A Hemagglutinin 1 Carrying Plant-Based Virus-like Particle Vaccine Generates an Efficacious Cellular Response by Exploiting IL-1 Signaling in Both Adult and Aged Mice

Fernando Alvarez, Roman Istomine, Hilary Hendin, Breanna Hodgins, Stephane Pillet, Jörg H. Fritz, Nathalie Charland, Brian J. Ward and Ciriaco A. Piccirillo

ImmunoHorizons 2022, 6 (6) 384-397
doi: https://doi.org/10.4049/immunohorizons.2200036
http://www.immunohorizons.org/content/6/6/384

This information is current as of July 11, 2022.

Supplementary Material
http://www.immunohorizons.org/content/suppl/2022/06/23/immunohorizons.2200036.DCSupplemental

References
This article cites 67 articles, 14 of which you can access for free at:
http://www.immunohorizons.org/content/6/6/384.full#ref-list-1

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://www.immunohorizons.org/alerts
A Hemagglutinin 1 Carrying Plant-Based Virus-like Particle Vaccine Generates an Efficacious Cellular Response by Exploiting IL-1 Signaling in Both Adult and Aged Mice

Fernando Alvarez,*† Roman Istomine,*† Hilary Hendin,†‡ Breanna Hodgins,†‡ Stephane Pillet,†§ Jörg H. Fritz,‡§ Nathalie Charland,‡§ Brian J. Ward,†‡§ and Ciriaco A. Piccirillo,*†‡
*Department of Microbiology & Immunology, McGill University, Montreal, Quebec, Canada; †Research Institute of McGill University Health Center, Montreal, Quebec, Canada; ‡Department of Experimental Medicine, McGill University, Montreal, Quebec, Canada; §Medicago Inc., Quebec, Quebec, Canada; and ††McGill University Research Center on Complex Traits, McGill University, Montreal, Quebec, Canada

ABSTRACT

Inactivated influenza vaccines have struggled to provide consistent protection in older individuals. Circumventing immune senescence, an aging of the immune response characterized by weak humoral responses to vaccines, and unchecked inflammation during infection require novel immunization strategies. Plant-based virus-like particles (VLPs) bearing recombinant hemagglutinin proteins have been shown to provide protection in older animals in preclinical challenge studies, despite eliciting relatively low or absent humoral responses. The nature of the cellular response induced by these vaccines and its evolution during infection have not yet been fully characterized, however. Using a murine model that recapitulates features of human immune senescence, we assessed T cell responses to vaccination with a VLP bearing the hemagglutinin of H1N1/California 07/2009 (H1-VLP) before and after challenge in young and aged BALB/c mice (2 and 18 mo old, respectively). We report that two i.m. doses of H1-VLP (3 µg) vaccine 21 d apart generated H1-specific Th1 and Th2 cells associated with the prevention of prolonged pulmonary inflammation and mortality in both adult and aged mice. While investigating the regulation of cellular immunity, we identified a unique IL-1R1+ tissue-adapted regulatory T cell population in the lungs of both H1-VLP–vaccinated adult and aged mice, suggesting a novel regulatory T cell population associated with vaccine-mediated protection. Collectively, this study provides preclinical evidence that the plant-based H1-VLP vaccine may act, in part, by preventing exacerbated immune responses against influenza A. ImmunoHorizons, 2022, 6: 384–397.

Received for publication May 12, 2022. Accepted for publication May 13, 2022.

Address correspondence and reprint requests to: Dr. Ciriaco A. Piccirillo, Research Institute of McGill University Health Centre, Centre for Translational Biology, Bloc E, Room E-M2.3248, 1001 Boulevard Décarie, Montreal, QC H4A 3J1, Canada. E-mail address: ciro.piccirillo@mcgill.ca

ORCIDs: 0000-0002-3864-5790 (F.A.), 0000-0002-0205-5692 (H.H.), 0000-0002-7703-6779 (C.A.P.).

This work was supported by Fonds de Partenariat pour un Québec Innovant et en Santé and Canadian Institutes of Health Research Operating Grant PJT-148821 (to C.A.P.), as well as by an academic-industry team award led by B.J.W. that was supported by Medicago Inc. and the Ministère de l’Économie et de l’Innovation du Québec with project oversight by Genome Québec.

F.A. and C.A.P. designed the conceptual framework of the study, designed experiments, and wrote the paper. B.J.W., J.H.F., and N.C. helped edit the paper and provided important conceptual input. F.A. designed and performed experiments and analyzed data. R.I. contributed to the in vivo influenca experiments. B.H., H.H., and S.P. contributed to the preparation of the virus and the early in vivo experiments. All authors provided valuable input throughout the study and the writing of the manuscript.

Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; HA, hemagglutinin; H1-VLP, HA1-bearing plant-based VLP; IIV, inactivated influenza vaccine; i.n, intranasally; LN, lymph node; MDCK, Madin–Darby canine kidney; MFI, mean fluorescence intensity; QVLP, quadrivalent HA-VLP; ROR, retinoic acid–related orphan receptor; Teff, effector T; Treg, regulatory T; VLP, virus-like particle.

The online version of this article contains supplemental material.

This article is distributed under the terms of the CC BY-NC 4.0 Unported license.

Copyright © 2022 The Authors

ImmunoHorizons is published by The American Association of Immunologists, Inc.
Introduction

Influenza A virus is a highly contagious member of the Orthomyxoviridae family that causes an acute respiratory infection and remains a major public threat worldwide. Vaccination can significantly reduce morbidity and mortality associated with seasonal and pandemic outbreaks. However, vaccine efficacy varies widely between seasons and age groups and is often relatively modest (30–50%) (1). This lack of efficacy is particularly important in the elderly, as the risk of influenza-associated complications and mortality increases sharply after 65 y of age (2). The modest efficacy of influenza vaccines in the elderly is likely to be multifactorial, although aging of the immune system, termed immune senescence, is thought to be a major influence (3). Immune senescence in the elderly is characterized by both uncontrolled inflammatory responses and a weak adaptive immune response upon vaccination, a phenomenon referred to as inflamm-aging. In both humans and mice, exacerbated inflammatory responses can prevent the quick return to immune homeostasis in the respiratory tissues and contribute to postinfection complications (4, 5). New vaccine strategies that not only elicit Ag-specific immune responses but also facilitate a quick return to immune homeostasis if infection occurs may be particularly advantageous for the elderly.

Prevention of pulmonary immunopathology in the elderly, who can suffer from prolonged and uncontrolled inflammatory responses during influenza A infection (6–9), is an important but understudied consideration for vaccine strategies that target this age group. FOXP3-expressing regulatory T (TREG) cells are a subset of CD4$^{+}$ T cells that possess a potent suppressive phenotype shown to limit pathogen-induced damage while facilitating the development of immune memory (10, 11). During an influenza A infection, pulmonary TREG cells contribute to tissue protection and repair (12) and can facilitate a quick return to immune homeostasis (13). In recent years, considerable insight has been gained into the heterogeneous nature of mucosal TREG cells, revealing distinct phenotypes that play different roles in immunity and tissue repair (14). Distinct TREG cell subsets were shown to sense danger-associated molecular patterns generated during influenza, including the alarmins IL-1 and IL-33 (12, 15). There is now emerging evidence that IL-1R1$^{+}$ and IL-33R$^{+}$ (ST2) TREG cells possess distinct functional properties, compete during influenza to determine the balance between permissive and suppressive TREG cells in situ, respectively, and ultimately alter the clinical outcome of local immune responses in infection (15). As such, these subsets may represent important components of the protective cellular response generated by immunization.

The delivery of viral Ags using virus-like particles (VLPs) has several advantages, including presentation of the Ag in a biologically relevant form, protection from degradation, and accelerated delivery to the regional lymph nodes (LNs) (16). Plant-based VLPs are a novel strategy to produce nonliving influenza vaccines (17–20). These plant-based VLPs bearing hemagglutinin (HA) trimers of either seasonal or pandemic strains have been designed to mimic the external structure of enveloped influenza virions without any genetic material or unwanted animal, bacterial, or viral contaminants (21). Following footpad injection in mice, these VLPs traffic to the neighboring LN in a matter of minutes (22). The mammalian immune system effectively recognizes these particles and APCs efficiently internalize the plant-based VLPs and process HA peptides for both MHC class I and II presentation (23). Monovalent HA-bearing VLPs typically elicit both humoral and cellular responses in mice and ferrets and confer good protection against challenge (24–26). Of relevance to the current work, we have previously shown that HA1-bearing plant-based VLP (H1-VLP) immunization can protect against H1N1 infection in aged mice (27), including better protection against frailty compared with an inactivated influenza vaccine (IIV) in extremely old mice (>22 mo of age) (28). In many of the older mice, protection was not linked to the development of detectable HA inhibition or microneutralization Ab responses, suggesting that components of cellular immunity may underlie this response (27, 29). This finding is consistent with the detection of polyfunctional CD4$^{+}$ T cells in human trials of monovalent as well as quadrivalent HA-VLP (QVLP) formulations (18, 30), and a recent large field study with QVLP has demonstrated comparable protection to a commercial IIV in older adults (>65 y of age) despite generally lower HA inhibition and microneutralization Ab titers (31). Interestingly, the relative vaccine efficacy of QVLP compared with IIV was highest in the oldest subjects (>75 y of age) who had the lowest Ab titers but consistently strong cellular responses (31). These results prompted us to better characterize cellular immune responses, and their regulation, to a plant-based VLP vaccine in the aged mouse model.

Given the prominent role that prolonged inflammation plays in the response of older individuals to influenza, investigating the vaccine-induced tissue adaptation of both effector and TREG cells may offer a novel approach to enhance vaccine design for the elderly. In this report, we hypothesized that plant-based VLPs elicit a potent, but well-regulated, cellular response to an influenza infection in mice, contributing to a quick return to immune homeostasis. Moreover, H1-VLP immunization induced the generation of both Ag-specific Th1 and Th2 T cell populations in vivo and modulated the abundance of TREG cells responding to either IL-1 or IL-33 in the lungs during infection. Finally, the number of IL-1–responding TREG cells correlated with increased protection following H1-VLP vaccination in both young adult and aged mice. Collectively, this study highlights the generation of a broad T cell and TREG cell response by plant-based VLPs in both adult and aged mice and suggests that IL-1R1 expression on mucosal TREG cells may be a novel correlate of vaccine-induced protection through the prevention of prolonged pulmonary inflammation.

Materials and Methods

Vaccine preparation and formulations

The H1-VLP and QVLP vaccines (Medicago, Ste-Foy, QC, Canada) were produced in Nicotiana benthamiana using Agrobacterium...
infiltration-based transient expression, as previously described (17, 26, 30). HI-VLP was based on the A/California/07/2009 HA sequence whereas QVLP was generated 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 (30). The concentrations of 3 μg/dose (50 μl) were obtained through dilution in sterile PBS. A commercial IIV (FluLaval Tetra, GlaxoSmithKline, Montreal, QC, Canada) contained the same viral Ants as QVLP and was kept at the manufacturer’s concentration (15 μg/500 μl). Mice were anesthetized i.m. with 50 μl at two separate sites to obtain a total of 3 μg of H1 protein/dose (total dose of 100 μl).

**Virus preparation**

Wild-type A/California/07/2009 H1N1 virus was obtained from the National Microbiology Laboratory, Public Health Agency of Canada and propagated in a Madin–Darby canine kidney (MDCK) cell line (American Type Culture Collection no. PTA-6503). The 50% tissue culture-infective dose of H1N1/CA required to achieve 50% lethality in our models (LD_{50}) was determined by titration experiments in both 2- and 18-mo-old mice as previously described (27) using the Spearman–Karber method (32). For the plaque reduction neutralization assay, the lungs of infected mice at day 4 postinfection were homogenized in HyClone SFM4MegaVir medium; 50% tissue culture-infective dose (50% TCID50) of HI-VLP before the

**Generation of bone marrow–derived dendritic cells**

Mice were euthanized and the femurs were isolated and sterilized in 70% ethanol. The bone marrow was mechanically ejected by inducing pressure on one end of the femur with complete RPMI 1640, and 1 × 10^6 bone marrow cells were cultured in 48-well plates in the presence of 10 ng/ml GM-CSF for 7 d (Miltenyi Biotec, Bergisch Gladbach, Germany). The frequency of CD11c+ expanded bone marrow–derived dendritic cells (BMDCs) was determined, and the BMDCs were then incubated for 18 h in the presence of 1 μg/ml HI-VLP before the CD4+ T cells were added.

**T cell culture**

Popliteal LNs from PBS or HI-VLP immunized mice were isolated, single-cell suspensions were prepared, and CD4+ T cells were isolated by automated magnetic bead separation (MACS, Miltenyi Biotec) using anti-CD4 beads (L3T4; Miltenyi Biotec). The cells were then plated in the presence of H1-VLP–primed BMDCs in flat 48-well plates at a ratio of 1:3 (2 × 10^5 T cells/well). The cells were incubated at 37°C in 5% CO2 for 96 h and then restimulated for 3 h with 20 ng/ml PMA (Sigma-Aldrich) and 1 nM ionomycin (Sigma-Aldrich) in the presence of BD GolgiStop (1:1000; BD Biosciences, Amersham, U.K.), and supernatants were added in serial dilutions to MDCK monolayers and left for 16 h to incubate before fixing and staining as previously described (33).

**Flow cytometry**

After lymphocyte isolation, single-cell suspensions were stained with the following fluorescence-conjugated mAbs, purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated: anti-CD4 Alexa Fluor 700 (GKL5), anti-CD8 V500 (S3-6.7) (BD Biosciences), anti-ST2 PerCP-eFlour 710 (RMST2-2), anti-CD25 PE-Cy7 (PC61) (BD Biosciences), anti-Foxp3 FTTC or PE (FJK-16s), anti-IL-17A allophycocyanin (eBio1B7), anti–IFN-γ PE-Cy7 (XM12), anti-retinoic acid–related orphan receptor (ROR)γt PE (AFKJS-9), anti–T-bet PE-Cy7 (BD Biosciences), anti-Helios Pacific Blue or PE (22F6) (BioLegend), anti-CD122a/IL-1R1 Alexa Fluor 647 (35F5) (BD Biosciences), and anti-Ki67 V450 (B56). Nonviable cells were excluded using the fixable viability dye reagents eFlour 780 or 506 (Thermo Fisher Scientific). Data were acquired using a FACS LSRFortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 9 software (Tree Star, BD Biosciences).
Statistical analysis
For all experiments, the mean and SD are shown. Multiple comparisons were tested using a one- or two-way ANOVA, and statistical hypothesis testing was done using Tukey's multiple comparison test. For single comparisons, an unpaired Student t test was used, unless otherwise stated, with the p value expressed in the figure legends. Best-fit linear regression was performed to establish correlates between the observed variables. All statistical analysis was performed with GraphPad Prism version 6 software (GraphPad Software, San Diego, CA).

RESULTS

Plant-based H1-VLP vaccination protects adult mice from influenza A–induced morbidity
Although plant-based VLPs have been shown to prevent acute mortality in models of influenza infection, little attention has been given to the impact of these vaccines on the pulmonary immunological landscape following challenge. Importantly, rapid viral clearance, accompanied by an appropriate contraction and control of CD8\(^+\) CTLs and IFN-γ–CD4\(^+\) (Th1) T cells can lead to a better clinical outcome after influenza infection (10, 34, 35). In this study, we studied the effects of an H1-VLP vaccine on the lung immune response to a low infectious dose (0.5xLD\(_{50}\)) of the pandemic H1N1/Ca influenza A virus. Because Ag availability is a key feature for the fine-tuning of successful cellular and humoral responses (36), we investigated the effects of a high (3 μg) or low (0.03 μg) dose of H1-VLP on the survival of mice (Fig. 1A). Compared to low dose– or PBS-treated mice, all mice immunized with the higher VLP dose survived the infection (Fig. 1B), suggesting that the full 3 μg/dose was required to provide full protection. Similarly, we observed that surviving mice immunized with high-dose H1-VLP lost less weight than did low dose–immunized and PBS control groups and were quicker to return to their original weight (Fig. 1C). Histology of the lungs shows that prior immunization with 3 μg of H1-VLP led to a reduction in cellular infiltration, characterized by decreased interstitial space infiltration and alveolar collapse by day 6 postinfection (Fig. 1D). Lung viral load by plaque assay at day 4 postinfection confirmed a significant reduction of infectious viral particles in the high dose–vaccinated mice (Fig. 1E). We then assessed the nature and dynamics of the T cell response in the lungs of H1-VLP (3 μg)– and PBS-treated (control) groups using flow cytometry at days 3, 6, and 12 postchallenge. Compared to the control adult group (2 mo of age), mice immunized with H1-VLP showed significantly reduced lung infiltration of CD4\(^+\) and CD8\(^+\) T cells during the infection (Fig. 1F, 1G). This was associated with lower numbers of pulmonary IFN-γ– or IL-17A–producing, but not IL-4–producing, CD4\(^+\) T cells in the lungs of immunized mice (Fig. 1H–J, Supplemental Fig. 1A), resulting in a balanced Th1/Th2 response in the lungs (Fig. 1K). Finally, we assessed whether these immune changes differed between the plant-based formulations (i.e., H1-VLP, QVLP) and the comparator vaccine, IIV. We observed a similar protection from weight loss and in the pulmonary infiltration of cells in the groups that received H1-VLP (3 μg/dose), QVLP (3 μg of H1/dose), or an egg-based quadrivalent IIV (3 μg of H1/dose) vaccines (Fig. 1L, Supplemental Fig. 1B). Moreover, the accumulation of IFN-γ–CD4\(^+\) T cells in the lungs was reduced by immunization (Fig. 1M). Importantly, the numbers of actively replicating T-bet–CD8\(^+\) T cells, which are associated with pulmonary pathology when maintained after viral clearance (35), were low in the lungs by day 8 in all vaccinated groups (Supplemental Fig. 1C). Collectively, plant-based H1-VLP and its quadrivalent equivalent (QVLP) are efficient at preventing a ramping of Th1 and CD8\(^+\) T cells responses in the lungs.

HI-VLP vaccination influences the functional adaptation of pulmonary T\(_{REG}\) cells during disease
Foxp3-expressing T\(_{REG}\) cells have been proposed to play a key role in the control of prolonged pulmonary inflammation during influenza (37) and contribute to tissue repair (12). Indeed, Foxp3\(^+\) T\(_{REG}\) cells accumulate in the lungs of infected mice, but not in H1-VLP–immunized mice (Fig. 2A), confirming our observations that a successful immunization prevents inflammation in the lungs. Concomitantly, the ratio of Th1 (CD4\(^+\)IFN-γ–) cells to T\(_{REG}\) cells was better maintained in H1-VLP–immunized mice compared with the control mice (Fig. 2B). In the lungs, we observed a distinct subset of pulmonary T\(_{REG}\) cells that expressed Helios, a transcription factor suggested to distinguish T\(_{REG}\) cells of thymic origin from peripherally induced T\(_{REG}\) cells (38) (Fig. 2C). Importantly, Helios reinforces a transcriptional program in T\(_{REG}\) cells that promotes robust and durable suppressive functions in inflammatory environments (39–41). During influenza, a steady accumulation of Helios\(^+\) T\(_{REG}\) cells in the lungs of unvaccinated compared with H1-VLP–vaccinated adult mice was observable (Fig. 2D). In our infection model, we observed that a significant portion of Helios\(^+\) T\(_{REG}\) cells adopted the transcription factor T-bet (Fig. 2E, 2F), a population of influenza-specific T\(_{REG}\) cells previously described to play a role in late Th1 cell suppression (42). Prior vaccination with H1-VLP prevented the accumulation of Helios\(^+\) T-bet\(^+\) T\(_{REG}\) cells, suggesting early control of immune inflammation in these mice.

Influenza A infection causes the release of the alarmins IL-1 and IL-33 (43, 44), cytokines involved in inflammation that can signal directly in tissue-localized T\(_{REG}\) cells (15). We have previously shown that Helios\(^+\) IL-33–responding (expressing the IL-33 receptor, ST2\(^+\)) and Helios\(^+\) IL-1α/β–responding (IL-1R1\(^+\)) T\(_{REG}\) cells represent two distinct and competing subpopulations of T\(_{REG}\) cells (15) that can either promote local suppression or immunity, respectively. To determine the effect of H1-VLP vaccination on these T\(_{REG}\) cell subpopulations postchallenge, we followed the accumulation of ST2\(^+\) and IL-1R1\(^+\) T\(_{REG}\) cells in the lungs. We observed that the frequency of Helios\(^+\) ST2\(^+\) T\(_{REG}\) cells gradually increased after challenge in adult mice, consistent with a prior account (15). We observed an increased frequency of ST2\(^+\) T\(_{REG}\) cells by day 3 in the lungs of H1-VLP–immunized
mice (Fig. 2G, 2H). Concomitantly, the level of expression (mean fluorescence intensity [MFI]) of ST2 was higher in TReg cells of H1-VLP–immunized mice, suggesting that the vaccine potentiates pulmonary ST2+Helios+TReg cells that suppress local inflammation (12, 15, 45). In contrast, IL-1RI+Helios−TReg cells gradually decreased in the lungs of PBS-treated mice but not H1-VLP–immunized mice (Fig. 2I, 2J) whereas the expression of IL-1R1 by both TReg and effector T (TEff) cells was higher in immunized mice (Supplemental Fig. 2E, 2F), illustrating that H1-VLP immunization favors IL-1 signaling in T cells in the lungs. Overall, these results indicate that vaccination by plant-based H1-VLP influences the balance of distinct functional subsets of pulmonary TReg cells by altering their response to IL-33 and IL-1 in the lungs.

**HI-VLP vaccination elicits an Ag-specific Th1 cellular response by exploiting IL-1 signaling in mice**

Because H1-VLP vaccination favored the induction of IL-1RI+TEff and TReg cells in the lungs, we next investigated how H1-VLP generates Ag-specific T cell responses. We have previously shown that i.m. immunization can elicit T cell–specific responses, although the resolution of this response in the spleen limited our capacity to analyze in depth (46). Thus, we immunized mice s.c. under the right footpad in a prime-boost experiment and assessed the expansion and differentiation of Th1, Th2, and Th17 T cells in the draining popliteal LNs, to which our plant-based VLPs migrate (22) (Fig. 3A). We observed an increase in expanding (Ki67+) CD4+ T cells (Fig. 3B) as well as a significant increase in the total numbers of both CD4+ and CD8+...
FIGURE 2. H1-VLP immunization promotes the accumulation of alarmin-adapted T<sub>REG</sub> cells in the lungs during infection in 2-mo-old adult BALB/c mice.

Two-month-old BALB/c mice were immunized with a conventional dose (3 μg) of H1-VLP by i.m. injection at days 0 and 21 and infected with 1/4 LD<sub>50</sub> of H1N1/Ca i.n. at day 42. (A) Number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (T<sub>REG</sub> Cells) in the lungs of infected mice during the course of disease. n = 4–5, representative of three experiments. Two-way ANOVA with Tukey correction: ***p < 0.001. (B) Ratio of Th1 over T<sub>REG</sub> cells (no. of IFN-γ<sup>+</sup> T cells/no. of T<sub>REG</sub> cells) during the course of disease between H1-VLP–immunized (red line) and PBS-treated (black line) mice. Two-way ANOVA: **p < 0.01, ***p < 0.001. (C) Representative flow cytometry of the expression of Foxp3 and Helios in lung-isolated T<sub>REG</sub> Cells at day 6 of infection in PBS-treated mice at day 6 postinfection. (D) Frequency of Helios<sup>+</sup> T<sub>REG</sub> cells in the lungs of PBS-treated compared with (Continued)
T cells (Fig. 3C–E). The frequency of IFN-γ–, IL-17A–, or IL-13–producing CD4+ T cells upon immunization also increased draining popliteal LNs (F1 F3) (Fig. 3F–I). When stimulating T cells with H1-VLP–pulsed BMDCs, we observed a significant portion of expanding IFN-γ– and IL-4–producing but not IL-17A–producing CD4+ T cells (Fig. 3J), revealing that H1-VLP immunization effectively induced both Ag-specific Th1 and Th2 cells, but not Th17 cells, suggesting that IL-17A–producing T cells might occur as an effect of VLP–induced inflammation. To confirm that the H1-VLP–specific CD4+ and CD8+ T cells can effectively migrate to the lungs during infection and in the absence of any influence of the humoral response generated by the vaccine, we made use of a transfer experiment in which cells from H1-VLP–immunized mice were transferred into naive recipient mice infected with influenza A. To this end, C57BL/6 mice (CD45.2+/−) were immunized with two s.c. doses 21 d apart of 3 μg of H1-VLP, and splenocytes isolated 4 d after the second immunization (Supplemental Fig. 3A) were then injected i.p. into naive congenic (CD45.1+/−) C57BL/6 mice infected with 1/2 LD50 of H1N1/Ca. We then assessed the infiltration of donor, H1-VLP–exposed T cells (CD45.2+/−) in the lungs 6 d postinfection. Recipient mice of H1-VLP–exposed T cells had increased numbers of CD45.2+ T cells in the lungs compared with recipient mice of cells from PBS-treated mice (Supplemental Fig. S3B). Moreover, lung-infiltrating T cells were predominantly from the CD4+ compartment in the H1-VLP–vaccinated group, suggesting an accumulation of Ag-specific T cells (Supplemental Fig. 3C–E).

The role of IL-1 in the development of T cell immunity has not been fully addressed, although there is evidence of its importance in the generation of Ag-specific T cells during vaccination (47, 48). Because IL-1R1+ T cells accumulated in the lungs of H1-VLP–immunized mice compared with PBS controls, we investigated the role of IL-1 in the development of a H1-VLP–specific immune response by immunizing wild-type mice and in mice lacking IL-1 signaling (IL-1RI−/−). Immunizing mice lacking IL-1RI caused increased expansion of TREG and CD4+Foxp3+ T cells in IL-1RI−/− mice (Fig. 3K), an increase in the frequency of TREG cells, and a decrease in IFN-γ+ Th1 cells (Fig. 3L, 3M). Concomitantly, the frequency of IL-13+ Th2 cells was not affected by the absence of IL-1 signaling (Fig. 3N) whereas the frequency of IL-17A+ T cells was significantly reduced (Fig. 3O), revealing that IL-1 is a critical signal regulating Th1 and Th17 responses to the vaccine. Indeed, the absence of IL-1 signaling caused a shift in the ratio of TREG to Th1 T cells in the LN (Fig. 3Q). Collectively, these results highlight the key role of IL-1 in establishing the balance between Ag-specific TREG and Th1 cells following H1-VLP vaccination.

**HI-VLP immunization confers protection from influenza A in aged mice**

As influenza-related morbidity and mortality are highly associated with age (49), we investigated whether H1-VLP immunization generates a protective cellular response in an aging mouse model (27). To do so, we vaccinated aged mice (18 mo old ± 14 d at time of infection) with two doses of H1-VLP (3 μg/dose), as described for the adult mice (Fig. 1). To investigate the contraction of the immune response after challenge, we infected both adult (2-mo-old) and aged (18-mo-old) mice with their respective 1/2 LD50 of H1N1/Ca strain (27) because the LD50 of aged mice is lower than that for their adult counterparts. As in adult mice (Fig. 4A, 4B), we observed a protective effect of H1-VLP in aged mice in terms of weight loss and survival (Fig. 4C, 4D). Indeed, the H1-VLP–immunized aged mice lost significantly less weight than did the PBS-treated mice during the course of infection (Fig. 4C). However, in contrast to adult mice, we observed a significant T cell infiltration in the lungs of H1-VLP–immunized aged mice that decreased significantly in the lung by day 12 of infection (Fig. 4E, 4F), revealing that vaccinated mice were undergoing immune contraction. Moreover, prior to the infection, serum titers of H1-specific IgG1 and IgG2a were significantly lower in the aged mice (Fig. 4G), suggesting that cellular immunity, rather than humoral immunity, is a major determinant of vaccine efficacy in this age group. Indeed, although both PBS-treated and H1-VLP–vaccinated aged mice displayed an early infiltration of CD8+ T cells, only vaccinated aged mice had a decrease of these cells by day 12 postinfection (Fig. 4H). Similarly, the numbers of IFN-γ–producing CD4+ T cells in the lungs decreased sharply by day 12 postinfection in vaccinated aged mice (Fig. 4I). In contrast, we did not note a significant difference in the accumulation of IL-4–producing CD4+ T cells between PBS-treated and H1-VLP–vaccinated aged mice throughout infection (Fig. 4J), even at peak inflammatory responses (day 6) in immunized...
mice, revealing that the abundance of Th2 cells in response to challenge was not altered by prior H1-VLP immunization. Finally, a population of IL-17A$^+$ CD4$^+$ T cells, a known factor associated with influenza-related morbidity in the elderly (50), contracted more sharply postchallenge in the lungs of vaccinated aged mice (Fig. 4K). These observations are consequential because, contrary to IFN-γ and IL-17A, IL-4 production is associated with the suppression of prolonged cytotoxic CD8$^+$ T cell responses during influenza (51), suggesting that the control of exacerbated Th1 responses by H1-VLP immunization enables a protective Th2 T cell response in the lungs. Collectively, these results suggest a generally similar effect of H1-VLP immunization in determining the nature of the infiltrating cellular response in both adult and aged mice upon subsequent challenge.

**H1-VLP facilitates the accumulation of IL-1R1$^+$ T<sub>REG</sub> cells in the lungs of influenza-infected aged mice**

A dysregulation of T<sub>REG</sub> cell function has been associated with prolonged pulmonary inflammation following influenza A in aging mice (52). Because the plant-based H1-VLP is protective in aging mice, we assessed how it influenced T<sub>REG</sub> cell populations. In aged mice, both PBS-treated and H1-VLP–vaccinated groups showed similar frequencies of Foxp3$^+$ T cells isolated at day 4. Student t test: ***p < 0.001 (individual mice shown). (K) Representative flow cytometry of the expression of Foxp3 and Ki67 in CD4$^+$ T cells in the draining LN of B6 (WT) and IL-1R1KO mice at day 4. (L–O) Frequency of Foxp3$^+$, IFN-γ$^+$, IL-13, and IL-17A$^+$ CD4$^+$ T cells in the draining LN of B6 (WT) and IL-1R1KO mice at day 4. Student t test: *p < 0.05, ***p < 0.001. (P) Ratio of the number of CD4$^+$ Foxp3$^+$ T<sub>REG</sub> cells over the number of IFN-γ$^+$ CD4$^+$ Foxp3$^-$ (Th1) cells in the draining LN at day 4. Student t test: ***p < 0.001.

**FIGURE 3.** H1-VLP immunization promotes the generation of Ag-specific Th1 cells in an IL-1–dependent manner.

(A) Methodology of the immunization protocol. Eight-week-old BALB/C mice were immunized with 3 μg of H1-VLP by s.c. injection under the footpad at days 0 and 21 and necropsied at day 4 after the second injection. The lymphocytes from the draining popliteal (draining LNs) and contralateral popliteal LNs (non-draining LNs) were isolated and analyzed (n = 4). (B) Representative flow cytometry of the expression of Foxp3 and Ki67 in CD4$^+$ T cells in the draining and non-draining popliteal LNs at day 4. (C) Total cell counts of cells isolated are shown. Student t test: ***p < 0.001. (D and E) Number of CD4$^+$ and CD8$^+$ T cells isolated at day 4. Student t test: **p < 0.01. (F) Representative flow cytometry of IFN-γ and IL-17A production in CD4$^+$ T cells. (G–I) Number of IFN-γ$^+$, IL-17A$^+$, and IL-13$^+$ producing CD4$^+$ (Foxp3$^-$) T cells upon secondary immunization. Student t test: **p < 0.01. (J) Frequency of IFN-γ$^+$, IL-17A$^+$, and IL-4$^+$ producing CD4$^+$ (Foxp3$^-$) T cells after they were cultured for 4 d in the presence of H1-VLP–primed mature bone marrow–derived dendritic cells (BMDCs). Student t test: ***p < 0.001 (individual mice shown). (K) Representative flow cytometry of the expression of Foxp3 and Ki67 in CD4$^+$ T cells in the draining popliteal LNs of B6 (wild-type [WT]) and IL-1R1KO mice at day 4. (L–O) Frequency of Foxp3$^+$, IFN-γ$^+$, IL-13, and IL-17A$^+$ producing CD4$^+$ (Foxp3$^-$) T cells in the draining LN of B6 (WT) and IL-1R1KO mice at day 4. Student t test: *p < 0.05, ***p < 0.001. (P) Ratio of the number of CD4$^+$ Foxp3$^+$ (T<sub>REG</sub>) cells over the number of IFN-γ$^+$ CD4$^+$ Foxp3$^-$ (Th1) cells in the draining LN at day 4. Student t test: ***p < 0.001.

https://doi.org/10.4049/immunohorizons.2200036
Two-month-old (adult) and 18-mo-old (aged) mice were immunized with 3 μg of H1-VLP i.m. at 21-d interval and infected at day 42 after the first immunization with their respective 1/2 LD50 of H1N1/Ca i.n. The mice were monitored for weight loss and survival (limit point established at loss of >20% of their initial weight). Results are representative of three experiments (n = 4–5 per group). (A and B) Weight loss and survival of 2-mo-old mice. One-way ANOVA with Tukey correction: **p < 0.01, ***p < 0.001. (C and D) Weight loss and survival of 18-mo-old mice. One-way ANOVA with Tukey correction: **p < 0.01. (E) Representative histology of the lungs of 18-mo-old mice at day 6 postinfection. H&E stain. Black arrows point to interstitial cell infiltrates and alveolar collapse; red arrows point to peribronchial cell infiltrates. (F) Count of total live cells isolated from the lungs of infected mice before peak (day 3), at peak (day 6), and after peak (day 12) of infection. Two-way ANOVA with Tukey correction: *p < 0.05, **p < 0.01, ***p < 0.001. (G) H1-specific ELISA to detect IgG1 (left) and IgG2a (right) titters of adult and aged (red) mice prior to infection (day 42). One-way ANOVA with Tukey correction: **p < 0.01. (H) Count of live CD8+ T cells isolated from the lungs of infected mice in the course of infection. (I–K) Counts of IFN-γ-, IL-4-, and IL-17A-producing live CD4+ (Foxp3+) TREG cells in the lungs of infected mice. Two-way ANOVA with Tukey correction: **p < 0.01, ***p < 0.001.

In adult mice, the frequency and numbers of IFN-γ-producing TREG cells in the lungs of adult mice correlate with peak weight loss by day 6 of infection (Fig. 6A, Supplemental Fig. 4A), revealing how the magnitude of the Th1 response is a strong determinant of clinical outcome. However, we did not observe this correlation in aged mice (Fig. 6B, Supplemental Fig. 4B). Foxp3+ TREG cell frequencies did not correlate with reduced peak weight loss or IFN-γ+ T cell numbers in the lungs (Fig. 6C, 6D). Interestingly, increased frequencies of IL-1R1+ TREG cells, but not ST2+ TREG cells (Supplemental Fig. S4C), observed in H1-VLP-vaccinated mice correlated with reduced frequencies of IFN-γ+ TREG cells (CD4+Foxp3+) in both adult (R² = 66; p < 0.001) (Fig. 6E) and aged mice (R² = 64; p < 0.001) (Fig. 6F). This finding, combined with the lack of correlation with conventional markers, reveals a potentially novel correlate of vaccine efficacy in the aged mouse. Indeed, we were able to extend this correlate to plant-based QVLP formulations as well as to the commercial IIV (Supplemental Fig. S4D). Collectively, these data confirmed that plant-based H1-VLP can facilitate the establishment of specialized Foxp3+ TREG cells and identified a potential novel correlate of vaccine efficacy.

**DISCUSSION**

Age at the time of infection is an important risk factor for severe outcomes following influenza infection. This phenomenon

https://doi.org/10.4049/immunohorizons.2200036
is widely attributed to the presence of comorbidities and to a general decline in immune competence, often called immune senescence (53). This decline in immune competence is multifaceted and complex and is characterized by, among other things, a reduced ability to respond to new Ags, a poorly sustained memory response, and an immune system in a chronic inflammatory state (54). Moreover, dysregulated Foxp3$^+$ TREG cells responses are attributed to prolonged inflammation in aging mice (52), indicating that targeting TREG cell populations could lead to immunization success in the elderly. Indeed, immune senescence is a major complicating factor in the development of vaccines for the elderly (54). Several novel vaccine strategies with the potential to improve immune responses and protection in older adults have recently been introduced (55) and others are in development (56). Among those rapidly approaching the market are the plant-based VLP vaccines that are the focus of the current work (31). Both preclinical and clinical trials have demonstrated the effectiveness of plant-based VLPs in protecting against influenza infection (18, 26, 31, 46, 57, 58). Because the elderly may depend more on cellular than humoral responses for protection from influenza and be less able to control nonprotective T cell responses (59, 60), a better understanding of the cellular responses, and their regulation, elicited by the plant-based VLP vaccines at different ages was required. To address these questions, we exploited facility-aged mice as a model of immune aging. We used H1N1/
California/07/2009 in these studies for its ability to infect mice without prior adaptation (27) and because this Ag was available both as a monovalent VLP and in both QVLP and IIV formulations.

In this study, we described the protective effect of plant-based monovalent H1-VLP immunization on H1N1/Ca influenza infection in mice. We showed a dose-dependent effect of H1-VLP immunization on the survival, overall morbidity, and efficient generation of Th1 and CD8+ T cell responses in infected mice. To understand how the vaccine influenced the regulatory branch of the adaptive immune response, we characterized Foxp3+ TREG cells, a unique subset of CD4+ T cells that possess the ability to suppress both innate and adaptive immunity, required for maintaining airway tolerance (61) and controlling inflammation in the lung (62). We observed that H1-VLP–immunized mice did not experience the steady accumulation of thymic-derived (38), functionally stable TREG cells (40) expressing the transcription factor Helios that was seen in the PBS control group. Because Helios+ TREG cells possess a TCR repertoire with increased self-reactivity compared with the Helios− subset (41), it is possible that the H1-VLP was able to generate Helios− TREG cells that had the ability to migrate to the lungs and actively contribute to the control of the disease. Indeed, H1-VLP immunization appeared to drive the expansion of Helios− TREG cells prior to infection.

Helios has also been closely associated with the ability of TREG cells to adapt their suppressive functions during infection to sense local inflammatory signals (15, 63). Among the inflammatory cytokines produced early during influenza A infections, we and others have shown that Helios+ and Helios− TREG cell are particularly apt at responding to IL-33 and IL-1β, alarmins of the same family (43, 64), to differentially modulate their function in the lung (15). Although ST2+Helios+ TREG cells were shown to produce amphiregulin to engage early repair mechanisms during influenza (12), little is known about the role of IL-1RI+Helios− TREG cells during influenza, although we have shown that they represent a less stable and more inflammatory-prone subset (15). Interestingly, although we confirmed the accumulation of ST2− TREG cells in unimmunized young mice, the frequency of ST2+ TREG cells in H1-VLP–immunized mice increased moderately at day 3, before returning to preinfection levels, suggesting a rapid control of inflammation in these mice. Indeed, ST2− TREG cells were shown to produce amphiregulin, a member of the epidermal growth factor family, which engages early repair mechanisms upon cellular damage in the lungs (12). Moreover, in both adult and aged mice, we observed a distinct expression of IL-1RI in TREG cells residing in the lungs during influenza, suggesting that IL-1 is important for local TREG cell homeostasis. It remains to be understood whether, in this case, IL-1 signaling contributes to the local homeostasis of the TREG cell pool, as the contraction of the immune response suggests, or whether these TREG cells are contributing to effector immune response by transiently relieving their suppressive functions (15), in turn favoring a quick return to immune homeostasis.

We discovered that IL-1RI expression was also increased in conventional CD4+ T cells in the lungs of immunized mice, suggesting a role for this alarmin in the modulation of global T cell responses in infection. The role of IL-1 during influenza remains largely ill defined. Although it is associated with increased immunopathology by promoting innate and adaptive immune dysregulation (44), it is also an important signal for the migration and expansion of Ag-specific CD4+ T cells (65). Interestingly, when we investigated the role of IL-1 in the generation of H1-specific T cells induced by H1-VLP vaccination, we found that the generation of Ag-specific Th1 cells was particularly reduced by the lack of IL-1 signaling, suggesting that H1-VLP exploits IL-1 signaling to generate a robust Th1 response.
Our aging vaccination-challenge model recapitulated that some hallmarks of vaccine-induced responses and influenza illness in the elderly reduced vaccine-induced humoral responses and postchallenge weight loss, as well as overt and prolonged activation of adaptive immune responses in the lungs (6, 27). Despite these challenges, H1-VLP was sufficient to provide protection in these mice, implying that cellular immunity plays a prominent role in conferring immune protection in aged mice. Importantly, the vaccine effectively reduced prolonged type 1 (IFN-γ) and type 3 (IL-17) responses by day 12 postinfection, in contrast to unimmunized mice. Moreover, H1-VLP immunization led to an overall decrease in Th1 (T-bet IFN-γ) and CD8+ T cell infiltrates but not Th17 (RORγt IL-17A) frequencies in the lungs postchallenge, suggesting that immunization does not directly promote the development of HA-specific Th17 cell responses. This difficulty in identifying such early T cell responses in aged mice may reflect the accumulation of memory T cells in the lungs and will require Ag-specific activation methods to be detected.

Indeed, in contrast to adult mice, we observed a ramping up of CD8+ and Th1 responses in aged mice upon challenge, but the magnitude of this inflammatory response remained lower and contracted faster in immunized compared with PBS-treated mice, suggesting that, although an effector T cell response takes place in the aged mice, it remains better controlled in mice that received the H1-VLP vaccine. Interestingly, H1-VLP immunization also contributed to increased the frequency of IL-4–producing Th2 cells in the lungs of both adult and aged mice. This may illustrate how H1-VLP effectively modified the Ag-specific T cell immune landscape prior to infection by promoting Th2 responses that, in turn, facilitate Ab production and may even provide support to control influenza-related immunopathology (51). Indeed, we could detect these Ag-specific Th2 cells after H1-VLP immunization prior to challenge.

In this study, we showed that H1-VLP vaccine elicits an IL-1–dependent cellular response that confers protective immunity against H1N1 influenza in both young and aged mouse models of infection with the pandemic H1N1/Ca strain. Incidentally, we observed a correlation between the presence of IL-1R1+ T_REG cells and the protection conferred by the H1-VLP vaccine in aged mice. Importantly, although humoral and cellular effector responses likely remain key determinants of vaccine efficacy, they are not consistently associated with a better outcome in the elderly (66), implying that T_REG cell–based biomarkers associated with the control of prolonged inflammation may represent an important and novel consideration in this vulnerable population. Finally, prior immunization with egg-based influenza A vaccine might positively affect the effect of subsequent H1-VLP administration (67), although this remains to be confirmed. A better understanding of the processes that influence the efficacy of vaccines in the elderly is essential to develop novel immunization strategies including the use of plant-based VLPs. Collectively, this study illustrates the ability of plant-based VLPs to elicit both effector and regulatory cellular response that effectively promote early, Ag-specific responses while simultaneously preventing prolonged immunopathology.

DISCLOSURES

B.J.W. has served as the medical officer for Medicago Inc. since 2011 and has held peer-reviewed grants with the company from various sources. S.P. and N.C. are currently employees at Medicago Inc. The other authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank Annie Beauchamps, Angela Brewer, Helen Mason, Marie-Hélène Lacombe, and Ekaterina Yurchenko for technical help.

REFERENCES


