Soluble Proteins Induce Strong CD8⁺ T Cell and Antibody Responses through Electrostatic Association with Simple Cationic or Anionic Lipopeptides That Target TLR2

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Soluble Proteins Induce Strong CD8+ T Cell and Antibody Responses through Electrostatic Association with Simple Cationic or Anionic Lipopeptides That Target TLR2

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The low immunogenicity exhibited by most soluble proteins is generally due to the absence of molecular signatures that are recognized by the immune system as dangerous. In this study, we show that electrostatic binding of synthetic branched cationic or anionic lipopeptides that contain the TLR-2 agonist Pam2Cys markedly enhance a protein’s immunogenicity. Binding of a charged lipopeptide to oppositely charged protein Ags resulted in the formation of stable complexes and occurs at physiologic pH and salt concentrations. The induction of cell-mediated responses is dependent on the electrostatic binding of lipopeptide to the protein, with no CD8+ T cells being elicited when protein and lipopeptide possessed the same electrical charge. The CD8+ T cells elicited after vaccination with lipopeptide–protein Ag complexes produced proinflammatory cytokines, exhibited in vivo lytic activity, and protected mice from challenge with an infectious chimeric influenza virus containing a single OVA epitope as part of the influenza neuraminidase protein. Induction of a CD8+ T cell response correlated with the ability of lipopeptide to facilitate Ag uptake by protected mice from challenge with an infectious chimeric influenza virus containing a single OVA epitope as part of the influenza neuraminidase protein. Induction of a CD8+ T cell response correlated with the ability of lipopeptide to facilitate Ag uptake by DCs followed by trafficking of Ag-bearing cells into draining lymph nodes. Oppositely charged but not similarly charged lipopeptides were more effective in DC uptake and trafficking. Very high protein-specific Ab titers were also achieved by vaccination with complexes composed of oppositely charged lipopeptide and protein, whereas vaccination with similarly charged constituents resulted in significant but lower Ab titers. Regardless of whether similarly or oppositely charged lipopeptides were used in the induction of Ab, vaccination generated dominant IgG1 isotype Abs rather than IgG2a.

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Generally, soluble protein Ags are poorly immunogenic when administered in the absence of adjuvants or through the use of sophisticated delivery modalities, as measured by their ability to elicit Ab and especially CD8+ T cells. This property has been attributed to size (i.e., they are nonparticulate), susceptibility to proteolytic degradation in serum, and a lack of molecular signatures or “danger signals” that dictate a response from the innate immune system. The shortcoming of poor immunogenicity of protein and subunit-based vaccines requires their coadministration with an adjuvant to elicit an immune response, a requirement that is of considerable importance to the vaccine industry. Of the many experimental adjuvants currently available, only squalene oil water emulsions and aluminum-based salt adjuvants have been approved for human use (1, 2). The use of these adjuvants, however, is usually limited by the fact that although they may be effective at eliciting humoral responses, they are often ineffective at stimulating cell-mediated immunity, particularly by CD8+ T cell. These limitations apply to many current vaccination strategies and can be attributed to the inadequate delivery of Ag to the appropriate APC, particularly the dendritic cell (DC) and subsequently to the appropriate Ag presentation pathway. Together with the lack of danger signals that are required to activate DCs for T cell priming, the poor immunogenicity of soluble protein vaccine candidates poses a significant challenge to their rationale.

Cellular immune responses are particularly important for generating protective immunity against a number of current infectious diseases (e.g., HIV, tuberculosis, malaria) (3, 4) that are causing high global mortality rates and for which there are either no or ineffective vaccines. A key requirement for the development of novel and effective vaccine strategies against such indications must be the development of robust vaccine technologies that are capable of inducing both humoral and cell-mediated immune responses.

Antigenic receptors that are expressed by DCs, such as the TLR family, have evolved to recognize a diverse range of microbial components. The engagement of TLRs by their appropriate ligands or agonists initiates a cascade of events that can result in the induction of Ab and cell-mediated responses. The use of TLR agonists as adjuvants to increase the efficacy and response to immunization with an Ag are well documented (5, 6) and form the vanguard of a rationally designed, new generation of vaccines. In many of these studies, vaccine formulations consisting of an admixture of Ag and TLR agonist are used, whereas others have used Ags covalently attached to TLR agonists. Some examples include Pam3Cys-SK4-conjugated-carbohydrate (7) and peptide-based (8) Ags for targeting TLR1/2 heterodimer, proteins linked to CpG oligodeoxynucleotides (9–11) and to DNA plasmids (12) for targeting TLR9, and imidazoquinoline-conjugated proteins (13, 14) for delivery to TLR 7/8.

Although the use of these agonists, whether admixed or covalently coupled to Ags, are effective at promoting vaccine efficacy,
LIPOPOLYPEPTIDE-MEDIATED ENHANCEMENT OF PROTEIN IMMUNOGENICITY

Covalent coupling has proved to be superior at eliciting Th1-type cell-mediated immunity and enhancing cross-presentation of Ags (11, 12, 15). This improvement in immunogenicity is attributed to the simultaneous delivery of both Ag and agonist to DCs, which leads to enhanced uptake, activation (16), and subsequent Ag presentation to T cells (17). We have shown previously that the covalent attachment of dipalmitoyl-S-glycerly-cysteine (Pam2 Cys), a synthetic analog of macrophage-activating lipoprotein-2 derived from the cytoplasmic membrane of Mycoplasma fermentans (18), to peptide-epitopes can facilitate their delivery into DCs via TLR2 and induce the activation of transcription factors including NF-κB leading to DC maturation, secretion of proinflammatory cytokines, and eventual activation of Ag-specific naïve T cells. Use of this TLR2 agonist has allowed us to design vaccine candidates that elicit protective humoral and cellular immune responses against pathogenic infection or tumorigenic challenge (8, 19–22).

With the aim of developing novel ways of adjuvanting otherwise nonimmunogenic and soluble proteins, we investigated whether the electrostatic linkage of Pam2Cys to protein Ags as opposed to a covalent conjugation results in the enhancement of protein immunogenicity. Robust, noncovalent association between protein and adjuvant would provide a manufacturing advantage compared with the chemistries involved for covalent attachment and would provide a “plug and play” approach to vaccine preparation. To facilitate firm association of Pam2Cys with protein Ag, the lipid moiety was assembled with a branched cationic arginine or an anionic glutamic acid-based structure designed to bind electrostatically to negatively or positively charged Ags, respectively. The physiochemical interactions between these simple lipopeptides and protein Ags were studied, and the uptake of Pam2Cys–Ag complexes by DCs was investigated. The ensuing Ab- and cell-mediated Ag-specific responses were evaluated by measuring Ab titer and determining the ability of the CD8+ T cells that were induced to produce cytokines and protect inoculated mice against challenge with a live infectious chimeric influenza virus engineered to contain the OVA CTL epitope SIINFEKL in its neuraminidase protein.

Materials and Methods

Synthesis of cationic and anionic lipopeptides

The synthesis of a branched cationic peptide construct containing four N-terminal arginine (R4) using traditional Fmoc chemistry has been described previously (23). Synthesis was performed manually using PEG-SRAME (24) to generate R4(Pam2Cys) or E8(Pam2Cys) (Fig. 1A). To prevent lipilation of the N-terminal positions of R4, the N-terminal amino groups of the arginines were first acylated using a 10-fold molar excess of di-tert-butyl dicarboxylate (Fluka Chemika, Buchs, Switzerland) in the presence of diisopropylethylamine.

After assembly, lipopeptides were cleaved from the solid phase support, and all side-chain protecting groups removed with 88% trifluoroacetic acid (TFA), 5% phenol, 2% triisopropylsilane, and 5% water for 3 h at room temperature. Lipopeptides were analyzed by reversed-phase (RP) HPLC using a Vydac C4 column (4.6 × 300 mm) installed in a Waters HPLC system. The chromatogram was developed at a flow rate of 1 ml/min using 0.1% TFA in H2O and 0.1% TFA in acetonitrile as the limit solvent. Lipopeptides were purified if necessary, with all purified products presented as a single major peak on analytical RP-HPLC, and had the expected mass when analyzed using an Agilent series 1100 ion trap mass spectrometer.

Sedimentation and HPLC analysis of lipopeptide–Ag complexes

Increasing amounts of the branched R4(Pam2Cys) or E8(Pam2Cys) lipopeptides were mixed with 1 nmol of OVA or hen egg lysozyme (HEL) in a total volume of 100 μl PBS in a flat-bottom 96-well plate. The opalescence of the solution was then measured by determining the OD at 450 nm. Mixtures containing 100 nmol of either R4(Pam2Cys) or E8(Pam2Cys) and 1 nmol OVA or HEL in a total volume of 100 μl PBS were centrifuged (1.2 × 10^7 g) for a period of 5 min, and the distribution of lipopeptide and Ag present in the supernatant and sedimented material was performed by RP-HPLC analysis. The material sedimented from solutions containing only lipopeptide, or protein or mixtures of both, was dissolved in a solution of 50% acetonitrile in water and before analysis by HPLC. The mass of the major peak was verified by mass spectrometry in all samples containing lipopeptides.

ELISAs

All experimental procedures involving animals were approved by the University of Melbourne’s animal ethics committee (animal ethics approval identification number 0707207). Groups of five female, 6–8-wk-old, BALB/c mice were inoculated subcutaneously in the base of the tail on day 0 and again on day 22 with saline containing different amounts of R4(Pam2Cys) or E8(Pam2Cys) lipopeptide added to 25 μg OVA or HEL in a total volume of 100 μl. Separate groups of animals also received OVA or HEL formulated as an emulsion (Sigma-Aldrich, St. Louis, MO). Sera were prepared from blood taken 3 wk after the primary inoculation and 2 wk after the secondary inoculation.

Flat-bottom well polyvinyl plates were coated with Ag (5 μg/ml) in PBS for 18–20 h at room temperature in a humidified atmosphere. The Ag was removed and BSA (10 mg/ml) in PBS added for 1 h before washing with PBS containing s/s 0.05% Tween-20 (Aldrich, Milwaukee, WI). Serial dilutions of sera obtained from immunized mice were added to wells coated with R4(Pam2Cys) or E8(Pam2Cys) lipopeptide, or protein or mixtures of both, and washed and held overnight at room temperature. After washing, bound Ab was detected using HRP-conjugated rabbit anti-mouse IgG Abs (Dako, Glostrup, Denmark) or rat anti-mouse IgG1 and IgG2a Abs (Southern Biotech) in conjunction with enzyme substrate (0.2 mM 2,2-azino-bis-3-ethylbenzthiazoline-sulfonic acid in 50 mM citric acid containing 0.004% hydrogen peroxide).

The titers of Ab are expressed as the reciprocal of the highest dilution of serum to achieve an OD of 0.2. All p values in this study were obtained using one-way ANOVA nonparametric statistical analysis performed with Prism 4 (GraphPad Software).

Intracellular cytokine staining assay

For the measurement of Ag-specific cell-mediated immune responses following vaccination, groups of three female, 6–8-wk-old C57BL/6 mice were inoculated subcutaneously in the base of the tail with saline containing different amounts of R4(Pam2Cys) or E8(Pam2Cys) lipopeptide, or added to 25 μg OVA in a volume of 100 μl per dose.

After 7 d, spleens were obtained from inoculated mice and splenocytes (1 × 10^6) derived from them were then exposed to the H2Kk restricted OVA-derived peptide epitope OVA257 (SIINFEKL; 1 μg/ml) in RPMI 1640 medium (Invitrogen) supplemented with 10% heat inactivated FCS (CSL, Parkville, Australia), gentamicin (12 μg/ml), 2 μg/ml sodium pyruvate, penicillin (100 IU/ml), streptomycin (100 μg/ml) and 55 μM 2-ME, Brefeldin A (1 μg/ml) in the form of BD GolgiPlug from the Cytofix/Cytoperm Plus Kit (Becton Dickinson) and recombiant IL-2 (10 U/ml; Roche, Mannheim, Germany). After 6 h, lymphocytes were washed with FACS Wash Buffer (1% FCS 5 mM EDTA in PBS) and stained with a PerCP-conjugated rat anti-mouse CD8 Ab (Clone 53-6.7; Becton Dickinson) for 30 min at 4°C. Fixation and permeabilization was then performed for 20 min at 4°C using Cytofix/Cytperm solution (Cytofix/Cyperm Plus Kit, Becton Dickinson) according to the manufacturer’s instructions. Cells were washed once with Perm/Wash solution

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and stained with a FITC-conjugated rat anti-mouse IFN-γ Ab (Clone XMG1.2; Becton Dickinson) or an allophycocyanin-conjugated rat anti-mouse TNF-α Ab (Clone MP6-XT22; BioLegend) for 30 min at 4°C before flow cytometric analysis. Data analysis was performed using FlowJo software (Treestar, Ashland, OR). Live viable cells were gated based on their forward and side scattering properties, and analysis was performed on total of 1 × 10^6 CD8^+ cells.

**In vivo cytotoxic T cell-killing assay**

The in vivo cytotoxic activity of CTLs elicited in inoculated mice was measured using peptide-pulsed target cells. Splenocytes obtained from naive mice were either untreated or pulsed with peptide OVA257 or the unrelated H-2^d peptide epitope NP147 (TYQRTRALV), which is derived from influenza virus nucleoprotein. Peptide-pulsed cell populations were incubated with 1 μg/ml peptide at 37°C in HBSS for 60 min. The untreated cell population was also incubated under similar conditions, but in the absence of any peptide. Cells were then washed once in supplemented RPMI 1640 medium and then resuspended in 20 ml of 0.1% BSA in PBS. Each cell population was labeled at 37°C for 10 min with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) at a final concentration of 5 μM for the nonpulsed population, 1 μM for OVA257 pulsed populations and 100 nM for NP147 pulsed populations. Afterward, the cells were washed once with supplemented RPMI 1640 prior to determining cell numbers. An equal number (1 × 10^7) of cells from each population was mixed together and injected i.v. into each test mouse. Specific lysis was assessed in organs 18 h after adoptive transfer of cells and analysis performed with flow cytometry. The percentage specific lysis of fluorescent target cells in each mouse was calculated according to the formula:

\[
\text{percentage specific lysis} = 1 - \left( \frac{r_{(\text{naive})}}{r_{(\text{vaccinated})}} \right) \times 100,
\]

where ratio \(r = \frac{\% \text{CFSE}_{\text{low cells}}}{\% \text{CFSE}_{\text{high cells}}} \)

**Influenza virus challenge and lung virus assay**

C57BL/6J mice were inoculated twice, 14 d apart, either subcutaneously in the base of tail or intranasally with 25 10^4 PFU of A/HKx31 influenza virus containing the K^bOVA257 epitope in the neuraminidase stalk (25) 9 d after receiving the second dose of Ag. Lymphocytes obtained from lung bronchoalveolar lavage (BAL) washes were obtained 5 d later by incising the trachea and inserting a plastic cannula with a 1-ml syringe attached. The respiratory tract was then washed with RPMI 1640 using three separate aliquots (1 ml), each being infused and withdrawn three times. OVA257-specific IFN-γ and TNF-α production by CD8^+ T cells were then enumerated in an intracellular cytokine staining assay.

To determine lung viral titres, lungs of mice challenged with virus were homogenized using a tissue homogenizer in 3 ml RPMI and centrifuged at 300 × g for 5 min. Titers of virus in the lung supernatants were then determined using a Madin-Darby canine kidney plaque assay as described previously (26).

**Labeling OVA with FITC**

FITC (6 mg; Sigma-Aldrich) was dissolved in 100 μl DMSO (Sigma-Aldrich) and added in a drop-wise manner to 1 ml of 0.1 M phosphate buffer (pH 8.3) containing 6 mg OVA (Sigma-Aldrich) while being vortexed. This solution was held overnight at 4°C in a light-proof container. The fluoresceinated protein was then purified by gel permeation chromatography on a Superdex 75 10/300 GL column (Amersham Biosciences, Sweden) installed in an AKTA design FPLC system (Amersham Biosciences, Uppsala, Sweden). Fluoresceinated material was concentrated using a Vivaspin 20 ultrafiltration spin column (Sartorius Stedim, Göttingen, Germany). The labeled protein in solution was determined using the expression:

\[
\text{protein concentration (M)} = \frac{A_{280} - (A_{\text{max}} \times CF)}{e}
\]

**FIGURE 1.** Schematic representation of the branched cationic lipopeptide R4(Pam2Cys) and the anionic lipopeptide E8(Pam2Cys) and precipitate formation with protein Ags under physiologically normal and high salt concentrations. A. Lipopeptide R4(Pam2Cys) consist of a two-tier branching lysine (K) scaffold to which are attached four arginine (R) amino acid residues and lipopeptide E8(Pam2Cys) consists of a three-tier branching lysine scaffold to which are attached eight glutamic acid (E) residues. In each lipopeptide, the lipid moiety dipalmitoyl-S-glyceryl cysteine (Pam2Cys) is attached to the ε-amino group of the C-terminal lysine residue. In the case of R4(Pam2Cys), the guanidinium side chains and N-terminal amino groups of the terminal R residues impart an overall negative charge of 8 -. B. Increasing amounts of R4(Pam2Cys) or E8(Pam2Cys) lipopeptide were mixed with 1 nmol of OVA or HEL in a total volume of 100 μl PBS (0.137 M NaCl) in a flat-bottom, 96-well plate. The turbidity of the solution was then measured by determining the OD of the solution at 450 nm. C. R4(Pam2Cys) or E8(Pam2Cys) (100 nmol) were mixed with 20 nmol OVA or HEL in a total volume of 60 μl PBS of varying NaCl concentrations before determining the OD of the solution.
where $A_{280}$ represents the absorbance of the labeled protein solution at a wavelength of 280 nm, $A_{	ext{max}}$ is the absorbance at 495 nm, $CF$ is the correction factor of 0.3 to adjust for the amount of $A_{280}$ contributed by FITC, and $\varepsilon$ is the extinction coefficient of OVA ($E_{1\text{cm}^1\%} = 6.99$).

**Uptake of FITC-OVA by murine DCs**

A line (D1 cells) of murine DCs was prepared and propagated according to Winzler et al. (27) as modified by Chua et al. (23). After a minimum of 21 d in culture, D1 cells were stained for MHCII using FITC-conjugated anti-IA/IE Ab (Clone M5/114.15.2; Becton Dickinson) and PE-conjugated CD11c (Clone 2G9; Becton Dickinson) prior to use. Cells ($2 \times 10^5$) were seeded onto a Petri dish in 1 ml fresh D1 media containing 5 $\mu$g/ml of FITC-conjugated OVA (OVA–FITC) alone or with the same amount of OVA–FITC that had been preincubated with an equimolar amount of R4-(Pam2-Cys). Cells were harvested at various time points after exposure to OVA–FITC and washed extensively with FACS Wash (1% FCS per 5 mM EDTA in PBS). Extracellular fluorescence was quenched as performed previously (23) by the addition of an equal volume of 0.1 M citrate buffer (pH 4.0) containing 250 $\mu$g/ml trypan blue before analysis by flow cytometry.

Cells were examined using a confocal scanning laser system (Zeiss LSM-510 Meta) installed on a Zeiss Axioplan2 microscope. Fluorophores were visualized using a 488-nm excitation filter and a 522/535-nm emission filter for fluorescein and a 561-nm excitation and a 605/632-nm emission filter for PE. The same pinhole and photomultiplier gain and laser power level settings were used for all samples examined. Single images ($1024 \times 1024$ pixels; 0.5-mm optical section) were captured using a 100× objective lens.

For DC maturation experiments, $2 \times 10^5$ cells were incubated with 1 ml fresh D1 media containing OVA (5 $\mu$g/ml) alone or mixed with increasing amounts of lipopeptide. As a positive control, cells were also exposed to 5 $\mu$g LPS (Sigma-Aldrich, Milwaukee, WI). After an incubation period of 16 h, cells were stained for MHCII using FITC conjugated anti-murine I-A/I-E Ab (Clone M5/114.15.2; Becton Dickinson) or an irrelevant FITC-conjugated Ab. After fixation with 1% paraformaldehyde in PBS, cells were analyzed by flow cytometry.

For the analysis of Ag uptake by DCs in vivo, BALB/c mice were inoculated s.c. in the footpad with 25 $\mu$g OVA–FITC alone or OVA–FITC that had been preincubated with a 5-fold molar excess of R4(Pam2Cys). Popliteal lymph nodes were removed after 24 h, and single-cell suspensions were prepared. CD11c+ cell enrichment was performed by magnetic

**FIGURE 2.** HPLC analysis of protein-lipopeptide solutions. HPLC analysis was performed on supernatants (A–C, G–I) of solutions containing either 100 nmol of branched R4(Pam2Cys) or E8(Pam2Cys) lipopeptide (G), 1 nmol OVA (B), or HEL (H), a mixture of R4(Pam2Cys) and OVA (C) or E8(Pam2Cys) and HEL (I) in a total volume of 100 $\mu$l PBS following centrifugation (1.2 $\times$ 10^5 G). Sedimented material from solutions containing only lipopeptide (D, J), protein (E, K), or mixtures of both (F, L) were dissolved in a solution of 50% acetonitrile in water and then analyzed by HPLC. In all samples containing lipopeptides, the identity of the peak corresponding to the lipopeptide was verified by mass spectrometry.
cell selection using anti-CD11c microbeads according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) prior to flow cytometric analysis for FITC uptake.

Results

Lipopeptide binding to protein is inhibited by high salt concentrations

Predicted isoelectric point values were used to determine the net electrical charge of OVA and HEL and were obtained using Lasergene 8.0 software (DNASTAR). For OVA the net charge is predicted to be $-11$, and for HEL the charge is $+8$. On the basis of these predicted charges and a knowledge of the charge exhibited by the synthetic lipopeptides, we predicted that R4(Pam2Cys) would bind to OVA, but not to HEL, and that E8(Pam2Cys) would bind to HEL and not to OVA.

These predictions were supported by experiments in which increasing amounts of lipopeptide, either R4(Pam2Cys) or E8(Pam2Cys), were added to a constant amount of OVA or HEL, and the OD of the resulting solutions were determined (Fig. 1B). In both cases where protein and lipopeptide had opposite charge, the solutions showed increasing opalescence tending to a plateau as the appropriate lipopeptide was added. These results suggested that lipopeptide–protein complexes were formed through electrostatic interactions.

Support for this idea came from experiments in which quantities of lipopeptide and protein that would generate the greatest amount of complex were mixed in the presence of increasing amounts of sodium chloride, and the amounts of complexes formed were measured by determination of OD. The results (Fig. 1C) showed that increasing concentrations of NaCl resulted in a progressive decrease in OD until negligible readings were obtained at $\sim 1$ M NaCl, suggesting an inability to form lipopeptide–protein complexes at this concentration.

The components of complexes formed between lipopeptide and protein Ag were identified by HPLC analysis carried out on the

FIGURE 3. Cell-mediated responses elicited by vaccination R4(Pam2Cys) or E8(Pam2Cys)-mixed OVA. A, Groups of C57BL/6J mice ($n = 3$ per group) were inoculated s.c. with 25 $\mu$g OVA alone or preincubated with a 5-fold molar excess of R4(Pam2Cys) or E8(Pam2Cys). Spleens of vaccinated animals were removed 7 d later, and IFN-$\gamma$ and TNF-$\alpha$ secretion from OVA257-specific CD8$^+$ T cells were enumerated in an intracellular cytokine-secreting assay. Bar graphs show the total amount of Ag-specific cytokine secreting cells and is representative from two separate experiments. B, OVA257-specific in vivo cytolytic responses were also assessed 7 d after vaccination by i.v. injections of CFSE$^{high}$ OVA257-pulsed and CFSE$^{low}$ irrelevant peptide-pulsed splenocytes from naive mice. Spleens of vaccinated animals were removed 16 h later and analyzed for the presence of CFSE-labeled cells by flow cytometry. The percentage of specific target cell lysis was measured by determining the amount of CFSE$^{high}$ cells in relation of the CFSE$^{low}$ cells in each animal. The results are presented as the mean value and SD and are representative of two separate experiments. Mice were also inoculated with 25 $\mu$g OVA alone or preincubated with a 5- or 10-fold molar excess of R4(Pam2Cys). OVA257-specific IFN-$\gamma$ and TNF-$\alpha$ production from CD8$^+$ T cells in the spleen (C) and in vivo cytolytic responses were then determined (D).

FIGURE 4. Lipopeptide-mediated enhancement of OVA dendritic cell uptake. A, CD11c$^+$ MHCI$^+$ splenocyte-derived cultured dendritic cells ($2 \times 10^5$ cells) were incubated with 5 $\mu$g/ml of FITC-conjugated OVA (OVA–FITC) alone or preincubated with an equal amount of R4(Pam2Cys). Cells were harvested after 3 and 24 h, washed extensively, and treated with trypan blue to quench extracellular fluorescence before flow cytometric analysis to determine levels of intracellular green fluorescence. B, Cells were also examined under a confocal microscope for OVA–FITC (green) localization after staining with anti–MHCI-PE Ab (red) and DAPI (blue) without extracellular fluorescence quenching. Original magnification $\times 100$. The presence of orange staining observed at the surface of OVA–FITC incubated cells is due to the colocalization of OVA–FITC and the PE-labeled anti–MHCI Ab.
supernatant and material sedimented from solutions containing lipopeptide only, protein Ag only, or a mixture of lipopeptide plus protein Ag. The results show that the supernatants obtained from solutions containing only lipopeptide or protein Ag contained most or all of the original component (Fig. 2A, 2B, 2G, 2H) with little or none of the material appearing in the sediment (Fig. 2D, 2E, 2F, 2K). In contrast, solutions containing both lipopeptide and protein Ag contained a significant amount of the protein Ag and lipopeptide in the sediment, indicating formation of macromolecular complexes between OVA and R₄(Pam₂Cys) (Fig. 2F) or HEL and E₈(Pam₂Cys) (Fig. 2L). The sizes of the peaks corresponding to the individual protein in the respective supernatant were significantly smaller than that present in the sedimented material, confirming that most of each protein Ag had associated with the appropriate lipopeptide forming a macromolecular complex.

**Inoculation with lipopeptide–protein complexes elicits Ag-specific CD₈⁺ cytotoxic T lymphocytes**

C57BL/6J mice were inoculated with OVA mixed with lipopeptide. Seven days later the numbers of OVA₂₅₇-specific TNF-α⁺, IFN-γ⁺, and TNF-α⁺IFN-γ⁺ producing CD₈⁺ T cells present in the spleen were determined in an intracellular cytokine staining assay. The presence of Ag-specific CTLs was also determined by measuring in vivo lysis of CFSE-labeled splenocytes derived from naive mice that had been pulsed with OVA₁₅₇₋₂₆₄ peptide. The results (Fig. 3A) demonstrate that inoculation with R₄(Pam₂Cys) complexed with OVA resulted in the induction of CD₈⁺ T cells secreting TNF-α only, IFN-γ only, and those producing both TNF-α and IFN-γ. Fewer CD₈⁺ T cells producing TNF-α or IFN-γ only were induced after inoculation with OVA alone, and only a small population of TNF-α and IFN-γ producing CD₈⁺ T cells were elicited after inoculation with OVA in the presence of E₈(Pam₂Cys). The results of an in vivo cytolysis assay (Fig. 3B) show that a strong cytolytic T cell response is also associated with inoculation with OVA in the presence of R₄(Pam₂Cys). The hierarchy of cytototoxic activities observed in the in vivo cytotoxicity assay paralleled the numbers of TNF-α⁺IFN-γ⁺–producing CD₈⁺ T cells detected, although in the case of the in vitro cytotoxic activity, there was little significant difference observed between animals that received OVA alone and those that were inoculated with OVA in the presence of E₈(Pam₂Cys).

To further investigate the characteristics of the responses induced by R₄(Pam₂Cys)–OVA, mice were inoculated with OVA complexed with a 5:1 and 10:1 molar excess of lipopeptide to OVA, and numbers of cytokine-secreting CD₈⁺ T cells present in the spleen were determined. Of the total number of cytokine-secreting cells elicited by inoculation with R₄(Pam₂Cys)–OVA complexes, significantly greater numbers of cells secreting IFN-γ only or TNF-α and IFN-γ were elicited after inoculation with a 5:1 ratio of lipopeptide to protein compared with a 10:1 ratio of lipopeptide to protein (Fig. 3C). This trend was also reflected in the in vivo cytolytic responses in which lower levels of target cell lysis resulted when a 10:1 ratio of lipopeptide to protein (Fig. 3D).

**Oppositely charged lipopeptide and protein Ag are efficiently taken up by DCs in vitro and in vivo**

A key requirement for the induction of cell-mediated responses is that Ag is presented to T cells by APCs. We therefore determined the extent to which lipopeptide–protein complexes, formed by electrostatic interaction, were taken up by DCs.

The intracellular accumulation of noncomplexed or R₄(Pam₂Cys)–complexed OVA–FITC was assessed in vitro using DC1 cells, a murine TLR₂⁺ DC line derived from splenocytes (27). The results of an experiment in which the uptake of FITC-labeled OVA by DCs was measured at 3 and 24 h (Fig. 4A) shows that uptake occurred in the presence and absence of R₄(Pam₂Cys), but that the presence of lipopeptide caused more protein to be internalized. At each time point examined, approximately twice the amount of intracellular OVA–FITC was detected in cells incubated with R₄(Pam₂Cys)–OVA compared with those incubated with OVA–FITC alone. The uptake of both lipopeptide–OVA complexes and OVA alone occurred within 3 h of exposure, with maximum uptake achieved after 24 h. There was no further increase in intracellular accumulation of Ag after this time (data not shown).

Examination of cells by confocal microscopy (Fig. 4B) showed that in cells incubated with R₄(Pam₂Cys)–OVA complexes, the FITC–OVA was distributed within the cell cytoplasm apparently accumulated in vesicles. This finding was in contrast to the Ag distribution in

**FIGURE 5.** Effect of lipopeptide charge on maturation of DCs, uptake of OVA, and in vivo trafficking to the draining lymph node. A, CD₁₁c⁺ MHCII⁺ splenocyte-derived cultured DCs (2 × 10⁵ cells) were incubated with 5 μg LPS or 5 μg OVA alone or were preincubated with increasing amounts of R₄(Pam₂Cys) or E₈(Pam₂Cys). Cells were harvested 16 h later, and surface expression of MHCII molecules was determined by flow cytometry after staining with a FITC-conjugated anti–I-α₁/I-E Ab. Cells expressing low levels of MHCII molecules were deemed to be immature, whereas those expressing high levels were considered to be mature. B, In another experiment, the intracellular fluorescence of cells was measured after 24 h incubation with 5 μg OVA–FITC alone or OVA–FITC that had been mixed with an equal amount of either R₄(Pam₂Cys) or E₈(Pam₂Cys). C, Groups of BALB/c mice (n = 3) were inoculated s.c. via the footpad with 25 μg OVA–FITC alone or OVA–FITC that had been mixed with a 5-fold molar excess of either R₄(Pam₂Cys) or E₈(Pam₂Cys). Popliteal lymph nodes were obtained 24 h later and analyzed for the presence of FITC⁺ cells. The data show the average amount of FITC⁺ cells in the lymph nodes of each mouse and are representative of three separate experiments.
cells exposed to OVA–FITC alone where Ag was localized at the cell surface with little intracellular cytoplasmic or vesicular presence. Exposure of DCs to R₄(Pam2Cys)–OVA complexes also resulted in the activation of >80% of cells based on the number of cells that were found to express high levels of surface MHCII molecules (Fig. 5A). At all concentrations of R₄(Pam2Cys) used, the level of DC maturation attained was more than twice that resulting from exposure to OVA alone, but comparable to levels of maturation obtained with LPS. Irrespective of lipopeptide charge, similar levels of DC activation was also observed using OVA premixed with E₈(Pam2Cys).

A comparison of the levels of intracellular Ag present in DCs after incubation with OVA mixed with each lipopeptide, however, showed that R₄(Pam2Cys) was considerably more effective than E₈(Pam2Cys) at facilitating Ag uptake (Fig. 5B). In fact, the percentage of DCs exhibiting intracellular fluorescence after incubation with OVA mixed with E₈(Pam2Cys) was not significantly different from that observed in cells exposed to OVA alone. To investigate whether the in vitro differences that we observed were reflected by in vivo differences, mice were inoculated in the footpad with lipopeptide–Ag mixtures followed 24 h later by examination of the popliteal lymph nodes for cells containing FITC–OVA. The results (Fig. 5C) show that more FITC-positive cells were present in the draining lymph nodes of animals that had received R₄(Pam2Cys) than in those that had received E₈(Pam2Cys) or OVA alone.

Inoculation with R₄(Pam2Cys)–protein complexes induces an accelerated clearance of chimeric OVA-influenza virus from the lung

To determine whether the CD₈⁺ T cells induced by lipopeptide–OVA complexes exhibited biologic function, mice were inoculated twicewith OVA alone or R₄(Pam2Cys)–OVA complexes. Nine days after receiving the second dose, mice were challenged via the intranasal route with A/HK×31 (H3N2) influenza virus containing the K₅⁰OVA₂₅⁷ epitope in the neuraminidase stalk.

Analyses of lymphocytes contained in BAL washings demonstrated that significant numbers of TNF-α/IFN-γ double-positive T cells were obtained from mice inoculated with R₄(Pam2Cys)–OVA complexes. It was also apparent that administration of R₄(Pam2Cys)–OVA complexes by the subcutaneous also induced significant numbers of cytokine-producing T cells in the lung. These observations indicate that the CD₈⁺ T cells induced by R₄(Pam2Cys)–OVA complexes accelerated OVA-specific T cell response at the site of virus challenge. In mice that received PBS, OVA alone, or R₄(Pam2Cys) only, challenge with virus could only evoke low numbers of cytokine-secreting OVA₂₅⁷-specific T cells in BAL. washes (Fig. 6A).

To determine whether the T cells elicited by R₄(Pam2Cys)–OVA complexes possessed additional biologic function, the titers of influenza virus present in the lungs of mice were determined at the same time point as cytokine profiles (Fig. 6B). Similar viral titers (∼10⁴.⁹ PFU) were found in the lungs of mice that had been inoculated intranasally with OVA alone or R₄(Pam2Cys) alone. Although small numbers of Ag-specific cytokine-producing T cells were detected in mice at this time (Fig. 6A), they were insufficient to significantly reduce the viral load compared with the control group of animals that had received PBS prior to challenge. In contrast, viral titers in mice that had received R₄(Pam2Cys)–OVA complexes were ∼40-fold less (10³.⁹ PFU) than in the PBS control group. This finding is equivalent to a better than 90% reduction in lung virus titer (Fig. 6C). Vaccination via the base of the tail resulted in ∼80% reduction in lung virus titer relative to the PBS control group.
viral titer. We conclude that the presence of increased numbers of activated T cells in the lungs of these mice and their ability to produce IFN-γ correlates with their ability to clear virus infection regardless of the vaccination routes examined.

**Inoculation with lipopeptide–protein complexes elicits Ab**

BALB/c mice were inoculated with OVA or HEL mixed with R₄(Pam₂Cys) or E₈(Pam₂Cys), respectively, at a 5:1 molar ratio of lipopeptide to protein. Ab titers in sera obtained after one or two doses of lipopeptide–protein Ag complex were then determined by ELISA.

Neither OVA nor HEL when administered alone in saline elicited significant titers of Abs after a single dose, but some Abs were apparent after the second dose, although the reproducibility in titer between individual animals was poor. Administration of these proteins with CFA, however, resulted in healthy Ab titers following primary and secondary inoculations, demonstrating that these proteins are strongly immunogenic under the right conditions.

In mice that received a single dose of OVA alone (Fig. 7A) or HEL alone (Fig. 7B), and in cases where the Ags were mixed with oppositely charged or with nonlipidated peptides (i.e., R₄ or E₈), little or no Ab was induced. Even after two doses, only low levels of Ab were detected, but the amounts detected were not consistent in all mice within a group.

Significant titers of Ab were, however, detected in mice that had been inoculated with R₄(Pam₂Cys)–OVA or E₈(Pam₂Cys)–HEL complexes. The Ab titers resulting from inoculations with these lipopeptide–protein complexes were comparable to the titers observed in mice vaccinated with OVA or HEL coadministered with CFA. Slightly lower titers of Ab were elicited in mice that had been inoculated with a mixture of R₄(Pam₂Cys) and HEL, each of which are positively charged and in mice that had been inoculated with a mixture E₈(Pam₂Cys) and OVA. This result is surprising given the requirement for the successful induction of a CD8⁺ T cell response on electrostatic association between lipopeptide and Ag.

We also observed that IgG1 was the dominant Ab isotype induced (Fig. 7C, 7D). Furthermore, the Ab isotype elicited was not dependent on the charge of the lipopeptide or protein used, indicating that the presence of Pam₂Cys enabled Th2 and Th-1 type responses.

**Discussion**

The use of TLR ligands as adjuvants is an effective way to enhance the immunogenicity of soluble Ags such as proteins, carbohydrates, DNA, and peptides. To date there have been many that have been investigated extensively for this purpose (5, 6). Although these ligands are effective at promoting vaccine efficacy, superior immune responses are generated when both TLR ligand and Ag are covalently linked (11, 12, 15, 16). From the series of experiments conducted in this study, we have found that the immunogenicity of otherwise nonimmunogenic proteins could also be markedly enhanced if linked not covalently but electrostatically to lipopeptides containing the TLR2 targeting ligand Pam₂Cys.

We reason that a lipopeptide that has charged amino acids fashioned in a branched manner would provide a tentacle-like structure capable of associating electrostatically with oppositely charged portions of a protein. The binding of positively charged protein Ags such as OVA was achieved using the arginine-based cationic lipopeptide R₄(Pam₂Cys), while the glutamic acid-based anionic lipopeptide E₈(Pam₂Cys) was used to bind to the positively charged protein HEL. It is possible that other amino acids such as lysine and aspartic acid could also be used to facilitate electrostatic interactions.

For cell-mediated responses, only inoculation with electrostatically associated lipopeptide–Ag complexes was effective in

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**FIGURE 7.** Ab responses elicited by vaccination with R₄(Pam₂Cys) and E₈(Pam₂Cys)-mixed protein Ags. Groups of BALB/c mice (n = 5 per group) were inoculated s.c. with 25 μg OVA (A) or HEL (B) alone, emulsified in CFA, or preincubated with a 5-fold molar excess of R₄(Pam₂Cys) or E₈(Pam₂Cys) lipopeptides or their nonlipidated versions (R₄ or E₈). Sera were obtained for blood taken 21 d after the primary (○) and 13 d after the secondary (●) inoculation. Ab levels were then determined by ELISA. Individual animal titers are presented with the mean value represented by the horizontal bar, and the data are representative of four separate experiments. Sera from secondary responses were also assayed for OVA- (C) or HEL-specific (D) Abs of the IgG1 (gray) or IgG2a isotypes (black).
eliciting CD8+ T cells that have the ability to secrete multiple proinflammatory cytokines and able to cause in vivo cytosis of target cells. Furthermore, inoculation with lipopeptide–Ag complexes resulted in accelerated clearance of virus from the lungs of mice challenged with influenza virus.

Although protein complexes containing either lipopeptide were similarly effective at inducing DC maturation, the CD8+ T cell responses that were elicited after inoculation with these complexes correlated with the ability of the lipopeptides to mediate Ag uptake by DCs and subsequent trafficking of Ag to the draining lymph node. When we consider that the internalization of Ag by DCs is a prerequisite for the priming of cell-mediated responses, it is not surprising that antigenic uptake facilitated by electrostatically associated constituents culminated in significantly higher responses than those elicited by similarly charged lipopeptide and Ag—that is, E8(Pam2Cys)-and OVA—where Ag uptake by cells was low. Similar observations correlating the magnitude of cell-mediated responses and DC Ag uptake with the nature of association between Ag and TLR-agonist have been reported previously in studies where more efficient Th1-type responses result from inoculation with TLR-ligands that are covalently conjugated to Ags compared with formulations where nonconjugated constituents are used (13, 16). Colocalization of TLR agonist-conjugated Ag in the same APC not only results in increased uptake and cross-presentation of Ag to T cells (9, 11, 15), but also requires much smaller doses to create the same level of response elicited by nonconjugated Ag (28). Although the strength of covalent bonds involved in the attachment of Ag and agonist in the above-mentioned studies would be greater than electrostatic bonds, the combined strength of the presumably multiple ionic bonds formed between the charged amino acids of the lipopeptides and Ag are clearly sufficient to facilitate internalization of bound Ag into DCs through the TLR2 endocytic pathway.

The dominant Ab isotype elicited in the secondary responses to the Ags that we examined was IgG1, indicating that vaccination with complexes between lipopeptide and Ag could generate Th2-type responses. The highest Ab titers were once again achieved by vaccination with oppositely charged constituents, although this response was not totally dependent on electrostatic binding because vaccination with similarly charged constituents also resulted in the induction of strong, albeit lower, Ab titers.

The role of the DC in Ab responses is centered on the uptake of Ag and the cognate activation of CD4+ T helper cells that provide the necessary signals required for differentiation of B cells into Ab secreting cells. Several recent in vitro studies, however, have found that TLR ligands can directly induce Ab secretion from both naive and memory B cells (29) and induce their differentiation into plasma cells (30) in the absence of T cell help. Conjugation of Ag to TLR ligands also appears to be beneficial for Ab responses where simultaneous triggering of B cell receptor together with TLR engagement can lead to enhanced Ag-specific B cell responses (31). A role for TLR ligands in the preferential activation of B cells and differentiation into IgM- and IgG-secreting plasma cells in the absence of T cell help is further supported by observations that memory B cells express higher levels of TLRs and associated adaptor molecules (32, 33).

B cell responses may also be mediated directly by DCs independently of T cell-mediated facilitated CD40 ligation (34, 35). In addition, extracellular Ag captured by DCs can also be retained in an unprocessed format that can then be transferred directly to naive B cells resulting in the initiation of an IgG-dominated Ab response (36). Somewhat relevant is the presence of a DC subset that has the ability to migrate into the primary B cell follicles to provide antigenic stimulation to both B and T cells (37). Together, the small disparity between the Ab responses elicited by vaccination with either lipopeptide is therefore not surprising, because the processes governing the induction of these responses might not be exclusively reliant on the internalization of Ag by DCs and recruitment of CD4+ T cell help.

There are several advantages in using these lipopeptides to enhance protein immunogenicity in a charge dependent manner. Because most TLR ligands are nonpeptide components of infectious agents, linking them covalently to antigenic epitopes or proteins may prove to be chemically challenging, whereas electrostatic association between lipopeptide and protein occurs spontaneously upon mixing, reducing the need for any further chemical intervention or modification. Furthermore, only small amounts of these lipopeptides are needed to elicit robust humoral and cell-mediated immune responses (0.5–2.5 nmol lipopeptide per animal) and could therefore be dose sparing and economical in use. This aspect is also advantageous for avoiding any side effects or toxicity that may normally be associated with the use of an adjuvant as well as overcoming the limited range of responses that can be induced. Given the findings of this study, this approach could therefore constitute a particularly efficient way of inducing immune responses against soluble microbial, viral, and tumorigenic protein Ags and may be effective for generating immunity against diseases, particularly those that require both Ab- and cell-mediated responses.

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References


