gen Virol, 2004. **85**(Pt 8): p. 2339-46.
433. Nguyen, H.H., et al., *Heterosubtypic immunity to influenza A virus infection requires a properly diversified antibody repertoire.* Journal of virology, 2007. **81**(17): p. 9331-9338.
434. Morens, D.M., et al., *The 1918 influenza pandemic: lessons for 2009 and the future.* Crit Care Med, 2010. **38**(4 Suppl): p. e10-20.

435. Kerr, J.R., *Swine influenza.* J Clin Pathol, 2009. **62**(7): p. 577-8.
436. Keynan, Y., et al., *Cellular immune responses to recurring influenza strains have limited boosting ability and limited cross-reactivity to other strains.* Clinical Microbiology and Infection, 2010. **16**(8): p. 1179-1186.
437. Davenport, F.M., A.V. Hennessy, and T. Francis, Jr., *Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus.* J Exp Med, 1953. **98**(6): p. 641-56.
438. Halstead, S.B., S. Rojanasuphot, and N. Sangkawibha, *Original antigenic sin in dengue.* Am J Trop Med Hyg, 1983. **32**(1): p. 154-6.
439. Kim, J.H., et al., *Original antigenic sin responses to influenza viruses.* J Immunol, 2009. **183**(5): p. 3294-301.
440. Fazekas de St, G. and R.G. Webster, *Disquisitions of Original Antigenic Sin. I. Evidence in man.* J Exp Med, 1966. **124**(3): p. 331-45.
441. Virelizier, J.L., A.C. Allison, and G.C. Schild, *Antibody responses to antigenic determinants of influenza virus hemagglutinin. II. Original antigenic sin: a bone marrow-derived lymphocyte memory phenomenon modulated by thymus-derived lymphocytes.* J Exp Med, 1974. **140**(6): p. 1571-8.
442. Frasca, D., et al., *Effects of age on H1N1-specific serum IgG1 and IgG3 levels evaluated during the 2011-2012 influenza vaccine season.* Immun Ageing, 2013. **10**(1): p. 14.
443. Toellner, L., et al., *Virus-coated layer-by-layer colloids as a multiplex suspension array for the detection and quantification of virus-specific antibodies.* Clin Chem, 2006. **52**(8): p. 1575-83.
444. Esposito, S., et al., *Live attenuated intranasal influenza vaccine.* Hum Vaccin Immunother, 2012. **8**(1): p. 76-80.
445. Osterholm, M.T., et al., *Efficacy and effectiveness of influenza vaccines: A systematic review and meta-analysis.* The Lancet Infectious Diseases, 2012. **12**(1): p. 36-44.
446. Ambrose, C.S., M.J. Levin, and R.B. Belshe, *The relative efficacy of trivalent live attenuated and inactivated influenza vaccines in children and adults.* Influenza Other Respi Viruses, 2011. **5**(2): p. 67-75.
447. Eick, A.A., et al., *Comparison of the trivalent live attenuated vs. inactivated influenza vaccines among U.S. military service members.* Vaccine, 2009. **27**(27): p. 3568-3575.
448. Doherty, P.C. and A. Kelso, *Toward a broadly protective influenza vaccine.* J Clin Invest, 2008. **118**(10): p. 3273-5.
449. McMurry, J.A., B.E. Johansson, and A.S. De Groot, *A call to cellular and humoral arms: Enlisting cognate T cell help to develop broad-spectrum vaccines against influenza A.* Human Vaccines, 2008. **4**(2): p. 148-157.
450. Nichol, K.L., et al., *Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial.* JAMA, 1999. **282**(2): p. 137-44.

451. Pearce, M.B., et al., *Efficacy of seasonal live attenuated influenza vaccine against virus replication and transmission of a pandemic 2009 H1N1 virus in ferrets.* Vaccine, 2011. **29**(16): p. 2887-94.
452. Stittelaar, K.J., et al., *Efficacy of live attenuated vaccines against 2009 pandemic H1N1 influenza in ferrets.* Vaccine, 2011. **29**(49): p. 9265-70.
453. Gao, R., et al., *Cytokine and Chemokine Profiles in Lung Tissues from Fatal Cases of 2009 Pandemic Influenza A (H1N1): Role of the Host Immune Response in Pathogenesis.* Am J Pathol, 2013.
454. Hagau, N., et al., *Clinical aspects and cytokine response in severe H1N1 influenza A virus infection.* Crit Care, 2010. **14**(6): p. R203.
455. Kwon, B.K., et al., *Progressive junctional kyphosis at the caudal end of lumbar instrumented fusion: Etiology, predictors, and treatment.* Spine, 2006. **31**(17): p. 1943-1951.
456. Kang, Y.M., et al., *Pandemic H1N1 influenza virus causes a stronger inflammatory response than seasonal H1N1 influenza virus in ferrets.* Arch Virol, 2011. **156**(5): p. 759-67.
457. Bermejo-Martin, J.F., et al., *Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza.* Critical care (London, England), 2009. **13**(6): p. R201.
458. Paquette, S.G., et al., *Interleukin-6 is a potential biomarker for severe pandemic H1N1 influenza A infection.* PLoS One, 2012. **7**(6): p. e38214.
459. Zhang, J., L. Patel, and K.J. Pienta, *Targeting chemokine (C-C motif) ligand 2 (CCL2) as an example of translation of cancer molecular biology to the clinic.* Prog Mol Biol Transl Sci, 2010. **95**: p. 31-53.
460. McLaren, P.J., et al., *Association Study of Common Genetic Variants and HIV-1 Acquisition in 6,300 Infected Cases and 7,200 Controls.* PLoS Pathog, 2013. **9**(7): p. e1003515.
461. Tyner, J.W., et al., *CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection.* Nature Medicine, 2005. **11**(11): p. 1180-1187.
462. Rodriguez, A., et al., *Characterization in vitro and in vivo of a pandemic H1N1 influenza virus from a fatal case.* PLoS One, 2013. **8**(1): p. e53515.
463. Weiss, I.D., et al., *Ccr5 deficiency regulates the proliferation and trafficking of natural killer cells under physiological conditions.* Cytokine, 2011. **54**(3): p. 249-57.
464. Keynan, Y., et al., *Evaluation of influenza-specific humoral response by microbead array analysis.* Canadian Journal of Infectious Diseases & Medical Microbiology, 2011. **22**(1): p. 25-29.
465. Thompson, L.H., et al., *Serological survey of the novel influenza A H1N1 in inner city Winnipeg, Manitoba, 2009.* Canadian Journal of Infectious Diseases & Medical Microbiology, 2012. **23**(2): p. 65-70.