Comparing Pooled Peptides with Intact Protein for Accessing Cross-presentation Pathways for Protective CD8+ and CD4+ T Cells*[S]

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To better understand the mechanisms of intracellular trafficking and presentation of exogenous peptides by antigen-presenting cells (APC), we compared the handling of overlapping 24-mer peptides from HIV Nef either mixed or covalently linked in tandem in one protein. Once internalized, peptides trafficked not only to endosomes but also to cytosol, and activated CD8+ and CD4+ T cells. In contrast, whole protein was found to traffic only to the endosomal compartments, and primarily activated CD4+ T cells. Finally, with adjuvant, overlapping peptides were capable of protecting against lethal viral challenge, whereas the intact protein was less protective. These data suggest that overlapping long peptides are cross-presented through more varied intracellular routes and are more efficient in priming protective immunity than the whole protein.

Despite much progress in vaccine development, there are still several challenges for design of subunit vaccines. Against intracellular pathogens, it is especially important to immunize protective CD4+ and CD8+ T cell responses. The former recognize epitopes presented by major histocompatibility complex (MHC)[3] class II molecules that are normally loaded mainly with peptides derived from exogenous antigens. Natural processing and loading are catalyzed by HLA- or H2-DM in acidic lysosomes (1).

By contrast, CD8+ T cell responses typically depend on epitope loading via the class I pathway. This "endogenous" route starts from the cytosol of any cells naturally infected by the relevant virus, which may not include professional antigen-presenting cells (pAPCs). Once processed by cytosolic proteases, viral peptide fragments are transported into the endoplasmic reticulum, and loaded onto MHC class I molecules, which then traffic to the surface where they can present to CD8+ T cells (2, 3). For exogenous antigens, "cross-presentation" by pAPCs is essential for priming CD8+ as well as CD4+ T cells.

Although they must be crucial for most CD8+ T cell vaccination strategies, the mechanisms underlying cross-presentation are not understood. First, internalized antigens are known to enter the cytoplasm, although the mechanisms remain unknown. Once in the cytoplasm, they can follow the conventional MHC class I pathway, *i.e.* processing by cytosolic proteasomes or proteases, transport into the endoplasmic reticulum, loading onto MHC class I molecules, and then trafficking to the surface for recognition by T cells (2, 3). Second, antigens internalized into endocytic compartments could be degraded by the local proteases into peptides and then loaded onto MHC class I molecules in the endocytic compartments that are recycled to and from the cell surface (4). Third, endosomes may fuse with the endoplasmic reticulum, allowing direct access of such peptides for loading onto nascent MHC class I molecules there before presentation at the cell surface (5); however, this theory remains controversial (6, 7).

It has been hypothesized that endocytic and cytosolic proteases process antigens differently. If so, exogenous antigens that have been processed in the endocytic compartments in un-infected APC might yield epitopes distinct from those processed in the cytosol of infected cells (8).

It is not yet clear how to optimize antigens for cross-presentation. Although intact proteins are preferred to nonamer epitopes (9), there is recent evidence that long 15–20-mer peptides are even more suitable (10, 11). However, it is not known whether long peptides and intact proteins access the same intracellular route(s) for cross-presentation. Here we designed constructs to enable us to address that issue and test whether these differences are important for eliciting protective immune responses *in vivo*. Because an intact protein usually contains multiple epitopes, it would not be fair to compare a whole protein with a single peptide. It is also not ideal to compare a set of overlapping peptides with their correspondent native protein because overlapping peptides, which include extra overlapped sequences, may contain relatively more epitopes than their cor-

[3] The abbreviations used are: MHC, major histocompatibility complex; pAPC, professional antigen-presenting cell; CFA/IFA, complete/incomplete Freund's adjuvant; ROP, recombinant overlapping-peptide protein; ppROP, protease-processed ROP (peptides); DC, dendritic cells; vv-Nef, vaccinia virus expressing Nef; HIV, human immunodeficiency virus; MALDI, matrix-assisted laser desorption ionization; IFN, interferon; Ni-NTA, nickel-nitrilotriacetic acid.

respondent native protein. Consequently, we engineered and expressed a protein with 20 tandem-linked overlapping long peptides that could be separated by clipping with protease Factor Xa. We compared the recombinant protein with the enzymically cleaved overlapping peptides from the same protein.

Previous publications have shown that overlapping synthetic peptides are capable of generating immune responses (12, 13). Our recombinant overlapping peptide protein (ROP) covers the complete sequence of HIV Nef. We compared its uptake and immunogenicity either intact or clipping into its constituent 24-mers, and tested whether the resulting responses can protect animals against lethal viral challenge.

EXPERIMENTAL PROCEDURES

DNA Design—A synthetic gene was designed to include a His tag and a series of 20-mer peptides, each overlapping by 10 amino acids, and separated by IEGR clip sites for the protease Factor Xa, it covers the full 206 amino acids of Nef from the Lai strain of human immunodeficiency virus (HIV) (Fig. 1*A*). The resulting construct was optimized for *Escherichia coli* expression, and restriction enzyme sites NdeI and BamHI were added at the ends for subsequent subcloning and expression in the pET16b vector. The gene was commercially synthesized by GeneArt, Regensburg, Germany.

Subcloning of the Target Gene into the pET16b Expression Vector—The synthesized target gene, provided in cloning vector pPCR-Script, was restriction-digested with NdeI and BamHI. The DNA was gel-purified using QIAquick® Gel Extraction kit (Qiagen, Germany). Meanwhile, the pET vector was digested with the same restriction enzymes, and the ends were de-phosphorylated with shrimp alkaline phosphatase (Roche). The vector was then gel-purified as described above. The target gene was ligated into vector DNA with T4 DNA ligase (New England Biolabs, UK), and the ligation product was transformed into DH5α bacteria. Colonies were inoculated from overnight LB agar plate cultures and incubated in LB medium with 100 mg/ml ampicillin. After overnight culture, plasmid DNA was extracted by the QIAprep Spin Miniprep Kit (Qiagen, Germany), and sequenced to confirm the presence of the correct insert.

Protein Expression—Miniprep DNA was transformed into BL21(DE3) bacteria, and colonies were inoculated and grown in 30 ml of low salt LB medium overnight at 37 °C. The culture was 1:30 diluted in low salt LB medium and grown at 37 °C with shaking for 2–3 h, when the bacteria reached exponential growth stage, as determined by $A_{600} = 0.6 – 1.0$. 1 mM Isopropyl 1-thio-β-D-galactopyranoside was added to induce protein expression. After a 4-h shaking at 28 °C, bacteria were pelleted at 4000 rpm, and frozen at −70 °C until further extraction and purification, when they were re-suspended in cold phosphate-buffered saline and lysed by sonication. The inclusion bodies, which contain the expressed protein, were washed with 1:100 B-Per solution (Pierce), pelleted at $15,000 \times g$ for 20 min, and extracted with 8 M urea in 50 mM Tris buffer (pH 7.8) containing 500 mM NaCl.

Protein Purification—The protein was further purified by nickel affinity chromatography using Ni-NTA purification kit (Qiagen, Germany) according to the manufacturer's operation

manual. After the purification, the eluting buffer containing imidazol was exchanged to 25 mM ammonium bicarbonate buffer by extensive dialysis overnight at 4 °C. The purified protein (1.5–2 mg/ml) was either lyophilized for further use or digested into peptides.

Protease Digestion—The purified protein in 25 mM ammonium bicarbonate buffer containing 5 μM $CaCl_2$ was incubated with protease Factor Xa (Qiagen, UK) at 1 mg of protein/1 unit of protease at 4 °C for around 120 min. The digestion was stopped by adding 1 μM phenylmethylsulfonyl fluoride, and the protease was removed in the YM10 filter tube (Millipore, Bedford, MA). The free His tag and the trace salts were removed on Ni-NTA-agarose and C18 columns (Waters, Milford, MA). The resulting mixture of the proteinase-processed recombinant overlapping peptides (ppROP) was lyophilized for mass spectrometry analysis and future experiments.

Mass Spectrometer Analysis—The protease-digested sample was analyzed on a Bruker Daltonics Ultraflex TOF/TOF mass spectrometer to confirm the identity of the digested peptides. All MALDI spectra were obtained in reflectron mode. A nitrogen laser, emitting 337 nm light in 3-ns pulse, was the ionization source. The accelerating voltage in the ion source was 30 kV.

For MALDI analysis, the peptide mixture was purified and desalted on a C18 column. About 10 μM peptide solution was premixed with the matrix: α-cyano-4-hydroxycinnamic acid (10 mM in 35% aqueous acetonitrile, 0.1% trifluoroacetic acid) at a 1:1 ratio and 1 μl of mixture applied directly to the sample plate. The droplet was air-dried before analysis in the mass spectrometer.

Endotoxin Test—Endotoxin tests were carried out using Charles River Laboratories' Endosafe kit (Charleston, SC) following the instructions in the kit. An endotoxin level greater than 0.25 EU/ml in the samples is considered positive.

Mice and Immunization—C57BL/10 or BALB/c mice were primed subcutaneously with 200 μg of Nef antigens emulsified with 100 μl of complete or incomplete Freund's adjuvant (CFA or IFA). They were boosted subcutaneously twice at 3-week intervals with the same vaccine emulsified with IFA. Three weeks after the last boost, splenocyte suspensions were prepared for IFN-γ Elispot, intracellular cytokine staining, vv-Nef challenge, and other assays. Control groups were immunized with CFA-IFA-IFA (adjuvant alone). All procedures were done in compliance with local and national animal ethics guidelines.

C57BL/10 or BALB/c mice were also primed subcutaneously with 200 μg of Nef antigens emulsified with an adjuvant composed of monophosphoryl lipid A + trehalose dicorynomycolate + cell wall skeleton (Sigma). However, this adjuvant is no longer available from the supplier.

Growth of Dendritic Cells in Bone Marrow Cultures—Mouse (H-2^b or H-2^d) bone marrow cells from femurs and tibias were washed and cultured in 10 ml of RPMI1640 medium containing 10% fetal calf serum, 15–20 ng/ml murine granulocyte-macrophage colony-stimulating factor (R & D Systems, UK) in 10-cm culture dishes for at least 6 days. On day 3, another 10 ml of fresh granulocyte-macrophage colony-stimulating factor-containing medium was added to the culture. Then 10 ml of culture medium were replaced by 10 ml of fresh granulocyte-macro-

phage colony-stimulating factor containing medium every other day, until they were harvested on day 7.

IFN-γ Elispot and Intracellular Staining Assays—Assays were performed using ELISPOT kits (Mabtech, Sweden). Briefly, splenocytes were restimulated overnight with 10 μM ppROP, ROP, or individual peptides (as indicated) in anti-IFN-γ-Ab precoated plates (Millipore, Bedford, MA), and challenged with overlapping synthetic peptides covering the Lai or NL4-3 Nef sequences (NIBSC Centralized Facility for AIDS Reagents, NE6 3QG, UK). Cells were discarded, and biotinylated anti-IFN-γ antibodies were added for 2 h at room temperature, followed by another 1 h of incubation at room temperature with anti-biotin antibody labeled with enzyme. After color developed, the reaction was stopped by washing plates with tap water and plates were air-dried. Spots were counted with an Elispot reader (Autoimmun Diagnostike, Strasburg Germany). Results were expressed as spot forming units/10^6 cells.

For intracellular cytokine staining, mouse splenocytes were cultured at 5×10^6 cells/ml with or without 10 μM overlapping synthetic peptides in 24-well culture plates for 6 h. Four hours before harvesting, cells were treated with Golgistop (BD Pharmingen) according to the vendor's protocol. Splenocytes were then stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (BD Pharmingen) or an immunoglobulin isotype control for 20 min. Splenocytes were then subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit (BD Pharmingen) and fluorescein isothiocyanate-conjugated anti-IFN-γ antibody (20 μg/ml) according to the manufacturer's instructions. Samples were acquired on a CyAn flow cytometer (Beckman Coulter, Fullerton, CA), and data were analyzed using Summit software (Dako Colorado).

Antigen Presentation—One million DCs or DC2.4 cells were pulsed with 5 μM ppROP or ROP at different time points. The DCs were irradiated (3000 rad) followed by 3 washes, before co-culture with CFA/ppROP- or CFA/ROP-immunized splenocytes at a ratio of 1:10, overnight at 37 °C in 5% CO$_2$. An IFN-γ ELISPOT assay was performed on the second day. The DCs were incubated ± 10 μg/ml of brefeldin A 1 h before and during co-culture with CFA/ppROP- or CFA/ROP-immunized splenocytes.

Cell Lines, Fluorescence Conjugation, and Confocal Microscopy—Dendritic cell line DC2.4 was kindly provided by K. Rock, University of Massachusetts (14).

For fluorescence conjugation of ppROP or ROP, we used LIVE/DEAD® Fixable Dead Cell Stain Kits (Invitrogen). 500 μl (1 mg/ml) of ppROP or ROP were incubated with 2.5 μl of green fluorophore (in dimethyl sulfoxide) at room temperature for 30 min. The conjugated ppROP and ROP can then be stored at −20 °C before use.

To observe the uptake of ppROP or ROP by the DC2.4 cells, we incubated 1×10^5 DC2.4 cells/0.5 ml of RPMI, no fetal calf serum in 8-chamber polystyrene tissue culture slides (BD Falcon, Bedford, MA) overnight at 37 °C, 5% CO$_2$. One hour before the incubation with ppROP or ROP, 50 μl of 1:1000 diluted (red) Lysotracker (Invitrogen) was added to the cell culture. The culture was washed 5 times with phosphate-buffered saline and made up to 445 μl of RPMI1640 with no fetal calf serum before adding 55 μl of fluorophore-conjugated ppROP or ROP.

Green fluorophore alone was added as a control. After various times of incubation at 37 °C in 5% CO$_2$, cells were washed and fixed with 4% paraformaldehyde. The cells were analyzed either by flow cytometry or as above by confocal microscopy, on a Radiance 2000 laser-scanning confocal microscope and Velocity software (Improvision, UK).

Challenge with vv-Nef—vv-Nef (vvTG1147), a gift from Dr. Yves Rivière of Pasteur Institute, Paris (15), were grown in baby hamster kidney (TK−) cells. BALB/c mice were vaccinated with CFA/ppROP or IFA/ppROP as above. Three weeks after the last immunization, mice were challenged intraperitoneally (*intraperitoneal*) with 3×10^8 plaque forming units of vv-nef. The survival rate of the mice was observed daily for 8 days.

RESULTS

Design, Expression, Purification, and Digestion of the Recombinant Protein—The ROP was designed to yield cleaved 20-mer peptides (overlapping by 10) covering the complete HIV Nef sequence (Lai strain; Fig. 1*A*, Table 1). Between each peptide is a clip site for Factor Xa protease (Fig. 1*A*), which allows them to be separated, leaving each with a C-terminal IEGR (a sequence not found in Nef itself). Before the N-terminal peptide, there is a His$_6$ tag (followed by the first clip site) to aid in protein purification.

The gene encoding ROP was transformed into *E. coli* BL21 cells and high expressing clones were isolated. After expression, the 55-kDa ROP protein was extracted from inclusion body and purified by affinity chromatography (Fig. 1*B*). It then appeared as a monomer on reducing SDS-PAGE (containing dithiothreitol), but as a monomer, dimer, and tetramer on non-reducing SDS-PAGE (Fig. 1*B*), indicating the protein is present as monomers and disulfide-linked multimers.

Digestion of the ROP by Factor Xa successfully generated the constituent overlapping peptides, as indicated by mass spectrometry (Fig. 1*C*). In addition, there were two unexpected peaks that may have resulted from nonspecific cleavage by Factor Xa. For example, peak 2254.919 (Fig. 1*C*) may have come from nonspecific cleavage of the ppROP-9 (supplemental Table S1). Similarly, Factor Xa infidelity may have cleaved ppROP-18 6 residues from its C terminus (supplemental Table S1). Endotoxin tests were negative for both ppROP and ROP.

ppROP Versus ROP: Differences in Intracellular Trafficking in DCs—To assess the uptake ROP and ppROP by APC, they were incubated with the dendritic cell line DC2.4 (H-2^b) (14) *in vitro*. First, they were labeled with the Live/Dead cell staining kit from Invitrogen, which covalently links fluorophore to free amino groups with a green fluorescent dye (530/30 nm), and the excess dye was removed. The DC2.4 cells were first stained with trypan blue to exclude positive cells, then the remaining DC2.4 cells were stained with Lysotracker red (Invitrogen). Finally, the fluorescent ROP or ppROP were incubated with the cells that were sampled serially for confocal microscopy and flow cytometry.

With ppROP, internalization was detectable after only 5 min (supplemental Fig. S1). Peptides were found both within and outside lysosomes before 40 min (Fig. 2*A*). At 120 min these peptides were mostly detected in the lysosomes (Fig. 2*A*), but only traces of peptides were detected after 24 h (supplemental Fig. S1). Co-localization with Lysotracker-labeled lysosomes
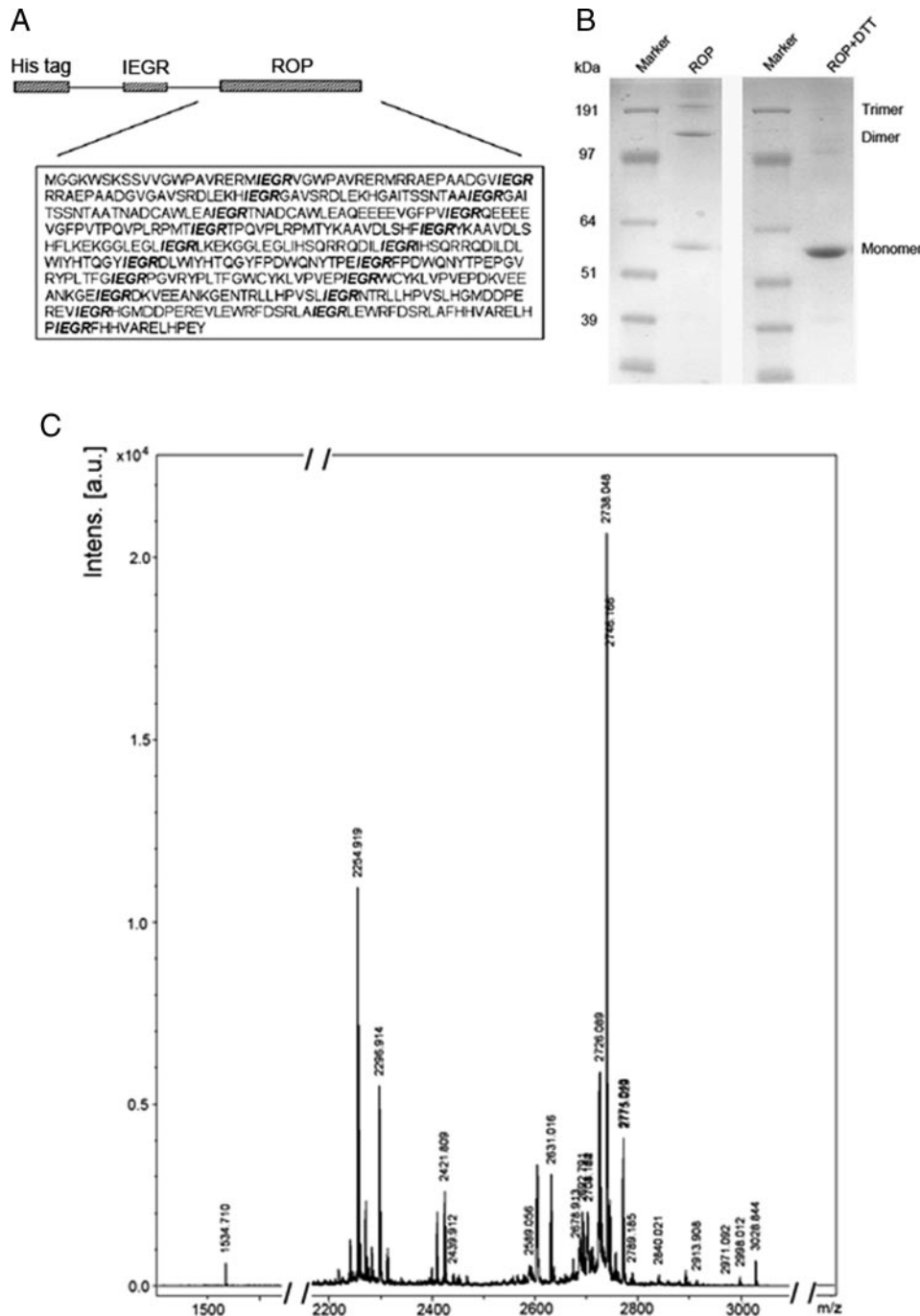
FIGURE 1. *A*, schematic representation of the recombinant protein composed of 20 overlapping peptides covering the sequence of Nef (Lai strain) + intervening Factor Xa clip sites (IEGR) between each peptide. *B*, purification of ROP. After purification on a Ni-NTA affinity column, the protein was analyzed by SDS-PAGE gel (*left panel*). The *top two bands* (dimer and tetramer) disappeared on reduction with DTT (*right panel*). *C*, mass spectrometry profile after ppROP was digested by Factor Xa. The spectrum was collected on a Bruker Daltonics Ultraflex TOF/TOF mass spectrometer. Peptide peaks appear as $[M + H]^{+1}$ ions. The peaks appearing at +23u are sodium adducts. Peptides that matched the expected ones are indicated by their molecular weights. The figure is representative of more than 20 similar results.

co-localization was almost complete from 40 min until at least 24 h (supplemental Fig. S1). Evidently, ROP is internalized much more slowly than ppROP, and almost all of it traffics to the lysosomes, whereas ppROP appears to access more compartments, likely including the cytoplasm. The more transient intracellular detection of ppROP may indicate a faster turnover than with ROP. The uptake of ppROP was similar when incubated with CFA (data not shown).

ppROP Versus ROP: Antigen Presentation—To determine whether ppROP and ROP can be presented by DCs, DC2.4 cells were pulsed at 37 °C with ppROP or ROP for 1 h followed by 3000 rad irradiation. After three washes, the cells were incubated overnight with splenocytes from CFA/ppROP- or CFA/ROP-immunized C57BL/10 mice (H-2ᵇ). Interferon-γ Elispot assays showed that both ppROP- and ROP-pulsed DCs stimulated splenocytes from CFA/ppROP-immunized mice. Both ppROP- and ROP-pulsed DCs stimulated only weak responses from CFA/ROP-immunized mice (Fig. 3). Their IFN-γ secretion was significantly reduced if the DCs were incubated with brefeldin A prior to and during pulsing with ppROP (Fig. 3). This experiment was repeated with fresh bone marrow-derived DCs and a similar result was achieved (data not show). These data are further evidence that ppROP require internalization for efficient presentation.

Priming with ppROP in Vivo Generated Diverse Immune Responses in Two Strains of Mice—We tested whether ppROP were able to elicit T cell responses in C57BL/10 (H-2ᵇ) and BALB/c (H-2ᵈ) mice. Response against individual overlapping synthetic peptides or entire pools thereof (Fig. 4) were measured by

increased gradually, reaching a maximum of about 75% by 120 min (supplemental Fig. S1). These data indicate that there is always a proportion of peptides not in the lysosomes. With ROP, by contrast, intracellular accumulation was detected only after 40 min, whereas the labeled protein co-localized even stronger with Lysotracker (Fig. 2*B*). Moreover, after 24 h, the labeled ROP was still accumulating in DC2.4 cells (Fig. 2*B*). This

IFN-γ Elispot. In both strains, ppROP injected with CFA (CFA/ppROP) evoked stronger responses than did ROP. Recognition of the mixture of overlapping synthetic peptides was similar in both strains (Fig. 4, *A* and *B*), but their epitope hierarchies were very different. Peptides 8, 9, and 17 were the highest among peptides recognized by CFA/ppROP-immunized C57BL/10 mice, whereas BALB/c cells only recognized peptides 2, 6, and

TABLE 1
Three sets of overlapping peptides used in this study

Peptides	Sequences
Lai-1	M G G K W S K S S V V G W P A V R E R M
NL4-3-1	- - - - - - - - - - I - - - - - - - - -
ppROP-1	- I E G R
Lai-2	V G W P A V R E R M R R A E P A A D G V
NL4-3-2	I - - - - - - - - - - - - - - - - - - -
ppROP-2	- I E G R
Lai-3	R R A E P A A D G V G A V S R D L E K H
NL4-3-3	- - - - - - - - - - - - - - - - - - - -
ppROP-3	- I E G R
Lai-4	G A V S R D L E K H G A I T S S N T A A
NL4-3-4	- - - - - - - - - - - - - - - - - - - -
ppROP-4	- I E G R
Lai-5	G A I T S S N T A A T N A D C A W L E A
NL4-3-5	- - - - - - - - - - - N - - A - - - - -
ppROP-5	- I E G R
Lai-6	T N A D C A W L E A Q E E E E V G F P V
NL4-3-6	N - - A - - - - - - - - - - - - - - - -
ppROP-6	- I E G R
Lai-7	Q E E E E V G F P V T P Q V P L R P M T
NL4-3-7	- - - - - - - - - - - - - - - - - - - -
ppROP-7	- I E G R
Lai-8	T P Q V P L R P M T Y K A A V D L S H F
NL4-3-8	- - - - - - - - - - - - - - - - - - - -
ppROP-8	- I E G R
Lai-9	Y K A A V D L S H F L K E K G G L E G L
NL4-3-9	- - - - - - - - - - - - - - - - - - - -
ppROP-9	- I E G R
Lai-10	L K E K G G L E G L I H S Q R R Q D I L
NL4-3-10	- - - - - - - - - - - - - - - - - - - -
ppROP-10	- I E G R
Lai-11	I H S Q R R Q D I L D L W I Y H T Q G Y
NL4-3-11	- - - - - - - - - - - - - - - - - - - -
ppROP-11	- I E G R
Lai-12	D L W I Y H T Q G Y F P D W Q N Y T P E
NL4-3-12	- - - - - - - - - - - - - - - - - - - G
ppROP-12	- I E G R
Lai-13	F P D W Q N Y T P E P G V R Y P L T F G
NL4-3-13	- - - - - - - - - G - - - - - - - - - -
ppROP-13	- I E G R
Lai-14	P G V R Y P L T F G W C Y K L V P V E P
NL4-3-14	- - - - - - - - - - - - - - - - - - - -
ppROP-14	- I E G R
Lai-15	W C Y K L V P V E P D K V E E A N K G E
NL4-3-15	- - - - - - - - - - - - - - - - - - - -
ppROP-15	- I E G R
Lai-16	D K V E E A N K G E N T R L L H P V S L
NL4-3-16	- - - - - - - - - - - - - S - - - - - -
ppROP-16	- I E G R
Lai-17	N T R L L H P V S L H G M D D P E R E V
NL4-3-17	- - S - - - - - - - - - - - - - - - - -
ppROP-17	- I E G R
Lai-18	H G M D D P E R E V L E W R F D S R L A
NL4-3-18	- - - - - - - - - - - - - - - - - - - -
ppROP-18	- I E G R
Lai-19	L E W R F D S R L A F H H V A R E L H P
NL4-3-19	- - - - - - - - - - - - - - - - - - - -
ppROP-19	- I E G R
Lai-20	F H H V A R E L H P E Y
NL4-3-20	- - - - - - - - - - - - F K N C
ppROP-20	- - - - - - - - - - - -

FIGURE 2. **Intracellular trafficking of ppROP or ROP in DC2.4 cells.** Confocal microscopy shows the uptake by DC2.4 cells of ppROP (*A*) or ROP (*B*) at 40 and 120 min. These antigens were labeled with green fluorophore. Lysosomes were stained with Lysotracker red.

FIGURE 3. **Antigen-presentation of ppROP and ROP by DC2.4 or DC cells.** ELISPOT responses of CFA/ppROP- (*n* = 3) or CFA/ROP-immunized (*n* = 3) splenocytes stimulated with ppROP- or ROP-pulsed DC2.4 cells. These DCs were incubated ± brefeldin A (*BFA*). Statistics: Student's *t* test. The figure represents two independent experiments.

19 (Fig. 4, *A* and *B*). The hierarchy of preferences was very similar for the Lai and NL4-3 peptides in C57BL/10 mice, but cross-reactions were much weaker in BALB/c, even though the amino acids differences were very few, and close to the N of peptides 2 and 6 (Fig. 4, *C* and *D*). Thus, ppROP elicited a diverse response in C57BL/10 mice, and it cross-reacted with at least one other viral strain.

ppROP-immunized T cells from C57BL/10 mice responded to ROP in addition to the native HIV Nef (Lai) protein *in vitro* (supplemental Fig. S2), as in Fig. 3. This indicates that immunization with overlapping peptides is able to generate immunity against epitopes processed from the corresponding full-length protein.

To determine whether ppROP- or ROP-specific T cells from C57BL/10 mice belong to the CD4⁺ or CD8⁺ subpopulation, we tested T cells responding to peptides Lai-1, Lai-8, Lai-17, and NL4-3-1 by IFN-γ intracellular cytokine staining (Fig. 5, *A–D*). Both CD4⁺ and CD8⁺ T cells from ppROP-immunized mice showed significantly elevated peptide-specific responses (Fig. 5, *A*, *B*, and *D*), except for Lai-17, where the increase was
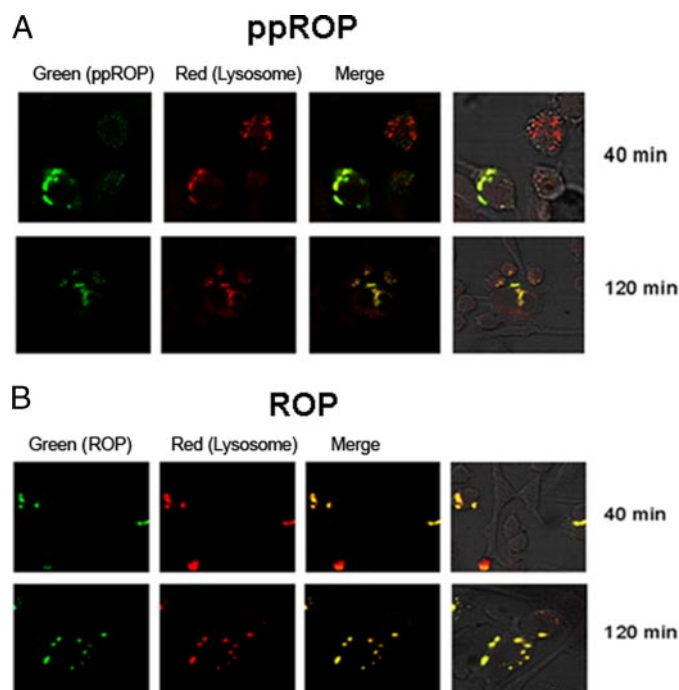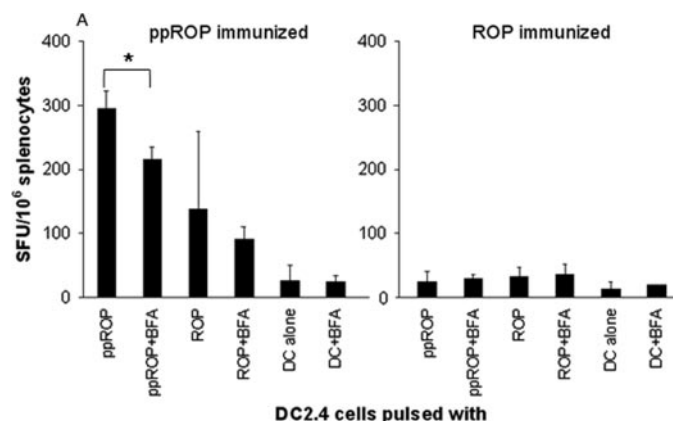
significant only for CD4⁺ T cells (Fig. 5*C*). In ROP-immunized mice, CD4⁺ T cell responses were also significantly higher against Lai-1 and NL4-3-1 than in the controls (Fig. 5, *A* and *D*), but they were consistently less prevalent than in ppROP-immunized mice. For CD8⁺ cells, they were significant only against NL4-3-1 (Fig. 5*D*).

Vaccination of Mice with ppROP/CFA Protects Mice against Lethal Viral Challenge—To determine whether the immunity induced in mice by CFA/ppROP is biologically significant, we challenged vaccinated BALB/c mice as above with a lethal dose of Nef-containing virus. Because HIV, SIV, and SHIV do not infect mice, we used 3×10^8 plaque forming units of vaccinia virus expressing Nef (vv-Nef). Eighty percent of the mice immunized with CFA/ppROP survived the challenge *versus*
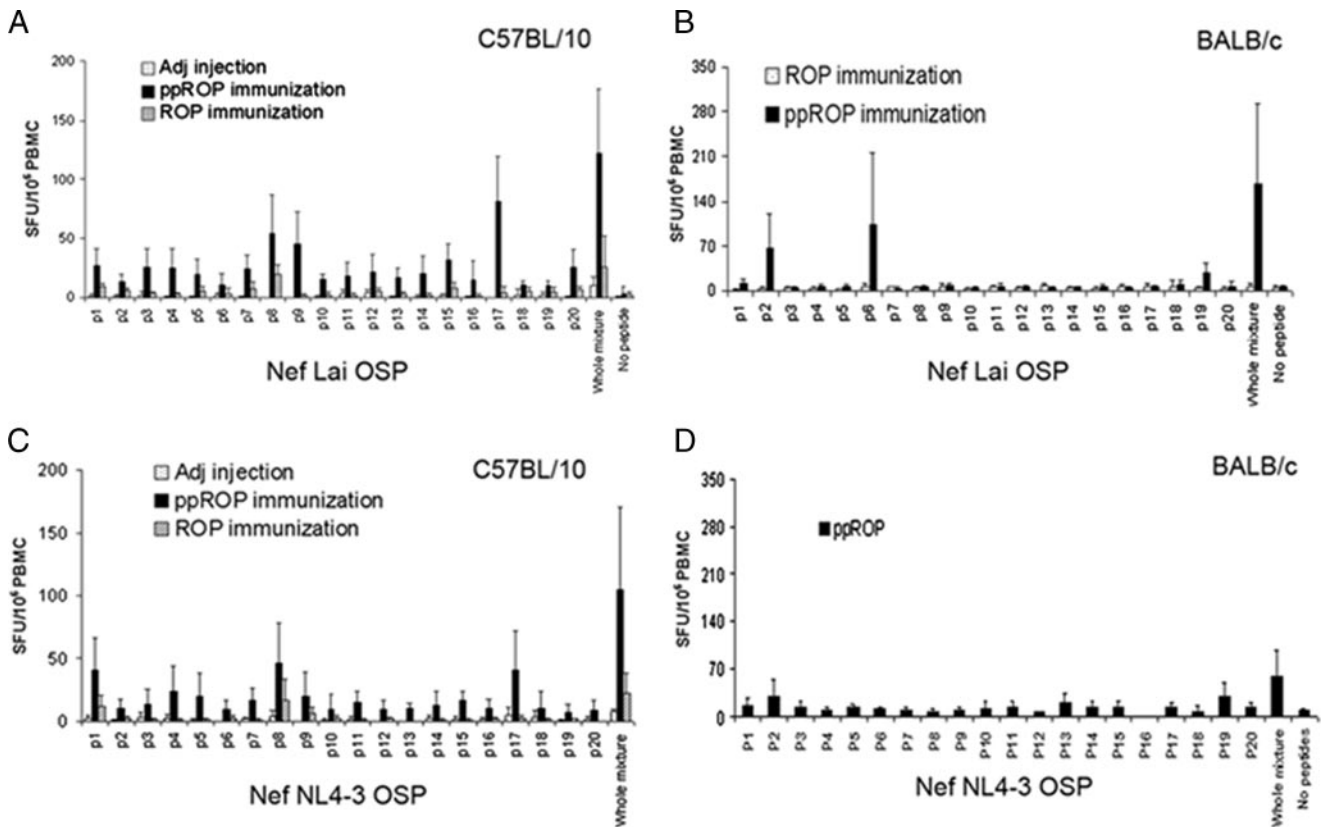
FIGURE 4. **Immunogenicity of ppROP.** C57 BL/10 ($n = 5$) and BALB/c mice ($n = 5$) were immunized with ppROP or ROP with CFA/IFA as described under "Experimental Procedures." *A* and *B*, mouse splenocytes were assayed for IFN-γ ELISPOTs against overlapping synthetic peptides of Nef (Lai) in C57 BL/10 (*A*) and BALB/c (*B*) mice. *C* and *D*, cross-reactivity of ppROP- or ROP-specific splenocytes against overlapping synthetic peptides Nef NL4-3 in C57 BL/10 (*C*) and BALB/c mice (*D*) mice.

none of the IFA/ppROP-immunized or naïve mice, and only 20% of those given adjuvant alone ($p = 0.0496$) (Fig. 6). The 40% survival in the CFA/ROP group was not statistically higher than adjuvant alone. This suggests that immunity induced by vaccination with CFA/ppROP could protect against a lethal viral challenge, and that this effect was dependent on the mycobacteria in the CFA.

DISCUSSION

We show here that recombinant overlapping peptides (ppROP) can access the endogenous intracellular pathway for cross-presentation much more efficiently than the intact ROP protein. With the assistance of CFA, priming *in vivo* with the overlapping peptides induces both CD8$^+$ and CD4$^+$ T cell responses that protect animals from lethal viral challenge.

When viruses infect cells, there is synthesis within the cytosol of viral antigens whose epitopes presented through the classical endogenous MHC I pathway to be expressed on the surface of the same cell. However, in many situations, viruses do not infect pAPCs, which are essential for priming CD8$^+$ and CD4$^+$ T cells. pAPCs have to take antigens in from exogenous sources such as infected cells, soluble proteins, or peptides in extracellular fluids. Ideally, these antigens would be taken into the cytosol to access the endogenous pathway, so the mechanism of antigen presentation would mimic that of infected cells. CD8$^+$ T cells activated by such pAPC would bear TCRs recognizing and killing the same antigenic epitopes as are presented by the

infected cells. However, for large antigens such as infected cells or soluble proteins, pAPC will internalize them through phagocytosis to phagosomes and endosomes, which normally channels them into the MHC class II pathway to activate CD4$^+$ T cells (16). In addition, both the pH and the enzymes in the phagosomes/endosomes are different from those in the cytosol. Therefore, unless the antigens are transported to the cytosol or through special routes binding to MHC class I molecules (8), epitopes generated in the phagosomes/endosomes may differ from those on the infected cells. Perhaps this is one reason why cross-presented soluble proteins are less efficient immunogens (17, 18).

Although peptides are small fragments of proteins, they can be presented quite differently, especially when loaded directly into empty surface class II molecules (19). Moreover, because many peptide vaccines are in clinical trials or have been approved for clinical applications (20, 21), it is important to understand how exogenous peptides are loaded and presented. Previous studies have shown that high affinity short nonameric MHC class I epitopes can bind directly to surface MHC class I molecules, whereas presentation of lower affinity nonamer requires internalization (22). As immunogens, short peptides (8–10-mer) are exceptional because they do not require trimming, but they nevertheless have many disadvantages compared with longer peptides (18). There are few reports on mechanisms of presentation of long peptides, although their uptake
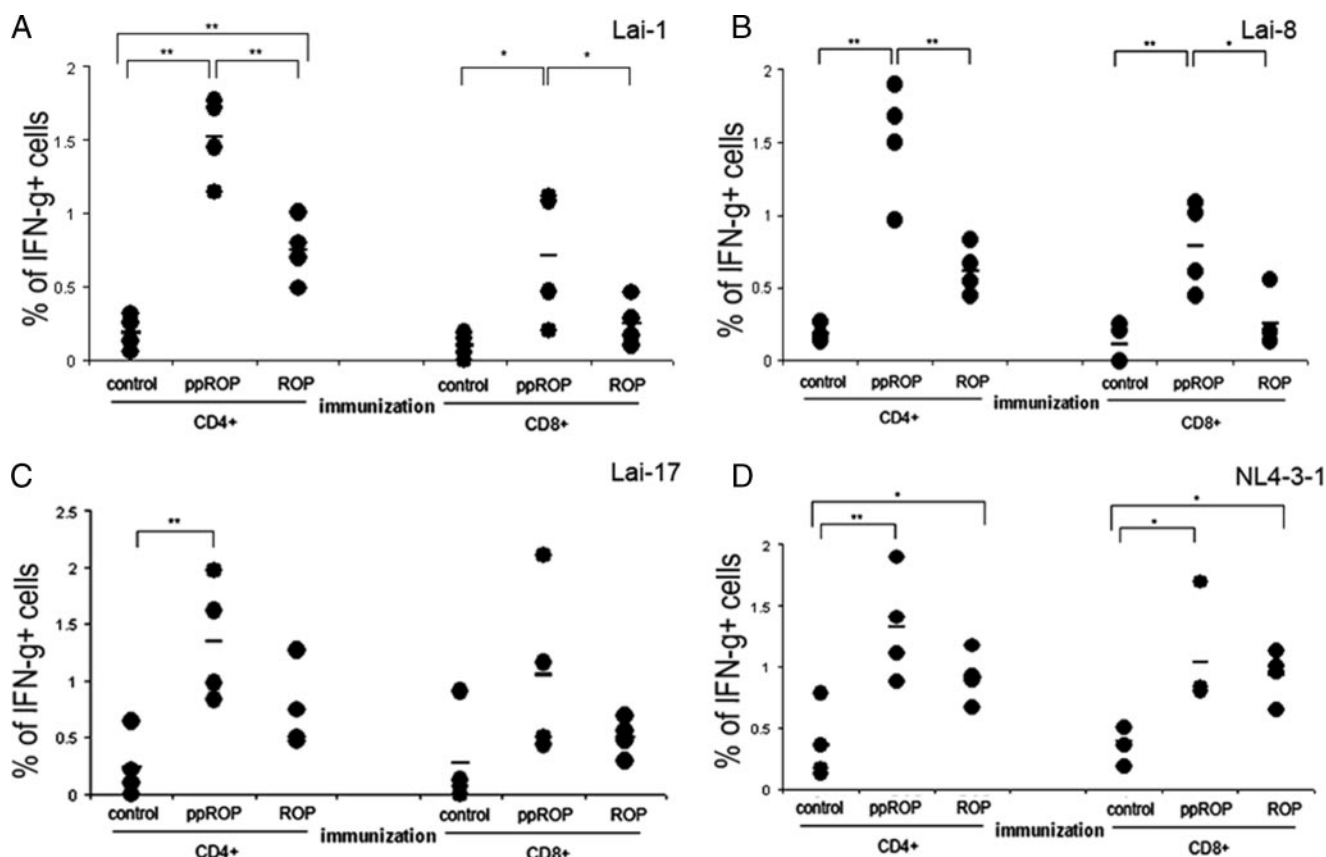
FIGURE 5. **Phenotypes of ppROP/ROP-specific T cells.** Splenocytes from CFA/ppROP or CFA/ROP immunized C57BL/10 mice ($n = 4$) were restimulated *in vitro* with HIV Nef peptides Lai-1 (*A*), Lai-8 (*B*), Lai-17 (*C*), and NL4-3-1 (*D*) and stained for intracellular IFN-γ in CD8⁺ and CD4⁺ cells. Statistics: one-way analysis of variance repeated measures test followed by Tukey's Multiple Comparison test. *, $p \leq 0.05$; **, $p \leq 0.01$. *SFU*, spot forming unit.
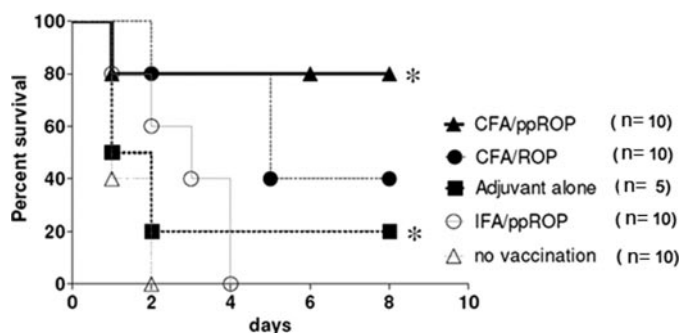

FIGURE 6. **Challenge of mice with 3×10^8 plaque forming units vv-Nef (Lai).** Five groups of BALB/c mice (number as indicated in the figure) immunized as described under "Experimental Procedures" were challenged with 3×10^8 plaque forming units vv-Nef (Lai). Statistics: *, χ square $= 3.854$, $p = 0.0496$ (df $= 1$).

by DC can be enhanced by conjugation with a Toll-like receptor agonist (23). However, the way in which natural long peptides are presented is not known.

In this study, we have compared antigen presentation of mixed or tandem-linked overlapping 24-mer peptides. We found that they took different intracellular routes: peptides were taken in early into both the endosomes and other compartments likely including the cytosol, and turned over rapidly; proteins were taken in later, stayed only in the endosomes, and persisted longer (Fig. 2, *A* and *B*, and supplemental Fig. S1).

The implication of this result is that long peptides may go through both MHC class I and class II pathways to stimulate

both CD8⁺ and CD4⁺ T cells, whereas protein may mainly stimulate CD4⁺ T cells. Indeed, immunizing mice with these peptides induced immune responses that involved both CD8⁺ and CD4⁺ T cells (Fig. 5, *A–D*), whereas immunization with the protein did not generate strong immune responses. Moreover, DCs can present both ppROP and ROP to ppROP-immunized lymphocytes, whereas ROP-immunized lymphocytes responded weekly. This implies that the protein antigen is not efficient in priming T cells (Fig. 3 and supplemental Fig. S2).

Interestingly but not surprisingly, priming with ppROP *in vivo* induced immune responses in two different strains of mice; the magnitude of the responses in the two strains of mice was similar, but the epitope hierarchies were distinct (Fig. 4, *A* and *B*). The peptide-immunized T cells also responded to Nef or ROP stimulation (supplemental Fig. S2). These results make the recombinant overlapping peptides promising as economic vaccine candidates that can be given to both animals and humans with no need for prior knowledge of MHC phenotypes.

Vaccination with ppROP clearly induced responses to epitopes processed naturally from the natural Nef protein that protected against viral challenge. To compare the biological significance of *in vivo* priming with the overlapping peptides and protein, we challenged the immunized mice with vaccinia viruses expressing Nef protein. Eighty percent of the ppROP/CFA-immunized mice were protected from lethal challenge *versus* only 40% of the CFA/ROP-immunized mice (Fig. 6).

Most non-replicating vaccines need the help of an adjuvant to induce an effective immune response (24). In our study, we found that ppROP generated immune responses and the resulting protection depended on CFA (Fig. 6). Although the mechanism of adjuvant action is not fully understood, recruitment of the adjuvant-induced innate responses by danger signals such as bacterial or viral pathogen or damage-associated molecular patterns (PAMPs or DAMPs) (25, 26), greatly enhances the adaptive immune responses (27). Moreover, although not universally accepted (22, 28), reports have suggested that adjuvants induce cytokine secretion and up-regulation of co-stimulatory molecules through TLR stimulation (21, 29, 30). In fact, TLRs can be critical for induction of both B and T cell immune responses (31, 32); their stimulation results in activation of DCs and other APCs, release of cytokines, and up-regulation of surface co-stimulatory molecules such as CD80 or CD86. CFA contains inactivated mycobacteria that can stimulate strong TLR responses (33). In our experiments, some of its components enhanced serine phosphorylation of DC proteins and up-regulated the expression of CD86.[4] The immune response against ppROP may be enhanced through CFA-TLR stimulation.

In summary, we have found in this study that the intracellular pathway of cross-presenting recombinant overlapping peptides is different from that of their correspondent protein. With the assistance of CFA, priming *in vivo* with the overlapping peptides induces both CD8+ and CD4+ T cell responses that protect animals from lethal viral challenge.

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