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Protective CD8 T Cell–Mediated Immunity against Influenza A Virus Infection following Influenza Virus–like Particle Vaccination

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The development of influenza A virus (IAV) vaccines capable of inducing cytotoxic CD8 T cell responses could potentially provide superior, long-term protection against multiple, heterologous strains of IAV. Although prior studies demonstrated the effectiveness of baculovirus-derived virus-like particle (VLP) vaccination in generating Ab-mediated protection, the role that CD8 T cell immunity plays in overall VLP-mediated protection is less-well understood. In this article, we demonstrate that intranasal vaccination of mice with a VLP containing the hemagglutinin and matrix 1 proteins of IAV/PR/8/34 leads to a significant increase in hemagglutinin 533–specific CD8 T cells in the lungs and protection following subsequent homologous challenge with IAV. VLP-mediated protection was significantly reduced by CD8 T cell depletion, indicating a critical role for CD8 T cells in protective immunity. Importantly, our results show that VLP vaccine–induced CD8 T cell–mediated protection is not limited to homologous IAV strains. VLP vaccination leads to an increase in protection following heterosubtypic challenge with a strain of IAV that avoids vaccine-induced neutralizing Abs but contains conserved, immunodominant CD8 T cell epitopes. Overall, our results demonstrate the ability of influenza protein–containing VLPs to prime IAV-specific CD8 T cell responses that contribute to protection from homo- and heterosubtypic IAV infections. These results further suggest that vaccination strategies focused on the development of cross-protective CD8 T cell responses may contribute to the development of "universal" IAV vaccines. ***The Journal of Immunology*****, 2013, 191: 2486–2494.**

Influenza A virus (IAV) causes significant seasonal illness, leading to ~200,000 hospitalizations and 36,000 deaths annually in the United States during nonpandemic years (1, 2). This high rate of severe illness, along with the constant threat of a pandemic influenza outbreak, has renewed interest in developing novel influenza vaccination strategies. Following primary IAV infection, the development of a cytotoxic, influenza-specific CD8 T cell response is important in terminating the acute infection and contributes to long-term immunity (3, 4). The current influenza subunit vaccine leads to the production of influenza-neutralizing Abs (5). However, whether a robust influenza-specific CD8 T cell response is generated following vaccination remains unclear (5, 6). Importantly, as the virus drifts, neutralizing Abs induced by either previous vaccination or IAV infection can rapidly become ineffective as the result of constant Ab-mediated selective pressure on IAV's hemagglutinin (HA) and neuraminidase (NA) (7). Conversely, CD8 T cell responses to IAV are often generated against influenza-derived proteins/epitopes that are more highly conserved and, thus, are able to contribute to heterosubtypic protection (8, 9). Therefore, the development of an IAV vaccine capable of inducing a cytotoxic CD8 T cell response could potentially provide superior, long-term protection against multiple, heterologous strains of IAV (10, 11).

Recently, influenza virus-like particles (VLPs) were developed from recombinant baculoviruses containing influenza virus proteins, such as HA and/or NA, on the surface and matrix 1 protein (M1) as a core of the VLP. Influenza VLPs resemble influenza virions in both size and morphology (12). These influenza VLPs are an attractive vaccination candidate because they are replication deficient (i.e., they contain no IAV genomic material) and, therefore, could be administered to the young and elderly populations that exhibit the highest risk for complications from seasonal influenza virus infection. Influenza VLPs induce potent Ab responses and provide heterosubtypic cross-protection from morbidity and mortality during lethal IAV challenge following a single, intranasal (i.n.) treatment (12–17). However, the immune component mediating heterosubtypic protection following influenza VLP vaccination remains unknown. Given this cross-protection, VLP-induced CD8 T cell responses may contribute to protection from subsequent infections. Indeed, one in vitro study (17) highlighted the ability of dendritic cells (DCs) pulsed with influenza VLPs to stimulate human influenza–specific CD8 T cells. However, whether i.n.-administered influenza VLPs can induce influenza-specific CD8 T cell responses in vivo remains unknown. Further, the role of VLP-induced CD8 T cell immunity in mediating protection following homo- and heterosubtypic IAV challenges remains to be elucidated.

In this study, we examine the development and contribution of influenza VLP-induced CD8 T cells to IAV immunity following a single, i.n. vaccination with VLPs containing HA and M1 of

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Abbreviations used in this article: CL-4, clone-4; DC, dendritic cell; dLN, lung-draining lymph node; HA, hemagglutinin; IAV, influenza A virus; i.n., intranasal (ly); M1, matrix 1 protein; ma, mouse adapted; NA, neuraminidase; NP, nucleocapsid protein; Penh, enhanced pause; p.i., postinfection; TCIU, tissue culture infectious unit; VLP, virus-like particle; WT, wild-type.

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A/PR/8/34. Our findings demonstrate a small, but significant, increase in HA_{533}-specific CD8 T cells immediately following influenza VLP administration, which is sustained for ≥1 mo. Our results also indicate HA_{533}-specific CD8 T cells primed by influenza VLP vaccination are increased in the lungs upon subsequent IAV challenge. These vaccine-induced CD8 T cells are crucial in providing protection from mortality during lethal, homologous IAV challenge because mice depleted of CD8 T cells 30 d following vaccination succumb to the challenge infection. Further support for vaccine-induced CD8 T cells in mediating protection is demonstrated by the ability of VLP-induced, HA_{533}-specific CD8 T cells to aid in protection from high-dose, heterosubtypic IAV challenge. Together, our findings highlight the potential use of influenza VLPs to induce effective, cross-protective CD8 T cells that can contribute to protective immunity during particularly severe seasonal and pandemic outbreaks of influenza virus infections.

Materials and Methods

Mice

Six- to eight-week-old wild-type (WT) BALB/c mice were obtained from The National Cancer Institute (Frederick, MD). Clone 4 (CL-4) $CD90.1^+$ mice containing TCR-transgenic T cells specific for the HA_{533}/HA_{529} epitope of A/PR/8/34 or A/Japan/305/57 were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed and maintained in the specific pathogen–free animal care facility at the University of Iowa. All experiments were performed in accordance with regulatory standards and guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

VLP vaccination

VLPs containing HA and M1 of mouse-adapted (ma) A/PR/8/34 were produced as described previously (12). Mice were anesthetized i.n. with isoflurane and administered 2.5 μg VLPs in PBS or 50 μl PBS (as a control).

Influenza virus infection

Mouse-adapted IAVs A/PR/8/34 (maH1N1), A/HK/1/68 (maH3N2), and A/Japan/305/57 (maH2N2) were prepared from stocks, as described previously (18). Mice were anesthetized with isoflurane and infected i.n. with 5.5×10^4 tissue culture infectious units (TCIU) of virus. For day-8 analysis, mice were challenged with 5.5×10^3 TCIU A/PR/8/34.

MHC class I tetramers

MHC class I tetramers $HA_{533–541}$ [H-2K(d)/IYSTVASSL] and nucleocapsid protein $(NP)_{147–155}$ [H-2K(d)/TYQRTRALV] were obtained from the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility (Atlanta, GA).

Surface staining

Single-cell suspensions of lungs, lung-draining LNs (dLNs; i.e., mediastinal and peribronchial), and spleens were prepared by pressing the tissues through wire mesh screens, plating 1×10^6 cells/well in a 96-well plate, and blocking with 2 μl rat serum in FACS buffer for 30 min at 4°C.

Following blocking, cells were incubated with FACS buffer containing rat anti-mouse CD8α conjugated to FITC (53-6.7), rat anti-mouse CD3ε conjugated to PerCP Cy5.5 (145-2C11) purchased from BD, or HA_{533} or NP_{147} tetramers conjugated to allophycocyanin and PE for 1 h at 4°C. Cells were fixed in FACS Lysis Buffer (BD), per the manufacturer's instructions, and resuspended in PBS. Data were acquired on a BD FACSCanto II and analyzed with FlowJo software (TreeStar).

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described (19). Briefly, 5×10^5 cells from lung single-cell suspensions were incubated for 6 h with $HA_{533–541}$ or $NP_{147–155}$ peptides in complete media containing recombinant human IL-2 (Novartis) and brefeldin A (Sigma). Following incubation, cells were surface stained with anti-mouse CD8α and anti-mouse CD3ε mAbs. Following fixation, cells were permeabilized by incubation for 30 min at 4°C in FACS buffer containing 0.5% saponin (ACROS) and subsequently stained with rat anti-mouse IFN-γ mAb (XMG1.2) conjugated to allophycocyanin (BD) for 30 min at 4°C in FACS buffer containing 0.5% saponin. Data were acquired on a BD FACSCanto II and analyzed with FlowJo software (TreeStar).

Purification and adoptive transfer of CL-4 T cells

Single-cell suspensions of spleens from CL-4 $CD90.1^+$ mice (containing TCR-transgenic T cells specific for the HA_{533}/HA_{529} epitope of A/PR/8/34 and A/Japan/305/57, respectively) were labeled with CD8α MicroBeads and purified using LS columns, according to the manufacturer's instructions (Miltenyi Biotec). A total of 5×10^5 purified donor $CD90.1^+/CD8\alpha^+$ CL-4 cells was labeled with 2.5 μM CFSE (Invitrogen) and adoptively transferred i.v. into BALB/c $CD90.2^+$ host mice (20). Mice were vaccinated with VLPs 24 h later. Three days following VLP vaccination, dLNs were collected, and proliferation was determined by analyzing CFSE dilution in $CD3\varepsilon^+/CD8\alpha^+/CD90.1^+$ cells.

CD8α depletion

An anti-CD8α–depletion strategy described to reduce splenic IAV-specific memory CD8 T cells, but allow naive T cell repopulation, was modified to ensure depletion of HA_{533}-specific memory CD8 T cells within the lungs and dLNs (21). Briefly, mice were treated with anti-CD8α mAb (clone 2.43) i.p. (250 μg) and i.n. (100 μg) 30 d following VLP vaccination or sublethal (0.1 LD_{50}) A/PR/8/34 challenge. The repopulation of naive CD8 T cells in the spleen, lungs, and dLNs was monitored for 14 wk. In subsequent experiments, all memory CD8 T cell–depleted mice were used 14 wk after anti-CD8α Ab treatment (i.e., a time point when naive CD8 T cells had re-established and stabilized). Control mice were given equivalent volumes of PBS i.n. and i.p.

Pulmonary virus titer

Lungs were harvested on days 2, 4, 6, and 8 postinfection (p.i.), homogenized, and snap-frozen. Pulmonary virus titers were determined as previously described (22). Briefly, 5×10^5 MDCK cells in Iscove's complete medium containing 50 μg/ml gentamicin and 100 U/ml penicillin/100 μg/ml streptomycin (all from Life Technologies) were infected with 10-fold serial dilutions of lung homogenates, as well as stock IAV (A/PR/8/34) as a control, and incubated at 37°C. Twenty-four hours later, medium was removed from all wells and replaced with Iscove's complete media containing 0.0002% TPCK-Trypsin (Worthington), 50 μg/ml gentamicin (Life Technologies), and 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies). Three days later, 0.5% chicken RBCs (Colorado Veterinary Products) were mixed 1:1 with supernatant from each of the cell culture wells, and agglutination was measured after incubating for 1 h at room temperature. Subsequently, TCIU were calculated using the Reed–Muench method.

Measurement of airway resistance

A whole-body plethysmograph (Buxco Electronics) was used to measure enhanced pause (Penh), an indicator of airway resistance, during respiration (23). Penh values for each mouse were recorded daily following IAV infection. Day-0 baseline Penh values were recorded prior to infection.

Statistical analysis

Data were compiled in graphical format using Prism software (GraphPad Software, San Diego, CA). Error bars represent the SEM. Statistical significance was determined using unpaired, two-tailed Student *t* tests, log-rank tests, or ANOVAs followed by the Bonferroni posttest.

Results

Influenza VLP vaccination leads to an increase in HA_{533}-specific CD8 T cells in the lungs following vaccination

We first determined whether vaccine-specific CD8 T cells accumulate in the lungs 7 and 14 d following a single, i.n. VLP vaccination. Given the expected low frequency of Ag-specific CD8 T cells present at baseline and following vaccination, we used a dual tetramer–labeling strategy to identify influenza-specific CD8 T cells that had expanded following vaccination (Fig. 1A) (24–26). No change in the frequency or numbers of influenza-specific lung CD8 T cells was observed on day 7 postvaccination (data not shown). The frequency and number of HA_{533}-specific CD8 T cells were increased significantly in the lungs on day

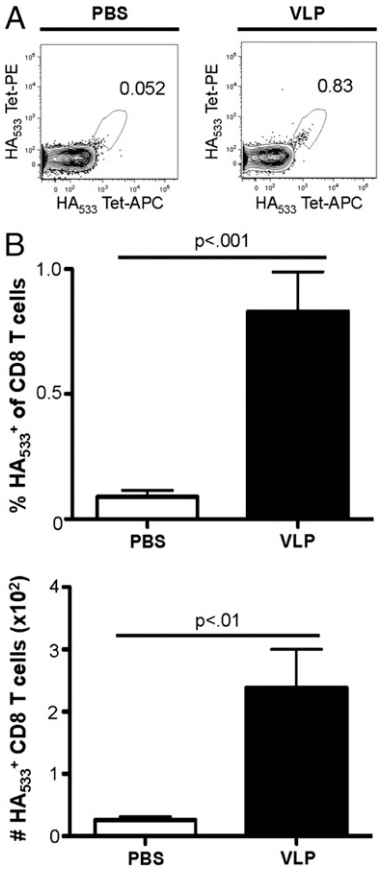

FIGURE 1. VLP vaccination boosts the HA_{533}^+ CD8 T cell response in the lungs. BALB/c mice were treated i.n. with 2.5 μg VLPs or 50 μl PBS as a control. On day 14 postvaccination, lungs were harvested, and the frequency and total numbers of HA_{533}^+ CD8 T cells were determined by flow cytometry. (**A**) Representative tetramer gating of $CD8\alpha^+/CD3\varepsilon^+$ lung samples on day 14 post–VLP vaccination or PBS administration. (**B**) Cumulative frequency and total numbers of HA_{533}^+ CD8 T cells. The data are the mean values ± SEM of two independent experiments ($n = 9$ mice/group). Data were analyzed using the Student t test.

14 postvaccination compared with mice that were given PBS i.n. as a control (Fig. 1). Further, this expansion on day 14 post–i.n. vaccination was specific for the IAV proteins contained within the VLPs, because no change in the NP_{147}-specific CD8 T cell population was observed when comparing vaccinated mice with controls (data not shown). (Note: IAV NP is not present in the VLP preparation used in these studies.) We also observed no changes in the total number of CD8 T cells present in the lungs at day 7 or 14 following VLP vaccination (Supplemental Fig. 1, data not shown). We observed no increase in the frequency or total numbers of HA_{533}-specific CD8 T cells in the dLNs at day 7 or 14 following vaccination (data not shown). Together, the above results indicate that the expansion of HA_{533}-specific CD8 T cells was specific to the HA protein contained within the VLP and was not an unknown, nonspecific effect of influenza VLP vaccination on total, or influenza-specific, CD8 T cell expansion.

Influenza VLP vaccination leads to proliferation of HA_{533}-specific CD8 T cells in dLNs

Given that we observed an increase in HA_{533}-specific CD8 T cells only in the lungs, but not dLNs, following VLP vaccination, we next sought to determine the location where these T cells were primed. During infection with influenza virus, naive CD8 T cells are primed in dLNs and then migrate to the site of infection (i.e., lungs) to kill virally infected cells (19, 27–30). Although the dLN is the initial site of expansion, activated CD8 T cells continue to proliferate in the lung during IAV infection (31). Thus, analysis of dLNs at day 7 postvaccination may have been too late to observe vaccine-induced CD8 T cell expansion (20). Further, recent studies demonstrated that influenza-specific CD8 T cells can be primed and expand in the lungs of mice lacking dLNs, spleen, and Peyer's patches when challenged with IAV (32). Therefore, it was determined whether HA_{533}-specific CD8 T cells were activated and expanded in dLNs or lung tissue. To monitor this expansion, 5×10^5 CFSE-labeled, naive CL-4, $CD90.1^+$ CD8 T cells (transgenic CD8 T cells specific for the HA_{533} epitope) were adoptively transferred into host WT ($CD90.2^+$) mice. The host mice were vaccinated 24 h later with either HA/M1 containing VLPs or a control VLP containing only M1. At 2.5 and 3 d following vaccination, the dLNs, lungs, and spleens were harvested and analyzed to determine CL-4 proliferation. Compared with M1-VLP controls (in which we observed limited CFSE dilution in donor CL-4 cells), HA/M1-VLP vaccination induced substantial proliferation of HA_{533}-specific CD8 T cells in the dLNs by day 2.5 postvaccination (Fig. 2, *left panels*). On day 3 postvaccination, a greater frequency of CL-4 cells was $CFSE^{lo/-}$ in the dLNs of mice treated with HA/M1-VLP versus M1-VLP. Further, limited divided CL-4 cells were present in the lungs, but not in the spleen, at day 2.5 postvaccination (Fig. 2, *upper panels*). However, by day 3, divided CL-4 cells had begun to accumulate in the lungs of mice vaccinated with HA/M1-VLP (Fig. 2, *lower middle panel*), suggesting that these cells are trafficking to the site of vaccination. These results imply that activation and early division of influenza VLP-specific CD8 T cells initially occur in dLNs following i.n. influenza HA/M1-VLP administration and that these influenza-specific cells subsequently migrate to the lung tissue.

HA_{533}-specific CD8 T cells are increased in dLNs and lungs following lethal IAV challenge 30 d post–HA/M1-VLP vaccination

Following infection with influenza virus, IAV-specific, long-lived memory CD8 T cells are generated and can reside in both the local lung tissue and in secondary lymphoid organs (33–41). These local, tissue-resident memory cells are key in early (days 0–3 p.i.) control of viral infection, because they are present at the site of initial Ag encounter and are able to respond rapidly to mediate protective immunity (35, 36). At later times following infection (day 5 p.i. and beyond), memory T cells that reside in dLNs during the steady state dominate the response. These dLN-resident memory CD8 T cells have a greater proliferative and cytotoxic capacity compared with local, tissue-resident CD8 T cells (38).

To determine whether CD8 VLP–induced T cell–mediated protection occurs following challenge, we examined the frequency and numbers of the influenza-specific CD8 T cells in both the lungs and dLNs prior to challenge, as well as at various times postchallenge. In the dLNs, we observed no significant difference between VLP-vaccinated or control mice with regard to the frequency or numbers of HA_{533}-specific CD8 T cells at any of the times analyzed postchallenge (data not shown). Similarly, at 30 d following VLP vaccination (i.e., day 0 of challenge), no difference in the number of HA_{533}-specific CD8 T cells present in the lungs was detected between the two groups (Fig. 3A, 3B). However, as early as 1 d following lethal IAV challenge, we observed a significant increase in HA_{533}-specific CD8 T cells in the lungs that continued through at least day 8 postchallenge (Fig. 3). In contrast, we observed no significant differences in the NP_{147}-specific CD8 T cell response (i.e., an epitope not contained within

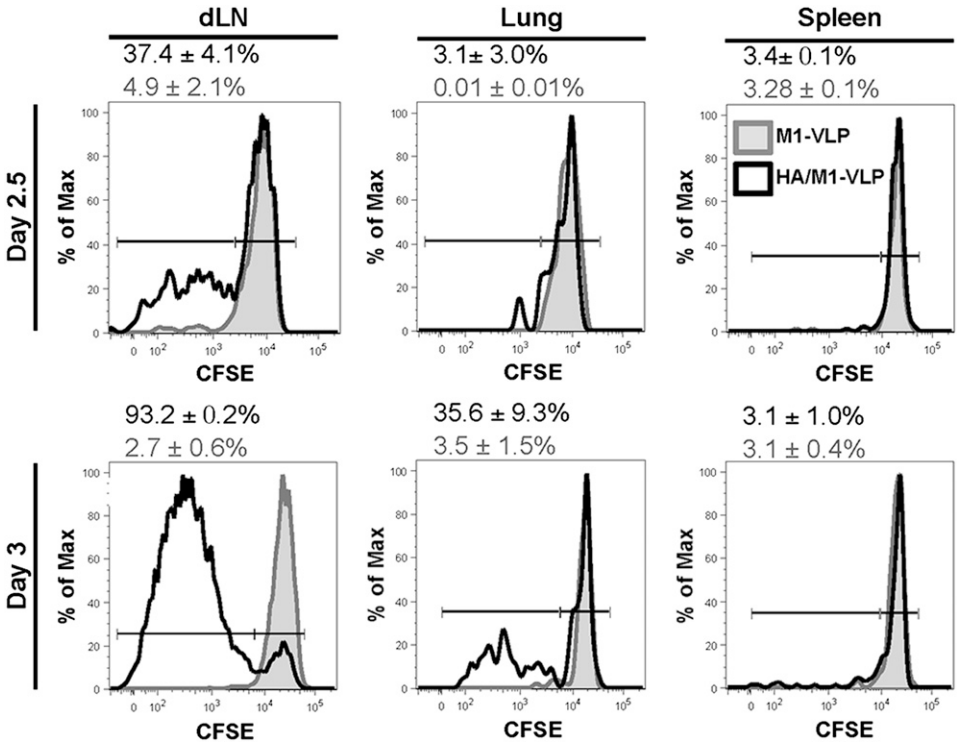

FIGURE 2. VLP treatment leads to proliferation of IAV-specific CD8 T cells in dLNs. CD90.2$^+$ BALB/c mice were given 5 $\times$ 10^5 MACS-purified, CFSE-labeled CL-4 CD90.1$^+$ cells i.v. and treated i.n. with 2.5 μg of VLP (open graph), PBS (data not shown), or 2.5 μg of VLP containing only M1 (shaded graph) 24 h later. dLNs, lungs, and spleens were collected 2.5 and 3 d following vaccination and analyzed via flow cytometry for proliferation (i.e., CFSE dilution). Data are representative of two independent experiments (n = 3–6 mice pooled/group).

the VLPs) in the lungs and dLNs of VLP-vaccinated versus non-VLP–primed mice (Supplemental Fig. 2, data not shown).

HA_{533}-specific CD8 T cells generated during VLP vaccination provide protection during lethal IAV challenge

Influenza-specific CD8 T cells generated following IAV challenge are crucial in the clearance of virus from the host during primary IAV infections (3, 4). However, neutralizing anti-H1 Ab responses are important in providing sterilizing immunity following subsequent homologous IAV challenge (42, 43). To evaluate whether CD8 T cells primed during influenza VLP vaccination were required for protection against subsequent lethal influenza virus infection, we first determined the efficiency of anti-CD8α depletion in a model generating a robust CD8 T cell response. We

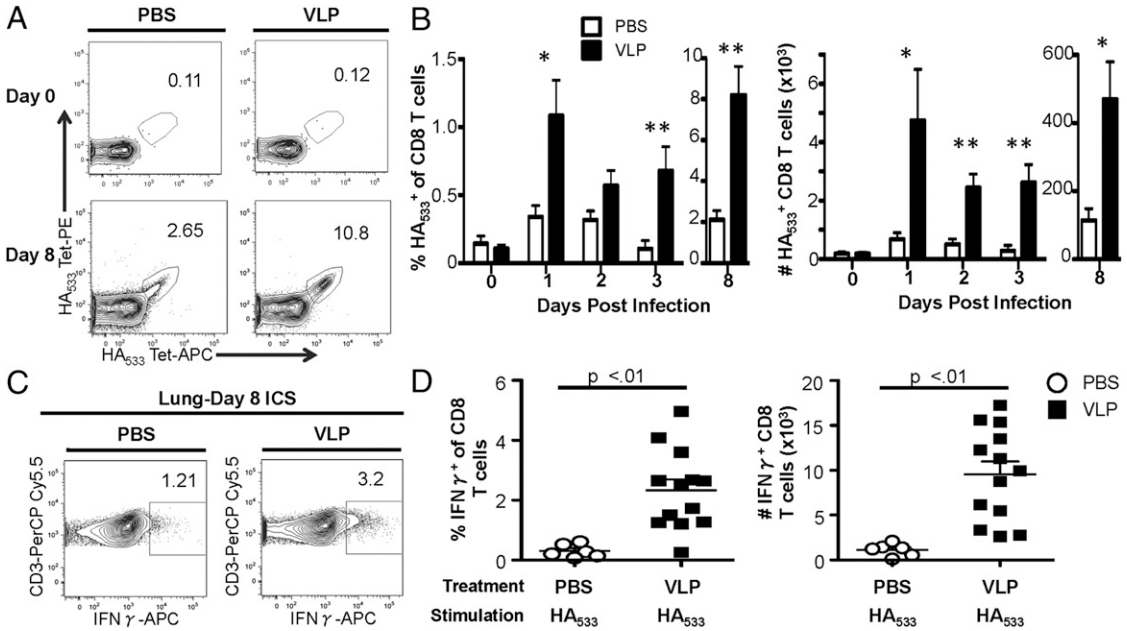

FIGURE 3. Kinetics of the HA_{533}-specific CD8 T cell response primed by VLP vaccination following lethal IAV challenge. BALB/c mice were treated i.n. with 2.5 μg VLPs or 50 μl PBS as a control. Thirty days later, all mice were infected with 5 LD_{50} of A/PR/8/34, with the exception of mice that were to be analyzed on day 8 p.i., which were challenged with 1 LD_{50} dose to ensure the survival of control animals at this time point. (**A** and **B**) Lungs were harvested at the indicated time points prior to infection (day 0) or p.i., and HA_{533}-specific CD8α$^+$/CD3ε$^+$ cells were identified by flow cytometry with HA_{533}-tetramers. (A) Representative tetramer gating of CD8α$^+$/CD3ε$^+$-gated lung samples on days 0 and 8 post–IAV challenge. (B) The frequency and total numbers of HA_{533}-specific CD8α$^+$/CD3ε$^+$ cells. (**C** and **D**) Lungs were harvested on day 8 p.i., and single-cell homogenates and HA_{533} cells were measured by intracellular cytokine staining. (C) Representative IFN-γ gating of CD8α$^+$/CD3ε$^+$-gated lungs on day 8 post–IAV challenge. (D) The frequency and total numbers of IFN-γ$^+$CD3$^+$CD8$^+$ T cells. The data are the mean values ± SEM from three independent experiments (n = 6–13 mice/group for lungs). Data were analyzed using two-way ANOVA followed by the Bonferroni posttest. *$p < 0.05$, **$p < 0.01$.

challenged mice with a sublethal dose of IAV, depleted CD8α$^+$ cells 30 d later, and monitored the total and HA_{533}-specific CD8 T cell response. Forty-eight hours following depletion, virtually no CD8 T cells remained in the blood, dLNs, lungs, or spleen of mice (Supplemental Fig. 3). Fourteen weeks following CD8α depletion, total CD8 T cell recovery had plateaued in the blood, dLNs, lungs, and spleen of mice (Supplemental Fig. 3). Further, when HA_{533}-specific CD8 T cells were examined, we observed that anti-CD8α administration also depleted these cells. However, there was no significant increase in the HA_{533}-specific cells within the recovered, total CD8 T cell population, suggesting that IAV-induced CD8 T cell immunity had been ablated (Supplemental Fig. 4).

After determining the efficiency of our depletion strategy, HA/M1-VLP–vaccinated mice were challenged 14 wk following CD8 T cell depletion with a lethal dose of A/PR/8/34. Although mice that were vaccinated and challenged without CD8 depletion survived following lethal IAV challenge, only 60% survival was observed when vaccinated mice were depleted of CD8 T cells and then lethally challenged with IAV (Fig. 4B). Further, we observed a slight, but not significant, increase in weight loss and Penh in vaccinated mice that had been depleted of CD8 T cells and allowed to recover total CD8 T cell numbers prior to lethal challenge compared with mice that were vaccinated and challenged with IAV (Fig. 4C, 4D). These data indicate that HA_{533}-specific CD8 T cells generated following VLP vaccination are required to mediate complete protection from lethal IAV challenge. It is likely that the remaining protection observed after removal of memory CD8 T cells is due to the IAV-specific Ab previously demonstrated to be induced following VLP vaccination (16).

Influenza VLPs provide protection from high-dose, heterosubtypic IAV challenge

Influenza-specific CD8 T cells were demonstrated to provide cross-protection against heterosubtypic strains of influenza virus in cases in which previously generated neutralizing Abs may not be able to control IAV infection (11, 44, 45). To determine whether CD8 T cells primed following i.n. influenza VLP administration could mediate a similar protection from heterosubtypic IAV challenge, groups of mice were vaccinated with HA/M1-VLPs and challenged 30 d later with homo- or heterosubtypic strains of IAV. When mice were challenged with a lethal dose of homosubtypic maH1N1 (A/PR/8/34) following influenza VLP vaccination, 86.6% of the mice survived the challenge and had significantly reduced morbidity compared with PBS-treated controls (Fig. 5A). We also observed significantly lower virus titers on days 2 and 4 post–IAV infection in mice that were administered influenza VLPs i.n. compared with PBS-treated controls (Fig. 5A). To determine whether influenza VLP vaccine–induced CD8 T cells play a role in mediating protection during heterosubtypic IAV infection, we next challenged mice vaccinated i.n. with VLPs containing HA and M1 from A/PR/8/34 with maH2N2 (A/Japan/305/57). Challenge with A/Japan/305/57 avoids neutralizing Ab responses directed against HA, but a modified, cross-reactive version of the HA_{533} epitope is still present within A/Japan/305/57 (Table I) (46). Following challenge with maH2N2, 50% of control mice survived infection, whereas significantly more mice (90%) survived infection when they were given A/PR/8/34 HA/M1-VLPs 30 d prior to challenge (Fig. 5B). Interestingly, despite this difference in lethality, we observed no difference in morbidity or virus titer at days 2 and 4 p.i. (Fig. 5B). To ensure that these results were specific to the conserved CD8 T cell response generated by vaccination and not due to a highly conserved component of the immune response or another nonspecific effect mediated by VLP vaccination, we also challenged HA/M1 (H1)-vaccinated mice with maH3N2 (A/HK/1/68). Challenge with A/HK/1/68 avoids neutralizing Ab responses along with the majority of memory CD4 and CD8 T cell responses that could have been generated following i.n. VLP administration specific for HA and M1 of A/PR/8/34 (8). Further, the HA_{533} epitope is altered in the maH3N2 strain, such that the peptide is no longer recognized by CD8 T cells (Table I) (47). As expected, all of the vaccinated and control mice succumbed to infection, and we observed no difference in weight loss or virus titer in the lungs of these mice (Fig. 5C).

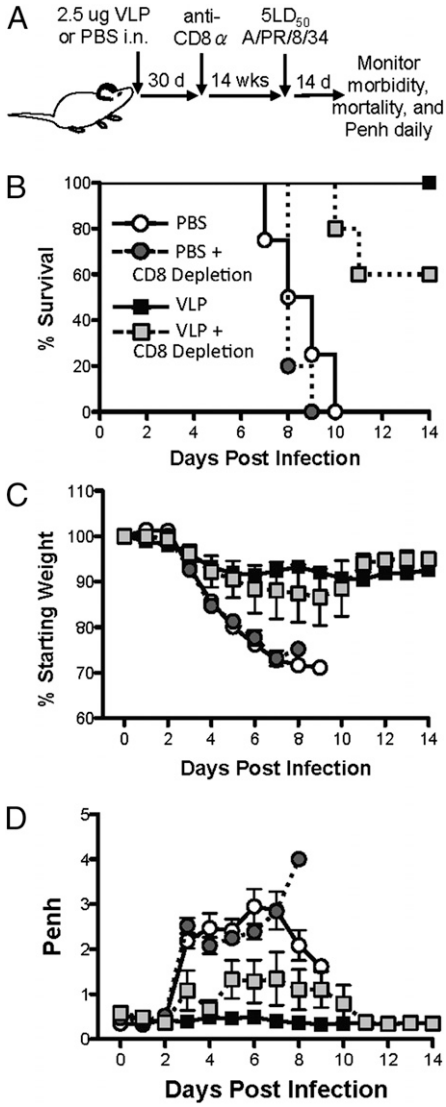

FIGURE 4. CD8 T cells primed by VLP vaccination are required for complete protection during IAV infection. (**A**) BALB/c mice were treated i.n. with 2.5 μg VLP in 50 μl PBS or 50 μl PBS as a control. Thirty days later, mice were treated i.n. and i.p. with anti-CD8α. Fourteen weeks later, mice were challenged with 5 LD_{50} A/PR/8/34. Mortality (**B**), morbidity (**C**), and airway resistance (**D**) were monitored daily for 14 d (n = 4–5 mice/group).

To further investigate differences that could account for the survival of influenza VLP-vaccinated mice challenged with maH2N2, even though no early difference in viral titer was detected, we challenged mice and monitored their airway resistance daily for 8 d using a whole-body plethysmograph. We observed significantly higher Penh values in mice that were not vaccinated and challenged with maH1N1 compared with vaccinated mice on days 3–8 p.i. (Fig. 5D). In contrast, we observed no difference in Penh when mice were challenged with maH3N2 following i.n.

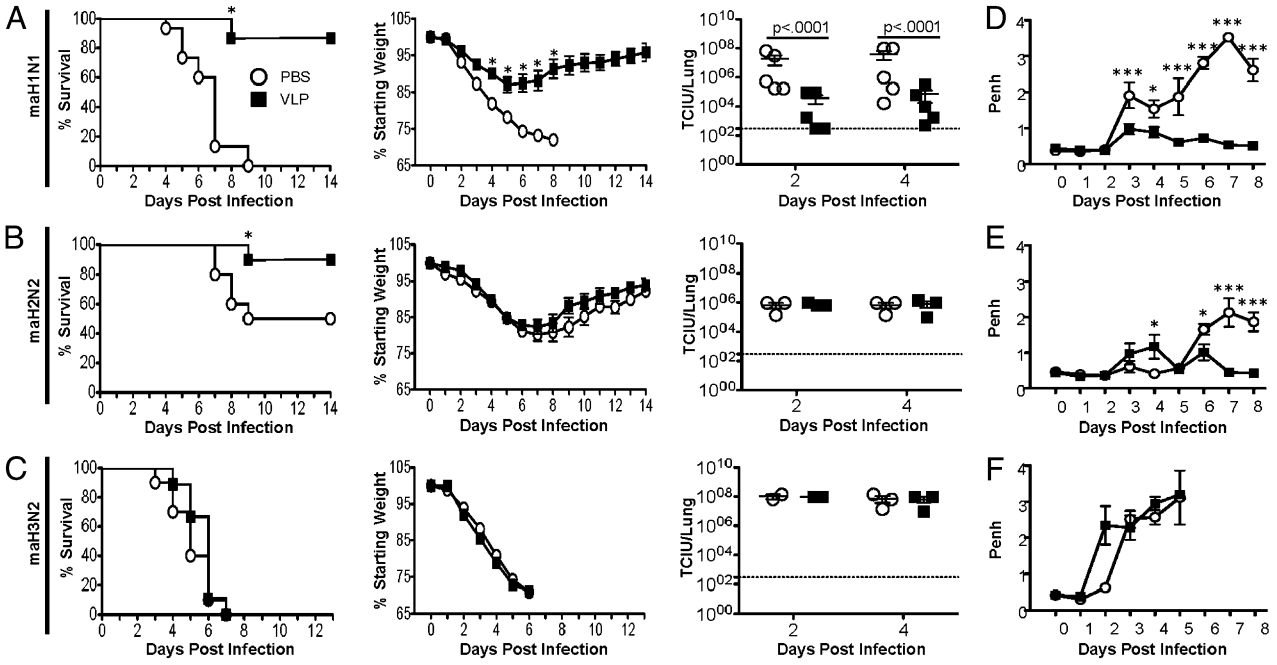

FIGURE 5. VLP vaccination leads to protection from mortality during heterosubtypic IAV infection. BALB/c mice were treated i.n. with 2.5 μg VLP or 50 μl PBS as a control. Thirty days later, mice were challenged with 5 LD_{50} PR/8 [(**A**), maH1N1] or equivalent TCIU (~5.5×10^4 TCIU/mouse) doses of A/Japan/305/57 [(**B**), maH2N2] or A/HK/1/68 [(**C**), maH3N2], and morbidity (*middle panels*) and mortality (*left panels*) were measured daily for 14 d. Lung homogenates ($n = 3$/group) were also collected on days 2 and 4 p.i. to determine viral titers (*right panels*). Airway resistance following challenge with maH1N1 (**D**), maH2N2 (**E**), or maH3N2 (**F**) was measured using a whole-body plethysmograph daily for 8 d ($n = 6$ mice/group for maH1N1 and maH2N2; $n = 3$ mice/group for maH3N2). The morbidity and mortality data are pooled from three independent experiments ($n = 10$–15 mice/group). Titer data were analyzed using the Student *t* test. Morbidity data were analyzed using two-way ANOVA followed by the Bonferroni posttest. Mortality data were analyzed using the log-rank test. $*p < 0.05$, $***p < 0.001$.

VLP vaccination or PBS administration (Fig. 5F, *lower right panel*). When mice were challenged with maH2N2 following VLP vaccination, a significant reduction in Penh was observed starting on day 6 p.i. compared with PBS-treated controls (Fig. 5E). This reduction in Penh correlates with the period of time during which unvaccinated mice challenged with maH2N2 succumbed to infection (Fig. 5B).

Because Penh was reduced significantly at day 6 post-heterosubtypic challenge in H1 influenza VLP–vaccinated mice, we also determined lung viral titers at days 6 and 8 p.i. At both days 6 and 8 after heterosubtypic IAV challenge, virus titers were significantly reduced in mice that had been vaccinated compared with PBS controls (Fig. 6A). The lack of early control of infection, but reduced Penh and virus titers at day 6 following infection, suggests that early Ab-mediated clearance of virus does not lead to increased survival in HA/M1-VLP–vaccinated mice challenged with maH2N2. Given this, we sought to determine whether A/PR/8/34 HA/M1-VLP vaccination led to a significant increase in protective HA_{533}-specific CD8 T cells following A/Japan/305/57 challenge. We did not detect any significant difference in HA_{533}-specific CD8 T cells in the lungs on day 2 p.i. between mice that were vaccinated with HA/M1-VLPs or control M1-VLPs (data not shown). This finding correlates with the lack of difference in Penh and virus titers observed at day 2 postchallenge. However, we observed a significant increase in both the frequency and number of HA_{533}-specific CD8 T cells in the lungs of HA/M1-VLP–vaccinated mice versus M1-VLP–vaccinated mice at day 6 post-challenge (Fig. 6B). Together, the results from Figs. 5 and 6 strongly suggest that the cross-reactive HA_{533}-specific CD8 T cells primed by HA/M1-VLP vaccination are key to mediating protection during maH2N2 (A/Japan/305/57) challenge.

Discussion

Vaccination continues to be the best defense against influenza virus infection. However, it remains unclear whether current subunit vaccination strategies induce influenza-specific CD8 T cell responses (17). Our findings suggest that HA_{533}-specific CD8 T cells generated following influenza VLP vaccination are important in providing full protection during lethal IAV challenge, because mice depleted of CD8 T cells displayed reduced survival and increased airway resistance compared with nondepleted, vaccinated mice (Fig. 4). Although a characterization of the full breadth of the immune response generated following influenza VLP vaccination has not been completed, recent work demonstrated the ability of influenza VLPs to generate Abs specific to the stalk domain of A/PR/8/34 HA (48). These HA stalk Abs were

Table I. The HA_{533} epitope

IAV Strain	Epitope	Amino Acid Sequence
A/PR/8/34 (maH1N1)	$HA_{533–541}$	IYSTVASSL
A/JAPAN/305/57 (maH2N2)	$HA_{529–537}$	IY**A**TVA**G**SL
A/HK/1/168 (maH3N2)	None (aa 533–541 of HA)	I**SFAISCF**L

Bold residues differ from the A/PR/8/34 amino acid sequence.

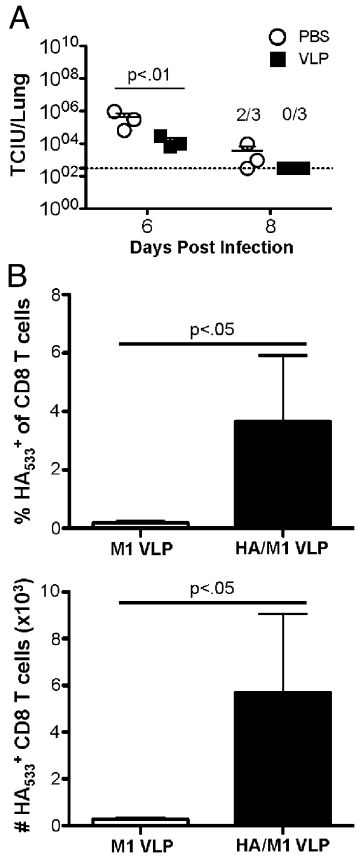

FIGURE 6. VLP vaccination leads to a reduction in virus titer and a significant increase in HA_{533}-specific CD8 T cells at later time points following heterosubtypic maH2N2 challenge. BALB/c mice were treated i.n. with 2.5 μg VLP or 50 μl PBS as a control. Thirty days later, mice were challenged with ~5.5×10^4 TCIU A/Japan/305/57. (**A**) Mice were sacrificed, and lungs (n = 3/group) were collected on days 6 and 8 p.i. to determine viral titers. 2/3 and 0/3 indicate the number of mice with detectable IAV titers in the lungs at day 8 p.i. (**B**) Lungs were harvested at day 6 post–A/Japan/305/57 infection, and tetramer-specific $CD8\alpha^+/CD3\varepsilon^+$ cells were identified by flow cytometry (n = 4 mice/group). Data were analyzed using the Student t test.

found to broadly neutralize groups 1 and 2 influenza virus and were suggested as a universal influenza vaccination candidate (49, 50). However, reduced airway resistance and virus titers following maH2N2 heterosubtypic challenge in HA/M1-VLP–vaccinated mice does not occur until day 6 p.i., when we detected significantly greater HA_{533}-specific CD8 T cell responses in the lungs (Figs. 5B, 6). This delayed protection and lack of reduction in early viral titers suggest that stalk-specific Ab-mediated protection is likely not responsible for the protection observed following VLP vaccination and heterosubtypic maH2N2 challenge. These findings, along with a loss of protection observed during homologous IAV challenge when mice have been vaccinated and subsequently depleted of IAV-reactive CD8 T cells (Fig. 4), suggest that investigation of IAV vaccine candidates that elicit CD8 T cell responses will also be important in developing broadly cross-reactive IAV vaccines.

Our study demonstrates that influenza VLPs drive the proliferation of naive, HA_{533}-specific CD8 T cells in dLNs as early as 2.5 d postvaccination (Fig. 2). Although influenza VLPs were administered in this study without adjuvant, recent publications highlighted the wide range of baculovirus and Sf9 host cell proteins present within the VLPs (48, 51). Both WT baculovirus and baculovirus-derived influenza VLPs administered i.n. led to similar mRNA upregulation of IFN-β and Mx1 in the lungs 6 h postadministration (48). Although this indicates that baculovirus particles alone prime the system to respond, our results still suggest the protective effect that we observed is specific to the influenza proteins present in the virus-like particles. HA/M1-VLPs induced the proliferation of transgenic, transferred HA_{533}-specific CD8 T cells (CL-4 cells) in dLNs, whereas influenza VLPs containing only M1 did not (Fig. 2).

The ability of influenza VLP vaccination to prime CD8 T cell responses that play a role in protection during lethal and heterosubtypic IAV infections is surprising, especially considering that these VLPs are nonreplicative. During IAV infection, dLN-resident $CD8\alpha^+$ DCs and migratory $CD103^+$ DCs (the known cross-presenting DC populations) are the primary DC subsets responsible for presentation of IAV Ags to CD8 T cells within dLNs (27–29, 52). Although acquisition of Ag by these DC subsets can occur via direct (infection) or indirect (cross-presentation or trogocytosis) methods, data demonstrated that cross-presentation alone is sufficient to induce functional CD8 T cell responses during IAV infection in vivo (53–55). Our results support the idea that cross-presentation alone is sufficient to induce functional and protective CD8 T cell responses following influenza VLP vaccination because the influenza VLPs used in these studies have no internal, IAV genomic material and cannot replicate. Interestingly, DC activation and cross-presenting ability have been linked to type I IFN responses. Natural influenza virus infection is known to induce type I IFN within the lungs, and type I IFN has been well documented to boost the ability of DCs to activate naive, influenza-specific CD8 T cell responses (56–60). At this time it remains unclear which mechanisms "license" cross-presentation during VLP vaccination (61). Whether experimental induction of type I IFN at the time of influenza VLP vaccination would improve the ability of DCs in activating IAV-specific CD8 T cell responses remains to be elucidated. Furthermore, it will be important to determine the APC subsets (presumably $CD103^+$ DCs) priming this VLP-specific CD8 T cell response, as well as whether these APCs are similar to those responsible for activating CD8 T cells during IAV infection and live attenuated influenza vaccination. Determining the differences in the ability of DC subsets to regulate CD8 T cell responses following different methods of i.n. IAV vaccination will allow for the development of IAV vaccines that are better able to induce broadly cross-protective CD8 T cell responses.

Vaccines that generate broadly cross-protective CD8 T cell responses may aid in the development of human IAV vaccines in the future. Phase I and II clinical trials testing a baculovirus-derived VLP containing HA, NA, and M1 of A/California/04/2009 (H1N1) and A/Indonesia/05/2005 (H5N1) independently demonstrated that these vaccines are safe and elicit Ab responses (62, 63). However, it remains unknown whether these vaccinations elicit CD8 T cell responses in humans. The Immune Epitope Database and Analysis Resource identifies 456 positive T cell epitopes within the M1 protein of various IAV strains contained in the database (http://www.iedb.org). Therefore, in humans, the M1 and HA proteins may elicit cross-reactive CD8 T cell populations, which could mediate protection from homo- and heterologous IAV infection in addition to HA-and M1-specific Abs. In contrast to humans, there are no identified T cell epitopes contained within the M1 protein of A/PR/8/34 in BALB/c mice (http://www.iedb.org). Given this, it is not surprising that M1 VLPs alone did not mediate protection from lethality in our BALB/c murine model (data not shown). In the future it will be important to determine in human trials whether CD8 T cell responses are generated in response to VLP vaccination in addi-

tion to Ab titers, as well as whether these CD8 T cells are capable of killing virally infected cells.

In summary, to our knowledge, the results presented in this article demonstrate for the first time that i.n. vaccination with influenza VLPs containing HA and M1 leads to proliferation of HA_{533}-specific CD8 T cells in dLNs and subsequent accumulation of these T cells in the lungs. These HA_{533}-specific CD8 T cells rapidly accumulate in the lungs following IAV challenge and are crucial in mediating complete protection from IAV challenge. Furthermore, influenza VLP vaccination induces heterosubtypic protection correlated with a late (day 6 p.i. and beyond) decrease in airway resistance and Penh, as well as a significant increase in vaccine-induced CD8 T cell responses. These results support continued investigation into influenza vaccines capable of generating protective CD8 T cell responses and the potential use of influenza VLPs as a cross-protective IAV vaccine.

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Disclosures

The authors have no financial conflicts of interest.

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