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RESEARCH ARTICLE

Inactivated Influenza Vaccine That Provides Rapid, Innate-Immune-System-Mediated Protection and Subsequent Long-Term Adaptive Immunity

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ABSTRACT **The continual threat to global health posed by influenza has led to increased efforts to improve the effectiveness of influenza vaccines for use in epidemics and pandemics. We show in this study that formulation of a low dose of inactivated detergent-split influenza vaccine with a Toll-like receptor 2 (TLR2) agonist-based lipopeptide adjuvant (R_4Pam_2Cys) provides (i) immediate, antigen-independent immunity mediated by the innate immune system and (ii) significant enhancement of antigen-dependent immunity which exhibits an increased breadth of effector function. Intranasal administration of mice with vaccine formulated with R_4Pam_2Cys but not vaccine alone provides protection against both homologous and serologically distinct (heterologous) viral strains within a day of administration. Vaccination in the presence of R_4Pam_2Cys subsequently also induces high levels of systemic IgM, IgG1, and IgG2b antibodies and pulmonary IgA antibodies that inhibit hemagglutination (HA) and neuraminidase (NA) activities of homologous but not heterologous virus. Improved primary virus nucleoprotein (NP)-specific $CD8^+$ T cell responses are also induced by the use of R_4Pam_2Cys and are associated with robust recall responses to provide heterologous protection. These protective effects are demonstrated in wild-type and antibody-deficient animals but not in those depleted of $CD8^+$ T cells. Using a contact-dependent virus transmission model, we also found that heterologous virus transmission from vaccinated mice to naive mice is significantly reduced. These results demonstrate the potential of adding a TLR2 agonist to an existing seasonal influenza vaccine to improve its utility by inducing immediate short-term nonspecific antiviral protection and also antigen-specific responses to provide homologous and heterologous immunity.**

IMPORTANCE **The innate and adaptive immune systems differ in mechanisms, specificities, and times at which they take effect. The innate immune system responds within hours of exposure to infectious agents, while adaptive immunity takes several days to become effective. Here we show, by using a simple lipopeptide-based TLR2 agonist, that an influenza detergent-split vaccine can be made to simultaneously stimulate and amplify both systems to provide immediate antiviral protection while giving the adaptive immune system time to implement long-term immunity. Both types of immunity induced by this approach protect against vaccine-matched as well as unrelated virus strains and potentially even against strains yet to be encountered. Conferring dual functionality to influenza vaccines is beneficial for improving community protection, particularly during periods between the onset of an outbreak and the time when a vaccine becomes available or in scenarios in which mass vaccination with a strain to which the population is immunologically naive is imperative.**

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The most effective way to curb the seasonal impact of influenza, which causes an average of 250,000 to 500,000 deaths annually (1), is vaccination. The "split" influenza virus vaccine, which is prepared from purified virions that have been inactivated and then disrupted (split) with detergent, induces neutralizing antibodies directed against viral hemagglutinin (HA) and neuraminidase (NA). Because circulating viruses mutate readily and new antigenically distinct strains are continuously selected in the presence of neutralizing antibody elicited by previous strains, the effectiveness of a seasonal vaccine diminishes with time. These vaccines must therefore be reformulated annually to include the HA and NA of the viral strains that are predicted to circulate in the forthcoming influenza season.

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The recent emergence of swine-origin H1N1 virus as well as ongoing zoonotic spillover events involving H7N9 and highly pathogenic H5N1 viruses have highlighted the real and potential burden that pandemics pose to global health and national economies in the absence of an effective vaccine. Although interventions, such as those employing the neuraminidase inhibitors, against novel strains can be effective at alleviating symptoms and at reducing disease severity when used preemptively (2, 3) or when initiated promptly after illness onset (4, 5), their efficacy is reliant on a daily dosing regimen (6), and their benefit does not extend beyond the course of treatment. Furthermore, widespread or prolonged use is associated with the emergence of drug-resistant strains (7, 8). The need for continual development of effective short-term intervention strategies as well as vaccination approaches that can induce broad cross-reactive immunity, particularly against heterologous virus subtypes, therefore remains a priority.

The induction of regulated mucosal cellular innate responses, particularly those mediated by macrophages (9), neutrophils (10, 11), and NK cells (12) as well as cytokines (13, 14), during the early stages of infection plays a crucial role in limiting viral replication and disease severity. This has led to the idea that an influenza vaccine that can be designed to induce short-acting innate protection before longer-lasting adaptive responses come into effect could be even more effective in limiting infection (15). Moreover, vaccines that prime or boost memory $CD8^+$ T cell responses to epitopes conserved across heterologous strains, i.e., those not subject to antibody-mediated selection pressure, could provide an additional level of cross-protective immunity to serologically diverse influenza virus strains, including novel subtypes with pandemic potential (16, 17). Cross-reactive $CD8^+$ cytotoxic T lymphocyte (CTL) responses induced in humans as a result of infection (18, 19) are closely associated with better prognosis after reinfections (20, 21), but these responses can wane over time (22) and can differ among different age groups (23, 24). Although CTL immunity does not prevent viral infection, it can significantly reduce viral load and moderate disease severity (25).

A number of studies have investigated ways of improving the level of protection that can be elicited by inactivated split virus vaccines in both mice and ferrets (26–31). While these approaches are effective at inducing primarily antibody-mediated heterosubtypic or cross-clade protection, the modification of a split virus vaccine formulation to induce innate immunity to provide immediate protection as well as subsequent adaptive immunity, including heterologous $CD8^+$ T cell-mediated responses, has yet to be realized. Such an intervention would extend the spectrum of immunity that can be induced by a seasonal influenza vaccine and would be beneficial in reducing disease burden during the period between the onset of a pandemic and the time at which a vaccine becomes available.

We have previously shown that inoculation of soluble protein antigens formulated with anionic or cationic lipopeptide-based adjuvants containing the Toll-like receptor 2 (TLR2) agonist S-[2,3-bis(palmitoyloxy)propyl]cysteine (Pam_2Cys) induces not only robust antibody but also strong $CD8^+$ T cell responses (32–34). Electrostatic association between the cationic lipopeptide R_4Pam_2Cys and the model antigen ovalbumin (OVA) induced endogenous $OVA_{257–264}$-specific $CD8^+$ T cell responses that mediated rapid clearance of a chimeric influenza virus containing the $K^bOVA_{257–264}$ epitope following viral challenge (34). In addition, our studies in animals have since demonstrated that intranasal (i.n.) inoculation of Pam_2Cys alone can rapidly induce pulmonary innate responses that protect against challenge with different influenza A virus (IAV) strains (35). These effects are induced within a day of inoculation and can reduce disease severity, lung viral titers, and the transmission of virus for up to a week. Importantly, the generation of adaptive immune responses is not compromised by intranasal treatment with Pam_2Cys (35).

In this study, we evaluated the advantages of formulating a split influenza virus vaccine with a modification of Pam_2Cys and examined its ability to confer immediate short-term protection against challenge with the homologous virus and with a serologically distinct virus as well as its ability to confer long-term protection by the induction of both virus-specific antibody and $CD8^+$ T cell responses. We also investigated the role of induced adaptive responses in mediating vaccine efficacy against these viral strains and report on the effects of vaccination on transmissibility of virus from infected animals.

RESULTS

Inoculation with split IAV vaccine formulated with R_4Pam_2Cys confers rapid antiviral protection. Our previous work (35) demonstrated that treatment of animals with Pam_2Cys in the absence of antigen at up to 7 days prior to influenza virus challenge results in a reduction in viral loads postchallenge. To assess whether these protective effects extended to the use of an influenza vaccine formulation containing R_4Pam_2Cys, we used a split influenza virus vaccine derived from PR8 influenza virus. This vaccine preparation contained proteins corresponding to the molecular weights of the major viral surface glycoproteins, as well as the internal nucleoprotein (NP) and the M1 matrix protein (see Fig. S1 in the supplemental material). By determining lung viral titers in mice challenged within 7 days of vaccination, i.e., before any adaptive immune response would have a significant effect, we were able to determine any protective effects mediated by the innate immune system (Fig. 1A).

The results (Fig. 1B) show that mice inoculated with a single dose of split virus vaccine formulated with R_4Pam_2Cys and challenged after 24 h with PR8 or X31 virus had significantly reduced lung viral titers 5 days postinfection compared to naive mice or those inoculated with split virus in the absence of R_4Pam_2Cys. A greater reduction in viral titers in mice inoculated with split virus plus R_4Pam_2Cys was detected when vaccinations were carried out 3 days prior to challenge. These protective effects were still evident 7 days following vaccination.

To evaluate protection against a lethal viral infection, vaccinated animals were challenged with a higher dose (500 PFU) of PR8 (Fig. 2A). While lung viral titers were not reduced in mice vaccinated in the presence of R_4Pam_2Cys and challenged a day after vaccination (Fig. 2B), significant reductions in viral titers in these mice compared to the control animals were detected when vaccinations were carried out 3 to 7 days prior to challenge.

Mice vaccinated with split virus plus R_4Pam_2Cys 3 days before lethal viral challenge were also protected from the substantial weight loss (Fig. 2C) and development of severe disease symptoms associated with infection compared to mice that received split virus alone, which by day 7 all had to be culled, having reached the defined humane endpoint (Fig. 2D). No protective effects were observed in similarly vaccinated $TLR2^{-/-}$ mice, indicating that protection mediated by R_4Pam_2Cys was dependent on recogni-

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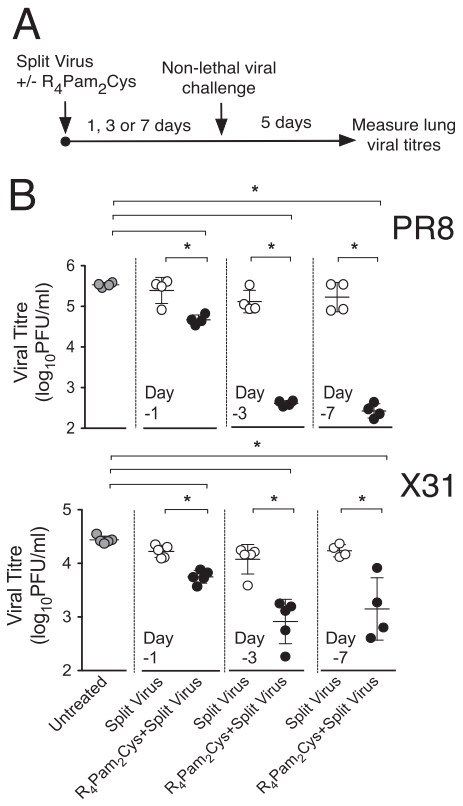

FIG 1 Rapid protective antiviral effects induced by inoculation with split virus vaccine formulated with R_4Pam_2Cys. (A) BALB/c mice ($n = 4$ to 5 per group) were inoculated via the intranasal route with PR8-derived split virus vaccine alone or combined with 5 nmol of R_4Pam_2Cys at 1 day (day −1), 3 days (day −3), or 7 days (day −7) prior to challenge with either 50 PFU of PR8 or $10^{4.5}$ PFU of X31. (B) Lungs were harvested 5 days later and viral titers determined. Symbols represent the titers obtained from individual mice, and horizontal lines indicate the mean virus titers (± standard deviations [SD]) of the group. Groups are defined at the bottom of panel B. Asterisks (*) indicate *P* values of <0.05.

tion and signaling operating through TLR2. These observations demonstrate that an influenza vaccine delivered with Pam_2Cys can induce rapid protection to reduce the impact of infection irrespective of the viral subtype. These protective effects are also achieved with a lesser dose of Pam_2Cys than was previously used to confer antiviral protection (35).

Antibody responses induced by vaccination of minimal doses of split IAV vaccine formulated with R_4Pam_2Cys. The induction of subsequent adaptive (antigen-specific) immunity was studied by measuring $CD8^+$ T cell and antibody responses at several time points after vaccination. Animals were also challenged 35 days after vaccination, and recall $CD8^+$ T cell responses and viral loads were analyzed 5 days postinfection (Fig. 3A). To evaluate antibody responses, mice were inoculated via the intranasal, subcutaneous, or intramuscular route. A low dose of split vaccine alone was unable to induce titers of antibody as high as those seen with adjuvanted split vaccine irrespective of the inoculation route used (Fig. 3B). In contrast, administration of the vaccine with R_4Pam_2Cys resulted in significant titers of antibody, with an ~600-fold improvement in titers following inoculation via the intranasal route and an ~20-fold to 60-fold improvement following inoculation by the subcutaneous or intramuscular route.

Serum antibody isotype profiles induced by intranasal inoculation of R_4Pam_2Cys-formulated vaccine were dominated by IgM, IgG1, and IgG2b (Fig. 3C). Similar isotype profiles were observed in the lung with the additional presence of IgA, indicating a significant mucosal antibody response. Taken together, these results indicate that formulation of an otherwise ineffective low dose of split virus vaccine combined with R_4Pam_2Cys elicits strong antibody responses characterized by the presence of a number of antibody isotypes, including significant levels of IgA, especially when administered intranasally.

Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) titers in sera obtained from mice inoculated with split virus plus R_4Pam_2Cys were found to be comparable to or higher than the antibody titers induced by PR8 infection (Table 1). In contrast, titers were not detected in sera of animals vaccinated with split virus alone. Not surprisingly, no detectable HI or NI reactivity was observed using sera from mice inoculated with vaccine alone or with vaccine formulated with R_4Pam_2Cys when tested against the serologically distinct H3N2 X31 virus. These results demonstrate that antibodies induced by inoculation of split virus can prevent viral HA and NA activity of homologous but not heterologous strains.

To investigate the induction of T cell responses, lymphocytes from the mediastinal lymph nodes (MLNs) and lungs were analyzed for the presence of $NP_{147–155}$-specific $CD8^+$ T cells by tetramer straining 7 days after vaccination. Compared to vaccination of split virus alone, vaccination of split virus in the presence of R_4Pam_2Cys resulted in the detection of higher numbers of $NP_{147–155}$-specific $CD8^+$ T cells at both these sites, especially in the lungs (Fig. 3D). Furthermore, higher numbers of gamma interferon (IFN-γ)-, tumor necrosis factor alpha (TNF-α)-, and interleukin-2 (IL-2)-producing $NP_{147–155}$-specific $CD8^+$ T cells were also detected in lymphocyte populations derived from animals vaccinated with split virus plus R_4Pam_2Cys following *in vitro* restimulation with $NP_{147–155}$ peptide for a further 7 days (Fig. 3E).

Protection against homologous and heterologous viral challenge. To evaluate the *in vivo* protective efficacy of split virus formulations, mice were challenged 35 days following inoculation with split virus vaccine alone or formulated with R_4Pam_2Cys and lung viral titers were determined 5 days later. Mice challenged with the homologous PR8 virus (Fig. 4A) were not protected if vaccinated with split virus or with R_4Pam_2Cys alone as these groups of mice had lung viral titers not significantly different from those of phosphate-buffered saline (PBS)-vaccinated control mice. In contrast, animals that received low-dose split virus formulated with R_4Pam_2Cys showed complete elimination of virus from the lungs.

A similar approach was used to determine if vaccination could clear infection with X31 (Fig. 4B). As with PR8 challenge, inoculation of mice with split virus alone or R_4Pam_2Cys alone provided no significant reduction of viral loads in lungs. The titers of virus in the lungs of mice that had been inoculated with split vaccine formulated with R_4Pam_2Cys, however, were significantly reduced. These results demonstrate that the presence of R_4Pam_2Cys in a split virus formulation not only enhances virus-clearing adaptive immune responses to the homologous virus strain but also enables the vaccine to provide cross-reactive virus-clearing immunity to heterologous virus.

Ability of antibodies induced by vaccination to mediate heterosubtypic protection. To investigate the possible presence of

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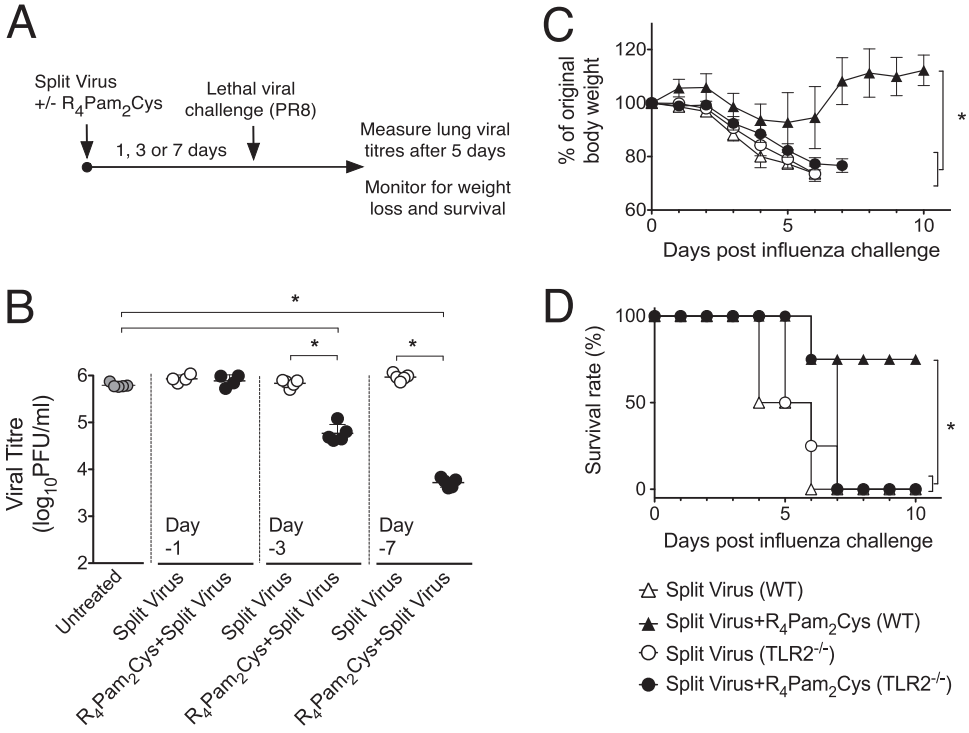

FIG 2 Inoculation with split virus vaccine formulated with R_4Pam_2Cys also provides rapid protection against lethal viral challenge in a TLR2-dependent manner. (A) BALB/c mice ($n = 5$ per group) were inoculated via the intranasal route with PR8-derived split virus vaccine alone or combined with 5 nmol of R_4Pam_2Cys 3 days prior to challenge with 500 PFU of PR8. (B) Lungs were harvested 5 days later, and viral titers were determined. Symbols represent the titers obtained from individual mice, and horizontal lines indicate the mean virus titers (± SD) for the group. C57BL/6 or $TLR2^{-/-}$ mice ($n = 4$ per group) were similarly inoculated 3 days prior to viral challenge and monitored daily for signs of illness and weight loss. Mice were killed when a humane endpoint was reached as characterized by >20% weight loss accompanied by signs of severe disease. (C and D) The mean body weight of mice is represented as a percentage of the original weight at the time of challenge as depicted in panel C, and survival over a 10-day period is shown in panel D. Asterisks (*) indicate *P* values of <0.05.

X31-neutralizing antibodies that were not detected in standard HI and NI assays, sera from vaccinated mice were tested for their ability to inhibit infection of MDCK cell monolayers in an *in vitro* virus neutralization assay. The results from these experiments (Table 2) demonstrated that sera obtained from naive mice or from mice inoculated with split virus alone were ineffective at specifically preventing PR8 and X31 infection of cells. In contrast, sera obtained from mice inoculated with split virus administered with R_4Pam_2Cys or from mice previously primed with PR8 virus effectively neutralized PR8 infection. They did not, however, inhibit infection by X31, indicating the absence of cross-neutralizing antibodies. Neutralization of X31 was achieved only using sera from mice previously infected with the homologous (X31) virus strain. These results are consistent with the presence of HI and NI antibodies in these sera (Table 1). We conclude that, although coformulation of the split vaccine with R_4Pam_2Cys results in a strong homologous neutralizing antibody response, detectable levels of neutralizing antibodies against a heterologous strain could not be demonstrated *in vitro*.

Protection against heterosubtypic viral challenge in μMT antibody-deficient mice. To provide evidence that the immunity to heterologous virus challenge occurred independently of any antibody-mediated effects, μMT B cell-deficient mice were vaccinated and we evaluated their ability to clear virus following challenge with X31. Prior to challenge, vaccine-specific antibody titers in these mice were compared to those obtained in congenic C57BL/6 mice. In contrast to the significant levels of antibody detected in the serum and lung homogenates of C57BL/6 mice inoculated with split virus formulated with R_4Pam_2Cys, antibody levels in μMT mice that had been similarly immunized were below the limits of detection of the assay (Fig. 5A).

When pulmonary viral titers were measured in vaccinated μMT mice following challenge with X31, significantly lower viral levels were present in mice vaccinated with split virus in the presence of R_4Pam_2Cys than in its absence (Fig. 5B). It therefore appears that, despite a lack of antibody, μMT mice can mount a virus-clearing response to a heterologous virus, indicating that the cross-protective immunity generated in the presence of R_4Pam_2Cys is antibody independent.

Pulmonary $CD8^+$ T cell responses in vaccinated animals following viral challenge. We next investigated whether protection against X31 was associated with $CD8^+$ T cell responses. Following vaccination, lymphocytes in the lungs were analyzed for virus-specific IFN-γ-producing $CD8^+$ T cells following *ex vivo* stimulation with syngeneic antigen-presenting cells (APCs) infected with X31 or pulsed with the nucleoprotein-derived H-$2K^d$-restricted immunodominant epitope $NP_{147–155}$. Our analyses found very few virus- or NP-specific IFN-γ-producing $CD8^+$ T cells in the lungs of mice 34 days after vaccination with split virus in the absence or presence of R_4Pam_2Cys (Fig. 6A). Following challenge with X31, however, the number of virus-specific IFN-γ-producing $CD8^+$ T cells that were detected in mice inoculated with split virus formulated with R_4Pam_2Cys was approximately 3-fold higher than the number detected in mice that received split

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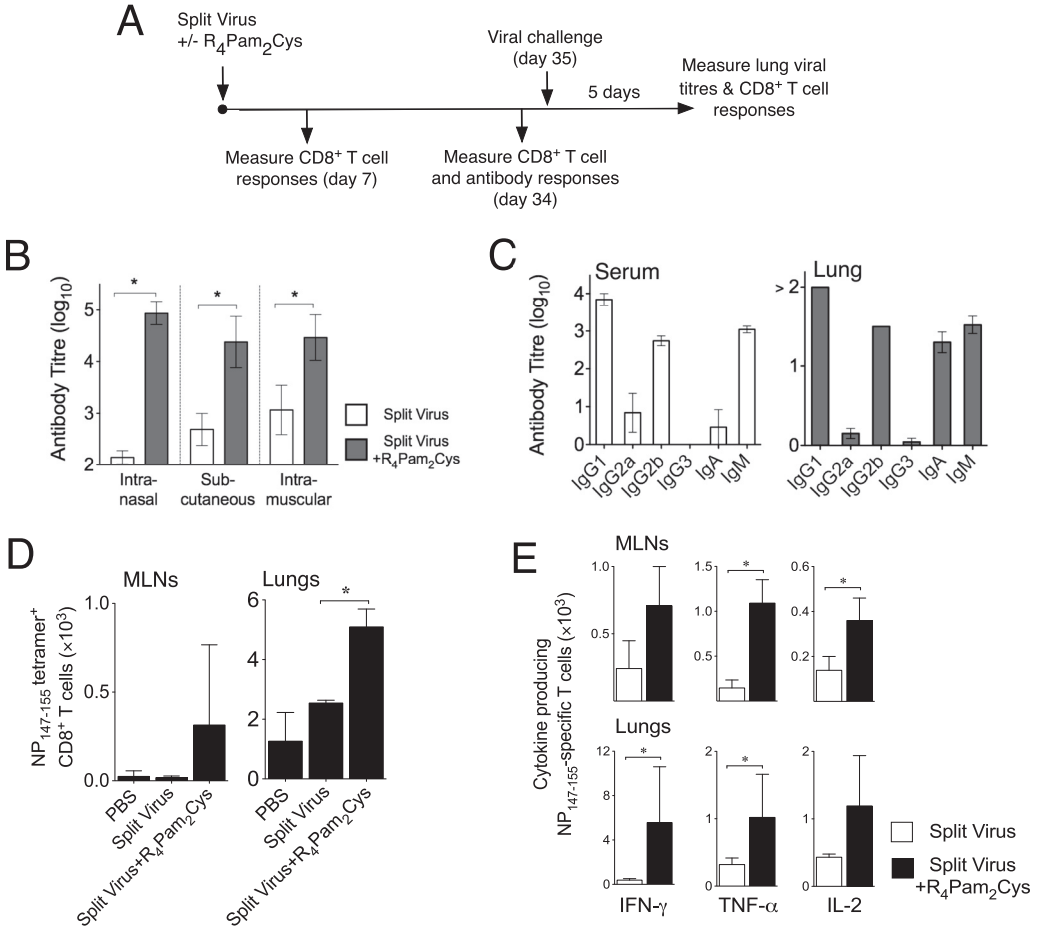

FIG 3 Induction of antibody and CD8$^+$ T cell responses by vaccination. (A) Adaptive immunity induced by vaccination was studied by measuring antigen-specific immune responses at various time points after inoculation and viral loads following challenge 35 days postvaccination. (B) To measure antibody titers, BALB/c mice (n = 5 per group) were inoculated via the intranasal, subcutaneous, or intramuscular route with split virus vaccine alone or with split virus combined with R_4Pam_2Cys. Sera were obtained from blood taken 34 days later, and vaccine-specific antibody levels were determined by ELISA using split virus vaccine as a coating antigen. (C) Isotypes of vaccine-specific antibodies in sera and lung homogenate supernatants from intranasally vaccinated mice were also assayed using isotype-specific detection antibodies. Results are presented as the mean antibody titer (± SD) from all serum samples in each group. (D) To analyze CD8$^+$ T cell responses, BALB/c mice (n = 3 per group) were inoculated via the intranasal route and lymphocytes in the lungs and MLNs were stained with fluorochrome-conjugated anti-CD8 antibody and H2K^d tetramer to detect $NP_{147–155}$-specific cells. (E) Lymphocytes were also restimulated *in vitro* in the presence of $NP_{147–155}$ for 7 days before analysis for IFN-γ-, TNF-α-, and IL-2-producing CD8$^+$ T cells by ICS. Bar graphs depict the means numbers of $NP_{147–155}$ tetramer$^+$-producing (D) and antigen-specific cytokine-producing (E) CD8$^+$ T cells per group, and asterisks (*) indicate P values of <0.05.

virus alone or PBS (Fig. 6A and B). A large proportion of this recall response also appeared to be specific for $NP_{147–155}$ (Fig. 6A).

To determine the importance of these responses in mediating heterologous protection, vaccinated animals were depleted of CD8$^+$ T cells prior to X31 challenge. Non-CD8$^+$ T cell-depleted mice vaccinated with split virus plus R_4Pam_2Cys exhibited significantly higher recall responses to $NP_{147–155}$ than mice vaccinated with split virus alone (Fig. 6C) at 5 days postinfection. In compar-

TABLE 1 Hemagglutination and neuraminidase inhibition (HI and NI) titers of sera from animals vaccinated against or infected with PR8 or X31[a]

Mouse vaccination or infection category	Titer (log_{10}) of virus strain[b]:			
	PR8		X31	
	HI	NI	HI	NI
PBS	<1.0	<1.5	<1.0	<1.5
Split virus	<1.0	<1.5	<1.0	<1.5
Split virus + R_4Pam_2Cys	2.92 ± 0.3	2.58 ± 0.3	<1.0	<1.5
PR8 infected	2.21 ± 0.0	2.71 ± 0.0	ND	ND
X31 infected	ND	ND	2.58 ± 0.4	2.98 ± 0.0

[a] Hemagglutination (HI) and neuraminidase inhibition (NI) tests were performed with PR8 or X31 virus on sera (n = 5) obtained 35 days after vaccination or infection.
[b] HI titers (log_{10}) are expressed as the reciprocal of the highest dilution of sample that inhibited 4 hemagglutinating units of virus. NI titers (log_{10}) are expressed as the reciprocal of the highest dilution of sample that caused a 50% inhibition of NA activity.

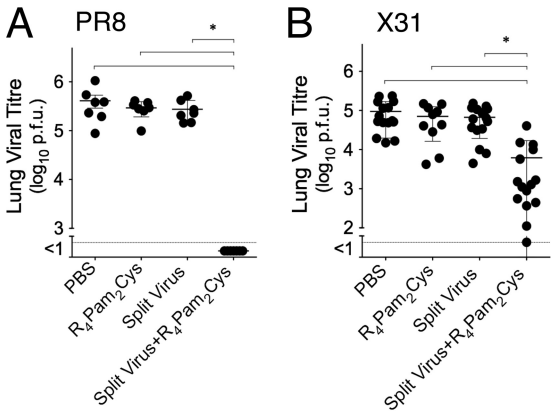

FIG 4 Protection of vaccinated animals against homologous and heterologous virus. BALB/c mice ($n = 7$ to 15 per group) were intranasally inoculated with PR8-derived split virus vaccine alone or formulated with R_4Pam_2Cys. A separate group of mice also received a similar dose of R_4Pam_2Cys alone. Animals were challenged intranasally 35 days later with 50 PFU of PR8 (A) or $10^{4.5}$ PFU of X31 (B). Titers of virus in lung homogenates collected 5 days after viral challenge were determined by plaque formation. Viral titers of individual animals are presented, with the mean value of the group represented by the horizontal bar. Error bars represent SD, and asterisks (*) indicate P values of <0.05.

TABLE 2 Ability of sera from vaccinated or infected mice to neutralize X31 and PR8 infection of MDCK cells[a]

Mouse vaccination or infection category	Titer (log_{10}) of virus strain[b]:	
	PR8	X31
PBS	<1	<1
Split virus	<1	<1
Split virus + R_4Pam_2Cys	3.95 ± 0.4	<1
PR8 infected	4.50 ± 0.0	<1
X31 infected	<1	3.52 ± 0.2

[a] Viral neutralization tests were performed using sera ($n = 5$) taken 35 days after vaccination or infection.

[b] Titers (log_{10}) are expressed as the highest average dilution (± standard deviation) that reduced plaque numbers to 50% of the titers obtained in the presence of serum from unvaccinated mice.

ison, responses in similarly vaccinated but $CD8^+$ T cell-depleted animals were absent and correlated with an absence of a reduction in viral titers (Fig. 6D). Taken together, these results support the view that the ability of mice inoculated with split virus formulated with R_4Pam_2Cys to clear virus from the lung is at least in part due to the presence of a strong NP-specific $CD8^+$ T cell-mediated recall response.

Reduction in pulmonary viral loads of contacts cohoused with R_4Pam_2Cys-vaccinated index mice. To determine if the cross-protective responses observed in mice inoculated with split virus plus R_4Pam_2Cys (Fig. 4B) also inhibited transmission of virus to an unvaccinated population, we used a mouse contact-dependent transmission model (36). Mice vaccinated with split virus alone or combined with R_4Pam_2Cys were infected with a transmissible strain (X31) 35 days after vaccination (vaccinated index mice) and were cohoused for 54 h with naive animals (unvaccinated contact mice). Irrespective of whether mice were vaccinated with split virus in the presence or absence of R_4Pam_2Cys, we found similar viral titers (>6 log_{10} PFU/ml) in the lungs of all vaccinated index mice (Fig. 7) after the cohousing period. This was expected because the recall and virus-clearing effects of pulmonary cytotoxic T cells take at least that long to manifest. Nevertheless, unvaccinated contact mice cohoused with index animals vaccinated with split virus plus R_4Pam_2Cys had undetectable or very low levels of virus in the lung. This is in contrast to the 1 to 5.4 log_{10} PFU/ml of virus found in more than 60% of contacts cohoused with index mice vaccinated with split virus alone or combined with PBS. It should be noted that transmissibility in this model is known to be dependent on the level of virus present in the saliva of index mice. These results therefore suggest that, despite equivalent pulmonary viral loads early after challenge, reduced levels of virus may be present in the saliva of mice vaccinated with split virus plus R_4Pam_2Cys compared to mice vaccinated with split virus alone (36), resulting in less viral shedding.

DISCUSSION

R_4Pam_2Cys associates electrostatically with oppositely charged regions on vaccine antigens, providing a means of forming complexes of the antigen and the TLR2 agonist without resorting to

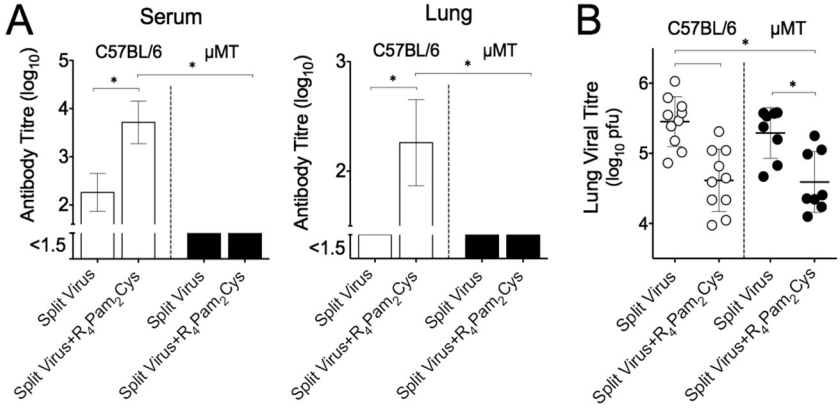

FIG 5 Cross-reactive immunity and antibody responses in vaccinated μMT mice. (A) C57BL/6 mice (white bars) or congenic μMT mice (black bars) were inoculated via the intranasal route with split virus vaccine alone or combined with R_4Pam_2Cys ($n = 8$ to 10 mice per group). Vaccine-specific antibody levels in serum and lung homogenates were measured 34 days later by ELISA. The means and SDs of the results determined for each group are shown. (B) Animals were challenged 35 days after vaccination with $10^{4.5}$ PFU of X31. Titers of virus in individual lung homogenates collected from C57BL/6 mice (white circles) and μMT mice (black circles) 5 days after viral challenge were determined by plaque formation. The mean values are represented by a horizontal bar. Error bars represent SD, and asterisks (*) indicate P values of <0.05.

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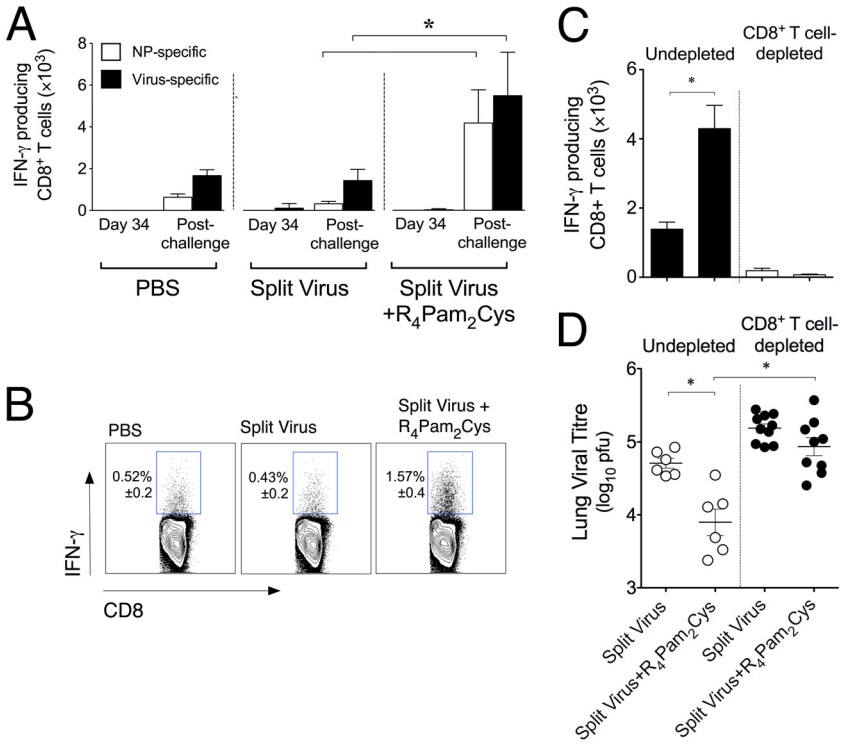

FIG 6 Induction of $CD8^+$ T cell responses in vaccinated mice following X31 challenge and its role in mediating cross-reactivity immunity. (A) BALB/c mice ($n = 5$ per group) were inoculated with split virus vaccine alone or premixed with R_4Pam_2Cys. Lymphocytes in lungs of vaccinated mice were analyzed for virus- or $NP_{147–155}$-specific IFN-γ-producing T cells in an ICS assay on day 34 postvaccination. Animals were then challenged on day 35 with $10^{4.5}$ PFU of X31, and specific IFN-γ-producing T cells in the lungs were enumerated 5 days later. Each bar represents the mean and SD of the results determined for each group. (B) Concatenated dot plots of samples from virus-challenged animals are also shown, depicting the percentages (± SD) of responsive IFN-γ^+ $CD8^+$ T cells in the lung following stimulation with virus-infected APCs. (C) Vaccinated mice ($n = 5$ to 10) were also depleted of $CD8^+$ T cells prior to X31 challenge. The total numbers of $NP_{147–155}$-specific IFN-γ^+ $CD8^+$ T cells in BAL fluid samples from $CD8^+$ T cell-depleted or undepleted vaccinated mice were enumerated in an ICS assay 5 days later. (D) Titers of virus in lung homogenates were also determined by plaque formation. Titers in individual animals are presented, with the mean values for the groups represented by the horizontal bars. All error bars represent SD, and asterisks (*) indicate P values of <0.05.

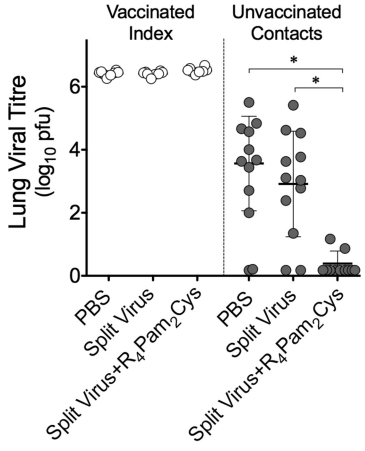

FIG 7 Pulmonary infection of unvaccinated contact mice cohoused with vaccinated index mice. BALB/c mice ($n = 4$ per group) were inoculated with PBS or with split virus vaccine alone or premixed with R_4Pam_2Cys. Mice were then challenged 35 days later with $10^{4.5}$ PFU of X31. After 6 h, 2 vaccinated index mice were cohoused with 3 naive unvaccinated contact mice for 48 h in a box that allowed direct contact. Vaccinated index mice were culled at 54 h postchallenge, while contact mice were culled at 96 h after the cohousing. Lungs were collected to assess viral loads as determined by plaque assay. Averaged geometric mean viral titers (± SD) in the lungs of mice determined in two separate experiments that included 8 index mice and 12 contact mice are shown.

the complexities of covalent chemistry. Directing antigens to TLR2, particularly on dendritic cells (DCs), using R_4Pam_2Cys results in improved antigen uptake and maturation of the DC and promotes proinflammatory cytokine secretion, resulting in the induction of both humoral and cellular immunity (32–34). By formulating inactivated detergent-split influenza virus vaccine with R_4Pam_2Cys, significant enhancement of vaccine immunogenicity through the induction of stronger neutralizing antibody responses and $CD8^+$ T cell-mediated cross-protective immunity results. Such a vaccination regimen also mitigates contact-dependent transmission.

These findings build on our previous work, which showed that the breadth and effectiveness of responses induced with a suboptimal dose of this same vaccine are improved when the vaccine is coadministered with a Pam_2Cys-peptide immunogen containing a cross-protective T cell epitope (37). The difference in this study was that, when R_4Pam_2Cys was used, the addition of extra antigen was not required to induce the immunogenic and protective effects observed. An additional benefit of a vaccine formulation that contains Pam_2Cys is its ability to stimulate innate cellular and cytokine-mediated responses which provide short-lived resistance to different influenza virus strains in an antigen-independent manner (35). This protective effect has been shown to last for up to 7 days and is characterized by neutrophils and macrophages infiltrating the lung as well as by elevated levels of IL-2, IL-6,

IFN-γ, and TNF-α, among other inflammatory mediators (35). Unlike the use of antivirals such as oseltamivir, which has been reported to affect the induction of adaptive immune responses (38, 39), the use of Pam_2Cys, besides conferring immediate protection, also permits the generation of subsequent and robust adaptive immune responses. Furthermore, and because the immediate protective effect of Pam_2Cys is mediated by host responses rather than by targeting a structural feature of the virus, resistance to the use of Pam_2Cys is less likely to occur. One could therefore envisage the use of a split virus formulation containing R_4Pam_2Cys which would be administered intranasally as a spray to provide immediate short-term antiviral protection in the event of an outbreak while the antigenic component of the vaccine would extend protection well beyond this time frame via the establishment of adaptive immunity.

In our efforts to define the immune mechanisms mediating cross-protection, we investigated the role of antibodies that were induced by vaccination using R_4Pam_2Cys. Although antibodies that were capable of inhibiting the HA and NA activity showed no cross-reactivity with heterologous virus, it was possible that antibodies against other conserved regions of the virus, e.g., the highly conserved stalk domain of HA (40–42) and the fusion active subunit of HA2 (43, 44), may have been involved. Antibodies against NP (45) and the ectodomain of the virus M2 protein (46), which exert their activity by binding to infected host cell surface-associated viral antigens and engage antibody-dependent cellular cytotoxicity (47), have also been implicated in facilitating cross-protective immunity. Nonetheless, results from our *in vitro* neutralization assay and the fact that cross-protection was demonstrated in antibody-deficient animals but not in $CD8^+$ T cell-depleted animals indicated that the cross-protective immunity afforded by the presence of R_4Pam_2Cys is $CD8^+$ T cell mediated.

The induction of NP-specific primary $CD8^+$ T cell responses following vaccination using split virus in the presence of R_4Pam_2Cys is associated with a strong recall response elicited following challenge, suggesting that T cells are recalled from a pool of memory cells established following vaccination and recruited from extrapulmonary sites such as the mediastinal lymph nodes to help facilitate viral clearance. Moreover, it is also possible that $CD4^+$ T cell responses to HA and NA are primed by vaccination to help support both antibody- and cell-mediated responses. Support for this hypothesis is based on our unpublished observations which showed that vaccination of OVA formulated with R_4Pam_2Cys also results in the proliferation of $CD4^+$ T cells and promotes their differentiation into T follicular helper cells to help drive germinal B cell formation. Moreover, both vaccine-induced OVA-specific antibody responses and $CD8^+$ T cell responses are absent in $CD4^+$ T cell-deficient animals (GK1.5 mice).

The use of virus-infected APCs in the intracellular cytokine staining (ICS) assays to examine $CD8^+$ T cell recall responses allows presentation of an array of virus-derived CTL cell epitopes potentially induced by the multiple antigens contained in the vaccine formulation. Although it appears that the CTL responses detected are directed largely against an epitope derived from NP that has been shown to be immunodominant in the response to viral infection, it remains to be seen what other T cell specificities are involved in this response; the presence of multiple and different viral proteins in the split virus vaccine suggests that an array of other subdominant responses (48) could also be elicited. The potential to boost cross-reactive $CD8^+$ T cell responses in humans by vaccination was exemplified in recent phase I and 2a clinical trials demonstrating the use of an NP and M1 proteins delivered by a vaccinia virus vector to increase antigen-specific T cell responses (49) and reduce disease symptoms and viral shedding following exposure to an influenza virus strain to which subjects had not previously been exposed (50). Virus vector-based vaccines that target conserved antigens have also been shown to reduce transmission in animals (51). The use of Pam_2Cys described here therefore not only provides similar benefits but also has advantages over such delivery systems due to its nonbiological nature and ease of manufacture and use.

The adaptive responses induced using R_4Pam_2Cys were achieved using a considerably smaller dose of split virus vaccine than is usually required to induce biologically active antibody responses in mice. This dose-sparing effect of R_4Pam_2Cys is an important point to be considered in the economy of vaccine manufacture, distribution, and administration in the event of influenza pandemics and epidemics. Alterations to current seasonal vaccine formulations to include R_4Pam_2Cys might also be attractive to manufacturers because such alterations would not require development of a new vaccine *per se* and could have the potential to significantly reduce the time periods that are usually associated with new vaccine development, expediting introduction.

These findings highlight the benefits of using a TLR agonist to improve the utility and also to extend the spectrum of immunity induced by influenza split virus vaccines against homologous and heterologous subtypes. A cost-effective method for dose sparing and at the same time extending efficacy could be of great benefit in scenarios in which there is an imperative for mass vaccination with a virus strain to which the population is immunologically naive. The innate- and adaptive-immune-system-mediated protective effects achieved through use of R_4Pam_2Cys could be especially beneficial for improving community protection, particularly during the period between the time of an outbreak and the time when a vaccine becomes available.

MATERIALS AND METHODS

Synthesis of the cationic lipopeptide R_4Pam_2Cys. The synthesis of the cationic lipopeptide R_4Pam_2Cys was carried out manually using conventional solid-phase methodologies as described previously (34). Synthetic lipopeptide preparations contained no detectable lipopolysaccharide (LPS) (<0.05 endotoxin units [EU]/ml) as determined using the Limulus amebocyte lysate assay (Lonza, Walkersville, MD).

Vaccination and infection of mice. All animal experimentation was performed with approval from the University of Melbourne's Animal Ethics Committee. C57BL/6, BALB/c, $TLR2^{-/-}$, and B cell-deficient (μMT) mice (52) were bred and maintained under specific-pathogen-free conditions and used when the mice were 6 to 10 weeks of age. μMT mice carry a disrupted gene encoding the μ-chain constant region of IgM which arrests B cell development and the ability to mount antibody responses (52). Mice were anesthetized with isoflurane and inoculated via the intranasal (i.n.) route with split virus vaccine that was provided by bioCSL Limited, Australia. The vaccine contained 1 μg of HA and was derived from A/Puerto Rico/8/34 (PR8; H1N1) virus. The vaccine was administered either in saline solution or formulated with 5 nmol of R_4Pam_2Cys (9.91 μg) in a total volume of 40 μl. For some experiments, mice were inoculated via the subcutaneous route at the base of the tail (50 μl at each side) or via the intramuscular route (40 μl into each quadriceps muscle).

After 1, 3, 7, or 35 days, mice were challenged intranasally with $10^{4.5}$ PFU of A/HK×31 (X31) (H3N2) influenza virus or with 50 or 500 PFU of PR8 influenza virus. X31 is a recombinant virus containing the HA and NA segments from a 1968 Hong Kong influenza virus but sharing

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the internal viral proteins of the PR8 virus. Lungs were harvested 5 days later, homogenized in 3 ml RPMI 1640 (Invitrogen, Australia), and centrifuged at 300 × *g* for 30 s, and supernatants were stored at −80°C. Titers of virus in the supernatants of lung homogenates were then determined using a Madin-Darby canine kidney (MDCK) plaque assay as previously described (53).

ELISAs. Levels of antibody present in serial dilutions of sera or the supernatants of lung homogenates obtained from mice 34 days following vaccination were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (34). A panel of horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1-, IgG2a-, IgG2b-, IgG3-, IgA-, and IgM-specific antibodies (Southern Biotech, USA) were used in a separate assay to determine the isotypes of antibodies. Titers of antibody are expressed as the reciprocal of the highest dilution of serum required to achieve an optical density of 0.2.

Hemagglutination inhibition (HI), neuraminidase inhibition (NI), and virus neutralization assays. The presence of hemagglutination inhibition (HI) antibodies and of neuraminidase inhibition (NI) antibodies was determined as described elsewhere (54, 55). Virus neutralization assays were carried out as described previously (55).

***In vivo* depletion of CD8$^+$ T cells.** Mice were inoculated via the intraperitoneal route with 100 μg (200 μl) of anti-CD8α antibody (clone 2.43; National Cell Culture Center, USA) 29 days after vaccination. Antibody was administered daily for 3 consecutive days and then once every 3 days thereafter for the duration of the experiment.

Intracellular cytokine staining (ICS) assay and tetramer staining. Lungs from mice were finely minced and treated with 2 mg collagenase A (Roche Diagnostics, Germany)–2 ml RPMI 1640 per lung for 30 min at 37°C. Cell suspensions were passed through a wire sieve before centrifugal sedimentation of cells and treatment with 0.15 M NH_4Cl–17 mM Tris-HCl at pH 7.2 for 5 min at 37°C. In some experiments, cells were obtained from bronchoalveolar lavage (BAL) fluids or mediastinal lymph nodes (MLNs). Lymphocyte cell suspensions (1×10^6) were cultured in the presence of the H2K^d-restricted influenza virus nucleoprotein (NP)-derived immunodominant epitope $NP_{147–155}$ (TYQRTRALV; 1 mg/ml) at 37°C in a mixture with 200 μl of supplemented RPMI 1640 containing 55 mM 2-mercaptoethanol (2-ME), BD GolgiPlug (1 mg/ml) from a Cytofix/Cytoperm Plus kit (Becton, Dickinson), and recombinant IL-2 (Roche, Mannheim, Germany) (10 U/ml). For some experiments, lymphocyte cell suspensions were cultured in the presence of 2×10^5 virus-infected syngeneic P815 target cells. These cells (4×10^6/ml) had been infected previously with X31 (at a multiplicity of infection of 20) for 1 h at 37°C in Opti-MEM (Life Technologies, Australia) supplemented with gentamicin, glutamine, penicillin, and streptomycin at the concentrations described above and had been washed extensively in fetal calf serum (FCS) before use. Infection of cells was confirmed by the phenotypic expression of surface HA as verified by flow cytometry.

After 6 h, lymphocytes were washed with fluorescence-activated cell sorter (FACS) wash buffer (1% FCS–5 mM EDTA–PBS) and stained with a peridinin chlorophyll protein (PerCP) Cy5.5-conjugated rat anti-mouse CD8 antibody (clone 53-6.7; Becton, Dickinson) for 30 min at 4°C. Fixation and permeabilization were then performed for 20 min at 4°C using Cytofix/Cytoperm solution (Becton, Dickinson) according to the manufacturer's instructions. Cells were washed once and stained for intracellular IFN-γ, IL-2, or TNF-α with fluorochrome-conjugated antibodies for 30 min at 4°C before flow cytometric analysis (FACSCanto II or LSR II; BD Biosciences). Data analysis was performed using FlowJo software (Treestar, USA).

For *in vitro* restimulation of lymphocytes over 7 days, cells (2×10^6) were cultured in 200 μl of supplemented RPMI 1640 media containing $NP_{147–155}$ peptide (5 μg/ml) and IL-2 (10 U/ml) with medium changes performed every 2 days. ICS assays were then performed as indicated above.

Tetramer staining of lymphocytes from the MLNs and lungs was performed at room temperature using a 1:200 dilution in a mixture with 50 μl of 10% FCS–PBS for 30 min followed by the addition of fluorochrome-conjugated anti-CD8 antibody for 30 min. Cells were extensively washed before analysis.

Contact-dependent virus transmission. The transmissibility of virus was evaluated in a mouse model developed for the study of contact-dependent viral transmission (36). Vaccinated mice were challenged after 35 days by the i.n. route with $10^{4.5}$ PFU of X31 virus. After 6 h, these index mice were introduced into a clean cage and cohoused with naive contact mice (2 index mice for 3 contact mice) for 2 days. Index mice were euthanized directly after the cohousing period, and contact mice were euthanized 4 days after their initial exposure to the index mice. Viral titers in lung homogenates of all animals were then determined in a standard plaque assay.

Statistical analyses. Analysis of variance (ANOVA) and *P* values were obtained using nonparametric one-way ANOVA; Tukey's *post hoc* range tests, two-way ANOVA, and Bonferroni posttests were performed using the Prism 5 software package (GraphPad Software, La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01024-15/-/DCSupplemental.

Figure S1, EPS file, 1.6 MB.

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