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Comparison of DNA vaccines producing HIV-1 Gag and LAMP/Gag chimera in rhesus macaques reveals antigen-specific T-cell responses with distinct phenotypes

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ABSTRACT

Optimized DNA expression vectors encoding the native HIV-1 Gag or a fusion of Gag with the lysosomal membrane associated protein 1 (LAMP) were compared for immunogenicity upon intramuscular DNA delivery in rhesus macaques. Both vaccines elicited $CD4^+$ T-cell responses, but with significant differences in the phenotype of the Gag-specific cells: the native Gag induced $CD4^+$ responses with a phenotype of central memory-like T cells ($CD28^+$ $CD45RA^-$), whereas the LAMP/Gag chimera induced $CD4^+$ responses with effector memory phenotype ($CD28^-$ $CD45RA^-$). Antigen-specific T cells producing both IFN-γ and TNFα were found in the animals receiving the native Gag, whereas the LAMP/Gag chimera induced humoral responses faster. These results demonstrate that modification of intracellular Gag trafficking results in the induction of distinct immune responses. Combinations of DNA vectors encoding both forms of antigen may be more potent in eliciting anti-HIV-1 immunity.

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1. Introduction

The major histocompatibility complex type II (MHC II) processing compartment (MIIC) is a lysosomal-like organelle that plays an important role regulating T-cell antigen processing and presentation. Targeting antigens directly to the MIIC compartment of professional antigen-presenting cells (APCs) have been shown to successfully enhance and modulate the immunogenicity of several antigens [1–15]. Lysosomal associated membrane protein-1 (LAMP-1) normally is present in MIIC of immature APCs. The effects of LAMP-1 targeting on the elicited immune responses have been analyzed in immunization studies in several mouse strains, macaques, and in humans. Several laboratories have reported that targeting antigens with the tyrosine based motif (YQTI) of LAMP-1 [1–3] can enhance the immune responses against a variety of antigens from many pathogens, including HPV-16 E7 and E6; HIV-1 Gag, Env gp120, Env gp160, and Nef; West Nile virus preM-E; dengue 2 preM-E; SARS coronavirus N; listeriolysin "O"; and tumor antigens such as the thyroid hormone receptor (TSHR), human telomerase reverse transcriptase (hTert) and human melanosomal antigen (MAGE-3) [4–15]. In general, the LAMP-1/antigen chimeras were shown to elicit broader repertoire of antigen-specific $CD4^+$ T-cell responses, greater functional avidity, augmented proliferative response and ability to secrete a variety of interleukins. The increased $CD4^+$ T-cell mediated responses produced by the LAMP-1/antigen formulation are thought to medi-

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ate enhancement in B cell and $CD8^+$ T-cell responses, as well as the development of immunological memory. When compared to the non-targeted molecules, LAMP-1/antigen chimera elicited higher antibody titers, increased antibody affinity and neutralizing activity, as well as expansion of the number of recognized B-cell epitopes. Similarly, the $CD8^+$ responses were found elevated in several LAMP-1/antigen chimeric systems, as assessed by tetramer staining, IFN-γ enzyme-linked immunospot (ELISPOT), cytotoxicity assays, and the functional avidities and T-cell response repertoires of $CD8^+$ cells. The longevity of the immunological memory of B cells and $CD8^+$ cells was also increased in animals immunized with LAMP-1/chimeras [4–15].

In previous studies, vaccination of mice with DNA plasmid expressing the LAMP/Gag chimera showed strong, broad cellular and humoral immune responses [9,11–14]. This vaccine construct was also tested in a pilot study using five rhesus macaques, and induced humoral and cellular immune responses, which were associated with a sustained activation of B lymphocytes as well as $CD4^+$ and $CD8^+$ T cells [12].

Here, we performed a direct comparison of the immune responses elicited upon intramuscular DNA injection of plasmids producing the native Gag or the human LAMP/gag chimera in a cohort of 22 Indian rhesus macaques. Both of the plasmids contain the RNA/codon optimized HIV-1 *gag* sequence [16–18], and were cloned into the same plasmid backbone, suitable for clinical studies. The results show that each of the HIV-1 antigen formulation delivered as naked DNA vaccines have unique immunogenic properties and induce qualitatively distinct cellular and humoral immune responses.

2. Materials and methods

2.1. Plasmids

pCMV-gag.kan plasmid contains the RNA optimized HIV-1 *gag* p55 gene from HXB2 inserted into the mammalian expression plasmid pCMV.kan [16,19], which consists of a plasmid backbone optimized for growth in bacteria, the human cytomegalovirus (CMV) promoter, the bovine growth hormone (BGH) polyadenylation site and the kanamycin resistance gene. The RNA optimized *gag* gene contains multiple nucleotide changes that destroy the previously identified RNA inhibitory/instability sequences but do not affect the coding potential [16–18]. The pCMV-LAMP/gag.kan plasmid contains the optimized HIV-1 *gag* between the human LAMP luminal domain and the LAMP transmembrane and cytoplasmic (TM/cyt) tail domain [12]. This modified LAMP/gag DNA vector has a backbone that does not contain the AAV-ITR sequences or the penicillin resistance gene used in previous studies [9,11–14]. Plasmids used for vaccination of rhesus macaques had DNA purity of 96% and endotoxin levels less than 0.33 EU/mg and were obtained with support from the NIH/NIAID *Reagent Resource Support Program for AIDS Vaccine Development*, Quality Biological, Inc. (Principal Investigator, Ronald Brown).

2.2. Analysis of protein expression

DCEK cells, a line of murine fibroblasts doubly transfected with class II MHC E^k and type 1 intercellular adhesion molecule (ICAM-1) [20,21], were plated in 6-well plates (2×10^6 cells/well) and transfected with plasmid DNA (4 μg) using the FuGENE™ 6 (Roche Applied Science, Indianapolis, IN) transfection reagents according to the manufacturer's instructions. The protein concentration for each cell extract was quantified prior to Western blot analysis and equal amounts of proteins were loaded per well. Western immunoblot analysis and antigen capture enzyme-linked immunosorbent assay (ELISA) were performed as described previously [11].

2.3. Immunization of macaques

Twenty-two healthy 4- to 8-kg male Indian rhesus macaques were maintained in the non-human primate facility of Southern Research Institute, Frederick, MD, USA. Animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee, according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, National Research Council, National Academy Press, 1996. Each animal was immunized via the intramuscular (IM) route five times at weeks 0, 4, 14, 24 and 36 with 2 mg of optimized *gag* or LAMP/gag plasmid using a biojector. HIV-1 Gag-specific humoral and cellular immune responses were determined as described in Fig. 2. Isolation of peripheral blood mononuclear cells (PBMCs) and HIV-1 Gag-specific IFN-γ ELISPOT assay were performed as described [12]. The ELISPOT assays were considered valid only when the negative controls had less than 5 spot forming cells (SFC) and the positive controls more than 500 SFC. Responses were considered positive when the sample produced more than 10 spot and the signal in the presence of peptides minus two standard deviations was higher than the signal in the medium control sample plus two standard deviations. Mucosal samples including mouth swabs, nasal and rectal washes were collected periodically. Antibody titers were determined as described [14], using either a 1:100 dilution of the individual sera samples collected over time or end-point dilutions using a pool of the sera collected at 2 weeks post vaccination 3 and 5, respectively. The reported optical density (OD) corresponds to the value minus three times the OD value of a non-immune serum. The reported titers correspond to the reciprocal of the highest serum dilution that gave a three times higher OD value than the corresponding dilution of a non-immune serum. The determinations were performed in duplicate.

2.4. Flow cytometric analysis

PBMC were resuspended at a density of 10^6 cells/ml in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine, in the presence or absence of a pool of HIV-1 gag 15-amino acid (aa) peptides overlapping by 11-aa (114 peptides spanning the entire Gag sequence, at a final concentration of 1 μg/ml for each peptide). The peptide stimulation was performed without the addition of costimulary anti-CD49 or antiCD28 antibodies. As positive control, cells were treated with the enterotoxin B of *Staphylococcus aureus* (SEB) at a final concentration of 20 ng/ml. Cells were treated for 6 h with monensin to prevent protein secretion, and cell surface staining was performed using the following antibody cocktail: CD3, CD4, CD8, CD45RA and CD28. Cells were washed twice, and fixed and permeabilized with Cytofix/Cytoperm (BD, Pharmingen) according to the manufacturer's instructions. In some experiments, an alternative surface staining adding to the cocktail a monoclonal antibody against CCR7 was performed. Intracellular cytokine detection was performed using a cocktail of antibodies against IFN-γ, IL-2 and TNFα labeled either with the same fluorochrome (APC) or with FITC, APC and PE Cy7, respectively. For each sample at least 10^5 T cells were acquired in the FACSAria flow cytometer (BD, Pharmingen) and the data were analyzed using the FlowJo platform (Tree Star, Inc., Ashland, OR). Typical background in the absence of peptide stimulation was below 0.02%. Peptide stimulated samples were considered positive if they were at least two-fold higher than the medium control.

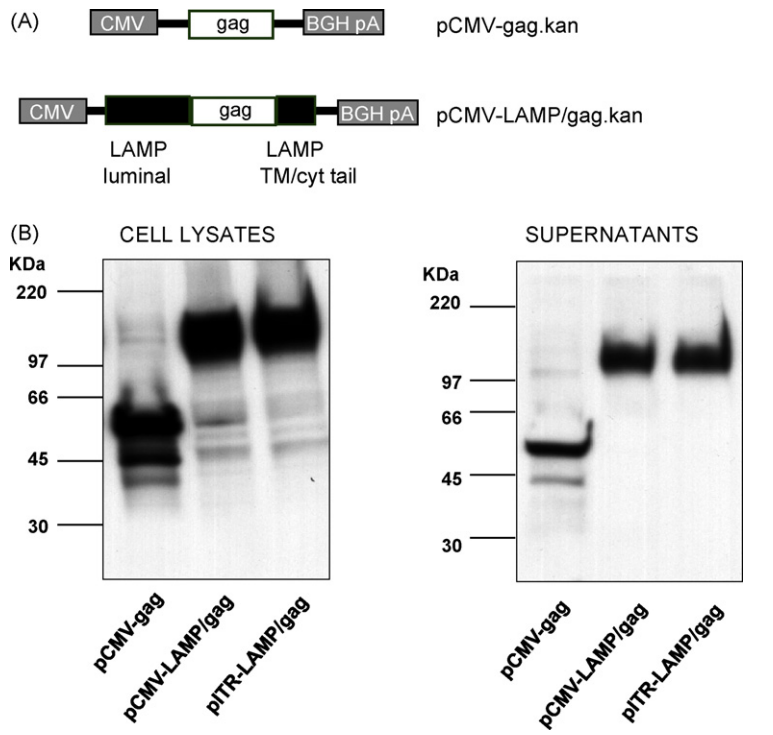

Fig. 1. HIV-1 Gag expression in vitro. (A) Schematic representation of the HIV-1 gag expression plasmids. The RNA optimized HIV-1 p55 gag gene was expressed as the authentic viral Gag or as LAMP/Gag chimera using the pCMV.kan expression vector containing the CMV promoter and the bovine growth hormone polyadenylation site (BGH pA). The luminal domain of LAMP, HIV-1 p55 gag (open box), and the LAMP transmembrane and cytoplasmic domains (TM/cyt) are indicated. (B) HIV-1 Gag expression in cell lysates (left panel) and supernatants of transfected DCEK cells (right panel). Western blot analysis of cell lysates and supernatants probed with anti-Gag monoclonal antibody. The molecular weight markers are shown on the left side. The lanes show: pCMV-gag.kan encoding the native Gag protein from an optimized *gag* gene; pCMV-LAMP/gag.kan encoding optimized Gag as a chimera inserted proximal to the transmembrane domain of the complete LAMP cDNA); pITR-LAMP/gag.Amp encoding the same LAMP/Gag protein chimera inserted in the pAAV-ITR vector [12].

2.5. Statistical analyses

Statistical analyses (two-tailed non-parametric *t* test) and graphs were produced using GraphPad Prism Version 4.0a for Macintosh (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Gag expression in vitro by cells transfected with optimized gag and LAMP/gag DNA plasmids

Eukaryotic expression plasmids were constructed as described in Section 2 using an RNA/codon optimized HIV-1 *gag* gene and LAMP/*gag* chimera inserted into the pCMV.kan vector (Fig. 1A). Gag expression in transfected DCEK cells was examined by Western immunoblot analysis (Fig. 1B) using an anti-Gag antibody. pCMV-LAMP/gag.kan, like the previously reported AAV-ITR-plasmid expressing LAMP/gag [12] produce high levels of Gg protein. Gag was found in both the cell-associated and the extracellular fractions of transfected cells. We noted further that the fusion to LAMP led to a small increase in protein accumulation in the supernatant (Fig. 1B, right panel) compared to the levels obtained from pCMV-gag.kan encoding the native Gag protein, possibly due to protein stabilization of the LAMP chimera. We have made similar observations studying other HIV-1 and SIV proteins fused to LAMP (V. Kulkarni, C. Bergamaschi, M. Rosati, G.N. Pavlakis, B.K. Felber, unpublished). LAMP/Gag chimera is identical to the protein produced from the previously published vector and was found to colocalize with MHC class II molecules as expected [14]. Electron microscopy analysis further showed that virus-like particles were only produced from

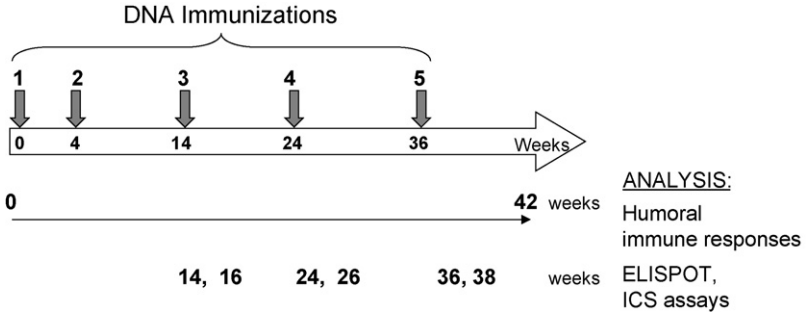

Fig. 2. Schedule of HIV-1 gag DNA vaccination. Twenty-two Indian rhesus macaques were immunized IM by a biojector using 2 mg/ml of pCMV-gag.kan or pCMV-LAMP/gag.kan on weeks 0, 4, 14, 24 and 36. Blood samples were assessed for Gag-specific cellular (ELISPOT, ICS) at the indicated time points and humoral immune responses were assessed every 2 weeks. Data are shown in Figs. 3–7.

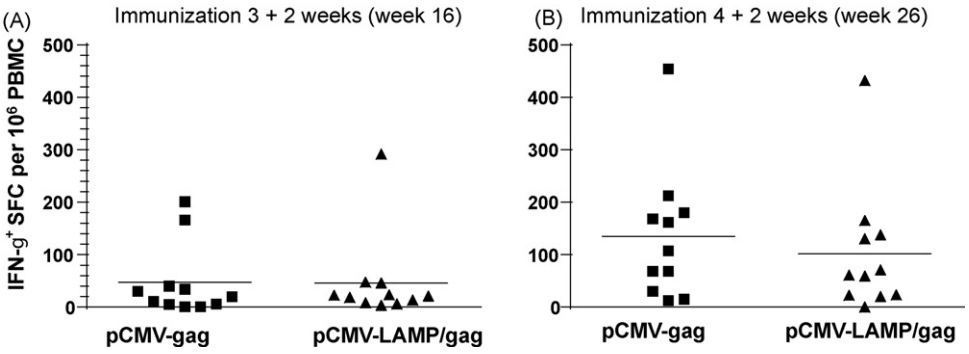

Fig. 3. HIV-1-specific IFN-γ ELISPOT responses in the immunized rhesus macaques. PBMCs were analyzed by IFN-γ ELISPOT assays 2 weeks after the 3rd (A) and 4th (B) immunization. The individual ELISPOT values and the mean values are shown.

Gag as expected [16] and not from LAMP/Gag protein (data not shown).

3.2. Vaccination protocol

Twenty-two Indian rhesus macaques divided into two experimental groups were vaccinated with 2 mg of the plasmids pCMV-gag.kan or pCMV-LAMP/gag.kan as outlined in Fig. 2. The DNAs were injected via the intramuscular route (IM) using biojector™ (Bioject Medical Technologies Inc, Oregon), a needle-free injection devise. Each animal received 5 vaccinations at weeks 0, 4, 14, 24, and 36, respectively. Cellular immune responses were analyzed by ELISPOT assay and intracellular cytokine staining (ICS) at the day of the 3rd, 4th, and 5th immunization, as well as 2 weeks later. Humoral immune responses were monitored every 2 weeks for a total follow-up time of 42 weeks.

3.3. HIV-1-specific cellular immune responses induced after DNA immunizations

We analyzed cellular immune responses by both ELISPOT and Flow cytometric analysis. For the ELISPOT assay, PBMC were stimulated with HIV-1 Gag peptide pool (15-aa overlapping by 11-aa) and the frequency of IFN-γ secreting cells was measured. All the macaques immunized with the DNA plasmids showed <100 IFN-γ spot forming cells (SFC)/10^6 PBMCs up to the 3rd immunization (Fig. 3A). The ELISPOT values increased after the 4th DNA vaccination in both the gag and LAMP/gag chimera groups (Fig. 3B). No further increase was observed after the 5th immunization. We also examined the ELISPOT responses to an amino- and carboxy-terminal pool of Gag peptides and found that the antigen-specific immune responses induced by Gag and LAMP/gag were primarily targeted to peptides spanning the amino-terminal portion of the protein (data not shown).

3.4. Analysis of cellular immune responses in T-cell subsets

Next, we performed flow cytometric analysis to detect HIV-1 Gag-specific cytokine positive T cells in peripheral blood from the immunized macaques. As outlined in Fig. 4A, $CD3^+$ T cells were identified within the main lymphocyte population, gated based on their forward and side scatter characteristics. The T cells were also divided in $CD4^+$ or $CD8^+$ cells (middle panel). In addition, subsets of memory T cells were identified based on the presence or absence of CD28 and CD45RA staining: $CD3^+CD45RA^-CD28^+$ represents the population of central memory-like (CM) T cells and $CD3^+CD28^-$ represents effector memory (EM) T cells (middle panel). In some experiments, staining for CCR7 was also performed, and we found that more than 90% of the T cells show a similar pattern of CD28 and CCR7 expression in all the animals analyzed (data not shown). This is in agreement with the designation of $CD3^+CD45RA^-CD28^+$ cells as T cells with central memory-like surface markers. Using a combination of directly conjugated monoclonal antibodies for cell surface immunophenotyping and intracellular cytokine staining (IFN-γ, TNFα, IL-2) of the T cells, the frequency of cytokine$^+$ cells (as sum of IFN-γ, TNFα, and IL-2 secreting cells) was established within each of these populations (Fig. 4A, bottom).

Fig. 4B and C shows the analysis of the cytokine$^+$ T cells in the two vaccine groups at two time points (immunization 3 and immunization 5 + 2 weeks). At the time of the 3rd DNA immunization there was no difference in the frequency of antigen-specific CD3+ T cells between the two groups (Fig. 4B, upper panel). The number of cytokine$^+$ HIV-1 Gag-specific T cells increased at two weeks after the 3rd DNA vaccination in both the gag and LAMP/gag chimera groups (data not shown), and these levels remained similar in the two groups for the entire follow-up period including at 2 weeks post 5th immunization (Fig. 4C, upper panel). Although the magnitude of the cellular responses was higher by the intracellular cytokine staining assay compared to the ELISPOT, both assays were qualitatively in agreement in that they did not show any significant difference in the amount of Gag-specific T cells between the two groups of immunized macaques.

We further evaluated the $CD4^+$ (middle panel) and $CD8^+$ (bottom panel) subsets of T cells. We did not find any significant differences between the animals vaccinated with native Gag and the LAMP/Gag chimeras in the number of $CD4^+$ cells (Fig. 4B and C, middle panels). Analysis of antigen-specific immune responses in the $CD8^+$ T-cell population showed a significant increase in Gag-specific immune responses over the course of the immunization (day of 3rd immunization compared to 2 weeks post 5th immunization; $p = 0.0140$) in animals that received the CMV-gag.kan plasmid (Fig. 4B and C, bottom panels). At 2 weeks post 5th immunization, the group vaccinated with the native Gag had significantly more cytokine-producing $CD8^+$ T cells, compared to the LAMP/Gag group ($p = 0.0464$, Mann–Whitney test). These data suggest that the fusion to the LAMP signal may induce a more polarized type of immune response, which is controlled predominantly by $CD4^+$ T helper responses and results in earlier antibody production (see below). On the other hand, our analysis shows that vaccination with the native Gag expressing plasmid induces both $CD4^+$ and $CD8^+$ T-cell responses (Fig. 4B and C).

3.5. Distinct Gag-specific immune responses in T-cell subsets

We further identified the subpopulation of the cytokine$^+$ Gag-specific $CD4^+$ T cells by separating the cells with Central Memory-like (CM; $CD28^+$ $CD45RA^-$) and Effector Memory (EM; $CD28^-$ $CD45RA^-$) phenotype as outlined in Fig. 4A. Despite the similar frequency of the Gag-specific T cells (Fig. 4C, top panel) and $CD4^+$ T cells (Fig. 4C, middle panel and Fig. 5, top panel), a

significant qualitative difference in the phenotype of the antigen-specific CD4+ T cells was found (Fig. 5, middle and lower panels) at 2 weeks post 5th immunization. Macaques that received the vector expressing the native Gag protein produced antigen-specific CD4+ T cells with a CM phenotype (Fig. 5, middle panel). Since we did not use CCR7 in these stainings, we cannot exclude that some of the CD28+CD45RA− antigen-specific T cells are transitional cells lacking CCR7 expression. This population of cells was significantly increased at 2 weeks post 3rd vaccination and remained at elevated levels up to the 5th immunization (Fig. 5, middle panel). In contrast, macaques that received the LAMP/Gag chimera showed antigen-specific CD4+ T cells with EM phenotype (Fig. 5, lower panel). A significant increase in the EM cell population was detected after the 5th immunization with the LAMP/Gag chimera but these changes were absent in animals immunized with the vector encoding the native Gag protein.

Thus, comparison of macaques immunized with DNA vectors expressing the native Gag and LAMP/Gag chimera, respectively,

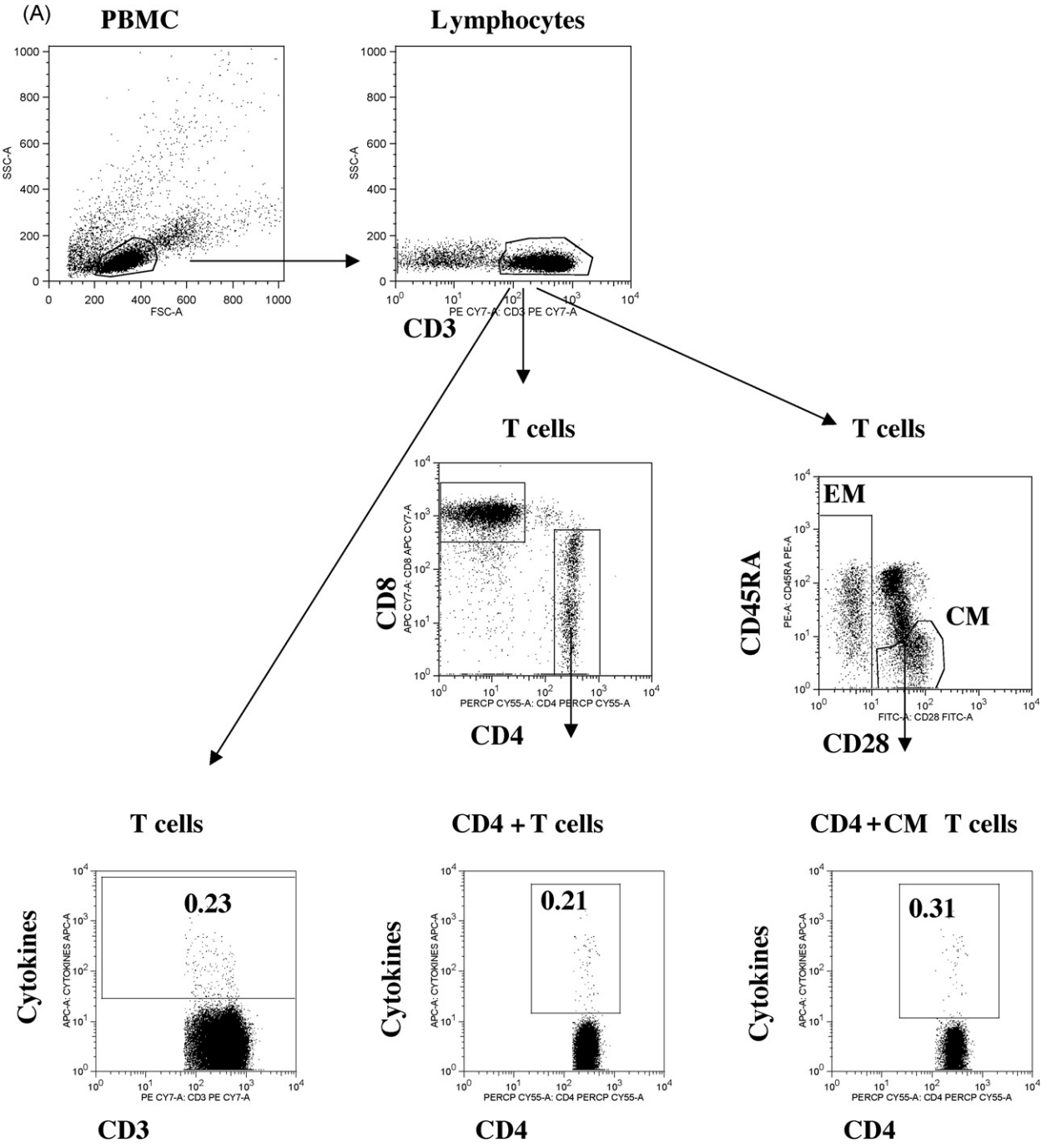

Fig. 4. Identification of cytokine positive Gag-specific T cells. (A) Outline of flow cytometric analysis to detect cytokine positive T-cell subsets. The main lymphocyte population was gated according to the forward and side scatter and T cells were identified by the expression of CD3. The T cells were analyzed according to their CD4 and CD8 expression, and identified as CM (CD3+CD45RA−CD28+) or EM T cells (CD3+CD28−). Expression of cytokines (using a combination of APC-conjugated IFN-γ, TNFα and IL-2) is shown in CD3+ T cells, total CD4+ and CM CD4+ T lymphocytes from peptide stimulated rhesus macaque PBMC. (B and C) Frequency of cytokine+ Gag-specific lymphocytes in vaccinated animals. PBMC were isolated from the vaccinated animals at the following time points: 3rd immunization (week 14, B), and 2 weeks after the 5th immunization (week 38, C). The plots show the frequency of HIV-1 Gag-specific cytokine positive total CD3+, CD4+ and CD8+ T cells (upper, middle and lower panels respectively) determined by flow cytometric analysis after in vitro stimulation with a Gag peptide pool.

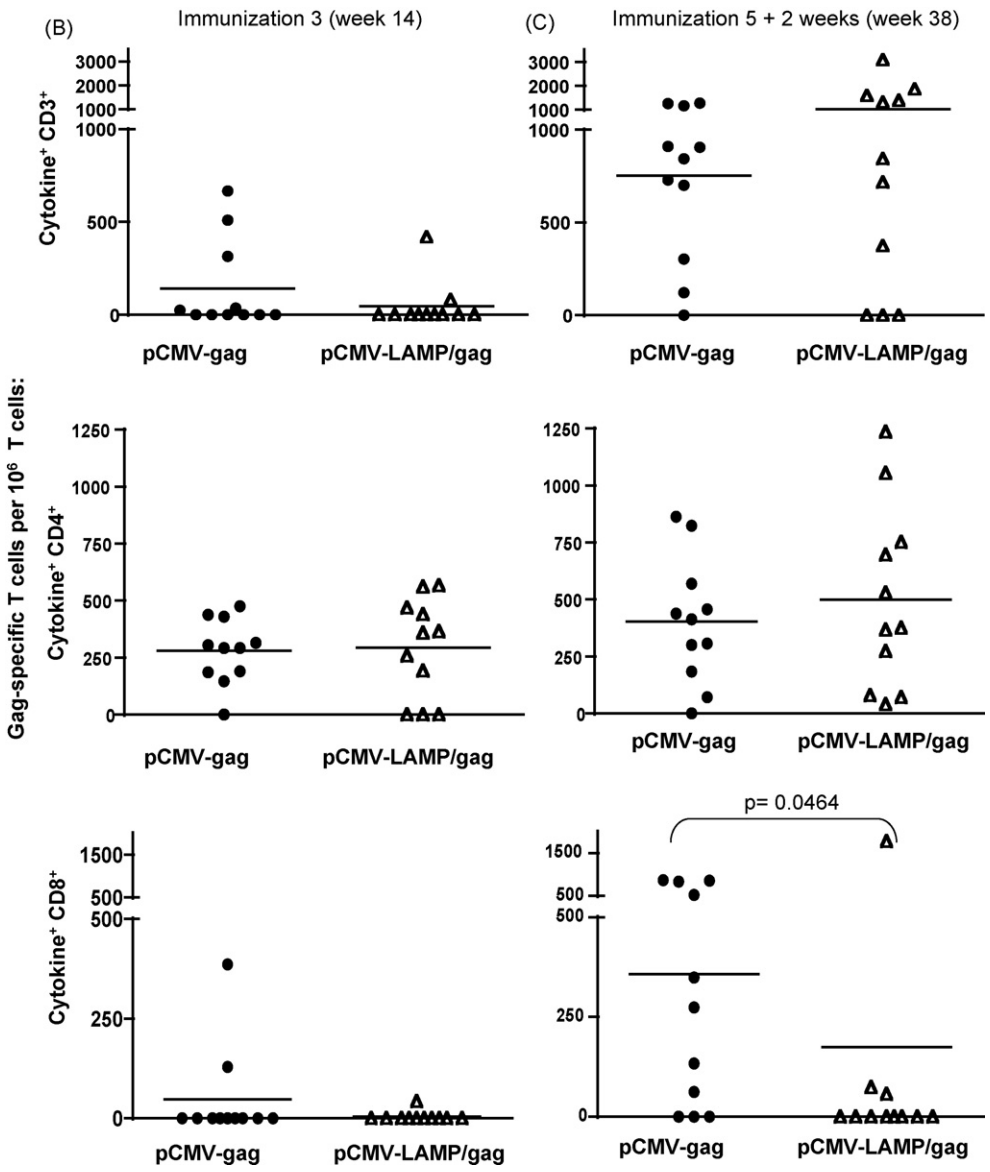

Fig. 4. (*Continued*).

revealed that they induce significantly different phenotypes of cytokine-producing Gag-specific $CD4^+$ T cells, although both vaccine groups showed similar frequencies of Gag-specific $CD3^+$ T cells (Fig. 4C) as well as cytokine-producing $CD4^+$ T cells (Fig. 5) in the circulating T lymphocytes. These findings suggest that this qualitative difference is indeed related to the manner in which Gag is processed and presented (native protein or fusion with LAMP).

3.6. Induction of IFN-γ and TNFα-producing antigen-specific T cells upon DNA vaccination with the Gag expression plasmid

In addition to measuring the production of the combination of cytokines (Figs. 4 and 5), we also measured the frequency of the Gag-specific T-cells secreting individual cytokines (Fig. 6). Using Flow cytometric analysis, $CD3^+$ T cells were identified within the main lymphocyte population, gated based on their forward and side scatter characteristics, and the frequency of IFN-γ, TNFα, or IL-2 secreting cells was established as outlined in Fig. 6A. Upon SEB stimulation, total T cells produce different levels of cytokines, with IL-2 > IFN-γ > TNFα.

Production of IFN-γ, TNFα and IL-2 upon stimulation with Gag peptides was monitored in PBMC samples by flow cytometry as outlined above (Fig. 6A) at 2 weeks post the 4th immunization. We analyzed the Gag-specific T cells in the two groups of vaccinated animals (Fig. 6B). The levels of Gag-specific IFN-γ producing T cells in both vaccine groups were not significantly different, as expected

Table 1
Humoral immune response end-point titers.[a].

Groups	Week 16 2 weeks post vaccination #3	Week 38 2 weeks post vaccination #5
pCMV-gag.kan ($n = 11$)	<1:100	1:2700
pCMV-LAMP/gag.kan ($n = 11$)	1:2700	1:8100

[a] End-point titer of pooled plasma at weeks 16 and 38, after 3rd and 5th DNA immunizations, respectively. The reported titers correspond to the reciprocal of the highest serum dilution that gave a three times higher OD value than the corresponding dilution of a non-immune serum.

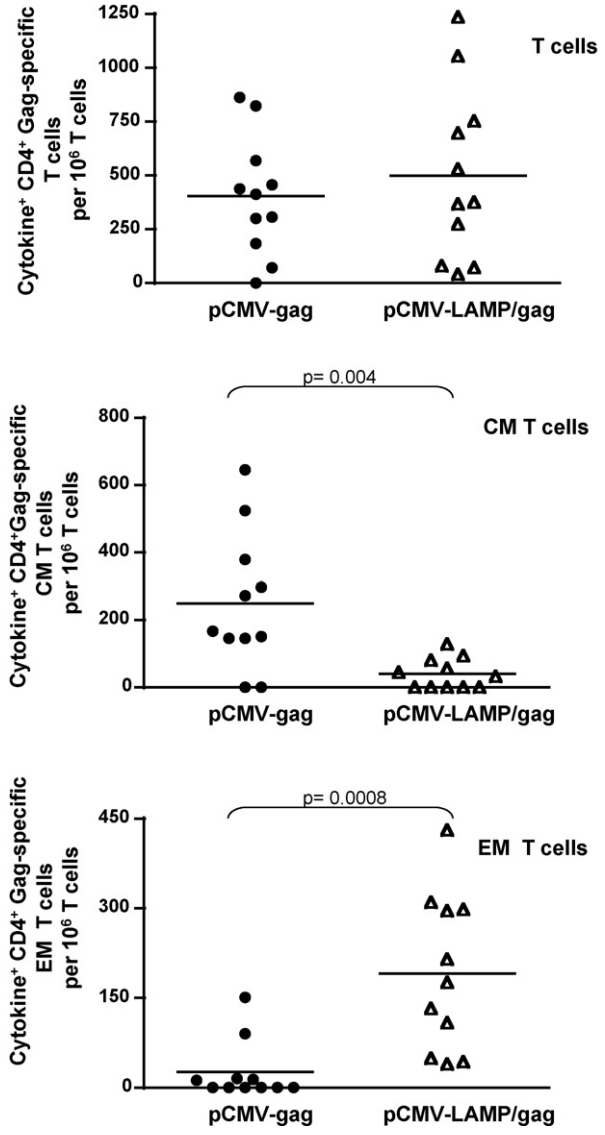

Fig. 5. Phenotype of cytokine$^+$ Gag-specific CD4$^+$ T-cell subsets in vaccinated animals. PBMC from immunized animals were analyzed by flow cytometry as described in Fig. 4A. Comparison of the measurements at 2 weeks post 5th immunization of Gag-specific cytokine$^+$ CD4$^+$ T cells (top panel), CD4$^+$ central memory T cells (CM, middle panel) and CD4$^+$ effector memory T cells (EM, bottom panel).

(Fig. 3). We did not detect a Gag-specific IL-2 producing population, despite its high frequency among the SEB stimulated T cells (Fig. 6A). In contrast, we detected significantly increased levels of TNFα producing T cells only in macaques vaccinated with the plasmid expressing the native HIV-1 Gag (Fig. 6B). We further found that vaccination with this plasmid induced T cells producing both TNFα$^+$ and IFN-γ$^+$ (Fig. 6B, bottom panel). This data shows that vaccination with the native Gag is able to induce dual functional antigen-specific memory T cells.

Thus, immunization with the two different Gag expression plasmids producing native Gag and LAMP/Gag induced different types of immune responses (CD4 and CD8 versus CD4 only; CD4 CM T cells versus CD4 EM T cells, respectively). Interestingly, we noted that only vaccination with native Gag elicited dual functional antigen-specific immune responses characterized by the production of both IFN-γ and TNFα.

3.7. Higher humoral immune responses induced by LAMP/Gag

One remarkable feature of the DNA-encoded LAMP-targeted antigens has been the increase in antibody-mediated responses of immunized macaques after two DNA immunizations [12]. These results were verified and extended in the present study, where macaques immunized with the LAMP/Gag chimera, showed higher humoral immune responses (Fig. 7A and Table 1). Fig. 7A shows the analysis of the plasma antibody levels measured at a 1:100 dilution of the individual animals sampled over time, and Table 1 shows the end-point titers of the pooled sera at 2 weeks after 3rd and 5th vaccination, respectively. All the LAMP/Gag immunized macaques developed antibodies against HIV-1 Gag after the 3rd immunization (Fig. 7A). On the other hand, 3/11 (27%) of native Gag vaccinated macaques developed antibodies against HIV-1 Gag after the 3rd immunization and this percentage was gradually elevated to 36% (4/11) after the 4th and 82% (9/11) after the 5th immunization. High anti-Gag immunoglobulin G (IgG) antibody titers (1:2700) were present in the pooled sera after 3 DNA immunizations with LAMP/*Gag* chimera as compared to <1:100 with the native Gag, indicating more effective antibody priming with the LAMP/*Gag* chimera (Table 1). The antibody titers reached their highest values (1:8100) after the 5th immunization (at week 38) in the LAMP/Gag vaccinated animals. Upon repeated vaccination, the group of the Gag vaccinated animals showed greatly improved responses with a titer of 1:2700. Thus, humoral immune responses to Gag develop faster in the LAMP/Gag vaccinated animals.

Serum samples of the immunized macaques were also assayed for IgA production. Significant but low levels of serum IgA were detected in all the vaccinated macaques (Fig. 7B). In addition to serum, there was presence of low levels of Gag-specific IgA in nasal washes in macaques vaccinated with both the optimized Gag and the LAMP/Gag chimera. Other secretions, including mouth swabs and rectal washes did not show the presence of IgA antibodies.

4. Discussion

In this study, we have analyzed the immunogenicity of optimized DNA vectors upon intramuscular inoculation in rhesus macaques by comparing plasmids encoding two different forms of the HIV-1 Gag protein: (i) the native myristoylated Gag, which forms secreted virus-like particles [16] and (ii) the LAMP/Gag chimera which targets the antigen to the lysosomal compartment and may promote enhanced processing and association with MHC class II molecules. The development of humoral and cellular immune responses was monitored.

We found that both forms of Gag induced humoral immune responses. The measurements of anti-Gag IgG demonstrated high antibody titers in animals receiving the LAMP/gag plasmid two weeks after the 3rd DNA inoculation. Additional DNA inoculations of the plasmid producing the native Gag protein were required to reach comparable levels to those obtained with the LAMP/gag vector after three inoculations. Thus, humoral immune responses developed with different kinetics and were detectable significantly earlier in animals vaccinated with the LAMP/gag construct. The increase in antibody production observed by LAMP/Gag was similar to that observed by DNA vaccines using Gag fusion to MCP-3 chemokine [19].

Analysis of the cellular immune responses showed that the frequency of total Gag-specific T cells in peripheral blood was similar in both groups and that Gag-specific CD4$^+$ cells were induced by both antigens. It is noteworthy that, in spite of similar frequency of the antigen-specific CD4$^+$ T cells, major differences were found in the memory phenotype and cytokine response profile between the two groups. Animals vaccinated with the LAMP/Gag chimera

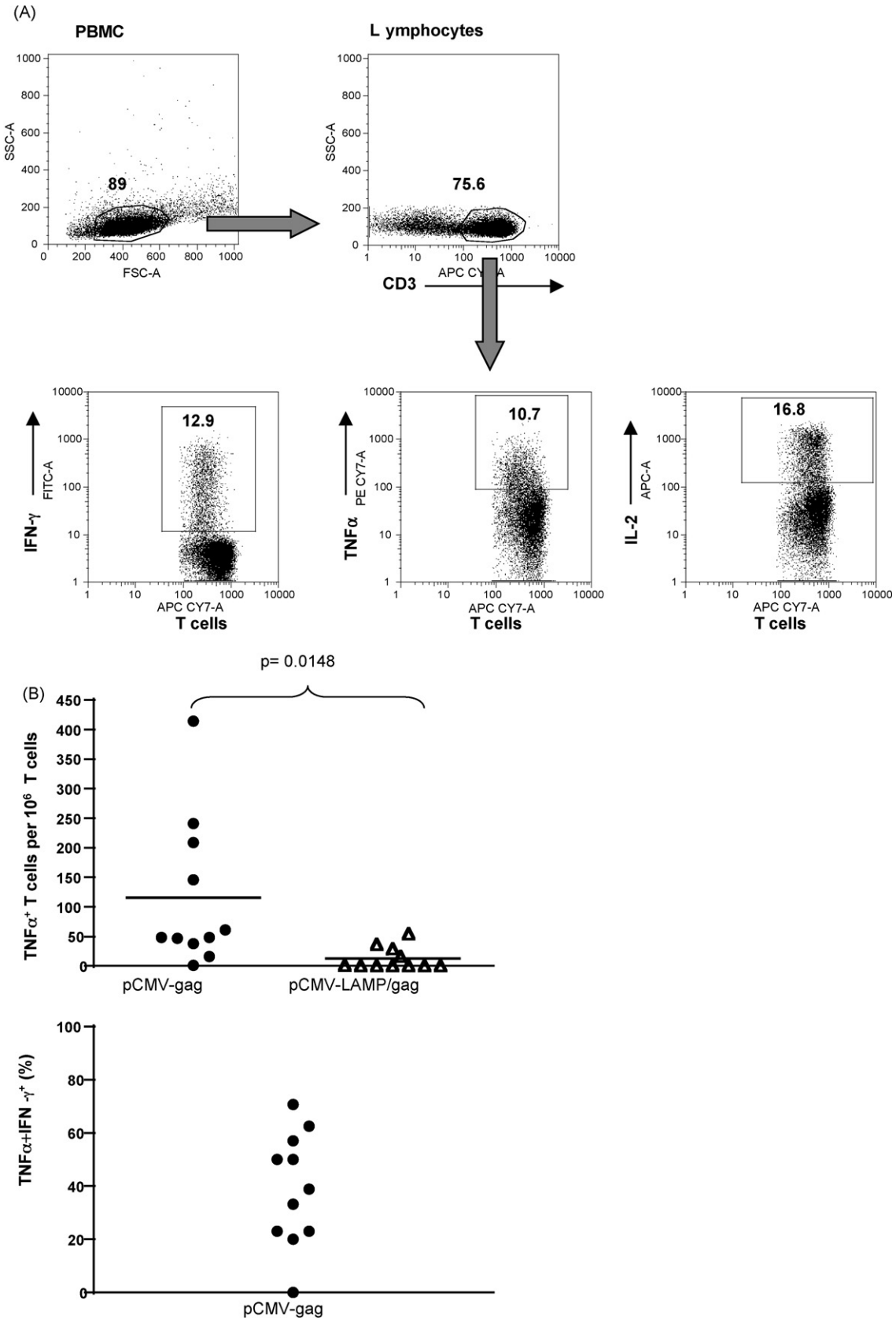

Fig. 6. Detection of single and dual cytokine$^+$ T cells. (A) The main lymphocyte population was gated according to the forward and side scatter and T cells were identified by the expression of CD3. Expression of IFN-γ, TNFα or IL-2 is shown in total CD3$^+$ T lymphocytes from SEB stimulated rhesus macaque PBMC. (B) The frequency of Gag-specific TNFα secreting T cells at 2 weeks post 4th immunization in the DNA-vaccinated macaques is shown. The bottom panel shows the percent of dual functional (IFN-γ and TNFα positive) T cells induced by the immunization with the native Gag expression plasmid as determined by Boolean gates analysis.

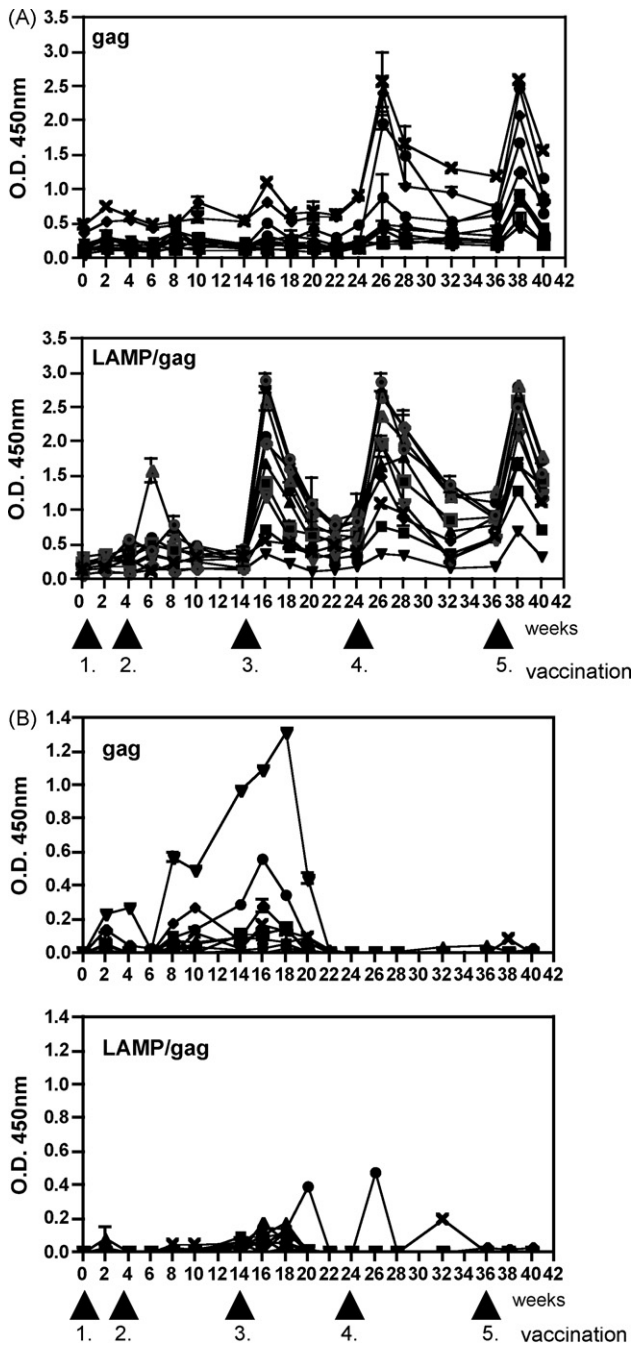

Fig. 7. Development of humoral immune responses in vaccinated rhesus macaques. Gag-specific humoral responses of rhesus macaques immunized with plasmids expressing either HIV-1 Gag or LAMP/Gag chimera. (A) Plasma samples were collected before vaccination and every 2 weeks thereafter. Gag-specific IgG responses were measured in individual macaques by ELISA using 1:100 dilution of the plasma. (B) Measurement of IgA antibody responses in the plasma of the vaccinated macaques. OD values are reported after background (corresponding to three times the values obtained with a non-immune serum) subtraction.

developed predominantly effector memory $CD4^+$ T-cell responses, characterized by the lack of CD28 expression among the antigen-specific T cells. In contrast, animals vaccinated with the plasmid encoding native Gag developed $CD4^+$ T-cell responses with a central memory-like phenotype ($CD45RA^-$ $CD28^+$). The mechanisms leading to the differences in the frequencies of CM and EM elicited by these antigens formulations are not understood. Whether or not these differences have an impact in the protection induced by the vaccine upon challenge with pathogenic virus will require additional studies using similar vectors encoding SIV antigens and subsequent virus challenge.

The presence of HIV-1-specific central memory T cells induced by DNA vaccination should be considered a positive aspect of the response, because these cells are responsible for the long-term immunological memory. It has been reported that the preservation of T cells with central memory phenotype correlates with lower viremia and lack of progression towards immune deficiency in SIV infected macaques [22–24]. On the other hand, depletion of $CD4^+$ central memory T cells is a landmark of disease progression [22,25,26]. Clearly, a broad and balanced immune response including both central and CD8 effector memory T cells is desirable in a vaccine against HIV-1. Although central memory cells have been associated with viral control, effector memory cells disseminated in mucosal sites may be a first line of defense. The different properties of the two gag plasmids used in this study open the possibility of combining plasmids encoding native and modified antigens for the generation of optimal responses, which may combine central and effector memory cells.

Another significant difference between the two groups of immunized macaques was the presence of antigen-specific TNFα-producing and IFN-γ- plus TNFα-producing T cells only in animals receiving the plasmid encoding the native Gag. The presence of these dual functional cells secreting two cytokines could be of great importance, because it has been shown that the preservation of these cells correlate better with protection during natural infection than the magnitude of the total antigen-specific T-cell responses [24]. It is noteworthy that the T-cell responses elicited by the intramuscular inoculation of Gag-encoding DNA vectors result mainly in IFN-γ producing cells, with low frequency of $TNF\alpha^+$ cells and absence of IL-2 production. These findings do not appear to be related to the detection sensitivity of our assay because the SEB-stimulated cells used as positive control produced all three cytokines and the frequency of $IL\text{-}2^+$ cells was higher than that of either IFN-γ or TNFα secreting T cells (see Fig. 6A).

HIV-1 infected individuals progressing towards AIDS have low frequency of multifunctional antigen-specific T cells, in spite of having a good frequency of effector T lymphocytes [27]. In contrast, HIV-1 infected individuals classified as long-term non-progressors typically have multifunctional memory T cells producing IFN-γ and TNFα [27], which is similar to the macaques immunized with the DNA expressing the native Gag. IL-2 production by memory T cells has also been associated with better prognosis [28]; we did not find significant numbers of Gag-specific $IL\text{-}2^+$ T cells in any of the vaccinated animals. The lack of IL-2 production could be overcome by improving the DNA delivery method. In fact, our recent results using DNA immunization of rhesus macaques by electroporation demonstrate increased cell-mediated responses with generation of IL-2 secreting cells [33,34].

The route and method of DNA delivery (i.e. intramuscular injection, electroporation) influences the breadth of the immune responses and the type of the induced antigen-specific T cells [29–35]. Recent data indicate that electroporation of DNA plasmids encoding SIV or HIV-1 Gag increased DNA vaccination efficiency in rhesus macaques [29–35] and led to the induction of antigen-specific multifunctional T cells [31,33,34]. In the present report, we demonstrate that different forms of HIV-1 Gag (the native HIV-1 Gag and LAMP/Gag chimera) are able to induce diverse immune responses in rhesus macaques. To our knowledge, this is the first report directly comparing different DNA encoded HIV-1 Gag proteins in the macaque model, demonstrating that altering antigen intracellular trafficking modulates immune responses. In addition, the use of 11 rhesus macaques per group allowed for statistical analysis and demonstrated statistically significant difference in immune responses. Previous immunogenicity experiments in mice using vectors encoding the native HIV-1 Gag or chimeras with several

forms of LAMP proteins support this observation [9]. In the mouse study, we found broader epitope recognition in animals immunized with either LAMP-1 or DC-LAMP chimeras compared to the native form of Gag. In addition, we also found higher frequency of Gag-specific T cells, measured by either ELISPOT or tetrameric staining of $CD8^+$ T cells, induced by the LAMP/Gag vaccine. Furthermore, we reported that mice immunized with the LAMP-1/Gag chimera showed a predominant Th type 2 response with IgG1 production and presence of IL-4^+ Gag-specific T cells, as compared to mice immunized with the DC-LAMP/Gag construct in which the antibody response was predominantly mediated by IgG2a and the frequency of IL-4 producing T cells was significantly lower. These immunogenicity studies in mice demonstrated the critical role of the specific antigen targeting in eliciting distinct immune responses. In another DNA vaccination study in macaques, we further reported that fusion of SIV Gag with the chemokine MCP-3 led to increased humoral immune responses compared to the native Gag [16,19]. Future studies are required to address the question whether combining DNA plasmids expressing different modified forms of an antigen provides better protection, as suggested by some experiments [16,19]. In summary, modification of the posttranslational fate of an antigen, in addition to optimization of RNA expression, provides another approach to improve immunogenicity of antigens, to amplify desirable vaccine responses, and to further increase the efficiency of DNA vaccines.

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