Better protection from influenza in the elderly with a plant-derived virus-like particle vaccine: novel approaches in an aged mouse model

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Abstract

Influenza virus infections are a major public health threat and worldwide epidemics cause 3-5 million cases of severe illness and 250,000-500,000 deaths every year, including 300-600 fatal cases in Canada. Influenza-associated complications and deaths mainly occur in the elderly. Adults >65 years of age often have age-associated immune defects (immunosenescence) that partly explain their poor response to available influenza vaccines. The efficacy of even the vaccines recommended for the elderly is far from optimal. It is therefore important to develop new vaccines that can provide better protection for this vulnerable population. Many studies suggest that vaccines based on virus-like-particles (VLPs) can stimulate both cellular and humoral responses compared to the detergent-split, inactivated influenza vaccines (IIV) that elicit primarily a humoral response. Medicago Inc. (Quebec, QC) has developed a candidate influenza vaccine based on plant-made VLPs expressing the hemagglutinin (HA) proteins of influenza viruses on their surface. Nothing was known about how this vaccine would act in the elderly but older subjects likely rely more on cellular than humoral immunity for protection. After developing an aged mouse model for vaccination and challenge, our first goal was to characterize the immune response and protection levels of one dose of the VLP candidate compared to IIV in young (6-8 weeks) and healthy aged mice (16-20 months of age). We demonstrated that the old mice were better protected with the VLP candidate compared to IIV despite low/absent anti-HA antibody titres. To make our mouse model more relevant to the elderly, we aged the mice close to the end of their natural life-span and assessed the immunogenicity of the VLPs (versus IIV) in the presence of natural occurring comorbidities. Again, we demonstrated that the VLP candidate was superior to IIV and that those with fewer comorbidities tended to mount a more robust immune response. Because VLPs have the flexibility to be given at different anatomical sites, we explored different routes and schedules of administration of the VLP candidate vaccine and showed that some of these alternate vaccination strategies could strongly influence both the magnitude and the pattern of the immune response in the aged mice. Delivery of the VLP by intramuscular (IM) and intranasal (IN) simultaneously led to better protection and a more balanced cellular and humoral response. To further characterize the effectiveness of these alternate vaccination strategies, we developed a frailty assessment based on 29 parameters and applied it in our aged mouse model after vaccination and challenge. We found that the animals given simultaneous IM+IN vaccination had the lowest increases in

frailty after infection compared to other strategies and IIV. Overall these studies demonstrate that the plant-derived VLP vaccines can elicit a balanced immune response in aged mice and provide better protection against influenza challenge both in terms of frailty and survival. These observations strongly suggest that both the superior flexibility and immunogenicity of the VLP vaccine may confer an advantage in the elderly.

Résumé

Les infections grippales causées par les virus influenza représentent un problème majeur en santé publique. Ces infections causent 3-5 millions de cas sévères chaque année et sont responsables de 250 000-500 000 morts à travers le monde, dont 300-600 au Canada. Les personnes âgées de plus de 65 ans sont particulièrement sensibles aux complications médicales associées à ces infections. Une diminution de la réponse immunitaire s'observe généralement chez les ainés (immunosenescence), ce qui pourrait en partie expliquer leur moins bonne réponse aux vaccins antigrippaux et l'efficacité limitée de ceux-ci chez les personnes âgées. Il est donc important de développer des nouveaux vaccins mieux adaptés à cette population vulnérable. Plusieurs études indiquent que les vaccins antigrippaux à base de particules pseudo-virales (PPV) stimulent tant la réponse immunitaire humorale que cellulaire, alors que les vaccins 'traditionnels' à virions fragmentés tendent surtout à produire une réponse humorale. Medicago Inc. a développé un candidat vaccin contre la grippe à base de PPV exprimant l'hémagglutinine virale (HA) à leur surface. Notre premier but était d'évaluer la réponse immunitaire et les niveaux de protection suite à l'administration d'une seule dose de ce vaccin PPV et de les comparer avec un vaccin à virions fragmentés. Notre but était également de comparer la réponse immunitaire et la protection chez les souris jeunes avec celles observées chez les souris âgées. Nous avons donc dû développer un modèle de souris âgées pour la première fois au Centre Universitaire de Santé McGill. Nous avons découvert que le vaccin PPV procurait une meilleure protection des souris âgées comparativement au vaccin à virions fragmentés; et ce même avec peu voire aucun anticorps contre HA. Pour mimer un modèle humain âgé, nous avons maintenu les souris dans nos installations jusqu'à un âge proche de leur fin de vie. Nous avons évalué l'immunogénicité des PPV en comparaison avec le vaccin à virions fragmentés sur ces souris atteintes de comorbidités naturelles. Nous avons découvert que généralement les PPV assuraient une meilleure réponse, particulièrement chez les souris avec moins de comorbidités. Afin d'encore améliorer la réponse immunitaire chez les souris âgées, nous avons exploré différentes voies d'administration des PPV. La voie d'administration des vaccins influence significativement l'amplitude et le type de réponse immunitaire, particulièrement chez les individus âgés. L'administration intramusculaire (IM) suivie directement par l'administration intranasale (IN) procuraient une meilleure protection ainsi qu'une réponse cellulaire et humorale plus équilibrées. Pour examiner plus profondément l'efficacité des vaccins et des voies

d’administration, nous avons évalué les changements de la vulnérabilité et de la ‘fragilisation’ chez souris âgées après immunisation et infection. Nous avons découvert que celles immunisées IM/IN versus la voie conventionnelle (IM) étaient en meilleure condition (basé sur l’examen de 29 paramètres) après l’infection. En général, ces études démontrent que les PPV sont supérieures aux vaccins conventionnels à virions fragmentés dans un modèle de souris âgées.

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Contribution of authors

The candidate has chosen to present a manuscript-based thesis. This thesis contains four manuscripts and is in accordance with the "Guidelines for Thesis Preparations", provided by the Faculty of Graduate and Post-Doctoral Studies of McGill University. The candidate is recognized as the principal author and to have performed the majority of the work in the manuscripts presented. The specific contributions of authors are as follows:

Chapter 2: Hodgins B, Yam KK, Winter K, Pillet S, Landry N and Ward BJ (2017), A single intramuscular dose of a plant-made virus-like-particle vaccine elicits a balanced humoral and cellular response and protects young and aged mice from influenza H1N1 challenge despite a modest/absent humoral response Clin Vaccine Immunol. 2017 Dec 5;24(12)

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Contributions to original knowledge

The work in this thesis contributes original knowledge to the fields of influenza vaccines in an aged mouse model and the development of new influenza vaccine strategies for the elderly with a plant-derived virus-like particle (VLP) vaccine. The specific contributions are as follows:

1. We showed that protection and immunogenicity differ between young and aged BALB/c mice vaccinated with plant-derived VLP and standard 'split' or inactivated influenza vaccines (IIV).
2. We developed an aged mouse model and demonstrated its potential importance when assessing vaccines designed for the elderly.
3. One dose of influenza vaccine led to 100% survival in the young mice and most of the older mice were protected.
4. We demonstrated that a small portion (~8%) of aged mice had pre-existing antibodies that appeared to be H1-specific in the ELISA but that these antibodies may not be functional.
5. We showed that very old mice with co-morbidities can still mount a humoral and cellular ($CD4^+$ and $CD8^+$ T cells) immune response to the plant-derived VLP vaccine.
6. Frailty changes in mice are greater in those not vaccinated, which confirms what is observed in humans.
7. Frailty changes may be lowest in animals that received an intranasal dose of the plant-derived VLP vaccine in either multi-modality or prime-pull schedules.

List of abbreviations

ADCC– Antibody mediated cell cytotoxicity
Ag – Antigen
AID – Activation-induced cytidine deaminase
ANOVA – Analysis of variance
APC – Allophycocyanin
APCs – Antigen-presenting cells
ARDS – Acute respiratory distress syndrome
AS03 – Adjuvant system 03
BALF – Bronchoaveolar lavage fluid
Bcl-2 – B-cell lymphoma 2
BD – Becton Dickinson
BEI –Biodefense and emerging infections
BSA – Bovine serum albumin
BUV – Brilliant ultraviolet
BV – Brilliant violet
C – Celsius
C-di-GMP – 3',5'-Cyclic diguanylic acid
CA – California
CD – Cluster of differentiation
cDNA – complementary deoxyribonucleic acid
CI – Confidence interval
CIHR – Canadian Institutes of Health Research
CMV – Cytomegalovirus
CO_2 – Carbon dioxide
ConA – Concanavalin A
COX-2 – Cyclooxygenase-2
CPA – Cell proliferation assay
CPE – Cytopathic effect
CpG – Cytosine triphosphate deoxynucleotide and guanine triphosphate deoxynucleotide
cpm – Counts per minute
cRPMI – complete Roswell Park Memorial Institute medium
CT^1 – Connecticut
CT^2 – Computed Tomography
CTL – Cytotoxic T lymphocytes
CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4
Cy5 – Cyanine 5
d0/3/21/42 – days
DC – Dendritic cell
DiD – 1,1' dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine
DMEM – Dulbecco's modified eagle's medium
DNA – Deoxyribonucleic acid
DNAse – deoxyribonuclease
dpi – Days post-infection
e.g – exempli gratia
eGFP – Enhanced green fluorescent protein
EIA – Enzyme immunoassay
ELISA – Enzyme-linked immunosorbent assay

EMEM – Eagle's minimum essential medium

FBS – Fetal bovine serum

Fc – Fragment, crystallizable

FI – Frailty index

Fig – Figure

FITC – Fluorescein isothiocyanate

Flt3 – fms-like tyrosine kinase 3

G – Gauge

g – gram

GE – General Electric

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GMT – Geometric mean titer

H & E – Hematoxylin and eosin

H_2SO_4 – Sulfuric acid

HA – hemagglutinin

HAI – Hemagglutination inhibition assay

HBSS-/- – Hank's balanced salt solution without calcium or magnesium

HBV – Hepatitis B virus

HD – High dose

HHS-32 – Harris Hematoxylin

HI –hemagglutination inhibition

HIV – human immunodeficiency virus

HPV – Human papillomavirus

hr – hour

HRP – Horseradish peroxidase

IAV – Influenza A virus

ID – Intradermal

ie – id est

IFN– Interferon

Ig – Immunoglobulin

IIV – Inactivated influenza vaccine

IL – Interleukin

ILT-2 – Ig-like transcript 2

IM – Intramuscular

IN – Intranasal

Inc – Incorporated

IP – Intraperitoneally

KIR – Killer-cell inhibitory receptor

KLRG-1 – Killer cell lectin like receptor G1

LAG-3 – Lymphocyte-activation gene 3

LAIV – live attenuated influenza vaccine

LD_{50} – Lethal dose 50

LN – Lymph node

Log – Logarithm

LRT – Lower Respiratory Tract

M – Molar

M1/2 – Matrix-1 or 2 protein

M2e – Matrix-2

MA – Massachusetts

MCP-1 – Monocyte chemoattractant protein 1

MDCK – Madin-Darby canine kidney cells

mDCs – Myeloid dendritic cells

mg – milligram

MHC I/II – Major histocompatibility complex

MIP-1 α – Macrophage inflammatory protein 1 alpha

mL – milliliter

mLD50 – Mouse lethal dose 50

mM – millimolar

MN – Microneutralization

MO – Missouri

N/A – Not applicable

NA – Neuraminidase

ng – nanogram

NK – Natural killer cell

NLR – Nod-like receptors

nm – nanometer

NS-1 – Nonstructural protein 1

NY – New York

OD – optical density

ODN – Oligodeoxynucleotide

OH – Ohio

ON – Ontario

OVA – Ovalbumin

PA – Pennsylvania

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

PD-1 – Programmed cell death protein 1

Pdm H1N1 – pandemic H1N1

pdm09 – pandemic H1N1 2009

PE – Phycoerythrin

PerCP – Peridinin chlorphyll protein

PET – Positron emission tomography

pFL – cDNA of Flt3 ligand

pg – picogram

pH – pouvoir hydrogène

PMA – Phorbol 12-myristate 13-acetate

PO – Per os

PR8 – A/Puerto Rico/8/1934 H1N1

PRR – Pattern recognition signals

QC – Quebec

QIV – Quadrivalent influenza vaccine

RANTES – Regulated on activation, normal T cell expressed and secreted

RBC – Red blood cells

RDE – Receptor destroying enzyme

rHA- recombinant hemagglutinin

RIG-I – Retinoid

RNA – Ribonucleic acid

RNP – Ribonucloprotein

ROUT – Robust regression and outlier removal

RPMI – Roswell Park Memorial Institute medium

RT – Room temperature

S9f – Spodoptera frugiperda Sf21

SA – Sialic acid

SAIL – Small Animal Imaging Labs

SC – Subcutaneous

SD – Standard dose

SHM – Somatic hypermutation

SI – Stimulation index

SIgA – secretory IgA

SPECT – Single photon emission computed tomography

Suppl – Supplemental

TCID50 – 50% Tissue culture infective dose

Th1/2 – Type 1/2 T helper

TIM-3 – T-cell immunoglobulin and mucin-domain containing-3

TIV – Trivalent influenza vaccine

TLR – Toll-like receptor

TMB – 3,3',5,5'-Tetramethylbenzidine

TNFα – tumor necrosis factor alpha

TPCK – tolylsulfonyl phenylalanyl chloromethyl ketone

Tregs – Regulatory T cells

TRMs – Tissue resident memory T cells

U – Units

U.S. – United States

µg – microgram

µL – microliter

µm – micrometer

URT – Upper respiratory tract

UT – Utah

VA – Virginia

VE – Vaccine efficacy

VLP – Virus-Like-particle

vs – Versus

VT – Vermont

WHO – World health organization

WSN – A/WSN/1933 H1N1

x g – Times gravity

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Chapter 1: Literature review

1.1 Influenza Background

1.1.1 Influenza Viruses

Influenza viruses are a major public health threat and worldwide epidemics result in 3 to 5 million cases of severe illness and 250,000 to 500,000 deaths annually, including 300 to 600 fatal cases in Canada (1, 2). However, these numbers may be much higher since influenza-related deaths can be difficult to quantify. Patients can present with a wide variety of symptoms and many possible/probable cases are not laboratory-confirmed as influenza positive. Influenza viruses belong to the *Orthomyxoviridae* family (3). These are negative sense single stranded RNA viruses with 7-8 segments in their genomes, each of which encodes one or two proteins (3). There are four different types of influenza virus: A, B, C and D. Types A and B are the main causes of human disease and are responsible for seasonal epidemics every year (4). Influenza C viruses mostly cause a mild respiratory illness. Influenza D is a pathogen of cattle and is not known to infect humans (3). Influenza A viruses are divided into subtypes based on their surface proteins: hemagglutinin (HA) and neuraminidase (NA). These viruses are further categorized into different clades. Current influenza A strains found circulating in humans are H1N1 and H3N2. Influenza B viruses are classified as two distinct lineages: B/Victoria and B/Yamagata based on HA gene sequences (5). During the 'flu season' in temperate regions of the world, several different types or subtypes of influenza A and B viruses can co-circulate in the population and cause illness (6, 7).

Like most RNA viruses, influenza viruses are not genetically stable and mutations can accumulate rapidly, particularly when a strain is adapting to a new host/population or is under immunologic 'pressure' (8). This process, termed antigenic 'drift' is responsible for the seasonal changes that occur from one year to the next (9). Another aspect of the genetic flexibility of influenza viruses is attributable to the fact that the gene segments can segregate independently. This process (ie: wholesale exchange of one or more gene segments) is termed genetic 'shift' (9). Pandemics are thought to be caused by 'shifts' with the addition of one or more gene segments from a different species (e.g: avian, porcine) and creation of a chimeric virus that is new to the

human population. The H1N1 pandemic of 1918-1919 (also known as “Spanish Flu”) that caused over 50 million deaths worldwide is thought to have resulted from such a shift (4, 10). For reasons that are still not completely understood (11), the highest mortality rate for this pandemic was in young adults, which is a very unusual pattern for influenza infection (12). Since this pandemic, influenza A viruses have been monitored closely by global health authorities in an effort to prevent any repetition of the very high mortality seen in this pandemic. Exchange of gene segments can happen in both A and B viruses (13) but not *between* A and B viruses outside of the laboratory (14).

1.1.2 Influenza A

Influenza A virus (IAV) is the only influenza that can cause pandemics and the virus can infect a wide spectrum of animal species (15). The genome is approximately 13,500 bases in length organized into 8 gene segments (ribonucleoprotein, RNP) that can produce up to 14 proteins (16, 17). As noted above, influenza A strains are classified based on their surface glycoproteins: HA and NA, of which 18 and 11 subtypes exist in nature respectively (3, 18). The HA protein plays a critical role in influenza virus biology; mediating attachment to the host cell that leads to viral entry. The HA protein is also responsible for entry into the cytoplasm by mediating fusion of the viral and endosomal membranes. The different HA proteins play a significant role in tissue tropism and pathogenicity by targeting different sialic acid receptors in various animal species (19). This surface protein is a major target for neutralizing antibody production and is the principle target for influenza vaccinations. However, influenza A viruses have developed several mechanisms to escape from the host immune surveillance through genetic drift and shift (20). This variability is the reason influenza vaccinations must be repeated annually. The second major surface protein, NA also plays a critical role in viral biology, as it is required for the release of budding virions and assists with virus dispersion (21). Recently, NA has been shown to help with viral entry. NA can bind to receptors on target cells and complement the process of HA receptor binding (22, 23). Other viral proteins with well-defined activities are the nonstructural protein 1 (NS-1) and matrix protein 2 (M2). NS-1 contains two functional domains (N-terminal RNA-binding domain and C-terminal effector domain) and it is a major inhibitor of host innate immune responses by suppressing Type I interferon which can lead to apoptosis (24). M2 helps maintain pH across the viral envelope and facilitates release of the

uncoated viral RNP into the cytoplasm and nuclear import to start viral replication (25). Although all of these viral proteins are required for survival, the two major surface proteins are the main cause for pandemics (20, 26, 27). In the last hundred years there have been four influenza pandemics (28). The most recent pandemic was H1N1 Influenza/A/California/07/2009.

1.1.3 Influenza A H1N1 2009 Pandemic

In mid-February of 2009, a novel H1N1 virus (A/California/07/2009) emerged that caused severe respiratory illness in Mexico (29, 30). By the end of April 2009, this virus had spread internationally to initiate a worldwide pandemic (pdmH1N1) (31). It was rapidly found to be composed of six genes from a triple-reassortant North American virus of swine lineage and two genes from a Eurasian swine virus (32). While this virus was distinct from other human and swine influenza A viruses, it was genetically related to much older H1N1 viruses that had circulated in the first half of the 20th century (32). To date, emergent sub-strains of pdmH1N1 have all been antigenically homogenous, such that antibodies from A/California/07/2009 can provide good homo-subtypic immunity (7, 33, 34). Although the 2009-10 pandemic is considered to have been relatively 'mild', during the first year that pdmH1N1 circulated, this new virus still caused 151,700 to 575,500 deaths worldwide (35).

1.1.4 Life Cycle of Influenza A

Once influenza has entered the respiratory tract, the virus infects airway epithelial cells and macrophages (36) that contain either alpha 2,6 or alpha 2,3 sialic acid glycan receptors depending on the viral strain (37, 38). For example, human-adapted viruses (e.g. H1N1) preferentially bind to α 2-6 sialic acids that are distributed in the upper airways of humans whereas the avian virus (e.g. H5N1) preferentially bind to α 2,3 SA which is the most prominent receptors of the duck gut and the lower airway of humans (4). These different IAVs have distinct patterns of attachment to the upper respiratory tract of humans. For example, H1N1 rapidly attaches to ciliated epithelial cells and goblet cells, whereas H5N1 barely interacts with these cells (39, 40). Intracellular entry of the virus leads to epithelial injury and can eventually cause failure of gas exchange leading to acute respiratory distress syndrome (ARDS) and death (41, 42).

HA is expressed on the surface of the virion as a homotrimer and each monomer contains two domains: HA1 and HA2 (43). After binding to SA receptors, hydrolysis of HA0 gives rise to a HA1-HA2 dimer (44). The HA1 domain binds more tightly to the cellular SA receptor and HA2 facilitates fusion of the virus to cellular membrane (45). The HA1 globular head is the variable region of the HA and is the main site of antigenic drift (27). Virus attachment prompts endocytosis by the clatherin- and calveolin-dependent mechanism. Once endocytosed, the low pH causes fusion of the viral and endosomal membranes (46). This low pH allows the M2 proton channel to open for proton flux into the virus. This influx causes viral uncoating and leads to release of viral RNPs (47). The free viral RNA is transported to the nucleus for transcription and replication. The surface proteins: HA, NA and M2 are transported to the plasma membrane where they accumulate in lipid rafts. RNPs and M1 are exported from the nucleus and are transported to the under-surface of the lipid rafts to form the new viral particle through budding (46). The viral M1 protein is located immediately underneath the host cell lipid bilayer and is required for the attachment of RNP to the cytoplasmic tails of the surface glycoproteins and for viral budding. Once the particle begins to bud from the infected cell, the NA protein cleaves nearby sialic acid residues on cell surface glycoproteins to facilitate viral release (Figure 1.1) (4, 46). Soon after infection, the innate immune response is initiated.

1.1.5 Immune Response to Influenza Virus Infection

1.1.5.1 Innate Response

When the virus first infects an individual, the innate immune response is strongly activated within a few hours and this activation typically lasts for at least 1-2 days. The viral ssRNA is recognized by pattern recognition receptors (PRRs) such as toll-like receptor 7 (TLR), RIG-I and nod-like receptors (NLR) (48, 49). The signaling cascades downstream of these PRRs lead to the induction of interferon-mediated antiviral responses and the secretion of pro-inflammatory cytokines (50). Interferons are important cytokines produced by first-responder cells that have a critical role in anti-viral functions such as stimulating intracellular anti-viral proteins and inhibiting synthesis of cellular protein to prevent viral replication and recruiting natural killer (NK) cells, monocytes and macrophages (51). Some innate cells that are important

in viral clearance are alveolar macrophages, NK cells and dendritic cells (DCs) which lead to phagocytosis and apoptosis (52, 53). DCs are antigen-presenting cells (APCs) that form an important link between the innate and adaptive arms of the immune system (54, 55). Cytokines produced as part of the innate response such as TNFα, type-1 interferons (IFNα & β), IL-1β and IL-6 are critical to stimulate adaptive immune responses and are responsible for many of the systemic symptoms of influenza infection (56). Some typical symptoms include fever, body aches, fatigue, headache, myalgia, dry cough, sore throat and nasal congestion (57).

1.1.5.2 Adaptive Response

The second line of defense against influenza viruses is the adaptive immune response. This response takes longer to initiate; however once immunological memory is established it is very fast acting when confronted by the same or a closely-related strain (58). Upon first exposure, the adaptive response typically begins in the lung approximately 5 days after infection, and consists of a humoral response (B cells) and cellular response ($CD4^+$ T cell and $CD8^+$ cytotoxic T lymphocytes (CTLs)) which work together to clear the virus (59, 60). $CD4^+$ T cells can be divided into at least four major categories: Th1, Th2, Th17 and Tregs. Natural infection with influenza often leads to Th1 response that typically produces IFNγ, TNFα and IL-2 as well as a Th2 response (61). These cytokines can facilitate viral clearance directly as well as indirectly through the support and activation of $CD8^+$ CTLs and cytotoxic $CD4^+$ cells (62). Although Th1 cells are not absolutely required for viral clearance, they help to improve the efficiency of the immune response and viral clearance (63). In particular, CTLs recognize influenza antigens on MHC I molecules and kill the infected cells by releasing granzymes and perforin to cause apoptosis (64). On the other hand, Th2 cells engage with B cells to generate antibodies that usually target the immunodominant surface proteins: HA and NA. Strain-specific anti-HA antibodies readily neutralize the virus (65) while antibodies directed against NA inhibit viral budding which limits viral spread (66). Antibody against the minor surface glycoprotein M2 can also have protective effect by facilitating antibody mediated cell cytotoxicity (ADCC). Non-neutralizing antibodies can mediate ADCC and complement cytolysis (67).

After influenza viral infection, memory B and T cells are produced for the specific strain. However, since the circulating strain(s) can differ each year due to antigenic drift, this memory

response doesn't always prevent future infection. In some cases, the B and T cell memory from prior influenza infection can be cross-reactive (68) but these responses may not always be strong enough to provide complete, or 'sterile', immunity (68). One example of cross-reactivity in T cells are tissue-resident memory $CD4^+$ and $CD8^+$ T cells (TRMs) (69, 70). These cells can be very fast acting upon secondary infection (69, 71, 72). Their daughter cells are typically poly-functional (ie: producing multiple cytokines/chemokines), and such cells have been shown to be beneficial in driving viral clearance (73, 74). The best method to prevent infection and help with viral clearance is vaccination.

1.2 Current Vaccines and Treatments for Influenza

1.2.1 Influenza Vaccines

Vaccination is the most efficient way to prevent disease. Most commercial influenza vaccines typically trigger the production of antibodies approximately two weeks after vaccination (75). The antibodies produced give variable protection against viruses included in the vaccine formulation (homotypic immunity) and, to a greater or lesser extent, against other related viruses (heterotypic immunity) (76). Due to the highly mutable nature of influenza viruses, seasonal influenza vaccines have to be 're-designed' every 6-12 months to provide protection against the strains that researchers predict will be the most common during the upcoming 'flu season' (typically over the winter months in temperate regions of the world) (77). In children and healthy young adults, antibodies against the HA protein are reasonably associated with protection. Licensing of influenza vaccines is primarily based on their ability to elicit hemagglutinin titres of $\geq$1:40 (seroprotection) in healthy adults which is the titre at which approximately 50% of individuals are protected from infection (78, 79). This criteria dates from 1972 and, although widely used, does not always correlate with vaccine efficacy (80). For example, anti-HA titres are not a good correlate for protection in either the elderly or very young children (81, 82). The most common influenza vaccines in current use worldwide are trivalent influenza vaccines (TIV), that consist of two influenza A viruses (H3N2, H1N1) and one B virus (either the Yamagata or Victoria lineage) (83). In 2014, quadrivalent influenza vaccines (QIV) were introduced into the Canadian market that contain both a Yamagata and a Victoria lineage

strain (84). The standard dose of the vaccine is 15 μg of HA per strain given by intramuscular (IM) injection without an adjuvant and is produced in eggs (85).

1.2.2 Egg-based Vaccines

The primary production platform for influenza vaccines at the present time is based on large-scale viral growth in embryonated hens' eggs. This method has remained essentially the same for over 70 years (86). However, there are real problems with this type of production including allergic reactions to egg proteins, insufficient egg supply and low viral growth in eggs necessitating viral 'adaptation' (e.g: mutation) for growth in eggs (87). Timelines for this platform do not allow for any adaptation to sudden changes in circulating strains. Furthermore, viral growth in eggs can reduce vaccine effectiveness by inducing mutations in these viruses to adapt to this substrate (88, 89). In the recent influenza season (2017-2018), a single mutation attributable to egg-adaptation is likely responsible for the poor vaccine effectiveness against the circulating H3N2 virus in adults (90-92). Egg-based vaccines are further categorized as split or subunit.

1.2.2.1 Split and Subunit Vaccines

There are two main types of inactivated vaccine: split and subunit. In split vaccines, one or more detergents is/are used to disrupt the virus and render it non-infectious (93). For subunit vaccines, HA and NA are partially purified from the detergent-treated whole virus (e.g: Sequiris' products in North America) or produced using recombinant technologies (e.g: Protein Science's new rHA-based vaccine) (93, 94). Most of the current influenza vaccines are composed of detergent-split, virions that are administered intramuscularly and offer protection based on the generation of antibody responses (77). An exception to this 'rule' is the recently approved FluMist vaccine, a live attenuated influenza vaccine (LAIV) that is used exclusively in young children (95).

1.2.2.2 Live Attenuated Influenza Vaccine (LAIV)

LAIV is an egg-based cold-adapted virus that grows optimally at 25°C which is the temperature of the nasal passages. This adaption inhibits growth in the lower respiratory tract, where the temperature is typically ~35°C. LAIV was licensed in 2003 as FluMist to be given by intranasal (IN) instillation (96, 97). It was shown to induce long-lasting antibody titres and was effective in children (98). In adults, serologic responses to LAIV are less robust than in children, almost certainly because pre-existing immunity in adult's hampers growth of the LAIV in the nose (99). In animal and human studies, LAIV induces both innate and adaptive immunity that is well-'balanced' between Th1(cellular)/ Th2 (humoral) responses (100). LAIV has been demonstrated to be more effective in inducing nasal IgA responses, whereas IIV is more effective at eliciting serum HA antibodies (98, 101). The fact that LAIV elicits little serum antibody response but induces a strong virus-specific $CD4^+$ responses in the respiratory mucosa suggests that localization of the immune response may be important parameter in the design of influenza vaccines. This 'local' $CD4^+$ T cell response has been shown to medicate broadly protective hetero-subtypic immunity (102, 103). Virus-specific $CD4^+$ and $CD8^+$ T cells secreting IFNγ are significantly higher in children vaccinated with LAIV compared to TIV (104). Surprisingly, LAIV was not recommended for the 2017-2018 influenza season due to low effectiveness compared with other TIVs especially for H1N1 strain (105, 106). Although egg-based influenza immunizations have dominated the market for over 70 years, alternative vaccines are starting to become popular (107, 108).

1.2.3 Alternatives to Egg-based Vaccines

Some alternative production methods for influenza vaccines are cellular and recombinant platforms such as the use of mammalian cells to grow viruses or transfected plant/insect cells to produce one or more influenza virus proteins (109, 110).

1.2.3.1 Cell-based Influenza Vaccine

The first cell-based influenza vaccine was introduce in 2012 and may (eventually) help reduce the potential constraints of egg-shortages (111). This cell-based vaccine is produced by Sequiris (Flucelvax™) and is currently only available in the U.S. It still requires growth of living virus in large, sterile (and expensive) bioreactors for production with the potential for low yields

and mutations. For these vaccines, viruses are grown in Madin-Darby Canine Kidney cells (MDCKs), split with detergent(s) and then further purified to produce a subunit vaccine (112). The immunogenicity of this formulation is similar to egg-based influenza vaccines (112). For the current influenza season (2017-2018), vaccine effectiveness was slightly better in cell-based vaccines (113). However, this platform is associated with a considerably higher cost and limited scalability (114).

1.2.3.2 Recombinant HA-based Vaccine

Another alternative to egg-base vaccines is the recombinant HA-based vaccine (FluBlok™) produced by Protein Sciences (now owned by Sanofi Pasteur). FluBlok has been licensed in the United States since 2013 but it is also not yet available in Canada (115). FluBlok is produced in a baculovirus-expression system (an insect cell line) and yields purified recombinant HA proteins. The HA proteins produced are the exact match to the circulating virus, unlike egg-based systems that usually require mutations for successful viral growth. This vaccine contains three times more antigen than the standard-dose (SD) split/subunit vaccines (45 μg vs 15 μg of HA/strain). FluBlok has demonstrated cross-protection against drift influenza viruses (116, 117) and to elicits higher HI antibody titres for influenza A compared to the split vaccine in the elderly (SD Fluzone™) (118, 119). However, as is the case for Flucelvax, this platform comes with a higher price-tag and it is not yet as scalable as egg-based systems (114). To date, an evaluation of the cell-mediated immune response to these recombinant vaccines has not yet been thoroughly explored. While HA is the major target for influenza vaccines, other targets are currently being further investigated.

1.2.4 Other Targets for Influenza Vaccines

Novel influenza vaccines that are in pre-clinical development or in clinical trials assess different targets or elicit different types of immune response. These new targets focus on the more conserved regions of the virus structural or surface proteins – so-called universal influenza vaccines (120, 121). Targeting these conserved regions may permit greater cross-reactivity and possibly eliminate the need for yearly influenza vaccination. Some of these targets include conserved regions in the M2e, NP, M1 or HA/NA proteins (120). The goals of these vaccines are

to induce strong, durable and cross-protective T cell responses and to produce broadly protective antibody responses towards epitopes shared by many virus strains.

1.2.5 *Alternative Routes of Administration*

Alternative routes of administration may be a potentially effective strategy to improve immunogenicity in the elderly. The most common routes for influenza vaccine administration are IN, IM and intradermal (ID) (122). As discussed above, IM is the most common route of administration for the split or subunit influenza vaccines. Generally, ID is better than IM, since less antigen is usually required to attain the same type of immune response (123-125). However, ID is no longer commonly used for influenza vaccines. Intranasal administration has only been successful with a live attenuated vaccine. To date, VLPs have been successfully delivered by IM, IN and ID (126-129).

1.2.5.1 Intradermal Administration

Intradermal (ID) administration, allows the antigen to interact with the abundant pool of professional APCs in the skin (130). ID has been demonstrated to be superior in the elderly compared to IM. There is currently one licensed influenza vaccine in the US that is delivered by ID as mentioned earlier. However, it is no longer recommended for the elderly as it did not prove to be superior to the current TIV (131, 132). Intradermal is also no longer used in Canada for either the young healthy adults or the elderly. An alternative to ID is microneedle delivery which functions similar to ID; however, it uses a small patch with many small needles. This method is not commercially available yet. In clinical trials, microneedle delivery has been shown to be easy to use, pain-less and effective at eliciting an immune response.

1.2.5.2 Intranasal Instillation

IN instillation has been a route of administration that has been widely researched especially for respiratory diseases. However, conventional influenza vaccines such as subunit/split vaccines by IN administration may not be able to mount an adequate local immune response, since many enzymes at the mucosal sites can easily degrade vaccine antigens.

Therefore, different compositions of vaccines should be used to help overcome this issue. It is thought to be preferable to generate mucosal immunity at the site of the local infection.IN administration will most often lead to higher IgA (133, 134). IN instillation is an attractive route of administration since it avoids the use of needles. To help overcome the challenges of intranasal administration, there are some effective mucosal adjuvants including cholera toxins, chitosan, c-di-GMP, *Escherichia coli* heat labile enterotoxin (LT), oil-in water adjuvants, immunostimulating complexes, type 1 interferons, bacterial second messengers and TLR agonists (135, 136). However, none of these have been licensed to use in Canada. Mice given a subunit vaccine with a mucosal adjuvant (chitosan or c-di-GMP) demonstrated better protection than mice administered the subunit vaccine alone or adjuvanted with alum (137). IN administration with an inactivated influenza virus vaccine achieved similar levels of virus-specific B cell memory responses as by intramuscular administration (138).

1.2.5.3 Multimodality and Simultaneous Vaccinations

In recent years, more complex immunization approaches have also been investigated. Although more difficult logistically, multimodality and simultaneous vaccinations have considerable promise to achieve better protection. These new strategies include multimodality vaccination (ie: immunization by ≥1 route simultaneously), prime-boost vaccination with ≥1 vaccine formulation (e.g: inactivated antigen prime followed by live-attenuated boost) and so-called "prime-pull" vaccination (139, 140). This last approach uses two different vaccination routes, with the booster being either cytokines or the vaccine itself at the site of natural infection (139, 141). For example, by priming the immune response through an intramuscular injection, then "pulling" to the site of natural infection (e.g: the lung with an intranasal instillation), it may be possible to mount a more protective immune response against influenza. Multimodality immunization is a similar concept to the prime-pull method in some respects but the individual receives two vaccines by different routes simultaneously. This method has the advantage of requiring only a single visit but has not been well explored to date with either existing or novel vaccines. To my knowledge, there has been only one clinical trial of multimodality immunization involving elderly subjects in a nursing home. In this study, the individuals who received both LAIV and the split inactivated vaccine at the same time were better protected than those who just received the split vaccine (142, 143). This area remains controversial, and many

vaccinologists consider these alternate strategies to be ‘inelegant’ even though they may actually be much more effective.

1.2.5.3.1 Tissue-Resident T Cells

One of the main reasons to consider these alternate approaches is the increasing appreciation that the best immune response may be a ‘local’ immune response. Intranasal instillation of vaccine antigens has been shown to induce a local cellular response including tissue-resident T cells. Since antibodies against influenza are directed primarily against the HA protein (which changes continuously) and are difficult to produce in the elderly, eliciting a lung-based cellular response in these individuals may provide better protection than the current intramuscular vaccination. $CD8^+$ and $CD4^+$ tissue-resident memory T cells (TRM) have been demonstrated to enhance viral clearance, to improve survival after lethal challenge and protect against heterosubtypic challenge in mouse models (69, 144). $CD4^+$ TRMs are distinct from T effector cells by expressing high levels of $CD69^+$ and $CD11a^+$, whereas $CD8^+$ TRMs express $CD69^+$ and $CD103^+$ (145, 146). Both $CD8^+$ and $CD4^+$ TRMs are thought to differentiate at tissue sites and they are recruited by local inflammation (147, 148). These tissue-resident T cells rapidly acquire effector functions when re-exposure to a pathogen (148). The “prime-pull” strategy has been shown to induce TRM in the specific mucosal layer (e.g: lungs or eyes) in other virus models (149, 150).

1.2.6 Antiviral Therapies

Antivirals (bind to the virus to prevent further development) can be used as a treatment or chemoprophylaxis for influenza infection. There are two general types of antiviral therapies commonly used: neuraminidase inhibitors and adamantanes (151). Neuraminidase inhibitors such oseltamivir, zanamivir, peramivir help limit viral spread and can act on both influenza A and B viruses. Adamantanes are only active against influenza A viruses where they inhibit the action of the M2 protein that releases viral RNA into the cell (151). Antiviral treatment is recommended as early as possible and the best clinical benefit is seen within 48 hours after infection (152). Antiviral therapies are typically used for patients with suspected influenza who are hospitalized, have severe progressive illness or who are at high risk for complications (152).

Chemoprophylaxis is usually only recommended for individuals at higher risk for influenza complications and under specific circumstances such as high-performance athletes. Chemoprophylaxis can also be used to prevent spread from infectious contacts to individuals at risk for complications during the first two weeks following vaccination (151). Although the elderly could benefit from treatment with antivirals, they cannot depend on them as a sole treatment.

1.3 Influenza and the Elderly

Influenza A epidemics and pandemics can affect all ages and all populations, but complications are most often seen in the elderly, in children younger than 2 years of age and in people with weakened immune systems (153). More than half of the 200,000 influenza-related hospitalizations that occur annually in the United States are people over the age of 65 (154). Influenza can be easily missed in this population since the elderly often have uncharacteristic complaints such as anorexia, alteration in mental status and can be afebrile or exacerbations of underlying chronic pulmonary or cardiovascular disease (155, 156). The elderly can make little or no antibody response to vaccination and have increased vulnerability due to general decline in immune function that comes with age (immunosenescence), chronic inflammation (inflammaging), frailty, persistent cytomegalovirus (CMV) infection and co-morbidities (81). A common complication from influenza in this age group is bacterial pneumonia, which can further increase morbidity (157). Influenza infections that lead to hospitalization or immobility can have a dramatic long-term impact on the elderly. Some common adverse outcomes following infection include loss of lower limb muscle strength (158), loss of independence in the activities of daily living and diminished quality of life (159). Even after a previously healthy older individual recovers, they are often weak from muscle loss and sometimes permanently disabled (158). The same trend is observed in animal models, where influenza-associated weight loss is mostly muscle loss (160). By moving less when infected, the elderly become frailer and more easily tired. Although there is currently no cure for muscle wasting in infectious diseases, COX-2 inhibitors such as celecoxib, valdecoxib and rofecoxib have been shown to slow muscle wasting in cancer patients (161, 162).

1.3.1 Inflammaging

Inflammaging is defined as chronic, sterile, low-grade inflammation that increases with age. Some processes that may contribute to inflammaging are cellular senescence, mitochondrial dysfunction, oxidative stress and age-associated changes in the microbiota (163, 164). It is thought that chronic low-grade inflammation is a physiological response to life-long antigen exposure and that it may be an efficient defense mechanism when controlled. Life-long antigen stimulation by microbes may lead to a relatively hypoactive innate immune system (165, 166). Macrophages have been suggested as the main cause of inflammaging (166, 167). M1 macrophages, which are known as inflammatory macrophages, consume much less energy than M2 macrophages which tend to have more anti-inflammatory properties (168). M1 macrophage dominance in the elderly may therefore be an evolutionary strategy to save energy expenditure as we age (166). Inflammaging may be involved in late progression of diseases such as Alzheimer's disease, frailty syndrome and cardiovascular disease (169-171). Chronic antigen stimulation drives both inflammaging and the increase of senescent T cells (see below) (172). Chronic antigen stimulation leads to immune cell exhaustion by increasing the expression of inhibitory receptors such as PD-1, CTLA-4, LAG-3, TIM-3 and others (173-175).

1.3.2 Immunosenescence

Immunosenescence is a decline in immune function that is associated with age and affects both innate and adaptive immune responses. This decline has broad implications for reduced responses to both natural infections and vaccines (81). Immunosenescence is thought to be one of the serious consequences of inflammaging. Immunosenescence and inflammaging likely impact many of the diseases that occur with greatest frequency in the elderly such as infections, cancer and chronic inflammatory diseases (166). These changes are thought to have evolutionary benefits in some respects by adapting and remodeling the immune system as we age (176, 177).

1.3.2.1 Effects of Age on Innate Immunity

Many innate cells are affected by ageing such as neutrophils, macrophages, DCs and NK cells. In monocyte derived DCs (mDCs) there is a decrease in antigen presentation, TLR-

mediated signaling and endocytosis. Moreover, dendritic cells from older individuals are less able to prime $CD4^+$ T cells to novel antigens (177, 178). The number of neutrophils and macrophages seems to be preserved in older individuals, however many of their functions are decreased including phagocytosis, chemotaxis and apoptosis. Additionally, macrophages isolated from older subjects have decreased TLR expression and function, MHC class II expression and cytokine production (179, 180). Innate immunity is only part of the immune response that is changed with age; adaptive immune responses are also affected.

1.3.2.2 Effects of Age on Adaptive Immunity

There are two main changes in adaptive immunity that occur with ageing: a decrease in naïve T cells with a largely commensurate increase in memory T cells. With age, clonal expansion, cytokine production and specific antibody production are compromised. Thymic involution occurs with increasing age and a decline in naïve T cell production from a lifetime of various pathogen exposures leads to a reduction in the naïve T cell pool and an increase in the proportion of memory T cells (181). Thymic involution may occur for evolutionary reasons, since the long-term maintenance of such a metabolically active organ may be increasingly taxing as we age (182, 183). A very similar phenomenon is observed in muscle and bone marrow.

For cellular responses, the most notable changes are in $CD8^+$ CTL (184). Ageing is associated primarily with a loss of naïve $CD8^+$ T cells rather than naïve $CD4^+$ T cells. However, the ability of $CD4^+$ T cells to differentiate into functional subsets is compromised leading to delayed viral clearance and prolonged inflammation that can contribute to pathology (185, 186). Whereas for $CD8^+$ T cells, both their number and their function are affected by age. Granzyme B levels in unstimulated T cells are significantly increased in some older adults. Granzyme B needs perforin to be internalized in the infected cells to cause apoptosis (187, 188). However, perforin is often not co-expressed in the $CD8^+$ T cells in older adults, therefore leading to an accumulation of granzyme B in the tissue microenvironment that can cause inflammation. CTL function is inhibited by terminally-differentiated (possible senescent) T cells. These terminally-differentiated T cells upregulate IL-10, a cytokine that suppresses CTLs (189, 190). The IFNγ: IL-10 ratio also seems to decrease with age leading to impaired T cell responses (191). IL-10 also decreases the expression of co-stimulatory molecules on antigens presenting DCs, which

limits the stimulation of T cell memory. Senescent T cells typically express CD57, KLRG-1, ILT-2, KIR and an antiapoptotic marker Bcl-2 (192-196). These cells are usually highly differentiated effector T cells and are often specific for latent viruses such as CMV. They secrete pro-inflammatory cytokines and are largely thought to be terminally differentiated and resistant to removal by apoptosis (197, 198).

Another surface molecule affected by ageing is CD28. The loss of this co-stimulatory surface molecule (CD28) is mostly seen in $CD8^+$ T cells (199). This decrease in CD28 has been correlated with poor antibody responses to influenza vaccination and a decrease in influenza-specific memory T cells (200). CD28 is required for activation, proliferation and differentiation of effector functions in T cells (201). High concentrations of TNFα may be the cause for the decreased expression of CD28 which can down-regulate clonal T cell expansion (202). Other cell types affected in the elderly are regulatory T cells (Tregs) and Th17 cells that typically increase with age leading to downregulation of T cell activation and increase in inflammation respectively (203, 204).

Although overall antibody levels tend to remain constant or increase with age, B cells are also affected by ageing (205). Some key changes are decreases in several crucial antibody qualities. These decreases are caused by lower activity of activation-induced cytidine deaminase (AID), class switching recombination binding and somatic hypermutation (SHM) that are all required for affinity maturation and class switching (206, 207). The elderly therefore tend to have low affinity antibodies and greater amounts of IgM. Many different health-related issues can often contribute to their decreased immunity(208).

1.3.3 Co-morbidities

Some chronic illnesses that increase the risk of bad outcomes in influenza include respiratory and heart diseases, diabetes or other metabolic diseases, chronic renal or adrenal gland failure, any type of cancer, hematological diseases, immunodeficiency, chronic inflammatory bowel disease, chronic hepatic diseases and neuromuscular disease (153, 209). Those with co-morbidities are strongly encouraged to accept annual vaccination since they can be at much higher risk for complications. Research on the broad implications of co-morbidities

overall and influenza vaccines has been limited. To our knowledge, only one clinical study has assessed both frailty and co-morbidities, comparing the relative efficacy of high-dose (HD) vs SD influenza vaccine (210). The effectiveness of the SD vaccines was significantly lower in adults with high-risk co-morbidities than in those with none. In contrast, the HD formulation had the same relative efficacy independent of the presence of co-morbidities (210).

1.3.4 Frailty

There is currently no universal definition of frailty. However, frailty theoretically can be defined as a state of increased vulnerability resulting from age-associated declines in reserve and functions across multiple physiologic systems (211). Frailty can be thought of as the clinical manifestation of biological age instead of chronological age. Immunosenescence and inflammaging are thought to be the cause of ageing and there is more and more evidence that inflammaging is the principle cause of frailty (212). Frailty has an impact on the overall health of an individual. In particular, frailty can influence immune responses in the elderly. Although there is some debate in the literature whether frailty can have an impact on the response to influenza vaccination (213), TIV vaccine effectiveness clearly declines in older adults as frailty increases (214). More recently however, a study conducted in veterans demonstrated that pre-existing immunity predicts influenza post-vaccination titres and not frailty. These authors did not observe a correlation between frailty and antibody titres (215). Assessing frailty in the elderly may help identify those who are less likely to respond to influenza vaccines and at higher risk for influenza and its associated complications. Moreover, frailty is very rarely measured when assessing vaccine effectiveness (216). Frailty also has an impact on other vaccinations and interventions (217).

1.3.4.1 Frailty Measurements

To date, there are two methods for measuring frailty: phenotype and frailty index (FI). Fried *et al* first described the phenotype method for measuring frailty in 2001 (218). This approach assesses the five following characteristics: weight loss, weakness, slowness, low level of physical activity and exhaustion. If an individual has 3 or more of these characteristics (ie: score ≥3), they are considered to be frail. In 2005, Rockwood *et al* introduced another method

for frailty measurements: the frailty index (219) that assesses deficits across different physiological systems and that can be measured over time in the same individual. FI is reported on a scale of 0-1.0 and can measure varying degrees of frailty, whereas each of the elements of the phenotype frailty measure is binary. In older adults, frailty seems to accumulate at a rate of ~3% per year. The highest FI observed in humans to date appears to be 0.67, after that individuals are no longer able to survive additional deficits. Adverse outcomes in humans can be predicted by FI, independent of age (220). There are several animal models used for ageing that could also be useful for frailty studies.

1.3.4.2 Animal Models for Frailty

Animal models of frailty are rare but are of high interest to investigators studying ageing. Candidate models for invertebrates include the fruit fly, *Drosophila melanogaster,* and the nematode, *Caenorhabditis elegans* (221, 222). Some consider these invertebrates to be ideal models due to their short life spans and the well-developed set of genetic tools associated with these organisms. They also have age-related behavior decline similar to those found in humans that can be used to assess frailty. For nematodes, physical activity assays have been indicators of muscle frailty in particular. The main mammalian frailty model used focus on mice (223-227). However, rat frailty models are starting to become developed (228, 229), as they also have relatively short lifespan (~2.5 years) and a great deal is already known about ageing in this species (230, 231).

Genetically modified mice such as IL-10 knockouts have many traits of early frailty such as increased inflammation (specifically increases in IL-6) and increased muscle loss (232). However, studies in genetically unmodified animals are more relevant for human beings. Early studies in aged mice or rats focused primarily on physical traits such as atrophy, cachexia and sarcopenia for basic frailty assessment (233, 234). Other studies have used specialized equipment, and measurements not very suitable for longitudinal studies (e.g: repeated X-rays, large volume blood draws). More recently, investigators have focused on body weight and a range of noninvasive measurements to assess frailty longitudinally (223). In particular, Whitehead *et al* created a frailty index for ageing C57BL/6 mice that assesses 31 parameters focused on the following systems: integument, musculoskeletal, ocular and nasal,

vestibulocochlear/auditory, digestive, urogenital and respiratory. Signs of discomfort, body weight and temperature are also assessed. These deficits are measured on a 3-point scale system: 0 = no deficit, 0.5 = mild deficit and 1 = severe deficit (227). This comprehensive analysis was compared to other more invasive methods and the results appear to be comparable but the FI allows for longitudinal studies to be performed. This system was also similar, in many ways, to human frailty index measurements (227). The submaximal FI for the mouse in this system is very close to 0.67 which is the maximum reported in humans (235). Despite the similarities between the murine and human frailty indices, there are still significant differences between mice and humans. For example, mice have much higher generative capacities, their muscle mass decreases minimally with age and they have higher telomerase activity (236). Mice are inbred which make them very different from humans and disease prevalence is also very different in the two species (237). Cancer and major neurologic decline are more common in inbred mice, whereas chronic inflammatory conditions like type 2 diabetes and cardiovascular disease are far more prevalent in humans (237). The exact cause of frailty remains unknown; however, it has been thought that CMV infection could increase frailty.

1.3.5 Cytomegalovirus (*CMV) Infection*

CMV is a beta-herpesvirus that is prevalent worldwide and persists in the human host for life (238). CMV is transmitted through direct contact with body fluids – most often passing from infected mothers to the child near the time of birth. The seroprevalence of CMV increases steadily with age and CMV may be a major driver of age-associated immune decline, particularly in T-cells (239). Chronic CMV infection has been associated with higher levels of highly differentiated T cells and lower levels of naive T cells. These changes are most prominent in $CD8^+$ T cells (184) but elderly subjects who are CMV-seropositive also have decreased numbers of naïve $CD4^+$ T cells (240). This loss of naïve $CD4^+$ T cells is correlated to an increase in effector/effector memory $CD4^+$ and $CD8^+$ T cells that is only seen in CMV-seropositive people. These changes have real-life consequences. For example, CMV-seropositivity is inversely correlated with antibody responses to influenza vaccination in the elderly (241). The negative impact on antibody responses to vaccination in individuals may be due to increased baseline TNFα production that can reduce B cell function (242). Chronic CMV infection also impacts other aspects of the immune response including DC function through the production of

an IL-10 ortholog (243). There are various parameters to consider in the elderly when evaluating vaccine candidates.

1.4 Strategies for Improving Influenza Vaccines in the Elderly

The elderly typically make a very poor immune response to 'standard' influenza vaccines (244). As a result, there have been many attempts to increase vaccine-induced protection in this vulnerable population. To date, these attempts have focused almost exclusively on improving antibody responses to vaccination. Although logical in some respects because the systemic antibody response to influenza vaccination clearly diminishes with age (245), natural influenza infection induces both antibody (B cell) and cellular (T cell) adaptive responses and both humoral and cellular responses can protect, either by neutralization of the virus and/or killing of influenza infected cells. Recent evidence suggests that the elderly may rely more on the cellular response for protection from influenza than younger subjects (245, 246). There is also some suggestion that responses at mucosal surfaces may be better maintained than systemic responses in the elderly (247). The decreased vaccine efficacy observed in the elderly could also be due to an imbalance in effector memory T cells and regulatory responses (244). So far, relatively little attention has been paid to addressing these 'other' aspects of the poor vaccine-induced response in the elderly.

1.4.1 Adjuvanted Vaccines

Squalene-based adjuvants such as MF59 and AS03 have been incorporated into influenza vaccines to help increase anti-HA antibodies and protection (248). To date, AS03 has been used primarily in pandemic vaccine candidates (including against the H1N1pdm virus in 2009-10), while MF59 is found in Fluad™, a seasonal influenza vaccine that has been using in the elderly for 10-15 years in Europe and more recently North America (248). Fluad, which contains the standard amount of HA (15ug/strain) plus MF59, was first made available in Canada in 2011. MF59 increases the local production of cytokines and chemokines at the site of injection, leading to increased cell recruitment and antigen uptake (249). These local effects are thought to drive the stronger antibody responses. Not only are more antibodies made in response to MF59-adjuvanted vaccines, the response is also broader with greater protection against heterovariant

viruses compared to the standard vaccine (250). An increase in antigen-specific polyfunctional T cells has been observed in children who receive the MF-59-adjuvanted vaccine, but no similar studies have been done in the elderly to date. In a field effectiveness study, Fluad was shown to reduce the risk of clinical influenza by 25% compared to the standard dose (251).

1.4.2 High Dose Vaccines

Another strategy to improve vaccine immune responses in the elderly is increasing the antigen dose, For example, Fluzone-HD, is identical to SD vaccines but contains 60 μg/strain instead of 15 μg/strain (252) . The HD formulation was approved in the USA and Canada in 2010 and 2016 respectively and has been demonstrated to improve HAI titres (252). This improvement occurs irrespective of age, presence of co-morbidities or frailty conditions (210, 253). It is thought that increased antigen delivery to antigen presenting cells following HD vaccination leads to the better antibody response. Indeed, HD vaccination increases the number of follicular DCs ornamented with antigen-antibody immune complexes (254). The immune response after HD vaccination has also been shown to be more durable (254, 255). Although the HD vaccine induces a strong and long-lived Th2 response, the Th1 response to this formulation remains relatively weak and the higher antigen dose may stimulate Treg-mediated suppression (256).

1.4.3 Intradermal Vaccines

Different routes of administration might also improve influenza vaccine responses in some populations. Intradermal (ID) administration (9 or 15 μg of HA/strain) has been approved in the US since 2011 (257) and may increase immunity by engaging the abundant pool of professional antigen-presenting cells (eg: DCs) in the skin (130, 258). Indeed, ID has been shown to increase recruitment of DCs and facilitate lymphatic drainage of free antigen (259). Direct delivery of antigen to the skin can induce cellular and humoral responses that are at least equal to IM delivery but with a lower dose of antigen (260, 261). Although the ID vaccine was initially considered a promising strategy for the elderly, it was not shown to be superior to the standard TIV (131, 132).

1.5 Virus-like Particles

Yet another strategy to make a more effective vaccine is to produce virus-like-particles (VLPs). These are non-infectious since they do not contain viral RNA and can be manufactured so they contain one or more viral proteins such as HA, NA, M1 and M2. Many VLPs are approximately the same size as influenza viruses, between 80-120 nm (262, 263). VLP-based vaccines have been shown to induce potent protective immune responses against a number of viral pathogens (264, 265). Among the reasons that VLP vaccines are more immunogenic than other subunit vaccines include their particulate nature and the fact that they display epitopes on their surface in a compact (immunologically-relevant) array. The structure of VLPs enables them to induce potent T-cell mediated immune responses through interactions with antigen-presenting cells (APCs), particularly dendritic cells (DCs) (266). VLPs can also induce strong B cell responses due to their repetitive and high-density display of epitopes (266). Currently there are several highly successful VLP-based vaccines (e.g: hepatitis B virus (HBV) and human papillomavirus (HPV)) that are approved by regulatory agencies and licensed commercially. VLPs can be produced in insect systems, in mammalian cells or in plants (262, 267, 268). Currently there is no VLP vaccine licensed for influenza. However, several different VLP vaccines produced using different platforms are being tested for this purpose in animals or are in clinical trials. Another potential advantage of VLPs is that they seem to induce a more durable immune response that the standard split-virus products. For example, Giles *et al* demonstrated that immunity acquired early in life with influenza VLPs can protect much older mice (269, 270). Furthermore, VLPs can, in some circumstances, be processed by antigen presenting cells for cross-presentation and the induction of $CD8^+$ T cells (271). Mice receiving influenza trivalent VLPs produce higher levels of HA-specific $CD8^+$ T cells than the commercial trivalent 'split' vaccine (272). Another promising feature of VLPs is the surface display of epitopes in a repetitive and high-density array; ideal for cross-linking B cell receptors and the induction of protective antibody responses. Most VLPs are currently produced in insect cells using the baculovirus expression system. These vaccines are inevitably contaminated with baculovirus particles however (about 5% of the total protein) (273). Although baculovirus does not replicate in mammalian cells, they have been shown to possible trigger innate immunity (274, 275). An alternative to these expression systems is plant-derived VLPs.

1.5.1 Plant-derived VLPs

Using a plant-based production platform, VLP vaccines can be made at relatively low cost and this system is more easily scalable than other production systems (276). Medicago Inc, a biotechnology company located in Quebec City, has created several novel influenza vaccine candidates that contain different viral HAs using its proprietary VLPs produced in plants. These plant-derived VLPs have none of the normal structural components of the virus but still assemble into 'empty' pseudovirions that share many similarities with living influenza viruses. These vaccines are produced by first cloning the HA gene of interest into an appropriate expression cassette. An attenuated strain of *Agrobacterium tumifasciens* is used to transiently insert the gene into the cells of young, rapidly growing *Nicotiana benthamiana* plants to drive high-level protein expression. Approximately five to six days after transfection, plants are harvested and the VLPs are purified (277). These VLPs only express HA on their surface. This method is much faster and more cost effective than other technologies. It allows the first doses of a candidate vaccine to be made only one month after the genetic sequence of a new strain has been identified (263, 278, 279). This system has obvious potential advantages in responding to a rapidly-moving pandemic. This type of VLP also has an equivalent or superior immunogenicity to the standard, inactivated vaccines in mice (280, 281). Enhanced and heterologous protection mediated by plant-made H5-VLP vaccination has also been demonstrated in ferrets challenged with A/Vietnam/1203/04 (282). Plant-made VLPs have the ability to bind antigen-presenting cells when the exposed HA interacts with the sialic acid receptors. They also have authentic display of trimeric HA spikes that imitate the surface of influenza viruses. The immunogenicity of the vaccines produced by Medicago Inc has proved to be comparable or superior to conventional vaccines in human clinical trials to date ((279) ,unpublished data). These plant-made influenza VLP vaccines not only produce efficient antibody responses, but they also elicit readily detectable and long-lasting, poly-functional T cellular responses in humans unlike the conventional influenza split vaccines (279). The strong cellular responses elicited by Medicago's VLP vaccines may be very advantageous for the elderly (279).

1.5.2 Alternative routes of VLPs

To date, influenza VLPs have been shown to effective by intranasal instillation, intramuscular, subcutaneous (SC) injection and intraperitoneally (IP) (269). Studies that have administered VLPs by IP demonstrated protection and sufficient immune response towards the HA protein. Furthermore, protection and immunity was shown to increase with the use of varying adjuvants (283, 284). Another alternative route SC injection have demonstrated that SC administration of VLPs, mounted adequate immune responses (285, 286). However, protection is inadequate or not tested with this route. One of the most popular alternative routes for influenza VLPs is IN instillation. Several studies have demonstrated protection in mice after lethal infection of influenza A receiving two or three IN instillations of VLPs (128, 138). Intranasal delivery with VLPs against the NA was also shown to be protective in mice (287, 288).

1.6 Rationale and Research Objectives

Influenza infection in the elderly can be catastrophic, leading to death, hospitalization, long-term disability and loss of autonomy (ie: frailty) among other serious outcomes. Influenza vaccines are currently the best method for protection; but available vaccines are far from optimal in this vulnerable population. Moreover, the outcomes associated with vaccination typically measure acute events (e.g: doctor's visits, Emergency Room visits, hospitalization, death) and do not consider long-term events like frailty after infection. Better vaccines need to be developed to prevent not only the acute events associated with infection but also frailty. The following chapters describe the candidate's contribution to novel research focusing developing new strategies to protect the elderly from influenza and its consequences using novel, plant-derived VLP vaccines that have many attractive properties.

In order to study novel strategies based on the plant-derived VLP vaccines, we first needed to develop an aged mouse model [Aim 1]. Although a small literature exists for influenza vaccine studies in aged mice, mostly due to the cost of these animals (>$250/mouse), so experiments had to be meticulously organized and coordinated to extract as much information as possible. We also wanted to compare protection levels and immunogenicity with one dose of influenza vaccine (H1-VLP vs H1N1 split vaccine) [Aim 1]. The older these aged mice get, the more co-morbidities we observe. In order to assess the effects of natural occurring co-

morbidities, we aged mice close to the end of their lifespan. Then we vaccinated them with two different influenza vaccines (split vaccine vs H1-VLP) and measured co-morbidities to evaluate the effects of co-morbidities on the immune responses [Aim 2].

Older human as well as older mice have lower immune responses towards vaccines. Different routes of administration may be beneficial to this population and boost immune responses. We compared routes and vaccine strategies in aged BALB/c mice [Aim 3]. Current vaccines strategies in the elderly help boost immune responses and increase vaccine effectiveness. However, most animal studies or clinical trials do not assess frailty and whether these vaccines geared towards the elderly can help prevent increase in frailty after infection. We assessed frailty changes in vaccinated mice after a sub-lethal infection [Aim 4].

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1.8 Figure and Legend

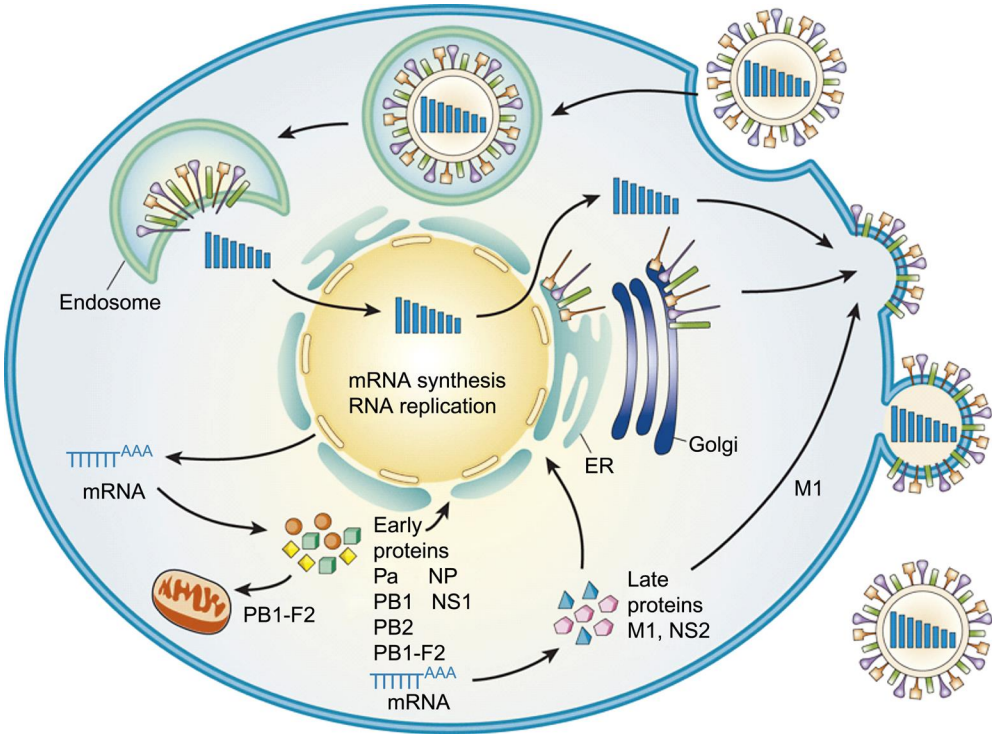

Figure 1.1 Schematic diagram of the influenza viral life cycle, with permission from Nature 459, 931–939

Chapter 2: A single IM dose of a plant-made VLP vaccine elicits a balanced humoral and cellular response and protects young and aged mice from influenza H1N1 challenge

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2.1 Preface

Virus-like particle vaccines bearing the influenza hemagglutinin protein produced in plants have been shown to induce a protective immune response in both ferrets and mice for different influenza strains including both seasonal and pandemic viruses. Nothing was known at the time about responses in older individuals, a population highly vulnerable to influenza. We developed an aged mouse model of influenza vaccination and challenge to assess immune responses and protection after a single dose of the H1-VLP without an adjuvant in both young and aged BALB/c mice and to compare these novel VLPs to an H1N1 inactivated split influenza vaccine. In addition, we also compared immune responses in the two age groups using different routes of administration (ie: intramuscular, intranasal).

2.2 Abstract

Background: Virus-like-particle (VLP) influenza vaccines can be given intramuscularly (IM) or intranasally (IN) and may have advantages over split-virion formulations in the elderly. We tested a plant-made VLP vaccine candidate bearing the viral hemagglutinin (HA) delivered either IM or IN in young and aged mice.

Methods: Young adult (5-8 weeks) and aged (16-20 months) female BALB/c mice received a single 3μg dose based on HA (A/California/07/2009 H1N1) content of a plant-made H1-VLP (IM or IN), split-virion vaccine (IM) or left naïve. After vaccination, humoral and splenocyte responses were assessed and some mice were challenged.

Results: Both VLP and split vaccines given IM protected 100% of the young animals but the VLP group lost the least weight, and had stronger humoral and cellular responses. Compared to split vaccine recipients, aged animals vaccinated IM with VLP were more likely to survive challenge (80% vs. 60%). Lung viral load post-challenge was lowest in the VLP IM groups. Mice vaccinated with VLP IN made little detectable immune responses but survival was significantly increased.

Conclusion: In both age groups, IM administration of the H1-VLP vaccine elicited more balanced humoral and cellular responses and provided better protection from homologous challenge than the split-virion vaccine.

2.3 Introduction

According to the World Health Organization, influenza epidemics account for 250,000 to 500,000 deaths worldwide every year (http://www.who.int/mediacentre/factsheets/fs211/en/). Although vaccines are widely recommended to protect against influenza, the elderly often respond poorly; in part due to prior experience with influenza antigens (1) but also as a result of immunosenescence (2). The latter affects both innate and adaptive immune responses and has broad implications for both natural infection and vaccination (1, 2).

Influenza vaccines for adults are administered by either intramuscular (IM) or intradermal injection of detergent-split virions at a fixed dose of 15μg HA /strain (3). These vaccines typically elicit strong antibody responses in healthy young adults and achieve vaccine

efficacy (VE) that varies between strains and years but averages 50-60% (4). These formulations work less well in the elderly (5). Recently, IM formulations with 60μg HA/strain (so-called high-dose or HD vaccine) or with an adjuvant have been shown to induce higher antibody responses in the elderly (4, 6) but only the former has been demonstrated to slightly improve VE (~24%) (6). Clearly there is room for alternate strategies to improve VE in adults and particularly in the elderly (7).

Virus-like particle (VLP) vaccines for influenza have many theoretical advantages including the delivery of an antigen bolus, presentation of viral antigens in an immunologically-relevant array and the possibility of both IM and IN delivery (8-11). These vaccines appear to elicit both strong antibody responses and long-lived and poly-functional $CD4^+$ T cell responses (12). The latter characteristic is of particular interest for the elderly since cellular responses may be more important for protection in this population (13).

In this work, we evaluated the immunogenicity and protective efficacy of a VLP vaccine bearing the HA of A/California/07/2009 H1N1 in young and aged mice. Our results demonstrate that a single 3 μg dose of this candidate vaccine delivered IM was superior to a standard split-virion vaccine in almost all measured outcomes at both ages. Although the same VLP vaccine delivered IN failed to elicit any detectable humoral or cellular responses, between 50% (aged) and 75% (young) of the animals were still protected from challenge.

2.4 Materials and Methods

2.4.1 Virus, Mice and Vaccines

Young adult (5-8 weeks) and aged (16-20 months of age) female BALB/c mice (Charles River Laboratories, Montreal, QC) were divided into the following groups: naïve, detergent-inactivated split vaccine given IM (A/California/07/2009 H1N1 or pdmH1N1: BEI resources, Manassas, VA), H1-VLP vaccine given either IM or IN (Medicago Inc., Quebec, Quebec). The H1-VLP was produced as previously described (14) using the wild-type sequence of HA protein from pdmH1N1. The complete study consisted of 6-15 mice per group in 5 separate experiments (Table 1). Mice received a single dose of vaccine (3 μg based on HA content) on day 0 or were

left naïve. For IM injections, 50 µL of vaccine was administered into the quadriceps muscle (right leg for VLP vaccine, bilaterally for split vaccine) using a 28G ½ needle. Intranasal instillation was performed in mice anesthetized with isoflurane (25 µL/nare).

Peripheral blood was collected from the lateral saphenous vein before immunization (d0) and twenty-one days post-vaccination (d21). Blood was collected in microtainer serum separator tubes (BD Biosciences, Mississauga, ON). Cleared serum samples were obtained by following the manufacturer's instructions and stored at -20°C. In each experiment, 6 mice per group were sacrificed in a CO_2 chamber at d21. Serum was collected by cardiac puncture, bronchiolar lavage fluid (BALF) was obtained in cRPMI then spleens were harvested from individual mice and splenocytes were isolated as previously described (15).

The remaining mice (10-11 animals/group) were challenged on d21 with 5x lethal $dose_{50}$ (LD_{50}) of wild-type A/California/07/2009 H1N1 virus (1800 $TCID_{50}$ in 50 µL: National Microbiology Laboratory, Public Health Agency of Canada) by IN instillation and weight loss was monitored daily for 12 days. In preliminary experiments, the mouse LD50 (mLD50) was found to differ between young and aged animals (approximately 663 and 105 tissue-culture infectious dose 50% [$TCID_{50}$], respectively) Three days post-infection, 7-14 mice/group were sacrificed to measure lung viral load. For these mice, serum, BALF and lungs were collected. All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

2.4.2 Antibody titre measurements

A hemagglutination inhibition assay (HAI) was performed to detect pdmH1N1-specific antibody in the mouse sera at d0 and d21 post-vaccination and 3 days post-infection as previously described (16). Briefly, mouse sera and receptor-destroying enzyme (RDE: Cedarlane, Burlington, ON) were mixed 1:4 and incubated for 18 hours at 37°C. The RDE was inactivated at 56°C for 30 minutes and sera were serially 2-fold diluted in phosphate buffered saline (PBS: pH: 7.4) to a starting dilution of 1:10 in 96-well V-bottom plates (Coring Inc. Costar, Corning, NY). Diluted sera (25 µL/well) were then incubated with 8 HA units of pdmH1N1 for 30 minutes at room temperature before 0.5% turkey erythrocytes diluted in PBS

(50 µL/well: Lampire biological Laboratories, Pipersville, PA) were added to wells. The HAI titre was determined by visual inspection as the highest dilution that inhibited erythrocyte agglutination using standard criteria.

Microneutralization (MN) titres were measured as previously described (17). Briefly, confluent monolayers of Madin-Darby canine kidney (MDCK: British Colombia Center for Disease Control) cells were incubated in 96 well, flat-bottom plates (Falcon Corning Life Science, Corning, NY) in MegaVir supplemented with 10 µg/mL gentamicin (Gibco Life Technologies, Burlington, ON), 0.25 µg/mL amphotericin B (Gibco Life Technologies, Burlington, ON), 100,000 U/mL penicillin G (Sigma, St. Louis, MO), and 10 µg/mL glutamine (Wisent, St. Bruno, QC). Sera were heat-inactivated at 56°C for 30 minutes, diluted 2-fold in MegaVir starting at 1:10 in duplicate wells (60 µL/well). Each well then received 100 infectious units of pdmH1N1 diluted in MegaVir 60 µL/well) and plates were incubated at 37°C in 5%CO_2. Cytopathic effect (CPE) was assessed at 4 days and the titre was defined as the reciprocal of the highest serum dilution to completely block CPE.

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (15). Briefly, U-bottom, high-binding 96-well plates (Greiner Bio-one, Frickenhausen, Germany) were coated overnight at 4°C with recombinant HA from pdmH1N1 (Immune Technologies, New York, NY) (0.5 µg/mL) in 100 mM bicarbonate/carbonate buffer at pH 9.5 (50 µL/well). Each plate contained a standard curve with 2-fold dilutions of purified mouse IgG (Sigma, St. Louis, MO) starting at 2000 ng/mL. Wells were blocked with 2% bovine serum albumin (BSA; Sigma) in PBS-Tween 20 (0.05%; Fisher Scientific, Ottawa, ON). Sera were heat inactivated (as above), diluted 1:50 in blocking buffer and added to four wells (50 µL/well). Plates incubated for 1 hour at 37°C; blocking buffer was added to the standard curves at this time. HRP-conjugated anti-mouse total IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:50,000 in blocking buffer was added (75 µl/well, 1 hr at 37 °C). 3,3' 5,5'-tetramethyl benzidine (TMB) substrate (100 µL/well: Millipore, Billerica, MA) was used for detection followed by 0.5 M of H_2SO_4 after 15 minutes (50 µL/well). Optical density (OD) was measured at 450 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). The concentration of HA-specific IgG was calculated using the mouse IgG standard curve.

2.4.3 Splenocyte Isolation, Stimulation and Cytokine Analysis

Individual spleens were harvested at 21 days before challenge into Hank's balanced salt solution at room temperature (RT) without calcium or magnesium (HBSS) (Wisent, St. Bruno, QC), and processed as previously described (15).

Supernatant was collected and measured the same as previously described, except at $3x10^5$ cells/well in 200 µL and with the following conditions: cRPMI alone (unstimulated) or with H1-VLP (2.5 µg/mL HA) in cRPMI for 72 hours at 37˚C *in vitro*. Spleens were isolated from a total of 13 mice per group from 2 experiments and tested in duplicates on multiplex ELISA by Quansys (Logan, UT). Cytokine/chemokine data are presented as radar graphs as previously described (18).

2.4.4 Splenocyte Stimulation and Cell Proliferation Assay (CPA)

Splenocytes were placed in duplicate in 96-well U-bottom plates (BD Falcon, Mississauga, ON) at 10^5 cells in 200 µl with cRPMI alone (unstimulated), with H1-VLP vaccine (2.5 µg/ml HA) or with ConA (2.5 µg/ml) for a stimulation control in cRPMI. After 72 hrs at 37˚C+5% CO_2, plates were spun down (300xg, 10 mins at RT) and supernatant was removed. Cells were pulsed with 1 µCi/well H^3-Thymidine (MP Biomedical, Solon, OH) for an additional 18 hrs. After one freeze-thaw, cells were harvested on glass-fiber filters with a Tomtec harvester 96 (Tomtec Inc., Hamden, CT[1]) and H^3-thymidine incorporation was measured by scintillation counter (Wallac Microbeta Trilux 1450 beta-counter; Wallec, Turku, Finland). Cell proliferation values were expressed as Stimulation Index (SI); for each mouse SI = (average Ag-stimulated cpm) / (average unstimulated cpm). IN administration was excluded from this analysis since in preliminary experiments, there was no evidence of a cellular response in the spleen (data not shown).

2.4.5 Lung viral load

Lungs were collected 3 days post-infection and individually homogenized in an equal amount of MegaVir medium (w:w) (VWR, Radnor, PA) using a tube homogenizer. The samples were

centrifuged at 14,000 x g for 5 minutes at 4°C and supernatants were collected and stored at -80°C. Virus titres were measured by $TCID_{50}$. MDCK monolayers were prepared in 96-well, flat bottom plates. On the day of inoculation, MegaVir was removed and the lung homogenates were serially diluted 1:10 with MegaVir TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Sigma at 2 mg/mL) (Megavir-trypsin). Each dilution was added to 6 wells (100 µL/well) and incubated at 37°C with 5% CO_2 for an hour. The lung-MegaVir mixtures were then removed and plates were replenished with fresh MegaVir-trypsin (200 µL/well). Plates were followed for 4 days for CPE. The $TCID_{50}$ was calculated using the Karber method (19). Lung viral loads were assessed 3 days (d3) post-infection from mice infected with 5x LD50.

2.4.6 Lung Histopathology

Lung samples were processed using the standard H&E stain. Briefly, lung samples were fixed in 10% formalin (FisherScientific, Ottawa, ON) then embedded in paraffin (Leica, Concord, ON). Sections (4 µm) were applied to slides then heated at 50-60°C. Samples were washed with Xylene (Chaptec, Quebec, QC) then were immersed in ethanol (Commercial Alcohols, Boucherville, QC) for 10 minutes. Slides were rinsed with distilled water, then briefly submerged into Harris Hematoxylin (HHS-32) (Sigma, St.Louis, MO) ½ solution in distilled water. Slides were washed under running tap water then washed 10 times with ethanol, followed by 1 minute of eosin-phloxin B (100 ml of Eosin of 1 % eosin Y; Sigma, St.Louis, MO, 10 ml of 1% of Phloxin B; Sigma, St.Louis, MO, 780 ml of ethanol, 4 mL of glacial acetic acid; FisherScientific, Ottawa, ON). Samples were immersed in ethanol for 10 minutes. Slides were dried then submerged in Xylene for 10 minutes. Slides were fixed with a couple of drops of acrytol (Leica, Concord, ON) with a cover slip and scored at 10X and 100X. Slides were scored blinded and the scoring system evaluated the following 5 parameters: 1) airway epithelial necrosis, attenuation or disruption, 2) airway inflammation, 3) peribronchiolar & perivascular lymphocytic cuffing, 4) alveolar cellular exudate/oedema and interlobular oedema and 5) alveolar septal inflammatory cells and cellularity (20). Each parameter was scored from 0-4 for a total possible score of 20.

2.4.7 Statistical Analysis

The geometric mean ratios between groups and their 95% confidence intervals (CI) were calculated using GraphPad Prism 6.0 software. For statistical analysis one-way ANOVA was performed on HAI and stimulation index values. All other statistical analysis was two-way ANOVA. All analyses were performed using GraphPad Prism 6.0 software.

2.5 Results

2.5.1 Antibody Response

Overall, the standard measures of influenza vaccine humoral response (HAI (Fig. 2.1A), MN (Fig. 2.1B) were weak regardless of the vaccine used in both young and old animals. Only the young animals that received the VLP vaccine IM consistently mounted detectable HAI (GMT: 14.51: $p<0.0001$) and MN responses (GMT: 17.5: $p<0.0001$) compared to the other groups. Very low HAI (≤10) and/or MN titres (≤10) were observed in a small number of young and aged animals across the other groups. When serum HAI and MN titres were found to be very low, we decided to assess the humoral response by ELISA as well (total pdmH1N1 HA-specific IgG). Antibodies measured by ELISA were readily detected in most groups (Fig. 1C) but were consistently higher in the young animals that had received the VLP vaccine IM (GMT 1771.1 ng/mL) compared to both the VLP IN group and the split virion group (GMT 109.1 ng/mL and 265.1 ng/mL: $p<0.01$ and 0.0001 respectively). Low ELISA titres were also detected in the aged animals that received either the H1-VLP (GMT 526.9 ng/mL) or the split vaccine IM (GMT 364.3 ng/mL) (Figure 2.1C). HA-specific IgA antibodies in the BALF were undetectable in all animals including the VLP IN group (data not shown).

2.5.2 Cellular Immune Response to Vaccines

Cytokine/chemokine and lymphoproliferative responses of splenocytes re-stimulated with H1 antigen *ex vivo* were also most consistently detected in the H1-VLP IM group (Figure 2.2 and 2.4) and were generally more robust in young compared to aged mice. Since split-virion formulations are the most common commercial vaccines, we compared the cytokine/chemokine responses of H1-VLP IM group to the IM split-virion group at the two ages. Splenocytes from the young H1-VLP recipients produced a greater amount (2- to 14-fold) of a broad range of

cytokines/chemokines than splenocytes from the split vaccine recipients including IL-2, GM-CSF, IL-3, IFNγ, IL-4, IL-17, IL-5, IL-10, MCP-1, MIP-1a and IL-6 (Figure 2.2A). Differences between the H1-VLP and split virion groups in the aged animals were far more modest such that animals that received the H1-VLP vaccine had slightly higher (1.1-1.4-fold) IL-2, GM-CSF, IFNγ, IL-3, IL-4, IL-5, TNFα and IL-6 production compared to the split-virion group (Figure 2.2B) but slightly lower production (0.9-0.6-fold) of IL-1α, IL-1β, IL-12, IFNγ and RANTES. None of these differences reached statistical significance. Antigen-specific cytokine/chemokine responses comparing young versus aged animals are presented in Figure 2.3. Overall, cytokine/chemokine responses of the young and aged animals to the VLP and split vaccine were similar with a few striking exceptions. For the H1-VLP given IM, the young animals made much greater quantities of IFNγ, while IL-1α production was much greater in the aged animals. In response to the split vaccine, the young mice produce significantly more IFNγ, IL-5 and IL-1α than the aged group. In preliminary experiments with either H1-VLP or split-virion formulations, the antigen used for re-stimulation (ie: H1-VLP, whole inactivated pdmH1N1, recombinant H1) did not significantly influence splenocyte proliferation or cytokine production (data not shown).

Compared to the split virion groups, splenocyte proliferation was consistently higher in the H1-VLP IM groups for both age groups, although significance was reach only in the young mice (Stimulation Index [SI] 7.38 ± 2.57 vs. 3.51 ± 1.38: $p<0.0001$ vs. aged mice SI 4.12 ± 0.87 vs 2.52 ± 0.95: $p = 0.3325$) (Figure 2.4).

2.5.3 Protection from Homologous Challenge

When challenged IN with 5 x mLD_{50}, of A/California/07/2009 H1N1 virus, young naïve mice rapidly lost up to 21.5% of their baseline weight and most were euthanized when a humane endpoint was reached when the mice lost more than 20% of their initial body weight. Only 1/11 (9.1%) of the young naïve animals survived (Figure 2.5A and C). Young mice given the H1-VLP IN also lost substantial weight (15.0%) but 80% of them recovered and survived. The young split vaccine and H1-VLP IM groups lost the least amount of weight (10.8% and 9.8%, respectively) and all of these animals survived. Overall, the aged animals lost more weight following challenge than younger mice. The aged naïve group also lost the most weight (20.2%) with kinetics similar to what was observed in the younger animals (Figure 25B and D). Most of these

animals met humane endpoints or died (22.2% survival). Aged mice that received the split-virion vaccine IM or the H1-VLP IN lost similar amounts of weight (17.8 % and 18.3%, respectively) (Figure 2.5B) and had similar rates of survival (60% and 55.6% respectively). The aged mice given H1-VLP IM lost the least amount of weight (14.2%) and 80% survived challenge in this age group. Although more than half of the aged animals in the VLP IN and split virion groups survived, most of these animals remained well below their baseline weights (15-18% loss) at 12 days after challenge. Only the H1-VLP vaccinated animals recovered a substantial amount of weight; reaching statistical significance in the last days of the experiment ($p<0.0001$ vs split vaccine group at day 12).

2.5.4 Lung Viral Load at 3 Days Post-Infection

Overall, vaccination had relatively little impact on d3 viral loads in either young or aged animals. In the young mice, the highest viral loads were found in the split vaccine group (mean of log values: 4.07 vs naïve:4.42) (Fig. 2.6). The greatest decrease in d3 viral load and the only decrease that reached statistical significance was observed in the young H1-VLP IM group (3.68: $p<0.05$) (Figure 2.6). H1-VLP IN had a slight decrease from the naïve and split vaccine (4.02). Among the aged animals, the split vaccine and H1-VLP IN mice had the highest viral loads (4.27 vs 4.29, respectively) compared to the naïve (4.50). The H1-VLP IM had a small decrease in the d3 viral loads (4.06) (Fig. 2.6).

2.5.5 Antibody Responses After Infection

At three days post-challenge, HAI titres were largely unchanged from day 21 titres (Figure 2.5A). All the other groups had undetectable HAI titres (<10) (Fig. 2.7A). ELISA titres rose 2- to 3-fold in the H1-VLP IM groups in both the young (GMT 2612.63 ng/mL) and aged mice (GMT 719.13 ng/mL). The GMT among the vaccine-naïve aged mice was 53.25 ng/mL suggesting that this range represents ‘background’ in this assay. HA-specific IgA antibodies were not detectable in the BALF at three days post-infection (data not shown).

2.5.6 Lung Histopathology

Lung histopathology total scores were the highest in the split vaccine in the young group (7.2 ± 5.1) and in both young and aged in the H1-VLP IM groups (7.0 ± 3.3 and 7.0 ± 4.6, respectively) from a total possible score of 20 (Table 2.2). It is interesting that the groups with the highest scores also had the best survival after lethal infection. The naïve groups in both young and aged animals had lower scores (3.0 ± 3.7 and 2.2 ± 1.3 respectively) that were very similar to the scores in other groups that also had lower survival (scores between 3.4-3.6).

2.6 Discussion

The development of more effective influenza vaccines for the elderly is a high priority since the available, split-virion formulations provide incomplete protection, even with higher antigen dose (4) or the addition of an adjuvant (21). One of the limiting factors in developing better vaccines for the elderly is the lack of a simple and affordable animal model (22). Although swine (23) and ferrets (24, 25) recapitulate many aspects of human influenza and can be infected with human isolates without adaptation, these models are expensive to start and become prohibitively so when age is included as a variable. Mice have many attractions because of their relatively short life-span (2-2.5 years), their affordability and the range of immunologic reagents available. Furthermore, some human isolates, including the A/California/07/2009 H1N1 strain used in these studies, can infect mice without pre-adaptation. Of course, none of these models have a lifetime of accumulated experience with influenza antigens when vaccinated or challenged in old age. Nonetheless, aged mice have been widely used to study age-related changes in immune responses to influenza challenge (26-28) as well as new influenza vaccination strategies for the elderly (29-33).

At the outset, we were optimistic that either IM or IN delivery of the plant-made VLP vaccine would protect aged mice better than a split-virion formulation. This optimism was based on the fact that VLPs can be delivered either IM or IN (34, 35) and the growing body of evidence that the plant-made VLPs stimulates the immune response a different way than split vaccines. For instance, we have recently shown that these VLPs rapidly access draining lymph nodes (36) where they associate with and activate immune cells including B cells, macrophages and dendritic cells (37). Furthermore, these vaccines elicit balanced humoral and cellular responses in both pre-clinical (young mice and ferrets) and clinical studies in healthy young adults (10-12,

38). The current work confirmed the earlier observation of a balanced humoral and cellular responses following plant-made VLP vaccination in the young mice and extended these findings to much older animals. All of the young animals and a significant proportion (80%) of the aged mice were protected from lethal challenge by a single IM dose of the VLP vaccine despite significant weight loss and obvious lung inflammation. Surprisingly, delivery of the VLP vaccine IN elicited minimal cellular or humoral responses but still protected almost 60% of the animals. Lung viral loads were only marginally decreased by immunization at d3 post-challenge but were most reduced (15-20% range) in animals that had received the VLP vaccine by IM. The kinetics of viral clearance in response to the VLP vaccines will be assessed in future experiments. Overall, there appeared to be a strong positive relationship between the presence of pathology at d3 post-infection and survival from lethal challenge. Among the aged animals, only the VLP IM group had regained their baseline weight two weeks after challenge.

This last observation is particularly interesting given the known impact of frailty in elderly subjects on both influenza vaccination (lower efficacy) and influenza infection (greater morbidity & mortality) (39, 40). The old mice used in this study (16-20 months of age) were still active and healthy in appearance but were obviously heavy (weight range 28-34g), many had significant loss of lung volume due to kyphosis (unpublished data) and were susceptible to lower doses of influenza virus at challenge (the LD_{50} was 6-fold lower than young mice). Although both the split-virion vaccine delivered IM and the VLP vaccine delivered IN increased survival of the old mice to 50-60%, they were clearly 'sicker' than the animals that had received the VLP vaccine IM. In on-going work, we have recently observed that even very old animals (22-26 month) with multiple co-morbidities have higher antibody titres induced by the plant-made VLP vaccine compared to a split-virion formulation (GMT 1212.5ng/ml vs 396.8 ng/ml respectively, $p<0.03$) (unpublished data).

Although both the split-virion and VLP formulations induced antibodies detectable by ELISA (Figure 2.1C), only the young animals that received the VLP vaccine IM mounted detectable antibody responses as measured by the standard HAI and MN assays (Figure 2.1A and B). In the case of the animals vaccinated IN with VLP, we found no evidence of antibody production (IgA or IgG) in any of the assays used. Given the higher IgG titres in the H1-VLP IM groups and the relatively low MN and HAI titres, the mechanism of protection may be antibody-

dependent cell-mediated cytotoxicity and preliminary data from human trials suggest that the plant-made VLP vaccines can indeed elicit anti-stem antibodies (data not shown). These data also strongly suggest that cellular immune responses contribute to protection in the VLP-vaccinated animals, both young and old. Certainly, H1 antigen specific proliferation of splenocytes and cytokine/chemokine production were stronger in both the young and aged VLP-vaccinated animals than in the split vaccine groups. Although cellular responses were generally of greater magnitude in the young compared to the older animals across all vaccine groups (Figure 2.2A and B, Figure 2.3), the patterns of antigen-specific cytokine/chemokine response were similar in young and old mice. When standardized against unstimulated splenocyte cultures, young animals that received the split-virion vaccine had 8-10-fold increases in IFNγ and IL-5 production while older animals had more modest responses (2-4-fold increases in a number of cytokines/chemokines) (Figure 2.3). When cytokine/chemokine production was standardized against the respective age-specific, split-virion groups however, both young and old animals IM vaccinated with H1-VLP were found to produce large amounts of IFNγ, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6 suggesting broad immune activation (4-14-fold in the young and 1.5-3-fold in the aged) (Figure 2.2). Despite the complete absence of antibody in the mice that received the H1-VLP vaccine IN, splenocytes from the young animals produced antigen-specific IFNγ in abundance (30-fold) and more modest amounts of IL-2, IL5 and TNFα (2-5-fold) (Figure 2.3). The aged mice immunized IN produced large amounts of antigen-specific IL1α (30-fold) and modest amounts of IFNγ and IL-2 (3-4-fold) (Figure 2.3). Given the fact that 50-60% of the IN immunized animals were protected from challenge, it is likely that the VLP-induced cellular responses would have been even more obvious had we studied either lung tissue or draining mesenteric lymph nodes. Overall, these results strongly support the idea that cellular immunity can provide protection against influenza challenge and that the importance of cellular responses may increase with advancing age (40). In light of these observations, it is interesting that Ramirez and colleagues have recently reported that CpG (a TLR9-targeted adjuvant that promotes cellular immunity) increases the efficacy of a single dose of a commercial split-virion vaccine (Fluzone™) against lethal H1N1 A/California/07/2009 virus challenge in young but not aged mice (41). Even with two doses of the CpG-adjuvanted vaccine, only 60% of the aged mice survived challenge.

In conclusion, we have shown that a single IM dose of the plant-made, H1 -VLP vaccine can elicit strong and balanced humoral and cellular immune responses in both young and old mice. Partial protection (50-60%) was achieved with a single dose of the same vaccine delivered IN even though no IgG or IgA responses were detected and systemic cellular responses were modest. These data suggest that the plant-made VLP vaccine may have important advantages over split-virion formulations in the elderly who currently derive only limited benefit from vaccination. Given the surprising protection provided by IN administration of the plant-made H1-VLP vaccine, further work is merited to explore possibly synergy between IM and IN delivery to protect this vulnerable population.

2.7 Acknowledgments

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2.9 Figures, Legends and Tables

Table 2.1 Number of mice per group used for each assay on day 21 and 3 days post-infection.

	Day 21	**3 Days Post-Infection**
Antibody Assays		
HAI	25-27	7-8
MNs	12-15	N/A
ELISA IgG	25-27	11-12
Cellular Assays		
Proliferation (Thymidine)	8	N/A
Cytokines/Chemokines Production (QUANSYS)	4-13	N/A
Viral Titres	N/A	7-14
Survival	N/A	10-11
Weight Loss	N/A	18-20
Histopathology	N/A	5

Table 2.2 Summary of total histopathology scores (20 points total) from H&E stain at 3 days post-infection.

	Total Score
Young	
Naïve	3.0 ± 3.7
Split Vaccine	7.2 ± 5.1
H1-VLP IM	7.0 ± 3.3
H1-VLP IN	3.6 ± 2.4
Aged	
Naïve	2.2 ± 1.3
Split Vaccine	3.6 ± 2.1
H1-VLP IM	7.0 ± 4.6
H1-VLP IN	3.4 ±2.3

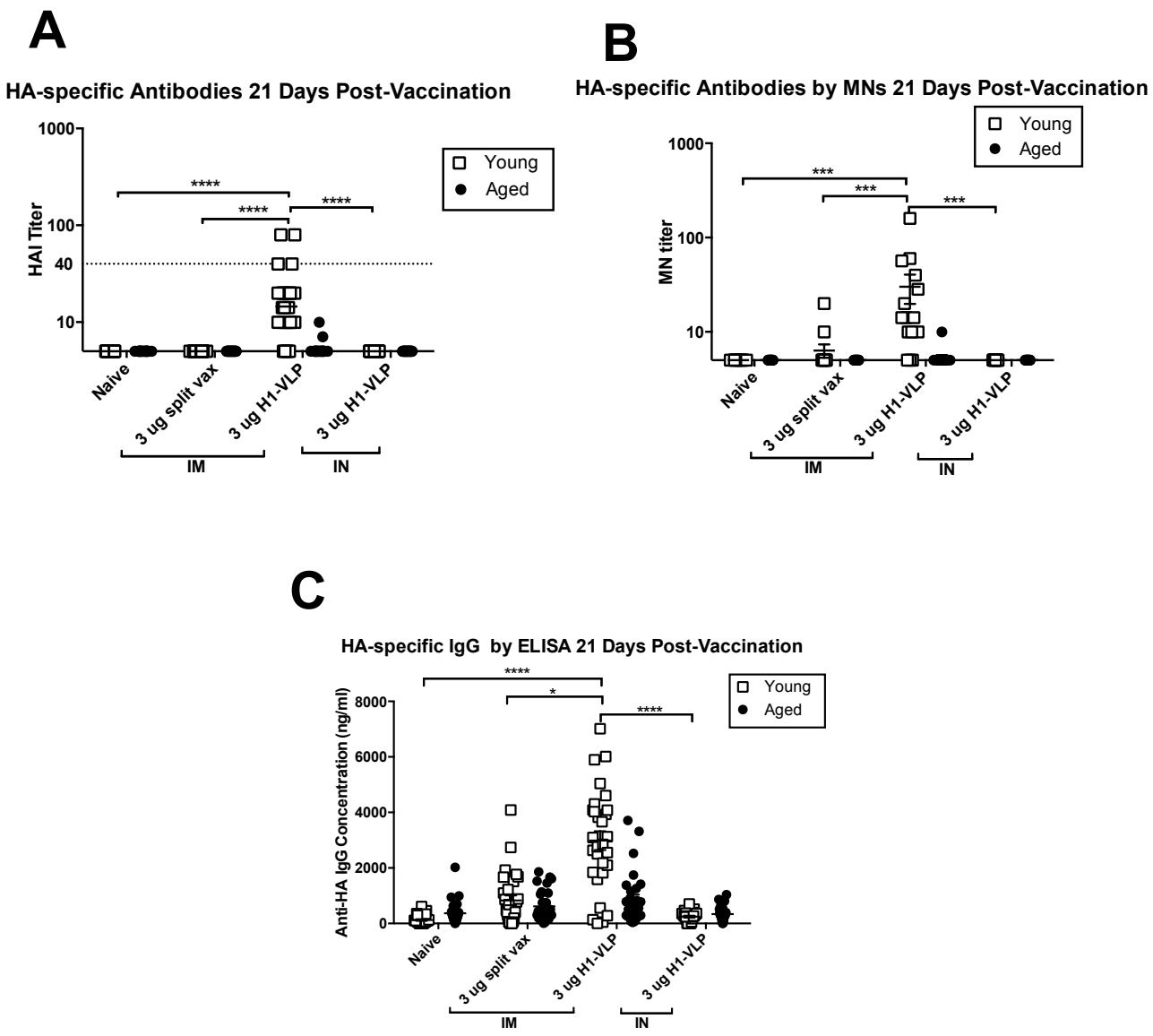

Figure 2.1 Serum antibody response after a single dose of H1-VLP (IN or IM) or split-virion vaccine (IM)

Young (5-8 weeks) and aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or intramuscularly with H1-VLP or split-virion vaccine. Three weeks post-vaccination sera from individual mice were analyzed by hemagglutination Inhibition (HI) (A) and microneutralization (MN) (B) titre against A/California/07/2009 H1N1. Influenza HA-specific IgG concentrations (C) by ELISA. Dotted line in A) represents 40 HAI which is considered the protection level in humans. Error bars indicate the standard error of the mean. For statistical analysis, one-way ANOVA was used on the log values for A). For B) and C) two-way ANOVA analysis was performed (**** $p<0.0001$, *** $p<0.001$, * $p<0.05$). A) and C) represent 25-27 mice/group combined from 3 studies. B) represents 12-15 mice/group combined from 2 studies.

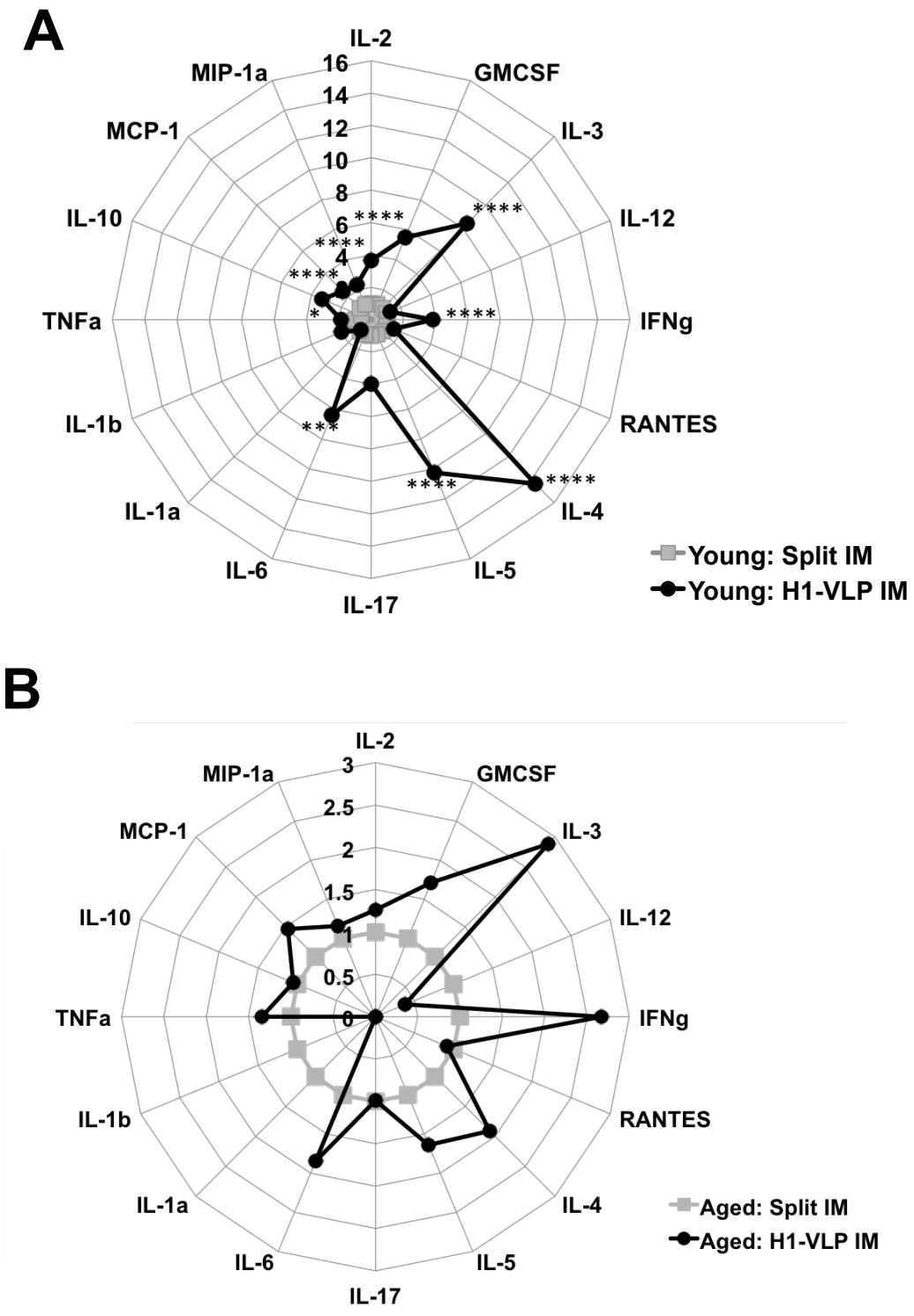

Figure 2.2 Splenocyte production of H1 antigen-specific cytokine/chemokines after ex vivo re-stimulation

A) Young (5-8 weeks) and B) aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or IM with H1-VLP or split-virion vaccine. Three weeks post-vaccination, splenocytes were collected and stimulated *ex vivo* for 72 hours with H1-VLP. Unstimulated splenocytes were pooled for each group and for the stimulated splenocytes 4-13 samples per group were run as singlets on multiplex ELISA. Supernatant concentrations were measured for the following cytokines/chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, MCP-I, IFNγ, TNFα, MIP-1α, GM-CSF and RANTES. For each age group, the average of the unstimulated of that group was subtracted and the split vaccine group was considered the 'standard' response and responses observed in the H1-VLP groups were calculated as fold changes from this group. A) is 4-fold greater than B). Error bars indicate the standard error of the mean. For statistical analysis, one-way ANOVA was performed (**** $p<0.0001$, *** $p<0.001$). A) and B) represent 4-13 mice/group combined from 2 studies.

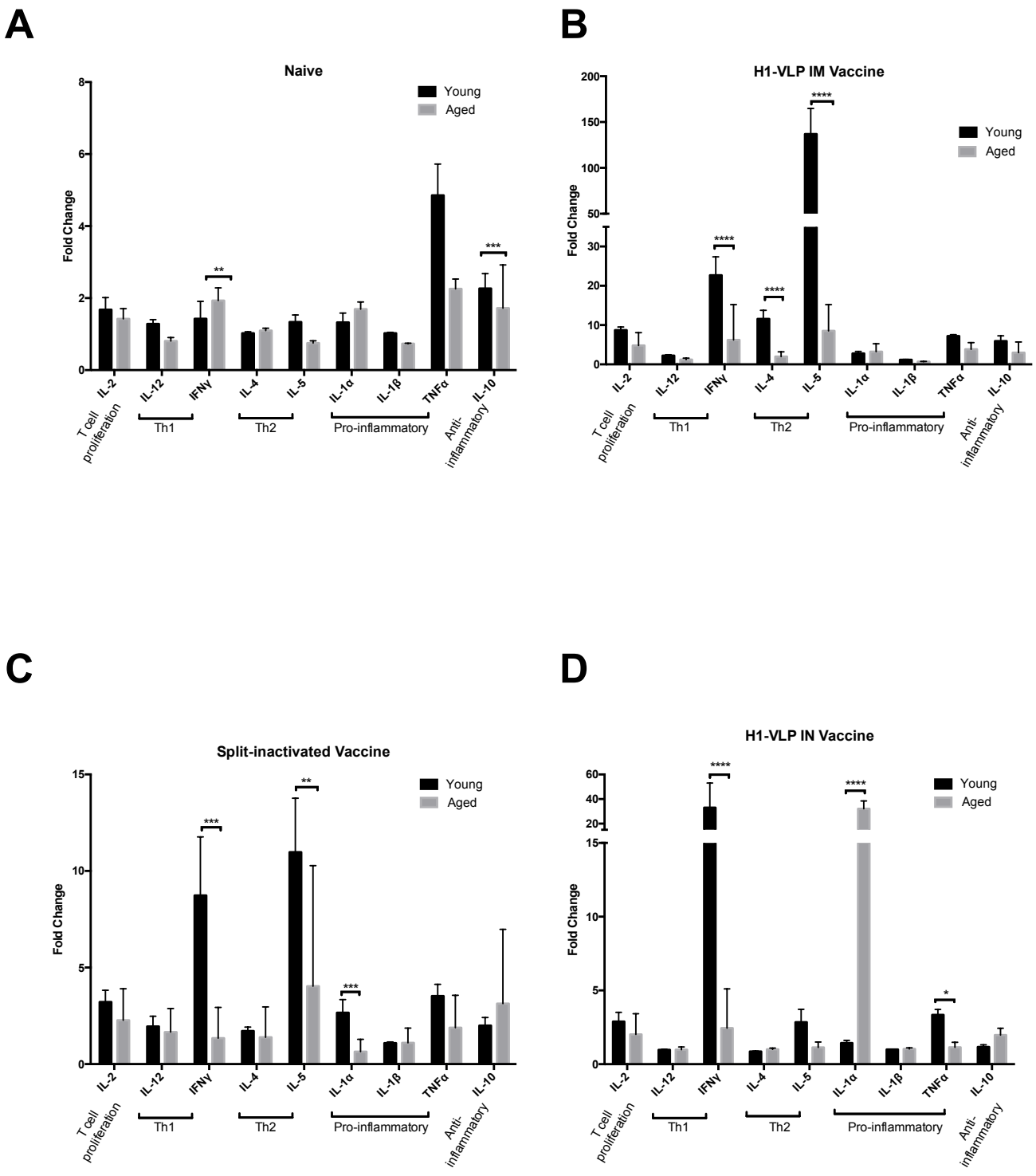

Figure 2.3 Cytokine/Chemokine production by splenocytes after ex vivo stimulation in young versus old mice

Young (5-8 weeks) and aged (16-20 months) BALB/c mice were immunized once by intranasal instillation with H1-VLP vaccine, IM with H1-VLP, split-virion vaccine or naïve. Three weeks post-vaccination, splenocytes were collected and stimulated *ex vivo* for 72 hours with H1-VLP. Concentrations were measured from the supernatant by multiplex ELISA for the following cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IFNγ and TNFα. The fold change for each group was calculated (stimulated/unstimulated) based on the unstimulated samples from the corresponding group. For statistical analysis, two-way ANOVA was performed on the log values of the fold change (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$). Data represent 4-13 mice/group combined from 2 studies.

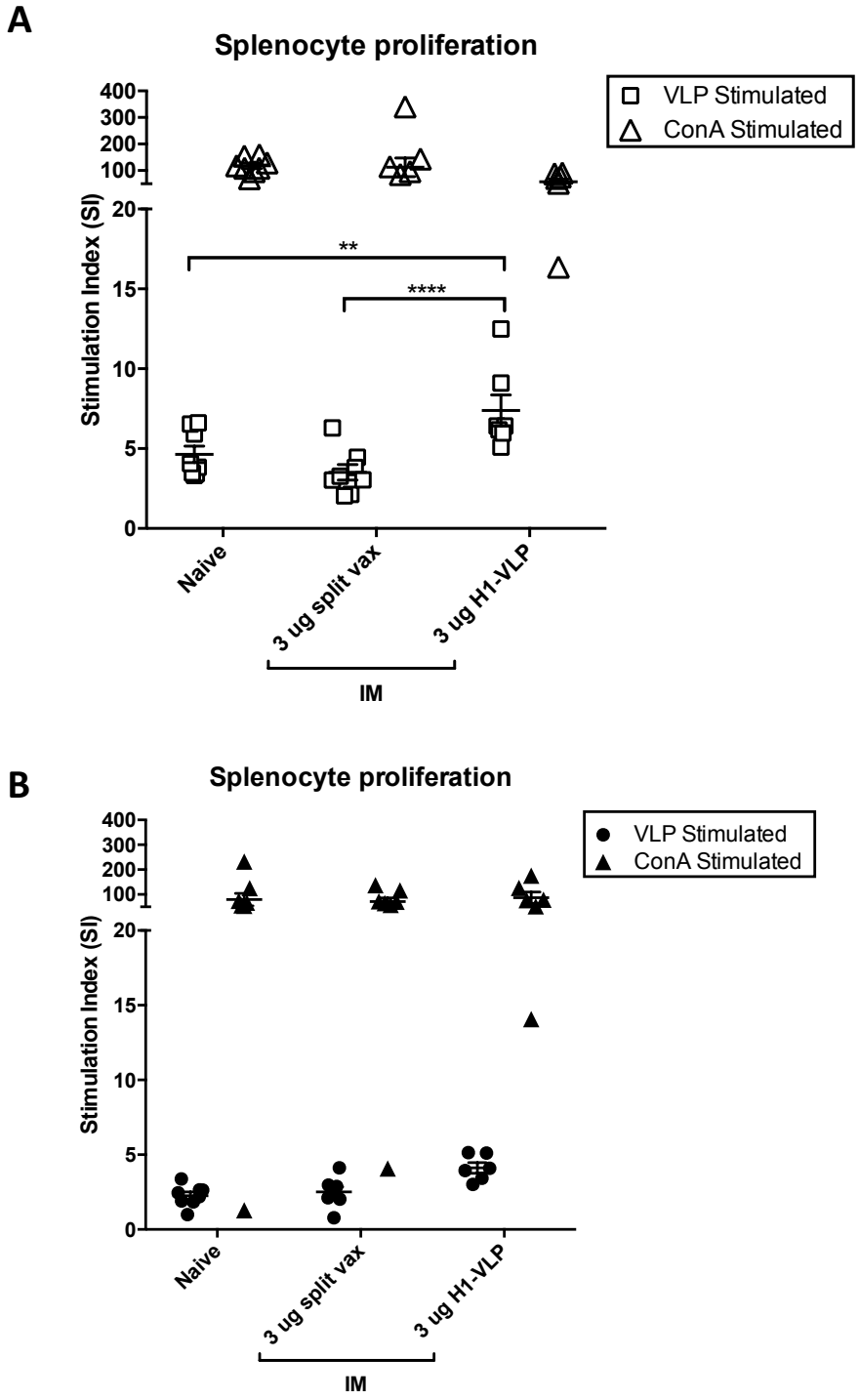

Figure 2.4 Splenocyte proliferation or stimulation index (SI) in response to H1 re-stimulation *ex-vivo*

A) Young (5-8 weeks) and B) aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or IM with H1-VLP or split-virion vaccine. Three weeks post-vaccination, splenocytes were collected and stimulated *ex vivo* for 72 hours with H1-VLP. At 72 hours, supernatants were removed and cells were pulsed with H^3-thymidine in complete media and incorporation of H^3-thymidine was measured by scintillation counter 12 hours later. Cell proliferation values were expressed as a Stimulation Index (SI) = (average antigen-stimulated cpm/ average unstimulated cpm). Error bars indicate the standard error of the mean. For statistical analysis, one-way ANOVA was performed (**** $p<0.0001$, ** $p<0.01$). A) and B) represent 8 mice/group from 1 study.

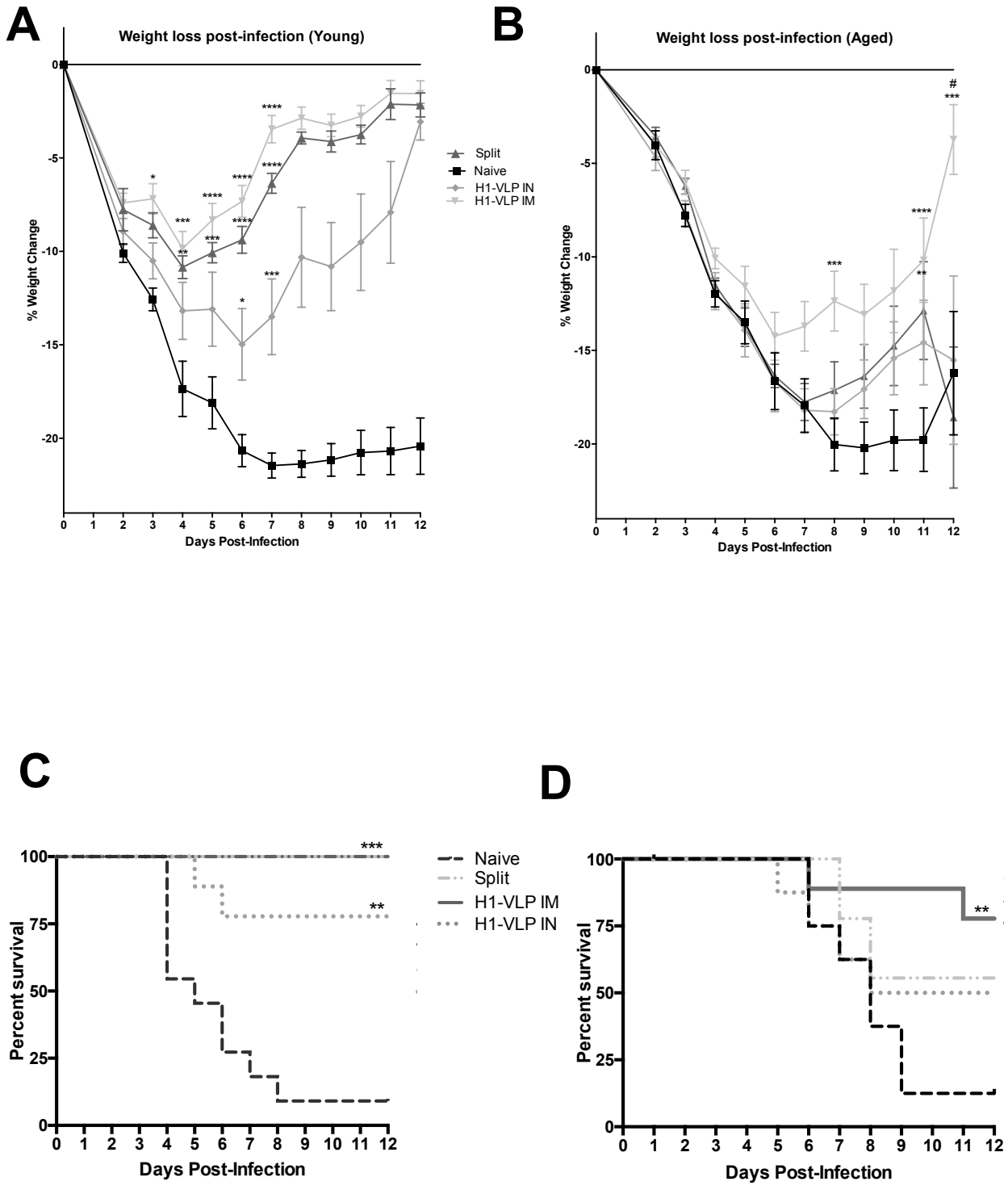

Figure 2.5 Weight loss and survival after A/California/07/2009 H1N1 challenge
Young (5-8 weeks) and aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or IM with H1-VLP or inactivated split vaccine. Three weeks after vaccination, mice were challenged with an age-appropriate 5x mouse lethal dose 50

(mLD50) of A/California/07/2009 H1N1. Weight loss for both the A) young and the B) aged mice, was monitored daily. Survival of young C) and aged animals D): mice were euthanized if they lost >20% of their initial weight. Error bars indicate the standard error of the mean. For statistical analysis, two-way ANOVA was performed (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared to naïve groups, # $p<0.0001$ compared to split vaccine). A) and B) represent 18-20 mice/group combined from 3 studies. C) and D) represent 10-11 mice/group combined from 2 studies.

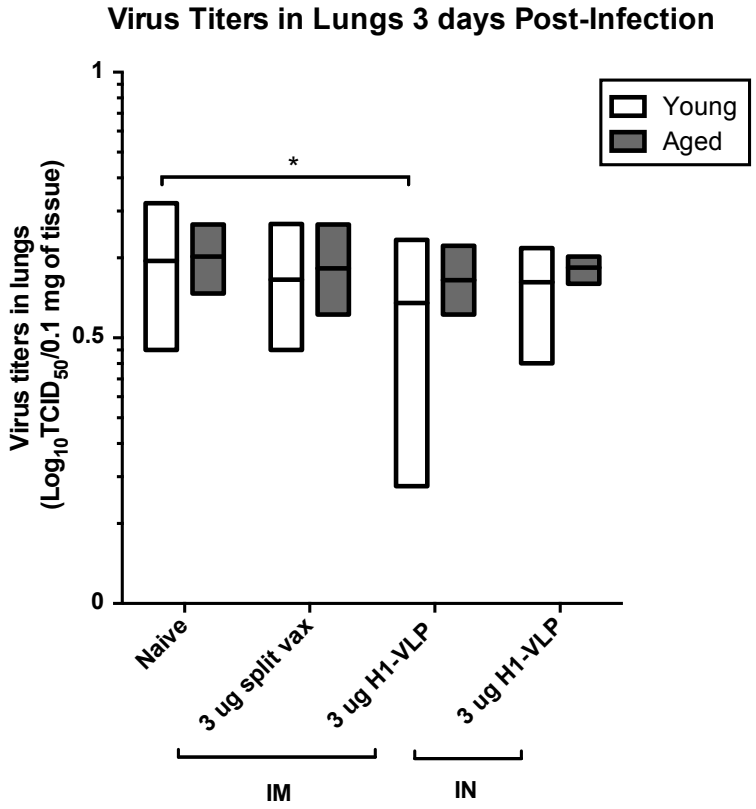

Figure 2.6 Lung viral loads after A/California/07/2009 H1N1 challenge
Young (5-8 weeks) and aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or IM with H1-VLP or inactivated split vaccine. Three weeks after vaccination, mice were challenged with an age-appropriate 5x mouse lethal dose 50 (mLD50) of A/California/07/2009 H1N1.$TCID_{50}$ values were log transformed and shown as floating bar graphs. Mean is depicted by a horizontal line within the bar graphs. The $TCID_{50}$ was calculated using the Karber method ($logTCID_{50}$/0.1mL = -1 - (total mortality% /100 – 0.5) x log10). For statistical analysis, two-way ANOVA was performed (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared to naïve groups,). There were 7-14 mice/group combined from 3 studies.

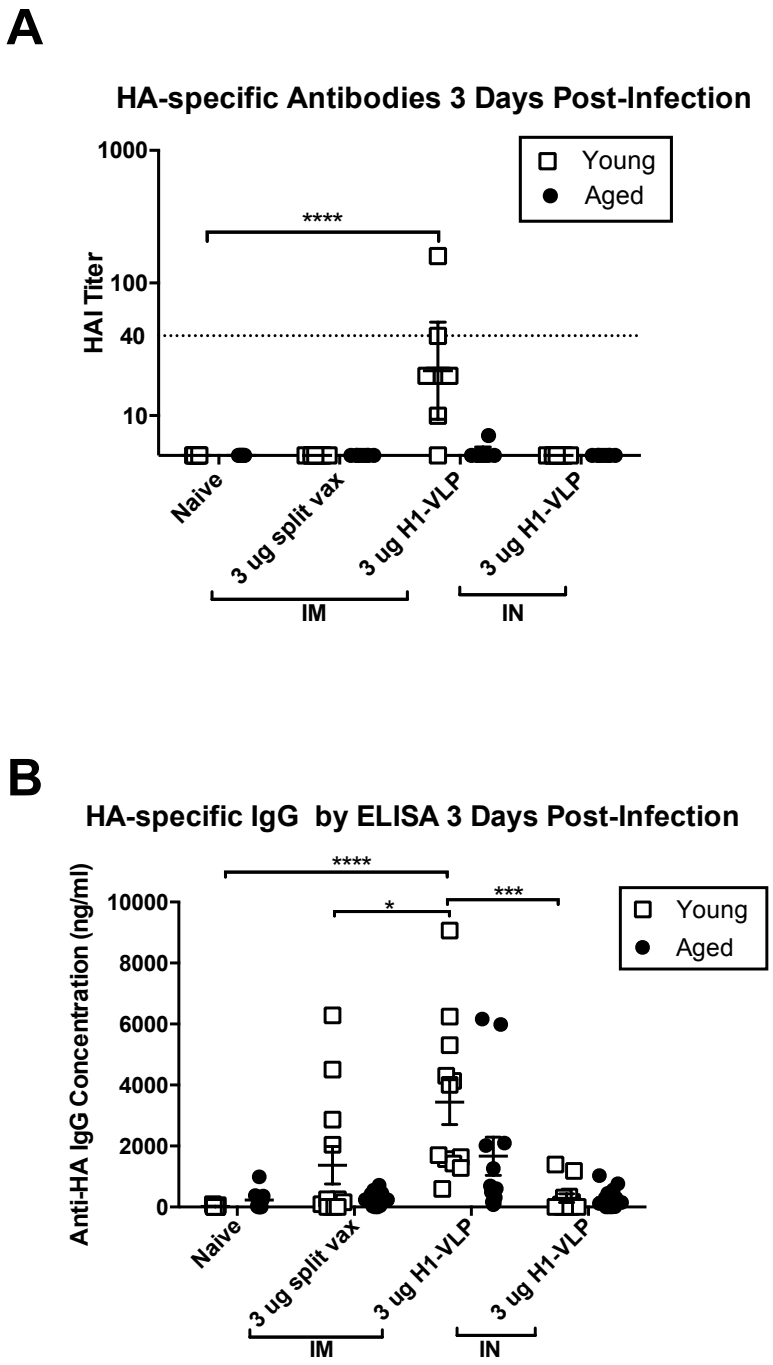

Figure 2.7 Serum antibody levels 3 days after challenge infection
Young (5-8 weeks) and aged (16-20 months) BALB/c mice were immunized once by IN instillation with H1-VLP vaccine or IM with H1-VLP or split-virion vaccine. Three weeks post-vaccination, mice were challenged with A/California/07/2009 H1N1 and sera from individual mice were analyzed. A) Hemagglutination Inhibition Assay (HI) and B) ELISA HA-specific IgG concentrations. Dotted line in A) represents 40 HAI which is considered the protection level in humans. Error bars indicate the standard error of the mean. For statistical analysis, one-way ANOVA was used on the log values for A) and B) (**** $p<0.0001$, *** $p<0.001$, * $p<0.05$). A)

represents 7-8 mice/group and combine from 2 studies. B) represents 11-12 mice/group and combine from 3 studies.

Chapter 3: An unexpected variable: pre-existing anti-influenza antibodies in aged mice with no known prior exposure to influenza antigens

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3.1 Preface

While trying to determine the mouse lethal dose 50 (mLD50) for old BALB/c mice, some of the mice not only did not succumb to infection; they appeared to be pre-immune. Even with very high challenge doses, we did not observe the same level of lethality as observed in previous studies. Therefore, we collected sera from all of the mice in our ageing mouse colony to determine whether or not they had any serologic evidence (ie: antibody titres) of exposure to A/California/07/2009 H1N1 prior to vaccination/infection.

3.2 Abstract

Although mice are not natural hosts for influenza viruses, unexpected results in an H1N1 vaccine challenge study prompted us to investigate whether or not the oldest animals in our ageing facility had evidence of prior exposure to HA1 antigen. Sera collected from 327 animals (mean age 19± 3 months) that had been housed at our site for 14± 4 months were assessed by ELISA, hemagglutination inhibition and microneutralization assays. Overall, 8.3% of the aged mice had anti-HA IgG levels ≥1000 ng/mL. These data suggest that prior exposure to natural influenza should be considered in influenza studies using aged mice.

3.3 Background

Although influenza affects people of all ages, the risk for complications and hospitalization are highest in the elderly, the very young and the immunocompromised. Compounding the natural vulnerability of the elderly to influenza, vaccine efficacy is also relatively low in this population. The development of better influenza vaccines for the elderly is therefore an important unmet need.

With a few notable exceptions (e.g: A/California/07/2009 (pdmH1N1) and some highly pathogenic avian viruses (1), mice are not natural hosts for influenza viruses. Nonetheless, mouse models have been extensively used in influenza vaccine research because of their low cost and manipulability, often using laboratory-adapted strains such as A/Puerto Rico/8/1934 (H1N1) [PR8] or A/WSN/1933 (H1N1) [WSN]. Although there is far less experience using aged mice to predict vaccine-induced responses in the elderly, such models are gaining traction (2). In many settings, including our own, investigators have no choice but to resort to ageing their own animals.

One issue we had not anticipated was the possibility of natural exposure to influenza. Although our mice were housed in a specific pathogen-free environment in a state-of-the-art animal facility, it was at least theoretically possible that routine handling by laboratory and animal facility staff could expose the mice to influenza antigens. Because we considered this to be such a low probability event, screening of the aged animals for pre-existing antibodies was typically only done on one animal per cage. When confronted by anomalous challenge results in a pdmH1N1 vaccination study however, we performed a more detailed serologic analysis of our vaccine-naïve, aged animals.

3.4 Methods

3.4.1 Mice

Mice were female BALB/c retired breeders purchased at 4-6 months of age (Charles River Laboratories, Montreal, QC) that were housed at the Research Institute of the McGill

University Health Centre (Royal Victoria or Montreal General Hospital sites, Montreal, QC) for 10 –18 months under specific pathogen-free conditions. Some animals were moved between sites during the ageing process. To our knowledge, all mice were influenza naïve, absent of influenza vaccination and infection. Peripheral blood (lateral saphenous vein) was collected from 327 mice between 16-22 months of age in microtainer serum separator tubes (BD Biosciences, Mississauga, ON). Sera were stored in aliquots at -20°C until used. All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

3.4.2 Serologic Testing

Sera from all animals were tested by enzyme-linked immunoabsorbant assay (ELISA) and a subset with high (n=7-9) or low (n = 20-22) ELISA titres was tested by hemagglutination inhibition (HI) and microneutralization (MN). All assays were performed as previously described (3, 4). However, for the ELISA, recombinant HA from A/California/07/2009 H1N1 (ImmuneTechnologies, New York, NY) (0.5 μg/mL) was used. The HI assay was performed as previously described (4, 5). Briefly, the mouse sera and receptor-destroying enzyme (RDE: Cedarlane, Burlington, ON) were mixed at a 1:4 ratio and incubated for 18 hours at 37°C. The RDE was inactivated at 56°C for 30 minutes and PBS was added to bring samples to a starting dilution of 1:10. Serial two-fold dilutions of sera (25μL/well) were placed in V-bottom 96-well plates (Corning Costar, NY) with PBS (25 μL/well). Each well then received 25 μL (8 HA) of A/California/07/2009. After 30 minutes at room temperature (RT), 50 μL/well of 0.5% turkey erythrocytes (Lampire, Pipersville, PA) was added. After a second 30 minute RT incubation, the plates were tilted to 90° for ~1 minute to assess hemagglutination using standard criteria. The highest dilution at which hemagglutination was inhibited (average of 2 wells) was the HI titre. Microneutralization was tested as previously described (3). However, each well was read for cytopathic effect 4 days post inoculation and the pandemic A/California/07/2009 strain was used.

3.4.3 Statistical Analysis

The geometric mean titres (GMT) and their 95% confidence intervals (CI) were calculated using GraphPad Prism 6.0 software. For statistical analysis, a Mann-Whitney test was

performed on the log10 values. ELISA values below detection of limit were set 1 and MN and HAI values below detection of limit (<10) were set to 5 for statistical analysis. All analyses were performed using GraphPad Prism 6.0 software.

3.5 Results

3.5.1 Seropositive Mice

The mice had been purchased at four times (Figure 3.1A): Group 1 animals (n= 16) were tested at 20-22 months of age and had experienced two full influenza seasons in our animal facility. Group 2 animals (n = 14) were tested at 20-22 months of age had experienced two full influenza seasons (the first season split between Charles River Laboratories and our facility). Group 3 animals (n = 192) were tested at 16-20 months of age and were born during a first flu season and had experienced another full influenza season in our facility. Group 4 animals (n=105) were tested at 16-18 months of age and had experienced only one flu season in our facility. To define ELISA 'positivity', we used an anti-HA cut-off of >1000 ng/mL based on the response of aged mice (18-22 months) to a single dose of A/California H1N1 vaccine. Using this cut-off value, we found that 6.3% (1/16), 14.3% (2/14), 8.9 % (17/192) and 6.7% (7/105) of the aged mice in our facility were ELISA positive in Groups 1-4 respectively (8.3 % overall: Figure 3.1B and 3.1C). The GMT for each group were 193.46 ng/mL ,112.78 ng/mL, 179.02 ng/mL and 227.72 ng/ml. Groups 1-4 respectively. The HA-specific IgG concentrations of these "seropositive" mice ranged from 1000-7013 ng/mL (mean 2145 ± 1449 ng/mL). The remaining ELISA-negative mice had a mean HA-specific IgG concentration of 307.5 ± 257 ng/mL (Figure 3.1B). HAI assays were run on a small subset of ELISA-positive (n=7) (GMT 1246.69 ± 1000.43 ng/mL) and ELISA-negative samples (n=20) (242.87 ± 148.30 ng/mL). A subset of animals (n=60) were also tested for H3N2 and using the cut-off value above 10% (6/60) were ELISA-positive (Supplemental Figure 3.1). MN assays were run on a small subset of ELISA-positive (n=9) (mean 1080.32 ± 486.51 ng/mL) and ELISA-negative samples (n=22) (393.76 ± 261.30 ng/mL). All of these samples had HI and MN titres below the limits of detection of these assays (both <10) data not shown.

3.6 Discussion

Few scientists working with mouse models of influenza, including ourselves, routinely consider serostatus of the animals in designing experiments. Although some low pathogenicity avian viruses can infect wild mice (6) and some inbred mouse strains (e.g: BALB/c, C57Bl/6) are susceptible to a small number influenza strains without adaptation (e.g: H1N1 1918, pdmH1N1 2009, H5N1, H7N9) (7-9), neither human-to-mouse nor mouse-to-mouse transmission is routinely considered (9). We were therefore puzzled by some inconsistencies in both the serologic responses to vaccination and survival rates during a routine challenge study of pdmH1N1 in aged mice. Our confusion evolved into surprise when we found that ~8% of the aged animals in our facility (16-22 months old) had high baseline levels of anti-HA antibodies by EIA for H1N1 (>1000 ng/ml).

Although most of the animals were old enough to have lived through at least one and, in most cases, two influenza seasons, they had been housed in a specific pathogen-free environment in airtight cages with a closed, filtered air system. It is therefore unclear how they might have been exposed to influenza viruses other than by aerosols generated by infected humans (10). Although it is mandatory for animal technicians in our facility to wear a standard surgical mask when opening cages and manipulating mice, N95-type masks are not required and the performance of surgical-type facemasks against aerosol transmission is far from perfect even when compliance is high (11). In North America, the 2014-2015 influenza season was relatively long (20 weeks) with H3N2 viruses predominating while the 2015-2016 season was more complex with an early predominance of H3N2 viruses, a long middle period of A(H1N1)pdm09 activity and a late surge of B virus activity (mostly Yamagata lineage) (12, 13). Hence, there is no reason to speculate that the mice in our ageing facility were exposed to unusual influenza transmission events from laboratory staff. Although early experiments suggested that some mouse-adapted influenza strains could be efficiently transmitted between mice (7), these observations were not confirmed in more recent work (2). Furthermore, we found no evidence for cage-clustering of seropositive animals in our study. The groups that had survived through two influenza seasons did not have a higher percentage of positive titres compared to the groups that only experienced one influenza season. One exception was group 2, which had a higher percent than the rest of the groups (14.3%).

Only small amounts of serum were available for most of the animals included in this study so the breadth of serologic testing was limited and not all assays could be performed for all animals. Hence, there are different sample numbers (n) for MN and HAI assays performed. Despite the presence of high HA-specific IgG detected by ELISA, none of the antibody-positive animals had HI or MN titres above the limit of detection of these assays. (HI ≥10 or MN ≥ 10). Although ELISA, HAI and MN titres are often highly correlated in mouse and human studies, this is not always the case, particularly with cross-reactive antibodies. For example, high levels of cross-reactive IgG detectable by ELISA despite low HI and MN responses have been observed in both children and adults exposed to pdmH1N1 (14). Although several 'exotic' Orthomyxoviruses that can infect rodents exist in Africa and Asia (e.g: Thogotoviruses, and Quaranjavirus) (15,16), it implausible that exposure to other *Orthomyxoviridae* contributed to the baseline seropositivity that we observed in some animals.

In conclusion, we report for the first time the presence of moderate to high anti-influenza IgG titres in some 16-22 month old mice housed in a state-of-the art animal facility despite no known prior exposure to influenza viruses or vaccine antigens. The fact that this occult exposure was sufficient to interfere with both vaccination and challenge studies suggests that testing of aged animals for pre-existing antibodies should be considered for all influenza studies in aged animal models and possibly for young mice housed during peak influenza seasons. These findings also raise interesting questions about possible exposure of aged animals to other human respiratory viruses that are routinely studied in small animal models (e.g: RSV, metapneumovirus).

3.7 Acknowledgments

There is no commercial or other association for conflict of interest for all authors. This work was supported in part by awards to BJW from the Canadian Institutes of Health Research (34469) and the Ministère des Finances et de l'Économie du Québec with project oversight by Genome Quebec. BH is a recipient of CIHR studentship award.

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3.9 Figure and Legend

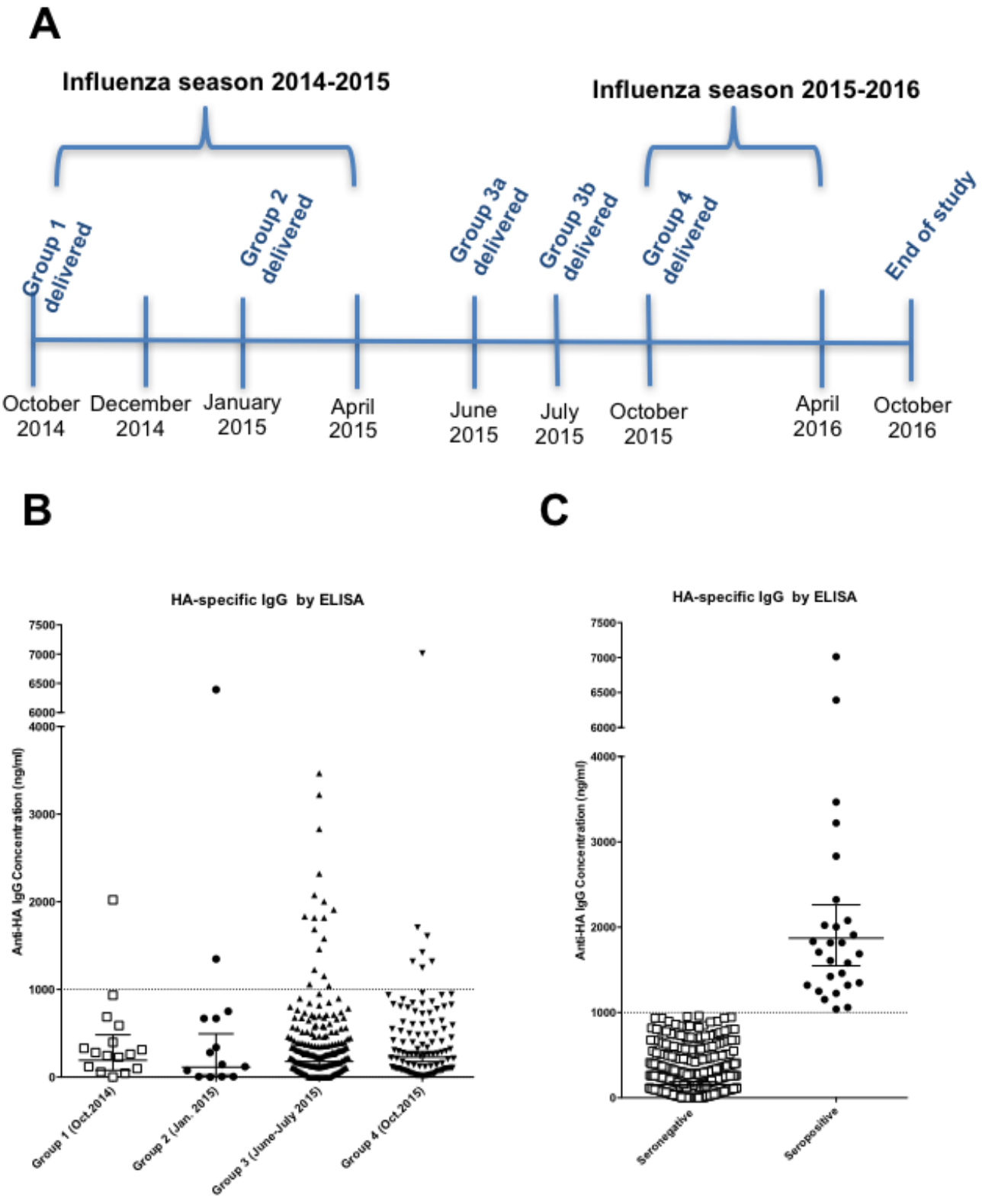
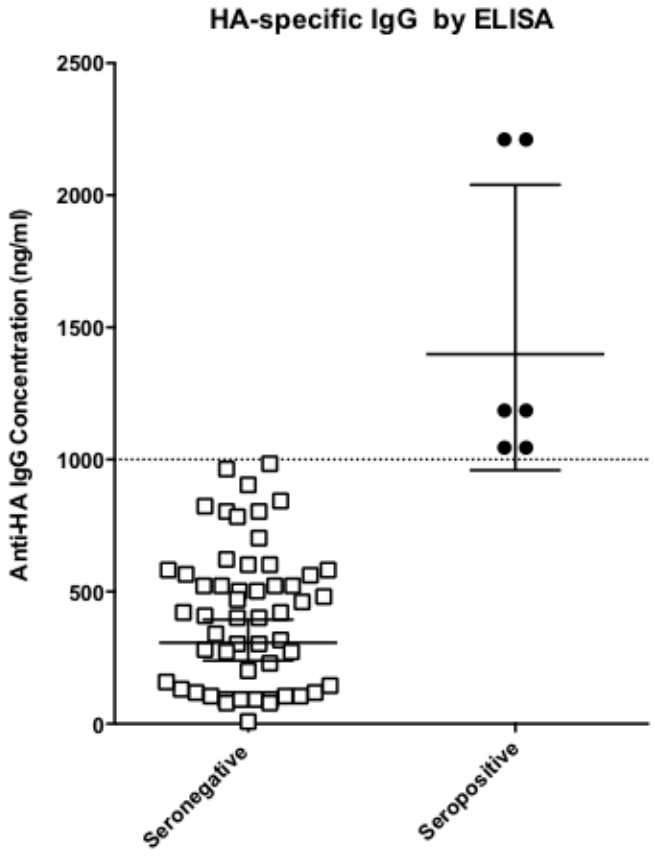


Figure 3.1 Timeline of mouse orders in relation to influenza seasons and baseline influenza HA-specific IgG measured by EIA in aged BALB/c mice for H1N1. A) All mice were 4-6 months of age at time of delivery. Group numbers correspond to date mice were received. To our knowledge, all mice were influenza naïve, and sera from individual mice were analyzed between 16-22 months of age. HA-specific IgG concentrations by ELISA with GMT (B) were considered seropositive if >1000 ng/mL. ELISA titres of individual groups: Group 1 (n= 16), Group 2 (n = 14), Group 3 (n = 192), Group 4 animals (n=105) (B). ELISA titres of all the animals categorized as seropositive or seronegative (C). Statistical analysis was performed using the Mann-Whitney test (B) on log-transformed values. Significant differences are indicted in brackets; ****, P<0.0001. Overall, 8.3% (n=27) of the aged mice were seropositive while the remainder (n = 300) were seronegative.

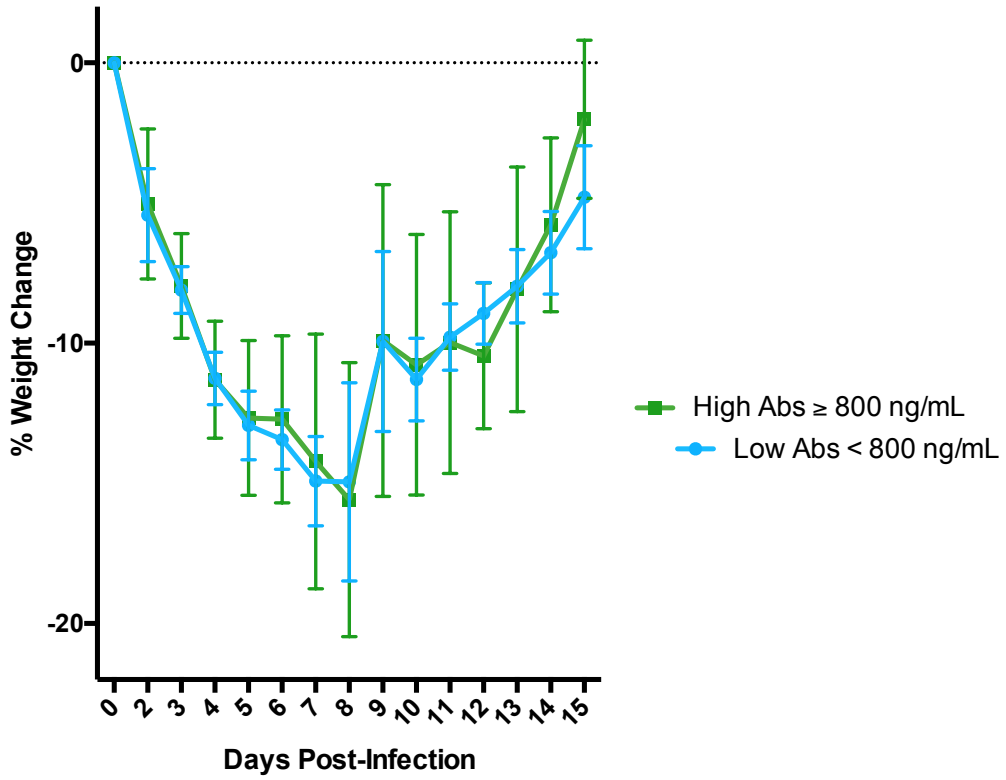
3.10 Postscript

This manuscript was submitted to PLOS One in 2017. In response to reviewers' comments, we performed further tests to establish whether or not the ELISA antibodies specific for H1 were truly biologically relevant. First, we challenged a small number of these mice with high titre influenza virus to determine if these antibodies were protective. All of the mice lost weight post-infection and there were no significant differences between those with high pre-existing H1 titres (≥ 800 ng/mL) or those with lower titres (Supplemental figure 3.2). Next, we gave an additional dose of split virus vaccine to mice with low vs high pre-existing titres (3µg, pdmH1N1). The animals with higher pre-existing titres did not have any obvious boosting effect suggesting that they had not previously 'seen' the H1 antigens (Supplemental Figure 3.3). Therefore, we can conclude that these H1-specific antibodies are not biologically relevant. The fact that the 'apparently ELISA positive' animals appear to have lost ELISA positivity after booster vaccination is very odd and difficult to understand.

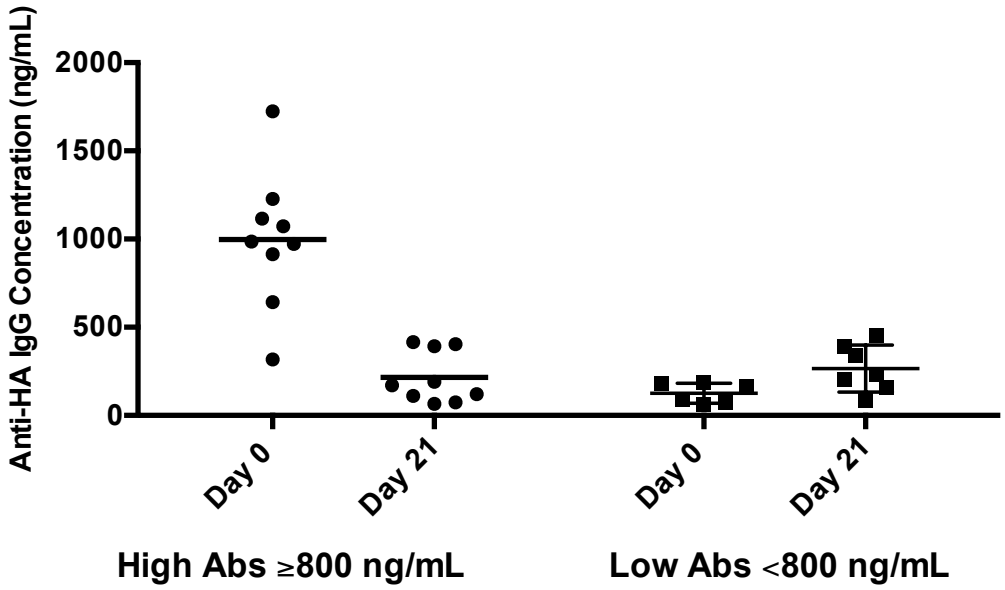
3.11 Supplemental Figures and legends



Supplemental Figure 3.1 Baseline influenza HA-specific IgG measured by ELISA in aged BALB/c mice for H3N2. All mice were 16-22 months of age. HA-specific IgG concentrations by ELISA with GMT were considered seropositive if >1000 ng/mL. Statistical analysis was performed using the Mann-Whitney test on log-transformed values. Significant differences are indicted in brackets; ****, P<0.0001. Overall, 10% (n=6) of the aged mice were seropositive while the remainder (n = 54) were seronegative.



Supplemental Figure 3.2 Percent weight loss after infection with H1N1 A/California/07/2009. Aged mice (16-20 months) were screened for H1-specific antibodies by ELISA and divided into two groups: low titres (<800 ng/mL) vs high titres (≥800 ng/mL). These mice were challenged with 5mLD50 of A/California/07/2009.



Supplemental Figure 3.3 Anti-HA IgG concentration by ELISA at Day 0 and Day 21. Aged mice (14-16 months) were screened for H1-specific antibodies by ELISA and divided into two groups: low titres (<800 ng/mL) vs high titres (≥800 ng/mL). Both groups received one dose of a split H1N1 A/California/07/2009 vaccine (3 μg).

Chapter 4: A plant-derived VLP influenza vaccine elicits a balanced immune response even in very old mice with co-morbidities

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4.1 Preface

To increase the relevance of our aged mouse model to humans, we decided to age mice until they had accumulated co-morbidities near the end of their natural life-span. Approximately half of human elderly between the ages of 65 and 84 years have co-morbidities. In our first study (Chapter 2), we demonstrated that the old mice (16-20 months) given one dose of the H1-VLP by IM were protected against weight loss and mounted a balanced (cellular and humoral) immune response. To assess the effects of co-morbidities, we used very old mice (24-26 months) with naturally occurring co-morbidities such as tumors, kyphosis, enlarged spleens and abscesses. We investigated the effects of these co-morbidities on the immune response generated after either VLP or IIV vaccination.

4.2 Abstract

The elderly are at high risk from influenza, in part because immunity wanes with age and through the accumulation of co-morbidities. A novel plant-derived virus-like-particle (VLP) vaccine bearing influenza hemagglutinin can induce a balanced humoral and cellular response in old mice (16-18 months) while split virion vaccines elicit mostly antibodies. Because mice also collect co-morbidities and lose immune competence as they age, we wished to determine how the plant-derived VLP vaccine would perform in animals approaching the end of their life-span.

Old (24-26 months) female BALB/c mice received two intramuscular doses of H1-VLP vaccine, an inactivated H1N1 vaccine (IIV) (both based on A/H1N1/California/07/09) (3μg each) or PBS. Serum was collected on day 42 and humoral responses were measured by enzyme-linked (ELISA), microneutralization (MN) and hemagglutination inhibition (HI) assays. Influenza-specific splenocyte $CD4^+$ & $CD8^+$ T cell responses were measured by flow cytometry. Full body computed tomography (CT^2) and structured necropsies were performed on day 42. Co-morbidities including reduced lung volume (kyphosis), masses, abscesses, etc. were assessed using a standard scoring system (1-21) and mice with scores ≥5 were considered to have important co-morbidities.

Overall, 53.3% of the animals had significant comorbidities. Three weeks post-boost, HI and MN titres were mostly undetectable but ELISA titres were significantly higher in the H1-VLP animals compared to the IIV group (GMT (95% CI): 961 (427, 2163) vs 425 (200, 903): $p = 0.03$). Both $CD4^+$ (TNFα, IFNγ) and $CD8^+$ (IFNγ) T cell responses were also greater in the H1-VLP group than the IIV.

Even in very old mice with co-morbidities, the plant-made H1-VLP vaccine elicited a stronger and more balanced immune response than IIV. Animals with fewer co-morbidities tended to have the better composite (humoral and cellular) responses. These novel vaccines have the potential to address some of the limitations of current vaccines in the elderly.

4.3 Introduction

The health risks of influenza increase steadily with age such that >70% of the mortality associated with seasonal outbreaks occurs in those ≥65 years of age (1). Unfortunately, the efficacy of current split-virion influenza vaccines typically diminishes as people grow older. Even with vaccines that have been designed, at least to some extent, for this population (e.g: high dose, MF59-adjuvanted), vaccine efficacy (VE) can still be very low (2). One factor that likely contributes to poor VE in older subjects is the fact that the vaccines used in adults and the elderly have all been optimized for antibody production, and specifically antibodies that react in the hemagglutination inhibition (HI) assay. In recent years, there has been increasing evidence that older individuals are protected from influenza primarily by cellular rather than antibody

responses (3-5). Although protection is far from optimal, the elderly can derive benefit from seasonal influenza vaccination despite making little-to-no antibody response (6-8). Other factors that contribute to low VE in the elderly are immunosenescence, a gradual weakening in multiple components of the immune system, and the accumulation of chronic inflammatory and co-morbidities conditions with age (e.g: arthritis, diabetes, cancer, degenerative diseases, etc.) (so-called 'inflamm-aging') (9, 10). There is a clear need to develop influenza vaccines that will provide better protection for the growing elderly population of the world.

Virus-like particles (VLPs) have features that theoretically make them attractive as vaccine candidates across all ages. These include rapid transport to lymphatic tissues, delivery of an antigen bolus to antigen-presenting cells (APC) and activation of APCs leading to stimulation of both humoral and cellular responses (11-14). We have recently demonstrated that many of these theoretical advantages can be realized with plant-made VLPs bearing the hemagglutinin proteins (HA) of pandemic and seasonal influenza viruses (15). In both pre-clinical studies with young animals and a series of clinical trials in healthy young adults, we have shown that these VLPs not only elicit strong and cross-reactive antibody responses but also induce long-lived and poly-functional $CD4^+$ T cell responses (14, 15). Based on these results, an obvious question to ask was whether or not some of these same advantages would also be seen at older ages. Like the elderly, old mice also suffer from chronic inflammatory conditions and co-morbidities, such as cancers, abscesses, rectal or uterine prolapse, skin lesions, renal disease, etc. (16). In this work, we compared the humoral and cellular responses to a candidate H1-VLP vaccine (H1N1 A/California/07/2009) to those seen with a split vaccine in very old mice with natural co-morbidities.

4.4 Materials and Methods

4.4.1 Virus, Mice and Vaccines

Female BALB/c mice (Charles River Laboratories, Montreal, QC) near the end of their life-spans (24-26 months age) were vaccinated with two doses of phosphate buffered saline (PBS), H1-VLP vaccine (Medicago, Quebec City, Quebec) or split inactivated H1N1 vaccine (BEI resources, Manassas, VA). The latter two were based on H1N1 A/California/07/2009

(pdmH1N1) – 3 μg/dose based on HA content in 50 μL for the H1-VLP and 100 μL for the split vaccines. Vaccines were administered intramuscularly (IM) 3 weeks apart (d0 and d21) and animals were sacrificed on d42. The H1-VLP was produced by transient expression in *Nicotiana benthamiana* plants as previously described (17) using the wild-type HA sequence from pdmH1N1. The H1-VLP or PBS were administered into the quadriceps muscle using a 28G ½ needle. The split vaccine was administered to both legs into the quadriceps muscle. The study included 9-17 mice per group.

Peripheral blood was collected in microtainer serum separator tubes (BD Biosciences, Mississauga, ON) from the lateral saphenous vein before immunization (d0), and at d21 post-vaccination. Sera were obtained using the manufacturer's instructions and stored at -20°C. At d42, mice were sacrificed by isoflurane followed by a CO_2 chamber. Serum was collected by cardiac puncture and splenocytes were isolated as previously described (18). All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

4.4.2 Antibody Titre Measurements

The hemagglutination inhibition (HI) (13), microneutralization (MN) (19) and enzyme-linked immunosorbent (ELISA) assays (18) were performed essentially as previously described. Both HAI and MN assays used H1N1 A/California/07/2009 (National Microbiology Laboratory, Public Health Agency of Canada) and cytopathic effect (CPE) in the MN assays was assessed at 4 days. The ELISA was performed using recombinant HA from pdmH1N1 as the coating antigen (Immune Technologies, New York, NY) (0.5 μg/mL).

4.4.3 Splenocyte Isolation and Stimulation

Individual spleens were harvested at d42 into Hank's balanced salt solution at room temperature (RT) without calcium or magnesium (HBSS -/-) (Wisent, St. Bruno, QC), and processed as previously described (18). Fresh splenocytes were seeded in 96-well U-bottom plates (BD Falcon, Mississauga, ON) in 200 μL per well ($1x10^6$ cells/well). Duplicate cultures were stimulated with cRPMI alone (unstimulated), H1-VLP (2.5 μg/mL HA) or PMA+

ionomycin (each 1 mg/mL) (Sigma, St. Louis, MO) for 18 hours at 37˚Cat 5% CO_2. After 13 hours, Golgi Plug™ was prepared according to manufacturer's instructions (BD Science, San Jose, CA) and added to samples (20µL /well). Five hours later, plates were spun (320xg, 8 minutes at 4˚C) and cells were processed for flow cytometry as described below. In our hands, we see no significant differences in T cell responses after splenocyte re-stimulation *ex vivo* with inactivated virions, VLPs or recombinant HA.

4.4.4 Flow Cytometry

Splenocytes were washed twice with 200 µL of cold PBS (pH: 7.4, Wisent) then centrifuged at 320xg for 8 minutes at 4˚C. Viability dye (50 µL/well diluted at 1:100) (Affymetrix ebioscience, Waltham, MA) was added and incubated for 20 minutes at 4˚C in the dark. Cells were washed as above and Fc block (1µL/well, BD Science, San Jose, CA) was added. Plates were incubated for 20 minutes at 4˚C. For the surface stains, each antibody was diluted at 1:100 in PBS and 50 µL/well of the extracellular 'cocktail' was added for 30 minutes at 4˚C protected from light. The following antibodies were used: CD3 –FITC (Clone:145-2C11, Affymetrix ebioscience), CD4-V500 (RM4-5, BD Bioscience) and CD8-PerCP-Cy5 (Clone:53-6.7, BD Bioscience). Cells were washed as above, then 100 µL/well of fixative was added (BD Science) for 30 minutes or overnight at 4˚C protected from light. For intracellular staining, cells were washed as above with 1X *permeabilization buffer* (BD Science) then stained with an intracellular 'cocktail' of antibodies as 1:50 dilution in *permeabilization buffer* (50 µL/well). The following markers were used: IL-2-Pe-Cy5 (Clone: JES6-5H4, Biolegend, San Diego, CA), IFNγ-PE (Clone: XMG1.2, BD Science) and TNFα-efluor450 (Clone:MP6-XT22, Affymetrix ebioscience) and incubated for 40 minutes in the dark at 4˚C. After washing with PBS, cells were fixed with intracellular fixative (Affymetrix ebioscience) and analyzed on BD LSRFortessa X-20 (BD Science) using Flowjo software (version 10.0.8r1). Our gating strategy is shown in supplemental Fig 4.1.

4.4.5 Co-morbidity Assessment

Immediately prior to sacrifice, co-morbidities were assessed by full body CT^2 scans (Mediso nanoScan SPECT/CT^2/PET, Budapest, Hungary) and each major organ was evaluated

as either normal (score 0), slightly abnormal (score 1) or grossly abnormal (score 2). At the time of sacrifice, a structured necropsy was performed and each major organ was visually assessed and scored (as above). An overall score for each animal was calculated (maximum score 18).

4.4.6 Computed Tomography

At d42, full body CT^2 scans were performed 5 minutes after tail-vein injection of a contrast agent (Omnipaque: GE Healthcare, Mississauga, ON). These images were analyzed with Amide software (version 1.0.4: sourceforge.net). Lung volumes were estimated by measuring the distance from the lung apices to diaphragm (vertical) on the coronal view and from the spine to the sternum (horizontal) on the sagittal view (population terciles scored 1-3). CT^2 images were blinded for analysis. CT^2 scans scores and necropsy scores were combined for a total score.

4.4.7 Statistical Analysis

For statistical analysis, one-way ANOVA or two-way ANOVA tests were performed on the log10 values for sera analysis. For statistical analysis, one-way ANOVA was used on the log values for A). For B) Mann-Whitney test was performed on the log values. Two-way ANOVA test was used for cytokine data. All analyses were performed using GraphPad Prism 6.0 software. Outliers were identified and removed by ROUT using GraphPad Prism.

4.5 Results

4.5.1 Humoral Response

Not unlike older humans, these very old mice had undetectable or limited humoral responses to vaccination using the standard influenza vaccine serologies (HI and MN, Fig 4.1A and B respectively). Only one or two mice in each vaccine group mounted any detectable HI response (GMT: 40 and 10 or 7.1 in the H1-VLP or IIV groups respectively). All other mice had HI titres below the limit of detection of our assay (≤10). No animal in the PBS or IIV groups mounted a detectable MN response. In contrast, 7/15 (46.7%) of the mice in the H1-VLP group had detectable MN responses although the overall MN response remained low (GMT:10: range

10-80). Antibodies measured by ELISA (Fig 4.1C) were detected in both active vaccine groups. If 2x the GMT of the PBS group is defined as the lower limit of detection in this assay, 11/15 (73.3%) of the IIV group and 12/15 (80.0 %) of the H1-VLP group mounted detectable responses. The overall GMT in the H1-VLP groups was 2 times higher than the IIV group (961 versus 425: $p<.03$).

4.5.2 $CD4^+$ and $CD8^+$ T Cell Responses

The PBS group had overall low T cell production of IFNγ, TNFα and IL-2. The $CD4^+$ and $CD8^+$ T cell responses following re-stimulation with H1 antigen *ex vivo* were generally higher in the H1-VLP group than the IIV animals (Fig 4.2) for most of the 'cytokine signatures' involving IFNγ, TNFα, IL-2. Differences between the H1-VLP and IIV groups reached statistical significance for $CD4^+$ T cells expressing IFNγ and TNFα (Fig 4.2A) and for $CD8^+$ splenocytes expressing IFNγ (Fig 4.2B). In the H1-VLP group, there was a higher percent of poly-functional $CD4^+$ T cells observed, compared to than the IIV group (0.012 vs 0.009, respectively) but this difference did not reach statistical significance (Fig 4.2A).

4.5.3 Co-morbidities

Mice in all of the groups had obvious but varied co-morbidities; enlarged and/or granular-appearing spleens, pale spleens (anemic), liver nodules, or abscesses, enlarged heart, inflammation/abscesses in ovaries and uterus, skin and tail erosions/ulcers. Masses (presumed tumors) were found in the liver, neck, lungs or uterus/ovaries of some animals (Fig 4.3). The abnormalities tended to be evenly distributed between the groups as demonstrated by the total co-morbidity scores (Table 4.1). Overall comorbidity scores were approximately the same between the H1-VLP group and the split-inactivated virion (5.33 ± 1.72 and 5.00 ± 2.54, respectively) and 53.3% (8/15) of mice in each group had at least 1 readily identified co-morbidity. There was no overall correlation between morbidity scores and either antibody or cellular responses.

4.6 Discussion

The ageing population is one of the major risk groups for influenza morbidity and mortality. Unfortunately, the currently available vaccines that target the elderly are far from optimal in the protection they provide. The development of vaccines that can stimulate cellular responses in addition to antibodies may be of particular interest for this population since older individuals appear to derive significant benefit from cellular memory (3, 20). We have recently demonstrated that VLP vaccines made in plants that carry HA trimers of either seasonal or pandemic influenza viruses can protect animals (mice, ferrets) in lethal challenge studies despite low and, in some cases, even completely absent antibody responses (21, 22). After footpad injection in a mouse vaccination model, these VLPs have been shown to move to regional lymph nodes (LN) within minutes where they preferentially associate with antigen-presenting cells (APC) and B cells (23) linked with a rapid increase in LN cellularity and dendritic cell activation (24). The *in vitro* interactions of these VLPs with human immune cells are also quite unusual. VLPs bearing HA trimers of seasonal strains cause rapid clustering of peripheral blood mononuclear cells (PBMC) leading to expression of activation markers and the release of pro-inflammatory cytokines by monocytes (25). In a process mediated by cell-surface sialic acid residues, HA-bearing VLPs are rapidly internalized by human APCs including monocyte-derived macrophages (26) and monocyte-derived dendritic cells (unpublished data). These animal and *in vitro* observations suggest that the plant-derived VLPs interact with immune cells very differently from inactivated split-virion vaccines (25, 26).

In clinical trials conducted to date, VLPs have been shown to elicit a more balanced humoral and cellular response than the Th2 (antibody)-dominated response typically seen with IIV comparators in healthy adults and older subjects (≥65 years of age) ((15), unpublished data). In all of these trials however, subjects with serious comorbid conditions were excluded. These clinical observations are consistent with previous studies showing that healthy BALB/c mice up to 16-20 months of age can be protected from lethal challenge with a single dose of the plant-derived VLP vaccine despite relatively weak (younger animals) or absent (older animals) antibody responses (21). In the current work, we extended our mouse model to the limit of the murine life-span. Under laboratory conditions, BALB/c mice are considered to be very old when they reach 18-20 months of age (27, 28). Even though BALB/c mice accumulate age-related

pathologies at a slower rate than other in-bred strains (29), more than half (53.3%) of the animals in our study had at least 1 obvious comorbid condition identified by either whole body CT^2 or structured necropsy at study termination. Although we observed a robust ELISA response in these very old mice after two doses of the VLP vaccine that was clearly superior to that seen in IIV recipients, even the VLP-vaccinated animals made almost no detectable HI or MN antibodies. However, the most striking differences between VLP- and IIV-treated animals were seen in the T cell responses. Based on antigen-specific intracellular cytokine responses, the VLP vaccinated animals had readily-detectable $CD4^+$ (ie: poly-functional and individual cytokines) and $CD8^+$ (ie: IFNγ) T cell responses that were clearly superior to those seen in the IIV and PBS control groups. Although not enough very old animals were available to perform a terminal challenge in this study, these data nonetheless suggest that the VLP-vaccinated animals would likely have been better-protected than the IIV recipients' due to the superior immunogenicity of the plant-derived VLPs.

Although influenza vaccine licensing strategies have long placed a heavy emphasis on a new product's ability to elicit high HAI titres (30), older adults typically make much lower HAI and neutralizing antibody responses than younger adults (6) and there is a clear inverse correlation between health status and the HAI response. Older adults with multiple co-morbidities have even lower antibody responses to IIV than the healthy elderly (31). Although a robust humoral response to influenza virus antigens can undoubtedly help to prevent infection, there is a growing appreciation that other factors need to be considered in assessing influenza vaccine-induced immunity including antibodies than those measured in the classical HI and MN assays (eg: anti-stalk) (32) and cellular immunity including NK and T cell responses (33). Such considerations may be particularly important in designing and evaluating new influenza vaccines for the elderly (3).

Our work has several obvious limitations, the most important of which were the relatively small numbers of very old animals available for study (ie: no challenge to directly assess efficacy) and the fact that morbidity evaluation in mice is very far from an exact science. Although we tried to standardize our co-morbidity assessment by using both whole body CT^2 and a structured autopsy, these very old animals were so fragile that substantial mortality occurred in performing the one CT^2 examination during a pilot study (25%). Therefore, we did

not want to risk losing mice with serial imaging and made extensive handling impossible (ie: frailty assessment) (34). Furthermore, although some of the parameters we measured have obvious parallels in the elderly (ie: kyphosis and restricted lung volume) (35), we cannot claim that the spectrum of co-morbidities observed in the very old mice are an accurate reflection of those experienced by ageing humans. Finally, although the overall co-morbidity 'score' was similar between groups in our study, we could not ensure that there was an equal distribution of the different kinds of co-morbidities across groups.

Although sufficient numbers of animals were not available in the current study to perform a challenge, our previous work with healthy old mice (21) strongly suggests that the pattern of immune response elicited by the VLP vaccine should provide better protection than IIV even in very old mice with multiple co-morbidities. These data also suggest that guarded optimism is appropriate for the outcome of an on-going efficacy study in subjects ≥65 years of age comparing the plant-derived quadrivalent VLP vaccine to a commercial comparator. Such an outcome would be highly desirable since the elderly are currently not well-served by the available vaccines against influenza.

4.7 Acknowledgments

We would like to thank Annie Beauchamp, Kaitlin Winter and Angela Brewer for animal work assistance. We would also like to thank Louis Cyr for technical assistance. We thank the SAIL Technology Platform and the immunophenotyping Core of the Research Institute of the McGill University Health Centre and staff for services provided.

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4.8 Figures, Legends and Table

Table 4.1 Summary of comorbidity scores assessed by necropsy and CT^2 scan after two immunizations of H1-VLP, split-virion vaccine of PBS

	Lung Volume Score	**Necropsy Score**	**Total**
PBS	2.25 ± 0.96	3.25 ± 1.89	5.5 ± 1.73
3 µg split vaccine	2.47± 0.92	2.53 ± 2.03	5.00 ± 2.54
3 µg H1-VLP	2.47 ± 0.83	2.87 ± 1.60	5.33 ± 1.72

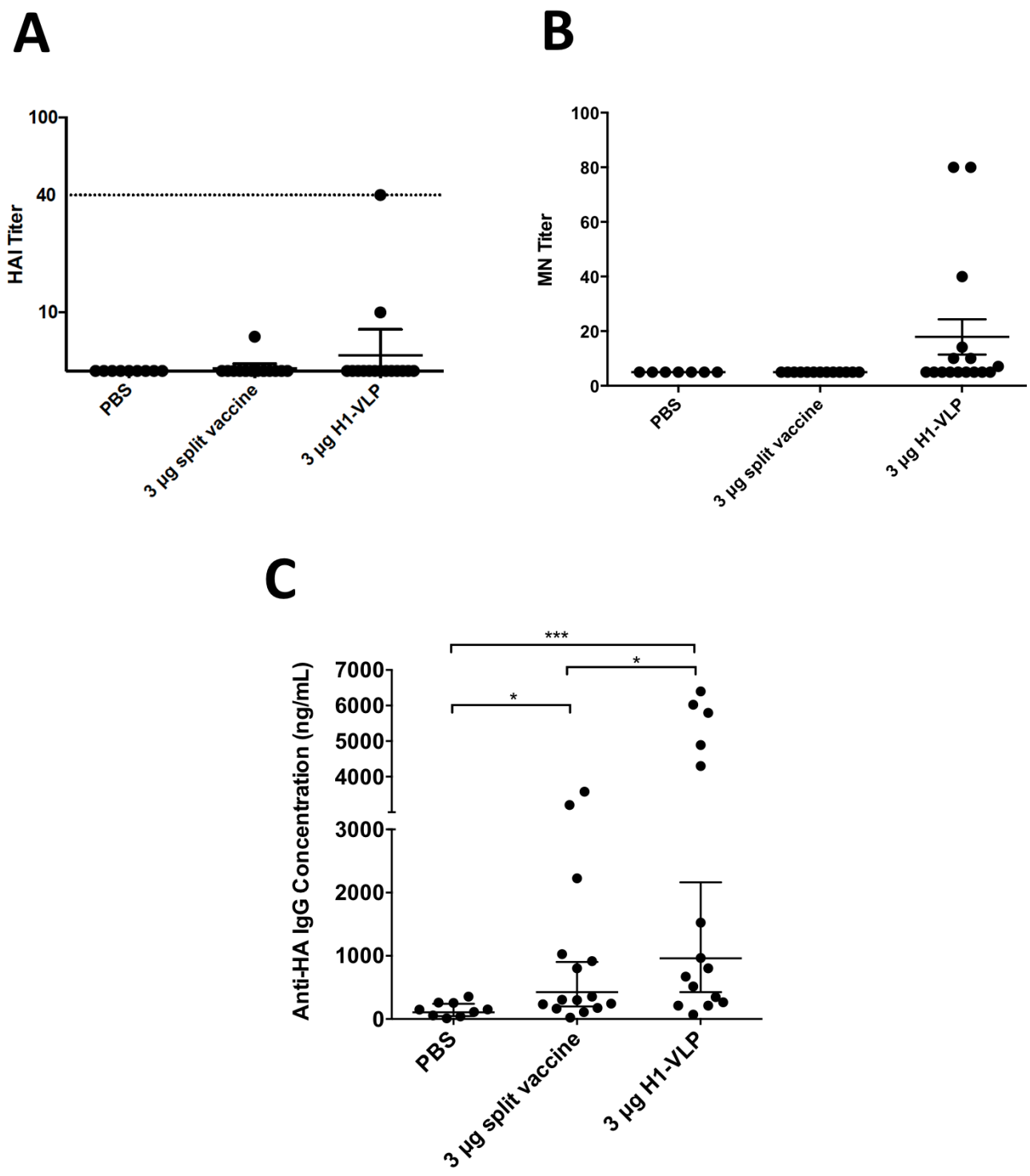


Figure 4.1 Serum antibody response after two doses of H1-VLP or split-virion vaccine

Aged (24-26 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or PBS. Three weeks post-boost sera from individual mice were analyzed by Hemagglutination Inhibition (HI) (A) and microneutralization (MN) (B) titre against A/California/07/2009 H1N1. Influenza HA-specific IgG concentrations (C) by ELISA. For statistical analysis, one-way ANOVA was used on the log values for A). For B) Mann-Whitney test was performed on the log values and C) two-way ANOVA analysis was performed (*** $p<0.001$, * $p<0.05$). A) and B) represent 9-15 mice/group and C) represents 9-15 mice/group.

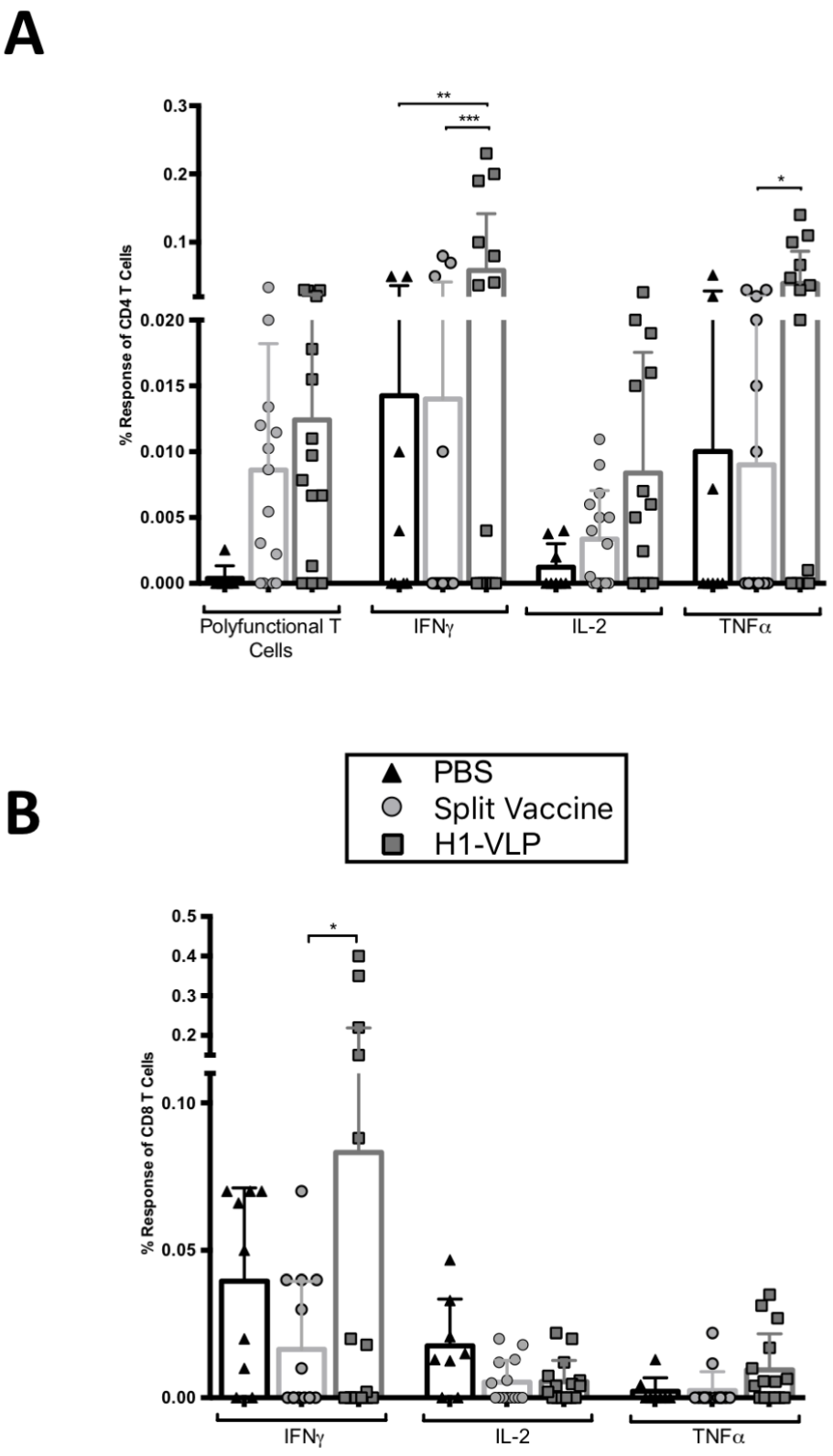


Figure 4.2 Cytokine production by splenocytes after *ex vivo* stimulation three weeks post-boost

Aged (24-26 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or PBS. Three weeks post-boost, splenocytes were collected and stimulated *ex vivo* for 18 hours with H1-VLP. Subtractive data was used (Stimulated - unstimulated). For statistical analysis, two-way ANOVA was performed (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$). Data represent 9-15 mice/group.

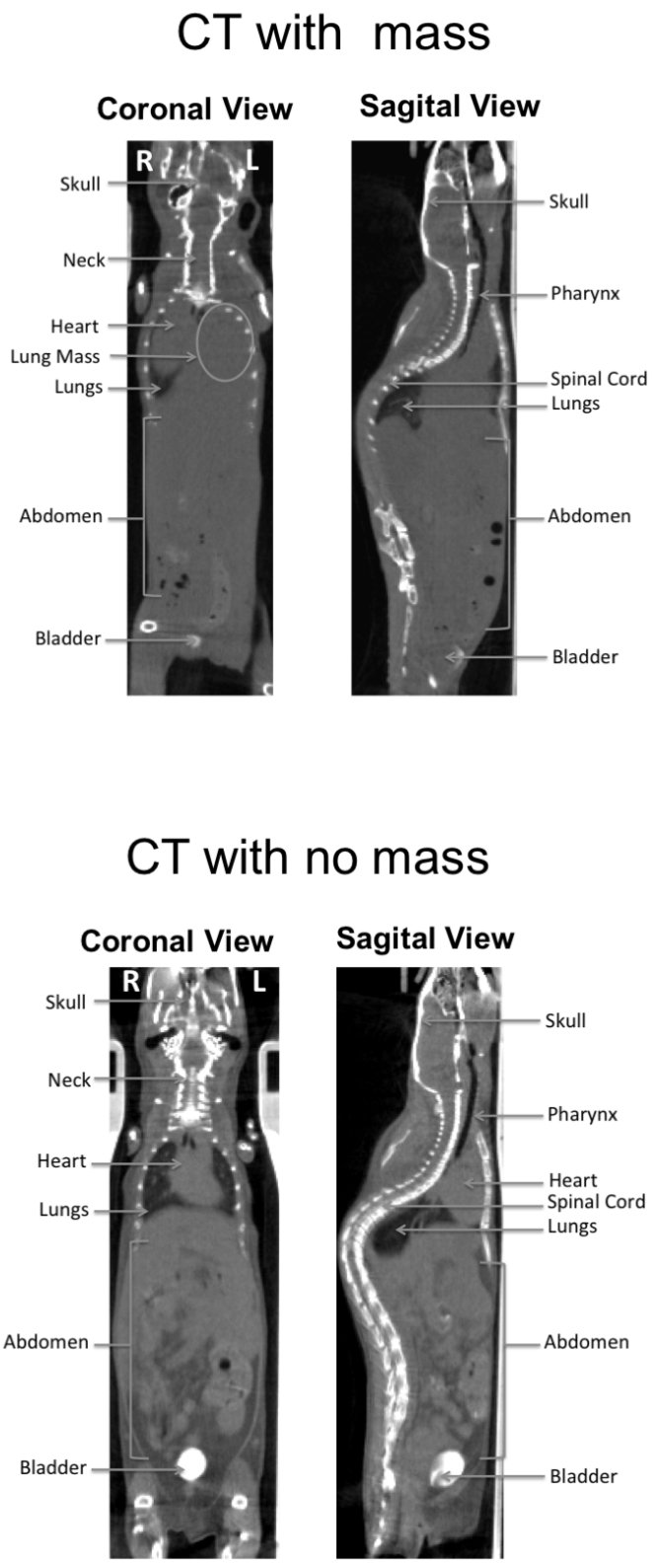
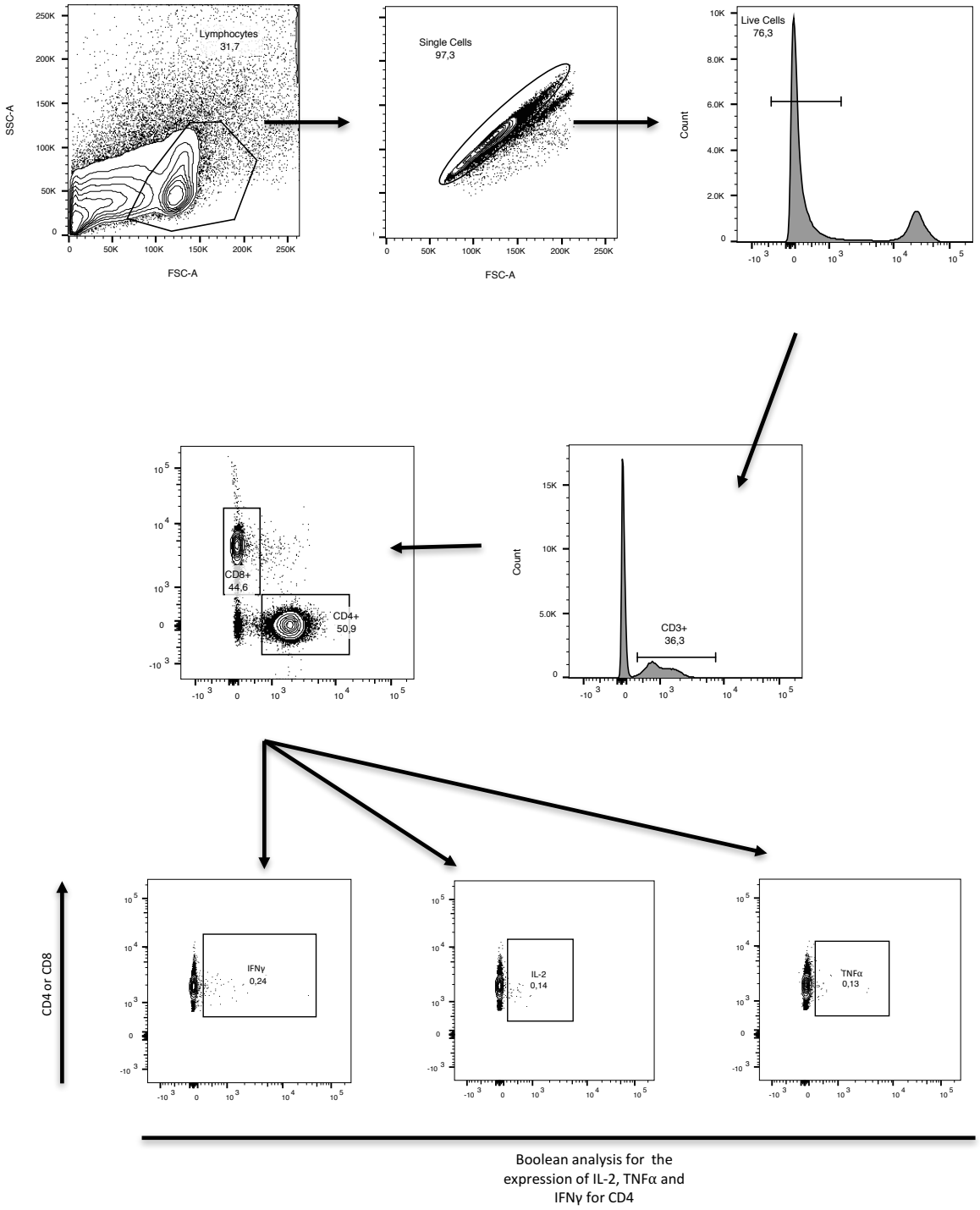


Figure 4.3 Full body CT[2] scans three weeks post-boost vaccination

Aged (24-26 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or naïve. Three weeks post-boost, 4-15 mice/group underwent full body CT^2 scans. A) Depicts a mouse with the presence of mass on the left side of its chest. B) demonstrates a relatively healthy aged mouse with no mass detected.

4.10 Supplemental Figure and Legend



Supplemental Figure 4.1 Flow cytometry-gating strategy for splenocytes

Aged (24-26 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or naïve. Three weeks post-boost (9-15 mice/group), splenocytes were collected and stimulated *ex vivo* for 18 hours with H1-VLP. This strategy was done for each cell type $CD4^+$ and $CD8^+$ T

cells and for each of those cell types the following cytokine expression was calculated: IFNγ, TNFα and IL-2. Subtractive data was used (Stimulated - unstimulated)

Chapter 5: Prime-pull vaccination with a plant-made virus-like particle influenza vaccine elicits a broad immune response and protects aged mice from frailty after a sub-lethal challenge

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5.1 Preface

To further develop our aged mouse model, we wanted to evaluate both the impact of frailty on immune responses and more fully explore the level of protection provided by the VLP vaccine (ie: increase in frailty as well as mortality). We also wanted to explore different approaches for the VLP vaccine, such a multi-modality (ie: different routes simultaneously) and prime-pull strategies (ie: sequential priming and boosting at different sites). Both of these approaches have been demonstrated to boost immunity and protection in some models. Because we observed more rapid recovery of weight after challenge in previous studies in old mice that had received VLP vaccination, we wanted to assess the potential impact of the VLP influenza vaccine on frailty as well as the potential impact of frailty on the success of the VLP vaccine. In humans, the combination of influenza and pneumonia is a major cause of frailty. Therefore, we incorporated an assessment of frailty before infection and 25 days post-infection to determine if any of the novel vaccine routes or strategies would help protect against frailty after infection.

5.2 Abstract

Background: Unlike split virus influenza vaccines, virus-like-particle (VLP) vaccines can be administered by different routes. Administered intramuscularly (IM), plant-derived VLPs based on influenza hemagglutinin (HA) elicit a balanced humoral and cellular responses that protects

aged mice from lethal challenge. We wished to determine if the flexibility of administration of the VLP vaccine (IM, intranasal (IN)) could be exploited to provide better quality protection in old mice after sub-lethal challenge (ie: immunogenicity, frailty and survival).

Methods: Old female BALB/c mice (18-22 months) received two doses of a plant-derived H1-VLP vaccine based on HA content (A/California/07/2009 H1N1) 21 days apart. Vaccines were administered IM or IN and were delivered either simultaneously (multi-modality: IM+IN: 1.5μg each route) or sequentially (prime-pull: IM/IN: 3μg each dose). Comparator groups included uninfected animals and animals given PBS (same routes) or H1N1 inactivated influenza vaccine (split) IM/IM (IIV: 3μg each dose). Six weeks after vaccination, humoral and cellular responses were assessed and mice were challenged with a sub-lethal dose of A/California/07/2009 H1N1 virus. Frailty was assessed by a blinded observer using a 29-parameter protocol immediately prior to challenge and at day 25 post-challenge.

Results: Survival rates were similar in all groups. Antibody responses were modest in all groups but tended to be higher in VLP groups compared to IIV recipients. All VLP groups had higher splenocyte T cell responses than the split virus group. Lung homogenate chemokine/cytokine levels and virus loads were lower in the VLP groups compared to IIV recipients 3 days after challenge ($p<0.05$ for viral load vs all VLP groups combined). The VLP-vaccinated groups also had less weight loss and recovered more rapidly than the IIV recipients. There was limited evidence of an immunologic or survival advantage with IN delivery of the VLP vaccine, but the lowest changes in the frailty index were seen in the IM+IN and IM/IN groups (versus split vaccine and IM/IM VLP).

Conclusion: Compared to IIV, the plant-derived VLP vaccine induced a broader immune response in aged mice (cellular and humoral) using both traditional (IM/IM) and novel schedules (multi-modality, prime-pull). The novel schedules that included IN delivery may also have reduced frailty after challenge in this aged mouse model.

5.3 Introduction

Influenza infection can be devastating in the elderly, resulting in both significant mortality and morbidity (1, 2). In most seasonal influenza outbreaks, those ≥65 years of age typically account for 71-85% of the deaths which are relatively easy to 'count'(3, 4). The impact of influenza–associated morbidity is more difficult to quantify since even a short period of forced

bed-rest, either at home or in hospital, can lead to major loss of muscle mass (ie: sarcopenia) (5) and accumulation of other physiologic and mental deficits (ie: increased frailty) (6, 7). More prolonged periods of bed-rest (ie: influenza complicated by pneumonia, intensive care admission) often lead to catastrophic disability with loss of independence in elderly subjects (8, 9). Vaccination is currently the best strategy to protect the elderly from influenza viruses (10) but this population often responds poorly to 'standard' inactivated influenza vaccines (IIV) due to prior experience with influenza antigens and immunesenescence (11-13). Although a number of vaccines that specifically target the elderly have been introduced in recent years (e.g: MF59-adjuvanted IIV, so-called 'high-dose' (HD)-IIV) (14), their impact in improving effectiveness (ie: preventing infection) have been relatively modest (15-20). Their potential advantages in preventing frailty have generally not been considered.

Several highly-successful virus-like particle (VLP) vaccines are in current use (ie: HBV and HPV vaccines) and VLP vaccines have many potential advantages for a wide range of targets (21-24). Several VLP vaccines for influenza are at various stages of pre-clinical and clinical development. One of the most advanced is produced by Medicago Inc (Quebec, QC) using transient production of the influenza hemagglutinin (HA) protein in *Nicotiana benthamiana* plants. After peripheral administration in mice, these plant-derived VLPs move rapidly to regional lymph nodes where they preferentially interact with B cells, NK cells and antigen-presenting cells (APC) (25). They also interact directly with human immune cells including B cells and APC leading to activation (26), internalization (27) and presentation (28). Indeed, these plant-derived VLPs appear to recapitulate many of the early interactions of intact influenza virions with host cells including fusion with host endosomal membranes (28). In animal models of pandemic infection, the plant-made vaccines can provide excellent protection despite eliciting little-to-no antibody response suggesting an unusual capacity to induce cellular responses (24, 29, 30). In clinical trials with healthy adults, the plant-derived VLP vaccines not only elicit good antibody levels against seasonal strains but also induce long-lived and poly-functional $CD4^+$ T cell responses (29). The latter characteristic is of particular interest for older individuals since this population may be protected primarily by cellular immunity (31).

In the context of the current work, one major advantage of VLP vaccines is their flexibility: they can be administered using different routes including intramuscular (IM),

intradermal (ID), oral (PO) and intranasal (IN) (32, 33). This flexibility makes alternate vaccination strategies possible including either simultaneous or sequential administration at different sites. The former can be considered a type of multi-modality immunization that, in theory, could stimulate different, tissue-specific immune mechanisms. The latter approach, also called 'prime-pull' consists of a systemic "prime" dose (e.g: IM) followed by a local "pull" dose given at the site of natural infection to 'recruit' the immune cells to that area (e.g: PO or IN) (34-36). These alternate vaccination strategies could potentially provide better protection in the elderly, inducing a long-lasting cross-protective cellular response (37-39) and boosting of local mucosal immunity (34, 40). Since 'standard' vaccination strategies based on IIVs that focus primarily on the induction of high systemic antibody titres have had only limited success in the elderly (31, 41), we were interested to know if the flexibility and unusual immunogenicity of the plant-derived VLP vaccines could be exploited to better protect older individuals. We have recently shown that a single dose of a plant-derived H1-VLP candidate vaccine could protect old mice from a lethal A/California/07/2009 H1N1 challenge. To our surprise, a single dose of the same VLP vaccine given by IN protected ~60% of the animals despite a complete absence of a detectable serologic response.

In the current work, we extended these observations by testing alternate VLP immunization strategies and following immunogenicity as well as protection against both frailty and death following a sub-lethal A/California/07/2009 H1N1 challenge. Our results demonstrated that the VLP vaccines elicit a broader immune response than IIV regardless of the vaccination strategy used. Animals that received a dose of the VLP vaccine IN had the most rapid weight recovery and the least change in frailty index after infection. Although preliminary, these data suggest that such alternate strategies of vaccination should at least be considered for elderly subjects when a vaccine with the flexibility to be administered via multiple routes becomes available.

5.4 Materials and methods

5.4.1 Virus, Mice and Vaccines

Female BALB/c mice (18-22 months of age: Charles River Laboratories, Montreal, QC) were vaccinated twice on day 0 (d0) and day 21 (d21). All active vaccinations were based on hemagglutinin (HA) content for H1N1 A/California/07/2009 (pdmH1N1). The plant-derived H1-VLP vaccine was produced by Medicago Inc. (Quebec City, QC) as previously described (42) using the wild-type HA sequence from pdmH1N1. Three groups of animals received the VLP vaccine: i) two 3µg doses intramuscularly (IM/IM) ii) a first 3µg dose IM boosted at d21 by a 3µg dose intranasally (IM/IN: Prime-pull group) or iii) two doses of 1.5µg IM plus 1.5µg IN (IM+IN: Multi-modality group). The active comparator group received two 3µg doses of a split H1N1 inactivated influenza vaccine (IIV: BEI resources, Manassas, VA). Control animals received similar volume IM and/or IN 'mock' vaccinations with phosphate buffered saline (PBS: pH:7.4, Wisent, Saint-Bruno, QC). For H1-VLP and PBS injections IM, 50µL was administered into the right quadriceps muscle using a 28G½ needle. For IIV injections, 50 µL was injected into each quadriceps muscle (100 µL total). Instillations of H1-VLP or PBS IN (25 µL/nare) were performed in lightly isoflurane anesthetized mice (50µL total).

Peripheral blood was collected using microtainer serum separator tubes (BD Biosciences, Mississauga, ON) from the lateral saphenous vein at d0 (pre-vaccination) and at d21 and d42 post-vaccination (Supplemental Figure 5.1). Serum was stored at -20°C in aliquots until used. At d42 (immediately pre-challenge), approximately $^1/_2$ of the animals were sacrificed within isoflurane and a CO_2 chamber (typically 6-8 mice/group). A terminal serum sample was collected by cardiac puncture. Spleens and lungs were harvested from individual animals and processed as described below. The remaining mice (typically 6-8 mice/group) were scored for frailty on d40-42 then challenged with a sub-lethal dose of wild-type pdmH1N1 virus (525 $TCID_{50}$ in 50µL: National Microbiology Laboratory, Public Health Agency of Canada) by IN instillation (25µL/nare). Weight loss was monitored daily for up to 28 days. At d45 or 3 days post-infection (dpi), 3-5 mice/group were sacrificed (isoflurane/CO_2) and serum (cardiac puncture) and lungs were collected (viral load and flow cytometry). At d67 (25 ± 4 dpi), surviving mice were scored for frailty and sacrificed as above to collect serum and lungs. All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

5.4.2 Antibody Titre Measurements

Serum antibody levels were measured by hemaglutination inhibition assay (HAI), microneutralization assay (MN) and enzyme-linked immunosorbent assay (ELISA) as previously described (43).

5.4.3 Lung T Cell Isolation and Stimulation

Lungs were perfused with 10mL of PBS and collected in in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS (both Wisent, St. Bruno, QC) stored on ice for approximately 2 hours before transfering to a lung digestion cocktail (DNAse I (10 mg/mL: Sigma, St. Louis, MO), Collagenase (12.5mg/mL, Sigma), Liberase (10 mg/mL: Roche, Basel, Switzerland), hyalurnidase I (50 mg/mL, Sigma)) prepared in DMEM. After a 30-40 minute incubation at 37°C in 5% CO_2, lungs were processed individually through a 70 μm cell strainer, resuspended in 10 mL of Hanks Buffered Salt Solution without calcium/magnesium (HBSS -/-: Wisent) then centrifuged at 320xg for 8 minutes at 4°C. Cells were passed through a cell strainer for a second time and washed with 5 mL of HBSS-/- before re-suspension and counting in complete Roswell Park Memorial Institute medium (cRPMI). Lung cells were seeded in duplicate at 1.5 x10^6 cells/well in 96-well U-bottom plates (BD Falcon, Mississauga, ON) in 200 μL. Cells were stimulated with cRPMI alone (unstimulated), PMA+ ionomycin (each 1 mg/mL: Sigma) or with 4 μg/mL of a previously-described (24) overlapping H1 peptide pool, from pdmH1N1 (BEI resources, Manassas, VA) (all stimuli at 80μL/well). Plates were incubated for 5 hours at 37°C in 5% CO_2.

5.4.4 Splenocyte Isolation and Stimulation

Individual spleens were harvested at d42 into HBSS -/- (Wisent) and processed at room temperature (RT) as previously described (44). Isolated splenocytes were seeded in duplicate in U-bottom 96-well plates (1x10^6 cells/well) as above and stimulated with cRPMI or alone (unstimulated), H1-VLP (2.5 μg/mL HA) or PMA+ ionomycin (each 1 mg/mL: Sigma) for 18 hours at 37°Cat 5% CO_2(all stimuli at 100μL/well).

5.4.5 Flow Cytometry

T cell responses was assessed in mononuclear cells isolated from the lungs and spleen at d42 (immediately pre-challenge). Golgi Plug™ (BD Science, San Jose, CA) and added to lung cells at the beginning of stimulation or 13 hours after stimulation for splenocytes (20µL /well). For flow cytometry, cells were washed twice with cold PBS then centrifuged at 320xg, 8 mins at 4°C. Viability dye (50 μL/well) (Affymetrix ebioscience, Waltham, MA) was added to each well (1:10 for lung cells and 1:100 for splenocytes in PBS) and incubated for 20 minutes at 4°C. Cells were washed as above and Fc block (1µL/well, BD Science, San Jose, CA) was added. The following cocktail was used for surface stains: CD3 –FITC (Clone: 145-2C11, eBioscience), CD4-V500 (Clone: RM4-5, BD Bioscience) and CD8-PerCP-Cy5 (Clone: 53-6.7, BD Bioscience), CD45-BUV495 (Clone: 30-F11, ebioscience), CD11a-APC (Clone: M17/4, Biolegend, San Diego, CA), CD103-BV711 (Clone:M290, BD Bioscience) and CD69-BV605 (Clone:H1.2F3, Biolegend). After 30 minutes, cells were washed as above, then fixed overnight at 4°C with 100 µL of fixative (BD Science). For the intracellular stains, cells were washed as above except with 1X permeabilization buffer (BD Science), then stained with an intracellular cocktail containing: IL-2-Pe-Cy5 (Clone: JES6-5H4, Biolegend), IFNγ-PE (Clone: XMG1.2, BD Science) and TNFα-efluor450 (Clone: MP6-XT22, Affymetrix ebioscience) and incubated for 40 minutes in the dark at 4°C. After washing with PBS as above, cells with fixed with an intracellular fixative (Affymetrix ebioscience) and analyzed on BD LSRFortessa X-20 (BD Science) using Flowjo software (version 10.0.8r1). Our gating strategy is shown in Supplemental Figure 5.2.

5.4.6 Frailty Measurements

Frailty measurements were adapted from *Whitehead et al* (45) using 29 of the original 31 parameters to adapt the procedure to BALB/c mice. The parameters assessed fell into the following categories: integument, musculoskeletal, ocular and nasal, vestibulocochlear/auditory, digestive, urogenital, respiratory. Signs of discomfort, body weight and temperature were also assessed. Supplemental Table 5.1 is the scoring sheet that was used at d0, d42 and d67 (25 dpi). Deficits were measured using a 3-point scale: 0 = no deficit, 0.5 = mild deficit and 1 = severe

deficit. All measurements were performed by the same operators (BH or AB) who were blinded to group assignment.

5.4.7 Lung Viral Load and Cytokine/Chemokine Levels at Day 45 (3 dpi)

Lungs were collected at 3 dpi and homogenized for viral load and cytokine/chemokine measurements as previously described (43, 46). Briefly, viral titres were calculated from the supernatants of lung homogenates using the Karber method and reported as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$): $logTCID_{50}$/0.1mL = -1 - (observed lysis of monolayer (as a percent(%) /100 – 0.5) x log10 (47). Viral load data are representative of 3-5 mice/group from two independent experiments. The lung homogenate supernatants were used (1:5 and 1:10) to measure 16 tissue cytokine/chemokine concentrations using a multiplex ELISA (Quansys, Logan, UT). Lung homogenates were collected from 4-7 mice/group in one experiment and tested in duplicate.

5.4.8 Statistical Analysis

The geometric mean ratios between groups and their 95% confidence intervals (CI) were calculated. For statistical analysis, one-way ANOVA was performed on HAI, ELISA, MNs, viral titres and frailty scores. For survival statistics, a log-rank (Mantel-Cox) test was used. All other statistical analyses used two-way ANOVA. All analyses were performed using GraphPad Prism 6.0 software.

5.5 Results

5.5.1 Sub-lethal Infection Survival Rates

Although the viral challenge dose was intended to be sub-lethal and was based on preliminary titration experiments, more than half of the PBS control animals nonetheless succumbed to infection (41.7% survival) (Figure 5.1). The vaccine groups with the highest survival rates were the IIV-IM/IM and VLP-IM/IM recipients (87.5% and 84.2%, respectively).

The VLP-IM/IN and VLP-IM+IN groups had a slightly lower survival (76.5% and 62.5%) but these differences did not reach statistically significance. All the naïve, uninfected mice survived.

5.5.2 Antibody Response

Antibody responses in these older mice were generally weak. The highest geometric mean HI titres were observed was the VLP-IM+IN group (GMT: 6.4± 4.0) but only 20% of the animals mounted a detectable response (Figure 5.2A). Only 5-7% of the animals in the other vaccine groups and none of the PBS control animals had a detectable HI response. Although geometric mean MN responses were slightly higher, only a small number of animals in each vaccine group had detectable responses (25-56 %). The highest MN response was in the VLP-IM/IM group (GMT: 15.3± 44.54; range 10-160 (Figure 5.2B) which was significantly higher than the VLP-IM+IN and placebo groups (GMT:6.8± 8.80 and <5, both $p<0.05$) The mean GMT in the IIV-IM/IM group was 10.21 (range 5-80) and was significantly greater than only the PBS group (<5: $p<0.05$). The ELISA assay demonstrated a more consistent antibody response (53-79 % of the animals in vaccinated groups mounted detectable levels). The VLP-IM/IN and VLP-IM/IM groups had the highest ELISA titres (GMT: 611.8 ng/mL; 95% confidence interval (CI): 420-891 ng/mL and GMT: 537.70 ng/mL; 95% CI: 322-899 ng/mL, respectively). These titres were significantly higher than only the PBS group (GMT: 209.1 ng/mL; 95% CI: 142-309 ng/mL: $p<.05$). (Figure 5.2C). Lower ELISA responses were seen in the VLP-IM+IN (GMT: 317.04 ng/mL; 95% CI: 220-458 ng/mL) and IIV-IM/IM groups but differences between groups did not reached statistical significance.

5.5.3 Cellular Immune Response

5.5.3.1 $CD4^+$ T Cells

In contrast to the relatively weak and inconsistent antibody responses seen in the aged mice, HA-specific T cell responses were readily detected in most of the animals that had received VLP vaccination, regardless of route or schedule. Overall, 59 % of the VLP-vaccinated animals had polyfunctional T cell responses above the mean of the PBS animals (Supplemental Table 5.3). At day 42 post-vaccination, 0.05% of splenocyte $CD4^+$ T cells in the VLP-IM/IM and

VLP-IM/IN groups had a polyfunctional response to H1-VLP stimulation *ex vivo* that was roughly twice as large as that seen in the VLP-IM+IN (0.02%), IIV-IM/IM (0.02%) and PBS groups (0.03%) (Figure 5.3A). Considering single-positive $CD4^+$ T cells, H1 antigen-specific responses were most convincingly seen in the VLP-IM/IM and VLP-IM/IN groups (IL-2, TNFα and IFNγ Figure 5.3A) but the VLP-IM+IN group also mounted a strong IFNγ response compared to the IIV-IM/IM and PBS groups ($p<0.05$). Overall, the IIV-IM/IM group did not elicit any $CD4^+$ T cell response above the baseline levels seen in the PBS group.

5.5.3.2 $CD8^+$ T Cells

Antigen-specific $CD8^+$ T cell cytokine responses were more variable than $CD4^+$ responses but were still much more consistently observed in the VLP-vaccinated animals than antibody responses. Overall, polyfunctional $CD8^+$ T cell responses above mean PBS levels were found in 40 % of the VLP-vaccinated animals (Supplemental Table 5.3). Again, the VLP-IM/IN and VLP-IM/IM groups had the most convincing $CD8^+$ T cell responses; generally, for the VLP-IM/IN groups (polyfunctional and all individual cytokines) and for IL-2 and IFNγ in the VLP-IM/IM group (reaching significance for IFNγ versus PBS: $p<0.05$) (Figure 5.3B). Again, the IIV-IM/IM group mounted little-to-no $CD8^+$ T cell response above baseline levels except for IL-2 production (0.03% versus 0.001% in the PBS group) although this difference did not reach statistical significance.

5.5.4 Lung Viral Loads and Lung Cytokine/Chemokine Concentrations 3 days post-infection

The VLP-IM/IM and VLP-IM/IN vaccinated groups had similar lung viral titres (log_{10} $TCID_{50}$: 4.4±0.3 and 4.5±0.6 respectively) (Figure 5.4A). The viral load in the VLP-IM+IN group was slightly higher (4.7±0.3). The highest viral loads were seen in the PBS (4.9 ±0.6) and the IIV-IM/IM groups (5.4±0.3). When the three groups that received a VLP vaccine were combined (VLP-IM/IM, VLP-IM/IN and VLP-IM+IN) the lung viral load (4.6±0.4) was lower than both the IIV-IM/IM and PBS groups (5.4±0.3 and 4.9±0.6 respectively) but statistical significance was only reached for IIV-IM/IM ($p<0.05$). Cytokine and chemokine concentrations in lung homogenates at 3 dpi tended to be lower overall in the VLP-vaccinated animals (independent of route) than the IIV-IM/IM and PBS groups (Figure 5.5). These differences

reached statistical significance for MIP1-α between the IIV-IM/IM and all of the VLP groups ($p < 0.01$ - < 0.05) (Figure 5.5A) and for IL-17 between the VLP-IM/IM and IIV-IM/IM groups (112.73 pg/mL vs 230.93 pg/mL, respectively, $p<0.05$) (Figure 5.5B). With the exception of low IL-5 levels seen only in the PBS animals, the highest cytokine/chemokine levels tended to occur in the PBS and IIV-IM/IM groups, perhaps as a reflection of the higher lung viral loads.

5.5.5 Weight Loss

Two of the groups that that received VLP vaccines (VLP-IM/IM and VLP-IM/IN) lost the least amount of weight (-9.4±1.5% and -9.9±1.5% respectively) and recovered most rapidly, returning to near baseline weights by 18 dpi (Figure 5.4B). The PBS control lost the most weight (-16.1%) and remained well below their baseline weight at 18 dpi (-6.8± 2.0 %). The VLP-IM+IN and IIV-IM/IM groups were intermediate in both their maximum weight loss (-13.3±1.9 % and -12.7±1.4 % respectively) and the timing of weight recovery (still -3.1±2.4 % and -1.5 ± 1.1 % at 18 dpi respectively).

5.5.6 Clinical Frailty

Changes in frailty between the time of infection and 25 dpi (± 4 days) are shown in Figure 5.6. The overall frailty of these old animals is obvious in the fact that the frailty score increased in even the uninfected control group. The PBS group had the greatest increase in the frailty index (8.3±4.0 %) which was significantly higher that the change in frailty in all of the other vaccinated groups combined (4.3±0.6%; $p=0.08$). This observation demonstrated that vaccines can help protect against increases in frailty after infection and, to some extent at least, validates the aged mouse model for this purpose. The lowest increase in the frailty scores after infection were seen in groups that had received intranasal administration for one of the two doses: either IM+IN (3.00 %) or IM/IN (3.36%). Both of the groups that received only received intramuscular doses (ie: VLP-IM/IM and IIV-IM/IM) had greater increases in frailty indices (4.93% vs 5.28%, respectively) although these differences did not reach statistical significance. It is also relevant that the increases in frailty index scores do not consider animals that succumbed to the infection prior to the second frailty assessment at 25 dpi (ie: a potential survivor effect).

5.6 Discussion

The development of better influenza vaccines for the elderly is not only a major problem; it is also a rapidly growing problem as many of the industrial countries of the world continue their epidemiologic transition towards the 'older end' of the age spectrum (48). Immune responses to influenza vaccination in older subjects are subject to a wide range of influences including a life-time of exposures to wild-type viruses as well as vaccines (13, 49) and loss of immune competence due to thymic involution, CMV infection, chronic inflammation and other factors (ie: immunosenescence and/or inflamm-aging) (50-53). It should therefore not be surprising when vaccines that work reasonably well in children and healthy young adults fail to work in the vulnerable elderly population. This is particularly true since a great deal of effort has been expended to optimize the ability of standard IIVs to elicit antibodies, and specifically antibodies detected by the classical hemagglutination inhibition (HI) assay (41, 54), when it seems increasingly clear that the elderly are protected primarily by other immune mechanisms including T cell responses (31).

Several important tools are needed for the development new vaccines and new vaccination strategies that will afford better protection in older individuals. One of the most critical, is new vaccine candidates that can elicit a different pattern of immunity than that induced by IIVs. Among the many novel influenza vaccine candidates (55), the plant-derived VLP vaccines that were the focus of our current work have many attractive features: particularly their flexibility regarding route of administration (43), their efficient delivery to lymph nodes and APCs (25-28) and their ability to elicit a more balanced humoral and cellular response than IIVs in both animal models and human clinical trials in healthy adults (23, 24, 30, 43). Another important tool is an appropriate animal model (56). Although ferrets are widely viewed as the best animal model for influenza infection (57), they live for much longer and are much more expensive than mice, not to mention the limited availability of immunologic reagents (58) and the difficulty working with these animals. Indeed, we are aware of only a single study of influenza vaccination or infection conducted in aged ferrets (59). In contrast, aged mice have been used in influenza and influenza vaccine research for at least 40 years due to their relatively short life-spans, their immunologic tractability and their relatively low cost. However, their limitations as models for human elderly should be acknowledged. For example, even very old

mice are typically influenza naïve when they are used in studies rather than having had a life-time of varied exposures to different influenza strains and vaccines (60). Of course, as with any complex human disease, immune responses in mice are not always fully predictive of response in humans (61). Nonetheless, the loss of antibody response despite strong antigen-specific cellular reactivity that we observed in our aged animals following VLP vaccination (Figures 5.2 and 5.3) is certainly consistent with Medicago's on-going studies of the Quadrivalent influenza VLP vaccine in older subjects (unpublished data). Knowing what to study in aged mice is also critically important. As recently pointed out by Miller et al, we must first 'know ourselves' (ie: have a better understanding of the immune correlates of protection) in order to know what kind of immune response we want following vaccination (62). Continuing this line of reasoning for a moment, it is also important that we know what outcomes to assess. Historically, almost all influenza vaccination studies in aged mice have focused on immunologic parameters (usually just antibody responses as discussed above) and survival. It is very likely that this relatively narrow focus has hampered the development of novel vaccines and vaccination strategies. The recent description of protocols to assess frailty in aged rodent models (63) is a major advance for influenza vaccine studies. Although complex and time-consuming, the inclusion of this assessment in our current work permitted us to recognize subtle differences between vaccines and vaccination schedules that may be highly relevant to protecting the elderly population.

Although these findings are best considered preliminary, our data showed that animals in the groups that had received one of the two doses IN (VLP-IM/IN and VLP-IM+IN) appeared to do better in terms of frailty than the animals that had received either IIV or VLP only by the IM route (Figure 5.6). However, a greater proportion of the animals in the VLP-IM+IN group succumbed to infection (37.5% versus 17.3% in all of the other groups) raising the possibility of either a 'survivor effect' confounding the frailty data or, more concerning, a risk of vaccine-enhanced disease in some animals (64). Although histopathologic studies to assess cellular infiltrates in the lungs were not included in our study and the viral loads at 3 dpi were similar in the two groups, it is interesting that the lung homogenate cytokine/chemokine profiles were strikingly different between the VLP-IM+IN and VLP-IM/IN animals (Figure 5.5). Despite considerable mouse-to-mouse variability, the VLP-IM+IN animals had much higher levels of several pro-inflammatory chemokines/cytokines (IL-Iβ, IL-6, MIP1-α, IFNγ) than the VLP-IM/IN group 3 dpi. Since very little is known about IN dosing of VLP vaccines, a small follow-

up study was performed to assess a higher dose of VLPs using the VLP-IM+IN schedule (3μg/route at each time-point instead of 1.5μg). Summary data comparing these two doses (Supplemental Figure 5.3) strongly suggest that the 1.5μg dose used in this study was likely sub-optimal for IN delivery.

Overall, the conventional IIV-IM/IM strategy was inferior to the two 3μg VLP strategies (VLP-IM/IM and VLP-IM/IN) for most of the assays except for survival which was between 75-87% for all groups except for VLP-IM+IN. It is interesting that increasing the dose in the VLP-IM+IN strategy to 3μg/route not only changed the cytokine/chemokine profile and significantly reduced viral titres at 3 dpi ($p<0.01$), but it also increased survival (84.2%) and dramatically increased lung-resident T cell responses (Suppl Figure 5.3). Although lung-resident T cells were barely detectable in other groups, the VLP-IM+IN (6μg) animals had a significantly higher number of lung tissue-resident memory (TRM), antigen-specific $CD4^+$ T cells ($p<0.01$) and a significantly higher proportion (%) of antigen-specific $CD8^+$ T cells expressing IFNγ (Suppl Figure 5.3). TRM $CD8^+$ T cells may be very important in viral clearance (38, 65, 66) and TRM $CD4^+$ are also thought to be important for influenza protection (67, 68). Indeed, TRM $CD4^+$ have been demonstrated to lead to protection from morbidity and mortality in murine models of influenza infection (39, 69). Moreover, $CD4^+$ T cell epitopes are conserved within different subtypes of influenza virus (70) and $CD4^+$ TRM are thought to be important for optimal protection against reinfection (39, 69). Pre-existing, antigen-specific, peripheral blood $CD4^+$ T cells have also been shown to be a good correlate of immunity in human challenge studies (19). Together, these data strongly support the concept of multi-modality vaccination even if the dose chosen for our primary study was sub-optimal.

In conclusion, we exploited the flexibility of the VLP format to compare the standard vaccine/standard route (IIV-IM/IM) to the novel vaccine/standard route (VLP-IM/IM) in our aged mouse model. The novel VLP vaccine was also used in multi-modality (VLP-IM+IN) and prime-pull (VLP-IM/IN) strategies. Each of these approaches was assessed using both conventional methods (e.g: viral loads, survival curves, classic serologies) as well as less common methods including splenocyte and tissue-resident memory T cell responses, lung cytokine/chemokine profiles and frailty to identify potential advantages for novel approaches to vaccination in the elderly. Our findings strongly support the further exploration of these

alternative vaccination strategies in older subjects as soon as a vaccine that can be used in this way is licensed.

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5.9 Figures and Legends

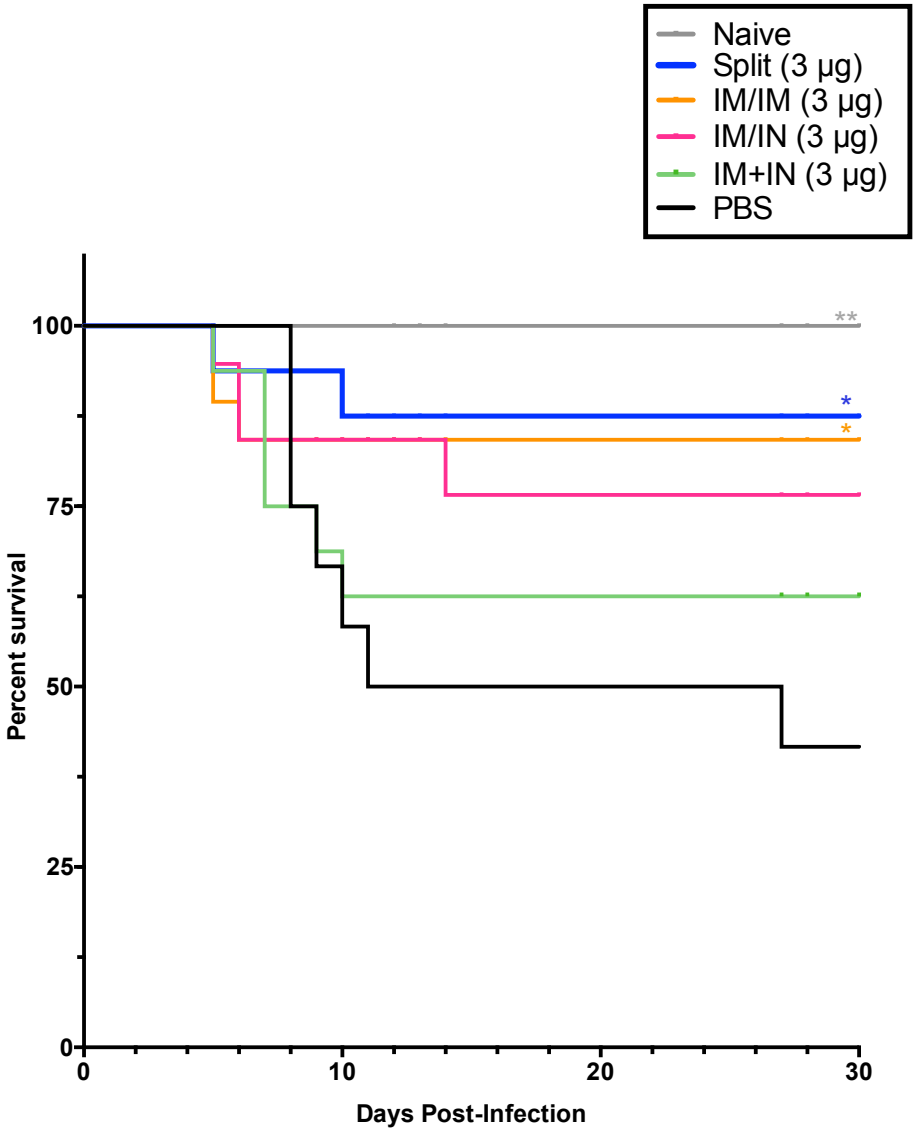


Figure 5.1 Survival after A/California/07/2009 H1N1 challenge

Aged BALB/c female mice (18-22 months of age) were immunized twice with H1-VLP vaccine or inactivated split vaccine. Six weeks after vaccination, mice were challenged with a sub-lethal dose of A/California/07/2009 H1N1 and were closely monitored for weight loss. Mice were euthanized if they lost >20% of their initial weight A log-rank (Mantel-Cox) test was used to compare survival curves with the PBS control group (** $p<0.01$, * $p<0.05$). This graph representative of 5-10 mice/group from two separate studies.

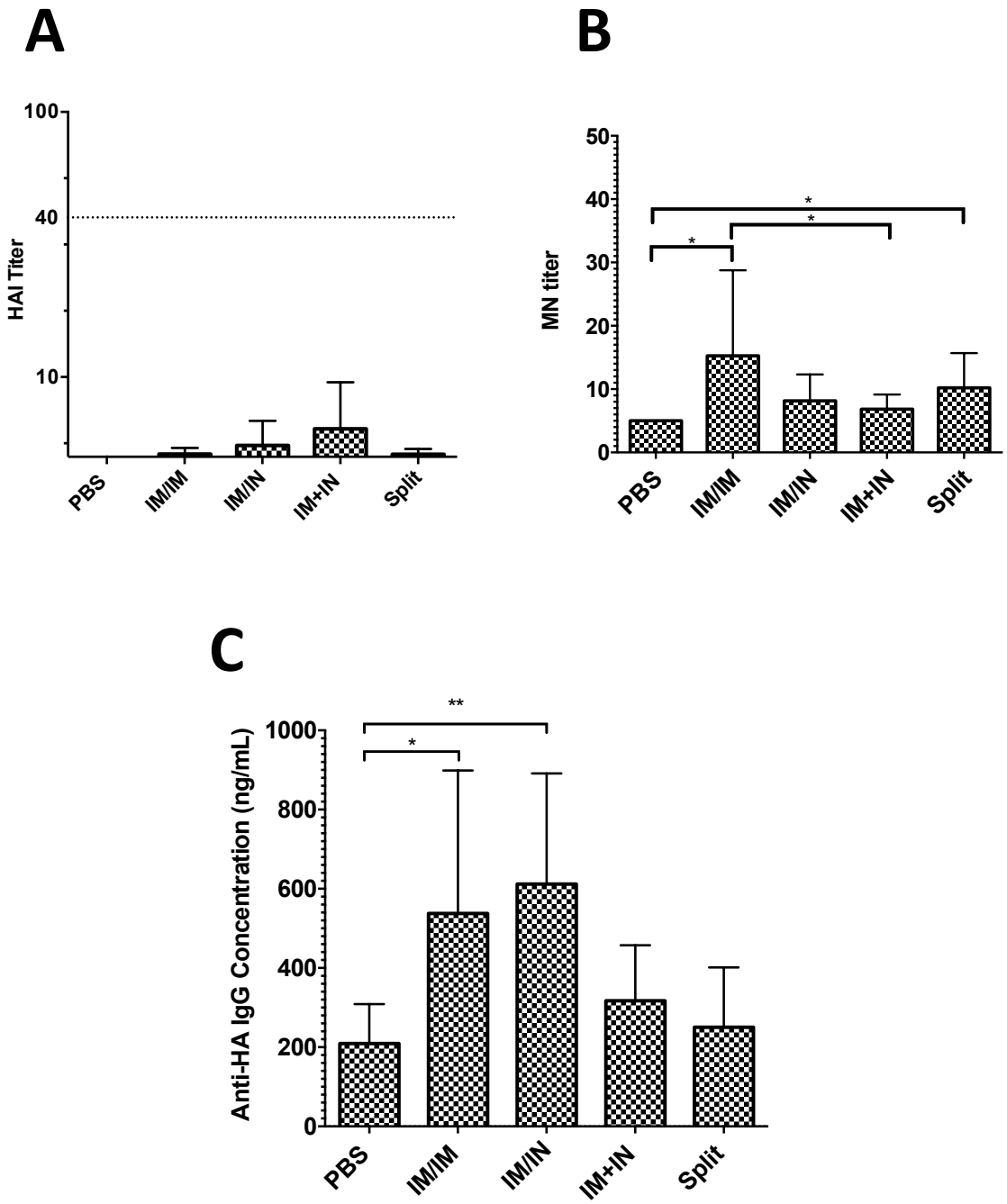


Figure 5.2 Antibody responses after two vaccinations towards H1N1 A/California/07/2009
Aged BALB/c female mice (18-22 months of age) were immunized twice with H1-VLP or inactivated split vaccine. Six weeks post-vaccination, the humoral response to the H1 of A/California/07/2009 H1N1 was analyzed in sera from individual mice by hemagglutination

inhibition assay (HAI: 8-10 animals/group) (A), microneutralization (MN: 6-8 animals/group) (B) and ELISA (C: 8-10 animals/group). The dotted line in A) represents an HAI titre of 1:40, which is considered protective in humans. Error bars indicate 95% CI. For statistical analysis, one-way ANOVA was used on log transformed values (*** $p<0.001$, ** $p<0.01$ * $p<0.05$). These data represent 2 independent studies.

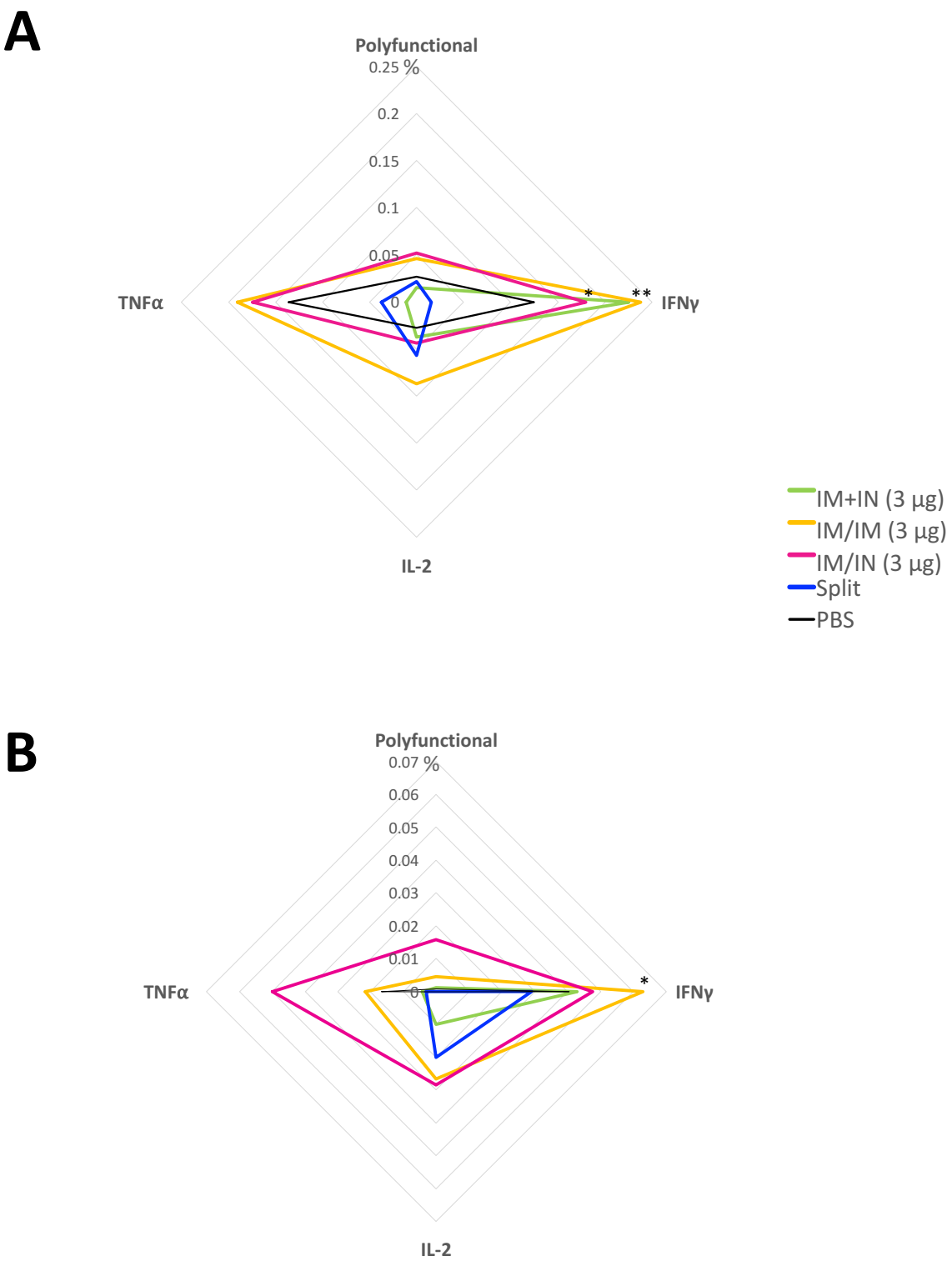


Figure 5.3 Splenocyte T cells expressing cytokines in response to H1 re-stimulation *ex-vivo*

Splenocytes were collected 3 weeks post-boost from aged female BALB/c mice (18-22 months of age). Percent of splenocytes A) $CD4^+$ T cells and B) $CD8^+$ T cells expressing 2 or more cytokines (polyfunctional) or single cytokines (IFNγ, IL-2 or TNFα)**.** For statistical analysis, two-way ANOVA was performed followed by Tukey's multiple comparison test (**** $p<0.0001$, *** $p<0.001$, * $p<0.05$) compared to the split vaccine. A) and B) are representative data from 6-8 mice/group from two studies.

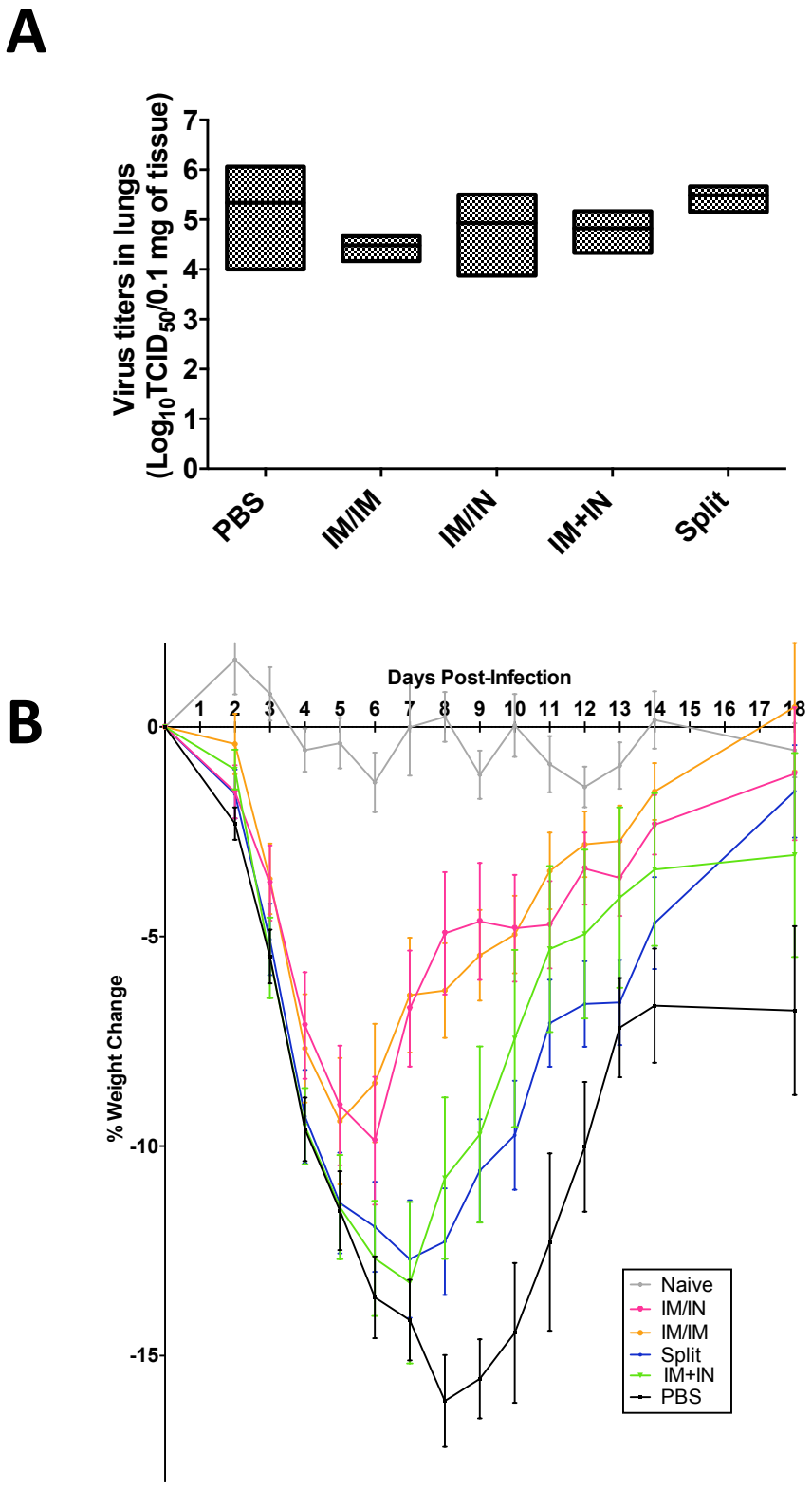


Figure 5.4 Lung viral loads and weight loss after challenge

Aged BALB/c mice (18-22 months) lungs were collected at 3 days post-infection after sub-lethal challenge with H1N1 A/California/07/2009. Three days post-infection A) lung viral loads were measured and throughout the infection mice were closely monitored for B) weight loss. A) is representative of 3-5 mice/group from two studies and B) are representative of 5-10 mice/group combined from two studies. For statistical analysis, A) One-way ANOVA was performed on the log10 values of the viral titres and the Tukey's multiple comparison test was performed. For B) Two-way ANOVA followed by the Tukey's multiple comparison test (**** $p<0.0001$, *** p 0.001, ** $p<0.01$, * $p<0.05$) (see supplemental Table 5.2). Error bars represent the standard error of the mean.

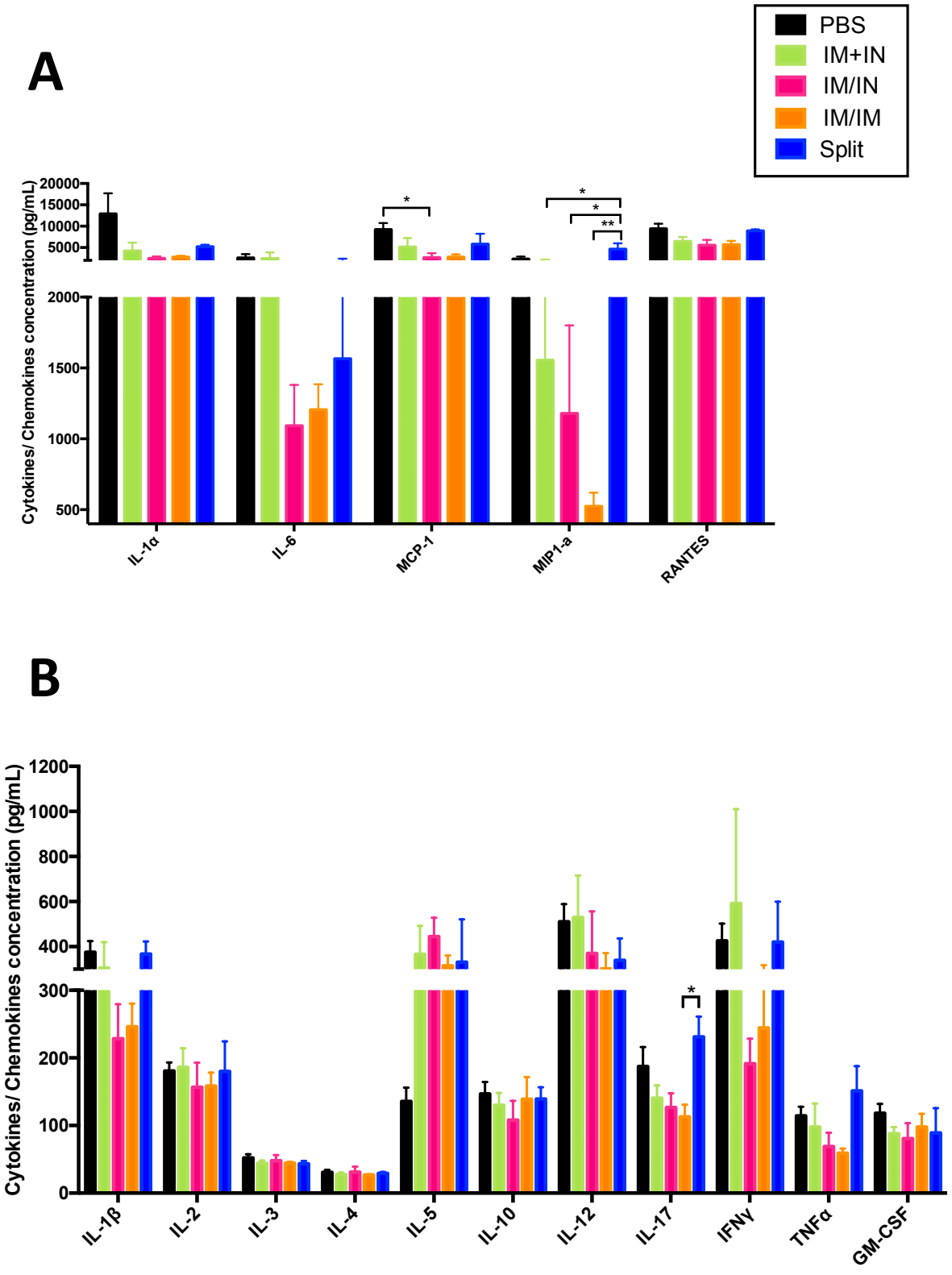


Figure 5.5 Cytokine and chemokine levels in lung homogenates 3 days after challenge

Six weeks post-vaccination, female BALB/c mice (18-22 months) were challenged with A/California/07/2009 H1N1 and lungs were collected and homogenized 3 days post-infection to measure cytokines/chemokines by multiplex ELISA for the following cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, MCP-I, IFNγ, TNFα, MIP-1α, GM-CSF and RANTES. For statistical analysis, one-way ANOVA followed by Tukey's multiple comparison test (**$p<0.01$, * $p<0.05$). Data represent 4-7 mice/group from one study. Error bars represent the standard error of the mean.

	PBS	IM+IN	IM/IN	IM/IM	Split	Naïve
	0.00	0.00	0.00	0.00	0.00	0.00
	2.00	0.00	0.00	0.00	0.00	0.00
	4.00	0.00	0.00	0.00	0.00	0.00
	6.00	1.00	0.00	0.00	0.00	3.00
	11.00	3.00	0.00	3.00	1.00	3.00
	27.00	4.00	1.00	3.00	3.00	5.00
		4.00	2.00	3.00	3.00	7.00
		5.00	2.00	3.00	3.00	12.00
		5.00	4.00	4.00	4.00	14.00
		8.00	4.00	9.00	6.00	
			5.00	9.00	7.00	
			6.00	11.00	7.00	
			10.00	12.00	8.00	
			13.00	12.00	15.00	
				14.00	17.00	
Average Change in Frailty Index (%)	8.33	3.00	3.36	5.53	4.93	4.89
Percent of Scores > 3%	66.67	50.00	42.86	46.67	46.67	44.44

Figure 5.6 Changes in clinical frailty index after challenge

At day 0 (post-vaccination but pre-challenge) and day 25 post-infection, clinical frailty indices were measured. The percent difference was calculated for each mouse that survived (25 dpi-0 dpi*100). A proportion of those with ≥ 3% change in each group was also calculated. Blank (grey) spaces represent mice that did not survive to 25 dpi. For statistical analysis, one-way ANOVA followed by Tukey's multiple comparison test was used to compared to groups. Data is representative of 6-8 mice/group from 2 studies.

5.10 Supplemental Figures, Legends and Tables

Supplemental Table 5.1 Frailty Index measurements for day 0 and day 25 post-infection

Date:_____ Randomized Cage #: _____ Actual Cage #: _____

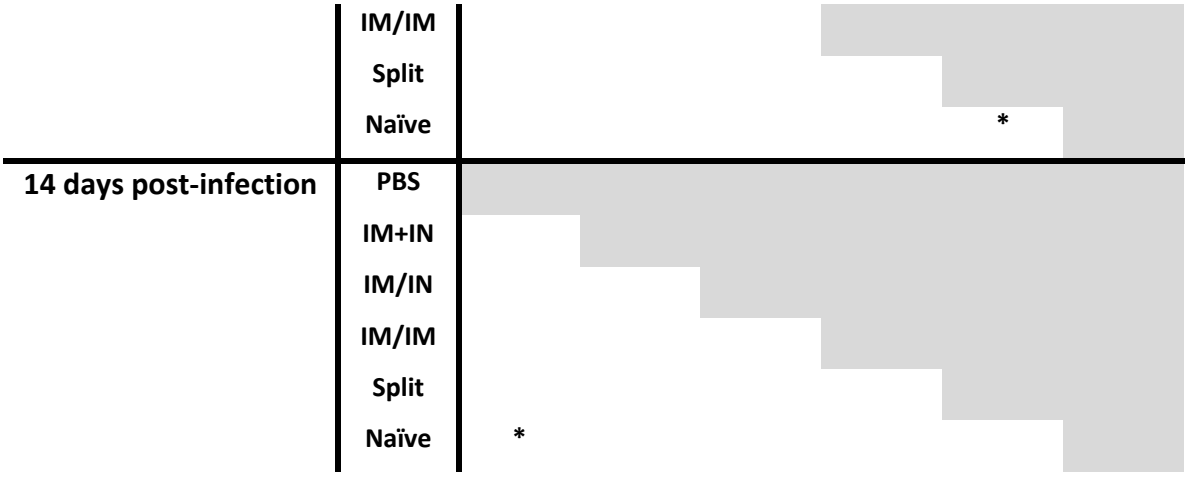
Mouse	1			2			3			4			5		
Body Temp (C)															
Temp score															
Body Weight (g)															
Body weight score															
1) Integument															
Alopecia	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Dermatitis	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Loss of Whiskers	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Coat Condition	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
2) Physical/Musculoskeletal															
Tumours	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Distended abdomen	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Kyphosis	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Tail Stiffening	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Gait disorders	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Tremor	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Forelimb grip strength	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Body Condition score	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
3)Vestibulocochlear/Auditory															
Vestibular disturbance	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Hearing Loss	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
4) Ocular/Nasal															
Cataracts	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Eye discharge/swelling	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Microphthalmia	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Corneal opacity	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Vision loss	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Menace reflex	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Nasal discharge	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
5) Digestive/Urogenital															
Malocclusions	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Rectal Prolapse	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Vaginal/uterine prolapse	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Diarrhoea	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
6) Respiratory system															
Breathing rate/depth	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
7) Comfort															
Mouse Grimace Scale	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Piloerection	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Total Score/Max Score:	/29			/29			/29			/29			/29		
Notes:															

Rating:0=absent, 0.5=mild, 1=severe Clinical Frailty Score Post-Vac.

Supplemental Table 5.2 Statistics comparing groups for weight loss, using Tukey's multiple comparison test

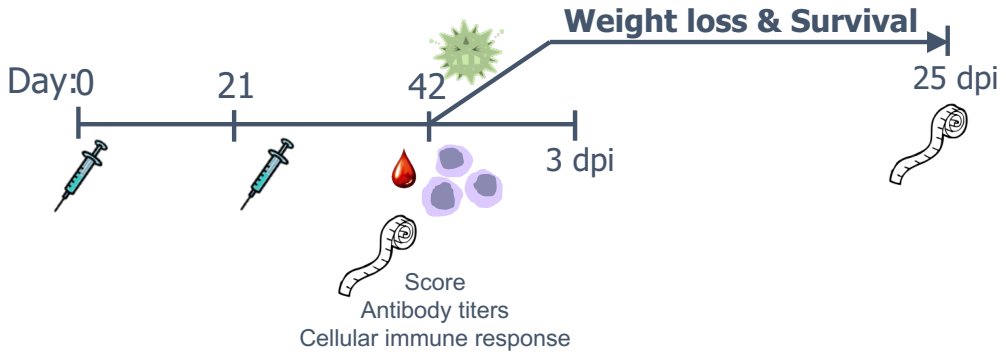
		PBS	IM+IN	IM/IN	IM/IM	Split	Naïve
3 days post-infection	**PBS**						
	IM+IN						
	IM/IN						
	IM/IM						
	Split						
	Naïve	**	**			**	
4 days post-infection	**PBS**						
	IM+IN						
	IM/IN						
	IM/IM						
	Split						
	Naïve	****	****	**	***	****	
5 days post-infection	**PBS**						
	IM+IN						
	IM/IN						
	IM/IM						
	Split						
	Naïve	****	****	****	****	****	
6 days post-infection	**PBS**						
	IM+IN						
	IM/IN						
	IM/IM	*					
	Split						
	Naïve	****	****	****	***	****	
7 days post-infection	**PBS**						
	IM+IN						
	IM/IN	****	***				
	IM/IM	****	****				
	Split			**	***		
	Naïve	****	****	**	**	****	
	PBS						

8 days post-infection	**IM+IN**	*					
	IM/IN	****	**				
	IM/IM	****					
	Split			****	***		
	Naïve	****	****	*	**	****	
9 days post-infection	**PBS**						
	IM+IN	*					
	IM/IN	****	*				
	IM/IM	****					
	Split			**	**		
	Naïve	****	****	****	***	****	
10 days post-infection	**PBS**						
	IM+IN	**					
	IM/IN	****					
	IM/IM	****					
	Split			*	*		
	Naïve	****	***		*	****	
11 days post-infection	**PBS**						
	IM+IN	*					
	IM/IN	**					
	IM/IM	****					
	Split						
	Naïve	****				**	
12 days post-infection	**PBS**						
	IM+IN						
	IM/IN	*					
	IM/IM	**					
	Split						
	Naïve	**				*	
13 days post-infection	**PBS**						
	IM+IN						
	IM/IN						

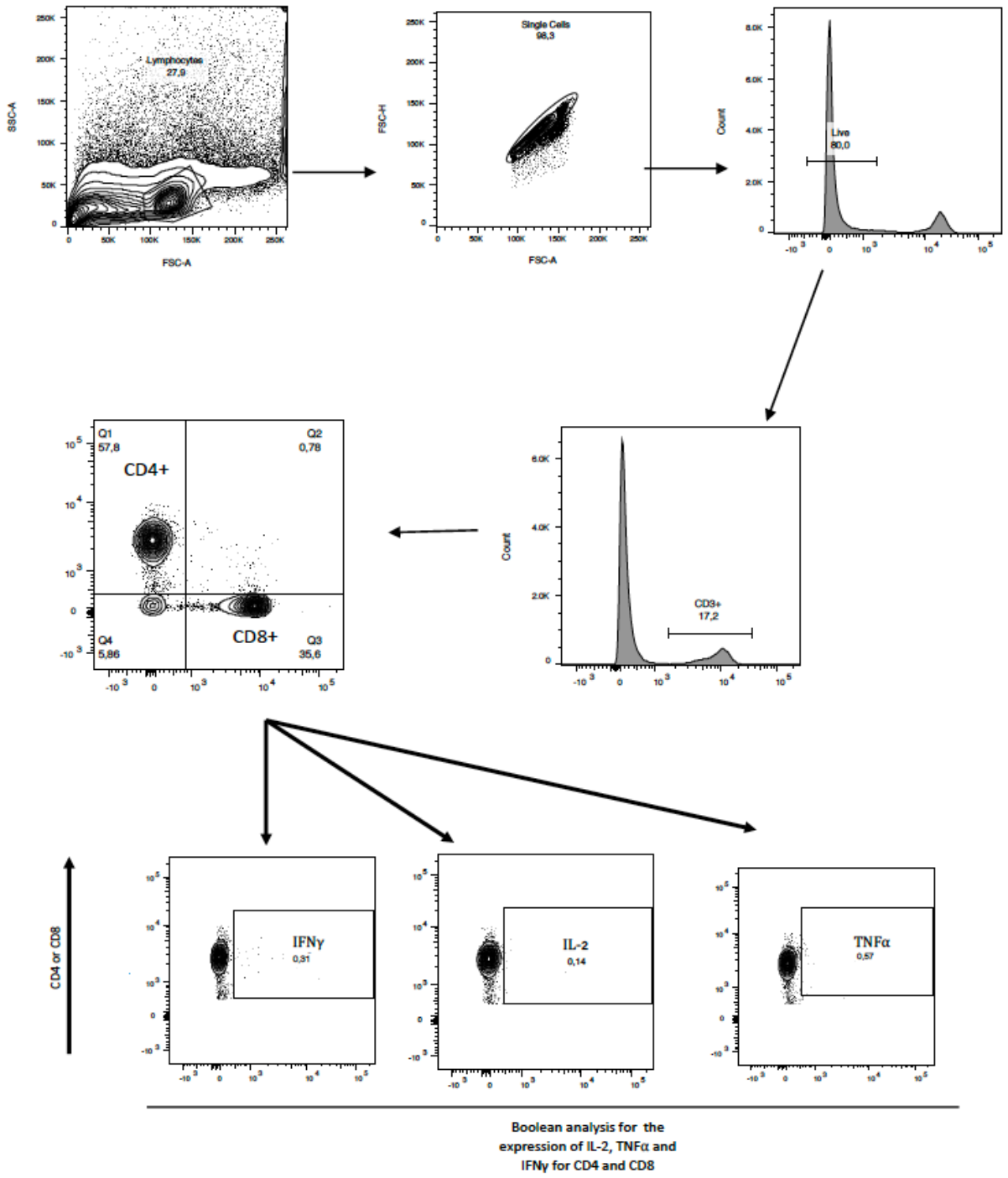


Supplemental Table 5.3 Percent of mice above PBS average in VLP-vaccinated animals

	$CD4^+$ T cells (%)	**$CD8^+$ T cells (%)**
Polyfunctional	59	40
IFNγ	53	35
IL-2	64	65
TNFα	30	38
Sum of Total (expressing 1 or more cytokines)	69	47

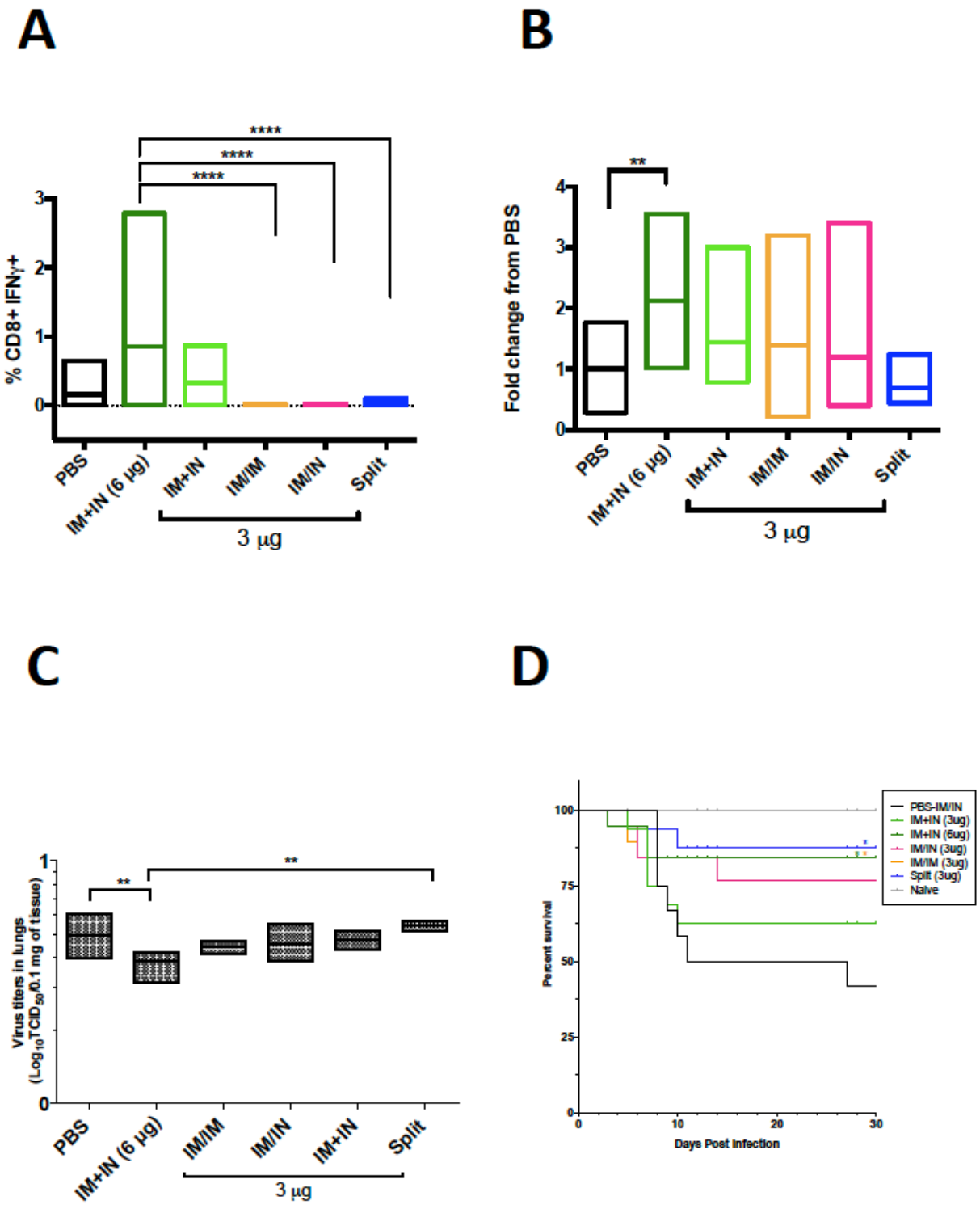


Supplemental Figure 5.1 Timeline for vaccine administration



Supplemental Figure 5.2 Flow cytometry-gating strategy for splenocytes

Aged (18-22 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or naïve. Three weeks post-boost (6-24 mice/group), splenocytes were collected and stimulated *ex vivo* for 18 hours with H1-VLP.



Supplemental Figure 5.3 Lung immune response 42 days after vaccination, viral load 3 days post-infection and survival curve after infection with A/California/07/2009

Aged (18-22 months) BALB/c mice were immunized twice with H1-VLP, split-virion vaccine or PBS. Lungs were collected six weeks after vaccination. Percent of lung A) $CD8^+$ T cells and expressing IFNγ presented as background subtracted (stimulated - unstimulated). Fold-change of tissue-resident B) $CD4^+$ T cells from the PBS group. For statistical analysis, two-way ANOVA was performed followed by Tukey's multiple comparison test (** $p<0.01$, * $p<0.05$). Six weeks after vaccination, mice were challenged with a sub-lethal dose of A/California/07/2009 H1N1 and were closely monitored for weight loss. Three days post-infection C) lung viral loads were measured. D) Survival curve: mice were euthanized if they lost >20% of their initial weight. A log-rank (Mantel-Cox) test was used to compare survival curves with the PBS control group. (** $p<0.01$, * $p<0.05$ compared to naïve group). Data are representative 5-10 mice/group from 2 studies. Error bars represent the standard error of the mean.

Chapter 6: General Discussion

The elderly are one of the most vulnerable populations that suffer from severe and complicated influenza. This infection often leads to hospitalization that can ultimately lead to significant losses in daily activity and autonomy. Infection can cause catastrophic disability eventually leading to long-term care placement; placing a huge burden on the healthcare system. The best way to prevent influenza infection is through vaccination. However, older individuals and particularly older individuals with co-morbidities tend to respond poorly to vaccines in general and to influenza vaccines in particular. Although there are currently two vaccines that target the elderly, they only increase vaccine effectiveness slightly and their cost is significant (1, 2). Sterilizing immunity does not appear work for this population, therefore new strategies should focus on increasing viral clearance and decreasing the severity of symptoms to prevent hospitalization. We have chosen to focus our work on a plant-derived virus-like-particle (VLPs) vaccine for the elderly. The goal of the current work was to develop an aged mouse model of influenza infection and to assess alternate vaccination strategies made possible by this novel vaccine to better protect the elderly from influenza.

6.1 Main findings

Any new influenza vaccine candidate is first tested in an animal model, typically in mice. These mice are usually approximately 8-12 weeks old (3). However, vaccines in general and even vaccines geared towards the elderly are rarely tested in aged animal models. Therefore, we established an aged mouse model and compared their immune responses to younger mice. Not surprisingly, the older mice had a much lower immune response to both the classical ('split' virion or IIV) and the novel VLP influenza vaccines and were not protected as well as their younger counterparts. Interestingly, the young mice were completely protected with either the H1N1 IIV vaccine or the H1-VLP whereas, in the older mice, the H1-VLP was clearly superior. This work was published in Clinical Vaccine Immunology and is detailed in Chapter 2 of this thesis.

While developing our aged mouse model (16-20 months of age), we discovered that, although the ages of the old mice were similar between experiments, there was a great deal of

variability in the susceptibility to infection and death. While trying to establish a mouse lethal dose 50 (LD50) for one of these experiments, it was clear that some of the older mice would not succumb to the infection. We retrieved stored sera from that group of aged mice and found that approximately 8% of them had pre-existing antibody titres towards the targeted H1 protein by ELISA. This work has been written as a brief report that was submitted and is presented in Chapter 3.

Building on the aged mouse model, we wanted to further investigate the effects of co-morbidities on the immune response to influenza vaccinations in very old mice (>22 months of age). To complete this work, we developed a protocol for quantifying co-morbidities using whole-body CT^2 scans and a structured autopsy. We found that VLPs were superior to the split vaccine even in these very old mice. We did not find any clear correlations with immune responses and co-morbidities, however those with fewer co-morbidities tended to have higher immune responses to the vaccine. This work is described in Chapter 4 as a manuscript and was submitted to PLOS One.

Last, we wanted to investigate novel vaccine strategies including alternate administration routes and schedules to obtain better immune responses and protection from infection in older mice. For protection, we not only assessed survival and weight loss, we also measured clinical frailty indices to assess the effects of infection on the overall well-being of the mice. We found that novel administration methods such as prime-pull vaccination (intramuscular boosted by intranasal) seemed to elicit a more balanced humoral and cellular responses as well as better protection from frailty after infection. This work has been written as a major manuscript and is presented in Chapter 5.

6.2 Future perspectives

6.2.1 Virus-like particles

In most of my studies, both cellular and humoral immune responses to the VLPs were assessed but initial work focused mostly on splenocyte responses. In later work, we found interesting T cell populations in the lungs of animals that had received simultaneous administration (IM and IN). In future studies, it would be worthwhile to expand the evaluation of 'local' (ie: lung) responses to further assess both innate and adaptive responses after alternate vaccination strategies. Collecting draining lymph nodes and lungs a few hours or days after vaccination could greatly improve our understanding of the type of immune response being induced.

6.2.1.1 Plant-derived VLPs vs other VLP systems

There are several other VLP expression systems in which influenza vaccine candidates are produced. An interesting future experiment, would be to compare the plant-derived VLPs with VLPs produced in both mammalian and insect cell culture systems. This comparison will not only help determine if the plant-based system has an advantage over the other systems, but may help further characterize the immune response to the VLPs. VLPs vary not only in the platform used to produce them but also in their antigen content (ie: HA alone versus HA+NA versus HA+M1) (4-6). Some VLPs have the potential to direct the immune response to more conserved regions of internal proteins of the virus such as the matrix 1/2 proteins or the nucleoprotein while others direct responses primarily to the major surface molecules such as HA or NA. Nonetheless, all of these targets seem to be able to induce protection in mouse models at least (5, 7, 8). It would be interesting to perform head-to-head comparisons of the immune responses, level of protection and with frailty changes between the different VLP systems. The insect cell line (s9f) used for VLP production is heavily contaminated with baculovirus components which have been shown activate the innate immune response (9). Currently, our lab is investigating if plant-derived VLPs have any adjuvant properties which may explain their ability to stimulate such a robust immune response.

6.2.1.2 Tracking of the VLPs

Our laboratory has previously produced enhanced green fluorescent protein (eGFP) H5-VLPs for imaging experiments by generating VLPs with a fusion protein (eGFP:H5). Using this reagent, the VLPs could be detected in the draining lymph node within 10 minutes after footpad injection (10). Unfortunately, this reagent proved to be very difficult to produce reliably in plants. Presently, the laboratory is working on producing another fluorescently-labeled VLP based on a more stable and structurally-compact fluorophore: mCherry. In theory, these new fluorescently-labeled VLPs could be used to track the VLPs *in vivo* after both intranasal and intramuscular administration. At our research facility, we have access to a sophisticated small animal imaging facility that has recently acquired an In-Vivo Xtreme imaging system. This type of imaging allows *in vivo* tracking with fluorescent dyes and bioluminescence and includes X-ray imaging (11). This imaging system should allow us to track the VLPs in a single mouse from several hours to several days. We have recently completed a pilot study with DiD-labeled VLPs with Xtreme in-vivo imaging but some optimization is still required. This new tracking capacity should help to identify the anatomical site(s) where the VLPs are located (e.g: muscle, mucosal surface, draining lymph nodes) and their persistence. Determining the movement of the VLPs in the various tissues will help to design future studies: ie: to decide which organs should be collected to further characterize the immune response elicited by these novel vaccines.

6.2.1.3 Different Routes of Administration

Many different VLPs have demonstrated the flexibility to be administered by different routes of administration (e.g: IM, IN, SC and ID) (12). Because influenza is a 'standard' vaccine model, many of these have also demonstrated an enhanced capacity to induce an immune response for influenza virus antigens. Interestingly, the concentration of antigen in some of these VLP studies has varied from 0.15-40 μg of protein per vaccination for intranasal administration (12-14). In our studies, the maximum IN dose we used was only 3 μg suggesting that one way to help improve immunogenicity of the plant-derived VLPs in aged mice would be to increase the dose: possibly up to 15 μg of HA. Although our data are quite limited, there is certainly a suggestion that the higher dose could elicit a stronger response (Chapter 5– Supplemental Figure 5.3).

6.2.1.4 Mucosal Adjuvants

Some mucosal vaccines are highly effective and this route of administration has obvious potential advantages to prevent mucosally-transmitted infections. Currently there are no adjuvants licensed for mucosal vaccines. However, as with other aspect of the immune response, ageing of the mucosal tissues leads to less robust mucosal immunity compared to young animals (15). Mucosal vaccines have the potential to induce HA-specific secretory IgA (SIgA) which plays a key role in protection and helps with cross-protection (16). Hence, the addition of a mucosal adjuvant may help increase the immune response we detect in lungs with the VLPs after IN administration. We did not see very high levels of IgA after two IN vaccinations with the H1-VLP vaccine or even 3 days post-infection in the aged mice. One of the most promising mucosal adjuvant candidates that might help overcome mucosal ageing is based on a combination of a plasmid encoding Flt3 ligand cDNA (pFL) and CpG ODN (17). This combination was shown to induce a balanced Th1 and Th2 responses in mice when administered with OVA. The CpG ODN activated pDC (presumably by TLR9) leading to a Th1 response whereas the pFL preferentially expanded DC-mediated Th2-type cytokines and antigen-specific SIgA antibody responses (17). Increasing the influenza-specific IgA response in the lung mucosa might be especially useful for the elderly, especially those in long-term care facilities by both decreasing illness in the vaccinated individual and limiting transmission. Outbreaks of influenza at these facilities can have devastating consequences for individuals and can place extra strain on health services (18).

6.2.2 Assessment of Cross-protection

6.2.2.1 Universal VLP vaccines

Although we see promising results with HA-VLPs in mice, ferrets and humans (19-23) and for the elderly (unpublished data), focusing the immune response on HA alone may not be ideal, especially for the induction of a cellular response and cross-protection (24, 25). Medicago's plant-derived HA-based VLPs have shown some cross-reactivity between strains (19, 20) – possibly by eliciting antibodies against the HA stalk domain - but targeting more conserved epitopes on other viral proteins may offer greater cross-protection and lead to long-

lasting memory. $CD8^+$ T cells can be long-lasting and cross-protective if they are directed towards more conservative regions of internal proteins of the influenza virus (e.g: matrix proteins, nucleoprotein) (26-29). Whether or not Medicago will pursue a version 2.0 of their VLP vaccine by adding additional proteins or epitopes is unknown at the current time.

6.2.2.2 Heterologous Protection

We demonstrated protective immune responses for the H1-VLPs in aged mice, with the best strategy incorporating both routes of administration (IM and IN). This type of immunization produced antibodies along with a strong T cell immune response similar to IM/IM. However, we did observe that mice that received an IN dose had smaller increase in frailty after infection. The strong T cell responses observed in these groups suggest we should further investigate the ability for cross-protection in this aged mouse model. Plant-derived VLPs should be able to confer heterologous protection as shown with other VLPs (14, 30). Medicago's plant-derived VLPs have been demonstrated to elicit a cross-reactive response for both antibody and $CD4^+$ T cells (19, 20). Therefore, it is likely that they will also elicit heterologous protection.

6.2.3 Effects of Frailty on Immune Responses

To assess influenza vaccine efficacy in animal models, it has been 'traditional' to assess only the following key assays: antibody responses towards the HA component (HAI, MN and ELISAs), survival and viral loads. However, older mice are similar to older humans in many ways and react differently from their younger counterparts. Complete or 'sterile' protection may not be achievable in older mice and avoiding disability is a major goal. This difference is one of the main reasons we started to incorporate frailty measurements into our aged mouse studies. To our knowledge, we are the first group to incorporate such measurements into influenza vaccination/challenge studies in aged mice. In these studies, we measured frailty at day 0, day 42 (post-vaccination) as well as 25 days post-infection (day 67) in surviving animals to measure both the impact of frailty on the immune response (day 0-42) and to assess protection from frailty after infection (day 67). Future studies could incorporate the measurement of other markers of immunosenescence/inflammaging such as TNFα, IL-6, c-reactive protein, etc (31, 32). Measuring these markers may further clarify correlations between frailty and immune

responses to vaccines as well as the role of these vaccines in protection against frailty after infection.

6.2.4 Pre-existing Antibody Titres

Mice are a relatively good model for humans for influenza vaccines, when cost, lifespan and available reagents are considered (33-35). However, unlike most human adults, mice are usually influenza-naïve. Another caveat when working in an animal model is that most viral strains are species specific, therefore most humans virus strains need to be adapted before infection (35). Surprisingly, we discovered that some of these aged mice do have pre-existing antibodies towards HA. These results are extensively discussed in Chapter 3 of this thesis. To reduce variability in our studies, we decided to exclude the mice that were considered to be influenza H1 seropositive. In retrospect, it would have been quite informative to specifically include these seropositive mice and to analyze them separately. These studies might have given insight into how older humans with prior influenza virus exposures would respond. Since all humans, especially the elderly have had multiple exposures to several different influenza viruses. Although we later demonstrated that these titres did not seem to have any biological relevance.

6.2.5 Ferret Model

Ferrets are a more representative model for humans than mice. The sialic binding pattern in both humans and ferrets are similar (36, 37). Ferrets have similar lung pathology to humans after influenza infection and they are also able to transmit from ferret to ferret unlike mice (38). However, an ‘old’ ferret costs significantly more than an aged mouse due to their initial price, housing requirements and their increased lifespan (2.5 years vs 5-6 years) (34, 39, 40). Nonetheless, the development of an aged ferret model in our laboratory (which is on-going) with the addition of clinical frailty assessment for ferrets (which has never been done) might help to further define the potential benefits of the plant-derived VLPs for older humans in general and the possible additional benefits of multimodality administration of VLPs in particular. Realistically, developing a frailty model for ferrets may not be practical due to the high cost of these animals. However, further development the mouse frailty model for other mouse strains may be useful not only for influenza research but also for other infections that can be detrimental

for the elderly (e.g: respiratory syncytial virus, *pneumococcal*, *clostridium difficile*, etc.) (41-43). Although the assessment of frailty in aged ferret may be unrealistic, testing simultaneous routes of VLP administration should still be performed in old ferrets since this model is more likely to predict what will happen in older humans.

6.3 Concluding Remarks

In this thesis, we describe the development and exploitation of an aged mouse model for influenza vaccination and challenge. Our work strongly suggests that, when developing vaccines for the elderly, it is important to use a relevant model. Furthermore, we have shown that, like humans, both death and frailty increase after influenza infection in the unvaccinated. We have also shown that novel vaccination strategies (simultaneous administration of IM and IN vaccine) may better protect older animals (viral load, inflammation, weight loss, frailty changes) through the induction of a balanced cellular and humoral immune response. Together, these findings give new insight into influenza-specific immune responses to vaccines in the elderly and may ultimately lead to better vaccination strategies for this vulnerable population.

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