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Influenza virus-like particle vaccines made in *Nicotiana benthamiana* elicit durable, poly-functional and cross-reactive T cell responses to influenza HA antigens

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Abstract Cell-mediated immunity plays a major role in long-lived, cross-reactive protection against influenza virus. We measured long-term poly-functional and cross-reactive T cell responses to influenza hemagglutinin (HA) elicited by a new plant-made Virus-Like Particle (VLP) vaccine targeting either H1N1 A/California/7/09 (H1) or H5N1 A/Indonesia/5/05 (H5). In two independent clinical trials, we characterized the $CD4^+$ and $CD8^+$ T cell homotypic and heterotypic responses 6 months after different vaccination regimens. Responses of VLP-vaccinated subjects were compared with placebo and/or a commercial trivalent inactivated vaccine (TIV:Fluzone™) recipients. Both H1 and H5 VLP vaccines elicited significantly greater poly-functional $CD4^+$ T cell responses than placebo and TIV. Poly-functional $CD8^+$ T cell responses were also observed after H1 VLP vaccination. Our results show that plant-made HA VLP vaccines elicit both strong antibody responses and poly-functional, cross-reactive memory T cells that persist for at least 6 months after vaccination.

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1. Introduction

After almost half a century of focusing on antibody responses to influenza, there is increasing interest in revisiting the role of T cell immunity after both influenza infection and vaccination [1,2]. Indeed, T cell memory may be central to understanding both infection- and vaccine-induced immunity in the elderly who often derive significant benefit from vaccination despite little evidence of a humoral response [3]. Although epidemiological studies of influenza-specific human T cell immunity are relatively rare, both $CD4^+$ and $CD8^+$ T cell responses can be readily detected [2,4,5]. Recent evidence suggests that cross-reactive T cells elicited by infection and vaccination can contribute to cross-strain protection in both animal models [6,7] and humans [8,9]. These observations have led to interest in adjuvants [10,11] and alternate delivery systems that can stimulate cellular as well as humoral responses. Virus-like particles (VLPs) vaccines, including VLP made in plants, have these characteristics [12–16].

Despite the growing appreciation that vaccines targeting cell-mediated immunity (CMI) may be critical to developing the next generation of more broadly protective vaccines [17–20], licensure criteria for influenza vaccines in all jurisdictions continue to focus on antibody responses [21]. This approach is based on historical precedent and the fact that T cell responses are difficult and expensive to measure compared to antibody levels. To our knowledge, there are currently no robust cellular response criteria (i.e.: correlate of protection) for any vaccine despite on-going international efforts related to human immunodeficiency virus (HIV) vaccine trials [22,23]. Despite these limitations, there is a growing consensus that long-term memory for monophasic viral infections such as influenza is likely to reside primarily in poly-functional, antigen-specific $CD4^+$ T cells [24,25]. Such cells are even being considered for adoptive immunotherapy in severe influenza [26].

In the context of two early Phase studies of candidate monovalent influenza A VLP vaccines made by transient transfection of *Nicotiana benthamiana* [16,27], we used multi-parameter flow cytometry to assess $CD4^+$ and $CD8^+$ T cell responses in subjects who received either one or two doses of plant-made VLP bearing influenza hemagglutinin (HA) at ~6 months after vaccination. The humoral immune response was assessed by a standard hemagglutinin inhibition assay (HI).

2. Materials and methods

2.1. Production of VLP vaccines

Briefly, whole *N. benthamiana* plants (41–44 days old) were vacuum infiltrated with an *Agrobacterium* inoculum containing the HA from A/California/7/09 (*H1*) or from A/Indonesia/5/05 (*H5*) expression cassette. Six days after infiltration, the aerial parts of the plants were harvested and homogenized in one volume of buffer (50 mM Tris, 150 mM NaCl, 0.04% (w/v) $Na_2S_2O_5$, pH 8.0) per kg biomass. The homogenate was pressed through a 400 μm nylon filter and the fluid was retained. The solution was brought to pH 5.3 ± 0.1 with 5 M acetic acid and heated to 41 ± 2 °C for 15 min to allow aggregation of insoluble material which was then pelleted at room temperature (RT) in a continuous-flow SC6 centrifuge at 1.2 L/min. The supernatant was mixed with diatomaceous earth (1% w/v), adjusted to pH 6.0 ± 0.1 with TRIS base and passed through a 0.45/0.2 micron filter. The extract was then concentrated by tangential flow filtration (TFF) on a 500,000 Da MWCO membrane and diafiltered against 50 mM $NaPO_4$, 500 mM NaCl and 0.005% (v/v) Tween 80 (pH 6.0). Formaldehyde was added to reach 0.0125% final concentration and the remaining insoluble fraction was removed by microfiltration.

This clarified extract was then passed through a Poros HQ column equilibrated at pH 7.5 with 50 mM Tris–HCl −0.01% Tween 80. The flow-through was captured on a Poros HS column equilibrated in 50 mM $NaPO_4$, 0.01% Tween 80 (pH 6.0) (Applied Biosystems, USA). After washing with 50 mM $NaPO_4$, 65 mM NaCl, 0.01% Tween 80 (pH 6.0), the VLPs were eluted with 50 mM $NaPO_4$, 500 mM NaCl, 0.01% Tween 80 (pH 6.0) and then captured on a Poros EP 250 coupled to bovine fetuin (30 mg fetuin/mL Poros EP 250 matrix) (Desert Biologicals, Australia) as recommended by the manufacturer and equilibrated in 50 mM $NaPO_4$, 150 mM NaCl (pH 6.0). The column was washed with 50 mM $NaPO_4$, 400 mM NaCl, (pH 6.0) and the VLPs were eluted first with 1.5 M NaCl, and then water containing 0.0005% Tween 80. The purified VLPs were concentrated by TFF on a 300,000 Da MWCO membrane, diafiltered against formulation buffer (100 mM PO_4, 150 mM NaCl, 0.01% Tween 80 at pH 7.4) and passed through a 0.22 μm filter for sterilization.

2.2. Clinical trials & study subjects

Aspires IRB (Rockville, MD) and IRB Services (Toronto, ON) approved the H1 and H5 study protocols respectively. These studies were conducted according to the Declaration of Helsinki, with written informed consent obtained from all participants. The Phase 1 trial was a randomized, double-blind, placebo-controlled, dose-ranging study to evaluate a single non-adjuvanted dose of an H1 VLP influenza vaccine in healthy adults 18–49 years of age (NCT01302990 at ClinicalTrials.gov)[1]. Subjects (n = 100) received 5, 13 or 28 μg of H1 VLP vaccine, a licensed trivalent vaccine (Fluzone®) or phosphate-buffered saline (PBS) placebo by IM injection into the deltoid muscle (20/group). Serum samples were obtained at day 0 and at 21 and 201 after vaccination for serologic testing (see below). Cellular responses were analyzed as described below on 88 subjects detailed in Table 1. The Phase II trial was a randomized, placebo-controlled, dose-ranging study to evaluate two doses, 21 days apart, of H5 VLP influenza vaccine ± Alhydrogel™ (Cedarlane Laboratory, Burlington, ON) in healthy adults 18–60 years of age (NCT01244867). The study included 255 subjects overall, 195 of whom received the H5 VLP vaccine mixed with Alhydrogel™ (150 at 20 μg H5 VLP/dose and 30 each at 30 or 45 μg H5 VLP/dose). An additional 30 subjects received the 45 μg H5 VLP dose without adjuvant and 45 received placebo (PBS). Serum samples were obtained on day 0, 21 days after each dose and at 228 days after the 1st vaccination. Cellular responses were analyzed as described below on 53 subjects detailed in Table 1.