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A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults

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ABSTRACT

Recent issues regarding efficacy of influenza vaccines have re-emphasized the need of new approaches to face this major public health issue. In a phase 1–2 clinical trial, healthy adults received one intramuscular dose of a seasonal influenza plant-based quadrivalent virus-like particle (QVLP) vaccine or placebo. The hemagglutination inhibition (HI) titers met all the European licensure criteria for the type A influenza strains at the 3 μg/strain dose and for all four strains at the higher dosages 21 days after immunization. High HI titers were maintained for most of the strains 6 months after vaccination. QVLP vaccine induced a substantial and sustained increase of hemagglutinin-specific polyfunctional CD4 T cells, mainly transitional memory and T_{EMRA} effector IFN-γ^+ CD4 T cells. A T cells cross-reactive response was also observed against A/Hong-Kong/1/1968 H3N2 and B/Massachusetts/2/2012. Plant-based QVLP offers an attractive alternative manufacturing method for producing effective and HA-strain matching seasonal influenza vaccines.

1. Introduction

Influenza A viruses are a major public health threat and, in the USA alone, seasonal epidemics account for >200.000 hospitalizations and >30.000 deaths annually [1]. Influenza B viruses can also cause seasonal epidemics in adults every two to four years. Based on data across four seasons, the clinical symptoms and hospital admission rates are similar in subjects infected with either influenza A or B [2]. Vaccination remains the most cost-effective means to control the health burden attributable to influenza. Seasonal influenza vaccines have historically included three strains: two influenza A strains and only one influenza B strain. As two antigenically-distinct influenza B lineages (B/Victoria and B/Yamagata) have co-circulated in North America since 2000, there have been frequent mismatches leading to a reduced vaccine effectiveness [3,4]. Indeed, split-virion vaccination against one B lineage provides little-to-no cross-protection against the alternate lineage [5,6]. It has been estimated that inclusion of both Victoria and Yamagata lineages in a quadrivalent inactivated vaccine (QIV) between 1999 and 2009 could have resulted in a major and significant reduction of the public health burden of influenza infections [7]. For the 2012–2013 Northern hemisphere influenza season, the World Health Organization (WHO) recommended the inclusion of a B strain from each lineage in the seasonal vaccine for the first time. Since that time, several different QIV formulations have been shown to be immunogenic for all four vaccine strains in children as young as 6 months and in adults [8–11]. Almost all of the currently used influenza vaccines have been designed to induce antibodies (Abs) against the viral surface glycoproteins, hemagglutinin (HA) in particular. These Abs, detected by HA inhibition (HI) assay, are thought to prevent viral attachment to host cells. The post-vaccination serum HI titer has been widely used as a surrogate marker for vaccine efficacy compliant with European Medicines Agency (EMEA) and Food and Drug Administration (FDA) guidelines. However there is mounting evidence that T cell-mediated immunity (CMI) plays an important role in controlling influenza. Studies in both animal models and humans demonstrate that protection can occur in absence or with very low level of Abs and HI titers, pointing out the role of the CMI in the control of the virus replication and the resolution of the disease. Moreover, CMI has recently been demonstrated to play a pivotal role in cross-protection against drifted and heterologous strains [12–15]. Although most of the work on CMI to date has focused on the conserved epitopes of internal influenza proteins like nucleoprotein (NP) and M protein, recent data demonstrate that the influenza HA molecule also contains class I- and class II-restricted epitopes and that HA-specific T cell responses can be detected after influenza infection or vaccination [16–20].

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Virus-like particle (VLP) influenza vaccines made in plants have proved to be immunogenic in both animal models and humans [21–24]. Plant-based VLP vaccines have the potential to address several of the limitations of currently licensed products including response time and scalability in the event of a pandemic. Furthermore, the plant-based vaccines target wild-type HA sequences in contrast to influenza strains grown in eggs or tissue culture that may have mutated for optimal growth, possibly compromising the effectiveness of the vaccine [25]. Finally, some highly-pathogenic avian influenza (HPAI) strains like H5N1 replicate very poorly in eggs thereby reducing vaccine production capacity.

Herein we described the results from a phase 1–2 randomized clinical trial conducted to assess the safety, tolerability, and immunogenicity of a non-adjuvanted plant-derived quadrivalent VLP influenza (QVLP) vaccine in healthy adults. This QVLP vaccine was well-tolerated and elicited sustained, cross-reactive humoral and cell-mediated immune responses.

2. Methods

2.1. Production of plant-derived HA VLP influenza vaccine

The QVLP vaccine was produced in *Nicotiana benthamiana* using the Agrobacterium infiltration-based transient expression plateform as previously described [21,26]. The HA protein in the VLPs were based on HA sequences of A/California/07/2009 H1N1 (A/H1N1 Cal), A/Victoria/361/11 H3N2 (A/H3N2 Vic), B/Brisbane/60/08 (B/Bris, Victoria lineage) or B/Wisconsin/1/10 (B/Wis, Yamagata lineage) influenza strains according to the recommendations of the World Health Organization for the 2013–2014 North Hemisphere influenza season. Drug Substances for each strain were combined into a quadrivalent Drug Product. Final doses were based on HA content. Each dose was administered in a volume of 0.5 ml in the deltoid muscle of the non-dominant arm.

2.2. Study design and objectives

Eligible male and female subjects between 18 and 49 years of age were enrolled for this phase 1–2 randomized, double-blind, placebo-controlled, dose-ranging clinical trial (NCT01991587 at ClinicalTrials.gov) conducted in the United States between May 2013 and February 2014. The study was carried out in accordance with the Declaration of Helsinki and the principles of Good Clinical Practices and was approved by the site's Ethics Review Board and by the Center for Biologics Evaluation and Research (CBER). Criteria of exclusion were detailed in the Suppl. Table 1. The primary objectives were to evaluate safety and tolerability of a single dose of the QVLP vaccine administered intramuscularly (IM) as well as the immunogenicity of the vaccine as measured by the HI antibody assay. Secondary and exploratory objectives focused on cross-reactivity of the HI antibody response, neutralisation of homologous and heterologous strains in the microneutralization (MN) assay and CD4 T cell responses against homologous and heterologous strains. Antibody responses (e.g.: IgG, IgE) to plant glycans were also measured by the ImmunoCap test (Pharmacia & Upjohn, Uppsala, Sweden).

2.2.1. Study procedures

The Phase 1 portion of this study used dose escalation with slow enrolment (staggered cohorts) for the 3 dose levels (3 μg, 9 μg, and 15 μg VLP per strain) with a placebo-controlled group. In the first Cohort, 13 subjects were randomized to receive the lowest QVLP dose (3 μg of each strain: $n = 10$) or placebo (phosphate buffer saline pH 7.4: 100 mM $NaKPO_4$, 150 mM NaCl, 0.01% Tween-80, $n = 3$). Once the 7-day safety data had been reviewed by the Data and Safety Monitoring Board (DSMB) the second cohort of 13 subjects was randomized to receive the medium dose of QVLP (9 μg of each strain: $n = 10$) or placebo ($n = 3$). Again, DSMB review of the 7-day safety data permitted randomization of the third cohort to receive either the high dose of QVLP (15 μg of each strain, $n = 10$) or placebo ($n = 4$). Upon satisfactory DSMB review the 7-day safety data for the third cohort, the Phase 2 portion of the study was initiated during which 80 subjects were randomised (1:1:1:1) to receive one of three QVLP vaccine doses or placebo. Blood samples for hematology and biochemistry and urine (routine & microscopic) were collected at screening, D3 and D201. Serum pregnancy tests (for female subjects) were performed at screening, D0, D21 and D201. Serum samples were obtained at D0, D21 and D201for HI and MN assays and measurement of antibodies against plant glycans. Anti-coagulated (EDTA) whole blood samples were collected in a subset of 10 subjects per group for analysis of T cell responses at D0, D21 and D201.

2.2.2. Safety/reactogenicity assessments

Subjects were observed for 30 min after vaccination for immediate reactions and were provided with diary cards to record solicited local or systemic reactions that occurred up to seven days after vaccination. Solicited local reactions included pain, swelling and redness (or erythema). Solicited systemic reactions included fever (≥38 °C), headache, muscle aches, joint aches, fatigue, chills, feeling of general discomfort or uneasiness and swelling (axilla, groin, neck, and/or chest). The severity of reported symptoms was assessed as mild, moderate or severe. Unsolicited adverse events (AEs) were collected from D0 through D21. All serious adverse events (SAEs), new onset of chronic disease (NOCD) and AEs leading to study withdrawal were collected throughout the study. Causality of all unsolicited AEs/SAEs/NOCDs was evaluated by the investigator and reported as definitely not related, probably not related, possibly related, probably related or definitely related.

2.2.3. HI assay

The HI assay was performed on serum samples as previously described according to WHO recommendations [23,27]. The homologous antigens (Ag) tested were A/H1N1 Cal propagated in Madin-Darby (Southern Research Institute), A/Texas/50/2012 H3N2 (A/H3N2 Tx, NYMCX-223 A, National Institute for Biological Standards and Control, NIBSC) a virus antigenically like the cell-propagated virus A/H3N2 Vic, B/Bris (NYMC BX-35, NIBSC) and B/Wis (NIBSC #12/110). The heterologous Ag tested were A/Brisbane/59/2007 H1N1 (A/H1N1 Bris, IVR-148, NIBSC #08/100), A/Uruguay/716/2007 H3N2 (A/H3N2 Uru, NYMC-175C, NIBSC #08/278), B/Malaysia/2506/2004 (B/Mal, NIBSC #08/184) and B/Massachusetts/02/2012 (B/Mass, NIBSC #13/152). Serum titers are expressed as the reciprocal of the highest dilution that showed complete inhibition of hemagglutination. Although some controversy subsists, HI titer ≥1:40 remains the reference standard indicative of seroprotection [28–30]. Seroconversion rate (SCR), seroprotection rate (SPR) and geometric mean fold rise (GMFR) were defined according to regulatory criteria [31] and were compared to the European Agency for the Evaluation of Medicinal Products' CHMP criteria i.e. SCR ≥ 40%, SPR ≥ 70%, GMFR ≥ 2.5 for healthy adults.

2.2.4. MN assay

MN antibody titers against the homologous strains A/H1N1 Cal, A/H3N2 Vic, B/Bris, B/Wis and the heterologous strain B/Mass were measured in serum at D0 and D21 according to the World Health Organisation guideline for the serologic diagnosis of influenza [27]. MN titers are expressed as the reciprocal of the highest dilution that showed complete neutralization of input virus. Sera that tested negative at a dilution of 1:10 were assigned a titer of 5 for statistical analysis. Samples were analyzed in duplicates and repeats were performed when acceptance criteria for the assay were not met.

2.2.5. Ex vivo T cell re-stimulation assay

Peripheral blood mononuclear cells (PBMCs) were collected at D0, D21 and D201 after vaccination. Cells were prepared and cryopreserved in liquid nitrogen until quickly thawed and prepared for the assays as

previously described [22]. Cells were stimulated with 2.5 μg HA/ml of VLP (homologous stimulations) for 20 h, or for 6 h with 2.5 μg/ml of peptide pool consisting in 15-mer peptides overlapping by 11 amino acids spanning the complete B/Mass or A/Hong-Kong/1/1968 H3N2 (A/H3N2 HK) HA proteins (heterologous stimulations, GenScript, Piscataway, NJ). A 6 h stimulation with a control peptide pool containing 32 peptides, 8–12 amino acids in length with sequences derived from the human Cytomegalovirus, Epstein-Barr and influenza viruses (CEF, 1 μg/ml, AnaSpec, Fremont, CA) or the staphylococcal enterotoxin B (SEB, 0.25 μg/ml, Toxin Technology, Sarasota, FL) as well as unstimulated (exposed to VLP or peptide pool vehicles') cells were included as a positives and negative controls respectively. Anti-human CD107a antibody (clone H4A3, Biolegend, San Diego, CA) was added at the beginning of the stimulations as recommended by Betts and Koup [32]. Expression of CD107a is associated with degranulation and serves as a functional marker for cell-mediated cytotoxicity. Five hours before the end of these incubations, 5 μg/ml of monensin and brefeldin A (Golgi blockers, BD Biosciences, Mississauga, ON) were 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 CD16/CD56-Brilliant Violet (clones 3G8 and HCD56 respectively, Biolegend), anti-human CD14-V500 (clone M5E2, BD Biosciences), anti-human CD3-V450 (clone SP34–2, BD Horizon, BD Biosciences), CD4-phycoerythrin-TexasRed (clone SFCI2T4D11, Beckman Coulter, Mississauga, ON), CD8-eFluor650 NC (clone RPA-T8, eBioscience, San Diego, CA), CD45RA-allophycocyanin-Cyanine 7 (clone HI100, BD Pharmingen), CD27-Brilliant Violet 605 and CCR7-phycoerythrin-Cyanine 7 (clone O323 and G043H7 respectively, Biolegend, San Diego, CA) for 30 min at 4 °C in the dark. Cells were washed twice with cold phosphate-buffered saline (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-fluorescein isothiocyanate (clone B27, BD Pharmingen), IL-2-Alexa700 (MQ1-17H12, Biolegend) and TNF-α-allophycocyanin (clone 6401.1111, BD Biosciences) 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 3×10^5 lymphocytes were acquired for each sample and data were analyzed using FlowJo™ (Tree Star, OR) and SPICE (http://exon.niaid.nih.gov/spice) software. The gating strategy is detailed in Suppl. Fig. 3. 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.

2.2.6. Antibodies to plant glycans associated with hypersensitivity/allergy reactions

In order to determine if QVLP vaccine can induce antibodies to plant-specific β(1–2) xylose and α(1–3) fucose carbohydrates found on the vaccine, serum IgE levels to plant-specific glycans were evaluated using samples collected at D0 and D21 as reported previously [33]. Briefly, the ImmunoCAP bromelain assays (Pharmacia & Upjohn, Uppsala, Sweden) was used to measure IgE directed against MUXF3, a carbohydrate motif found on many plant allergens that can trigger histamine release by mast cells and basophils in vitro. Positive responses in the ImmunoCAP assay (graded 0–4) are correlated with clinically-apparent allergic responses [34–36].

2.3. Statistical analysis

All safety endpoints were summarized by treatment groups using descriptive statistics. The GMFR and the geometric mean titer (GMT) were compared using analysis of variance (ANOVA) followed by a Tukey-Kramer post-hoc test for pairwise comparison inside each sampling day while the Sidak's multiple comparison test was used to compare the response at different time for a same vaccine dose or placebo. The SCR and SPR were compared using the Fisher's exact test. Two-group comparisons between CMI responses in placebo and 15 μg QVLP groups were assessed by parametric unpaired t tests or non-parametric Mann-Whitney test when data did not meet the condition for application of a parametric test. Multiple comparisons between the CD4 T cell functional signatures of placebo and 15 μg QVLP groups were assessed by multiple t tests using the Holm-Sidak method to correct for multiple comparisons. The statistical analyses were performed using SAS® software (version 9.2) and GraphPad Prism (version 6.03, GraphPad Software, La Jolla, CA).

Table 1
Summary of demographics and baseline characteristics (safety population).

Characteristic	Placebo ($N = 30$)	3 μg VLP vaccine ($N = 30$)	9 μg VLP vaccine ($N = 30$)	15 μg VLP vaccine ($N = 30$)	*p*-Value
Sex, n (%)					
Female	16 (53.3)	15 (50.0)	20 (66.7)	12 (40.0)	0.2264[a]
Male	14 (46.7)	15 (50.0)	10 (33.3)	18 (60.0)	
Race, n (%)					
White or Caucasian	24 (80.0)	19 (63.3)	22 (73.3)	24 (80.0)	0.4195[b]
Black or African American	6 (20.0)	11 (36.7)	8 (26.7)	6 (20.0)	
Ethnicity, n (%)					
Hispanic or Latino	24 (80.0)	21 (70.0)	21 (70.0)	25 (83.3)	0.5282[a]
Not Hispanic or Latino	6 (20.0)	9 (30.0)	9 (30.0)	5 (16.7)	
Age (years)					
Mean (SD)	34.4 (9.04)	33.5 (10.58)	34.9 (8.32)	31.9 (9.67)	0.6279[c]
Range	18–49	19–49	21–49	19–48	
BMI (kg/m^2)					
Mean (SD)	25.42 (3.544)	24.87 (4.069)	25.09 (3.795)	26.75 (3.294)	0.2000[c]
Range	19.8–31.7	18.4–31.1	18.9–32.1	19.4–31.1	
Influenza Immunization History, n (%)					
Yes	1 (3.3)	1 (3.3)	2 (6.7)	3 (10.0)	0.8340[d]
No	29 (96.7)	29 (96.7)	28 (93.3)	27 (90.0)	

[a] *p*-Value for the difference of the number of subjects between treatment groups and two levels of the demographic variable by Fisher's exact test.
[b] *p*-Value for the difference of the number of subjects between treatment groups and Caucasian vs other races by Fisher's exact test.
[c] *p*-Value for the difference between treatment groups from an analysis of variance with treatment group as factor.
[d] *p*-Value for the difference of the number of subjects between treatment groups and influenza immunized vs not immunized by Fisher's exact test.

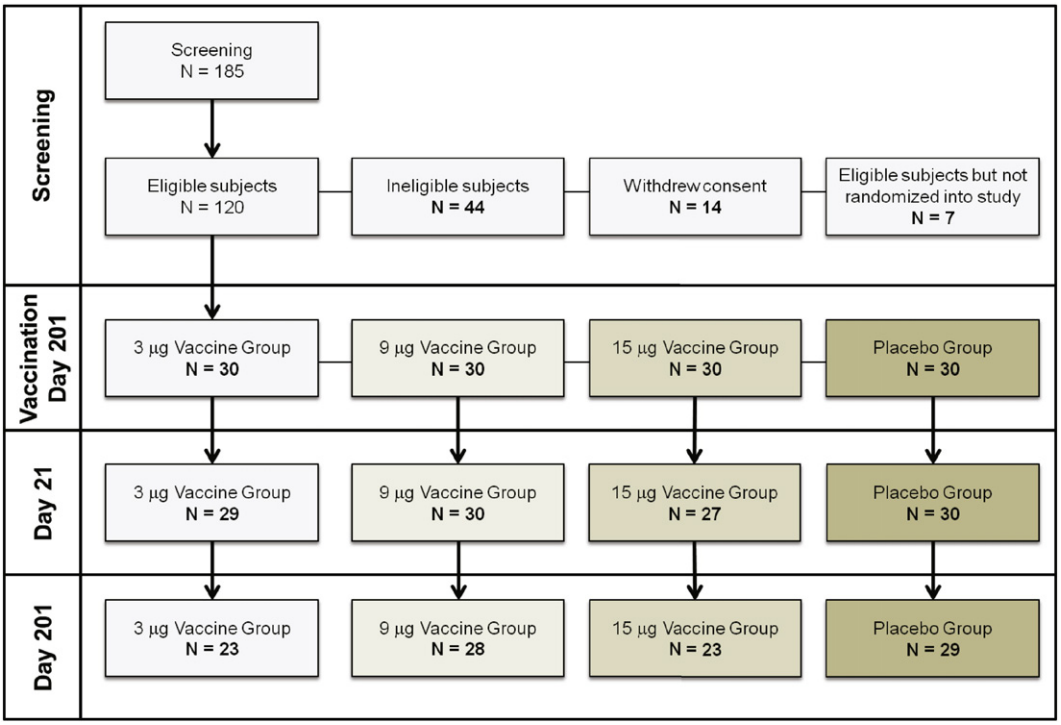

Fig. 1. Subject disposition from screening to day 201 visit.

3. Results

Gender was well-distributed between the groups except in the 9 μg QVLP group that had a higher proportion of female subjects (Table 1). Greater than half of the subjects (>63.3%) for all vaccine groups were white or Caucasian and Hispanic or Latino in origin; the remaining subjects were black or African American. The mean age and body mass index (BMI) were similar among all groups. The previous influenza vaccination rate was rather low in all groups (≤10%) considering the recommendation for annual influenza vaccination in the United States. As shown in Fig. 1, 120 subjects were randomized and received either QVLP vaccine or placebo. Overall, 96.7% of subjects completed the study through D21 and 85.8% through D201.

3.1. Safety

The QVLP vaccine was well-tolerated at each dose level (Table 2). The overall reactogenicity increased with increasing dose but did not achieve statistical significance. Pain at the injection site in the 7 days post-vaccination was reported the most common local solicited AE occurring in 50% of the 15 μg dose recipients and 36.7% in the 3 μg and 9 μg dose groups (versus 13.3% in the placebo group) (Table 2). Differences in the incidence of local symptoms were not statistically significant between the active dose groups, except for pain in the 15 μg dose group compared to placebo ($p = 0.0048$). Except for one case of severe fatigue and headache in the 15 μg group and one case of severe headache in the placebo group, the majority of systemic reactions were mild and of short duration across all vaccine doses and placebo. Headache was the most common systemic AE and was similar for all QVLP doses (3 μg = 13.3%, 9 μg = 13.3% and 15 μg = 23.3%) and placebo (23.4%). Symptoms of mild or moderate fatigue, muscle aches, and feelings of general discomfort or uneasiness were experienced in ≤10% of QVLP vaccinated subjects and were reported at a similar rate by the placebo group. During the 21 days after vaccination, there were no statistically significant differences in the rates of unsolicited AE between groups (4 in each of the 3 and 9 μg groups, 5 in the 15 μg group and 6 in the placebo group). There was no SAEs or new onset of chronic disease (NOCD) during this study.

3.1.1. Immunization with QVLP does not induce allergic symptoms or IgE responses

Given the particular nature of this vaccine, we have carefully considered the potential for allergic reactions from the outset. To date, more than 1000 people have received 1 or 2 doses of a plant–derived VLP vaccine without the induction or worsening of clinical allergies or the development of pathologic IgE responses [33]. No subject in any group reported either worsening or induction of allergic symptoms following QVLP vaccination. Overall, only 2 subjects had low-positive tests in the bromelain assay: one in the highest dose QVLP group prior to immunization and one in the placebo group post-vaccination (Table 3). It is noteworthy that the low titer to plant-origin carbohydrate motifs anti-MUXF3 IgE actually disappeared following vaccination in the subject who received 15 μg/strain QVLP. These observations likely reflect background (environmental) exposures to plant carbohydrates.

3.2. Antibody response

3.2.1. QVLP vaccine elicited a sustained HI antibody response

The HI titers against the four homologous strains increased as compared to baseline (D0) values 21 days after vaccination with the VLP vaccine. This increase was statistically significant for all the homologous strains at each dose of vaccine with the exception of titers against the two B strains in subject who received 3 μg of VLP vaccine (data not shown). There was no significant increase in HI antibody titer in the placebo group. Twenty-one days after immunization, GMT, SPR, SCR and GMFR in vaccinated subjects were significantly higher than the placebo group for the four homologous strains regardless of the vaccine dose (Figs. 2 and 3) with the exceptions of HI titers against B/Bris and SCR for B/Bris and B/Wis in the 3 μg group (Fig. 3). A small vaccine-dose effect was observed, particularly for the two B strains (Fig. 3). The SPR for B/Wis in the 15 μg group was significantly higher than in the 3 μg group (Fig. 3B). In addition, GMFR for B/Bris and B/Wis and SCR for B/Bris were significantly lower in subjects who received the 3 μg dose compared to

Table 2
Incidence of local and systemic solicited signs and symptoms through day 7 by severity (safety population).

	Placebo ($N = 30$)	3 μg VLP vaccine ($N = 30$)	9 μg VLP vaccine ($N = 30$)	15 μg VLP vaccine ($N = 30$)
	n (%)	*n* (%)	*n* (%)	*n* (%)
Subjects with at least one local or systemic solicited signs or symptoms	11 (36.7)	14 (46.7)	14 (46.7)	18 (60.0)
Mild	8 (26.7)	12 (40.0)	11 (36.7)	14 (46.7)
Moderate	2 (6.7)	2 (6.7)	3 (10.0)	3 (10.0)
Severe	1 (3.3)	0	0	1 (3.3)
Solicited local signs				
Pain at injection site	4 (13.3)	11 (36.7)	11 (36.7)	15 (50.0)
Mild	4 (13.3)	10 (33.3)	10 (33.3)	14 (46.7)
Moderate	0	1 (3.3)	1 (3.3)	1 (3.3)
Severe	0	0	0	0
Swelling at injection site	2 (6.7)	0	2 (6.7)	2 (6.7)
Mild	2 (6.7)	0	1 (3.3)	1 (3.3)
Moderate	0	0	1 (3.3)	1 (3.3)
Severe	0	0	0	0
Solicited systemic symptoms				
Headache	7 (23.3)	4 (13.3)	4 (13.3)	7 (23.3)
Mild	6 (20.0)	3 (10.0)	3 (10.0)	6 (20.0)
Moderate	1 (3.3)	1 (3.3)	1 (3.3)	0
Severe	0	0	0	1 (3.3)
Muscle aches	3 (10.0)	0	4 (13.3)	5 (16.7)
Mild	3 (10.0)	0	3 (10.0)	3 (10.0)
Moderate	0	0	1 (3.3)	2 (6.7)
Severe	0	0	0	0
Joint aches	2 (6.7)	0	0	4 (13.3)
Mild	2 (6.7)	0	0	1 (3.3)
Moderate	0	0	0	3 (10.0)
Severe	0	0	0	0
Fatigue	5 (16.7)	1 (3.3)	4 (13.3)	5 (16.7)
Mild	3 (10.0)	1 (3.3)	2 (6.7)	2 (6.7)
Moderate	1 (3.3)	0	2 (6.7)	2 (6.7)
Severe	1 (3.3)	0	0	1 (3.3)
Chills	1 (3.3)	1 (3.3)	2 (6.7)	2 (6.7)
Mild	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)
Moderate	0	0	1 (3.3)	1 (3.3)
Severe	0	0	0	0
Feelings of general discomfort or uneasiness	4 (13.3)	1 (3.3)	3 (10.0)	4 (13.3)
Mild	2 (6.7)	1 (3.3)	3 (10.0)	3 (10.0)
Moderate	2 (6.7)	0	0	1 (3.3)
Severe	0	0	0	0
Swelling in the axilla	0	0	1 (3.3)	0
Mild	0	0	1 (3.3)	0
Moderate	0	0	0	0
Severe	0	0	0	0
Swelling in the neck	1 (3.3)	0	0	1 (3.3)
Mild	1 (3.3)	0	0	1 (3.3)
Moderate	0	0	0	0
Severe	0	0	0	0

the 2 other QVLP groups although these measures remained significantly higher than placebo (Fig. 3C & D). The antibody response elicited by the 9 μg and 15 μg doses met all the CHMP criteria for the four homologous strains 21 days after vaccination (Figs. 2 and 3). SCR varied from 42.3 to 59.3%, SPR from 74.1.0 to 96.2% and GMFR from 3.8 to 8.0 depending on strain and dose received. All the CHMP criteria were also met for the influenza A strains in the 3 μg group but generally fell short for the B strains: only SPR for B/Wis (72.4%) met the criterion.

HI titers, SPR, SCR and GMFR remained significantly higher than the placebo group six months (D201) after immunization for the two A strains with the exception of HI titer, SPR and GMFR for A/H1N1Cal in the 3 μg group and GMFR for A/H1N1Cal in the 15 μg group (Fig. 2B & D). SPR and GMFR of the 9 μg and 15 μg groups still met the CHMP criteria for the two influenza A strains at D201. These same criteria were also met by the antibody response against A/H3N2 Vic at D201 even with the lowest dose (3 μg) of VLP vaccine. In contrast, the humoral response against the two B strains decreased substantially between D21 and D201 (Fig. 3). The GMT, SPR, SCR and GMFR for B/Wis were significantly lower at D201 than 21 days after immunization for all the vaccine doses tested. For the B/Bris, response differences reached statistical significance for SPR in the 9 μg group, SCR in the 9 μg and 15 μg groups as well as for GMFR at all vaccine doses tested. However, it is important to note that HI titers and SPR for B/Wis in the 15 μg group and GMFR for B/Bris in the 9 μg and 15 μg groups as well as GMFR for B/Wis in the 9 μg group remained significantly higher than placebo six months after vaccination (Fig. 3).

Table 3
IgE responses to bromelain glycans.

Group	Number of subjects with IgEs ≥ grade 1 to bromelain at screening	Number of subjects that showed an IgE increase 21 days after vaccination
3 μg QVLP vaccine ($n = 29$)	0% (0/29)	0% (0/29)
9 μg QVLP vaccine ($n = 30$)	0% (0/30)	0% (0/30)
15 μg QVLP vaccine ($n = 27$)	3.7% (1/27)	0% (0/27)
Placebo ($n = 30$)	0% (0/30)	3.3% (1/30)

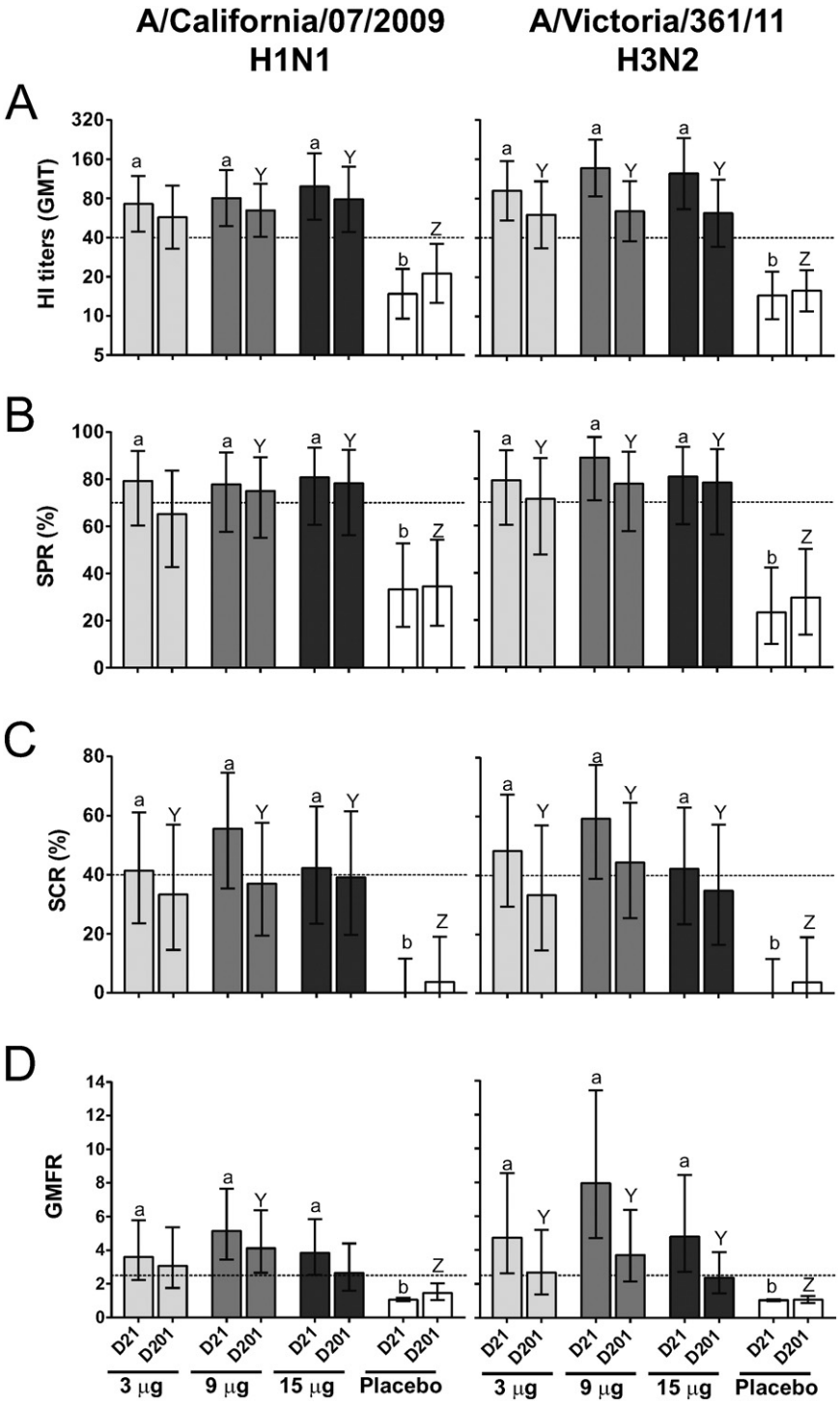

Fig. 2. Serum HI antibody response against the two homologous influenza A strains 21 and 201 days after vaccination with 3 μg, 9 μg and 15 μg of the plant-derived quadrivalent VLP vaccine. A) Geometric mean titer (GMT), B) Percent of seroconversion rate (SPR), C) Percent of seroconversion rate (SCR) and D) Geometric mean fold increase ratio (GMFR). The dotted lines mark the values of the CHMP criteria for licensure. The different letters indicate statistically significant differences ($p \leq 0.05$) between vaccine groups and placebo at D21 (lower case) and at D201 (upper case).

3.2.2. QVLP vaccine elicited a cross-reactive HI antibody response

Cross-reactive HI responses were measured against heterologous influenza strains 21 days after the vaccination. HI titers, SPR, SCR and GMFR for A/H3N2 Uru and B/Mass in the QVLP subjects were significantly higher than placebo at all vaccine doses with the exception of B/Mass titers in the 3 μg group (Figs. 4 and 5). The strong heterologous response against A/H3N2 Uru was maintained at D201 (Fig. 4) while only SCR and GMFR for B/Mass in the 9 μg and 15 μg groups remained higher than placebo (Fig. 5). Although of lower magnitude, cross-reactive responses were also detected against A/H1N1 Bris and B/Mal (Figs. 4 and 5). In particular, the HI titers, SPR, SCR and GMFR for B/Mal were significantly higher in the 15 μg QVLP recipients than the placebo 21 days after immunization (Fig. 5). HI titers met CHMP criteria for SPR (85.2–92.3%), SCR (55.6–61.5%) and GMFR (4.7–5.8) for B/Mass in the 9 and the 15 μg groups respectively. Similar results were observed for A/H3N2 Uru to which 55.6–50.0% of the 9 and 15 μg QVLP recipients

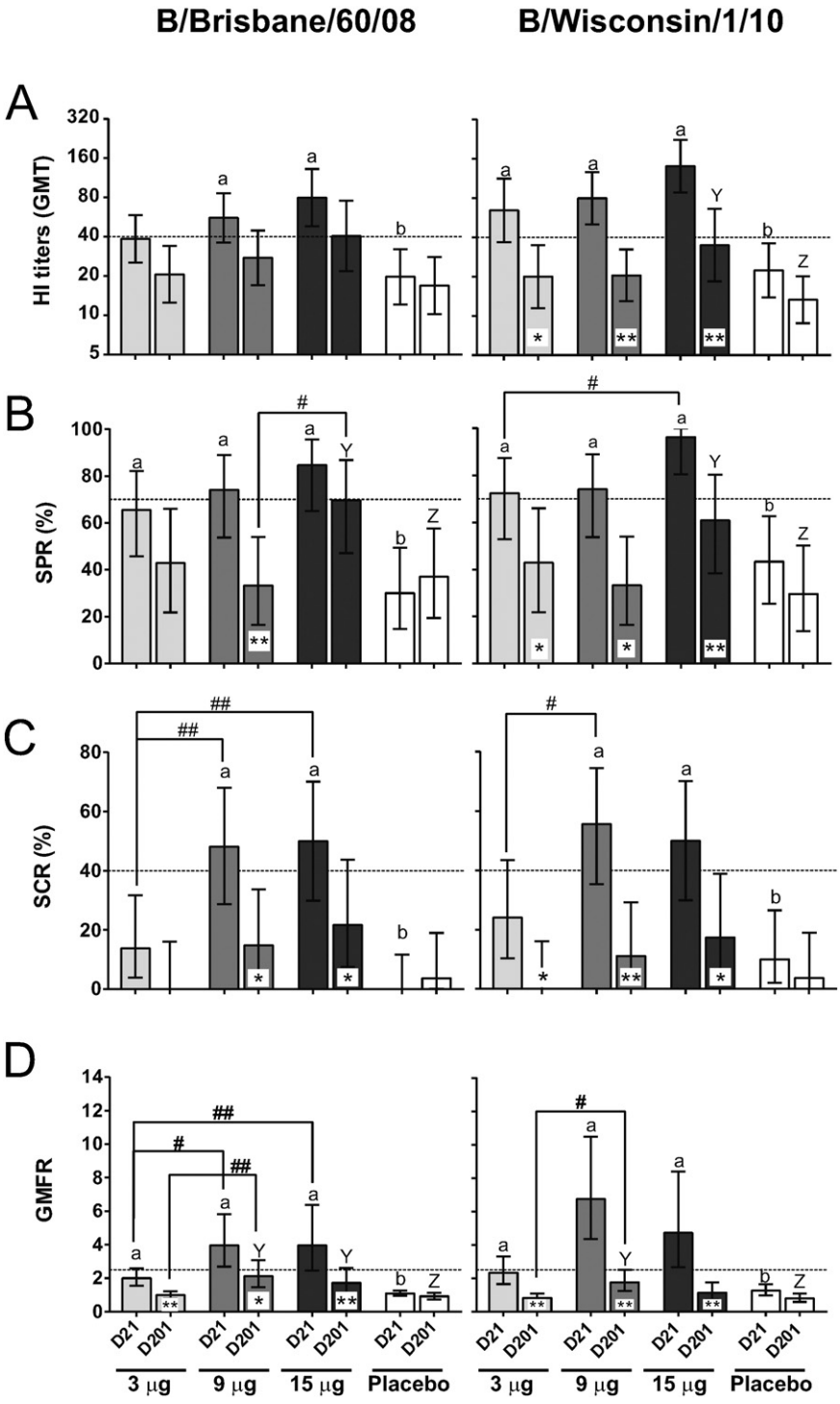

Fig. 3. Serum HI antibody response against the two homologous influenza B strains 21 and 201 days after vaccination with 3 µg, 9 µg and 15 µg of the plant-derived quadrivalent VLP vaccine. A) Geometric Mean titer (GMT), B) Percent of seroconversion rate (SPR), C) Percent of seroconversion rate (SCR) and D) Geometric mean fold increase ratio (GMFR). The dotted lines mark the values of the CHMP criteria for licensure. The different letters indicate statistically significant differences ($p \leq 0.05$) between vaccine groups and Placebo at D21 (lower case) and at D201 (upper case). Asterisks inserted inside the D201 bars indicate statistically significant differences between the response at D21 and D201 for each vaccine dose ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$). Hash signs indicate statistically significant differences between vaccine doses ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$) for each time point.

achieved a 4-fold increase in HI titer and GMFR between 5.2 and 5.3. SPR for A/H3N2 Uru at D21 reached 66.7–61.5% in the two high QVLP dose groups.

3.3. Neutralizing antibody response

The MN analyses were assessed 21 days post-vaccination against homologous influenza. Pearson's average correlation coefficient between the log-transformed HI and MN titers for the 3 QVLP doses were 0.8260, 0.8888, 0.8847 and 0.8451 for A/H1N1 Cal, A/H3N2 Vic, B/Bris and B/Wis respectively (all $p < 0.0001$). MN antibody titers increased with increasing vaccine dosages although not achieving statistical significance (data not shown). MN SCR, defined as 4-fold increase of baseline MN titer, obtained at each dose and for each strain were also quite similar to the HI results (data not shown). All doses tested achieved MN SCR >40% for

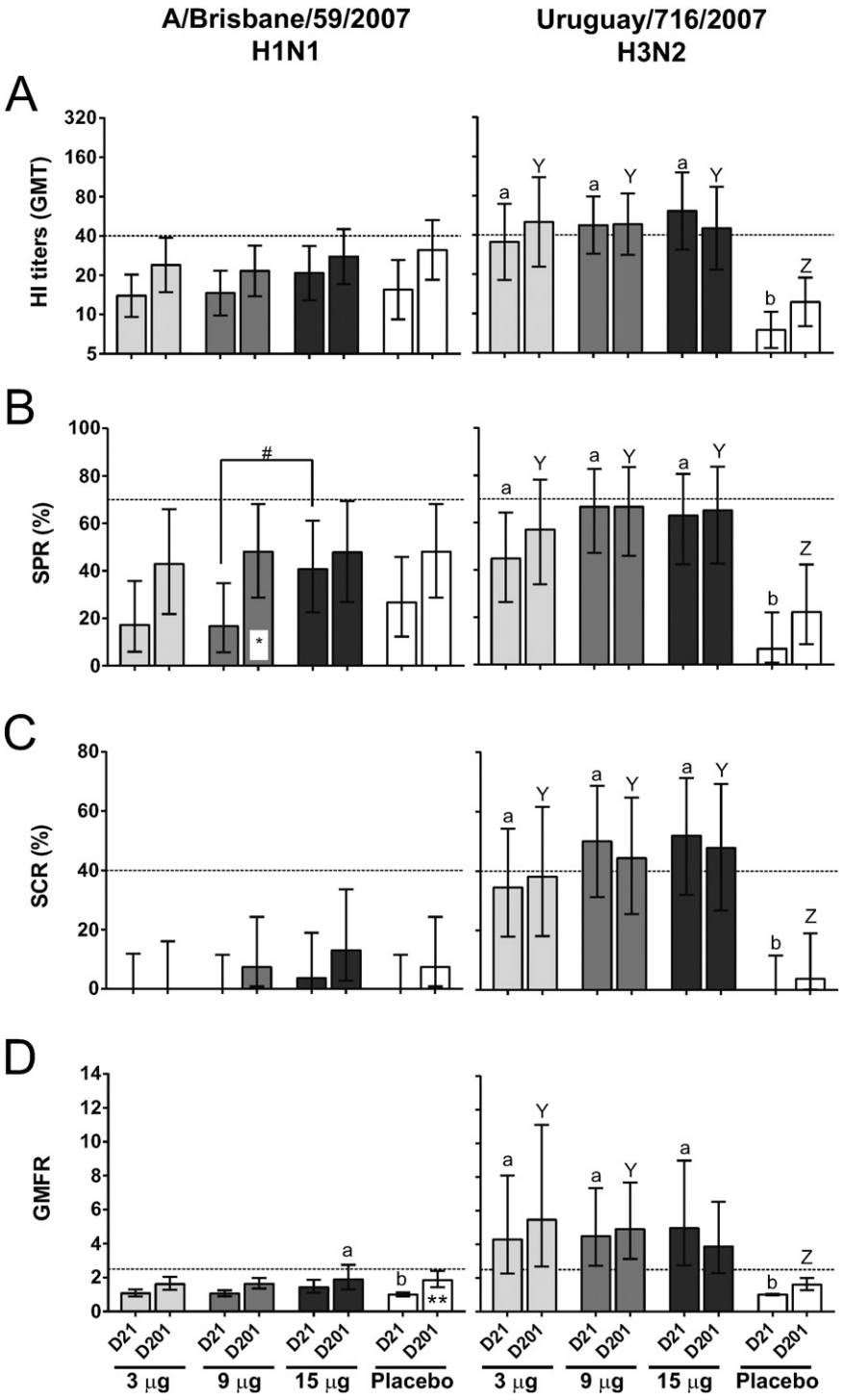

Fig. 4. Serum HI antibody response against two heterologous influenza A strains 21 and 201 days after vaccination with 3 μg, 9 μg and 15 μg of the plant-derived quadrivalent VLP vaccine. A) Geometric Mean titer (GMT), B) Percent of seroconversion rate (SPR), C) Percent of seroconversion rate (SCR) and D) Geometric mean fold increase ratio (GMFR). The dotted lines mark the values of the CHMP criteria for licensure. The different letters indicate statistically significant differences ($p \leq 0.05$) between vaccine groups and Placebo at D21 (lower case) and at D201 (upper case). Asterisks inserted inside the D201 bars indicate statistically significant differences between the response at D21 and D201 for each vaccine dose ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

the A strains. The highest dose (15 μg/strain) achieved MN SCR >40% for all 4 strains.

3.4. T-cell response

3.4.1. QVLP vaccine elicited a polyfunctional and cross-reactive CD4 T cell response 21 days after immunization

We observed an overall increase in the Ag-specific CMI between D0 and D21 in vaccinated subjects but not in placebo (data not shown). Detailed T cell responses following ex vivo Ag re-stimulation were measured in subjects who received 15 μg QVLP per strain and compared to the placebo group ($n = 9$ and 6 respectively). At 21 days after vaccination, there was a striking increase in the proportion of influenza HA-specific polyfunctional CD4 T cells following homologous Ag recall in the QVLP recipients compared to placebo. This increase was observed for each of the four strains included in the QVLP (Fig. 6). The polyfunctional CD4 T cells were mainly IFN-γ^{+} (Fig. 7A) but the specific CD107a/IFN-γ/IL-2/TNF-α signatures varied between strains (Suppl.

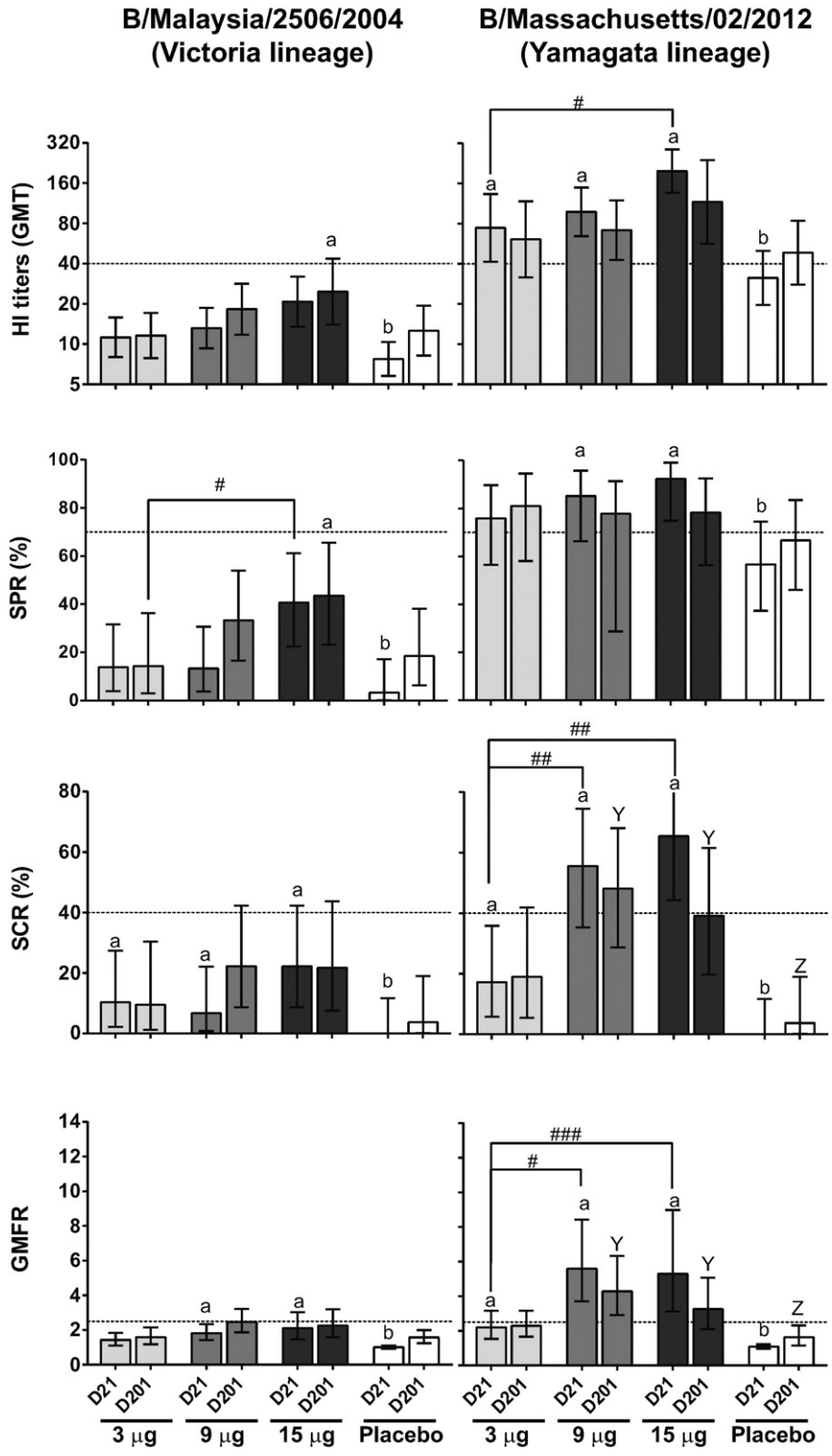

Fig. 5. Serum HI antibody response against two heterologous influenza B strains 21 and 201 days after vaccination with 3 μg, 9 μg and 15 μg of the plant-derived quadrivalent VLP vaccine. A) Geometric Mean titer (GMT), B) percent of seroconversion rate (SPR), C) percent of seroconversion rate (SCR) and D) geometric mean fold increase ratio (GMFR). The dotted lines mark the values of the CHMP criteria for licensure. The different letters indicate statistically significant differences ($p \leq 0.05$) between vaccine groups and Placebo at D21 (lower case) and at D201 (upper case). Asterisks inserted inside the D201 bars indicate statistically significant differences between the response at D21 and D201 for each vaccine dose ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$). Hash signs indicate statistically significant differences between vaccine doses ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$) at each time point.

Fig. 1). No statistically significant differences were observed in IFN-γ^+ CD4 T cells between QVLP recipients and placebo at D0 (data not shown). No significant CD8 T cell responses were observed against homologous strain Ag at 21 days after vaccination. Memory cells in the IFN-γ^+ CD4 T cell population elicited by QVLP vaccine were characterized according to the expression of CD45RA, CCR7 and CD27 as follows: naïve (CD45RA$^+$ CCR7$^+$ CD27$^+$), central memory (CM: CD45RA$^-$ CCR7$^+$ CD27$^+$), transitional memory (TM: CD45RA$^-$ CCR7$^-$

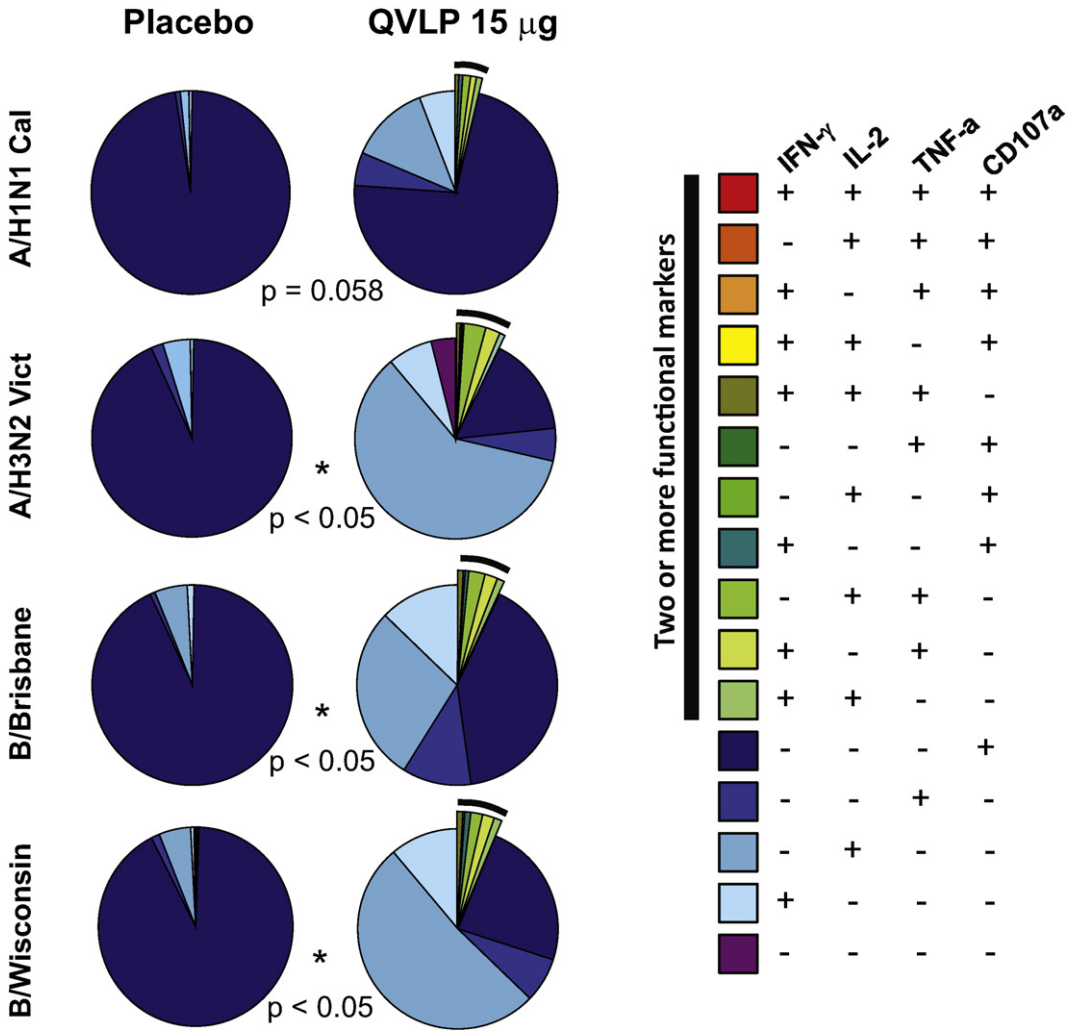

Fig. 6. Homologous CD4 T cell-mediated response 21 days after vaccination. Relative distribution of CD4 T cell subsets based on the expression of the IFN-γ, IL-2, TNF-α and CD107a after ex vivo Ag re-stimulated with homologous Ag. The black arcs highlighted polyfunctional CD4 T cells. The permutation analysis (SPICE software http://exon.niaid.nih.gov/spice) revealed significant (*$p \leq 0.05$) differences in the distribution of the functional response between placebo and 15 μg QVLP groups.

$CD27^+$), effector memory (EM: $CD45RA^-$ $CCR7^-$ $CD27^+$), effector memory $CD45RA^+$ (T_{EMRA}: $CD45RA^+$ $CCR7^-$) $CD27^-$ and T_{EMRA} $CD27^+$ [37,38]. At D21, the HA-specific IFN-γ^+ CD4 T cells were mainly T_{EMRA} $CD27^+$ (over 50%) and TM (between 20% to 30%), with the exception of A/H3N2Vic HA-specific CD4 T cells wherein the relative proportion of T_{EMRA} $CD27^-$ reach ~30% (Fig. 7A, detailed pie-charts).

To assess CMI directed against heterologous HA antigens, PBMCs were stimulated ex vivo with peptide pools from the B/Mass or A/H3N2 HK HA proteins. Increases of B/Mass and A/H3N2 HK cross-reactive CD4 T cells were noticed and we observed a significant increase of Ag-specific IFN-γ^+ CD4 T cells in the 15 μg QVLP recipients as compared to placebo (Fig. 7B). The frequencies of IFN-γ^+ IL-2^- TNF-α^+ $CD107^+$, IFN-γ^+ IL-2^+ TNF-α^+ $CD107^-$ and IFN-γ^+ IL-2^- TNF-α^+ $CD107^-$ A/H3N2 HK HA-specific CD4 T cell populations was significantly higher in vaccinated subjects as compared to placebo (Suppl. Fig. 2, upper panel). B/Mass cross-reactive CD4 T cells exhibited a similar pattern although the difference was only significant for the IFN-γ^+ IL-2^- TNF-α^+ $CD107^-$ (Suppl. Fig. 2, lower panel). The A/H3N2 HK HA-specific IFN-γ^+ CD4 T mainly (>75%) displayed a T_{EMRA} $CD27^-$ phenotype (Fig. 7B higher panel, detailed pie-chart) while B/Mass HA-specific IFN-γ^+ CD4 T cells displayed T_{EMRA} $CD27^+$ and TM phenotypes (Fig. 7B lower panel, detailed pie-chart).

3.4.2. The QVLP vaccine also elicits a long-term polyfunctional and cross-reactive CD4 T cell response

Six months after immunization, minimal expression of CD107 was observed on PBMC after ex vivo stimulation (data not shown). The analysis was therefore focused on the expression of IL-2, TNF-α and IFN-γ using the same three surface markers to define memory cell populations (CD45RA, CCR7 and CD27 as above). Memory $CD45RA^-$ CD4 T cells expressing at least one of these 3 cytokines upon homologous or heterologous stimulation were mainly TM. The proportion of Ag-specific CD4 TM cells expressing at least one cytokine (Responsive CD4 TM cells) was higher in 15 μg QVLP group than in placebo for both homologous (Fig. 8, left panel) and heterologous (Fig. 9, left panel) Ag although that difference was only statistically significant for A/H3N2 Vic HA-specific CD4 TM cells. The functional signatures of TM at 201 days after immunization are detailed on the right panel of Figs. 8 and 9. A/H3N2 Vic HA-specific double positive IFN-γ^+ TNF-α^+ as well as single positive IFN-γ^+ and TNF-α^+ CD4 TM cells were significantly increased in the QVLP group compared to placebo (Fig. 8 right panel). Similar trends, although not statistically significant, were observed with the other homologous strains. A significantly higher proportion of heterologous B/Mass HA-specific double positive IFN-γ^+TNF-α^+ was also observed in the QVLP group compared to placebo (Fig. 9, right panel).

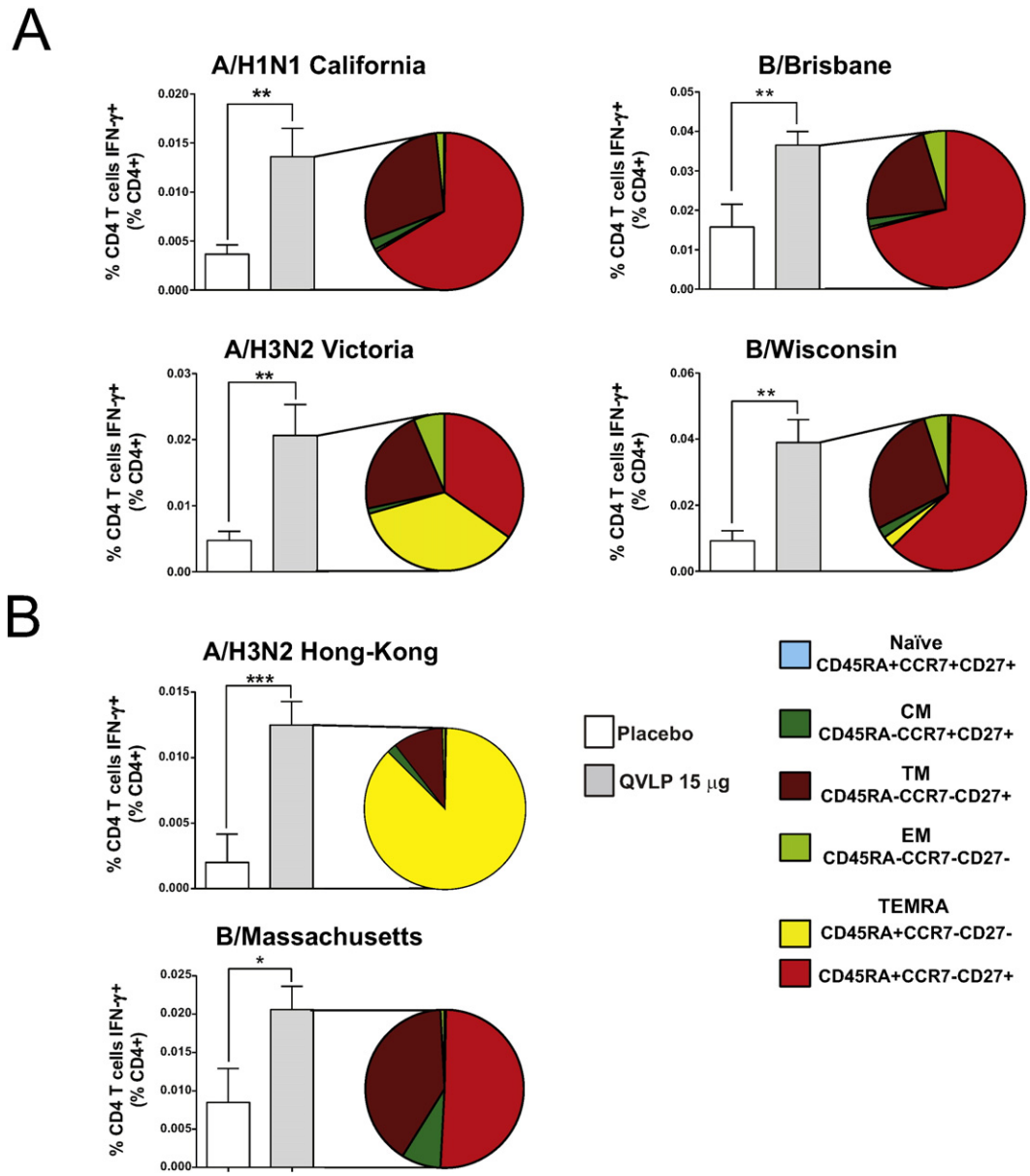

Fig. 7. Homologous (A) and heterologous (B) CD4+ T cell-mediated response 21 days after vaccination. Percentage of CD4 T cells expressing IFN-γ upon Ag ex-vivo re-stimulation (mean ± SE). The significant differences between placebo and 15 μg QVLP groups are represented ($p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). The pie-charts detailed the relative distribution of IFN-γ^+ $CD4^+$ memory T cell subpopulations based on the expression of surface markers CD45RA, CCR7 and CD27 in the 15 μg QVLP group.

4. Discussion

Although the use of influenza vaccines has clear health benefits in most seasons, recent events have highlighted serious limitations of both current products and manufacturing processes. In this context, plant-based manufacturing has considerable potential since molecular techniques are used to target wild-type Ag as soon as sequence data for a new strain becomes available [26]. As a result, this platform can be much more nimble than classical approaches based on egg- or tissue culture-adapted viruses. Herein we report the results of a phase 1–2 dose-escalation trial of a QVLP vaccine that was both well-tolerated and induced strong and cross-reactive humoral and cellular responses. Pain at the site of injection was the most common AE reported but was consistently described as mild-to-moderate and of short duration. Similarly, systemic solicited reactions such as headache, muscle pain and fatigue were reported to be mild-to-moderate and transient in nature with equal frequency in the QVLP recipients and placebo groups. Overall, the reactogenicity of the QVLP vaccine was similar to the reported profiles of split-virus QIVs and commercial TIVs [8,9,39,40]. It was also reassuring that we did not see either worsening or induction of allergic symptoms following QVLP exposure and there was no evidence of a potentially immunopathologic IgE response to plant-origin carbohydrate motifs present on the QVLP vaccine. These observations are consistent with our previous work with monovalent plant-based VLP vaccines [33].

The HI and MN titers were also consistent with previous studies of monovalent VLP vaccines bearing HA proteins of either seasonal (H1) or pandemic (H5) influenza strains [22,23]. Indeed, at 21 days after immunization, the two higher doses of QVLP (9 and 15 μg/strain) readily met all of the CHMP criteria for licensure for all four strains in the vaccine, including HI titer >40 defined as the humoral correlate of protection. Even the lowest QVLP dose (3 μg/strain) met these criteria for the influenza A strains. Despite the relative small sample size of this phase 1–2 study, these observations demonstrate that the QVLP vaccine can achieve standard immune correlates of protection at HA doses comparable to commercial TIV or QIV vaccines in healthy adults. The

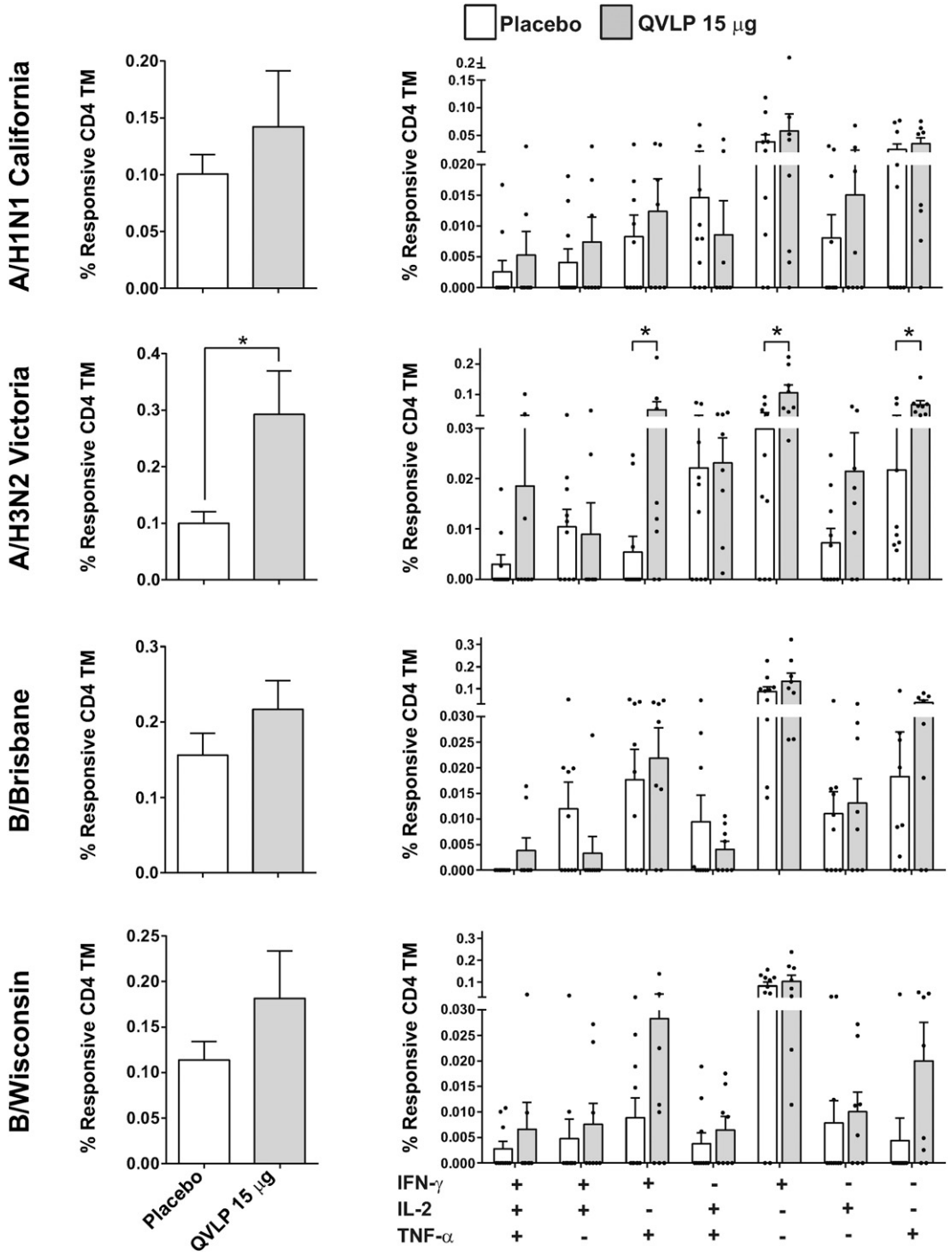

Fig. 8. Long-term homologous $CD4^+$ T cell-mediated responses 201 days after vaccination. Left panel: Percentages of transitional memory (TM, $CD45RA^-CCR7^-CD27^+$) CD4 T cells expressing at least one of the three functional markers (IL-2, TNF-α or IFN-γ) upon ex vivo Ag-specific re-stimulation (mean ± SE). Right panel: Qualitative analysis of the TM CD4 T cell response based on the expression of the three functional markers. The mean (bar ± SE) and individual results (dots) are represented for the seven functional signatures. The symbol * indicates significant differences between placebo and 15 μg QVLP groups ($p \leq 0.05$).

functional nature of the Abs induced by QVLP vaccination was confirmed by the MN results that correlated well overall with HI titers. Although occasional discrepancies between HI and MN results have been reported and there is some evidence that MN results may be more sensitive predictors of protection for children [41], our data suggest that the Abs induced in the first 3 weeks after QVLP vaccination are capable of both inhibiting hemagglutination and preventing viral entry into host cells (neutralization).

Durability of the sero-response is another important consideration for novel influenza vaccines. Although at least one commercial TIV has been reported to increase the number of influenza virus-specific memory B cells [42], there is a great deal of evidence that TIV/QIV-induced antibodies fall rapidly over time with some studies showing slightly greater persistence of antibodies directed against either A [43,44] or B strains [40]. In our study, GMT were maintained at ≥1:40 for the 4 strains included in QVLP for at least 6 months in the 15 μg group. At all QVLP doses, antibody persistence was generally greater for influenza A than the B strains. The mechanisms that underlie this long-lasting response following QVLP vaccination are not yet fully understood but may, in part, be attributable to the parallel induction of CD4 T cell help

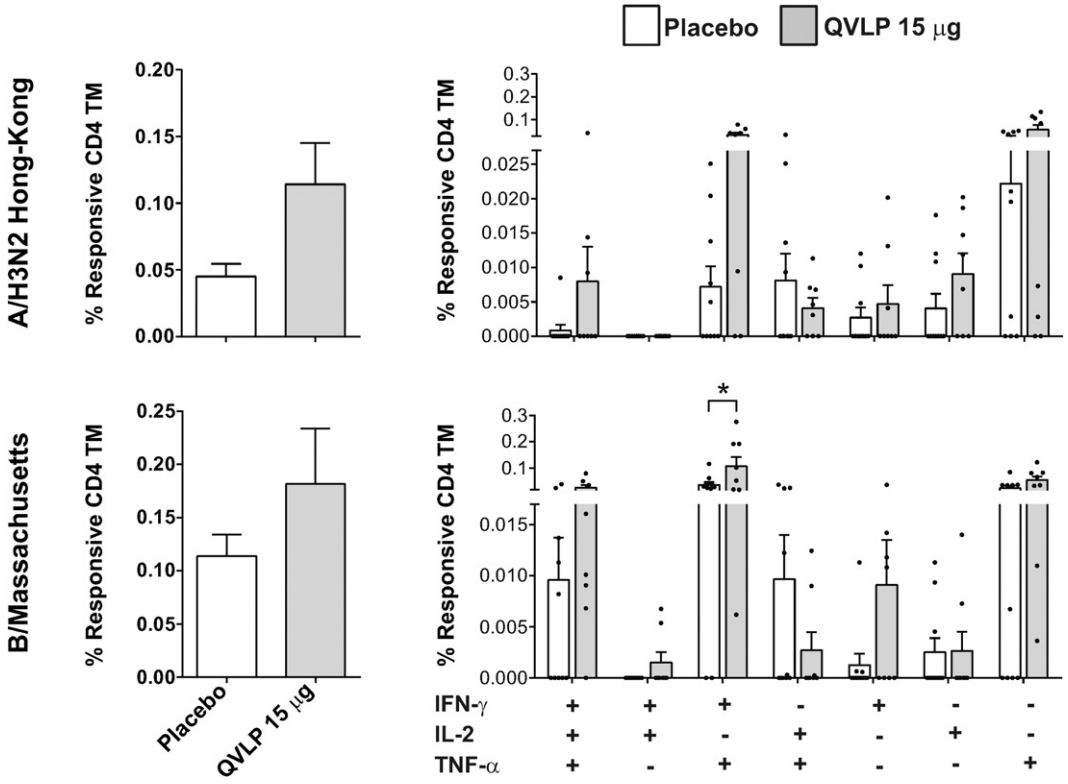

Fig. 9. Long-term heterologous cell-mediated responses 201 days after vaccination Left panel: Percentages of transitional memory (TM, CD45RA$^-$ CCR7$^-$ CD27$^+$) CD4 T cells expressing at least one of the three functional markers (IL-2, TNF-α or IFN-γ) upon ex vivo Ag-specific re-stimulation (mean ± SE). Right panel: Qualitative analysis of the TM CD4 T cell response based on the expression of the three functional markers. The mean (bar ± SE) and individual results (dots) are represented for the seven functional signatures. The symbol * indicates significant differences between placebo and 15 μg QVLP groups ($p \leq 0.05$).

by the plant-made vaccine (see below). Both increased [45] and decreased sero-response have been reported in subjects who have been previously vaccinated [46,47]. We do not yet know how the unusual persistence of the antibodies induced by QVLP vaccination may influence subsequent immunizations and/or exposure to natural disease. Since antigenic drift strains often occur during and between influenza seasons, cross-reactivity is another important consideration for any influenza vaccine. Although sera were not tested against a larger panel of heterologous antigens, the QVLP vaccine at higher doses (9 and 15 μg/strain) induced strong cross-reactive antibody responses against the B/Mass and A/H3N2 Uru strains at least. Lower levels of cross-reactive antibodies were induced against the A/H1N1 Bris and B/Mal strains. The A/H1N1 Bris results may be partially explained by the low HA sequence homology between A/H1N1 Cal and A/H1N1 Bris and H1/Bris (79.2%) compared to the H3 (97.3%) and B/Victoria antigens tested (99%). On the other hand, the B Yamagata HA antigens included in this study share 98.8% homology yet there was little evidence of cross-reactivity for B/Mal. Differences in HA glycosylation may also have contributed to the low cross-reactivity observed for the A/H1N1 Bris and B/Mal strains. The A/H1N1 Bris HA has two additional glycosylation sites at positions 122 and 157, both of which lie within the highly immunogenic receptor binding domain (RBD) [48]. The presence of additional glycans in this critical region could certainly interfere with binding of anti-HA Abs induced by the HA of A/H1N1Cal included in the QVLP [49–51]. Poor cross-reactivity for the heterologous B/Mal strain may be also have been influenced by the presence of an additional *N*-linked glycan site at position 197 that commonly occurs in egg-adapted B/Victoria-lineage viruses including the B/Bris strain included in the QVLP vaccine.

Given that HI titers have long been the major criteria for influenza vaccine licensure, it is not surprising that most commercially available influenza vaccines (e.g.: TIV/QIV) have been designed to induce strong humoral responses. However, protective responses for most viral illnesses result from a strong collaboration between the different elements of the immune system: innate and adaptive, humoral and cellular [52,53]. Indeed, while pre-formed antibodies can provide protection from many viral illnesses (i.e.: antibodies can be sufficient), CMI is generally required to effectively clear most viral infections and to maintain long-term immunity. Actually, there is a growing consensus that CMI, and CD4 T cell responses in particular [54], play a key role in long-term, cross-protective immunity to influenza. Such responses may be critically important in the elderly [55]. With the exception of the live-attenuated formulation (LAIV) commercial influenza vaccines are generally very poor inducers of robust anti-viral CMI [56–58]. It is noteworthy that, despite generally lower detectable serum antibody levels, LAIV also provides better cross-protection than inactivated TIV/QIV formulations [57]. Despite the non-living nature of plant-based VLPs and the fact that influenza HAs are generally not considered to be strong inducers of CMI, monovalent influenza VLP vaccines bearing either H1 or H5 have consistently elicited strong cellular responses in both animal models [23] and humans [22]. In the current study, these results have been confirmed and extended through a detailed characterization of both the short- and long-term cellular responses induced by QVLP in healthy adults.

Compare to the placebo recipients, the QVLP-immunized subjects mounted striking HA-specific poly-functional CD4 T cell responses at D21 that were still readily detected at D201. Poly-functionality has been associated with better protection against many infections including influenza [59–61]. CD4 T cells play diverse roles in response to infection. CD4 T cells provide necessary help for both CD8 T cells and B cells to achieve their full functional potential, as well as mediating direct effector functions through cytolysis of influenza-infected cells [62,63]. Most of the poly-functional CD4 T cells in the QVLP vaccinated subjects were IFN-γ$^+$, a widely-used parameter to assess effective anti-viral responses. In the context of influenza vaccination, it is particular interesting that such IFN-γ$^+$ CD4 T cells are an approved correlate of protection

for LAIV [64]. IFN-γ synthesis is considered to be an effector function, since this cytokine has an important role in the clearance of a wide range of viral, bacterial, parasitic and fungal infections [61]. In addition, the expression of IFN-γ in T cells has been associated with homing ability to lungs [65] and the presence of tissue-resident memory T cells in lungs has been associated with better protection against influenza [66,67]. IFN-γ produced by CD4 T cells is thought to be necessary for the generation of lung-resident $CD103^+$ CD8 tissue-resident memory T cells that provide enhanced protection against infection at mucosal sites [68]. Furthermore, Wilkinson et al. [69] showed that pre-existing influenza-specific IFN-γ^+ CD4 T cell correlate with disease protection in humans. Influenza Ag-specific $CD4^+$ T cells also likely play an important role in mediating cross-reactive protection after immunization and natural disease [18,70].

The Ag-specific IFN-γ^+ CD4 T cells elicited by the QVLP vaccine 21 days after immunization were essentially memory cells, either TM or T_{EMRA}. $CCR7^-$ effector memory cells were associated with better protection and have been proposed as one of the most desirable populations to be elicited by a vaccine [71]. Concurrently with the expression of IFN-γ, the presence of CD27 is also strongly correlated with the ability of T cells to home to the lungs [72] and Ag-specific T cells in the lungs of mice that survive influenza lethal challenge strongly express CD27 [73]. At D201, the HA-specific CD4 memory T cells detected in peripheral blood after vaccination with QVLP were mostly phenotyped as TM cells. These cells display an intermediary phenotype between CM and EM, having a longer lifespan than EM cells while maintaining the capacity to quickly differentiate into effector cells that can control virus replication. Although we have previously observed a limited $CD8^+$ T cell response following monovalent H1-VLP immunization [22] and saw hints of such a response in the QVLP recipients, we acknowledge that the timing of sample collection in the current study as wells as the use of VLP for ex vivo stimulation was not optimal for the identification of cytotoxic T cell (CTL) responses.

In conclusion, this first, dose-escalation clinical trial of a plant-made QVLP vaccine has demonstrated that a single, unadjuvanted dose of this candidate vaccine was well-tolerated and elicited strong and cross-reactive humoral and cellular responses that persisted for at least 6 months. At the higher doses (9 and 15 μg/strain), QVLP vaccine met all of the CMHP criteria for licensure. Unlike most other non-living influenza vaccines, QVLP vaccine also induced a striking polyfunctional and cross-reactive CD4 T cell memory response.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2016.03.008.

Author contributions

SP, EA, ST, BJW and NL participated in the conception, the design, and implementation of the clinical trial. ST, EA, BJW and NL were involved in the acquisition, analysis and interpretation of the safety data. SP, EA, JFP, BYD, BJW and NL were involved in the acquisition, analysis and interpretation of the CMI data. DB, MD, SP, EA, BJW and NL were involved in the acquisition, analysis and interpretation of the serological data. All the authors contributed in the development of this manuscript and gave final approval to submit for publication.

Conflict interest statement

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, Éric Aubin, Sonia Trépanier, Diane Bussière and Stéphane Pillet are employees of Medicago Inc.

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