Clinical Immunology (2014) 154, 164–177

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

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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Received 19 February 2014; accepted with revision 6 August 2014
Available online 14 August 2014

KEYWORDS
Influenza;
Virus-like particle;
Vaccine;
Cell-mediated immunity;
Long-term memory response;
Nicotiana benthamiana

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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http://dx.doi.org/10.1016/j.clim.2014.08.003
1521-6616/

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.

Table 1 Summary of H5 and H1 cohorts used for CMI assessment on cryopreserved PBMC at 6 months post-vaccine. The total number of subjects is indicated for H5 and H1 VLP vaccine cohorts. Groups are defined based on the dose of VLP (μg of HA content) with or without 1% Alhydrogel® as adjuvant (Adj) and compared with placebo controls or those who received a commercial trivalent vaccine (TIV: Fluzone™).

Cohort H5 (N = 53 subjects: 5 groups, ± adjuvants)		Cohort H1 (N = 88 subjects: 5 groups, no adjuvant)	
1. Placebo (No Adj.)	8 subjects	1. Placebo	18 subjects
2. H5 VLP, 45 μg-No Adj.	11 subjects	2. Fluzone™	18 subjects
3. H5 VLP, 20 μg + Adj.	13 subjects	3. H1 VLP, 5 μg	18 subjects
4. H5 VLP, 30 μg + Adj.	7 subjects	4. H1 VLP, 13 μg	16 subjects
5. H5 VLP, 45 μg + Adj.	14 subjects	5. H5 VLP, 28 μg	18 subjects

2.3. Assessment of humoral response

The HI assay was performed as previously described according to the WHO recommendation [27,28]. Homologous antibody responses were evaluated using the H1N1 X-179A reassortant strain for the Phase I (H1) trial. Due to the high pathogenicity of wild-type H5N1 viruses, the reassortant A/Indonesia/5/05 H5N1 (subclade 2.1 strain, CBER) was used for HI testing for the Phase II (H5) trial. An HI titre of 1:40 was used as the 'cut-off' for presumed seroprotection for H1 while a slightly lower titre (1:32) was used to assess H5 responses [29].

2.4. PBMC handling and flow cytometry

PBMC were collected and cryopreserved at 201 or 228 days (Phase I/Phase II study respectively) after the first dose of vaccine/placebo and held in liquid nitrogen until used in assays. Cryopreserved PBMC were quickly thawed with gentle flicking in a water bath at 37 °C, transferred into a conical polypropylene tube and re-suspended in pre-warmed R-20/BENZ medium consisting in RPMI-1640 (Invitrogen, Burlington, ON) supplemented with 20% heat-inactivated fetal bovine serum (hiFBS)(Wisent, St-Bruno, QC), 1% HEPES (Invitrogen), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate (Invitrogen), and 50 U/mL of benzonase (VWR, Ville Mont-Royal, QC). The cell suspension was centrifuged at RT (365 ×*g* for 10 min) and the supernatant was discarded. Pelleted cells were re-suspended at a concentration of 2.5×10^6 viable cells/mL for 6 h at 37 °C/5% CO_2 in RPMI-1640 containing 10% hiFBS, 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, and 10 mM HEPES. Following this resting period, cells were distributed in 96-well round bottom plate at 1.5×10^6 viable cells per well and stimulated with VLP for 20 h, or left without stimulation overnight prior to a 6 h exposure to 2.5 μg/ml of H1 or H5 peptide pools consisting in 14–15 mer peptides overlapping by 11 amino acids. The H1 and H5 peptide pools were provided by the Biodefense and Emerging Infection Research Resources Repository (Manassas, VA) and GenScript, (Piscataway, NJ) respectively. A 6 h stimulation with 5 ng/mL PMA (Sigma) and 500 ng/mL ionomycin (Invitrogen) stimulation was included as a positive control. Anti-human CD107a antibody (BD Pharmingen, Mississauga, ON) was added at the beginning of the stimulations as recommended by Betts et al. [30]. Expression of CD107a due to degranulation is a functional marker associated with cell-mediated cytotoxicity [31]. Five hours before the end of these incubations, 5 μg/mL of monensin and brefeldin A (Golgi blockers, BD Biosciences, Mississauga, ON) was added to the wells to inhibit protein secretion. Following ex vivo stimulation, cells were stained with LIVE/DEAD fixable Aqua Dead cell Stain (Invitrogen), anti-human CD3-V450 (BD Horizon, BD Biosciences), CD4-phycoerythrin-TexasRed (Beckman Coulter, Mississauga, ON), CD8-eFluor650 NC (eBioscience, San Diego, CA), and CD45RA-allophycocyanin-H7 (BD Pharmingen) for 30 min at 4 °C in the dark. Cells were then washed twice with cold PBS and fixed with Cytofix/Cytoperm (BD Biosciences) solution according manufacturer's instruction followed by 3 washes with Perm/Wash buffer (BD Biosciences). Intracellular cytokines were detected by staining with anti-human IFN-γ-phycoerythrin-cyanine7 (BD Pharmingen), IL-2-Alexa700 (Biolegend, San Diego, CA), TNF-α-allophycocyanin (BD Biosciences) and IL-17-peridinin chlorophyll protein-cyanine5.5 (eBioscience) in the Perm/Wash buffer for 30 min at 4 °C in the dark. Cells were then washed and re-suspended in PBS supplemented with 2% hiFBS + 2 mM EDTA and kept at 4 °C until data acquisition (within 6 h) on BD LSR II flow cytometer (BD, Franklin Lakes, NJ). Approximately 0.5×10^6 events were acquired and data were analyzed using FlowJo™ (Tree Star, OR) and SPICE (http://exon.niaid.nih.gov/spice) software. The total cell viability was always >85% and analyses were only performed on AQUA dye negative, live cells. SPICE-based functional analysis was performed on background-subtracted values from non-stimulated samples.

2.5. Statistical analysis

Unless otherwise specified, groups were compared by the Kruskal–Wallis non parametric one-way ANOVA followed by Dunn's Multiple Comparison Test using Prism™ Software (version 6.0, GraphPad Software, La Jolla, CA).

3. Results

3.1. Antibody response to H1 and H5 VLP vaccines

The humoral responses to one dose of 5, 13 or 28 μg of the plant-made H1 VLP vaccine without adjuvant or 45 μg of trivalent Fluzone® (15 μg per strain) or placebo are shown in Table 2. At enrolment, 34.5% of the subjects had pre-existing HI titers thought to indicate seroprotection (≥1:40), presumably due to exposure to natural influenza or prior vaccination (range 26.3–44.4%). Twenty-one days after

Table 2 Serum hemagglutination inhibition antibody response against A/H1N1/California/07/2009 at baseline and after the vaccination, by dose group.

	Placebo	5 μg H1 VLP vaccine	13 μg H1 VLP vaccine	28 μg H1 VLP vaccine	45 μg Fluzone®
Baseline	n = 19	n = 18	n = 19	n = 19	n = 18
Subjects with HI titer ≥1:40 (%) (95%CI)	26.3 (9.1–51.2)	33.3 (13.3–59.0)	42.1 (20.3–66.5)	26.3 (9.1–51.2)	44.4 (21.5–69.2)
Geometric mean titer (95%CI)	19.9 (10.1–39.3)	17.5 (7.9–38.5)	20.7 (10.1–42.6)	13.4 (7.2–25.0)	26.7 (12.8–55.6)
21 days after vaccination	n = 19	n = 18	n = 19	n = 19	n = 18
Subjects with HI titer ≥1:40 (%) (95%CI)	31.6 (12.6–56.6)	88.9 (65.3–98.6)	89.5 (66.9–98.7)	94.7 (74.0–99.9)	100 (81.5–100)
Subjects with seroconversion [a] (%) (95%CI)	5.3 (0.1–26.0)	72.2 (46.5–90.3)	68.4 (43.3–87.4)	89.5 (66.9–98.7)	94.4 (72.7–99.9)
Geometric mean titer (95%CI)	21.5 (10.7–43.0)	290.4 (139.1–606.3)	218.3 (102.4–465.3)	543.6 (224.6–1315.9)	870.9 (540.9–4102.1)
Mean geometric increase	1.1	16.6	10.5	40.6	32.7
6 months after vaccination	n = 17	n = 18	n = 16	n = 17	n = 17
Subjects with HI titer ≥1:40 (%) (95%CI)	29.4 (10.3–56.0)	88.9 (65.3–98.6)	75.0 (47.6; 92.6)	76.5 (50.1–93.2)	100 (80.5–100.0)
Subjects with seroconversion [a] (%) (95%CI)	5.9 (0.1–28.7)	66.7 (41.0–86.7)	50.0 (24.7–75.3)	70.6 (44.0–89.7)	94.1 (71.3–99.9)
Geometric mean titer (95%CI)	20.4 (9.0–46.3)	148.2 (72.7–302.4)	99.2 (48.0–205.1)	230.5 (86.1–616.7)	361.5 (226.6–576.8)
Mean geometric increase	1.0	8.5	4.8	17.2	13.6

[a] Seroconversion is defined as ≥4-fold from baseline or a final HI titer ≥1:40 when there is no detectable titer at baseline.

vaccination, 91.03% and 100% of the subjects who had received either the plant-made H1 VLP vaccine or Fluzone® respectively were considered to be seroprotected. There were no significant differences between the vaccine groups for seroconversion or seroprotection at 21 days and all vaccine groups were statistically different from the placebo group by two-tailed Fisher's exact test (p values < 0.001). Six months after vaccination, 80.1% of the H1 VLP vaccine recipients and 100% of the Fluzone recipients still had seroprotective antibody titers (P = 0.1016).

The humoral responses to two doses of 20, 30 or 45 μg mixed with Alhydrogel or 45 μg of H5 VLP without adjuvant or placebo given 21 days apart are shown in Table 3. At baseline, no subject had a detectable HI titer for the H5N1 strain (data not shown). After the first dose, none of the placebo recipients and only a small proportions of subjects who received any vaccine mounted a response ≥1:32 (range 6.7–10.7%). After the second dose, 53.3% of the subjects who had received the adjuvanted H5 VLP vaccine had seroprotective titers (range 46.7–58.6%) compared to only 21.4% among those who received the unadjuvanted vaccine Although there is no statistical significance in GMT titers between the adjuvanted dosages and the unadjuvanted vaccine, a trend seems to point towards the better immunogenicity of the adjuvanted dosages, with the 20 μg dosage having the lower *p* value (20 μ P = 0.170; 30 μg P = 0.289; 45 μg P = 0.548). Six months after the boost, only 8.8% of the H5 VLP vaccinated subjects (±adjuvant) still had seroprotective titers (range 6.7–10.7%).

3.2. Analysis of T cell response

Long-term T cell responses were evaluated in subsets of the H5 and H1 study subjects (n = 53 and 88 respectively). Groups of 7–18 subjects vaccinated with different doses ± adjuvant were compared to placebo controls as detailed in Table 1. For the H1 VLP trial, a commercial comparator was included (2009 Fluzone®). The magnitude and functionality of the specific cellular responses to H1 and H5 were determined using intracellular cytokine detection by flow cytometry on $CD4^+$ and $CD8^+$ T cell subpopulations after stimulation with cognate influenza H1 and H5 peptide pools or VLPs. Functional markers assessed included CD107a, IFN-γ, TNF-α, IL-2 and IL-17. A "responsive cell" is defined as a positive cell by flow cytometry analysis for at least one of these functional markers.

Table 3 Serum hemagglutination inhibition antibody response against A/H5N1/Indonesia/5/2005 after first and second vaccination, by dose group.

	Placebo	20 μg + Al H5 VLP vaccine	30 μg + Al H5 VLP vaccine	45 μg + Al H5 VLP vaccine	45 μg H5 VLP vaccine
After first vaccination	n = 14	n = 30	n = 29	n = 30	n = 28
Subjects with HI titer ≥1:32 (%) (95%CI)	0.0 (0.0–23.2)	6.7 (0.8–22.1)	0.0 (0.0–11.9)	10.0 (2.1–26.5)	10.7 (2.3–28.2)
Subjects with seroconversion [a] (%) (95%CI)	0.0 (0.0–23.2)	6.7 (0.8–22.1)	0.0 (0.0–11.9)	10.0 (2.1–26.5)	10.7 (2.3–28.2)
Geometric mean titer (95%CI)	4.0 (4.0–4.0)	5.5 (4.2–7.3)	5.1 (4.3–6.1)	5.9 (4.1–8.5)	5.4 (3.7–7.7)
Mean geometric increase (95%CI)	1.0 (1.0–1.0)	1.4 (1.1–1.8)	1.3 (1.1–1.5)	1.5 (1.0–2.1)	1.4 (0.9–1.9)
After second vaccination	n = 13	n = 29	n = 28	n = 30	n = 28
Subjects with HI titer ≥1:32 (%) (95%CI)	0.0 (0.0–24.7)	58.6 (38.9–76.5)	53.6 (33.9–72.5)	46.7 (28.3–65.7)	21.4 (8.3–41.0)
Subjects with seroconversion [a] (%) (95%CI)	0.0 (0.0–24.7)	58.6 (38.9–76.5)	53.6 (33.9–72.5)	46.7 (28.3–65.7)	21.4 (8.3–41.0)
Geometric mean titer (95%CI)	4.0 (4.0–4.0)	24.3 (14.6–40.5)	22.4 (14.0–35.8)	19.3 (12.1–30.5)	11.9 (7.3–19.5)
Mean geometric increase (95%CI)	1.0 (1.0–1.0)	6.1 (3.7–10.1)	5.6 (3.5–9.0)	4.8 (3.0–7.6)	3.0 (1.8–4.9)
6 months after vaccination	n = 13	n = 28	n = 28	n = 30	n = 27
Subjects with HI titer ≥1:32 (%) (95%CI)	0.0 (0.0–24.7)	10.7 (2.3–28.2)	10.7 (2.3–28.2)	6.7 (0.8–22.1)	7.1 (0.9–23.5)
Subjects with seroconversion [a] (%) (95%CI)	0.0 (0.0–24.7)	10.7 (2.3–28.2)	10.7 (2.3–28.2)	6.7 (0.8–22.1)	7.1 (0.9–23.5)
Geometric mean titer (95%CI)	4.9 (3.6–6.6)	9.4 (6.8–12.8)	7.7 (5.7–10.4)	8.2 (6.1–11.2)	6.3 (4.7–8.5)
Mean geometric increase (95%CI)	1.1 (1.0–1.3)	1.0 (0.8–1.1)	0.9 (0.8–1.0)	0.8 (0.7–1.0)	1.0 (0.9–1.1)

[a] Seroconversion is defined as a ≥4-fold from baseline or an HI titer ≥1:32 when there is no detectable titer at baseline.

3.2.1. H5 VLP vaccine elicits a long-term CD4⁺ T cell response to H5N1 HA antigen

PBMCs were stimulated ex vivo with the H5 VLP or a peptide pool covering 91% of the HA protein sequence. The correlation between the number of CD4⁺ responsive cells to the H5 VLP and the peptide pool was excellent although of higher magnitude with the former stimulus (correlation coefficient 0.75: Fig. 1A). These results strongly suggest that the observed CD4⁺ responses are specific to the H5 influenza virus HA protein. Six months after H5 VLP immunization, we observed a clear CD4⁺ T cell response ranging from 250 to 10,000 responsive cells per million CD4⁺ T cells (Figs. 1A & B). There were no significant differences in the magnitude of the CD4⁺ T cell responses between the adjuvanted vaccine groups (data not shown). All data from the patients who received 20, 30 or 45 μg of the adjuvanted vaccines were therefore grouped and compared to placebo and unadjuvanted vaccine groups. Based on a putative threshold of biological CMI response determined by placebo non-responders, readily-detectable CD4⁺ T cell responses were observed in 31/34 (91%) of those who had received an adjuvanted vaccine and in 9/11 (82%) of the non-adjuvanted vaccine recipients (Fig. 1B). Interestingly, 2/8 (25%) placebo recipients also had detectable CD4⁺ T cell responses. Patients who received any dose of the adjuvanted vaccine had significantly higher CD4⁺ T cell responses compared to the non-adjuvanted high dose (45 μg) and placebo groups (Fig. 1B). Not surprisingly given the timing of PBMC collection, most of the CD4⁺ T cell responses observed were found in the memory pool (CD45RA⁻: Fig. 1C).

The global functional T cell signatures based on expression of IL-2, TNF-α and IFN-γ are presented in Fig. 2 comparing placebo to other vaccine groups. The frequencies of all possible combinations of mono- or poly-functional IL-2, TNF-α and IFN-γ CD4⁺ cells are represented in the bar graph (Fig. 2, upper panel). Notably, we observed a significant increase of poly-functional IL-2⁺/TNF-α⁺ and, to a lesser extent, single positive TNF-α⁺ and IL-2⁺ CD4⁺ responding cells in adjuvanted H5 vaccine groups (Fig. 2, upper panel). The pie-charts represent the qualitative distribution of the functional response, the size of each chart being proportional to the magnitude of the CD4⁺ response (Fig. 2, lower panel). The permutation analysis (Fig. 2, Table), suggested significant differences in the qualitative distribution of the functional response between the placebo and adjuvanted H5 vaccine groups. No significant CD8⁺ T cell responses were detected in any of the H5 VLP vaccinated groups compared to placebo even though some of the vaccinated subjects had detectable responses (data not shown).

3.2.2. H1 VLP vaccine elicits long-term CD4⁺ and CD8⁺ T-cell responses to H1N1 HA antigen

CD4⁺ T cell responses were detected in the H1 VLP vaccinated groups at low frequencies varying from 100 to 1000 responding cells per million CD4⁺ T cells (Fig. 3). Although widely distributed, the magnitude of CD4⁺ T responsive cells were significantly higher in the H1 VLP groups (all doses) than the placebo group ($P \leq 0.05$) and were comparable to the active comparator (Fluzone) (Fig. 3). This effect held true for responses induced by ex vivo stimulation with both H1 VLP and the H1 peptide pool (Figs. 3A & B). The overall prevalence of detectable responses was about 40% in the placebo group, 50% with the Fluzone comparator and 70%

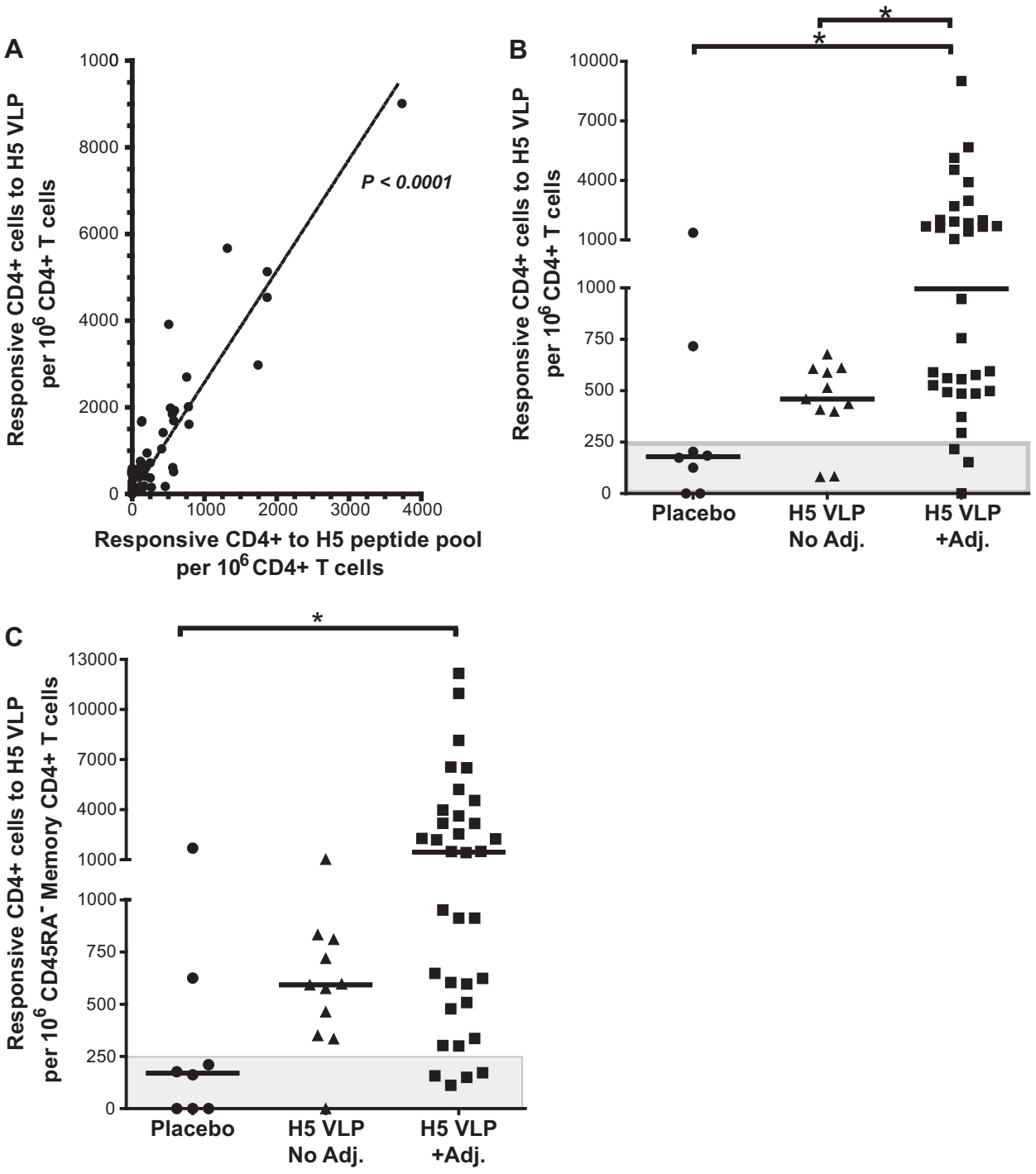
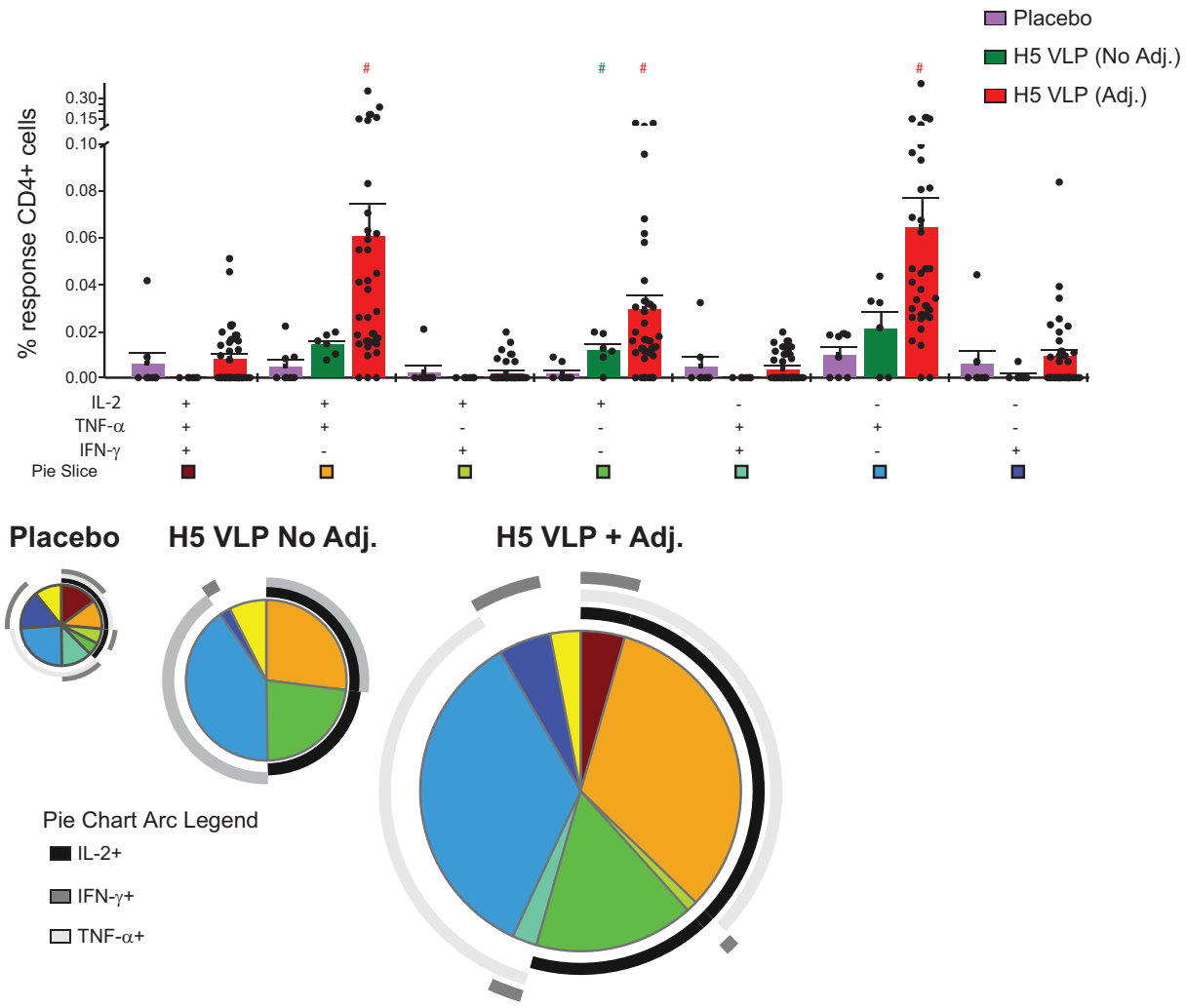


Figure 1 $CD4^+$ T cell responses from the H5 VLP-vaccinated patients (n = 53). Data are derived from SPICE-based functional analysis on background-subtracted values from non-stimulated samples that are summed-up and presented as "total" H5 VLP responsive cells per million $CD4^+$ T cells. Responsive cells are defined as positive cells by flow cytometry analysis for at least one of the functional markers CD107a, IFN-γ, TNF-α, IL-2 and IL-17. (A) Correlation between responses observed in $CD4^+$ T cells stimulated ex vivo with H5 VLP or the H5 peptide pool. (B) Comparison between the responses of CD4+ T cells from the Placebo group (dot), the H5 VLP 45 μg group without adjuvant (triangle) and the H5 VLP vaccinated groups (20, 30, 45 μg) with adjuvant (square) after ex vivo stimulation with H5 VLP. c Comparison between the responses of $CD4^+CD45RA^-$ memory T cells from the Placebo group (dot), the H5 VLP 45 μg group without adjuvant (triangle) and the H5 VLP vaccinated groups (20, 30, 45 μg) with adjuvant (square) after ex vivo stimulation with H5 VLP. The symbol * indicates statistically significant ($P \leq 0.05$) differences between groups (Kruskal–Wallis one-way ANOVA followed by Dunn's Multiple comparison test). The lower gray rectangle on the lower of the graph indicates a putative threshold of biologically significant CMI based on ROC analysis (data not shown), assuming that negative placebo (6/8) should have negative CMI responses.



Pie Category Test Results	Placebo	H5 VLP No Adj.	H5 VLP + Adj.
Placebo			
H5 VLP No Adj.	0.3742		
H5 VLP + Adj.	0.0236	0.7235	

Figure 2 Qualitative analysis of functional markers in total CD4⁺ T cells from the H5 VLP-vaccinated patients (n = 53) ex vivo stimulated with H5 VLP. The three functional markers IL-2, TNF-α and IFN-γ were detailed. The % functional response within CD4⁺ T cells (Y-axis) is shown on the bar graph representation (higher panel) with the mean (bar) and individual results (dots) represented for all possible combination of 1 to 3 functional markers and for each of the 3 groups (Placebo, H5 VLP No Adj. and H5 VLP + Adj.). The symbol # indicates statistically significant differences ($P \leq 0.05$, Wilcoxon rank test) from Placebo for each functional signature. The distribution of functional responses is highlighted in color-matched pie charts (lower panel) based on each functional signature following the color code on the bottom of the bar graph X-axis. The size of the charts is proportional to the magnitude of the CD4⁺ response. The arcs represent the different functional markers. The permutation analysis (table) highlighted the significant differences ($P \leq 0.05$) in the distribution of the functional response between the 3 treatment groups.

with the H1 VLP vaccine. The response of memory CD4⁺ in the VLP vaccinated subjects remained slightly higher than in the placebo group but the difference did not reach statistical significance (Fig. 3C). Due to technical problems resulting in aberrantly high background, TNF-α single positive cells had to be removed from the detailed analysis of the IL-2, TNF-α and IFN-γ signature of CD4⁺ responsive cells. Nevertheless, and similar to our observations with the H5 VLP vaccine, the functional profiling of the H1 VLP vaccine group revealed significantly more poly-functional IL-2⁺/TNFα⁺ (+/−IFNγ⁺) H1 responding cells in the H1 VLP vaccine than either placebo or Fluzone recipients (Fig. 4).

In contrast to the H5 VLP cohort, CD8⁺ T cell responses were clearly detectable in the H1 VLP cohort. Indeed, these responses occurred at higher frequencies than CD4⁺ T cells ranging from 50 to 2000 responding cells per million CD8⁺ T cells. The CD8⁺ response of patients immunized with Fluzone was significantly higher than placebo group (Figs. 5A & B).

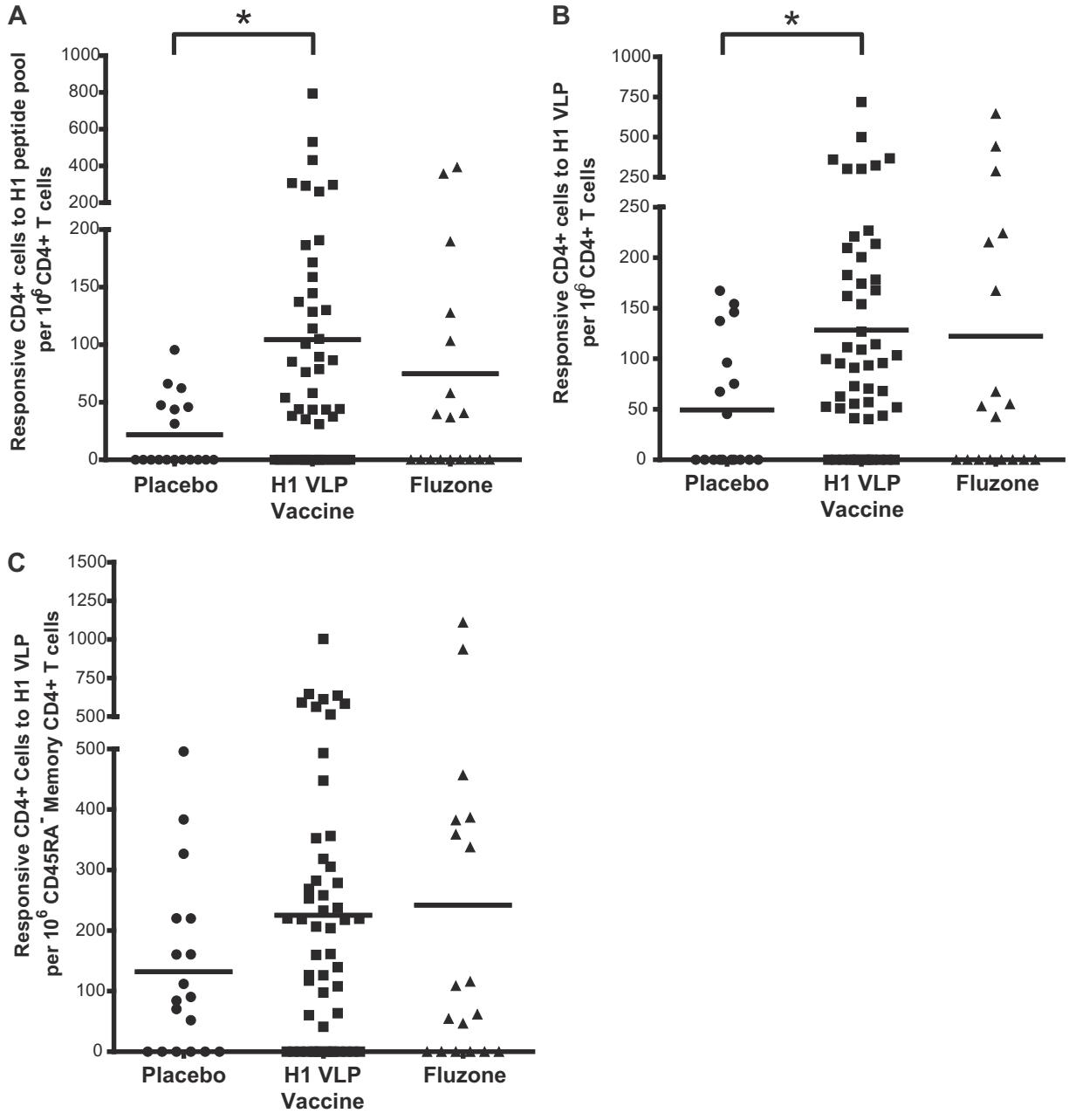
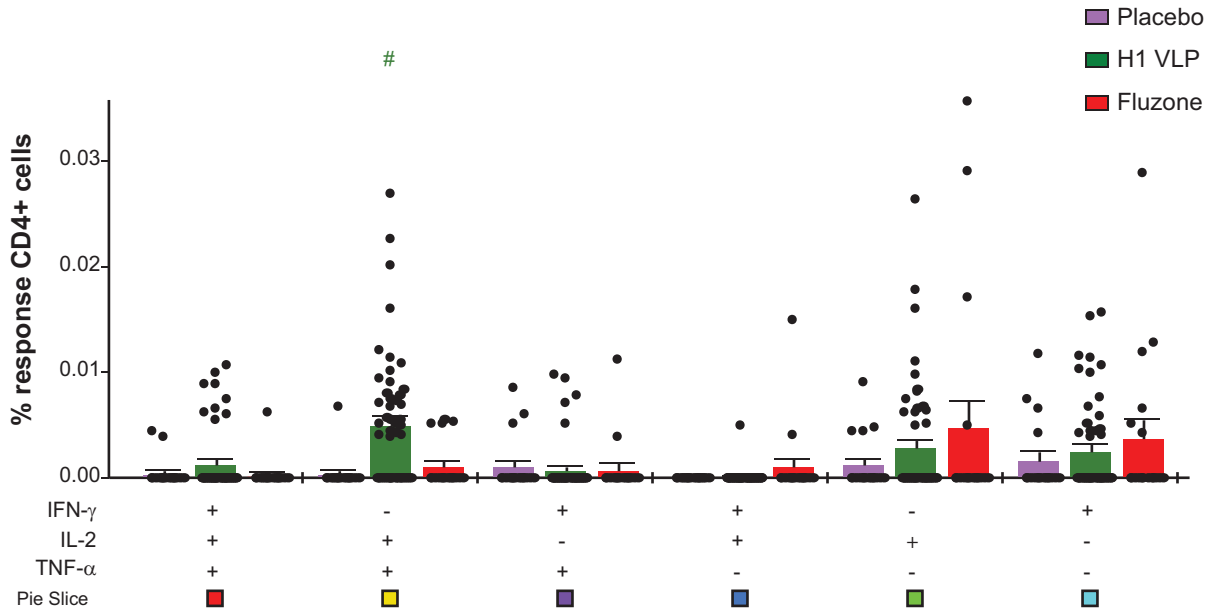
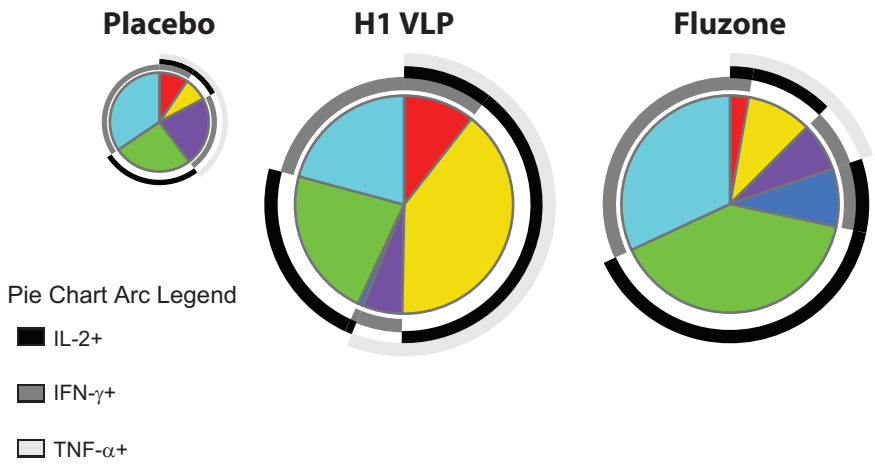


Figure 3 $CD4^+$ T cell responses from the H1 VLP-vaccinated patients (n = 88). Data are derived from SPICE-based functional analysis on background-subtracted values from non-stimulated samples that are summed-up and presented as "total" H1 VLP responsive cells per million $CD4^+$ T cells. Responsive cells are defined as positive cells by flow cytometry analysis for at least one of the functional markers CD107a, IFN-γ, TNF-α, IL-2 and IL-17. Comparison between the responses of $CD4^+$ T cells from the Placebo group (dot), the H1 VLP vaccinated group (5, 13, 28 μg) no adjuvant (square) and to the Fluzone comparator group (triangle) after ex vivo stimulation with (A) H1 peptide pool or (B) H1VLP. (C) Comparison between the responses of $CD4^+CD45RA^-$ memory T cells from the Placebo group (dot), the H1 VLP vaccinated group (5, 13, 28 μg) no adjuvant (square) and to the Fluzone comparator group (triangle) after ex vivo stimulation with H1 VLP. The symbol * indicates statistically significant ($P \leq 0.05$) differences between groups (Kruskal–Wallis one-way ANOVA followed by Dunn's Multiple comparison test).

Although widely distributed, the magnitude of the $CD8^+$ T cell response in the H1 VLP group was slightly higher than in the placebo recipients but the difference did not reach statistical significance. Responses were similar following ex vivo stimulation with either H1 VLP or the H1 peptide pool, suggesting that the measured $CD8^+$ response is mainly directed against the HA protein (Figs. 5A & B). However, the $CD8^+$ response to the H1 VLP was not qualitatively different from placebo (data not shown). The overall prevalence of detectable $CD8^+$ responses was ~60% with the H1 VLP vaccine versus 50% and 70% in the placebo and the Fluzone groups respectively.





Pie Category Test Results	Placebo	H1 VLP	Fluzone
Placebo			
H1 VLP	0.0042		
Fluzone	0.8281	0.0276	

Figure 4 Qualitative analysis of functional markers in total CD4$^+$ T cells from the H1 VLP-vaccinated patients (n = 88) ex vivo stimulated with H1 VLP. The three functional markers IL-2, TNF-α and IFN-γ were detailed. The % functional response within CD4$^+$ T cells (Y-axis) is shown on the bar graph representation (higher panel) with the mean (bar) and individual results (dots) represented for all possible combination of 1 to 3 functional markers and for each of the 3 groups (Placebo, H1 VLP and Fluzone). The symbol # indicates statistically significant differences ($P \leq 0.05$, Wilcoxon rank test) from Placebo for each functional signature. The distribution of functional responses is highlighted in color-matched pie charts (lower panel) based on each functional signature following the color code on the bottom of the bar graph X-axis. The size of the charts is proportional to the magnitude of the CD4$^+$ response. The arcs represent the different functional markers. The permutation analysis (table) indicated significant differences ($P \leq 0.05$) in the distribution of the functional response between the 3 treatment groups. Note: Due to technical problems resulting in a high background, TNF-α single positive cells were removed from this analysis.

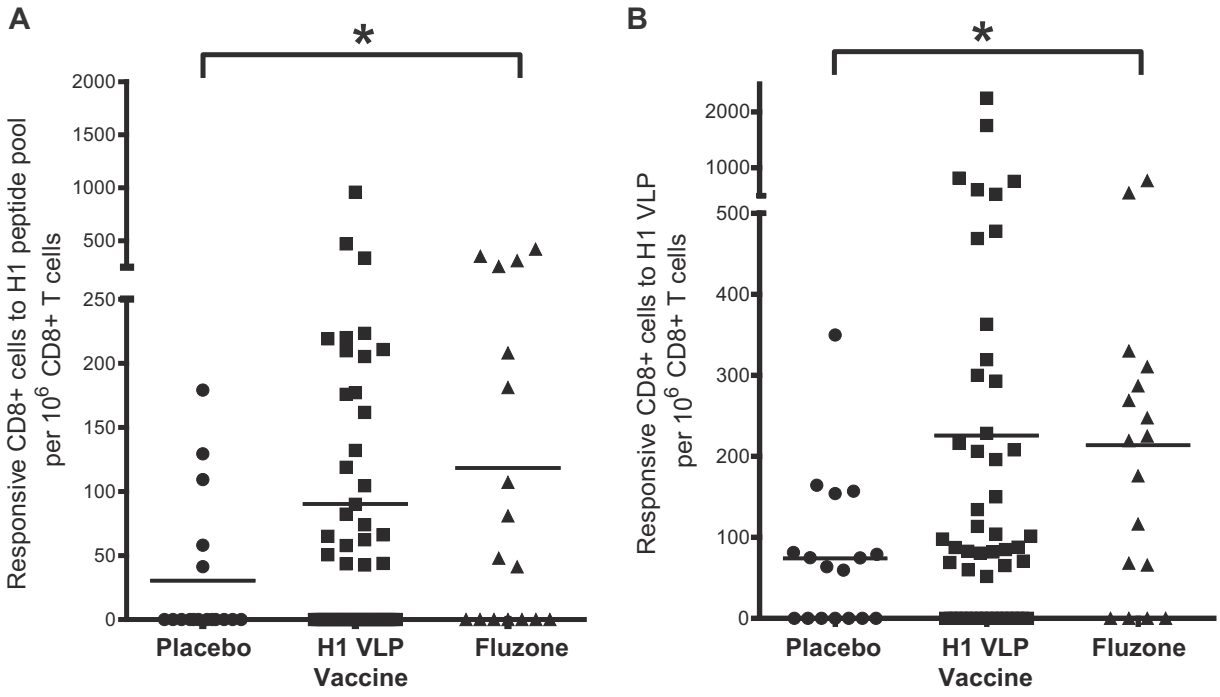


Figure 5 $CD8^+$ T cell responses from the H1 VLP-vaccinated patients (n = 88). Data are derived from SPICE-based functional analysis on background-subtracted values from non-stimulated samples that are summed-up and presented as "total" H1 VLP responsive cells per million $CD4^+$ T cells. Responsive cells are defined as positive cells by flow cytometry analysis for at least one of the functional markers CD107a, IFN-γ, TNF-α, IL-2 and IL-17. Comparison between the responses of $CD4^+$ T cells from the Placebo group (dots), the H1 VLP vaccinated group (5, 13, 28 μg) no adjuvant (square) and the Fluzone comparator group (triangle) after ex vivo stimulation with (A) H1 peptide pool or (B) H1VLP. The highest responders from each group were considered as outliers in this analysis and removed for statistical analysis. The symbol * indicates statistically significant differences between two-group groups analyzed by the Mann–Whitney test ($P \leq 0.05$).

3.2.3. H5 and H1 VLP vaccines elicit cross-reactive $CD4^+$ T cells

Using the heterologous VLP or peptide pools for ex vivo stimulation of PBMC, we found that recipients of the adjuvanted H5 VLP vaccine mounted cross-reactive $CD4^+$ T cell responses against H1 VLP compared to the placebo group (Fig. 6A). Similar cross-reactive responses were detectable in both $CD4^+$ and $CD8^+$ T cells following H5 VLP stimulation of PBMC isolated form in H1 VLP recipients (Figs. 6B & C). The cross-reactive $CD4^+$ cell response in the H1 VLP recipients was significantly higher that both placebo and Fluzone groups (Fig. 6B). Similar trends for the cross-reactive $CD8^+$ T cell response in the H1 VLP recipients did not reach statistical significance (Fig. 6C).

4. Discussion

It has long been believed that inactivated vaccines are weak inducers of cellular responses and are particularly limited in their capacity to generate $CD8^+$ T cell responses [32]. This dogma has shifted in recent years with the recognition that the appropriate combination of stimuli can drive strong T cell responses, in part through cross-presentation of antigens by dendritic cells [33]. Our data strongly suggest that plant-made VLPs can provide such stimuli for influenza HA antigens.

The last two decades have seen a growing awareness of the global threat posed by influenza A viruses [34]. These events have focused attention both on pandemic preparedness (e.g.: vaccine supply, timeliness of vaccine manufacturing) and on what constitutes a 'successful' immune response to influenza vaccination (e.g.: vaccine efficacy across the age range). After more than half a century of stagnation (i.e.: egg-based vaccines delivered IM), the last few years have witnessed a remarkable expansion in production platforms (e.g.: tissue culture, recombinant), formulations (e.g.: live attenuated, single protein HA), delivery routes (e.g.: IN, ID) and adjuvants (e.g.: MF59, AS03). Furthermore, this short list refers only to licensed products. If investigational vaccines are included, the range of innovations is truly remarkable. In parallel with this renaissance of interest in influenza vaccines, the scientific community has gained a much deeper understanding of immune responses to both influenza infection and vaccination. It is now clear that the historical emphasis on optimizing humoral responses [21] has hindered the development of more effective vaccines. This is particularly true for the elderly who derive significant benefit from vaccination despite the fact that they often make little to no antibody response. It is now clear that the cellular rather than humoral responses are central to successful vaccine-induced immunity in the elderly [3,35]. Indeed, there is growing evidence that T cell responses can be cross-reactive and confer significant benefit across the entire age range [36,37].

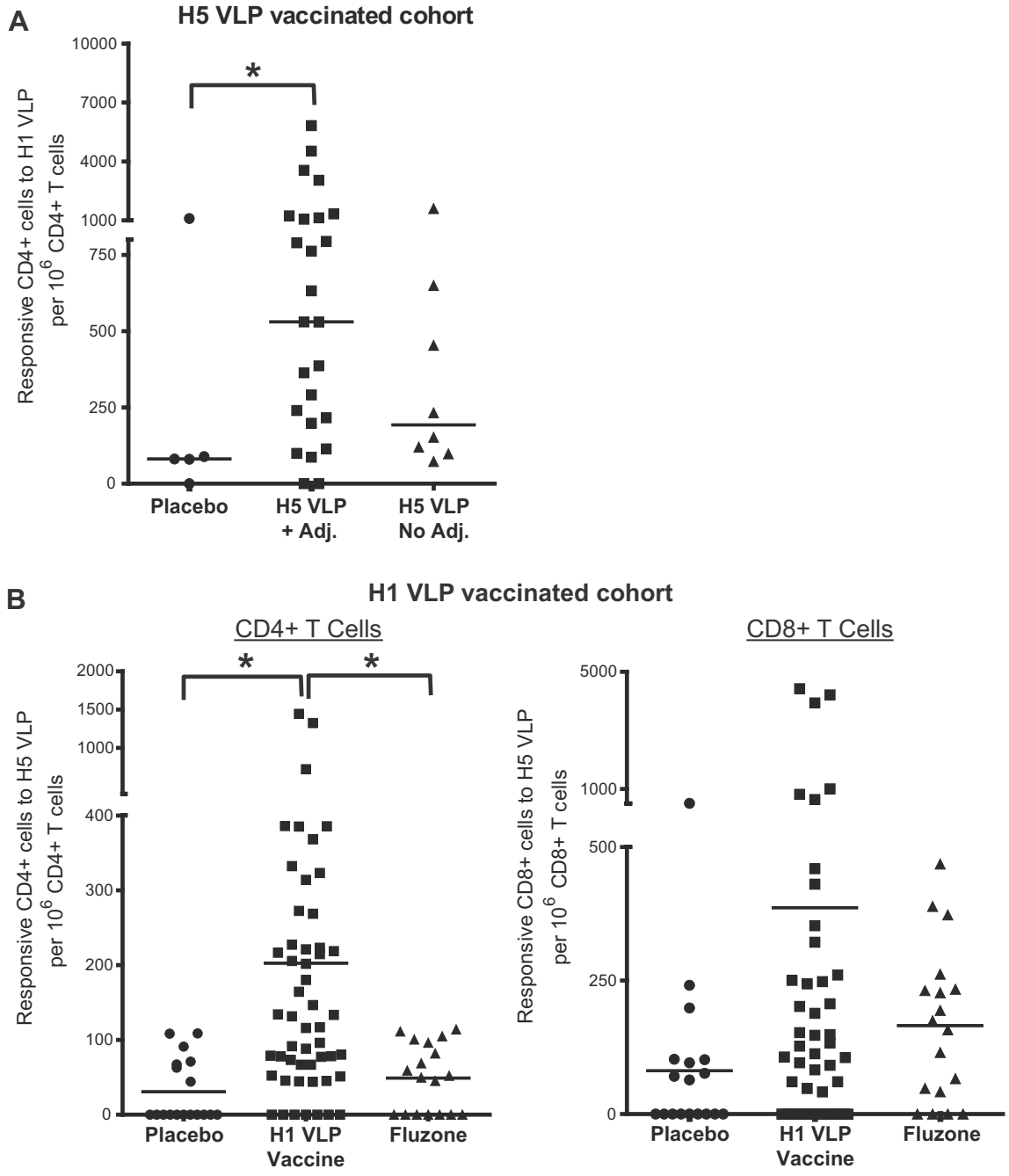


Figure 6 Cross-reactive responses. (A) $CD4^+$ T cells from the H5 VLP-vaccinated patients ex vivo stimulated with H1 VLP. (B) $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell responses from the H1 VLP-vaccinated patients ex vivo stimulated with H5 VLP. Comparison between the responses of the Placebo group (dots), the H1 VLP vaccinated group (5, 13, 28 μg) no adjuvant (square) and to the Fluzone comparator group (triangle). Data are derived from SPICE-based functional analysis on background-subtracted values from non-stimulated samples that are summed-up and presented as "total" H5 VLP responsive cells per million $CD4^+$ or $CD8^+$ T cells. Responsive cells are defined as positive cells by flow cytometry analysis for at least one of the functional markers CD107a, IFN-γ, TNF-α, IL-2 and IL-17. The symbol * indicates statistically significant differences between two-groups analyzed by the Mann–Whitney test ($P \leq 0.05$).

In both pre-clinical and clinical work to date [16,27,38], we have shown that our plant-made VLP vaccines bearing influenza HA can elicit antibody responses that are comparable to those induced by commercial trivalent inactivated vaccines (TIV) including Fluzone. The humoral response reported during the Phase II trial with the plant-made H5 VLP vaccine is very comparable to our previous data [27]. In our pre-clinical work, particularly with the H5 antigen that is notorious for low (antibody) immunogenicity [39], we were surprised to find that animals (e.g.: mice, ferrets) could be fully protected despite modest or even undetectable antibody titers. In some cases, these VLP-vaccinated animals

were even protected from heterotypic challenge [27] (Landry et al., unpublished data). Similar findings have been reported by other groups in both animal models and human challenge studies [27,36,37,39–41].

In the current work we demonstrated that, in addition to efficient antibody responses, these plant-made VLP vaccines elicit readily detectable and durable poly-functional T cellular responses in humans. Although most of the work on T cell responses to date has focused to conserved epitopes on internal influenza proteins like the nucleoprotein (NP) and the M proteins, recent data suggest that the influenza HA molecule contains class I and class II restricted epitopes able to induce cross-reactive T cells [42–45]. While we do not know the specific H1 or H5 peptides responsible for the T cell responses observed in the current study, our data support the growing consensus that epitopes on influenza HA can elicit cross-reactive responses in both $CD4^+$ and $CD8^+$ T cell subsets (Fig. 6).

Given the timing of sampling (six months after vaccination) it is not surprising that $CD4^+$ T cell responses (H1 and H5 studies) were more readily detected that $CD8^+$ responses (H1 study only) and that most of the responding $CD4^+$ T cells were $CD45RA^-$ memory cells (Figs. 1C & 3C). While H1 plant-made VLP vaccine elicited a humoral response comparable to Fluzone, significant qualitative differences were identified in the CMI response elicited by the 2 vaccines. Compared to Fluzone, the plant-made VLP vaccine clearly induced more poly-functional $CD4^+$ T-cells. Furthermore, while the numbers of H1-responsive $CD4^+$ cells (per million $CD4^+$ T-cells) were comparable between the 2 vaccines, the functional profiles were significantly different as pointed out by the permutation analysis (Fig. 4). This T cell response induced by VLP vaccination was therefore quite distinct from that elicited by a classic TIV produced in egg (e.g.: Fluzone) [46]. The effectiveness of the plant-made VLP vaccines appears to rely more on CMI, as illustrated by the protection of H5 VLP-vaccinated ferrets from challenge with $10LD_{50}$ of heterologous virus despite the very low HI titers [27]. The fact that the plant-made vaccines induced strong poly-functional $CD4^+$ T cell responses is particularly significant. The induction of such poly-functional T cells is thought to be highly desirable [47]. Antigen-specific $CD4^+$ T-cells have been shown to play a major role in mediating protective immunity against influenza virus, in part by providing cognate help to B-cells, a prerequisite for immunoglobulin switch, affinity maturation and the establishment of B-cell memory [48]. Of particular interest, the plant-made VLP vaccines induced a high frequency of IL-2^+/TNF-α^+ $CD4^+$ T cells (Figs. 2 & 4). Recently, Weaver and co-workers [49] observed a $CD4^+$ T cells immune response biased towards $CD4^+$/IL-2^+/TNF-α^+/IFN-γ^- T cells after recent and confirmed infection with 2009 pandemic H1N1 in human patients. These T helper primed, precursor cells were highly specific to 2009 pandemic H1N1 antigens and have been demonstrated to serve as a reservoir of memory $CD4^+$ T cells with effector potential [47,49]. The balance between humoral (T helper 2-type) and cellular (T helper 1-type) responses elicited by an influenza vaccine is likely to be an important characteristic for the optimization of vaccine efficacy. In our studies, the antibody response, as measured by HI titres, was generally more robust in the H1 VLP than the H5 VLP recipients while the frequency of responding $CD4^+$ T cells was 2–5 fold higher following H5 versus H1 VLP vaccination. Only some of the latter difference was attributable to the use of alum (Figs. 1 & 3). The development of both $CD4^+$ and $CD8^+$ T cell responses in the period immediately following vaccination and the relationship between humoral and cellular responses after plant-made vaccine administration are being investigated in on-going clinical trials.

Two of the unexpected observations in this work are related to the use of Alhydrogel® adjuvant in the H5 VLP trial. Aluminum adjuvants are known to enhance B cell responses but are not thought to be particularly potent in the induction of cellular immunity [50]. Furthermore, although alum is known to have an antigen-sparing effect for influenza vaccines in mice, ferrets and macaques, the impact of alum on human antibody responses to split–virus H5 influenza formulations have been disappointing [51,52]. It was therefore unexpected to see such a striking impact of alum on both humoral and cellular responses in our H5 VLP study (Table 3, Fig. 3). At least one group has reported a strong impact of aluminum hydroxide on cellular responses following inactivated influenza vaccination in mice [53]. The adjuvant effects we observed are similar to those reported for oil-in-water adjuvants with traditional, split-virus formulations [11,25]. Both the strength of antigen adsorption [54–56] and antigen phosphate content [57] have recently been implicated in modulating the effectiveness of aluminum-containing adjuvants (negative and positive effects respectively). Studies are on-going to examine the interaction between our plant-made VLPs and alum to better understand these findings.

This study raises several intriguing questions about the nature of immune responses to influenza vaccines, particularly the relationship between humoral and cellular responses. Our H1 VLP study was conducted in 2011, when many (perhaps most) of the subjects would have been exposed to the wild-type H1N1 virus or an H1N1-contating vaccine or both. Between 25 and 50% of these subjects had serologic evidence of exposure at baseline (Table 2). Such prior exposure likely explains the excellent antibody responses in the groups that received any vaccine as well as the presence of readily-detectable $CD4^+$ and $CD8^+$ T cell responses in some placebo recipients. Prior exposure may also have contributed to the apparent effect of the commercial comparator vaccine on T cell responses (Fig. 4). Certainly, the presence of a pre-existing population of antigen-specific $CD4^+$ cells is known to promote a faster and more efficient T cell response reactivation [11,25]. The existence of such a pre-existing pool of cross-reactive T cells may explain why both $CD4^+$ and $CD8^+$ responses were found in the H1 vaccine recipients while only $CD4^+$ T cell responses remained detectable at 6 months in the H5 study. Although no comparator vaccine was available for inclusion in the H5 trial, the presumed lack of prior exposure to any antigenically-similar viruses makes these results easier to interpret. Perhaps the most interesting aspect of this work was the observation that cross-reactive $CD4^+$ T cells could be demonstrated in both the H1 study (vs H5 VLPs) and in the H5 study (vs H1 VLPs). These data suggest that immunization with plant-made VLP vaccines can elicit broadly reactive memory T cell populations that could be highly advantageous both for drifted virus strains in the seasonal context as well as in the event of a pandemic.

At the current time, we cannot explain the particular immunogenicity of the plant-made VLP vaccines. However, the nature of the memory T cell response induced by these

vaccines appears to be quite different from that induced by inactivated influenza vaccines and similar, in some respects, to those formulated with oil-in-water adjuvants. Factors that might contribute to the differences observed include plant sphingolipids that could potentially bind to the CD1 receptor [58], Lewis glycans that are C-lectin type agonists, and small amounts of residual DNA and *Agrobacterium*-derived pseudo-endotoxins that are potential TLR agonists. The particulate nature of these vaccines may also contribute to their immunogenicity through facilitated transport and/or uptake by antigen presenting cells. The potential impact of the T cell responses induced by this new type of vaccine will only become apparent when plant-made VLP vaccine efficacy can be directly assessed in humans. Nonetheless, it seems likely that the induction of both antibodies and cross-reactive T cell responses will offer superior protection from severe influenza and may even provide a degree of protection in the case of mismatched strains.

Conflicts 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, Sonia Trépanier and Stéphane Pillet are employees of Medicago Inc.

Acknowledgments

We would like to thank Michèle Dargis, Diane Bussière and the serology group for HI titer determination. We are also truly thankful to John J. Treanor for his helpful comments on the manuscript.

Appendix A. Suppelementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2014.08.003.

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