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Nef modulates the immunogenicity of Gag encoded in a non-infectious HIV DNA vaccine

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Abstract

Gag-CD8⁺ T cell responses are associated with immune control of HIV infection. Since during HIV infection Nef impairs T cell responses, we evaluated whether deletion of *nef* from a non-infectious HIV DNA vaccine ($\Delta 4$ Nef(+)), creating $\Delta 5$ Nef(-), would affect its immunogenicity. When compared with $\Delta 4$, mice injected with $\Delta 5$ developed significantly lower CD8⁺ T cell responses to Gag, but no significant change in the responses to Env. *In vitro*, deletion of Nef abrogated the induced cell death, production of virus-like particles and release of Gag from transfected cells. Thus the effect of Nef in causing extrusion of Gag might adjuvant the CD8⁺ T cell responses to Gag in DNA vaccine.

Keywords

HIV; DNA vaccine; Nef; Apoptosis; CD8 T cells

INTRODUCTION

It is now well established that control of HIV in the early stages of the infection is associated with the development of cell mediated immune (CMI) responses against multiple antigens of the virus. The most direct evidence for importance of CMI responses has come from SIV-infected macaques which, following treatment with anti-CD8 antibodies, failed to control the infection. These infected animals developed massive virus burdens and rapidly succumbed to disease [1,2,3]. However, the specific viral proteins needed to induce the protective CMI responses are still unknown. More recent studies on the nature of the protective CMI responses against HIV and SIV showed that the most frequent epitopes recognized by cytotoxic T lymphocytes (CTLs) are located in the Gag and Nef proteins. Surprisingly, less are located in the Env which houses moieties responsible for antigenic variation of the viruses, as well as

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docking sites for viral binding to cellular receptors [4]. The Gag and Nef contain the highest epitope density of the virus [5-10,11,12]. Clinically, the magnitude and breadth of the CD8⁺ T cell responses to Gag and Nef in HIV-infected persons correlated inversely with plasma virus loads [13-15], with the lower virus load indicating control of the virus [16,17].

Examination of the immune responses induced by various vaccine strategies showed different results. Inactivated virus or individual viral proteins usually induced antibody rather than CMI responses. On the other hand, plasmid DNAs encoding viral proteins have been recognized as potent inducers of T cell responses. Most of these studies have been conducted in mice. In an earlier report, we had shown that an HIV DNA vaccine derived from the highly pathogenic SHIV_{KU2}, following deletion of the *int*, *rt*, and *vif* genes and substitution of the 3'LTR with polyA sequences of SV40, was non-infectious and capable of only half of a single cycle of replication of virus [18,19]. In a subsequent report, we showed that following exchange of the SIV *gag* and *nef* with those of HIV-1, the DNA now expressed the Gag, Env, and Nef of HIV. Immunization of mice with this DNA (Δ 4SHIV_{KU2}, Δ 4 Nef (+)) induced CMI responses dominated by anti-HIV Gag CD8⁺ T cells [20,21]. During natural infection, the viral protein Nef is well known to interfere with host immune response mechanisms. For example, in infected CD4⁺ T cells, Nef favors the retention of T-cell receptors and other adaptor molecules in the recycling endosomes thereby limiting the formation of the immunological synapse. Nef also acts to modify the status of antigen-presenting cells by triggering the secretion of multiple cytokines [22]. In addition, Nef modulates the apoptotic death of infected and bystander cells [23-26]. Consequently, the benefit of inclusion of a *nef* gene encoding a functional Nef in an HIV-based vaccine has been questioned. To minimize the undesirable effects of Nef, genetically engineered *nef* genes, in which the function was altered but the immunogenic epitopes were conserved, were used in HIV vaccine constructs [27,28]. In this report, we revisited the importance of a functional Nef in the induction of immune T cell responses by an HIV DNA vaccine. We examined whether deletion of *nef* from the Δ 4 Nef(+) DNA creating Δ 5 Nef(-) would affect the immunogenicity of this vaccine in immunized mice. Results of this study showed that the Δ 4 Nef(+) DNA induced strong CD8⁺ T cell responses to Gag, whereas Δ 5 Nef(-) DNA induced Gag-specific CD8⁺ T cell responses that were significantly reduced. Data from experiments in cell cultures pointed out some mechanisms that might explain the intriguing difference in the induced immune responses obtained with the two constructs. Since anti-Gag CD8⁺ T cell responses are important for protection, our results suggest that inclusion of *nef* with *gag* coding sequences in HIV DNA vaccine constructs may be of great importance in the future design of an efficacious HIV vaccine.

MATERIALS AND METHODS

DNA constructs

Details of construction of the Δ 4 plasmid DNA have been described in an earlier report [19, 20]. The inserted sequences were derived from SHIV-KU2 (GenBank data base accession # AY751799) and HIV-1_{SF2}. The DNA is comprised of *vpx* and *vpr* genes from SIVmac239, and *gag*, *pro*, *vpu*, *tat*, *rev*, *env*, *nef* and a portion of *rt* genes from HIV-1, all under the transcriptional control of the SIV 5'LTR promoter and the polyA sequences of SV40. The *pol* gene was truncated to remove 80% of *rt*, and the complete *integrase* and *vif* coding sequences together with the 3'LTR from SHIV-KU2. The Δ 5 plasmid was derived from Δ 4 DNA by deleting 562 bp of the 633 bp *nef* coding sequences following digestion with Bpu 1102I, removal of the fragment of 562 bp, and ligation of 9 kb (Fig. 1). Both viral constructs are carried by the pET9 plasmid (Novagen, EMD Chemicals, San Diego, CA).

Animals

BALB/c mice were purchased from Harlan Laboratories and housed in the Laboratory Animal Resources Building of KUMC. All mice were used in accordance with National Institute of Health and the KUMC Institutional Animal Care and Use Committee guidelines.

Inoculation of mice and splenocyte preparations

Two groups of six week old BALB/c mice were inoculated intramuscularly with a single dose of 200 μ g of either the $\Delta 4$ or the $\Delta 5$ DNA. All DNAs used for inoculation were more than 85% in supercoiled form. DNA solutions were prepared in phosphate buffered saline (pH 7.4) and contained 2 μ g/ μ l DNA. Each mouse was injected with a total of 100 μ l of DNA solution, 50 μ l in each gastrocnemius muscle. Four animals of each group were killed at 2 and 4 weeks, respectively, and spleens collected for CMI studies. Splenocytes were collected in Hanks solution, treated with BD lysing solution to remove the erythrocytes, and mononuclear cells counted as previously described [20]. A portion of the splenocytes from each mouse was used for ELISPOT assay, and a second portion was used as part of a pool comprised of cells from the four mice for flow cytometry as described below.

HIV peptides

Overlapping 15 mer peptides, with 11 amino acid overlaps, spanning the entire molecule of HIV Gag, Env, Tat, Rev, and Nef proteins were obtained from the NIH AIDS Research and Reference Reagent Program (Catalog # 8117, 6451, 5138, 6445 and 5189, respectively).

Detection of HIV-specific CMI responses in splenocytes of mice

Quantitative ELISPOT assays were performed as previously described [20] to measure IFN- γ -producing splenocytes in response to groups of overlapping peptides kindly provided by the AIDS Reference Reagent Program. Gag, Env, Tat+Rev+Nef pools of peptides were used at a concentration of 2 μ g/ml in duplicate wells of cells. Con-A-stimulated cells (0.5 μ g/well) were used as positive controls and cells lacking stimulation with the peptides were used as negative controls. Spots were counted with a stereo-microscope and reported as the number of spot forming splenocytes (SFS)/10⁶ splenocytes. The cut-off of positive ELISPOT was set up 10 SFS/10⁶ splenocytes, two times the background.

To examine and evaluate the proliferative responses of HIV-specific CD8⁺ T cells, we used a CFSE-based labeling assay as described earlier [21]. Briefly, splenocytes were labeled with CFSE, as recommended by the manufacturer (10⁷ cells/ml in 1 μ M CFSE for 10 min at 37°C) and suspensions containing a density of 2 $\times$ 10⁶ cells/ml were deposited in 96 Deep Well tissue culture plates (Nunc, Fisher Scientific, Pittsburgh, PA) in either 1 ml of medium alone or medium supplemented with 2 μ g/ml of indicated pools of HIV peptides. The suspensions were incubated for 4 days at 37° C. Cells were then collected and restimulated for 6 hours with relevant peptides in the presence of Brefeldin A and CD28 costimulatory antibody, after which they were stained with ethidium monoazide (EMA), anti-CD3, -CD4 and -CD8 antibodies. Following fixation and permeabilization, cells were stained with anti-IFN- γ for intracellular cytokine detection as previously described [21].

Polychromatic flow cytometry analysis was performed on a 3-laser BD LSRII instrument. Data files were collected and analyzed using the FACS Diva software (version 4.1.2, BD Biosciences, San Jose, CA). Data were displayed as two color dot plots to measure the proportion of the single positive or double positive cells in the highly CD3⁺ CD8⁺ population.

Cell Line and primary cells

The HEK 293-T cell line was purchased from ATCC. The primary human foreskin fibroblast (HFF) cultures were developed by trypsin dissociation and cultivation of cells from a normal baby's foreskin, kindly provided by the KUMC Obstetrics Department. Both cell types were cultured in D-MEM supplemented with glutamine, antibiotics, and 10% fetal bovine serum. Cultures were transfected with DNAs at 50-60% confluence for 293T and 70-80% for HFFs.

Transfection of HEK 293T and Primary Human Foreskin Fibroblasts (HFF)

Transfections were performed using a cationic polymer polyethylenamine, ExGen™ 500, according to the protocols provided by the manufacturer (Fermentas, Hanover, MD) for adherent cells. Transfection of pET plasmid DNA was used as a negative control and transfection of expression plasmid of GFP (pCG-GFP) was used to evaluate the proportion of transfected cells for each experiment. Subconfluent monolayers in 6 well plates were transfected with a mixture containing 5 µg of DNA and 15.5 µl of ExGen™ 500 in a final volume of 330 µl of PBS. The mixture was preincubated for 20 min at room temperature and then used to inoculate onto the cell cultures. Four hours later, monolayers were rinsed with medium and re-fed with maintenance medium. Every 24h a portion of transfected cells with pCG-GFP was dissociated by trypsin and evaluated by flow cytometry for the proportion of GFP+ cells. Transfections of HFFs ranged 25-30% of GFP+ cells and those of HEK-293T cells 90-95% of GFP+ cells following 3 independent transfection experiments. Supernatant fluids were then harvested from different transfected HFF wells, 18, 24, 48 and 76 hours later, and assessed for Gag p24 content. Cultures of HEK 293T-transfected cells were labeled with 100 µCi of ³⁵S-methionine at 48 hours post-transfection and used for immunoprecipitation of viral proteins from the cell lysate, supernatant and cytosolic compartment (Qiagen, Valencia, CA, USA) using a hyperimmune macaque serum that has antibodies against all of the viral proteins as previously reported [19,20]. To specifically immunoprecipitate the Nef protein from both cell lysate and culture medium supernatant, we used a Rabbit anti-HIV Nef polyclonal serum produced in our lab.

Quantitation of Gag p24 release in the culture medium of transfected cells

Gag p24 was assayed by the highly sensitive capture enzyme-linked immunosorbent assay (ELISA) kit (Coulter Laboratories, Hialeah, FL). A standard curve was prepared for each assay, as per the manufacturer's instructions. The concentrations of Gag p24 were determined from the OD₄₅₀ plotted against a standard curve by linear regression analysis as previously reported (19).

Electron microscopy

Forty eight hours post-transfection of HEK 293-T cells with Δ4 Nef (+) and Δ5 (-) DNAs, monolayers were treated with PBS/EDTA for 5 min at 37°C to dissociate the cells. Dissociated cells were rinsed twice with PBS, spun down and pellets were fixed for 1 h at 4°C with 2.5% glutaraldehyde in 0.1M Sodium cacodylate (pH 7.3). Fixed cells were rinsed with the cacodylate buffer, and then treated with 2% osmium tetroxide (OsO₄) buffered in 0.1M cacodylate for 1 hour at room temperature. Pellets were rinsed twice with distilled water and then dehydrated twice through a graduated series of ethanol, 30%, 70%, 80%, 95%, 100%. Pellets were infiltrated in a mixture of 50% propylene oxide, 50% resin overnight. Pellets were then embedded the following day in fresh Embed 812 resin medium mixture (EMS, Inc) and polymerized overnight in a 60°C oven. Sections were cut on a Leica UCT ultramicrotome at 70 - 80 nm and placed on nickel 300 mesh grids. Sections were stained with 1% uranyl acetate in 70% ethanol and lead citrate. Then sections were viewed in a J.E.O.L. 100CXII electron microscope at 80kv and photographed.

Detection and evaluation of induced cell death

We performed two assays to detect and to evaluate induced cell death. The TUNEL-based *in situ* cell death detection kit POD (Roche, Boulder, CO) based on enzymatic labeling that was used to assess the fragmentation of DNA in treated cells. Monolayers of HFFs on cover slips in 6-well plates were transfected with DNA constructs and 72 hours later cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X100 in 1% sodium citrate. Fixed cells were stained using the manufacturer's instructions (Roche, Boulder, CO). Briefly, following permeabilization for 2min in ice, cells were incubated for 30 min at 37°C with 50 µl of the TUNEL-reaction mixture (containing 5 µl of TUNEL enzyme and 45 µl TUNEL label solution). Slides were rinsed 3 times with PBS then incubated 10 min at room temperature with 100 µl of substrate solution and then rinsed 3 times with PBS. Stained cells were mounted under glass cover slips and then analyzed under light microscope and photos were taken at 200× and 400× magnification.

In the second assay, we examined the cultures for development of apoptosis by co-staining with Annexin V and propidium iodide (PI). Monolayers of cells were dissociated with trypsin and then suspended cells in culture medium were incubated in a tissue culture incubator for 1 hour at 37°C in polypropylene tubes. Cells were pelleted, rinsed in PBS, and then the pellet resuspended at a concentration of 5×10^6 /ml in 200 µl of binding buffer (10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 2.5mM CaCl₂). Five µl of Annexin V (Invitrogen, Carlsbad, CA) conjugated with FITC were added to the cell suspensions, mixed gently, and then incubated for 10 min at room temperature. Cells were rinsed with the binding buffer, resuspended into 200 µl of fresh binding buffer, and then adjusted to 1 µg/ml with propidium iodide (PI) just before flow cytometry analysis. UV-treated cells were used as positive control for each experiment.

Statistical analysis

We used the Wilcoxon rank sum test with the Statistix 8 software (Analytical Software, Tallahassee, FL) that gives exact p-values for small sample sizes to examine the statistical significance of the difference between CMI responses to Gag induced by Δ4 and Δ5 DNAs in mice.

RESULTS

Reduced IFN-γ and proliferative CD8⁺ T cells responses to Gag in mice immunized with Δ5 Nef(-) DNA

In earlier reports, we had shown that 200µg of Δ4 Nef (+) (Δ4SHIV_{KU2}) DNA injected into the gastrocnemius muscles of mice induced specific CD8⁺ T cell responses against all the HIV proteins expressed by the HIV DNA vaccine. These responses were mostly proliferative with a minor fraction of cells producing IFN-γ as measured by ELISPOT and intracellular staining (ICS) assays. Early T cell responses were detected 1-2 weeks post-immunization and declined to a minimal level after 4 weeks. Then, secondary antigen-specific T cell responses similar to the primary responses started to re-emerge as memory responses more than 30 weeks post-immunization [20,29]. Using this background, we focused on the primary responses at the 2 and 4 week time points, and we inquired whether the absence of Nef in Δ5(Nef-) DNA, (Fig. 1), would affect the initial responses to all expressed HIV antigens in injected mice. As shown in Fig. 2A, at 2 weeks post-inoculation, mice that were immunized with Δ5 Nef(-) DNA developed significantly lower ($P < 0.05$) IFN-γ ELISPOT responses to HIV Gag compared to mice injected with Δ4 Nef(+) DNA. There was no significant difference in the responses to Env, Tat and Rev, and as expected, no response was detected against Nef in Δ5-injected mice. At 4 weeks, as expected, the response to Gag decreased, but the response was still weaker in the Δ5 Nef(-) than the Δ4 Nef(+) animals (data not shown).

To further examine the influence of Nef on the CD8⁺ T cell responses induced by the HIV DNA vaccine, we used multiparametric flow cytometry to analyze antigen-specific proliferation (CFSE dilution) and IFN- γ secretion of splenocytes in response to HIV antigens. As shown in Fig. 2B, at the 2 week time point following immunization, 3.8% and 2.3% of the CD8⁺ T cells in splenocytes from mice immunized with $\Delta 4$ Nef(+) DNA underwent proliferation with HIV Gag and Env peptides, respectively, and about 1% of these cells produced IFN- γ (Fig. 2B, first row). At 4 weeks, the same magnitudes of proliferative responses were observed against both Gag and Env peptides, while the IFN- γ -producing cells dropped to 0.8% and 0.3% respectively (Fig. 2B, 3rd row). In contrast, mice immunized with $\Delta 5$ Nef(-) DNA did not develop any detectable Gag-specific proliferative CD8⁺ T cell responses at either the 2 or 4 week time points (Fig. 2B, 2nd & 4th rows) and only about 0.05% IFN- γ -producing cells were detected. In contrast, 1.5% and 5.5% of the splenocytes proliferated in response to Env peptides at week 2 and week 4, respectively. As we recently reported, only a minimal number of these CD8⁺ T cells produced IFN- γ . The absence of both types of CD8⁺ T cells against the TRN pool of peptides confirmed the results obtained by ELISPOT, as well as the absence of Nef antigen expression in the $\Delta 5$ construct.

Thus, immunization of mice with the deleted $\Delta 5$ Nef(-) DNA vaccine resulted in CD8⁺ T cells responses that were mostly restricted to Env, in contrast with the broad responses against Gag, Env, and Nef induced by the original $\Delta 4$ Nef(+) DNA vaccine.

Reduced Gag p24 antigen release by HFFs transfected with $\Delta 5$ Nef(-)DNA

In order to explore a potential mechanism for the different CMI responses induced by the $\Delta 4$ and $\Delta 5$ DNAs, we first examined the amount of Gag p24 antigen released by HFFs that were transfected with the two constructs by ELISA. The pET plasmid lacking the viral genome was used as a control. Culture supernatant fluids were harvested at 18, 24, 48 and 72 hours following transfection. Fig. 3 showed that only a minimal amount of the viral protein was produced at 18 hours, but, at the 24, 48 and 72 hour time points, there was at least 50% less p24 in the supernatant of HFFs transfected with the $\Delta 5$ Nef(-) DNA compared with that of the $\Delta 4$ Nef(+) DNA. As expected, no Gag p24 was detected in the supernatant of HFFs transfected with the empty pET9 plasmid. These data indicated that the absence of *nef* in the $\Delta 5$ DNA resulted in a significant reduction in the release of p24 into the culture medium of transfected cells.

In addition, we observed that the two DNAs had dramatically different effects in primary HFF cultures. Whereas the $\Delta 5$ Nef(-) DNA transfected cultures remained morphologically intact throughout the study period, the $\Delta 4$ Nef(+) DNA transfected cultures developed foci of degeneration that appeared first at 24 hours and increased in both number and size with further incubation. By 72 hours, more than 50% of the cultures had undergone degeneration. This degeneration correlated with the higher concentrations of Gag p24 in the clarified supernatant fluids from the $\Delta 4$ cultures as shown in Fig. 3.

Reduced Gag p24 release and deficit in VLP formation in HEK 293-T transfected with $\Delta 5$ Nef(-) DNA

To evaluate if this difference in Gag release between both constructs was due to a better expression of Gag and other viral proteins by the $\Delta 4$ Nef(+) DNA, we examined protein expression in the highly transfectable HEK-293 T cells. These cells were transfected with $\Delta 4$ and $\Delta 5$, and 48 hours later, the cells were labeled overnight with ³⁵S-methionine. Viral proteins were then immunoprecipitated with anti-SHIV monkey serum. In addition, we used serum from a rabbit immunized with HIV Nef to specifically detect Nef, since the separation of Gag (p24) and Nef (p27) on 10% SDS polyacrylamide gel is difficult due to the closeness of their sizes. Thus, in Fig. 4A (left panel), we confirmed that Nef was precipitated from both the supernatant and cell lysate of $\Delta 4$, but as expected, was absent in samples of $\Delta 5$ transfected

cells. We then collected cytosolic fraction and supernatant from transfected cells and used the anti-SHIV serum to compare the profile of their proteins. We found that the Gag protein and Env glycoproteins were significantly reduced in the supernatant of $\Delta 5$ -transfected cells in comparison with $\Delta 4$. In contrast, no reduction was observed in the cytosolic fraction of either construct and a slight increase of the total HIV proteins, specifically Gag p24, was noticed in the $\Delta 5$ -transfected cells (Fig. 4A, right panel). Altogether, these results showed that the removal of *nef*-coding sequences did not affect the expression of Gag and Env proteins in the cytosol of transfected HEK-293 T cells. However, it confirmed the initial ELISA results indicating that Gag release is reduced in the absence of Nef protein expression.

We next examined whether these differences in protein profiles in the cytosol and the supernatant could be further extended to the cytoplasmic membrane level knowing that Gag, Env and Nef are essential to formation and release of virus like particles (VLPs). Thus, HEK-293 T transfected cells with both constructs were examined at the ultrastructural level by electronic microscopy. As shown in Fig 4B, in the electron micrographs, VLPs were abundantly budding at the surface of cells transfected with the $\Delta 4$ (Nef+) construct while minimal VLPs were seen at the surface of cells transfected with the $\Delta 5$ Nef(-) construct. These data demonstrated that absence of Nef was associated with a defect of the assembly of VLPs at the surface of cells expressing the $\Delta 5$ Nef(-) construct.

Apoptosis and necrosis are associated with expression of Nef in HFFs

To determine whether the $\Delta 4$ DNA-induced degeneration of HFFs was of an apoptotic/necrotic nature, we used the *in situ* detection of cell death in the cultured cells 3 days after transfection. Transfected-HFFs with the pET plasmid and UV-treated non transfected cells were used as controls. As shown in Fig. 5, intense labeling was observed mainly in the nuclei of HFFs transfected with $\Delta 4$ Nef(+) DNA (Fig. 5A) and the UV-treated culture (Fig. 5F). The $\Delta 4$ -transfected culture, with extensive cytopathic effects, had stained cell debris that corresponded to apoptotic vesicles. In contrast, no apoptosis was observed in cells transfected with the $\Delta 5$ Nef(-) DNA (Fig. 5C) or the empty plasmid (Fig. 5E). Absence of staining in HFFs (Fig. 5B and 5D) following treatments with the same procedure, except for omission of the enzyme, confirmed the specificity of the apoptotic staining. These data clearly showed that expression of Nef was associated with induction of apoptosis in transfected HFFs.

TUNEL data were confirmed by double-staining of transfected HFFs after 24, 48 and 72 hours with Annexin-V and PI and examination of the cells by flow cytometry. Data in Fig. 6 demonstrated that HFFs transfected with $\Delta 4$ Nef(+) DNA had the highest numbers of cells positive for Annexin V (early apoptosis), and double positive for Annexin V and IP (late apoptosis) and positive for IP (necrosis) at all three time points of analysis. A total of 10%, 23% and 52% of apoptotic/necrotic HFFs were detected at 24, 48 and 72 hours post transfection with $\Delta 4$ Nef(+) DNA while only a maximum of 5.3% of apoptotic/necrotic HFFs was detected during the 3 time points in transfected cells with $\Delta 5$ Nef(-) or pET control plasmid DNAs.

Altogether, these data proved that fibroblasts transfected with the $\Delta 5$ Nef(-) DNA did not develop either cytopathic effects or signs of apoptosis. This absence of cell death correlated with reduced release of Gag p24 in the supernatant fluid of the $\Delta 5$ -transfected cells as evidenced in Fig. 3.

DISCUSSION

The present study shows that Nef protein encoded in a non-infectious HIV DNA vaccine plays an essential role in modulating primary CD8⁺ T cell responses against the capsid Gag protein. When compared with mice immunized with ice injected $\Delta 4$ with Nef(+), the $\Delta 5$ Nef(-) DNA m vaccine developed significantly lower CD8⁺ T cell responses to Gag, but no significant

change in the responses to Env. Although Gag and Env proteins were equally expressed in cell cultures transfected with both constructs, we observed intriguing differences in induced apoptosis of transfected primary fibroblasts and assembly and release of VLPs at the surface of transfected cells. Deletion of Nef impaired the induced cell death and production of VLPs in cells transfected with $\Delta 5$ Nef(-) DNA, in comparison to the massive apoptosis and large number of VLPs observed in cells transfected with $\Delta 4$ Nef(+) DNA. These differences also correlated with a reduced Gag release in the extracellular environment. Altogether, these results suggest that Nef-induced apoptosis and assembly of proteins in VLPs may provide mechanisms for enhanced release of Gag in favor of vigorous CD8⁺ T cell responses against this protein. These results illustrate the necessity of including functional Nef in experimental HIV DNA vaccine prototypes.

We previously reported that repeated immunizations of macaques with the $\Delta 4$ Nef (+) ($\Delta 4$ SHIV-KU2) DNA alone induced protection of animals from disease induced by the pathogenic SHIV 89.6P used as challenge [18]. In order to more fully investigate comprehensively the quality of T cell responses induced by the vaccine, we developed multiparametric flow cytometry based assays to measure multiple functions. Using the mouse model, we performed a longitudinal study following intramuscular injection of animals with a single dose of $\Delta 4$ Nef (+). We demonstrated that this vaccine strategy induced poly-functional and long-lasting CD8⁺ T cell responses specific for all expressed HIV antigens by the construct with the response against Gag being dominant [21].

A fundamental question arising from these observations is whether the natural genomic organization of the genes in $\Delta 4$ Nef(+) specifically promote an anti-CD8⁺ T cell response to Gag. The viral genome of the $\Delta 4$ Nef(+) plasmid is efficiently expressed by the LTR/Tat system and following multiple splicing, proteins are efficiently translated mimicking the expression of the infectious virus both *in vitro* ([20] and this study) and *in vivo* [20]. Expressed proteins are expected to assemble and be extruded as VLPs out of the transfected cells. Interestingly, we found that the absence of Nef expression in the $\Delta 5$ Nef(-) was associated with a defect in formation of VLPs in the transfected HEK 293-T cells. Nef was previously shown to interact with Gag during the virion assembly to ensure the stability and maturation of infectious virus particles [30]. In addition, Nef was also shown to enhance the incorporation of Env into late endosomes [31]. Thus, one possible explanation is that the modification of intracellular trafficking of viral proteins as a result of the absence of Nef in the $\Delta 5$ Nef(-) vaccine may influence the efficiency of antigenic presentation of Gag in immunized animals and consequently result in the decrease of Gag-specific CD8⁺ T cell responses. Interestingly, in a human phase I clinical trial using a combination of four HIV vaccine constructs with one expressing truncated Gag, Pol, Nef fusion protein, there was no detectable Gag-specific CD8⁺ T cell response [32,33]. Viral gene expression in the used constructs was driven by the CMV promoter that strongly expressed the proteins *in vitro*. In this context, since the protease (PR) was mutated, the maturation of viral proteins might have been compromised and consequently affected the interaction between Gag and Nef proteins. In addition, the expression of Env in separated plasmids might have contributed to the lack of assembly into VLPs. Altogether these observations suggested that assembly of Gag into VLPs that are extruded from the cells might be more effective for induction of Gag-specific CD8⁺ T cell responses. Indeed, VLPs have been already used as immunogens in both mouse [34] and primate [35,36] models, and have been shown to induce robust T cells responses against Gag and Env antigens. They also induce maturation of dendritic cells and macrophages [37] and allow cross-presentation of Gag-antigen [38]. This series of events are all believed to be effective for the development of efficient CD8⁺ T cell responses.

In a previous report, we detected massive infiltration of macrophages and dendritic cells in muscles sections, while doing a histological examination of muscles of mice injected with $\Delta 4$

Nef (+) DNA. In addition, we localized Gap p24 in the sarcoplasmic reticulum of skeletal muscles cells as well as in the cytoplasm of infiltrating mononuclear cells as soon as 3 days post-inoculation [20]. Whether the DNA plasmid has been taken by both types of cells and whether other mechanisms have participated in the transfer of Gag antigens to professional APCs have not been determined. However, VLPs extruded from transfected cells in the muscle might target dendritic cells and macrophages but also the inflammatory mononuclear cells that were shown to infiltrate the muscle shortly after DNA immunization. In addition, we observed an intriguing difference in the ability of each construct to induce cell death of transfected human primary cell cultures. Indeed, we detected about 50% of apoptotic HFFs at 72 hours post-transfection with $\Delta 4$ Nef (+). This percentage is higher than the proportion of transfected cells suggesting that the Nef-induced apoptosis is affecting not only the transfected but also, bystander HFFs. This massive apoptosis induced by $\Delta 4$ Nef (+) DNA was extremely reduced in transfected primary fibroblasts with $\Delta 5$ Nef(-) DNA lacking Nef. Thus, Nef induced apoptosis of transfected cells might synergize with VLP formation mechanisms to maximize the extrusion of Gag and its efficiency to induce Gag-specific CD8⁺ T cell responses. Apoptotic and necrotic cells expressing antigens are well known to be an important source for antigen cross-presentation and efficient priming of anti-viral CD8⁺ T cell responses by dendritic cells and macrophages [29,39,40]. In HIV DNA vaccine strategies, the expression of apoptotic genes, such as Fas-Ligand [41] or mutated Caspase-3 [42], in the same unit with viral genes or in a separate constructs has been associated with increased immunogenicity and protection against chimeric virus in the mouse model. In addition, using an Env-defective HIV virus pseudotyped with the envelope of the ecotropic MuLV, it has been shown that immunization with syngenic apoptotic infected splenocytes generated strong proliferative and IFN- γ -producing CD8⁺ T cell responses to Gag p24 and Nef. These responses protected the mice from infection with live cells producing MLV-pseudotyped HIV[43]. The induction of Gag-proliferative and IFN- γ -secreting CD8⁺ T cell responses are similar to the type of Gag responses found in mice immunized with $\Delta 4$ Nef (+) DNA ([21] and present study).

In contrast to the Gag results, we found no significant difference in Env-specific CD8⁺ T cell responses induced by the $\Delta 4$ Nef (+) and $\Delta 5$ Nef (-) DNA vaccines. However, there was an apparent slight accumulation of Env proteins in the cytosol of $\Delta 5$ Nef (-) transfected cells, probably due to the lack of efficient VLP (assembly and release. This result indicates that the processing of Env glycoproteins is likely less dependent on VLP formation and apoptotic cell death induction to be accessible to antigen presenting cells. One cannot exclude that the low ELISPOT responses to Env might limit the detection of possible differences.

There is much evidence now that Gag-specific CD8⁺ T cell responses are more important than previously thought in controlling viral replication and disease progression. In a recent study, examination of a large and genetically diverse cohort of 578 untreated HIV-infected individuals showed that only Gag-specific CD8⁺ T cell responses were associated with lowering viremia [4]. One potential mechanism to explain this *in vivo* finding is provided by the study of Sacha and coworkers. They demonstrated that Gag-specific CD8⁺ T cells recognize and eliminate HIV-infected cells as early as 2h post-infection, before integration of the proviral DNA and synthesis of viral proteins. In contrast, CD8⁺ T cells specific to Env did not appear until 18h post-infection, close to the onset of progeny virus production [44]. Taken altogether, these results clearly suggest that HIV vaccine prototypes should efficiently induce functional and potent CD8⁺ T cell responses against Gag to insure effective control of virus replication and pathogenesis.

In the present study, we highlighted the adjuvant role of Nef encoded by a non-infectious HIV DNA vaccine to enhance the CD8⁺ T cell responses toward Gag antigen.

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Figure 1. Schematic representation of $\Delta 4$ Nef(+) and $\Delta 5$ Nef(-) DNA constructs

HIV and SIV structural, regulatory, and accessory genes are indicated in boxes. Expression of viral genes is driven by the 5'LTR of SIV and RNA stop transcription is insured by the SV40 poly-A signal. $\Delta 5$ Nef(-) was derived from $\Delta 4$ Nef(+) DNA by additional deletion of *nef* coding sequences.

Figure 2. HIV-specific IFN- γ producing and proliferating CD8⁺ T cells in mice immunized with $\Delta 4$ and $\Delta 5$ HIV DNA vaccines

A) At two weeks post immunization of BALB/c mice with 200 μ g of $\Delta 4$ Nef(+) and $\Delta 5$ Nef(-) DNA constructs, splenocytes were isolated and examined for IFN- Δ ELISPOT responses using pools of Gag, Env, Tat+Rev and Nef HIV peptides as described in Materials and Methods. The data represent the measurements obtained from each group of 4 mice shown as average and standard deviation represented by the error bars. SFC: spot-forming cells. The response to Gag in the group of $\Delta 4$ -mice was significantly higher ($P < 0.005$) than that of Gag in the group of $\Delta 5$ -mice. NS: not significant. **B)** Pools of splenocytes from 4 mice immunized with $\Delta 4$ Nef(+) DNA and 4 others with $\Delta 5$ Nef(-) DNA were used at week 2 (first and second row) and week 4 (third and fourth row) to detect and evaluate specific T cell responses to HIV antigens. Total splenocytes were labeled with CFSE and cultured with specific pools of HIV peptides (Gag, Env, Tat, Rev and Nef combined, TRN) or grown in medium without antigen. After 4 days, cells were harvested and restimulated for 6h with indicated peptide in the presence of co-stimulatory antibodies and Brefeldin A. Restimulated cells were surface stained with anti-CD3, -CD8, -CD4 antibodies in presence of EMA (for exclusion of dead cells) and subsequently fixed, permeabilized, and stained with an anti-IFN- γ mAb. For each sample, 25,000 events were collected within low FSC/SSC, EMA-, CD3⁺ and high CD8⁺ T cell population. Data were then displayed as two color dot plots and the proportion of total live EMA- CD3⁺ CD8⁺ T cells producing IFN- γ and proliferating (CFSE dilution) in response to specific antigens were measured. Frequencies of proliferating cells only (middle number in each dot plot) and those of cells proliferating and producing IFN- γ (top number in each dot plot) are indicated. The frequencies for Ag-specific responses are reported as the percentage of cytokine-producing or proliferating CD8⁺ T cells after subtraction of background production or proliferation in the sample cultured for 4 days with medium only and restimulated for 6h with relevant mixes of peptides (medium (4d) Ag (6h)). Results are representative of 2-3 independent experiments.

Figure 3. Evaluation of HIV Gag-p24 released in the culture medium of transfected HFF cultures
Culture medium of transfected HFF monolayers with Δ4 and Δ5 DNA constructs and pET plasmid lacking viral genome were harvested at 18, 24, 48 and 72 hours post-transfection and examined by ELISA for detection of HIV Gag p24. Calculated concentrations of p24 protein per ml of culture medium were used to plot the graphs. The results are representative of two independent experiments.

Figure 4. Viral protein expression and VLP formation in HEK-293 T cells

A) HEK 293-T cells were transfected with Δ4 Nef(+) and Δ5 Nef(-) DNAs as described in Materials and Methods. After forty-eight hours, proteins of transfected cells were labeled with ^{35}S -methionine and then viral proteins were immunoprecipitated from the cell lysate (C), the supernatant fluid (S) and the cytosolic fraction with rabbit anti-Nef polyclonal serum (left panel) and monkey anti-SHIV plasma (right panel). pET plasmid DNA was used as negative

control (NC). The sizes of the major virus proteins are indicated in kD. **B)** Ultrastructural analysis of VLP production by $\Delta 4$ and $\Delta 5$ transfected HEK-293-T cells. Forty-eight hours after transfection, monolayers of HEK-293-T cells were harvested and treated for electronic microscopy as described in Materials and Methods. Sections were observed in a J.E.O.L. 100CXII at 80kv and photographs were taken as indicated at various magnifications (4.8 to $36 \times 1000(K)$).

Figure 5. Detection of apoptotic death in transfected HFF cultures

For *in situ* detection of cell death, HFFs were transfected with $\Delta 4$ Nef(+) and $\Delta 5$ Nef(-) DNA and 3 days later fixed with 4% paraformaldehyde and stained as described in the manufacturer's procedure, briefly reported in the method section. HFFs transfected with $\Delta 4$ Nef(+) (A) and (B) and $\Delta 5$ Nef(-) (C) and (D) stained in presence (A & C) or absence (B & D) of the terminal transferase enzyme, respectively. HFFs transfected with pET plasmid (E) lacking viral genome were used as negative controls and UV-treated HFFs as positive control (F). Both controls were stained in presence of terminal transferase enzyme.

Figure 6. Quantitative analysis of apoptotic and necrotic death in transfected HFF cells
HFFs were transfected with $\Delta 4$ Nef(+) DNA, $\Delta 5$ Nef(-) DNA, and the pET plasmid lacking viral DNA and 24, 48 and 72 hours later cells were stained with Annexin V-FITC and PI as described in Materials and Methods. Cells were examined by flow cytometry and data are shown as two color dot plots. The proportions of early apoptotic cells (positive for Annexin V only) are reported in the lower right. Necrotic cells (positive for PI only) are reported in the upper left. Late apoptotic cells (double positive for Annexin V and PI) are reported in the upper right quadrants of each dot plot.