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 Virus Challenge despite a Modest/Absent Humoral Response

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ABSTRACT

Virus-like-particle (VLP) influenza vaccines can be given intramuscularly (i.m.) or intranasally (i.n.) 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 i.m. or i.n. in young and aged mice. Young adult (5- to 8-week-old) and aged (16- to 20-month-old) female BALB/c mice received a single 3-μg dose based on the HA (A/California/07/2009 H1N1) content of a plant-made H1-VLP (i.m. or i.n.) split-virion vaccine (i.m.) or were left naive. After vaccination, humoral and splenocyte responses were assessed, and some mice were challenged. Both VLP and split vaccines given i.m. 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 i.m. with VLP were more likely to survive challenge (80% versus 60%). The lung viral load postchallenge was lowest in the VLP i.m. groups. Mice vaccinated with VLP i.n. had little detectable immune response, but survival was significantly increased. In both age groups, i.m. 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 (VLPs), plant-made vaccines

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 virus antigens (Ag) (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 (i.m.) or intradermal injection of detergent-split virions at a fixed dose of 15 μg hemagglutinin (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 to 60% (4). These formulations work less well in the elderly (5). Recently, i.m. formulations with 60 μg HA/strain (so-called high-dose [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 i.m. and intranasal (i.n.) delivery (8–11). These vaccines appear to elicit both strong antibody responses and long-lived and polyfunctional 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 virus in young and aged mice. Our results demonstrate that a single 3-μg dose of this candidate vaccine delivered i.m. was superior to a standard split-virion vaccine in almost all measured outcomes at both ages. Although the same VLP vaccine delivered i.n. failed to elicit any detectable humoral or cellular responses, between 50% (aged) and 75% (young) of the animals were still protected from challenge.

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RESULTS

Antibody response. Overall, the standard measures of influenza vaccine humoral response (hemagglutination inhibition [HAI] [Fig. 1A] and microneutralization [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 i.m. consistently mounted detectable HAI (geometric mean titer [GMT], 14.51; P < 0.0001) and MN (GMT, 17.5; P < 0.0001) responses compared to the other groups. Very low HAI (<10) and/or MN (<10) titers 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 enzyme-linked immunosorbent assay (ELISA), as well (total pandemic H1N1 [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 i.m. (GMT, 1,771.1 ng/ml) than in both the VLP i.n. group and the split-virion group (GMT, 109.1 ng/ml and 265.1 ng/ml; P < 0.01 and P < 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 (GMT, 364.3 ng/ml) vaccine i.m. (Fig. 1C). HA-specific IgA antibodies in the bronchoalveolar lavage fluid (BALF) were undetectable in all the animals, including the VLP i.n. group (data not shown).

Cellular immune response to vaccines. Cytokine/chemokine and lymphoproliferative responses of splenocytes restimulated with H1 antigen ex vivo were also most consistently detected in the H1-VLP i.m. group (Fig. 2; see Fig. 4) and were generally more robust in young mice than in aged mice. Since split-virion formulations are the most common commercial vaccines, we compared the cytokine/chemokine responses of the H1-VLP i.m. group to those of the i.m. split-virion group at the two ages. Splenocytes from the young H1-VLP recipients produced a greater (2- to 14-fold) amount of a broad range of cytokines/chemokines than splenocytes from the split-vaccine recipients, including interleukin 2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, gamma interferon (IFN-γ), IL-4, IL-17, IL-5, IL-10, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, and IL-6 (Fig. 2A). Differences between the H1-VLP and split-virion groups in the aged animals were far more modest, so that animals that received the H1-VLP vaccine had slightly (1.1- to 1.4-fold) higher IL-2, GM-CSF, IFN-γ, IL-3, IL-4, IL-5, tumor necrosis factor alpha (TNF-α), and IL-6 production than the split-virion group (Fig. 2B) but slightly (0.9- to 0.6-fold) lower production of IL-1α, IL-1β, IL-12, IFN-γ, and RANTES. None of these differences reached
statistical significance. Antigen-specific cytokine/chemokine responses comparing young versus aged animals are presented in Fig. 3. Overall, cytokine/chemokine responses of the young and aged animals to the VLP and split vaccines were similar, with a few striking exceptions. For the H1-VLP vaccine given i.m., the young animals made much greater quantities of IFN-γ, while IL-1α production was much greater in the aged animals. In response to the split vaccine, the young mice produced significantly more IFN-γ, IL-5, and IL-1α than the aged group. In preliminary experiments with either H1-VLP or split-virion formulations, the antigen used for restimulation (i.e., H1-VLP, whole inactivated pdmH1N1, or 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 i.m. groups for both age groups, although significance was reached only in the young mice (stimulation index [SI], 7.38 ± 2.57 versus 3.51 ± 1.38 [P < 0.0001]) versus the aged mice (SI, 4.12 ± 0.87 versus 2.52 ± 0.95 [P = 0.3325]) (Fig. 4).
Protection from homologous challenge. When challenged i.n. with 5 times the mouse 50% lethal dose (mLD50) of A/California/07/2009 H1N1 virus, young naive 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 naive animals survived (Fig. 5A and C). Young mice given the H1-VLP vaccine i.n. also lost substantial weight (15.0%), but 80% of them recovered and survived. The young split-vaccine and H1-VLP i.m. groups lost the least weight (10.8% and 9.8%, respectively), and all of the animals survived. Overall, the aged animals lost more weight following challenge than the younger mice. The aged naive group also lost the most weight (20.2%), with kinetics similar to what was observed in the younger animals (Fig. 5B and D). Most of these animals met humane endpoints or died (22.2% survival). Aged mice that received the split-virion vaccine i.m. or the H1-VLP vaccine i.n. lost similar amounts of weight (17.8% and 18.3%, respectively) (Fig. 5B) and had similar rates of survival (60% and 55.6%, respectively). The aged mice given H1-VLP i.m. lost the least weight (14.2%), and 80% survived challenge. Although more than half of the aged animals in the VLP i.n. and split-virion groups survived, most of the animals

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**FIG 2** Splenocyte production of H1 antigen-specific cytokines/chemokines after *ex vivo* restimulation. Young (5- to 8-week-old) (A) and aged (16- to 20-month-old) (B) BALB/c mice were immunized once by i.n. instillation with H1-VLP vaccine or i.m. with H1-VLP or split-virion vaccine. Three weeks postvaccination, splenocytes were collected and stimulated *ex vivo* for 72 h with H1-VLP. Unstimulated splenocytes were pooled for each group, and for the stimulated splenocytes, 4 to 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-1, IFN-γ, TNF-α, MIP-1α, GM-CSF, and RANTES. For each age group, the average of the unstimulated mice of that group was subtracted, and the response of the split-vaccine group was considered the standard response; responses observed in the H1-VLP groups were calculated as fold changes from this group. The magnitude of panel A is 4-fold greater than that of panel B. For statistical analysis, one-way ANOVA was performed. ****, P < 0.0001; ***, P < 0.001; **, P < 0.01. The data represent 4 to 13 mice/group combined from 2 studies.
remained well below their baseline weights (15 to 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 ($P < 0.0001$ versus the split-vaccine group at day 12).

**Lung viral load at 3 days postinfection.** Overall, vaccination had relatively little impact on day 3 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, versus naive, 4.42) (Fig. 6). The greatest decrease in the day 3 viral load and the only
A decrease that reached statistical significance was observed in the young H1-VLP i.m. group (3.68; \( P < 0.05 \)) (Fig. 6). The H1-VLP i.n. group had a slight decrease from the naive and split-vaccine groups (4.02). Among the aged animals, the split-vaccine and H1-VLP i.n. mice had the highest viral loads (4.27 versus 4.29, respectively) compared to the naive mice (4.50). The H1-VLP i.m. group had a small decrease in the day 3 viral loads (4.06) (Fig. 6).

**Antibody responses after infection.** At 3 days postchallenge, HAI titers were largely unchanged from the day 21 titers (Fig. 5A). All the other groups, except H1-VLP
i.m. young mice, had undetectable HAI titers (<10) (Fig. 7A). ELISA titers rose 2- to 3-fold in the H1-VLP i.m. groups in both the young (GMT, 2,612.63 ng/ml) and aged (GMT, 719.13 ng/ml) mice. The GMT among the vaccine-naive 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 3 days postinfection (data not shown).

Lung histopathology. Lung histopathology total scores were highest in the young split-vaccine group (7.2 ± 5.1) and in both young and aged mice in the H1-VLP i.m. 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

**FIG 5 Weight loss and survival after A/California/07/2009 H1N1 challenge.** Young (5- to 8-week-old) and aged (16- to 20-month-old) BALB/c mice were immunized once by i.n. instillation with H1-VLP vaccine or i.m. with H1-VLP or inactivated split vaccine. Three weeks after vaccination, the mice were challenged with an age-appropriate 5 times the mL50 of A/California/07/09 H1N1. (A and B) Weight loss for both the young (A) and aged (B) mice was monitored daily. (C and D) Survival of young (C) and aged (D) animals. Mice were euthanized if they lost >20% of their initial weight. The error bars indicate the standard errors 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 naive groups. #, P < 0.0001 compared to the split-vaccine group. The data in panels A and B represent 18 to 20 mice/group combined from 3 studies; the data in panels C and D represent 10 to 11 mice/group combined from 2 studies.
infection. The naive groups of 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 and 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 doses (4) or the addition of an adjuvant (14). One of the limiting factors in developing better vaccines for the elderly is the lack of a simple and affordable animal model (15). Although swine (16) and ferrets (17, 18) 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 to 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 preadaptation. Of course, none of these models have a lifetime of accumulated experience with influenza virus 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 virus challenge (19–21), as well as new influenza vaccination strategies for the elderly (22–26).

At the outset, we were optimistic that either i.m. or i.n. 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 i.m. or i.n. (27, 28) and on the growing body of evidence that the plant-made VLPs stimulate the immune response in a different way than split vaccines. For instance, we have recently shown that these VLPs rapidly access draining lymph nodes (29), where they associate with

FIG 6 Lung viral loads after A/California/07/2009 H1N1 challenge. Young (5- to 8-week-old) and aged (16- to 20-month-old) BALB/c mice were immunized once by i.n. instillation with H1-VLP vaccine or i.m. with H1-VLP or inactivated split vaccine. Three weeks after vaccination, the mice were challenged with an age-appropriate 5 times the mLD\textsubscript{50} of A/California/07/09 H1N1. TCID\textsubscript{50} values were log transformed and are shown as floating bar graphs. The means are depicted by horizontal lines within the bars. The TCID\textsubscript{50} was calculated using the Karber method [log TCID\textsubscript{50}/0.1 ml = −1 − (percent total mortality/100 − 0.5) × log\textsubscript{10}]. For statistical analysis, two-way ANOVA was performed. * P < 0.05 compared to naive groups. There were 7 to 14 mice/group combined from 3 studies.
and activate immune cells, including B cells, macrophages, and dendritic cells (30). Furthermore, these vaccines elicit balanced humoral and cellular responses in both preclinical (young mice and ferrets) and clinical studies in healthy young adults (10–12, 31). The current work confirmed the earlier observation of 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 i.m. dose of the VLP vaccine despite significant weight loss and obvious lung inflammation. Surprisingly, delivery of the VLP vaccine i.n. elicited minimal cellular or humoral responses but still protected almost 60% of the animals. Lung viral loads were only marginally decreased by immunization at day 3 postchallenge but were most reduced (15 to 20% range) in animals that had received the VLP vaccine i.m. 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 day 3 postinfection and survival from lethal challenge. Among the aged

![Graph A: HA-specific Antibodies 3 Days Post-Infection](image)

**FIG 7** Serum antibody levels 3 days after challenge infection. Young (5- to 8-week-old) and aged (16- to 20-month-old) BALB/c mice were immunized once by i.n. instillation of H1-VLP vaccine or i.m. with H1-VLP or split-virion vaccine. Three weeks postvaccination, the mice were challenged with A/California/07/09 H1N1, and sera from individual mice were analyzed. HAI assay titers (A) and ELISA HA-specific IgG concentrations (B) are shown. The dotted line in panel A represents 40 HAI units, which is considered the protection level in humans. The error bars indicate the standard errors of the mean. For statistical analysis, one-way ANOVA was used on the log values. ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.05$. The data in panel A represent 7 or 8 mice/group combined from 2 studies. The data in panel B represent 11 or 12 mice/group combined from 3 studies.
animals, only the VLP i.m. group had regained their baseline weight 2 weeks after challenge.

The 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 and mortality) (32, 33). The old mice used in this study (16 to 20 months of age) were still active and healthy in appearance but were obviously heavy (weight range, 28 to 34 g); many had significant loss of lung volume due to kyphosis (unpublished data) and were susceptible to lower doses of influenza virus at challenge (the LD₅₀ was 6-fold lower than for young mice). Although both the split-virion vaccine delivered i.m. and the VLP vaccine delivered i.n. increased survival of the old mice to 50 to 60%, they were clearly “sicker” than the animals that had received the VLP vaccine i.m. In ongoing work, we have recently observed that even very old animals (22 to 26 months) with multiple comorbidities have higher antibody titers induced by the plant-made VLP vaccine than by a split-virion formulation (GMT, 1,212.5 ng/ml versus 396.8 ng/ml, respectively; \( P < 0.03 \)) (unpublished data).

Although both the split-virion and VLP formulations induced antibodies detectable by ELISA (Fig. 1C), only the young animals that received the VLP vaccine i.m. mounted a detectable antibody response as measured by the standard HAI and MN assays (Fig. 1A and B). In the case of the animals vaccinated i.n. with VLPs, 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 i.m. 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-stalk 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 than in the older animals across all the vaccine groups (Fig. 2A and B and 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- to 10-fold increases in IFN-\( \gamma \) and IL-5 production, while older animals had more modest responses (2- to 4-fold increases in a number of cytokines/chemokines) (Fig. 3). When cytokine/chemokine production was standardized against the respective age-specific split-virion groups, however, both young and old animals i.m. vaccinated with H1-VLP were found to produce large amounts of IFN-\( \gamma \), GM-CSF, IL-2, IL-3, IL-4, IL-5, and IL-6, suggesting broad immune activation (4- to 14-fold in the young mice and 1.5- to 3-fold in the aged mice) (Fig. 2). Despite the complete absence of antibody in the mice that received the H1-VLP vaccine i.n., splenocytes from the young animals produced antigen-specific IFN-\( \gamma \) in abundance (30-fold) and more modest (2-5-fold) amounts of IL-2, IL-5, and TNF-\( \alpha \) (Fig. 3). The aged mice immunized i.n. produced large amounts of antigen-specific IL-1\( \alpha \) (30-fold) and modest amounts of IFN-\( \gamma \) and IL-2 (3- to 4-fold) (Fig. 3).

Given the fact that 50 to 60% of the i.n.-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 virus challenge and that the importance of cellular responses may increase with advancing age (33). In light of these observations, it is interesting that Ramirez and colleagues have recently reported that CpG (a Toll-like receptor 9 [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 (34). 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 i.m. 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 to 60%) was achieved with a single dose of the same vaccine delivered i.n., 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 i.n. administration of the plant-made H1-VLP vaccine, further work is merited to explore possible synergy between i.m. and i.n. delivery routes to protect this vulnerable population.

**MATERIALS AND METHODS**

**Virus, mice, and vaccines.** Young adult (5- to 8-week-old) and aged (16- to 20-month-old) female BALB/c mice (Charles River Laboratories, Montreal, QC, Canada) were divided into the following groups: naive, detergent-inactivated split-vaccine given i.m. (A/California/07/2009 H1N1 or pdmH1N1; BEI Resources, Manassas, VA), and H1-VLP vaccine given either i.m. or i.n. (Medicago Inc., Quebec, Quebec, Canada). The H1-VLP vaccine was produced as previously described (35) using the wild-type sequence of HA protein from pdmH1N1. The complete study consisted of 6 to 15 mice per group in 5 separate experiments (Table 1). The mice received a single dose of vaccine (3 μg based on HA content) on day 0 or were left naive. For i.m. injections, 50 μl of vaccine was administered into the quadriceps muscle (right leg for the VLP vaccine; bilaterally for the split vaccine) using a 28-gauge, 1/2-in. needle. Intranasal instillation (25 μl/nare) was performed in mice anesthetized with isoflurane.

Peripheral blood was collected from the lateral saphenous vein before immunization (day 0) and 21 days postvaccination (day 21). The blood was collected in microtainer serum separator tubes (BD Biosciences, Mississauga, ON, Canada). 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 day 21. Serum was collected by cardiac puncture, BALF was obtained in complete RPMI (cRPMI) medium, and then spleens were harvested from individual mice and splenocytes were isolated as previously described (36).

The remaining mice (10 or 11 animals/group) were challenged on day 21 with 5 times the LD50 of wild-type A/California/07/2009 H1N1 virus (1,800 50% tissue culture infective doses [TCID50] in 0.5 ml; National Microbiology Laboratory, Public Health Agency of Canada) by i.n. infection with isoflurane. The mLD50 was found to differ between young and aged animals (approximately 663 and 105 TCID50, respectively). Three days postinfection, 7 to 14 mice/group were sacrificed to measure the lung viral load. For these mice, serum, BALF, and lungs were collected. All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

**Antibody titer measurements.** An HAI assay was performed to detect pdmH1N1-specific antibody in the mouse sera at day 0 and day 21 postvaccination and 3 days postinfection, as previously described (37). Briefly, mouse sera and receptor-destroying enzyme (RDE) (Cedarlane, Burlington, ON, Canada) were mixed 1:4 and incubated for 18 h at 37°C. The RDE was inactivated at 56°C for 30 min, and the 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 (Corning Inc. Costar, Corning, NY). The diluted sera (25 μl/well) were then incubated with 8 HA units of pdmH1N1 for 30 min at room temperature before 0.5% turkey erythrocytes diluted in PBS (50 μl/well; Lampire Biological Laboratories, Pipersville, PA) were added to the wells. The HAI titer

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**TABLE 1 Numbers of mice per group used for each assay on day 21 and 3 days postinfection**

| Assay                             | No. of mice/group | Day 21 | 3 days postinfection |
|-----------------------------------|-------------------|--------|----------------------|
| Antibody                          |                   |        |                      |
| HAI                               | 25–27             | 7–8    |                      |
| MNs                               | 12–15             | NA     |                      |
| ELISA IgG                         | 25–27             | 11–12  |                      |
| Cellular                          |                   |        |                      |
| Proliferation (thymidine)         | 8                 | NA     |                      |
| Cytokine/chemokine production     | 4–13              | NA     |                      |
| (Quansys)                         |                   |        |                      |
| After infection                   |                   |        |                      |
| Viral titers                      | NA                | 7–14   |                      |
| Survival                          | NA                | 10–11  |                      |
| Weight loss                       | NA                | 18–20  |                      |
| Histopathology                    | NA                | 5      |                      |

*NA, not applicable.*
was determined by visual inspection as the highest dilution that inhibited erythrocyte agglutination, using standard criteria.

MN titers were measured as previously described (38). Briefly, confluent monolayers of Madin-Darby canine kidney (MDCK) cells (British Columbia Center for Disease Control) were incubated in 96-well flat-bottom plates (Falcon Conning Life Science, Corning, NY) in MegaVir (VWR, Radnor, PA) supplemented with 10 μg/ml gentamicin (Gibco Life Technologies, Burlington, ON, Canada), 0.25 μg/ml amphotericin B (Gibco Life Technologies, Burlington, ON, Canada), 100,000 U/ml penicillin G (Sigma, St. Louis, MO), and 10 μg/ml glutamine (Wisent, St. Bruno, QC, Canada). Sera were heat inactivated at 56°C for 30 min and diluted 2-fold in MegaVir starting at 1:10 in duplicate wells (60 μl/well). Each well then received 100 infectious units of pdmH1N1 virus diluted in MegaVir (60 μl/well), and the plates were incubated at 37°C in 5% CO₂. The 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.

ELISAs were performed as previously described (36). Briefly, U-bottom, high-binding 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4°C with recombiant 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 2,000 ng/ml. The wells were blocked with 2% bovine serum albumin (BSA) in PBS-Tween 20 (0.05%; Fisher Scientific, Ottawa, ON, Canada). The sera were heat inactivated (as described above), diluted 1:50 in blocking buffer, and added to four wells (50 μl/well). The plates were incubated for 1 h at 37°C; blocking buffer was added to the standard curves at this time. Horseradish peroxidase (HRP)-conjugated anti-mouse total IgG antibodies (Jackson Immuno Research Laboratories Inc., West Grove, PA) were diluted 1:50,000 in blocking buffer. The sera were added to the wells, and the plates were incubated for 1 h at 37°C; then a 1:500,000 blocking buffer was added and incubated at 75 μl/well; 1 h 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 H₂SO₄ after 15 min (50 μl/well). The 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 and stimulation and cytokine/chemokine analysis. Individual spleens were harvested 21 days before challenge in Hank’s balanced salt solution (HBSS) without calcium or magnesium (Wisent, St. Bruno, QC, Canada) at room temperature (RT) and processed as previously described (36).

Supernatant was collected and measured as previously described, except at 3 × 10⁵ cells/well in 200 μl under the following conditions: cRPMI alone (unstimulated) or with H1-VLP (2.5 μg/ml HA) in cRPMI for 72 h at 37°C in vitro. Spleens were isolated from a total of 13 mice per group from 2 experiments and tested in duplicate on multiplex ELISA by Quansys (Logan, UT). Cytokine/chemokine data are presented as radar graphs as previously described (39).

Splenocyte stimulation and cell proliferation assay (CPA). Splenocytes were placed in duplicate in 96-well U-bottom plates (BD Falcon, Mississauga, ON, Canada) at 10⁵ cells in 200 μl with cRPMI alone (unstimulated), with H1-VLP vaccine (2.5 μg/ml HA), or with concanavalin A (ConA) (2.5 μg/ml for a stimulation control in cRPMI. After 72 h at 37°C plus 5% CO₂, the plates were spun down (300 x g; 10 min at RT), and the supernatants were removed. The cells were pulsed with 1 μCi/well [³H]thymidine (MP Biomedical, Solon, OH) for an additional 18 h. After one freeze-thaw, the cells were harvested on glass fiber filters with a Tomtec harvester 96 (Tomtec Inc., Hamden, CT), and [³H]thymidine incorporation was measured with a scintillation counter (Wallac Microbeta Trilux 1450 beta-counter; Wallac, Turku, Finland). Cell proliferation values were expressed as the Sti for each mouse, the Sti was equal to the average Ag-stimulated counts per minute divided by the average unstimulated counts per minute. I.n. 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 postinfection and individually homogenized in an equal amount (wt/wt) of MegaVir medium (VWR, Radnor, PA) using a tube homogenizer. The samples were centrifuged at 14,000 × g for 5 min at 4°C, and the supernatants were collected and stored at −80°C. Virus titers were measured by the TCID₅₀. MDCK cell monolayers were prepared in 96-well flat-bottom plates. On the day of inoculation, the MegaVir was removed and the lung homogenates were serially diluted 1:10 with MegaVir TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Sigma; 2 mg/ml) (MegaVirus-trypsin). Each dilution was added to 6 wells (100 μl/well) and incubated at 37°C with 5% CO₂, the plates were replaced with fresh MegaVir-trypsin (200 μl/well). The plates were maintained with fresh MegaVir-trypsin (200 μl/well) for 5 min at 37°C plus 5% CO₂. The 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.

Lung histopathology. Lung samples were processed using the standard hematoxylin and eosin (H&E) stain. Briefly, lung samples were fixed in 10% formalin (Fisher Scientific, Ottawa, ON, Canada) and then embedded in paraffin (Leica, Concord, ON, Canada). Sections (4 μm) were applied to slides and then heated at 50 to 60°C. Samples were washed with xylene (Chaptec, Quebec, QC, Canada) and then immersed in ethanol (Commercial Alcohols, Boucherville, QC, Canada) for 10 min. The slides were rinsed with distilled water and then briefly submerged in Harris hematoxylin (HHS-32; Sigma, St. Louis, MO) one-half solution in distilled water. The slides were washed under running tap water and then washed 10 times with ethanol, followed by 1 min of eosin-xiloxin B (100 ml of 1% eosin Y [Sigma, St. Louis, MO, 10 ml of 1% phloxin B [Sigma, 780 ml of ethanol, and 4 ml of glacial acetic acid (Fisher Scientific, Ottawa, ON, Canada)]. Samples were immersed in ethanol for 10 min. The slides were dried and then submerged in xylene for 10 min. The slides were fixed with 2 drops of acryl (Leica, Concord, ON, Canada) a coverslip and scored at ×10 and ×100 magnification. The slides were scored blinded, and the scoring
system evaluated the following 5 parameters: (i) airway epithelial necrosis, attenuation, or disruption; (ii) airway inflammation; (iii) peribronchiolar and perivascular lymphocytic cuffing; (iv) alveolar cellular exudate/edema and interlobular edema; and (v) alveolar septal inflammatory cells and cellularity (41). Each parameter was scored from 0 to 4 for a total possible score of 20 (Table 2).

**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 analysis of variance (ANOVA) was performed on HAI and stimulation index values. All other statistical analysis was by two-way ANOVA. All analyses were performed using GraphPad Prism 6.0 software.

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**TABLE 2** Summary of total histopathology scores (20 points total) from H&E staining at 3 days postinfection

| Group          | Total score ± SD |
|----------------|------------------|
| Young          |                  |
| Naive          | 3.0 ± 3.7        |
| Split vaccine  | 7.2 ± 5.1        |
| H1-VLP i.m.    | 7.0 ± 3.3        |
| H1-VLP i.n.    | 3.6 ± 2.4        |
| Aged           |                  |
| Naive          | 2.2 ± 1.3        |
| Split vaccine  | 3.6 ± 2.1        |
| H1-VLP i.m.    | 7.0 ± 4.6        |
| H1-VLP i.n.    | 3.4 ± 2.3        |

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