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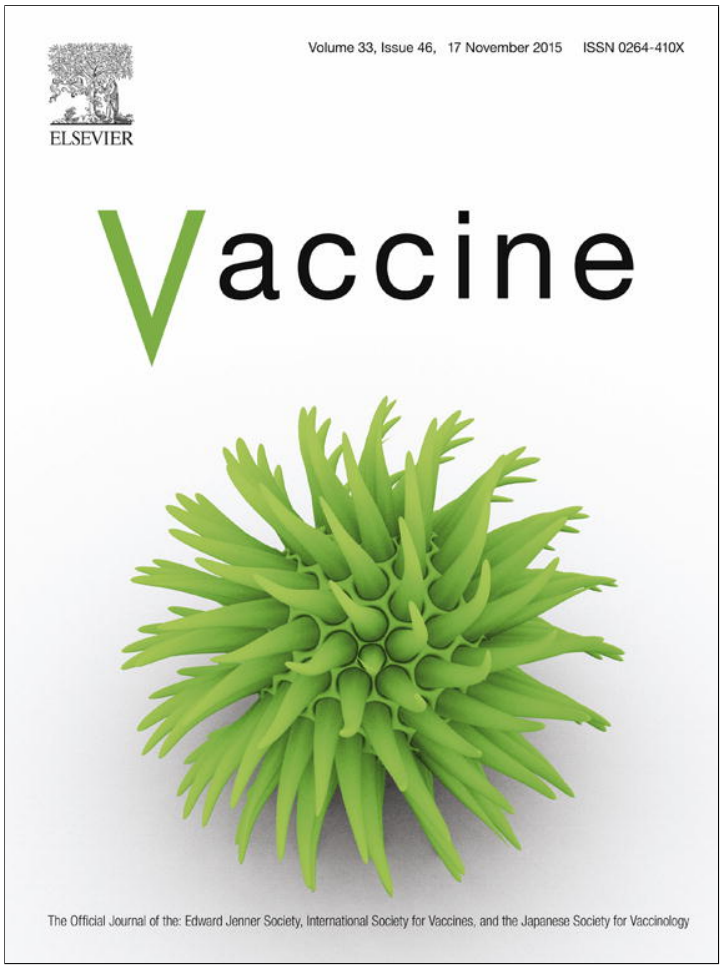

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Plant-derived H7 VLP vaccine elicits protective immune response against H7N9 influenza virus in mice and ferrets

S. Pillet[a,b], T. Racine.[c], C. Nfon[d], T.Z. Di Lenardo[b], S. Babiuk[d,e], B.J. Ward[a], G.P. Kobinger[c,e,f], N. Landry[a,*]

[a] Medicago Inc., 1020 Route de l'Église, Bureau 600, Québec, QC, Canada
[b] Research Institute of the McGill University Health Center, Montreal, QC, Canada
[c] Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada
[d] National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada
[e] Department of Immunology, University of Manitoba, MB, Canada
[f] Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

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ABSTRACT

In March 2013, the Chinese Centre for Disease Control and Prevention confirmed the first reported case of human infection with an avian influenza A H7N9 virus. Infection with this virus often caused severe pneumonia and acute respiratory distress syndrome resulting in a case fatality rate >35%. The risk of pandemic highlighted, once again, the need for a more rapid and scalable vaccine response capability. Here, we describe the rapid (19 days) development of a plant-derived VLP vaccine based on the hemagglutinin sequence of influenza H7N9 A/Hangzhou/1/2013. The immunogenicity of the H7 VLP vaccine was assessed in mice and ferrets after one or two intramuscular dose(s) with and without adjuvant (alum or GLA-SE^{TM}). In ferrets, we also measured H7-specific cell-mediated immunity. The mice and ferrets were then challenged with H7N9 A/Anhui/1/2013 influenza virus. A single immunization with the adjuvanted vaccine elicited a strong humoral response and protected mice against an otherwise lethal challenge. Two doses of unadjuvanted vaccine significantly increased humoral response and resulted in 100% protection with significant reduction of clinical signs leading to nearly asymptomatic infections. In ferrets, a single immunization with the alum-adjuvanted H7 VLP vaccine induced strong humoral and CMI responses with antigen-specific activation of CD3$^+$ T cells. Compared to animals injected with placebo, ferrets vaccinated with alum-adjuvanted vaccine displayed no weight loss during the challenge. Moreover, the vaccination significantly reduced the viral load in lungs and nasal washes 3 days after the infection. This candidate plant-made H7 vaccine therefore induced protective responses after either one adjuvanted or two unadjuvanted doses. Studies are currently ongoing to better characterize the immune response elicited by the plant-derived VLP vaccines. Regardless, these data are very promising for the rapid production of an immunogenic and protective vaccine against this potentially pandemic virus.

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1. Introduction

On 29 March 2013, the Chinese Center for Disease Control and Prevention confirmed the first reported case of human infection with an avian H7N9 influenza A virus. As of October 2014, 453 laboratory-confirmed human infections with H7N9 viruses, including 175 deaths, have been reported resulting in a case fatality rate of 38.6% [1]. In most of the laboratory confirmed cases, infection with this novel H7N9 strain resulted in severe pneumonia and acute respiratory distress syndrome (ARDS) that required intensive care [2]. Although human-to-human transmission remains highly controversial [3], the ability of the H7N9 hemagglutinin (H7) to bind weakly to alpha 2,6-linked sialic acid suggests that H7N9 viruses could pose a pandemic threat [4,5]. Moreover, A/H7N9 viruses appear to be transmissible between ferrets via respiratory droplets [6] adding to the concern of a possible pandemic threat as transmission of influenza virus in ferrets closely mimics that in humans [7].

The possible emergence of H7N9 is occurring in the context of the A/H1N1 2009 outbreak that challenged national and global pandemic plans. In particular, the H1N1 2009 experience reinforced the world's commitment to vaccination as the most-cost effective means to prevent infection and to control potential pandemics. Unfortunately, the full process from the identification of a new

* Corresponding author. Tel.: +418 658 9393; fax: +418 658 6699.
E-mail address: landryn@medicago.com (N. Landry).

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strain to release of the vaccine product is traditionally completed within 4–6 months. Seasonal and pandemic strains that kill or grow poorly in eggs can be even more challenging [8], highlighting some of the limitation of current egg-based vaccines. Despite substantial efforts to develop so-called 'universal' vaccines and/or to predict the nature of the next pandemic strain, the degree of homology between vaccine and the circulating strains remains a crucial determinant of vaccine efficacy as illustrated during the 2012–2013 season [9]. In the absence of a universal vaccine, there is a great deal of interest in technologies and platforms that can produce large amounts of strain-specific vaccine rapidly.

Among the most promising approaches to address these issues are nanoparticles vaccines including virus-like particles (VLP) and particularly VLP vaccines produced in plants [10–12]. VLP are assembled upon expression of specific viral proteins and can present an external surface that closely resembles that of the cognate virus (Fig. S1) but without any genetic material. Influenza VLP that incorporate one or more viral proteins resemble intact virions in structure and morphology, and contain functionally active and immunologically relevant structural proteins [13]. In particular, the highly immunogenic viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are usually in their native conformation within VLP due to the absence of possible modifications by fixatives or chemicals used to inactivate and/or split virions in other vaccines. The self-assembled macrostructure of VLP can therefore present native conformational epitopes of surface proteins to the immune system [13]. Furthermore, influenza viruses grown in embryonic eggs and, in a lesser extent mammalian tissue culture systems, have the potential to mutate for optimal growth in these environments. The number and location of mutations in egg- and tissue-culture-adapted strains varies widely from year to year [9]. The immune response generated by those vaccines could therefore diverge from the actual circulating strain particularly when mutations occur in critical areas of the surface proteins. In contrast, the proteins contained in VLP can be engineered to exactly match the amino acid sequence of the circulating wild-type strain. Finally, the non-infectious nature of VLP is also a desirable safety feature for a vaccine candidate.

Plant-derived VLP vaccines address several of the serious limitations of currently licensed vaccines [13]. First, the protein(s) included in the VLP is/are based on the genetic sequence of circulating human influenza viruses rather than influenza strains adapted for optimal growth in embryonated eggs or tissue culture. Moreover, among the novel recombinant antigen production platforms, Agrobacterium infiltration-based transient expression in *Nicotiana benthamiana* has shown unprecedented speed and productivity, each kilogram of infiltrated leaves potentially producing approximately 1500 vaccine doses, based on a theoretical human dose of 30 μg of antigen [13,14]. Finally, plant-based manufacturing systems avoid some of the scalability issues associated with tissue-culture and egg-based production platforms as well as other recombinant technologies (i.e. available capacity in large-scale bioreactors). In 2012, Medicago demonstrated its capacity to produce 10 million doses of a plant-based H1N1 influenza vaccine within a month [15].

Here, we described the rapid development of an H7N9 plant-derived VLP candidate vaccine based on the HA sequence of A/Hangzhou/1/2013. When an adjuvant was incorporated, a single intra-muscular (IM) injection of this plant-made H7 VLP vaccine induced a robust HI antibody response in both mice and ferrets. We also demonstrated clear induction of a strong, Ag-specific, T-cell response after immunization in ferrets. These humoral and cell-mediated immune (CMI) responses were observed in conjunction with 100% protection after a lethal challenge with H7N9 A/Anhui/1/2013 in mice, as well as a significant reduction of clinical signs and virus load in ferrets. Two doses of the unadjuvanted vaccine achieved an even better level of protection in the mouse model.

2. Material and methods

2.1.1. Plant-derived VLP vaccine production and characterization

The vaccine production and characterization is described in supplementary methods and results sections. The vaccine was ready for production 19 days after obtaining the H7 sequence. By mid-May 2013, we performed our first pre-clinical trials in mice and ferrets demonstrating the immunogenicity of the H7 VLP vaccine (Fig. 1).

2.2. Animal immunizations

Mice: Female BALB/c mice 6–8 weeks old (Charles River, Saint-Constant, QC) were immunized IM using a 27G1/2 needle with one or two dose(s) of 3 μg H7 VLP vaccine (lot #500-32-020) based on HA content (see supplementary methods for details) with or without either Alhydrogel® (alum, 0.5 mg/dose, Cedarlane Laboratory, Burlington, ON, USA) or the toll-like receptor 4 (TLR-4) agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE, 0.005 mg/dose, Immune Design, Seattle, WA, USA) adjuvant 21 days apart. Placebo groups received IM injection of the saline buffer used to dilute the vaccine with either alum or GLA-SE adjuvant. All mouse procedures performed at the Research Institute of the McGill University Health Centre prior to challenge were approved by the McGill University Animal Care and Use Committee.

Ferrets: Male ferrets (*Mustela putoris furo*) 10–14 weeks old (Marshall Farms, North Rose, NY, USA) were immunized IM using a 25G5/8 needle with one or two dose(s) of 15 μg H7 VLP vaccine (lot #500-32-020) based on HA content with or without either alum (0.5 mg/dose) or GLA-SE (0.005 mg/dose) adjuvant 21 days apart. All ferret procedures prior to challenge were performed in the Centre National de Biologie Expérimentale (Laval, QC, USA) after approval by the Institutional Animal Care and Use Committee.

2.3. Immune response assessment in mice and ferrets

Hemagglutination inhibition (HI) assay: HI assays were performed as previously described according to the WHO recommendations using turkey's red blood cells and H7 VLP as the target antigen [16,17].

CMI response in ferrets: Fourteen days after the first immunization (prime), ferret's peripheral blood was collected in potassium EDTA-containing Vacutainer blood collection tubes (BD). Peripheral blood mononuclear cells (PBMC) were isolated as described previously [18] and seeded at 10^6 cells/well in 96-well plate in RPMI supplemented with 0.1 mM MEM non-essential amino acid (Invitrogen), 1 mM sodium Pyruvate (Invitrogen), 10 mM HEPES, 2 mM L-glutamine (Invitrogen), 55 μM β-mercaptoethanol, 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin (Complete RPMI) for cell proliferation and flow cytometry assays. *Flow cytometry analysis*: PBMC were incubated at 37 °C in a 5% CO_2 atmosphere with H7 or H5 VLP in complete RPMI during 18 h. After incubation, 1 μl/mL of Brefeldin A solution (GolgiPlug, BD) was added to the PBMC according to the manufacturer's recommendation, and cells were incubated for an additional 5 h at 37 °C in a 5% CO_2 atmosphere. PBMC were then washed, fixed, permeabilized and stained with FITC-conjugated mouse anti-human CD3 (Santa Cruz Biotechnology) and PE-conjugated mouse anti-bovine IFN-γ [18]. *Lymphocyte proliferation*: PBMC were incubated at 37 °C in a 5% CO_2 atmosphere with H7 VLP in complete RPMI for 48 h. Mitogen (2.5 μg/mL concanavalin A, Sigma-Adrich)-stimulated PBMC were included as a positive control. After incubation, cells were pulsed with 1 μCi of ^{3}H-TdR/well (MP Biomedical, Solon, OH, USA) and

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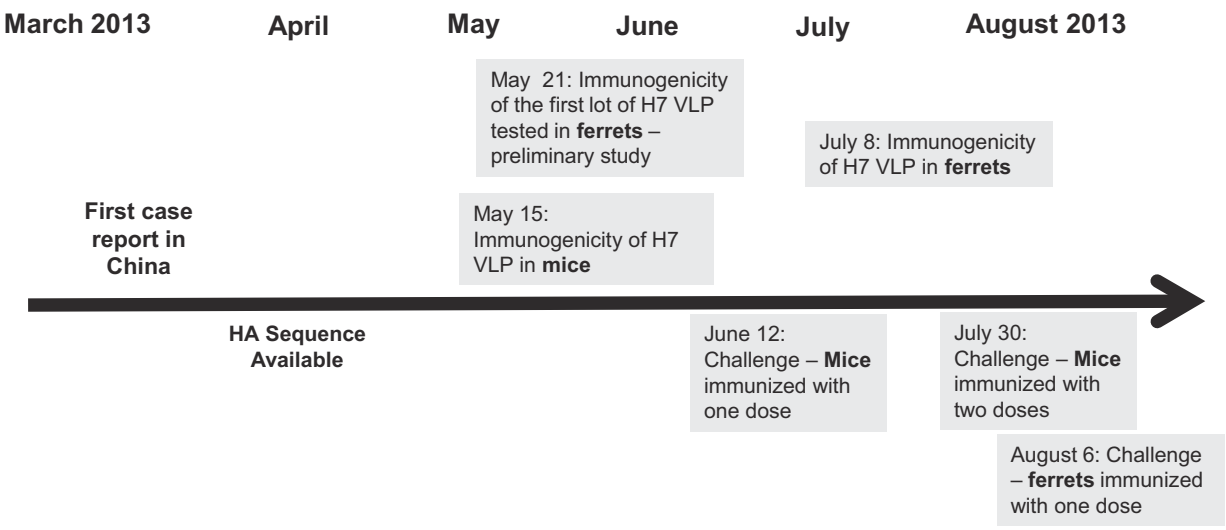

Fig. 1. Time line representing the milestones between the first cases of the H7N9 influenza infections in March 2013, the production of the plant-based H7 VLP vaccine candidate and the demonstration of its immunogenicity and protective effects in mice and ferrets.

incubated for an additional 18 h. After one freeze–thaw, cells were harvested on 96-well glass-fiber filters using a Tomtec harvester 96 (Tomtec Inc., Hamden, CT, USA) and ^{3}H-thymidine incorporation was measured by scintillation counter (Wallac Microbeta Trilux 1450 beta-counter, Wallac, Turku, Finland). Mean disintegration per minute (DPM) for triplicate cultures were calculated and data are expressed as stimulation indices (SI) representing the ratio of the mean DPM of antigen-stimulated cells divided by the mean DPM of unstimulated cells.

2.4. Animal challenges

Mice immunized with one dose of adjuvanted or unadjuvanted vaccines were challenged intranasally (IN) with 100 LD_{50} of influenza A/Anhui/1/13 at four weeks after immunization, while mice immunized with two doses of the unadjuvanted vaccine were challenged 8 weeks after the booster dose.

The challenges were performed in the biosafety level 4 laboratory at the National Microbiology Laboratory (Winnipeg, MB, USA) and were approved by the Canadian Science Centre for Human and Health Animal Care Committee, following the guidelines of the Canadian Council for Animal Care.

Weight loss was monitored daily for a minimum of 12 days after infection and animals that lost ≥30% of their initial body weight were humanely euthanized according to institutional guidelines.

Ferrets were challenged IN with 10^{6} $TCID_{50}$ of influenza A/Anhui/1/13 four weeks after receiving one dose of vaccine. The challenges were performed in the biosafety level 3+ laboratory at the Canadian Food Inspection Agency (Winnipeg, MB, USA) and were approved by the Canadian Science Centre for Human and Health Animal Care Committee, following the guidelines of the Canadian Council for Animal Care.

Weight loss was monitored daily for 6 days after the infection. Three days after challenge, half of the animals ($n = 4$) were sacrificed. Lungs and nasal washes were collected and virus titers were measured in 10% lung homogenates and nasal washes by virus isolation on MCDK cells as previously described [19].

2.5. Statistical analysis

Analysis of variance (ANOVA) and other statistical analysis were performed using Prism Software (version 6.0; GraphPad Software, La Jolla, CA, USA).

3. Results

The plant-made H7 VLP vaccine induces strong humoral response and protection against lethal challenge in mice

All animals in the Placebo groups had HI titers <10 regardless of the adjuvant used. A single immunization with 3 μg of H7 VLP adjuvanted with GLA-SE elicited HI titers ≥40 in all the vaccinated mice. We also detected HI titers against H7 in all mice immunized with 3 μg H7 VLP adjuvanted with alum and in 12 out of 16 (75%) mice that received the unadjuvanted vaccine (Fig. 2).

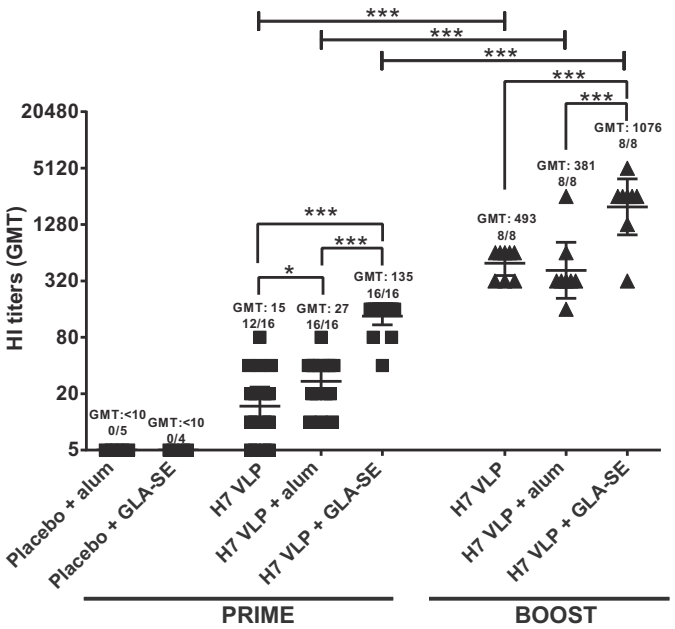

Fig. 2. HI titers of mice after one (Prime) and two (Boost) IM immunizations with the H7 VLP candidate vaccine. Mice were immunized with 3 μg HA content of H7 VLP alone, with Alhydrogel® 2%.
(alum) or GLA-SE. The HI assays were performed using the H7 VLP as the antigen. Geometric Mean Titer (GMT) with 95% confidence interval 16 days after prime and 29 days after the boost are represented. Ratios of positive responders are also indicated. Statistical differences between selected groups were measured using one-way ANOVA with post-hoc Bonferroni's test on the log2 transformed data. Significant differences are indicated: * $p \leq 0.05$, *** $p \leq 0.001$.

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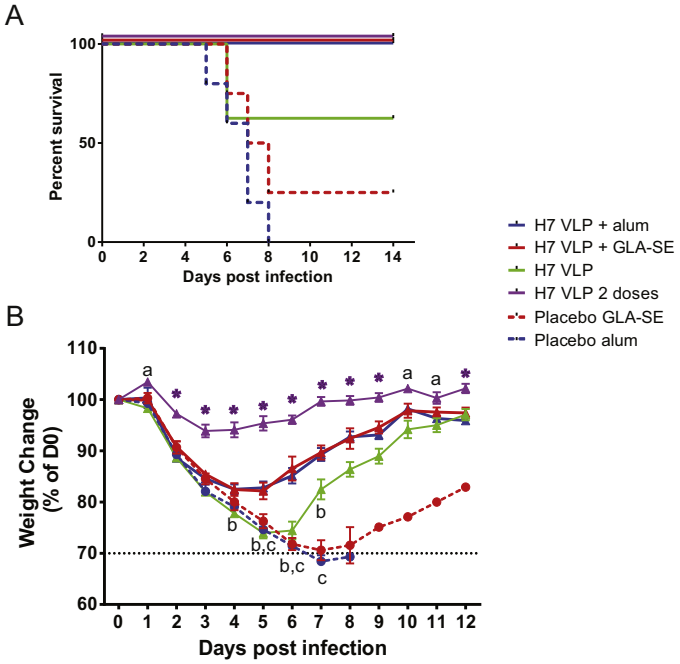

Fig. 3. Protection from H7N9 A/Anhui/1/2013 virus challenge in mice. Mice immunized with one dose of 3 μg HA of unadjuvanted H7 VLP (H7 VLP), one dose of 3 μg HA of alum-adjuvanted H7 VLP (H7 VLP + alum), one dose of 3 μg HA of GLA-SE-adjuvanted H7 VLP (H7 VLP + GLA-SE), two doses of 3 μg HA of unadjuvanted H7 VLP 21 days apart were challenged with 100 LD_{50} of A/Anhui/1/13 H7N9 virus. (a) Survival and (b) weight loss were recorded for up to 12 days. Data were plotted as percentage of the weight on day 0. A two-way ANOVA analysis followed by post-hoc Bonferroni's test was performed on day 0 to day 5 data. A one-way ANOVA analysis was performed on the data of the following days as some mice had to be euthanized for human reason after day 5. (a) indicates significant differences ($p \leq 0.05$) between mice that received one and two dose(s) of unadjuvanted vaccine. (b) indicates significant differences ($p \leq 0.05$) between mice that received one dose of adjuvanted and unadjuvanted vaccines. (c) indicates significant differences ($p \leq 0.05$) between mice that received adjuvanted vaccines and the placebo-matched control. That comparison was stopped after day 7 due to the euthanasia of all but one mice in the placebo groups. Asterisk (*) indicates significant differences ($p \leq 0.05$) between mice that received two doses of the unadjuvanted vaccine and all the others groups.

The two-way ANOVA analysis revealed that the second immunization significantly increased the humoral immune response of both the adjuvanted and unadjuvanted groups.

The mice were challenged with 100 LD_{50} of the H7N9 A/Anhui/1/2013. All the mice immunized with one dose of the adjuvanted formulations survived the challenge 4 weeks after the immunization while five out of eight (62.5%) mice that received the non-adjuvanted vaccine survived (Fig. 3a). All but one mouse that received placebo lost 30% of their body weight and had to be euthanized for humane reasons within 10 days after the infection (Fig. 3a). The only mouse from the placebo group that survived the challenge lost more than 25% of body weight, and did not fully recover 2 weeks after the challenge by contrast to the mice that received the unadjuvanted H7 VLP vaccines (Fig. 3b). Mice vaccinated with two doses of the non-adjuvanted vaccine and challenged 8 weeks after the boost were fully protected (100% survival, Fig. 3a) and only experienced minor weight loss (Fig. 3b). The impact of the infection on weight loss was very similar regardless of the adjuvant used and the significantly higher humoral response observed with the GLA-SE adjuvanted H7 VLP vaccine when compared with the alum adjuvanted H7 VLP vaccine did not translate into a better protection as measured by the impact on body weight. The beneficial effect provided by the administration of two doses of unadjuvanted vaccine compared with one dose was significant as soon as 1 day post-infection, while the difference with mice that received the adjuvanted formulations became significant 2 days after the infection (Fig. 3b).

3.1.1. Adjuvanted plant-made H7 VLP vaccine induces humoral and CMI responses in ferrets

The efficacy of the H7 VLP candidate vaccine was also evaluated in ferrets. A preliminary experiment involving three animals per group receiving two doses of 15 μg H7 VLP with or without adjuvant 21 days apart confirmed the benefit of a second dose on the humoral response for both GLA-SE- and alum-adjuvanted H7 plant-derived vaccines in ferrets and demonstrated that alum induced higher HI titers than GLA-SE in this animal model (geometric mean titers (GMT) after two doses = 806 and 50 for H7 VLP + alum and H7 VLP + GLA-SE respectively). Therefore, only alum was used in the subsequent experiments and animals were immunized with one or two dose(s) of 15 μg H7 VLP alum-adjuvanted and compared with unadjuvanted vaccine. Controls included ferrets that received adjuvanted (alum) or unadjuvanted placebo. Undetectable (<10) HI titres against H7 were observed in all the ferrets before the immunization with H7 VLP (data not shown). After one dose, no significant HI titers were measured in animals that received placebo (adjuvanted or unadjuvanted) or the unadjuvanted vaccine. However, one IM injection of 15 μg of the adjuvanted H7 VLP vaccine elicited HI titers ≥40 in 100% of the animals (GMT = 70) 14 days after the immunization. Titers continued to rise significantly until 21 days after the immunization, reaching a GMT of 247 (Fig. 4A) A second dose of adjuvanted vaccine on day 21 led to a further increase of the HI response (GMT = 1226) when measured 14 days later.

The CMI response was assessed in the PBMC 14 days after one dose of vaccine. The cell proliferation after *ex vivo* stimulation with H7 VLP (2.5 μg HA equivalent/mL) significantly increased in ferrets vaccinated with the adjuvanted vaccine, while the proliferation of PBMC from animals vaccinated with the unadjuvanted vaccine was comparable to animals that received placebo (Fig. 4b).

To better characterize the CMI response in ferrets, we determined the amplitude of H7-specific T cell activation by measuring the proportion of $CD3^+$ T cells that synthesized IFN-γ after *ex vivo* stimulation with H7 VLP. PBMC were also stimulated with an H5 VLP *ex vivo* to determine whether or not the $CD3^+$ T cell activation we observed is antigen specific. In parallel with both the humoral and lymphoproliferative responses, the percentage of $CD3^+$ T cells that produced IFN-γ following H7 VLP *ex vivo* stimulation was significantly higher in ferrets that received one dose of the alum-adjuvanted vaccine than either the unadjuvanted vaccine or control groups (Fig. 4c). The $CD3^+$ T cells responses after *ex vivo* stimulation with H5 VLP or placebo were similar in all groups (Fig. 4c).

3.1.2. A single dose of the alum-adjuvanted H7 VLP vaccine induces protection in ferret

The ferrets that received one dose of vaccine were challenged by IN inoculation with 10^6 $TCID_{50}$ of the H7N9 A/Anhui/1/2013 virus 28 days after vaccination. In contrast to the animals that received placebo or the unadjuvanted vaccine, ferrets immunized with even a single dose of 15 μg HA of the alum-adjuvanted H7 VLP vaccine experienced no weight loss (Fig. 5a) Furthermore, a single dose of the alum-adjuvanted vaccine was sufficient to significantly decrease viral loads in the nasal washes and lungs 3 days after the infection when compared with placebo matched controls (Fig. 5b and c).

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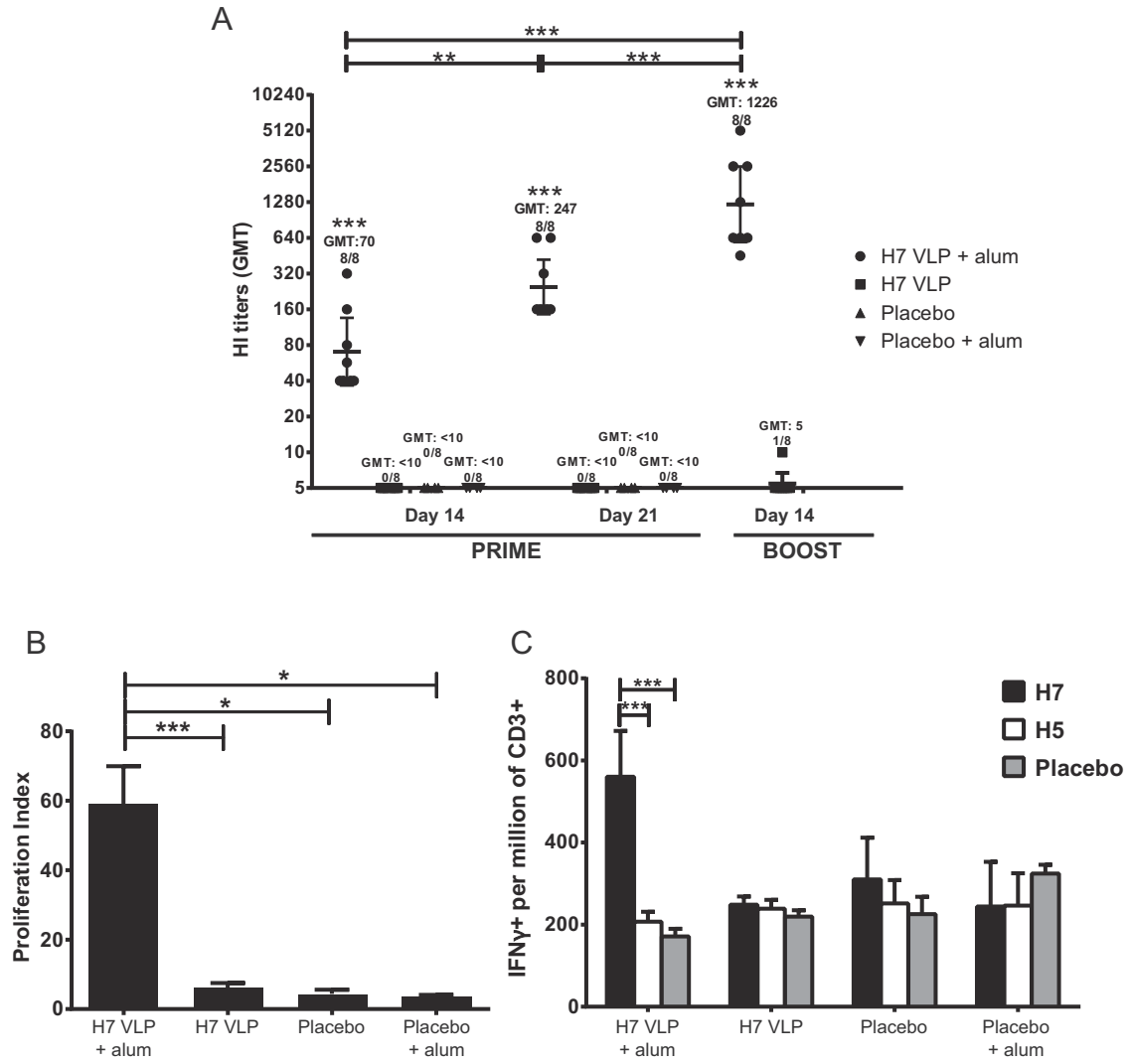

Fig. 4. Humoral and cellular immune response of vaccinated ferrets. (a) HI titers of ferrets after one (Prime) and two (Boost) IM immunizations with the H7 VLP candidate vaccine. Ferrets were immunized with 15 μg HA content of H7 VLP alone (H7 VLP) or with Alhydrogel® (H7 VLP + alum). The HI assays were performed using the H7 VLP as the antigen. Geometric Mean Titer (GMT) with 95% confidence interval 14 days and 21 days after prime as well as 14 days after the boost are represented. Ratios of positive responders are also indicated. (b) Proliferation activity of PBMCs isolated 14 days after prime and stimulated *ex vivo* with 2.5 μg of H7 VLP. The proliferation index represents the ratio between proliferation values of the H7 VLP-stimulated cells and placebo-stimulated cells. (c) Number of IFN-γ secreting cells per million of $CD3^+$ T cells after *ex vivo* stimulation with H7 VLP (black bar), H5 VLP (white bar) or placebo (grey bar). Statistical differences between selected groups were measured using one-way ANOVA followed by post-hoc Bonferroni's test. Significant differences are indicated: Asterisk (*) $p \leq 0.05$, *** $p \leq 0.001$.

4. Discussion

Previous studies with various candidate H7 vaccines have reported a generally poor immunogenicity suggesting that an H7-based H7N9 vaccine would likely require either a high dose or an adjuvant (or both) to provide protection [20–23]. In the current study, a single IM immunization with 3 μg of an alum-adjuvanted plant-made H7 VLP vaccine candidate was sufficient to elicit 100% protection against an otherwise lethal challenge in mice, and two doses of the unadjuvanted vaccine resulted in 100% protection with significant reduction of clinical signs leading to a nearly asymptomatic infection. Smith et al. [24] reported that protection of mice after two doses of a baculovirus-origin VLP vaccine containing full-length influenza HA and NA from the H7N9 A/Anhui/1/2013 strain and the M1 protein from the H5N1 A/Indonesia/05/2005. However, mice immunized with two doses of the unadjuvanted baculovirus VLP vaccine still lost 10–15% of their initial body weight after challenge with 3.6 LD50 A/Anhui/1/2013. More recently, weight lost and partial (80%) protection after challenge with 10 LD50 A/Anhui/1/2013 was observed in mice vaccinated IN with two doses of 5 μg of unadjuvanted recombinant baculovirus-origin H7 subviral particles 3 weeks apart [25].

The plant-origin VLP vaccine contains low biological reactivity lipopolysaccharide (LPS) of Agrobacterium origin that has recently been demonstrated to have immunological activities and may itself act as an adjuvant [26]. The presence of this LPS may, at least partially, be responsible for the better protection we observed after two immunizations with the unadjuvanted plant-derived H7 VLP. Indeed, Klausberger et al. [27] have reported full protection and an absence of any signs of disease in mice vaccinated with two doses of 3 μg HA content of a baculovirus-origin H7 VLP vaccine produced using a very similar platform to that used by Smith et al. [24] and it has been suggested that the presence of baculovirus 'contaminants' may be partially responsible for the superior protection observed by Klausberger et al. [27]. The impact of low biological reactivity LPS contained in the plant-made VLP vaccines on the immune response is currently under investigation.

Human influenza viruses, including A/H7N9, replicate efficiently in the respiratory tract of ferrets without prior adaptation [7,28,29]. Moreover, ferrets infected with human influenza viruses mimic the

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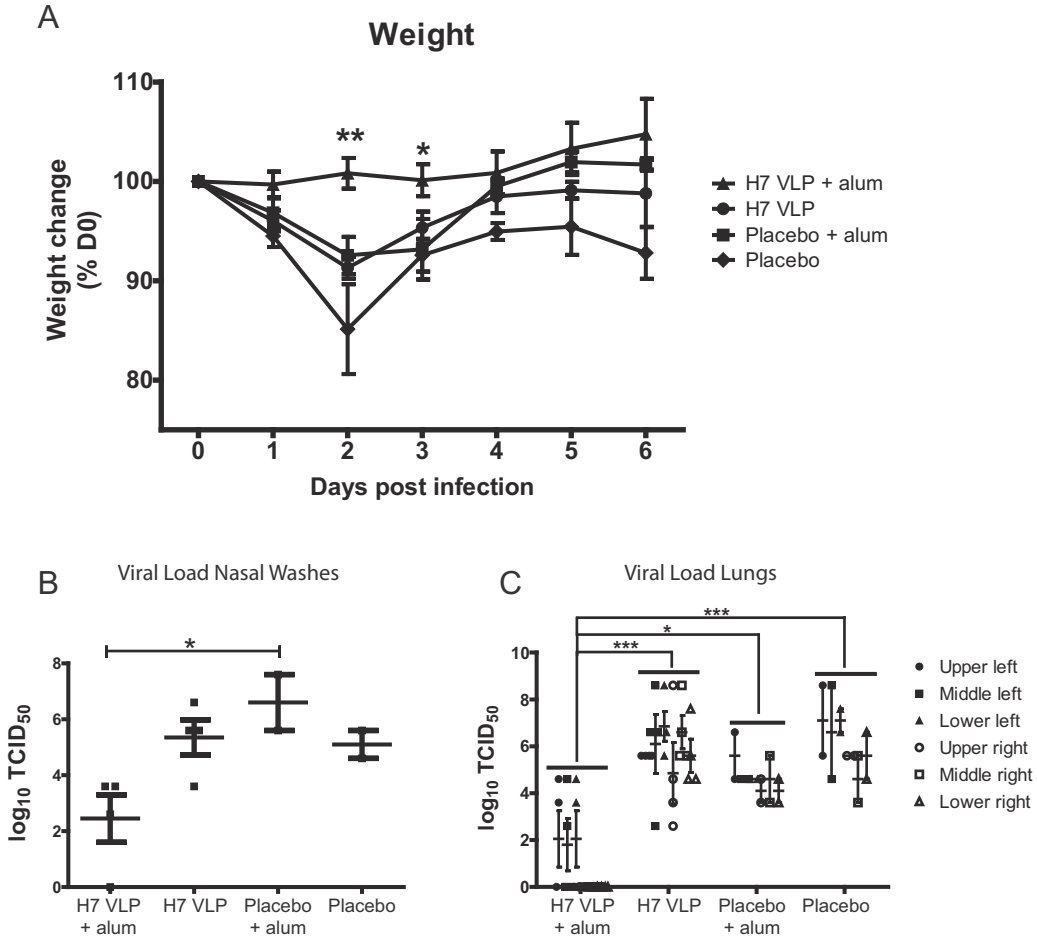

Fig. 5. Protection from H7N9 A/Anhui/1/2013 virus challenge in ferrets. Ferrets immunized with one dose of 15 μg HA of unadjuvanted H7 VLP ($n = 8$, H7 VLP), one dose of 15 μg HA of alum-adjuvanted H7 VLP ($n = 8$, H7 VLP + alum) or adjuvanted ($n = 4$) and unadjuvanted ($n = 4$) placebos were challenged with 10^6TCID$_{50}$ of A/Anhui/1/13 H7N9 virus 28 days after the immunization. (a) Weight loss was monitored during 6 days and data were plotted as percentage of the weight on day 0. Half of the animals from each group were sacrificed 3 days post-infection for viral load quantification in (b) nasal washes and (c) lungs. Left and right lungs were sampled at three different locations (i.e. Upper, Middle and Lower) and viral load was expressed per gram of tissue. Statistical differences were assessed using a one-way ANOVA analysis followed by post-hoc Bonferroni's test. Significant differences are indicated: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

major clinical signs observed during infection in humans [7,28]. This animal model has been proven to be instrumental in the evaluation of vaccine candidates against influenza [30]. Ferrets immunized IM with a single dose of 15 μg HA of alum-adjuvanted plant-derived H7 VLP vaccine had protective HI titers (≥40) as early as 14 days after the IM injection. These values continued to increase, rising to a GMT of 247 twenty-one days after the immunization. A second injection further increased the humoral response illustrating the strong immunogenicity of the adjuvanted vaccine formulation. As reported for H1N1 and H5N1 vaccines, adjuvant appears to be required to induce a detectable humoral immune response in ferrets, even after two doses [31,32].

Most recently, Yan et al. [33] have reported full protection against lethal challenge with H7N9 A/Anhui/1/2013 in mice immunized twice with 25 μg of a DNA vaccine expressing the H7 HA administered by IM injection followed by an *in vivo* electroporation boost. This vaccine regime induces relatively low HI titers but the mice have evidence of strong CMI that may play an important role in the observed protection [33]. In our study, we observed similar protection in mice that had received only one dose of either GLA-SE- or alum-adjuvanted vaccine despite highly variable HI titers. Indeed, all of the mice were protected despite relatively low HI titers (<40) in some animals. There is a growing body of literature reporting full protection against influenza challenge in mice, ferrets and humans despite low and sometimes undetectable HI titers, highlighting the role that CMI plays in mediating protection against influenza infection [34–36]. Although, De Groot's recent bioinformatics analysis predicted that the H7N9 strain might exhibit low immunogenicity and could act as a "stealth" virus due to its low content of T-cell epitopes [37], we measured substantial lymphoproliferation and CD3$^+$ T cell activation in the ferrets that had received one dose of the alum-adjuvanted plant-made H7 VLP vaccine. The ability of epitope-specific T cells to proliferate in response to antigenic stimulation has been identified as an important correlate of vaccine protection and impaired proliferation ability has been associated with an inability to clear viral infections [38,39]. To our knowledge, this represents the first demonstration of a strong, antigen-specific cellular immune response following immunization with plant-made VLP in ferrets. A higher frequency of HA-specific T cell activation after vaccination correlated with better protection against highly virulent H1N1 1918 Spanish influenza virus in ferrets [40]. As abovementioned, there is mounting evidence that T-cells play a key role in controlling influenza infection in humans. The CD8 T cells appear to mediate rapid clearance of influenza virus during the infection which strongly influences the duration of the disease [41]. The CD4 T cells are central components of protective immunity by providing cognate help for B cell affinity maturation and the establishment of memory [42]. The expansion and persistence of CD8 memory cells also benefit by the presence of effective CD4 T cells [35,43,44]. In

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this context, it is interesting that plant-derived H5 influenza VLP has recently been proven to induce strong, long lasting and heterosubtypic CMI in humans [45].

Ferrets can be readily infected with A/H7N9 viruses although this infection is typically non-lethal [29]. The strong humoral and cellular immune responses observed in the ferrets that received one dose of the alum-adjuvanted VLP vaccine in our study was associated with protection from weight loss as well as a significant reduction of viral loads in both nasal washes and lungs 3 days post-infection.

The viral load in nasal washes correlated with virus shedding and transmission while viral replication and colonization of the lower respiratory tract is closely associated with pathogenicity and clinical signs in animal models and humans [46], suggesting that a single dose of the adjuvanted plant-derived H7 vaccine could have a significant impact on both virus transmission and pathogenicity in humans.

The 2009–2010 H1N1 influenza pandemic highlighted some of the limitations of the current vaccine platforms, focusing attention on the need for more rapid and scalable influenza vaccine response capabilities. Herein we describe the rapid development of a candidate H7 plant-based VLP vaccine capable of eliciting a protective immune response following a single dose in mice and ferrets.

Conflict of interest

Brian J Ward has been a principal investigator of vaccine trials for several manufacturers, including Medicago Inc., for which his institution obtained research contracts. Since 2010, Dr. Ward served as Medical Officer for Medicago Inc. In addition, Dr. Ward has held and continues to hold peer-reviewed support from CIHR and other sources for collaborative, basic science work with Medicago Inc. Dr. Ward has received honoraria from several vaccine manufacturers for participation on Scientific Advisory Boards (including Medicago Inc.). Nathalie Landry and Stéphane Pillet are employees of Medicago Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.09.065.

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