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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\(^{2}\)Pam\(_{2}\)Cys) 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\(^{2}\)Pam\(_{2}\)Cys 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\(^{2}\)Pam\(_{2}\)Cys 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\(^{2}\)Pam\(_{2}\)Cys 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 naïve 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 naïve is imperative.

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.
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 (PamCys) induces not only robust antibody but also strong CD8+ T cell responses (32–34). Electrostatic association between the cationic lipopeptide R5PamCys and the model antigen ovalbumin (OVA) induced endogenous OVA257–264-specific CD8+ T cell responses that mediated rapid clearance of a chimeric influenza virus containing the K2OVA257–264 epitope following viral challenge (34). In addition, our studies in animals have since demonstrated that intranasal (i.n.) inoculation of PamCys 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 PamCys (35).

In this study, we evaluated the advantages of formulating a split influenza virus vaccine with a modification of PamCys 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 R5PamCys confers rapid antiviral protection. Our previous work (35) demonstrated that treatment of animals with PamCys 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 R5PamCys, 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 R5PamCys 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 R5PamCys. A greater reduction in viral titers in mice inoculated with split virus plus R5PamCys 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 R5PamCys 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 R5PamCys 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 R5PamCys was dependent on recogni
Inducing Innate and Adaptive Immunity to Influenza

Serum antibody isotype profiles induced by intranasal inoculation of R_Pam_Cys-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_Pam_Cys 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_Pam_Cys 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_Pam_Cys 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 NP147–155-specific CD8+ T cells by tetramer staining 7 days after vaccination. Compared to vaccination of split virus alone, vaccination of split virus in the presence of R_Pam_Cys resulted in the detection of higher numbers of NP147–155-specific CD8+ T cells at both 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 NP147–155-specific CD8+ T cells were also detected in lymphocyte populations derived from animals vaccinated with split virus plus R_Pam_Cys following in vitro restimulation with NP147–155 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_Pam_Cys 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_Pam_Cys 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_Pam_Cys 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_Pam_Cys 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_Pam_Cys, however, were significantly reduced. These results demonstrate that the presence of R_Pam_Cys 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...
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 R₄Pam₂Cys 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 R₄Pam₂Cys 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 R₄Pam₂Cys, 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 R₄Pam₂Cys 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 R₄Pam₂Cys 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-2Kd-restricted immunodominant epitope NP147-155. 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 R₄Pam₂Cys (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 R₄Pam₂Cys was approximately 3-fold higher than the number detected in mice that received split

FIG 2 Inoculation with split virus vaccine formulated with R₄Pam₂Cys 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 R₄Pam₂Cys 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.

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virus alone or PBS (Fig. 6A and B). A large proportion of this recall response also appeared to be specific for NP<sub>147–155</sub> (Fig. 6A).

To determine the importance of these responses in mediating heterologous protection, vaccinated animals were depleted of CD<sup>8</sup><sup>+</sup> T cells prior to X31 challenge. Non-CD<sup>8</sup><sup>+</sup> T cell-depleted mice vaccinated with split virus plus R<sub>4</sub>Pam<sub>2</sub>Cys exhibited significantly higher recall responses to NP<sub>147–155</sub> than mice vaccinated with split virus alone (Fig. 6C) at 5 days postinfection.

### TABLE 1

<table>
<thead>
<tr>
<th>Mouse vaccination or infection category</th>
<th>Titer (log&lt;sub&gt;10&lt;/sub&gt;) of virus strain&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PR8</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Split virus</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Split virus + R&lt;sub&gt;4&lt;/sub&gt;Pam&lt;sub&gt;2&lt;/sub&gt;Cys</td>
<td>2.92 ± 0.3</td>
</tr>
<tr>
<td>PR8 infected</td>
<td>2.21 ± 0.0</td>
</tr>
<tr>
<td>X31 infected</td>
<td>ND</td>
</tr>
<tr>
<td>X31 infected</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hemagglutination (HI) and neuraminidase inhibition (NI) tests were performed with PR8 or X31 virus on sera (<i>n</i> = 5) obtained 35 days after vaccination or infection.
<sup>b</sup> HI titers (log<sub>10</sub>) are expressed as the reciprocal of the highest dilution of sample that inhibited 4 hemagglutinating units of virus. NI titers (log<sub>10</sub>) are expressed as the reciprocal of the highest dilution of sample that caused a 50% inhibition of NA activity.
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 R₄Pam₂Cys (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 10₄.₅ 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.

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

<table>
<thead>
<tr>
<th>Mouse vaccination or infection category</th>
<th>Titer ((\log_{10})) of virus strain¹</th>
<th>Titer ((\log_{10})) of virus strain²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>PR8 (&lt;1)</td>
<td>X31 (&lt;1)</td>
</tr>
<tr>
<td>Split virus</td>
<td>PR8 (&lt;1)</td>
<td>X31 (&lt;1)</td>
</tr>
<tr>
<td>Split virus + R₄Pam₂Cys</td>
<td>3.95 ± 0.4</td>
<td>1</td>
</tr>
<tr>
<td>PR8 infected</td>
<td>4.50 ± 0.0</td>
<td>1</td>
</tr>
<tr>
<td>X31 infected</td>
<td>(&lt;1)</td>
<td>3.52 ± 0.2</td>
</tr>
</tbody>
</table>

A Viral neutralization tests were performed using sera \((n = 5)\) taken 35 days after vaccination or infection.

Titer \((\log_{10})\) 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.

**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 R₄Pam₂Cys. A separate group of mice also received a similar dose of R₄Pam₂Cys alone. Animals were challenged intranasally 35 days later with 50 PFU of PR8 (A) or 10⁴.₅ 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.

**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 R₄Pam₂Cys \((n = 8\) 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 10⁴.₅ 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.

**DISCUSSION**

R₄Pam₂Cys 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
the complexities of covalent chemistry. Directing antigens to TLR2, particularly on dendritic cells (DCs), using \( \text{R}_4\text{Pam}_2\text{Cys} \) 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 \( \text{R}_4\text{Pam}_2\text{Cys} \), 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 \( \text{Pam}_2\text{Cys} \)-peptide immunogen containing a cross-protective T cell epitope (37). The difference in this study was that, when \( \text{R}_4\text{Pam}_2\text{Cys} \) 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 \( \text{Pam}_2\text{Cys} \) 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$_{2}$Cys, besides conferring immediate protection, also permits the generation of subsequent and robust adaptive immune responses. Furthermore, and because the immediate protective effect of Pam$_{2}$Cys is mediated by host responses rather than by targeting a structural feature of the virus, resistance to the use of Pam$_{2}$Cys is less likely to occur. One could therefore envisage the use of a split virus formulation containing R$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys. 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys also results in the proliferation of CD4$^{+}$ T cells and promotes their differentiation into Th 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 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$_{2}$Cys 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys 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$_{P}$Pam$_{2}$Cys.** The synthesis of the cationic lipopeptide R$_{P}$Pam$_{2}$Cys 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 bioCIS 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$_{P}$Pam$_{2}$Cys (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$^{-5.5}$ PFU of A/HKx31 (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...
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 (53).

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 thereafter for the duration of the experiment. 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 NaH2CO3–17 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 NP345–365 (TYQRTRALV; 1 mg/ml) at 37°C in a mixture with 200 μl of supplemented RPMI 1640 containing 35 mM 2-mercaptoethanol (2-ME), BD GolgiPlug (1 mg/ml) from a manufacturer’s instructions. Cells were washed once and stained for intracellular cytokine staining (ICS) assay and permeabilization were then performed for 20 min at 4°C using Cytofix/Cytoperm Plus kit (Becton, Dickinson), and recombinant IL-2 (Treestar, USA). Data analysis was performed using FlowJo software (TreeStar, USA).

For intravenous restimulation of lymphocytes over 7 days, cells (2 × 10^6) were cultured in 200 μl of supplemented RPMI 1640 media containing NP345–365 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

Figure S1, EPS file, 1.6 MB.

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