

# Dendritic Cell-Lysosomal-Associated Membrane Protein (LAMP) and LAMP-1-HIV-1 Gag Chimeras Have Distinct Cellular Trafficking Pathways and Prime T and B Cell Responses to a Diverse Repertoire of Epitopes<sup>1</sup>

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Ag processing is a critical step in defining the repertoire of epitope-specific immune responses. In the present study, HIV-1 p55Gag Ag was synthesized as a DNA plasmid with either lysosomal-associated membrane protein-1 (LAMP/gag) or human dendritic cell-LAMP (DC-LAMP/gag) and used to immunize mice. Analysis of the cellular trafficking of these two chimeras demonstrated that both molecules colocalized with MHC class II molecules but differed in their overall trafficking to endosomal/lysosomal compartments. Following DNA immunization, both chimeras elicited potent Gag-specific T and B cell immune responses in mice but differ markedly in their IL-4 and IgG1/IgG2a responses. The DC-LAMP chimera induced a stronger Th type 1 response. ELISPOT analysis of T cell responses to 122 individual peptides encompassing the entire p55gag sequence (15-aa peptides overlapping by 11 residues) showed that DNA immunization with native gag, LAMP/gag, or DC-LAMP/gag induced responses to identical immunodominant CD4<sup>+</sup> and CD8<sup>+</sup> peptides. However, LAMP/gag and DC-LAMP/gag plasmids also elicited significant responses to 23 additional cryptic epitopes that were not recognized after immunization with native gag DNA. The three plasmids induced T cell responses to a total of 39 distinct peptide sequences, 13 of which were induced by all three DNA constructs. Individually, DC-LAMP/gag elicited the most diverse response, with a specific T cell response against 35 peptides. In addition, immunization with LAMP/gag and DC-LAMP/gag chimeras also promoted Ab secretion to an increased number of epitopes. These data indicate that LAMP-1 and DC-LAMP Ag chimeras follow different trafficking pathways, induce distinct modulatory immune responses, and are able to present cryptic epitopes. *The Journal of Immunology*, 2006, 177: 2265–2275.

The establishment of MHC-restricted CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses primarily depends on the repertoire of peptides generated by the proteases involved in Ag processing (1–5). Classically, CD8<sup>+</sup> T cells recognize peptides originating from cytosolic or nuclear proteins that are processed by the proteasomal and postproteasomal enzymes in the cytoplasm and endoplasmic reticulum (6, 7). CD4<sup>+</sup> T cells, on the other hand, typically recognize peptides originating from exogenous proteins captured by APCs and processed by the proteases along the endosomal/lysosomal pathway (1, 3, 5, 8, 9). However, several overlapping pathways have been shown to play an important role in the interweaving of these processes.

The processing of exogenous Ags for presentation by MHC class I (MHC I)<sup>3</sup> has been reported to occur in the vacuolar endosomal/exosomal compartment, as well as in the cytoplasm (5, 10–15). Also, endogenous proteins have been shown to be processed and presented by MHC class II (MHC II) molecules (8). APCs have a variety of specialized Ag uptake mechanisms that capture and transport the Ags to specific endocytic subcompartments (9, 16, 17), which contain distinct groups of chaperone molecules and proteases (18–21). For example, cathepsins B and Z have been shown to be present in early and late endosomes but absent from lysosomes, whereas cathepsin D is present in lysosomes and late endosomes (18, 19). This enzymatic compartmentalization is associated with specialized Ag capture and can also influence the immune response.

Several studies have demonstrated that targeting of an Ag to different cellular subcompartments can quantitatively and qualitatively influence the repertoire of CD4<sup>+</sup> and CD8<sup>+</sup> epitope-specific immune responses elicited by that Ag (5, 22–27), and it has been postulated that cleavage by the enzymes of a specific cellular compartment can determine the repertoire of epitopes generated by the Ag's processing. Some of these studies have addressed the impact of different targeting mechanisms on a limited number of known CD4<sup>+</sup> and CD8<sup>+</sup> epitopes; however, the impact of these strategies on the in vivo epitope-specific T cell responses and on the modulation of the whole repertoire of activated cells induced by a protein Ag has not been systematically investigated.

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<sup>3</sup> Abbreviations used in this paper: MHC I, MHC class I; MHC II, MHC class II; LAMP, lysosome-associated membrane protein; DC, dendritic cell; SFC, spot-forming cell; MAC, minimum stimulatory Ag peptide concentration.

We and others (22–24, 28–36) have shown that Ag sequences conjugated to lysosomal-associated membrane protein (LAMP)-1 can be targeted effectively to vacuolar endosomal/lysosomal compartments containing MHC II and will elicit enhanced Ab, CD4<sup>+</sup>, and CD8<sup>+</sup> responses and prolonged immunological memory to those Ags. LAMP-1 is expressed constitutively at high levels by all cells, including immature dendritic cells (DC), and is one of the most glycosylated proteins known to date. A recently identified member of the LAMP family, designated DC-LAMP, is specifically expressed in the endosomal/lysosomal compartments of human mature DC (37) and lung type II pneumocytes (38, 39). This human DC-LAMP differs biochemically from the other LAMP molecules primarily in bearing a mucin-like sugar structure containing several putative O-linked glycans that are localized to the amino-terminal half of its luminal domain. More importantly, its expression is associated with significant remodeling of the endosomal/lysosomal MHC II compartments of DC. Human DC-LAMP molecules migrate to adjacent tubules and vesicles (37, 40), while the MHC II molecules are transported to the plasma membrane, suggesting a critical role for DC-LAMP in human DC function that could make it extremely useful as a molecular vaccine adjuvant.

In this study, we have examined the cellular trafficking of HIV-1 Gag, which we have targeted to the vacuolar endosomal/lysosomal compartments containing MHC II and recycling MHC I molecules (41), by coexpressing it with each of two LAMP molecules, LAMP-1 (to form a chimera designated LAMP/Gag) and human DC-LAMP (DC-LAMP/Gag). We also analyzed the peptide-specific responses mediated by B lymphocytes and by CD4<sup>+</sup> and CD8<sup>+</sup> T cells after immunization of mice with these DNA plasmid chimeras. Both chimeras elicited equally potent immune responses that were several-fold greater than those elicited in response to the native Gag. Moreover, LAMP- and DC-LAMP-mediated targeting to the endosomal/lysosomal compartment induced T cell responses to the same CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes as did native Gag but also revealed additional epitopes not exposed in native Gag. These results indicated that Ag targeting to the endosomal/lysosomal compartment for processing increased the breadth of the epitope responses but did not alter the CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes, as compared with the native Gag protein processing.

## Materials and Methods

### Plasmids

Eukaryotic expression plasmids were constructed using nt 1–1503 of the HIV-1 HXB2 p55gag gene (GenBank accession no. K03455; HIV sequence database, Los Alamos National Laboratory Theoretical Biology and Biophysics) and inserted into pITR vector (42), which contains adeno-associated virus-inverted terminal repeats flanking the expression elements (CMV promoter and bovine growth hormone polyadenylation signal). The LAMP/gag construct was made by inserting the p55gag sequence between the luminal domain and the transmembrane domain and cytoplasmic tail of LAMP (GenBank accession no. J03881), as described previously (24). The DC-LAMP plasmid was constructed from cDNA obtained from a human lung tissue library (GenBank accession no. AB013924) (38). The DC-LAMP luminal domain was amplified by PCR using primers bearing an *NheI* site at the 5' end and an *XhoI* site at the 3' end; the transmembrane domain was inserted with *EcoRI* and *KpnI* sites at the 5' and 3' ends, respectively. The DC-LAMP/gag chimera was made by replacing the LAMP-1 luminal domain and the transmembrane and cytoplasmic domain sequences of LAMP/gag with the corresponding DC-LAMP sequences. The plasmids used for vaccination were produced in *Escherichia coli* and made endotoxin-free (Qiagen).

### Confocal microscopy

Mouse L fibroblast cell line expressing MHC II (I-E<sup>K</sup>) DCEK.ICAM.Hi7 cells (hereafter referred to as DCEK cells; provided by Dr. S. Swain, The

Trudeau Institute, Saranac Lake, NY) were plated onto poly-D-lysine-treated coverslips in 6-well plates (10<sup>6</sup> cells/well) and incubated overnight. The cells were transfected with the indicated plasmids using LipofectAMINE 2000 transfection reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. After 48 h, the coverslips were fixed with 2% paraformaldehyde and blocked with 4% normal goat serum and 0.1% saponin. For detection of DC-LAMP/Gag expression, the cells were incubated for 1 h with mouse anti-Gag supernatant medium (provided by Dr. J. K. Hildreth, Johns Hopkins School of Medicine, Baltimore, MD) at a 1/50 dilution, washed three times with 0.1% saponin, and stained with Texas red-conjugated anti-mouse-IgG at 1 mg/ml (Jackson ImmunoResearch Laboratories). Colocalization with MHC II was evaluated by incubating the cells for 1 h with FITC-labeled goat anti-mouse I-Ek (14-4-4S) (BD Pharmingen) at a 1/75 dilution. Endogenous LAMP was detected by incubating the coverslips for 1 h with rat anti-mouse LAMP-1 (1D4B) supernatant medium and diluted 1/50 (43, 44). The coverslips were washed three times with PBS solution containing 0.1% saponin, followed by incubation for 1 h with FITC-conjugated anti-rat IgG at 1 μg/ml (Jackson ImmunoResearch Laboratories). The cells were washed with PBS, and the coverslips were mounted onto glass slides using ProLong antifade reagent (Molecular Probes). Confocal microscopy was performed using the Wallac confocal laser scanning microscope, and the images were captured individually and digitally colored by using Photoshop 5.0 (Adobe).

### Subcellular fractionation

Approximately 5 × 10<sup>6</sup> cells from the 293 cell line, a human embryonic kidney cell line transformed with adenovirus 5 DNA (American Type Culture Collection), were transfected with the indicated plasmids, as described above, and incubated for 24 h. The cells were then washed with PBS, followed by two washes with homogenization buffer (0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl), and removed from the plates with a cell scraper. They were then homogenized on ice (40 strokes) in a Dounce homogenizer with a tight-fitting pestle. After centrifugation at 300 × g for 10 min, the postnuclear supernatant was diluted in Percoll to a final concentration of 30% and centrifuged at 20,000 rpm for 45 min, using a Beckman Sw41Ti rotor. The gradient was divided into 15 fractions of 0.5 ml collected from the bottom. The Percoll was removed by centrifugation at 28,700 rpm for 2 h in a Beckman Sw55Ti rotor, and the biological material above the Percoll pellet was collected. The fractions were diluted in PBS-0.05% Tween 20 (PBST) and analyzed by dot blotting: 100 μl of each fraction was applied, in duplicate, to Immobilon P membranes (Millipore), incubated for 30 min, and exposed to a vacuum. The membranes were blocked with PBST containing 5% milk for 2 h, then incubated with mouse anti-Gag Ab (for detection of LAMP/Gag or DC-LAMP/Gag chimeras) at a 1/50 dilution or with mouse anti-human LAMP-1 (H4A3) (45) (for detection of endogenous LAMP-1) for 1 h. After washing with PBST, the membranes were incubated with an HRP-conjugated goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories) at a 1/10,000 dilution for 1 h. The reaction was developed with an ECL detection system (ECL kit; Amersham Biosciences).

### Protein expression

293 cells were transfected with the indicated plasmids using the LipofectAMINE 2000 transfection reagent and a typical transfection by this protocol had ~40% efficiency. After 4 h, the medium was exchanged with complete DMEM (Invitrogen Life Technologies) containing 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin, with or without the following reagents: 50 μM chloroquine (Sigma-Aldrich); 80 μM E64c (Sigma-Aldrich); 1 μM MG132 (Sigma-Aldrich); or 100 nM brefeldin A (Calbiochem). After 48 h of culture, the supernatant and cells were harvested, the cells were disrupted with lysis buffer (10 mM Tris-HCl (pH 7.5) with 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and premixed protease inhibitors (Complete; Roche Applied Science)) for 15 min on ice, and cellular debris was removed by centrifugation. The amount of Gag protein in the cell lysate and supernatant fractions was analyzed by Western blotting. Initially, the samples were normalized according to the total protein concentration, as determined by bicinchoninic acid (Pierce). They were then resolved on 10% polyacrylamide gels, transferred to Immobilon membranes (Millipore), and blocked with PBS containing 5% nonfat dried milk. Molecular weight markers (Amersham Biosciences) were used as standards. After washing with PBST, the blot was probed with mouse anti-Gag at a 1/50 dilution for 2 h, washed three times, and then incubated with peroxidase-conjugated goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories) at a 1/10,000 dilution for 1 h. Gag was visualized using an ECL kit. Images were scanned digitally and exported to Photoshop 5.0.

### ELISA analysis of IFN- $\gamma$ production by immunized mice

Female BALB/c mice, 6–8 wk of age (Charles River Laboratories), were immunized twice, s.c., with the indicated DNA plasmids at 50  $\mu$ g/50  $\mu$ l/mouse at 4-wk intervals. Single-cell suspensions, depleted of RBC, were prepared from freshly isolated splenocytes in culture medium (RPMI 1640 medium supplemented with 5% v/v FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50  $\mu$ M 2-ME, and 1 M HEPES buffer). Splenocytes ( $10 \times 10^6$  cells/ml) were cultured in triplicate in a 96-well plate in the presence of recombinant baculovirus HIV<sub>SF2p55</sub> Gag (5  $\mu$ g/ml; National Institutes of Health AIDS Research and Reference Reagent Program) or medium alone, and the culture supernatants were harvested after 72 h for the quantitation of secreted IFN- $\gamma$  using an OPTEDIA ELISA kit (BD Pharmingen).

### CD8<sup>+</sup>-mediated T cell responses of immunized mice

Gag-specific CD8<sup>+</sup>-mediated responses were measured after a single DNA immunization, followed in 3 wk by in vivo expansion of Gag-activated T cells through inoculation, i.p., with recombinant VDK-1 vaccinia virus containing the *gag/pol* sequence ( $10^7$  PFU; National Institutes of Health AIDS Research and Reference Reagent Program) or the native vaccinia virus as control. Five days later, the mice were injected i.v. with an immunodominant H-2K<sup>d</sup>-restricted Gag peptide epitope (aa 197–211, AMQMLKETI) at 10  $\mu$ g/ml, and 2 h later, they were sacrificed and splenocytes were collected.

**Tetramer staining.** Splenocytes from mice immunized as described above to elicit Gag-specific CD8<sup>+</sup>-mediated responses were washed in FACScan buffer at 4°C and blocked with anti-mouse CD16/CD32, Fc $\gamma$ III/II receptor Ab (BD Pharmingen) at 10  $\mu$ g/ml for 10 min at 4°C. The splenocytes ( $1 \times 10^6$  cells/well) were stained in duplicate with PE-conjugated tetramer (H-2K<sup>d</sup>/AMQMLKETI; National Institute of Allergy and Infectious Diseases, National Institutes of Health) and the Ig isotype control rat IgG1 Ab (BD Pharmingen) at a dilution of 1/100 for 1 h at 4°C. The cells were washed twice with FACScan buffer and incubated with FITC-conjugated rat anti-mouse CD8 (BD Pharmingen) at a dilution of 1/100 for 30 min at 4°C. Finally, the cells were washed twice with FACScan buffer. Cells were analyzed on a BD Biosciences FACScan with CellQuest software. A minimum of 200,000 events were analyzed, and the results were expressed as a percentage of tetramer binding cells on total CD8<sup>+</sup> cells.

**Intracellular IFN- $\gamma$  staining.** Intracellular IFN- $\gamma$  staining to detect Gag-specific CD8<sup>+</sup>-mediated responses was performed using the Cytofix/Cytoperm Plus (with GolgiStop) kit (BD Pharmingen). In brief, splenocytes harvested from the immunized mice were incubated with GolgiStop containing monensin for 2 h at 37°C. After incubation, cells were washed twice with FACScan buffer, and nonspecific binding was blocked by incubating cells with anti-Fc $\gamma$ R Ab (BD Pharmingen) at 10  $\mu$ g/ml for 10 min at 4°C. The splenocytes ( $1 \times 10^6$  cells/well) were stained in duplicate with FITC-conjugated rat anti-mouse CD8<sup>+</sup> Ab (BD Pharmingen) at a dilution of 1/100 for 30 min at 4°C. The cells were washed twice with FACScan buffer and resuspended in 200  $\mu$ l of Cytofix/Cytoperm solution at 4°C for 20 min. Cells were then washed twice with Perm/Wash solution and stained with PE-conjugated rat anti-mouse IFN- $\gamma$  Ab or the Ig isotype control rat IgG1 Ab (BD Pharmingen) diluted 1/100. Analysis was done on a BD Biosciences FACScan with CellQuest software. A minimum of 200,000 events were analyzed, and the results were expressed as a percentage of IFN- $\gamma$ <sup>+</sup> cells on total CD8<sup>+</sup> cells.

**<sup>51</sup>Cr release assay.** P815 target cells ( $2 \times 10^6$ /ml) were pulsed overnight with the p55Gag peptide AMQMLKETI (10  $\mu$ M in culture medium). The cells were then labeled with 50  $\mu$ Ci/ml [<sup>51</sup>Cr]Na<sub>2</sub>CrO<sub>4</sub> (Amersham Biosciences) for 2 h, washed three times, resuspended in culture medium, and diluted to  $2 \times 10^5$  cells/ml. Then,  $1 \times 10^4$  cells were seeded onto V-bottom 96-well plates with an equal volume of 2% Triton X-100 (maximum release), medium alone (minimum release), or a suspension of splenocytes from mice immunized to produce Gag-specific CD8<sup>+</sup>-mediated responses (effector cells) at different E:T ratios, beginning at 100:1. All reactions were conducted in triplicate. After 4 h of incubation at 37°C, 50  $\mu$ l of supernatant was transferred to a LumaPlate (Packard), dried, and counted in a TOPCOUNT NXT scintillation counter (Packard). The percentage of specific lysis (percent killing) was calculated as:  $100 \times ((\text{cpm released with effector}) - (\text{minimum release})) / ((\text{maximum release}) - (\text{minimum release}))$ .

### Ab response

Mice serum was obtained from the tail vein before the first immunization (prebleed) and 10 days after the second DNA immunization (to evaluate the total Gag-specific IgG response) or after the vaccinia boost (for mapping epitope-specific responses), and levels of IgG recognizing the Gag

protein or individual Gag peptides were analyzed by ELISA: ELISA plates were coated with 100  $\mu$ l of either the HIVIIIB lysate (Applied Biosystems) at 5  $\mu$ g/ml or a collection of 12 pools of Gag peptides, each containing 10 peptides that are 15-mer with 11 overlapping amino acids (HIV-1 HXB2gag peptide, Complete set, catalog no. 5107; National Institutes of Health AIDS Research and Reference Reagent Program) at 10  $\mu$ g/ml, both diluted in 0.1 M carbonate buffer. After an overnight incubation at 4°C, the solution was removed, and the plates were washed six times with PBST. The plates were incubated for 2 h at 37°C with 200  $\mu$ l of blocking buffer (PBS with 0.05% Tween 20 and 5% FBS) and then washed three times. Serum samples were serially diluted (3-fold) eight times in blocking buffer, starting at 1/100. For the response to the Gag peptides, the serum samples were added at 1/500 only. A 100- $\mu$ l sample of each dilution was added to the blocked plate, and the plates were incubated overnight at 4°C. For IgG detection, the reaction was developed using goat anti-mouse IgG-HRP (Cappel) at a 1/5000 dilution. For IgG1 and IgG2a, biotinylated anti-mouse IgG1 and IgG2a Ab (BD Pharmingen) diluted 1/500 was conjugated with avidin-HRP (BD Pharmingen) diluted 1000-fold, and 100  $\mu$ l was added to each well for 2 h at 37°C. Turbo tetramethylbenzidine substrate solution (BD Pharmingen) was added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 100  $\mu$ l of 1 M sulfuric acid, and absorbance at 450 nm was measured in a Bio-Rad model 3550 microplate reader.

### ELISPOT analysis of activated T cell repertoires

Mice were immunized twice with the indicated DNA plasmids and then boosted once, i.p., with VDK-1 vaccinia virus containing the *gag/pol* sequence ( $10^7$  PFU, National Institutes of Health AIDS Research and Reference Reagent Program) at 3- to 4-wk intervals. IFN- $\gamma$  and IL-4 ELISPOT assays were performed, using an ELISPOT set from BD Biosciences, according to the manufacturer's protocol. Initially, ELISPOT plates were coated with anti-IFN- $\gamma$  or anti-IL-4 Abs at 5  $\mu$ g/ml and incubated at 4°C/overnight. The plates were blocked with RPMI 1640 medium containing 10% FCS for 2 h at room temperature, and total splenocytes ( $10^6$  cells/well) from immunized mice were then added. The cells were cultured at 37°C in 5% CO<sub>2</sub> with culture medium only (Hybridoma-serum-free medium; Invitrogen Life Technologies), or in the presence of Con A (2.5  $\mu$ g/ml; Sigma), recombinant baculovirus HIV<sub>SF2p55</sub> Gag (5  $\mu$ g/ml; National Institutes of Health AIDS Research and Reference Reagent Program) or the 122 individual HIV-Gag 15-mers of the HIV-1 HXB2gag peptide-complete set (10  $\mu$ g/ml; National Institutes of Health AIDS Research and Reference Reagent Program), as indicated in the Results. CD4<sup>+</sup> or CD8<sup>+</sup> T cell-populations were purified by negative selection with columns (R&D Systems) and cultured at  $5 \times 10^5$  cells/well with irradiated feeder cells ( $5 \times 10^5$  cells/well) obtained from nonimmunized mice under the same conditions. After 16 (IFN- $\gamma$ ) or 36 (IL-4) h of culture, the plates were washed and incubated with biotinylated anti-IFN- $\gamma$  or anti-IL-4 Ab for 2 h at room temperature, followed by HRP-conjugated avidin for 1 h at room temperature. Reactions were developed with AEC substrate (Calbiochem-Novabiochem). Analyses of the IFN- $\gamma$ /IL-4 levels were performed using the Immunospot series Analyzer software (BD Biosciences). The data indicate the number of spot-forming cells ((SFC)/ $10^6$ ) obtained with each stimulus minus the SFC number obtained with culture medium alone. The data were also compared with the number of SFC from mice immunized with control plasmid. The number of SFC from the plasmid control group was normally 0.

### Statistical analysis

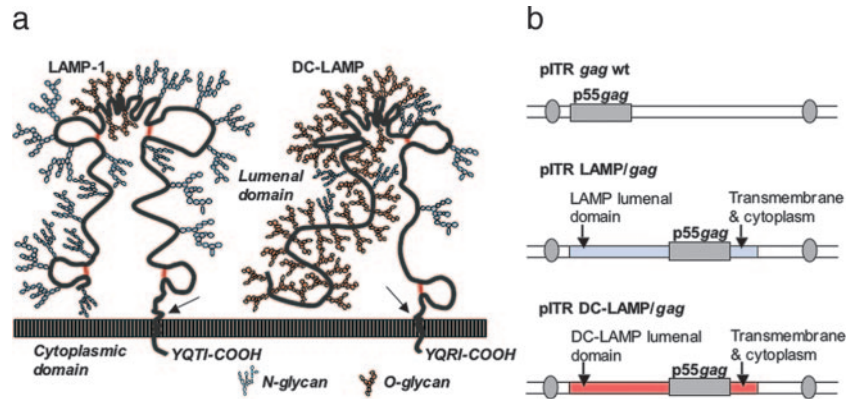
Statistical analysis of the results was based on unpaired *t* tests and  $\chi^2$  independence tests. Values of *p* < 0.05 were considered statistically significant.

## Results

### LAMP/Gag and DC-LAMP/Gag chimeras traffic differentially through the endosomal/lysosomal pathway

Our previous work has demonstrated that association of HIV-Gag with LAMP molecules induces Gag trafficking to MHC II compartments in transfected cells, in contrast to the cytoplasmic localization or the secreted virus-like particles of the native Gag. Although it has been documented that both LAMP-1 and DC-LAMP colocalize with MHC II molecules, some recent data have suggested that the intracellular trafficking of these two molecules is regulated by different mechanisms and that they do not follow the same pathways (40). In addition, LAMP-1 and DC-LAMP have

**FIGURE 1.** Schematic representation of LAMP and DC-LAMP structures and pITR *gag*, pITR LAMP/*gag*, and pITR DC-LAMP/*gag* plasmids. *a*, Structures of LAMP and DC-LAMP, including the N- (gray) and O-glycan residues (brown). *b*, Schematic representation of pITR plasmids. Boxed areas represent the open reading frames as indicated. The ovals indicate the adeno-associated virus-inverted terminal repeat sequences of the plasmids. Arrows indicate the schematic position of the various sequences: The HIV<sub>HXB2</sub>-p55*gag* (p55*gag*) sequence is indicated by a stippled box; LAMP and DC-LAMP luminal domains and transmembrane and cytoplasmic tails are indicated by blue and red boxes, respectively.



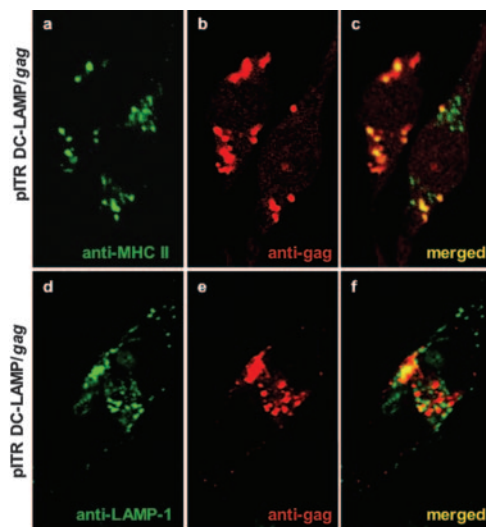
quite distinct glycosylation patterns (Fig. 1*a*). To verify whether the association with DC-LAMP would also alter the intracellular trafficking of HIV-Gag, we made *gag* chimeric plasmid constructs containing either LAMP-1 or DC-LAMP sequences (Fig. 1*b*). The LAMP/*gag* construct has been described previously (24). The DC-LAMP plasmid was constructed from cDNA obtained from a human lung tissue library (38). The DC-LAMP/*gag* chimera was made similarly by replacing the LAMP-1 luminal and transmembrane domain sequences with the respective DC-LAMP homologous sequences.

Steady-state localization of the DC-LAMP/Gag chimera was analyzed by confocal microscopy of transfected mouse DCEK cells (Fig. 2). The localization of the Gag protein (Fig. 2, *b* and *e*) was compared with that of endogenous MHC II (Fig. 2*a*) and LAMP-1 (Fig. 2*d*) molecules. The results indicated that the DC-LAMP/Gag chimera partially colocalizes with MHC II (Fig. 2*c*) and LAMP-1 (Fig. 2*f*). These analyses were also performed in human 293 cells and presented similar results (data not shown). Additional comparative analysis of the LAMP/Gag and the DC-LAMP/Gag localization was performed by subcellular fractionation of transfected 293 cells in Percoll density gradients (Fig. 3). The subcellular fractions obtained were analyzed by dot blotting and probed with specific Abs for each of the chimeric Ags and for

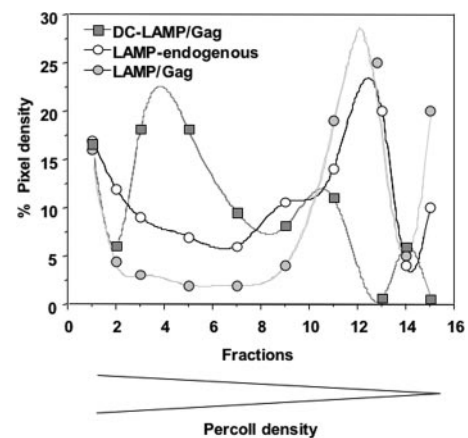
endogenous LAMP. The distribution of the LAMP/Gag protein followed the same pattern as that of the endogenous LAMP-1, whereas DC-LAMP/Gag showed a distinctly different distribution. DC-LAMP/Gag showed greater expression levels in fractions of intermediate density that were related to late endosomes, as verified by analysis of Rab9 localization (data not shown). It also showed a much lower level of expression in the lower density fractions representing the plasma membrane (fractions > 12). The confocal microscopy and the cell fractionation results showed that the DC-LAMP/Gag and LAMP/Gag chimeras had distinct distributions along the endosomal/lysosomal pathway, in agreement with data from electron microscopy studies (40).

#### *LAMP/Gag and DC-LAMP/Gag are susceptible to different protease and trafficking inhibitors*

Trafficking of the LAMP/Gag and DC-LAMP/Gag chimeras was further studied by analysis of the effects of proteases and traffic inhibitors on the intracellular degradation and protein secretion of Gag (Fig. 4). Cells transfected with LAMP/*gag* or DC-LAMP/*gag* were treated for 24–48 h with chloroquine, E64c, MG132, or brefeldin A, and the cell extracts were subjected to Western blot

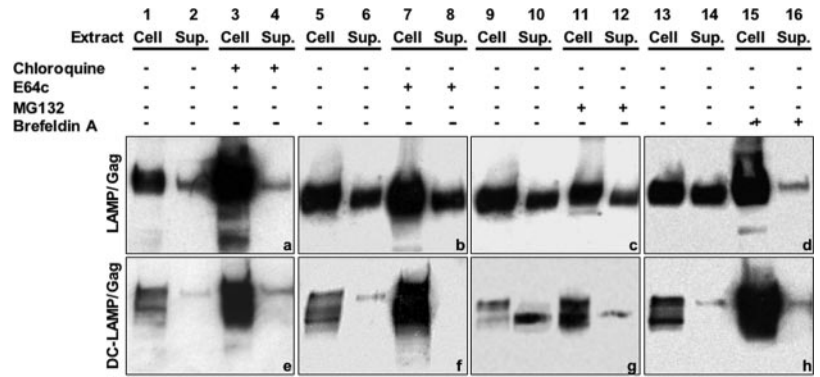


**FIGURE 2.** Colocalization of DC-LAMP/Gag with MHC II and endogenous LAMP-1 molecules in mouse DCEK cells. Confocal microscopy of mouse DCEK cells transfected with the DC-LAMP/*gag* plasmid and stained with: *a*, FITC-conjugated anti-MHC II (green); *b*, Texas red-conjugated anti-gag (red); *c*, merged images of *a* and *b*; *d*, anti-LAMP-1 mAb (green); *e*, anti-gag (red); and *f*, merged images of *d* and *e*.



**FIGURE 3.** LAMP/Gag and DC-LAMP/Gag chimeras traffic differentially through the endolysosomal pathway. 293 cells were transfected with LAMP/*gag* and DC-LAMP/*gag*, and after 24 h, the cells were fractionated on Percoll gradients, as described in *Materials and Methods*. The expression of LAMP/Gag and DC-LAMP/Gag in the various fractions was analyzed by dot blotting; the membranes were stained with anti-Gag Ab, followed by anti-mouse HRP-conjugated Ab. Endogenous LAMP was analyzed by staining the dot blot membranes with anti-human LAMP, followed by HRP-conjugated anti-mouse Ab. The amount of protein in each fraction was determined by measuring the pixel intensity and was plotted as percentage of pixel intensity in relation to the total intensity.

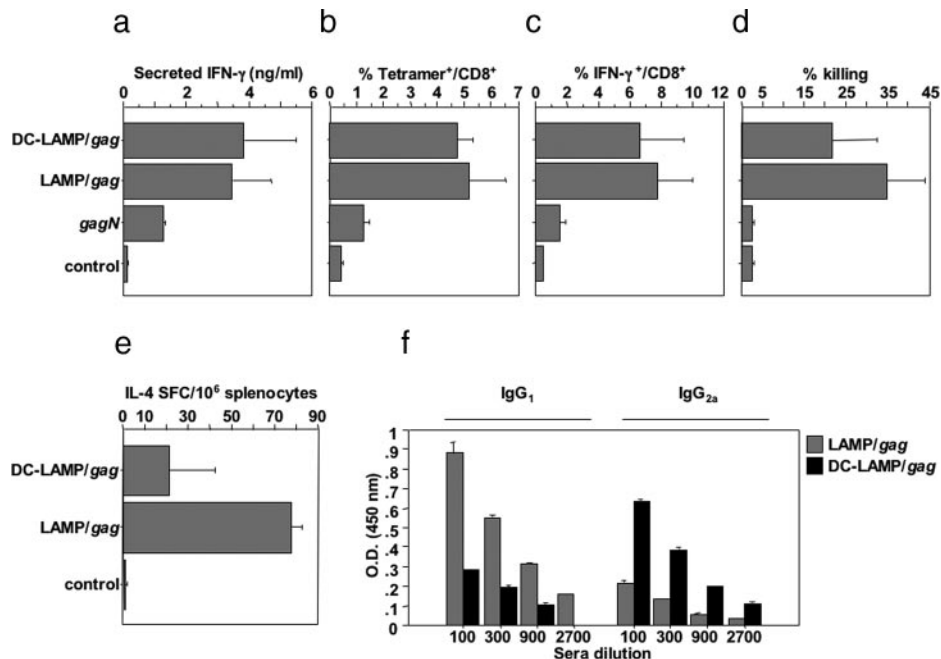
**FIGURE 4.** Different effects of distinct Ag-processing inhibitors on LAMP/Gag and DC-LAMP/Gag expression. 293 cells were transfected with LAMP/*gag* (*a–d*) and DC-LAMP/*gag* (*e–h*) and treated simultaneously with: *a* and *e*, chloroquine (50  $\mu$ M); *b* and *f*, E64c (80  $\mu$ M); *c* and *g*, MG132 (1  $\mu$ M); or *d* and *h*, brefeldin A (0.1  $\mu$ M). After 48 h, the supernatant (sup) and the cell extracts (cell) from these cultures were collected separately and analyzed by Western blotting. The membranes were stained with anti-Gag Ab, followed by HRP-conjugated anti-mouse IgG, and developed using the ECL system.



analysis. Chloroquine has a generalized inhibitory effect on acidic lysosomal proteases, and E64c is a cysteine protease inhibitor; MG132 inhibits proteasome degradation, and brefeldin A blocks transport from the endoplasmic reticulum to the Golgi. Experiments were performed on both DCEK and 293 cells, presenting similar results

Cell treatment with chloroquine (Fig. 4, lanes 3 and 4) resulted in increased accumulation of LAMP/Gag (Fig. 4*a*) and DC-LAMP/Gag (Fig. 4*e*) in the cellular extracts (Fig. 4, lane 3), as compared with the untreated cell extracts (Fig. 4, lane 1), suggesting that the lysosomal degradation plays a major role in LAMP/Gag and DC-LAMP/Gag processing. The treatment with E64c also

had a significant inhibitory effect on the degradation of the Gag chimeras (Fig. 4, *b* and *f*, lanes 7 and 8); however, this effect was much stronger in the case of the DC-LAMP/Gag chimera than for LAMP/Gag. In contrast, treatment with the proteasome inhibitor MG132 did not significantly alter the amount of LAMP/Gag or DC-LAMP/Gag present in the supernatant or cell extracts (Fig. 4, *c* and *g*). Although MG132 appeared to have a small effect on the accumulation of DC-LAMP/Gag in the cell extract, this result may reflect a nonspecific effect of the MG132 on lysosomal proteases (46). One caveat is that the protease inhibitors could be affecting cell viability and protein synthesis; however, additional control experiments indicated that the limiting step for Gag accumulation



**FIGURE 5.** Immune responses induced by LAMP/*gag* and DC-LAMP/*gag* immunization. *a*, *Gag*-specific induction of IFN- $\gamma$  production. Mice, in groups of two animals for each individual experiment, were injected on days 1 and 21 with 50  $\mu$ g of pITR plasmids encoding the *gagN*, LAMP/*gag*, or DC-LAMP/*gag* chimera. Animals were sacrificed on day 31, and splenocytes were prepared for assay of CD4<sup>+</sup> T cell responses as described in *Materials and Methods*. Supernatant IFN- $\gamma$  levels were measured by capture ELISA. *b–d*, *Gag*-specific CD8<sup>+</sup> response. Groups of mice were immunized with 50  $\mu$ g of the indicated pITR plasmids, then boosted i.p. 3 wk later with 10<sup>7</sup> PFU of rVV-*gag-pol*. Five days later, 10  $\mu$ g of the H-2K<sup>d</sup>-binding HIV-1 *Gag* peptide was injected i.v., and splenocytes were harvested after 2 h and analyzed for ex vivo activity. *b*, Flow cytometry quantification of H-2K<sup>d</sup> tetramer-binding CD8<sup>+</sup> T lymphocytes; *c*, flow cytometry quantification of *Gag*-specific CD8<sup>+</sup> T lymphocytes producing IFN- $\gamma$ ; *d*, cytolytic activity in a 4-h <sup>51</sup>Cr release assay using P815 target cells pulsed with the H-2K<sup>d</sup>-binding HIV-1 *Gag* peptide at a 100:1 E:T ratio. Nonspecific lysis (using unpulsed P815 target cells) was <5% for all groups (data not shown). *e*, *Gag*-specific induction of IL-4 production. Mice were immunized twice with the indicated plasmids and boosted once with rVV-*gag-pol*. *Gag*-specific IL-4 production was analyzed 15 days later by ELISPOT. Bars indicate the number of SFC per 10<sup>6</sup> total splenocytes. *f*, Ab response. Mice were immunized as in *a*, and 10 days after the second immunization, the sera were collected, and IgG1 and IgG2a were analyzed by ELISA. Bars indicate OD measured in the indicated dilutions. All the graphs represent the results of immune assays obtained from at least three individual experiments performed in a similar manner and combined according to each vaccine plasmid. No results were excluded from the analyses. The error bars indicate SD.

in these cells is the degradation and not the rate of protein synthesis.

It is noteworthy that in all the supernatant control extracts there was a much greater amount of LAMP/Gag than DC-LAMP/Gag (which was present in minute amounts), suggesting that the latter chimera is not secreted to the same extent as LAMP/Gag. This difference was further analyzed by treating the cells with brefeldin A to block the translocation of the chimeras from the ER to the Golgi (Fig. 4, *d* and *h*). This interference with trafficking resulted in higher intracellular levels of both Gag chimeras. Additionally, LAMP/Gag secretion was inhibited greatly by brefeldin A, which had no noticeable effect on the small amount of DC-LAMP/Gag present in the supernatant. We also observed that the Gag protein of DC-LAMP/Gag was much more susceptible to degradation than that of LAMP/Gag, as indicated by the increased concentration of DC-LAMP/Gag in extracts of cells incubated with the lysosome protease inhibitors or with brefeldin A (Fig. 4, *e*, *f*, and *h*).

In summary, these experiments indicate that LAMP/Gag and DC-LAMP/Gag follow the same pattern during their initial intra-

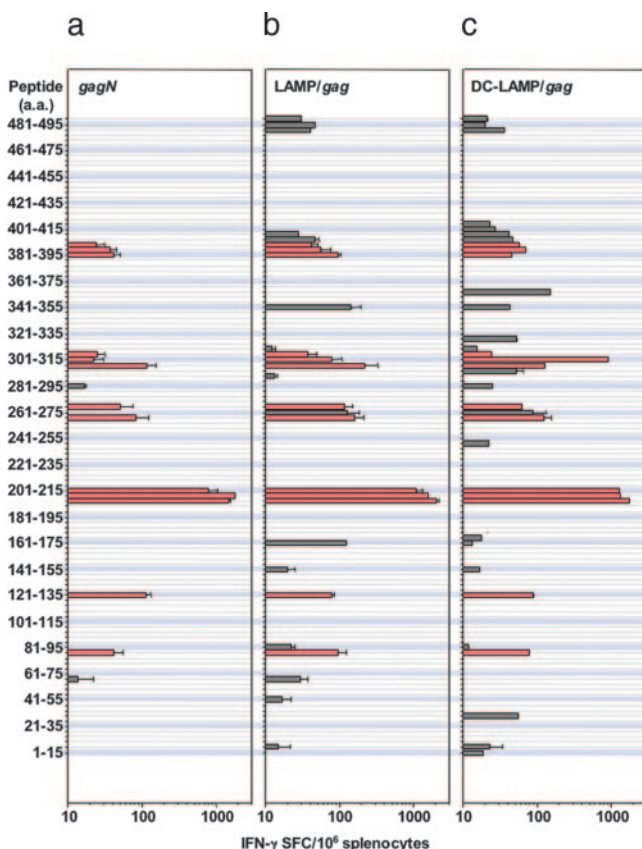
cellular trafficking, but their pathways diverge after they pass through the *trans*-Golgi.

#### Immunization with the DC-LAMP/gag plasmid induces an enhanced and predominantly type 1 Th immune response

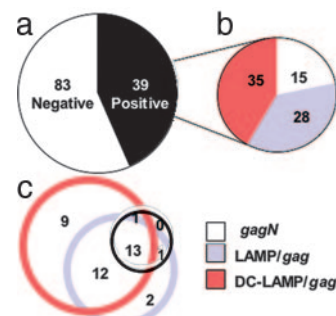
In previous studies, we have shown that Ag targeting with LAMP-1 to the MHC II-containing compartment results in an enhanced and balanced Th immune response (22, 24, 28). In the present study, we have compared the overall immune response elicited by DC-LAMP/gag and LAMP/gag plasmid DNA immunization. Both DC-LAMP and LAMP targeting promoted greater CD4<sup>+</sup> and CD8<sup>+</sup> responses than did the nontargeted native Gag Ag (Fig. 5), and there were no statistically significant differences in the magnitude of the CD4<sup>+</sup> IFN- $\gamma$  secretion in response to the Gag Ag (Fig. 5*a*). In addition, the CD8<sup>+</sup>-specific responses elicited by the DC-LAMP and LAMP chimeras were also similar, as determined by the tetramer staining (Fig. 5*b*), intracellular IFN- $\gamma$  staining (Fig. 5*c*), and killing detected by chromium release assay (Fig. 5*d*). However, marked differences were detected in terms of the number of IL-4 SFC elicited by the two chimeras (Fig. 5*e*). LAMP/gag immunization induced ~4-fold more IL-4 secreting cells than did the DC-LAMP/gag. In addition, the IgG isotype profiles of the LAMP/Gag and DC-LAMP/Gag chimeras were different (Fig. 5*f*). The response elicited by the DC-LAMP/gag plasmid was predominantly IgG2a (IgG1:IgG2a ratio = 0.33), whereas that elicited by LAMP/gag was predominantly IgG1 (IgG1:IgG2a ratio = 9.00). It is noteworthy that native gag elicit very little Ab responses and did not induce any detectable IL-4 by real-time PCR or ELISA in our previous studies (24). This difference in the Th profile has many possible causes, including differences in trafficking and cellular localization, greater secretion of the LAMP/Gag protein, faster degradation of the DC-LAMP/Gag, some specific immunomodulatory properties of the luminal domains of LAMP-1 and DC-LAMP molecules, or differences on epitope processing.

#### LAMP/gag and DC-LAMP/gag DNA plasmids induce a broader repertoire of T cells than does native Gag

Because the LAMP-1/gag and DC-LAMP/gag chimeras promote Gag trafficking through subcellular compartments distinct from those used by native Ags and because increased Gag expression (24), together with structural changes in the Gag protein chimeras and functions of the LAMP molecules, may promote significant differences in Gag processing, we investigated the effect of



**FIGURE 6.** DNA plasmid chimeras elicited a greater diversity and different pattern of T cell epitopes. Mice were immunized twice with the indicated DNA plasmids and boosted with rVV-Gag-Pol at 3- to 4-wk intervals. Fifteen days after the boost, splenocytes were cultured with 122 15-mer Gag peptides (with 11 overlapping amino acids), and the frequency of IFN- $\gamma$ -producing cells was analyzed by ELISPOT, as described in *Materials and Methods*. The average number of SFC/10<sup>6</sup> splenocytes, obtained from five different experiments, was calculated, and the results for each group were compared with the negative control (empty pITR immunized and boosted with rVV-gag-pol) group. The bars indicate the epitope responses that were significantly different ( $p < 0.05$ ) from that of the control group. Red filled squares represent the peptides that induced a response in all the three groups. *a-c*, The T cell response for each plasmid DNA vaccine.



**FIGURE 7.** Statistical analysis of the T cell IFN- $\gamma$  response to Gag peptides mediated by gag DNA plasmids. Venn diagram representation of the results obtained from the experiments in Fig. 6. *a*, Sum of the epitopes that induced positive response in all the analyzed groups; *b*, number of epitopes that induced a positive response in each specific group (gagN-white, LAMP/gag-blue, DC-LAMP/gag-red); *c*, number of epitopes that induced a positive response in one or more groups (intersection).

LAMP-1- and DC-LAMP-mediated Ag trafficking on the repertoire of T cells primed by a panel of Gag peptides. Mice were immunized twice with the pITR vector control, native *gag*, LAMP/*gag*, or DC-LAMP/*gag* DNA plasmids, then boosted with a vaccinia/*gag* preparation expressing Gag in its native form to expand the population of activated T cells. Fifteen days later, the spleens were harvested for immune assays.

The peptide-specific T cell responses induced by each of the DNA constructs were analyzed by IFN- $\gamma$  ELISPOT against 122 individual peptides (15 aa each, with an 11-aa overlap) encompassing the entire p55Gag sequence (Fig. 6). The average T cell responses against each peptide by the various groups of vaccinated mice were compared by a nonparametric *t* test to the background value for mice primed with the pITR control plasmid, followed by a vaccinia/*gag* boost. The responses were considered positive when the *p* value was <0.05 and the average response was >10 IFN- $\gamma$  SFC/10<sup>6</sup> splenocytes. One group of peptides was identified as dominant epitopes and induced T cell activation in all groups tested (Fig. 6, red bars); these included the already-described CD8<sup>+</sup> AMQMLKETI epitope (47). In addition, the mice immunized with LAMP/*gag* and DC-LAMP/*gag* showed a response to other peptides. Collectively, the results showed that 39 of the 122 peptides induced a positive IFN- $\gamma$  response (Fig. 7*a*). Among these, 15 induced responses in the native *gag*-immunized mice, 28 in the LAMP/*gag*-immunized mice, and 35 in the DC-LAMP/*gag*-immunized mice (Fig. 7*b*). Moreover, of the 39 peptides, only 13 induced a response in all three groups (Fig. 7*c*), whereas the others were unique to one or two groups of immunized mice. Taken together, these results indicate a broader range of T cell *Gag*-specific responses induced by chimeras bearing LAMP or DC-LAMP.

Further statistical analyses were performed to test whether each DNA plasmid induced activation of a different repertoire of T cells. We compared the average IFN- $\gamma$  SFC number to each Gag peptide for every two groups (Gag vs LAMP/Gag, Gag vs DC-LAMP/Gag, and LAMP/Gag vs DC-LAMP/Gag). The results in-

dicated that specific groups of peptides induced major differences in the frequency of IFN- $\gamma$ -producing cells, as much as ~100-fold (data not shown).

The results were also analyzed applying a higher threshold level (100 IFN- $\gamma$  SFC), and, in all cases tested, the repertoire of peptides recognized by the T cells elicited by each plasmid was significantly different (data not shown).

The differences on the peptide responses are not "all or nothing," and we think that the diversity on the ELISPOT responses reflect the processing efficiency of specific peptides. The chimeras probably produce different yields of a given peptide, and some peptides may seldom be produced in enough amounts to reach the T cell activation threshold whereas others more frequently do.

#### Threshold of CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation

MHC I and II presentation of Gag peptide epitopes was evaluated by measuring the IFN- $\gamma$  response to Gag peptides by purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells obtained from mice immunized with native *gag* or LAMP/*gag* (Table I). T cells responding to the individual peptides, including the immunodominant CD8<sup>+</sup> AMQMLKETI epitope (aa 197–205), were elicited in all cases by each of the immunogens, indicating that the epitopes generated by LAMP- and DC-LAMP-targeted Ags can access the MHC I and MHC II molecules and produce the same immunodominant epitopes as the native Gag Ag. Although Ag TCR are ideally expected to exhibit a single specificity, they actually show a level of degeneracy that permits a certain level of cross-reactivity between similar peptides (48). Considering this fact and the fact that epitopes are an average of 9 aa long, we can conclude that a 15-mer peptide can contain more than one MHC-restricted T cell epitope, implying that a positive response to a 15-mer peptide does not necessarily indicate that the T cells are responding to the same epitope.

To address at least in part the issue of the diversity of T cell clones activated by Gag peptides, we purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells from native *gag*- and LAMP/*gag*-immunized mice and then

Table I. Native *gag* and LAMP/*gag* elicit different T cell activation thresholds in response to a panel of Gag peptides<sup>a</sup>

Cell Type	Peptide (aa)	MAC		
		Plasmid Used		MAC Ratio ( <i>gag</i> /LAMP/ <i>gag</i> )
		<i>gagN</i> (peptide $\mu$ g/ml)	LAMP/ <i>gag</i> (peptide $\mu$ g/ml)	
CD8 <sup>+</sup>	57–71	5	7.5	0.7
	193–207	0.2	0.2	1
	197–211	0.2	0.2	1
	201–215	5	5	1
CD4 <sup>+</sup>	77–91	10	1.25	8
	121–135	2.5	15	0.2
	257–271	0.2	1	0.2
	265–275	12.5	0.2	6.2
	297–311	2	2	1
	301–315	20	2	10
	305–319	20	20	1
	381–395	2.5	0.2	12.5
	385–399	20	2.5	8
	389–403	20	2.5	8
	393–407	20	7.5	2.7

<sup>a</sup> Mice were immunized twice with *gagN* or LAMP/*gag* DNA plasmids, followed by a vaccinia/*gag* boost, as described in Figs. 6 and 7. Fifteen days after the boost, the splenocytes were cultured with different doses of the indicated Gag 15-mer, ranging from 0.2 to 20  $\mu$ g/ml. The results indicate the minimum Ag concentration necessary to induce 50% of the maximum response. In some experiments, CD4<sup>+</sup> and CD8<sup>+</sup> were purified and cultured with the indicated peptides, and the cell population involved in the IFN- $\gamma$  production was determined, as indicated. The MAC ratios were calculated to facilitate comparison of the affinities of each group. MAC ratios < 1 indicate a greater affinity of the T cells from *gag*-immunized mice; ratios > 1 indicate a greater affinity of the T cells from LAMP/*gag*-immunized mice.

incubated them with a range of concentrations of selected peptides that induced positive responses in both groups of immunized mice (Table I). In this sense, among the polyclonal T cell population, we may have clones with distinct affinities for a given MHC-peptide complex, and as we lower the concentration of peptide, only the T cell clones with higher affinity would be activated. The concentration required to induce 50% of the maximum IFN- $\gamma$  SFC response (minimum stimulatory Ag peptide concentration (MAC)) was determined for each peptide (Table I). Assuming that the affinity of the peptide for the MHC molecule was the same for both groups of immunized mice, the differences in the threshold of activation of the T cell response by a peptide could be attributed to differences in the affinity of the TCR for the MHC-peptide. We observed that some peptides at high concentrations activated similar numbers of IFN- $\gamma$  SFC in both groups of immunized mice. However, when the responses were compared at different peptide concentrations, some the peptides showed different thresholds of activation for the native *gag*- and LAMP/*gag*-immunized mice. In general, the native *gag* induced CD8<sup>+</sup> T cell clones that reacted with peptides 57–71, 193–207, 197–211, and 201–215 with affinities similar to those of the T cell clones elicited by LAMP/*gag* DNA immunization, as indicated by the MAC ratios of ~1:1 (0.7, 1, 1, and 1, respectively). In contrast, the MAC ratios for the majority of the CD4<sup>+</sup> responses were >1, with the exception of those for two peptides (121–135 and 257–271), indicating that the LAMP/*gag* plasmid immunization may elicit CD4<sup>+</sup> T cell clones with a higher affinity for the MHC-peptide epitopes than do the other two Ags.

#### LAMP-targeted p55Gag elicits a broader B cell epitope response

The effect of the association of *Gag* with LAMP-1 or DC-LAMP on the repertoire of activated B lymphocytes was also analyzed. Sera obtained from the immunized mice were tested by ELISA with 12 pools of *Gag* peptides, each containing 10 overlapping peptides. IgG specific for a dominant central region in the *Gag* sequence was detected in the sera from each group of immunized mice. However, IgG specific for a carboxyl-terminal region of *Gag* was found only in LAMP/*gag*- and DC-LAMP/*gag*-immunized mice (Table II), indicating that immunization with these DNA chimeras exposed additional B cell epitopes. Assays with individual peptides spanning aa 401–501 showed several *Gag* sequences reactive with serum of mice immunized with LAMP/*gag* and DC-LAMP/*gag* but not native *gag* (Table III). These epitopes at the carboxyl-terminal end of *Gag* may have been made available as a result of a higher-level expression of *Gag* in the form of the LAMP chimera or of structural distortions created by the fusion with LAMP protein. Once the mice are primed, these epitopes appear to be stimulated by native *Gag* protein because the titers of the Ab response against these epitopes were increased by inoculation with vaccinia/*gag*.

## Discussion

The data shown here demonstrated that targeting of HIV-*Gag* by LAMP-1 and DC-LAMP molecules results in Ag trafficking along distinct vacuolar endosomal/lysosomal pathways and that the Ag chimeras elicited distinct modulatory T cell helper responses. Ag processing by the endosomal/lysosomal mechanisms produced the same immunodominant CD4<sup>+</sup> and CD8<sup>+</sup> T cell epitopes as the native *Gag* Ag and revealed cryptic B and T cell epitopes that were not detectable in immunizations with native *Gag* Ag.

DNA plasmids are thought to generate immune response by three main processes: 1) direct transfection of bone marrow-derived APC; 2) transfer of protein Ags to bone marrow-derived

Table II. LAMP-targeted p55Gag elicits a broader B cell epitope response<sup>a</sup>

Gag Peptide Pools (10 15-mer peptides)	Plasmid Immunization		
	<i>GagN</i> (SI) <sup>b</sup>	LAMP/ <i>gag</i> (SI)	DC-LAMP/ <i>gag</i> (SI)
(aa 1–51)	– <sup>c</sup>	–	–
(aa 40–91)	–	–	–
(aa 80–131)	–	–	–
(aa 120–171)	–	–	–
(aa 160–211)	4+	3+	–
(aa 200–251)	4+	6+	2+
(aa 240–291)	4+	3+	3+
(aa 280–331)	3+	4+	3+
(aa 320–317)	–	–	–
(aa 360–411)	–	–	–
(aa 400–451)	–	10+	12+
(aa 440–501)	–	10+	2+

<sup>a</sup> Mice were immunized twice with the indicated DNA plasmids and then boosted with vaccinia/*gag* at 3- to 4-wk intervals: sera were collected 10–15 days after the boost, and IgG levels were analyzed by capture ELISA using 12 pools, each containing 10 *Gag* peptides (15 mer, with an 11 aa overlap) to coat the plates. The OD values obtained for each pool were compared with those obtained in pITR-immunized control mice. The results in the table represent the fold increase in the OD for the respective pool over that for the control group. Values > 2-fold were considered negative.

<sup>b</sup> SI, Stimulation index = OD vaccine/OD control mice.

<sup>c</sup> –, No Ab response was detected.

APC by cross-priming; and 3) direct presentation by modified muscle cells. Several studies have demonstrated that both direct transfection of APC and cross-priming are the major mechanisms in vivo, and, in the first case, the role of the trafficking signal in Ag processing is clear. In the case of cross priming, however, a possible mechanism for Ag transfer would be through exosomes, which can contain the peptide Ags already processed before delivering to the APC. Alternatively, when the chimeric Ag is transferred intact, it would traffic according to their molecular signal to the respective cellular compartment of the new cell. In ongoing studies, we have observed by biochemical and electron microscopy that exosome-like structures are being secreted from LAMP/Ag-transfected cells. More studies are being performed currently to identify processed peptide bound to the MHC molecule of the secreted exosomes.

The proteolysis of LAMP/*Gag* and DC-LAMP/*Gag* are highly affected by lysosomal inhibitors and brefeldin A but apparently not by MG132, confirming their traffic along the endosomal/secretory pathway and suggesting a minor role for the proteasomes on their processing. Moreover, the analysis of the intracellular trafficking of LAMP/*Gag* and DC-LAMP/*Gag* chimeras by confocal microscopy analysis (Ref. 24; Fig. 2) and subcellular density fractionation indicate that LAMP/*Gag* is present in the same compartments as the endogenous LAMP-1, whereas DC-LAMP/*Gag* is mainly present in fractions not related to the endogenous LAMP-1. It was also observed that LAMP/*Gag*, but not DC-LAMP/*Gag*, is secreted in large quantities by the transfected cells. These observations are in agreement with previous reports of the occurrence of LAMP-1 in secreted exosomes and as soluble molecules (49–53), whereas DC-LAMP have not been found in a secreted form. In addition, it was demonstrated recently that vacuolar endosomal/lysosomal compartments containing MHC II molecules are restructured from multivesicular bodies to long tubules in DC (40, 54, 55) and macrophages (56). DC-LAMP molecules, that are first located at the internal multivesicular bodies' membranes, migrate to specialized tubules upon APC maturation, whereas LAMP-1 is not observed in these differentiated tubular structures. On the other hand, the migration of MHC II molecules to the cell surface is related to the

Table III. Characterization of Gag peptides inducing a B cell response after immunization with LAMP/gag or DC-LAMP/gag<sup>a</sup>

Peptide (aa)	Plasmid Immunization			
	pITR (control) (SI) <sup>b</sup>	GagN (SI)	LAMP/gag (SI)	DC-LAMP/gag (SI)
401–414	– <sup>c</sup>	–	–	–
405–419	–	–	6+	4+
409–423	–	–	3+	3+
413–427	–	–	–	–
417–431	–	–	–	–
421–435	–	–	–	–
425–439	–	–	–	5+
429–443	–	–	–	–
433–447	–	–	–	–
437–451	–	–	–	–
441–455	–	–	–	–
445–459	–	–	–	–
449–463	–	–	–	–
453–467	–	–	–	–
457–471	–	–	–	–
461–475	–	–	–	–
465–479	–	–	–	–
469–483	–	–	12+	–
473–487	–	–	11+	3+
477–491	–	–	11+	–
481–495	–	–	–	–
485–501	–	–	–	–

<sup>a</sup> Mice were immunized, and sera were collected as described in Table II. Individual peptides from Gag peptide pools 11 and 12 (Table II) were used to coat the ELISA plates, and the serum IgG levels against these peptides were then analyzed by capture ELISA. The OD values obtained for each peptide were compared with those for the pITR-immunized control mice. The results in the table represent the fold increase in the OD for the respective pool over that for the control group.

<sup>b</sup> SI = Stimulation index = OD vaccine/OD control mice.

<sup>c</sup> –, No Ab response was detected.

exocytosis mechanisms, and LAMP-1 has been observed associated with the MHC molecules in vesicles and cell membrane (40, 55). These findings support the idea that these two proteins are regulated by distinct trafficking mechanisms in the vacuolar endosomal/lysosomal pathways.

Ag processing of the cytoplasmic Gag is expected to be mediated by the proteasomal complex or, in the case of the LAMP/Gag and DC-LAMP/Gag chimeras, by the vacuolar endosomal/lysosomal enzymes. Given that specific endosomal/lysosomal compartments vary in their proteolytic content and that protease-mediated cleavage of an Ag may destroy or generate T cell epitopes (57), Ag targeting along distinct endocytic pathways will determine the various types of proteases to which an Ag is exposed and, therefore, the peptides that will result from its degradation. In our system, it is important to note that the significant differences in the glycosylation patterns of LAMP-1 and DC-LAMP may also affect the accessibility of proteolytic enzymes to the Gag Ag (58).

In our study, the epitope-specific response generated by targeting of the Gag protein to different processing compartments was systematically investigated by using an overlapping peptide library. Groups of mice were primed with control LAMP-1 plasmid, native gag, LAMP/gag, or DC-LAMP/gag DNA and then boosted with the recombinant vaccinia-gag virus. Analysis of the ELISPOT IFN- $\gamma$  response to a panel of 122 Gag peptides encompassing the entire protein demonstrated that some of the peptides behaved as dominant epitopes and induced strong T cell activation in all tested groups, whereas the response to other peptides showed to be enhanced in or unique to mice primed with a specific plasmid DNA construct. The difference in the repertoire of the T cell responses that we observed as a result of this distinct Ag targeting is consistent with previous reports. Streptococcus protein Ags have

been shown to require different proteases for processing and presentation of two epitopes (59), and the Ag uptake mechanism influences their presentation by determining which subcellular compartment is targeted (60). It was also reported that association of lymphocytic choriomeningitis virus minigene epitopes with another member of the LAMP protein family, lysosomal integral membrane protein II, increased the CD4<sup>+</sup> response to one epitope but decreased another, suggesting that different Ag processing pathways can indeed influence on the priming of a T cell response to a specific Ag (61).

The different T cell repertoires primed by LAMP/gag and DC-LAMP/gag chimeras were also associated with a broader T cell response to the Gag protein. Fernandes et al. (23) have also demonstrated that association of OVA Ag with targeting signals to different cellular compartments, such as LAMP-1 and the transferrin receptor, influenced in the breadth of epitopes presented to cloned T cells, with the LAMP targeting signal being associated with a broader response. However, the broader response induced by LAMP/gag and DC-LAMP/gag chimeras may not be entirely attributed to qualitative differences in the peptide products after protein processing but could also reflect differences in the level of Ag expression and access to MHC loading compartments.

Regardless of the differences in the enzymatic processing of the Gag protein chimeras, the major immunodominant epitopes were not deleted or altered during processing. It is likely that other mechanisms, such as peptide-MHC affinity or TCR repertoire availability, directed the T cell immunodominance to the same CD4<sup>+</sup> and CD8<sup>+</sup> epitopes. The fact that native Gag protein is localized to the cytoplasm, whereas the chimeras trafficked through the endosomal/lysosomal pathway, could suggest a preferential generation of MHC II epitopes by these chimeras. However, our evaluation of CD4<sup>+</sup>- and CD8<sup>+</sup>-specific responses by tetramer staining, <sup>51</sup>Cr release assay (Fig. 5), and ELISPOT assays (Fig. 6) has demonstrated that immunization with the chimeric plasmid DNA constructs elicited consistent MHC I- and II-restricted epitopes, including the same major CD8<sup>+</sup> epitope AMQMLKETI.

In addition, we observed that immunization with these three constructs also influenced the B cell repertoire, a result that may be related to differential exposure of Gag protein sequences resulting from modifications in the protein folding structure and/or cellular localization caused by the chimerization with LAMP-1 or DC-LAMP.

It is interesting that the Th cytokine profile induced by DC-LAMP/gag appeared to be biased toward type I Th cytokines, with a predominantly IgG2a response and a lower number of IL-4 SFC cells than were elicited by LAMP/gag. In contrast, the LAMP-1/gag chimera showed a Th type II bias, with a greater IL-4 response and a predominantly IgG1 Ab response. These distinct immune modulatory effects of the LAMP-1 and DC-LAMP chimeras may reflect differences in their cellular trafficking or Ag processing or still the biological functions of the molecules.

Several mechanisms of HIV escape from immune system have been described, including mutations within the epitopes themselves (62–64) or in regions that interfere with epitope processing (65). Also, disease progression to AIDS is associated with viral escape from immune control. Therefore, eliciting an immune response to a maximally diverse repertoire of HIV epitopes may delay disease progression, since the loss of one epitope-specific immunodominant response could be compensated by the response to another. Indeed, the breadth of memory HIV-specific CTL has been found to be inversely correlated with viral load and to be important for the control of virus replication (66). In addition, heterozygosity in class I HLA-A, HLA-B, or HLA-C is correlated

with slower progression to AIDS, whereas homozygosity in one or more loci is associated with a more rapid progression (67). Thus, the increased T cell repertoire elicited by the LAMP-1 and DC-LAMP chimeras may be an efficient strategy for the design of effective anti-HIV vaccines. However, we need to perform additional experiments to address the biological significance of eliciting broader B and T cell responses in viral protection, and we are currently planning a nonhuman primate challenge study using the SIV model. Immunization against native simian HIV Ags with DNA or vaccinia virus preparations has been shown to increase the breadth of the CTL response after simian HIV challenge (68), and it is possible that endosomal/lysosomal targeting, such as that provided by the LAMP/gag or DC-LAMP/gag chimeras, could further enhance the diversity of the epitope response. Finally, Kavanagh et al. (69) recently showed that DC transfected with HIV Nef targeted to the lysosome by LAMP was able to expand a greater repertoire of T cells from HIV patients than the DC transfected with HIV Nef. These results imply that LAMP-targeted Ag generated a greater or distinct repertoire of peptides than the native Nef, and it was considered as a promising approach to HIV immunotherapy.

In summary, we have described an approach to modulating Ag intracellular trafficking that can generate an extended repertoire of activated T cells, a result that has potential application to HIV vaccine design.

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## Disclosures

The authors have no financial conflict of interest.

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