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

Running title: VLP influenza vaccine protects aged mice

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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.

Keywords: aged mouse model; influenza; Virus-like-particles (VLP); plant-made vaccines
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 15 µg 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 60 µg 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.

Materials and Methods

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 28G½ 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 CO2 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 of wild-type A/California/07/2009 H1N1 virus (1800 TCID50 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% [TCID50], 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.

**Antibody titer 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 titer was determined by visual inspection as the highest dilution that inhibited erythrocyte agglutination using standard criteria.

Microneutralization (MN) titers 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%CO₂.

Cytopathic effect (CPE) was assessed at 4 days and the titer 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 H₂SO₄ 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.
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).

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% CO₂, plates were spun down (300xg, 10 mins at RT) and supernatant was removed. Cells were pulsed with 1 µCi/well H³-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 (Tomtec Inc., Hamden, CT) and H³-thymidine incorporation was measured by scintillation counter (Wallac Microbeta Trilux 1450 beta-counter; Wallac, 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).
**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 titers 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.

**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.

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.

RESULTS

Antibody response

Overall, the standard measures of influenza vaccine humoral response (HAI (Fig. 1A), MN (Fig. 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 titers (≤10) were observed in a small number of young and aged animals across the other groups. When serum HAI and MN titers 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 titers 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 1C). HA-specific IgA antibodies in the BALF were undetectable in all animals including the VLP IN group (data not shown).

221 **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 and 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 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 2B) but slightly lower production (0.9-0.6-fold) of IL-1a, IL-1b, 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 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 IL1α 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 4).

Protection from homologous challenge

When challenged IN with 5 x mL}_{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 5A & 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 5B & 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 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).

### 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. 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 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. 6).

### Antibody responses after infection

At three days post-challenge, HAI titers were largely unchanged from day 21 titers (Figure 5A). All the other groups had undetectable HAI titers (<10) (Fig. 7A). ELISA titers 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).

### 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. 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).

**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 titers induced by the plant-made VLP
vaccine compared to a split-virion formulation (GMT 1212.5 ng/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 1C), only the young animals that received the VLP vaccine IM mounted a detectable antibody responses as measured by the standard HAI and MN assays (Figure 1A & 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 titers in the H1-VLP IM groups and the relatively low MN and HAI titers, 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 2A & B, Figure 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 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). 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 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 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/09 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.

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References:


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3 BH has presented this work in poster form Infectious Diseases and Immunity in Global Health research day April 20th 2016 and at International Society for Influenza and other Respiratory Virus Diseases 5th conference as an oral presentation.
7. (ACS) ACS, (NACI) NACoI. 2016. A Review of the Literature of High Dose Seasonal Influenza Vaccine for Adults 65 Years and Older.


Figure Legends

Figure 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) titer 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. 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 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-1, 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 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 4. Splenocyte proliferation or stimulation index (SI) in response to H1 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. At 72 hours, supernatants were removed and cells were pulsed with H³-thymidine in complete media and incorporation of H³-thymidine was measured by scintillation counter 12 hours later. Cell proliferation values were expressed as a Stimulation Index (SI) = (average antigen-stimulated cpmp/ 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 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/07/09 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 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 (mLD$_{50}$) of A/California/07/09 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 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/07/09 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.
Table 1. Number of mice per group used for each assay on day 21 and 3 days post-infection.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Day 21</th>
<th>3 Days Post-Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody Assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAI</td>
<td>25-27</td>
<td>7-8</td>
</tr>
<tr>
<td>MNs</td>
<td>12-15</td>
<td>N/A</td>
</tr>
<tr>
<td>ELISA IgG</td>
<td>25-27</td>
<td>11-12</td>
</tr>
<tr>
<td><strong>Cellular Assays</strong></td>
<td></td>
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<tr>
<td>Proliferation (Thymidine)</td>
<td>8</td>
<td>N/A</td>
</tr>
<tr>
<td>Cytokines/Chemokines</td>
<td>4-13</td>
<td>N/A</td>
</tr>
<tr>
<td>Production (QUANSYS)</td>
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<td></td>
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<tr>
<td><strong>After Infection</strong></td>
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</tr>
<tr>
<td>Viral Titers</td>
<td>N/A</td>
<td>7-14</td>
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<tr>
<td>Survival</td>
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<td>10-11</td>
</tr>
<tr>
<td>Weight Loss</td>
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<td>18-20</td>
</tr>
<tr>
<td>Histopathology</td>
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<td>5</td>
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</tbody>
</table>

Table 2. Summary of total histopathology scores (20 points total) from H&E stain at 3 days post-infection.

<table>
<thead>
<tr>
<th></th>
<th>Total Score</th>
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<tbody>
<tr>
<td>Young</td>
<td></td>
</tr>
<tr>
<td>Naïve</td>
<td>3.0 ± 3.7</td>
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<tr>
<td>Split Vaccine</td>
<td>7.2 ± 5.1</td>
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<tr>
<td>H1-VLP IM</td>
<td>7.0 ± 3.3</td>
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<tr>
<td>H1-VLP IN</td>
<td>3.6 ± 2.4</td>
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<tr>
<td>Aged</td>
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<tr>
<td>Naïve</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>Split Vaccine</td>
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</tr>
<tr>
<td>H1-VLP IM</td>
<td>7.0 ± 4.6</td>
</tr>
<tr>
<td>H1-VLP IN</td>
<td>3.4 ± 2.3</td>
</tr>
</tbody>
</table>
HA-specific Antibodies by MNs 21 Days Post-Vaccination

HA-specific Antibodies by ELISA 21 Days Post-Vaccination

HA-specific Antibodies by HAI 21 Days Post-Vaccination

**A**

**B**

**C**

Anti-HA IgG Concentration (ng/ml)

HAI Titer

MN titer

Young

Aged

Naive

3 ug split vax

3 ug H1-VLP

10

100

1000

0

2000

4000

6000

8000

Young

Aged

Naive

3 ug split vax

3 ug H1-VLP

10

100

1000

40

HAI Titer

MN titer

Young

Aged

Naive

3 ug split vax

3 ug H1-VLP

10

100

1000

40
A

Spleenocyte proliferation

Stimulation Index (SI)

** **

IM
VLP Stimulated
ConA Stimulated

B

Spleenocyte proliferation

Stimulation Index (SI)

VLP Stimulated
ConA Stimulated
Virus titers in lungs (Log_{10} TCID_{50}/0.1 mg of tissue)

- Naive
- 3 ug split vax
- 3 ug H1-VLP
- 3 ug H1-VLP

Virus Titers in Lungs 3 days Post-Infection

* Young
* Aged
A

HA-specific Antibodies 3 Days Post-Infection

<table>
<thead>
<tr>
<th></th>
<th>IM</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 ug split vax</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 ug H1-VLP</td>
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</table>

B

HA-specific IgG by ELISA 3 Days Post-Infection

<table>
<thead>
<tr>
<th></th>
<th>IM</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
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</tr>
<tr>
<td>3 ug split vax</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 ug H1-VLP</td>
<td></td>
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