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Anti-tumor CD8$^+$ T cell immunity elicited by HIV-1-based virus-like particles incorporating HPV-16 E7 protein

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ABSTRACT

Here we report a novel strategy for the induction of CD8$^+$ T cell adaptive immune response against viral and tumor antigens. This approach relies on high levels of incorporation in HIV-1 VLPs of a mutant of HIV-1 Nef (Nef^{mut}) which can act as anchoring element for foreign proteins. By *in vitro* assay, we found that VLP-associated Nef^{mut} is efficiently cross-presented by antigen presenting cells. Inoculation in mice of VLPs incorporating the HPV-16 E7 protein fused to Nef^{mut} led to an anti-E7 CD8$^+$ T cell response much stronger than that elicited by E7 recombinant protein inoculated with incomplete Freund's adjuvant and correlating with well-detectable anti-E7 CTL activity. Most relevantly, mice immunized with Nef^{mut}-E7 VLPs developed a protective immune response against tumors induced by E7 expressing tumor cells. These results make Nef^{mut} VLPs a promising candidate for new vaccine strategies focused on the induction of CD8$^+$ T cell immunity.

Introduction

Virus spread in infected host can be prevented and/or counteracted by virus-specific antibodies through the neutralization process (Klasse and Sattentau, 2002; Reading and Dimmock, 2007). However, in many instances, particularly in established infection, this cannot be sufficient, and the control and clearance of viral infection require the contribution of alternative immune mechanisms. Among these, specific CD8$^+$ cytotoxic T lymphocytes (CTLs) are crucial for virus clearance in view of their ability to recognize and kill virus-infected cells. The protective function of CTLs can also be relevant in virus-induced tumors, as well as in virus-independent cancers, due to the recognition of tumor-associated antigens.

Several immunogens have proven suitable for induction of specific CTLs, including attenuated viruses (Wang et al., 2006), recombinant viral vectors (He et al., 2007; Yang et al., 2007; Liniger et al., 2007), and naked DNA (Anderson and Schneider, 2007; Liu and Ulmer, 2005). However, both safety concerns and scarce efficacy have limited their approval for humans. Virus-like particles (VLPs) represent a promising tool for overcoming these limitations. They are self assembling, non-replicating, non-pathogenic, genomeless particles (Grgacic and Anderson, 2006). VLPs can be engineered to incorporate foreign antigens (chimeric VLPs) as in the case of human immunodeficiency virus type 1 (HIV-1) Pr55^{gag} -derived VLPs, whose immunogenicity can be broadened by the inclusion of chimeric Gag molecules (Buonaguro et al., 2006; Luo et al., 2007; Paliard et al., 2000; Wagner et al., 1994; Young and Ross, 2006). However, their assembling efficiency is affected by both domain of insertion (Kattenbeck et al., 1997) and length of exogenous sequences (Muller et al., 2004).

HIV-1 Nef is a 27-kDa myristoylated protein incorporating in virions at low levels, i.e., about 10 molecules for viral particle (Bukovsky et al., 1997; Pandori et al., 1996). We previously characterized a Nef mutant (Nef7) exhibiting unique efficiency of incorporation (about 100-fold higher than the wild-type protein) (Peretti et al., 2005). Moreover, this mutant lacks several anti-cellular effects typically induced by wild-type Nef, including CD4 down-

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regulation, increase of HIV-1 infectivity, PAK-2 activation (D'Aloja et al., 2001), and MHC Class I down-regulation (Green et al., 2009). Most importantly, Nef7 has been shown to act as carrier of heterologous proteins fused to its C-terminus (Green et al., 2009; Peretti et al., 2006). As we recently described, HIV-1 wild-type Nef carrying the $^{G}3^{C}$ mutation (creating a palmitoylation site at the N-terminus) shows an incorporation efficiency similar to Nef7 (Muratori et al., 2006). Here, these Nef mutants were combined in a single Nef molecule (here referred to as Nef^{mut}) and fused with the human papilloma virus (HPV)-E7 protein to recover VLPs incorporating high amounts of the Nef^{mut}/E7 fusion product.

The "high-risk" HPV (HR-HPV) genotypes are recognized as etiologic agents of invasive cervical cancer, its precursor lesions, and other kinds of anogenital tumor. The two early viral proteins E6 and E7 are the most frequently targeted antigens for immunotherapeutic approaches against HPV-related cancer. These proteins are constitutively expressed in HPV-associated lesions, and their expression is required for maintenance of the cell transformed state. They contribute to events leading to cellular malignant conversion by interacting with cellular partners. The E7 ultimate effect is up-regulation of genes required for G1/S transition of cell cycle and cellular DNA synthesis, whereas E6 targets cellular proteins involved in apoptosis control, growth arrest, terminal cell differentiation, and antiviral defense (Fehrmann and Laimins, 2003; Munger et al., 2004). Several lines of evidence suggest that CD8$^+$ T-cell-mediated immune response is important in controlling HPV infection and, by consequence, virus-associated neoplasia. Accordingly, immune suppression and/or poor anti-HPV E7 CTL response has been associated with viral persistence and disease progression (Bourgault et al., 2004).

Several vaccine platforms targeting E7 and/or E6 have been developed over the last decade (Brinkman et al., 2007) including peptide/protein-based therapies, viral vector-based therapies, and dendritic cell-based therapies (Bellone et al., 2007). Prophylactic vaccines preventing infection by the HR-HPV type-16 and type-18 are now available. However, these vaccines have no effect against pre-existing HPV infection and HPV-associated lesions. Furthermore, due to the long latency from infection to cancer, their eventual benefits will be visible within decades. Thus, developing new therapeutic strategies to cure the already infected individuals remains a priority.

Pseudotyping VLPs with the glycoprotein of vesicular stomatitis virus (VSV-G) is expected to improve their efficiency of delivery into antigen presenting cells (APCs) as well as to favor CD8$^+$ T cell immune response through cross-presentation and cross-priming mechanisms. In fact, VSV-G allows VLPs to enter APCs through endocytosis, thereby promoting the fusion between VLP envelope and endosome membranes. This guarantees efficient delivery of VLP contents into cytoplasm, thus facilitating their interaction with proteasome, protein degradation, and association with Class I MHC molecules (Marsac et al., 2002).

Here, we report that (VSV-G) VLPs incorporating Nef^{mut} fused with HPV type-16 E7 induced an anti-E7 CTL response correlating with protection from HPV-related tumor. This result represents the proof of principle that (VSV-G) Nef^{mut}-based VLPs can indeed represent an useful tool for the induction of CD8$^+$ T cell adaptive immune response against heterologous proteins for vaccination purposes.

Results

Construction of (VSV-G) HIV-1 based Nef^{mut} VLPs

Both Nef7 and $^{G}3^{C}$ Nef mutants incorporate at high levels in HIV-1-derived VLPs and act efficiently as carrier molecules (Peretti et al., 2005; Muratori et al., 2006). In Fig. 1, the design of the Nef^{mut}-based VLP system is summarized. In detail, the $^{G}3^{C}$ mutant of Nef7 (here referred to as Nef^{mut}) fused with a foreign antigen was packaged into

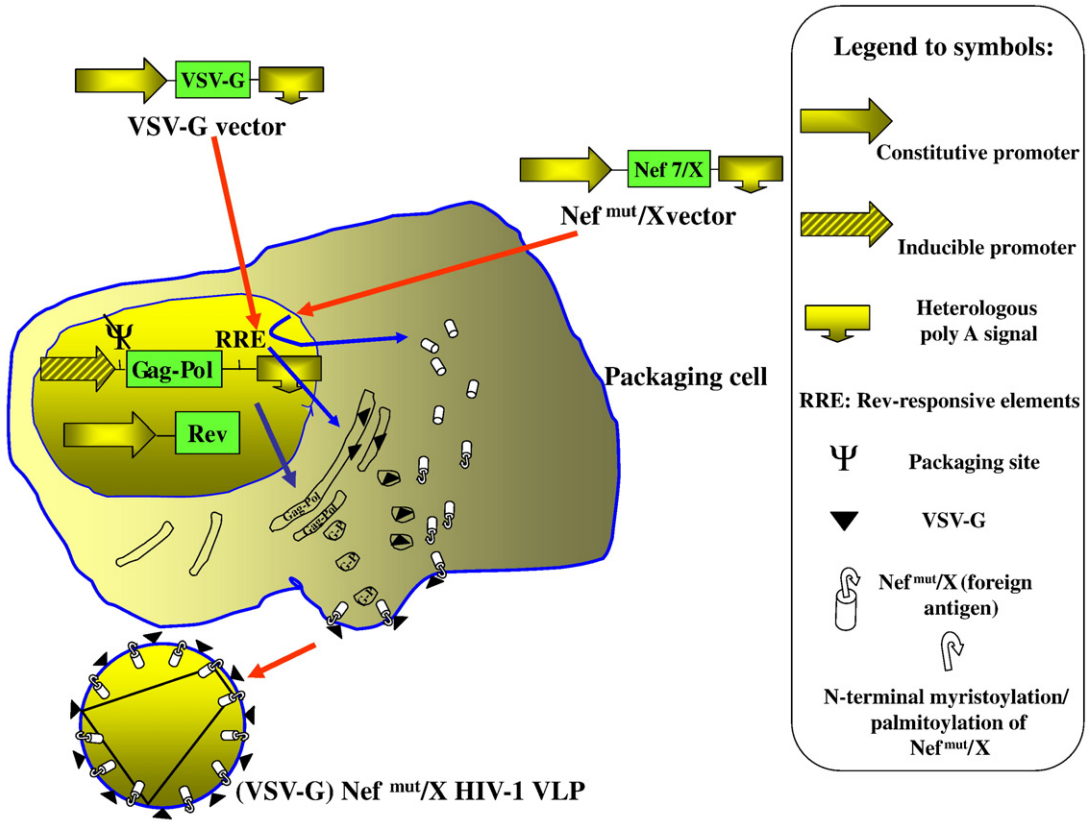

Fig. 1. Summary of the method for the production of Nef^{mut}-based VLPs through the transfection of vectors into 293 GPR HIV-1 packaging cells. Cell and VLP are not in scale.

VSV-G pseudotyped VLPs upon transfection of the respective expression vectors into 293 GPR packaging cells which express HIV-1 Gag-Pol in an inducible manner. After induction, the released VLPs were harvested and concentrated/purified by ultracentrifugation on sucrose cushion. VLPs were recovered as the pellet fraction and used for both molecular characterization and immunogenicity tests.

Cross-presentation of Nef^{mut} in APCs challenged with (VSV-G) Nef^{mut} HIV-1 VLPs

The cross-presentation in APCs of HIV-1 Gag products in the absence of viral replication has been already described (Buseyne et al., 2001). Conversely, nothing has been reported about Nef. We attempted to fill this gap taking advantage of the high virion incorporation extents of Nef^{mut}. Cross-presentation of Nef^{mut} delivered by VLPs was evaluated by an *in vitro* assay based on the co-culture of VLP-challenged human B-lymphoblastoid cell lines (B-LCLs) with MHC Class I-matched, Nef-specific CD8$^+$ T-cell lines. We first evaluated the efficiency of VLP entry into B-LCLs by challenging these cells with fluorescent (VSV-G) VLPs incorporating Nef^{mut}-GFP fusion molecules. In particular, 500-ng HIV-1 CAp24 equivalents of these VLPs were used to challenge 10^5 B-LCLs which, at different times, were harvested, incubated with trypsin 15 min at 37 °C, and fixed. As control, the challenge was performed at 4 °C or using receptor-less Nef^{mut}-GFP VLPs (null VLPs). FACS analysis (Fig. 2A) indicated the presence of high levels of fluorescent products into the B-LCLs as early as 4 h after challenge. Conversely, no cell-associated fluorescence was detected when the cells were incubated at 4 °C or using Null VLPs. These results demonstrated that (VSV-G) Nef^{mut}-based VLPs can be efficiently internalized by human B-LCLs.

To assess whether VLP-associated Nef^{mut} undergoes cross-presentation, we evaluated the cell activation in terms of IFN-γ production by Nef-specific CD8$^+$ T cells co-cultured with MHC Class I-matched B-LCLs previously challenged with VLPs. In particular, HLA-B7 B-LCLs were challenged with either 500-ng HIV-1 CAp24 equivalents of (VSV-G) Nef^{mut} VLPs/10^5 cells or the same amounts of VLPs not incorporating Nef (Void VLPs). After 3 h of absorption, cells were extensively washed and put in co-culture at 1:2 ratio with a CD8$^+$ T-cell line specific for the TPGPGVRYPL HLA-B7 restricted Nef epitope. As positive control, co-cultures including B-LCLs pre-treated for 16 h with the B7-restricted Nef peptide were also carried out. After overnight incubation, supernatants were harvested for the determination of IFN-γ contents, and the co-cultures were re-seeded in fresh medium in the presence of brefeldin A. Five hours later, the co-cultures were analyzed by FACS for the expression of both CD8 and IFN-γ. The results obtained by both FACS and ELISA assays (Fig. 2B) consistently documented the production of IFN-γ in the indicator CD8$^+$ lymphocytes. Conversely, no IFN-γ production was detectable in co-cultures with B-LCLs challenged with void VLPs. Of note, similar results were obtained using a CD8$^+$ T-cell line specific for the RRQDILDLWIY HLA-Cw7 restricted Nef epitope (not shown).

We concluded that (VSV-G) HIV-1 Nef^{mut}-based VLPs efficiently enter B-LCLs, and that VLP-associated Nef^{mut} molecules undergo effective antigen cross-presentation. These results appeared particularly significant also considering the moderate to low cross-presentation activity previously described in human B-LCLs (Munz et al., 2000; Sallusto and Lanzavecchia, 1994).

Recovery of HPV-E7 incorporating VLPs

The interest in the Nef^{mut}-based VLP system as novel vaccine platform relies on its ability to elicit effective immune response against the foreign moiety fused with Nef^{mut}. We selected HPV type-16 E7 protein as heterologous antigen in view of its potent immunogenicity and its involvement in the induction and maintenance of HPV-related cell transformation. Moreover, a well-characterized animal model for HPV-related tumors is available for evaluating the effectiveness of anti-HPV vaccination strategies.

We produced VLPs incorporating the products of fusion between Nef^{mut} and either wild-type or E7^{GGG} isoforms, the latter being a non-oncogenic mutant unable to bind the cellular pRb protein due to three amino acid substitutions within the pRb binding site, i.e., $^{D}21^{G}$, $^{C}24^{G}$, and $^{E}26^{G}$ (Smahel et al., 2001). The intrinsic stability of the fusion products was assessed by anti-Nef Western blot analysis of lysates from 293T cells transfected with the respective vectors (Fig. 3A). Both full-length Nef^{mut}-E7 and Nef^{mut}-E7^{GGG} fusion products were detected in amounts comparable to Nef^{mut} alone, which was suggestive of intrinsic stability of the fusion products comprising E7. This is of relevance since E7 has been described to be a quite unstable protein (Selvey et al., 1994). On the other hand, both anti-Nef and anti-E7 Western blot analyses of VLPs purified from supernatants of packaging cells showed that the fusion products were efficiently incorporated in their full-length forms and at similar extents (Fig. 3B).

To more accurately evaluate the VLP incorporation extents of Nef^{mut}-E7 fusion products, semi-quantitative Western blot assays on purified VLPs were also performed (Fig. 3C). In detail, the signals produced by scaled quantities of recombinant (r)Nef protein and of Nef^{mut}-E7 VLPs (measured in nanograms of VLP-associated HIV-1 CAp24) were evaluated by densitometric analysis (not shown). We observed that the signal from 62.5-ng CAp24 of Nef^{mut}-E7 VLPs had an intensity similar to that from 12.5 ng of rNef, indicating that Nef^{mut}-E7 associated with VLPs at a mass ratio of approximately 1:5 as compared with CAp24. Similar results were obtained for the VLP incorporation of Nef^{mut}-E7^{GGG} (data not shown).

Hence, both HPV-E7 and E7^{GGG} are incorporated at high levels in (VSV-G) Nef^{mut}-based VLPs, which are therefore suitable candidates for anti-HPV vaccination studies.

Nef^{mut}-E7 molecules delivered by VLPs do not affect Class I MHC expression

The expression of HIV-1 Nef in infected cells correlates with induction of relevant mechanisms of immune evasion mainly mediated by down-regulation of MHC Class I (Schwartz et al., 1996; Wonderlich et al., 2008), and similar effects have been demonstrated for HPV-E7 (Bottley et al., 2008). Thus, before analyzing the immunogenicity of Nef^{mut}-E7 VLP, the impact of the delivery of Nef^{mut}-E7 molecules on MHC Class I expression in professional APCs, e.g., human immature monocyte-derived dendritic cells (iDCs), was accurately evaluated. In this perspective, iDCs were preventively assayed for their ability to internalize fluorescent (VSV-G) VLPs (Fig. 4A). We observed VLP uptake in a significant part of the cell population 2 h after challenge, and in the most part of iDCs after 4 h. Identical results were achieved by challenging mature human DCs as well as primary murine iDCs (data not shown).

We analyzed the expression of MHC Class I on the surface of iDCs 16 h after challenge with 500 ng HIV-1 CAp24 equivalents/10^5 cells of Void, Nef^{mut}, or Nef^{mut} -E7 (VSV-G) VLPs. As shown in Fig. 4B, no modulation of MHC Class I expression was detectable in any challenged iDC population, and similar results were observed 4 and 8 h after challenge (data not shown). Experiments of Nef^{mut}-E7^{GGG} VLP uptake in iDCs generated analogous results (not shown).

These data indicate that the VLP-mediated delivery of Nef^{mut} alone or in combination with E7 does not induce detectable effects on the surface MHC Class I expression of iDCs, thus allowing unbiased analyses of the immunogenicity of Nef^{mut}-based VLPs.

In vivo induction of anti-E7 T cell immune response by (VSV-G) Nef^{mut}-E7 VLPs

Next, we sought to evaluate the efficiency of Nef^{mut}-based VLPs in inducing specific CD8$^+$ T cell adaptive immune response against HPV

E7 *in vivo*. For this purpose, we established an immunization schedule consisting in three subcutaneous inoculations in C57 Black/6 mice of 10 μg CAp24 equivalents of (VSV-G) VLPs at 2-week intervals. Mice were inoculated with VLPs incorporating Nef^{mut} alone or fused with HPV E7 or E7^{GGG} or with void VLPs. After two additional weeks, mice were sacrificed and cultures of explanted splenocytes were carried out for 4 days in the presence of E7 or control peptides. Finally, the presence of activated E7-specific CD8$^+$ lymphocytes was tested by IFN-γ ELISpot assay. As shown in Fig. 5A, we reproducibly detected a number of IFN-γ producing cells much higher in splenocyte cultures from mice inoculated with either Nef^{mut}-E7 or Nef^{mut}-E7^{GGG}VLPs than in splenocytes from control conditions. Concerning the humoral immune response, weak anti-E7 and anti-Nef antibody responses were observed in sera from inoculated mice (not shown), in the presence, however, of well-detectable anti-Gag antibodies with titers ranging from 1:800 to 1:1600, with no significant variations among the different groups.

Next, we compared the antigen-specific CD8$^+$ T cell immune responses induced by E7-based VLPs with that elicited by recombinant E7 protein. To this end, mice received three injections with 2-week intervals of 10 μg HIV-1 CAp24 equivalents of (VSV-G) VLPs incorporating either Nef^{mut} alone or the Nef^{mut}-E7 fusion protein. Meanwhile, another group of mice received three doses of 10 μg of rE7 protein emulsified in incomplete Freund's adjuvant. Two weeks after the last inoculation, mice were sacrificed, and splenocytes isolated and cultured in the presence of E7 or control peptides. After 4 days of incubation, cell activation was evaluated by IFN-γ ELISpot assay. As reported in Fig. 5B, we detected significantly higher E7-specific response in splenocytes from mice inoculated with Nef^{mut}-E7 VLPs with respect to the response detectable in splenocytes from rE7 inoculated mice. Of note, to properly compare the responses elicited by VLP-delivered E7 and rE7, it should be considered that mice received equivalent amounts of HIV-1 Gag and rE7 proteins, and that, as here reported, Nef^{mut}-E7 molecules associate with VLPs about five times less efficiently than HIV-1 Gag products. In addition, E7 represents only about one-third of the whole Nef^{mut}-E7 fusion molecule. Thus, the amount of rE7 administered to mice was about 15-fold higher than that of E7 delivered by VLPs. Notwithstanding, VLP-associated E7 generated a cell immune response about three times stronger than rE7.

In conclusion, these data strongly suggest that the incorporation into (VSV-G) HIV-1-based VLPs of a foreign antigen as part of a Nef^{mut}-based fusion product is an effective tool to elicit a potent CD8$^+$ T-cell-mediated immune response.

Detection of anti-E7 CTL activity in cultures of splenocytes from mice inoculated with (VSV-G) Nef^{mut}-E7 VLPs

Next, we asked whether the anti-E7 cell immune response induced by the Nef^{mut}-based VLPs associated with a detectable cytotoxic activity. To this end, splenocytes pooled from mice immunized with (VSV-G) VLPs incorporating either Nef^{mut} or Nef^{mut}-E7 were cultivated in the presence of E7 peptides for 4 days. Afterwards, the CD8$^+$ cell fraction was purified and co-cultivated for 6 h at scalar cell ratios from 40:1 to 5:1 with murine EL-4 cells previously loaded with either E7-specific or control peptides. The cytotoxic activity was finally evaluated by FACS analysis as the percentage of cell mortality in EL-4 target cells. We noticed that EL-4 cells were killed much more efficiently by the CD8$^+$ cells recovered from mice immunized with Nef^{mut}-E7 VLPs than by those from control mice (Fig. 6). Considering that EL-4 cells are resistant to the cytotoxic activity of CD8$^+$ NK cells (Fostel et al., 2006), our results strongly suggest that the inoculation of Nef^{mut}-E7 VLPs indeed induced anti-E7-specific CTLs.

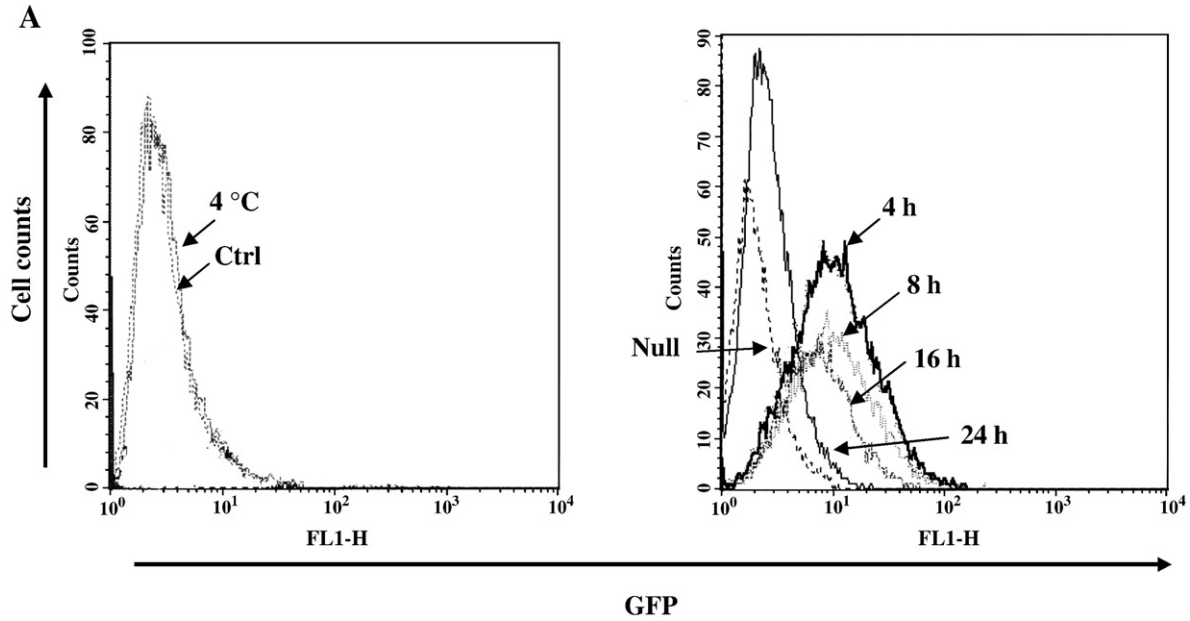

Fig. 2. Nef^{mut} delivered by VLPs is cross-presented by B-LCLs. (A) Kinetic of internalization of (VSV-G) Nef^{mut}-GFP VLPs in human B-LCLs. On the left: B-LCLs were untreated or challenged at 4 °C with (VSV-G) Nef^{mut}-GFP VLPs, maintained for 4 h at the same temperature, and finally FACS analyzed. On the right: B-LCLs were challenged with (VSV-G) Nef^{mut}-GFP VLPs or with the receptor-less counterpart (Null), and FACS analyzed after different times of incubation at 37 °C. Cells incubated with Null VLPs were analyzed only 4 h after challenge. Results are representative of three independent experiments. (B, C) HLA-B7 B-LCLs were challenged with either (VSV-G) Nef^{mut} VLPs or the void counterpart and then put in co-culture with a CD8$^+$ T-cell line specific for an HLA-B7 restricted Nef epitope. As control, B-LCLs were also treated with 5 μg/ml of the B7-restricted Nef peptide. Sixteen hours later, cells were FACS analyzed for the expression of both CD8 and IFN-γ upon treatment for 5 h with brefeldin A (B). In the top row, the cell populations selected for antigen-specific IFN-γ intracellular staining on the basis of the forward scatter/anti-CD8 profile are shown. Percentages of IFN-γ-positive cells are reported. FSC: forward scatter. In the bottom row, both CD8 and intracellular IFN-γ expression on the same cell populations are shown, and the percentages of IFN-γ/CD8-positive cells reported. (C) IFN-γ contents in the co-culture supernatants as measured by ELISA. Mock: supernatants from HLA-B7-restricted Nef-specific CD8$^+$ cells cultivated alone. Results are representative of two independent experiments carried out with triplicate conditions (B) or are reported as the mean values (C) from the same two experiments.

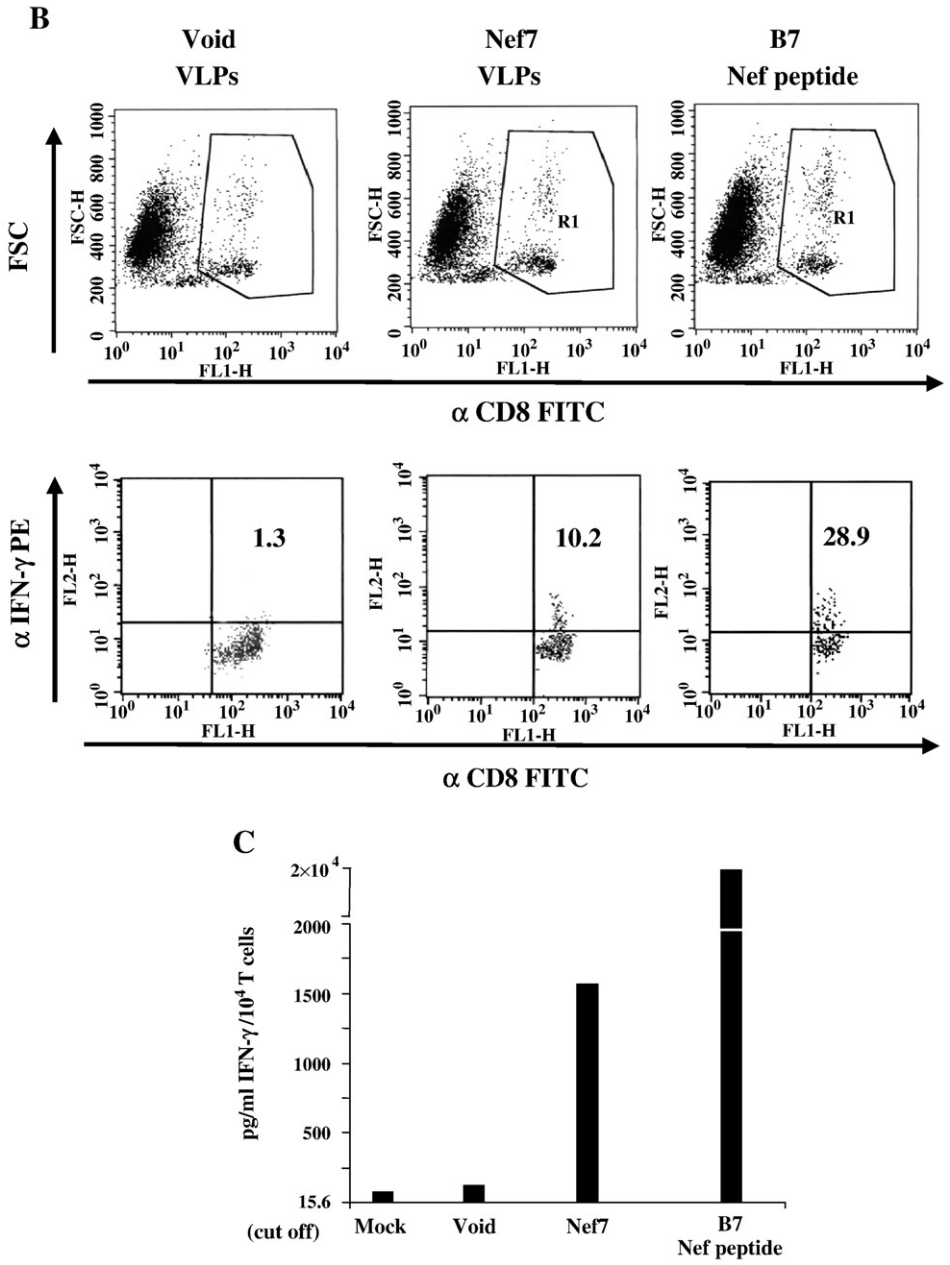

Fig. 2 (*continued*).

The inoculation of Nef^{mut}-E7 VLPs protects mice from HPV-related tumor

In view of the good cytotoxic activity exerted by splenocytes from mice immunized with Nef^{mut}-E7 VLPs, we were encouraged to evaluate whether this vaccination approach could induce a protective effect against experimental tumors induced by the inoculation of TC-1 cells, i.e., a C57 Black/6 mice-derived cell line expressing HPV-E7 (Lin et al., 1996). To this end, mice (nine per group) received three injections of VLP resuspension medium (control mice) or of 10-μg HIV-1 CAp24 equivalents of either Nef^{mut} or Nef^{mut}-E7 VLPs at 2-week intervals. After two additional weeks, mice were inoculated subcutaneously with 5×10^4 TC-1 tumor cells. As depicted in Fig. 7, the majority of mice inoculated with the Nef^{mut}-E7 VLPs rejected the TC-1 tumor cells, whereas control mice or mice immunized with Nef^{mut} VLPs developed visible and progressively growing tumors. Tumor growth appeared slightly delayed in mice inoculated with VLPs incorporating Nef^{mut} alone as compared with naïve mice. This observation suggests a low-grade, innate immune activation induced by the inoculation of (VSV-G) Nef^{mut} VLPs.

Taken together, these data are suggestive of the potential usefulness of Nef^{mut}-based VLPs as anti-tumor vaccines. In addition, these results are consistent with the assumption that a strong anti-E7 CTL response contributes to the eradication of HPV-related tumors.

Discussion

It was previously reported that HIV-1 Gag products can be cross-presented to induce efficient CD8$^+$ T-cell-adaptive immune response (Buseyne et al., 2001; Marsac et al., 2002; Moris et al., 2004). Extending this finding to products incorporated into HIV-1 virions allowed to conceive the Nef^{mut}-based VLP system as a platform for the preferential induction of CD8$^+$ T cell immune response.

We assayed the immunogenicity of the foreign moiety delivered by the Nef^{mut}-based VLPs in the context of the HPV model. HPV E7 is

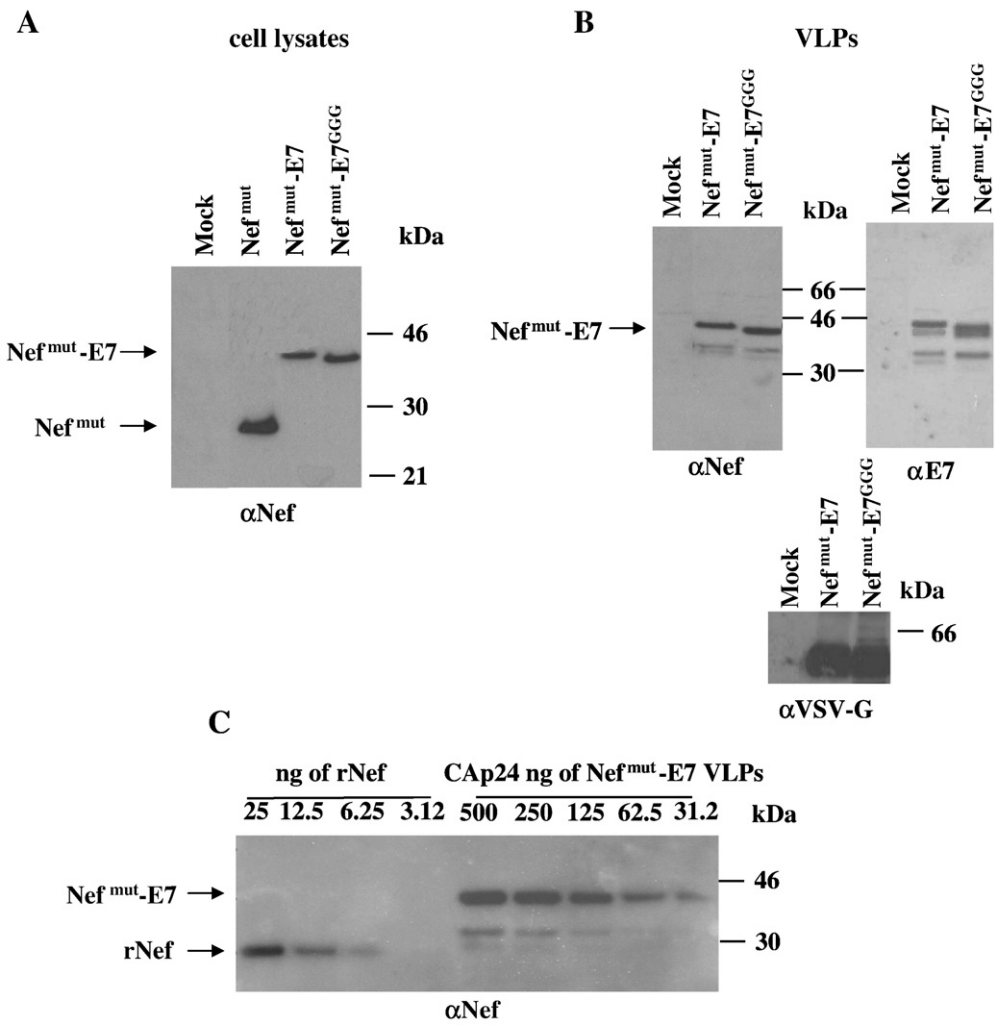

Fig. 3. Molecular characterization of (VSV-G) VLPs incorporating Nef^{mut}-E7 fusion products. (A) Anti-Nef Western blot analysis of 30 μg of proteins from lysates of 293T cells transfected with vectors expressing the indicated products. (B) Western blot analysis of 500 ng of HIV-1 CAp24 equivalents of (VSV-G) VLPs incorporating the indicated fusion products and carried out with either anti-Nef, anti-E7, and anti-VSV-G Abs. (C) Quantitation of Nef^{mut}-E7 virion incorporation. Two-fold serial dilutions of (VSV-G) Nef^{mut}-E7 VLPs were analyzed by anti-Nef Western blot for the presence of Nef^{mut}-E7 as compared with serial dilutions of rNef. The amounts, in nanograms, of both rNef and VLP-associated HIV-1 CAp24 are indicated on the top. In all panels, arrows indicate the specific signals, and molecular markers are given in kilodaltons (kDa). Results are representative of four (A), eight (B), and three (C) independent experiments.

considered a privileged therapeutic target since it is critically involved in the malignant transformation occurring upon persistent HPV cell infection and, together with E6, is stably expressed in transformed keratinocytes of HPV infected patients (Munger et al., 2004). The induction of E7-specific CD8$^+$ T cell response in mice inoculated with either Nef^{mut}-E7 or -E7^{GGG} VLPs is consistent with the idea that products fused with Nef^{mut} undergo efficient cross-presentation similarly to that observed for Nef^{mut} in the *in vitro* antigen cross-presentation assay. We have no obvious explanation for the lower anti-E7 cell immune response induced by VLP-associated E7^{GGG} compared with wild-type E7. Clearly, it did not depend on differences in VLP incorporation levels.

Nef^{mut}-E7 VLPs elicited a very poor antibody response against both Nef and E7 in immunized mice, suggesting that the VLP-associated fusion product preferentially enter the MHC Class I presentation pathway. On the other hand, the induction of sustained levels of anti HIV-1 Gag antibodies was likely due to free Gag products usually co-purifying with HIV-1 particles.

The anti-E7 cell immune response elicited by VLP-associated E7 appeared to be significantly stronger than that generated by recombinant E7. This result is of relevance since it represents the significant added value of Nef^{mut}-based VLP system over recombinant proteins in terms of induction of CD8$^+$ T cell immune response. Of course, it will be important to extend this finding to additional viral and tumor antigens.

In this study, we have tested the capacity of E7 delivered by Nef^{mut}-based VLP system to counteract an HPV-related tumor in a preventive setting. Our results strongly suggest a correlation between the development of E7-specific CTLs induced by the inoculation of Nef^{mut}-E7 VLPs and the growth inhibition of E7 expressing tumor cells. Similar results have been previously obtained in anti-E7 therapeutic experimental vaccinations. In particular, the eradication of established HPV-expressing tumors in animals was obtained by administration of vaccine composed of a liposome-encapsulated E7-specific CTL-T helper fusion peptide (Daftarian et al., 2006) or of chimeric HPV-like particles expressing L1-E7 fusion protein (Schafer et al., 1999). In both cases, vaccines induced E7-specific CTL response in mice, which thus is regarded as essential for a therapeutic approach focused on the eradication of established HPV E7-induced tumors in animal models. The cell-mediated immune response we obtained in animal vaccination with Nef^{mut}-E7 VLPs appeared efficient in counteracting the tumor cell growth. On this subject, it is important to point out that, even if in our system mice were challenged with tumor cells after VLP inoculation, this immunization strategy has some analogy to a true therapeutic approach

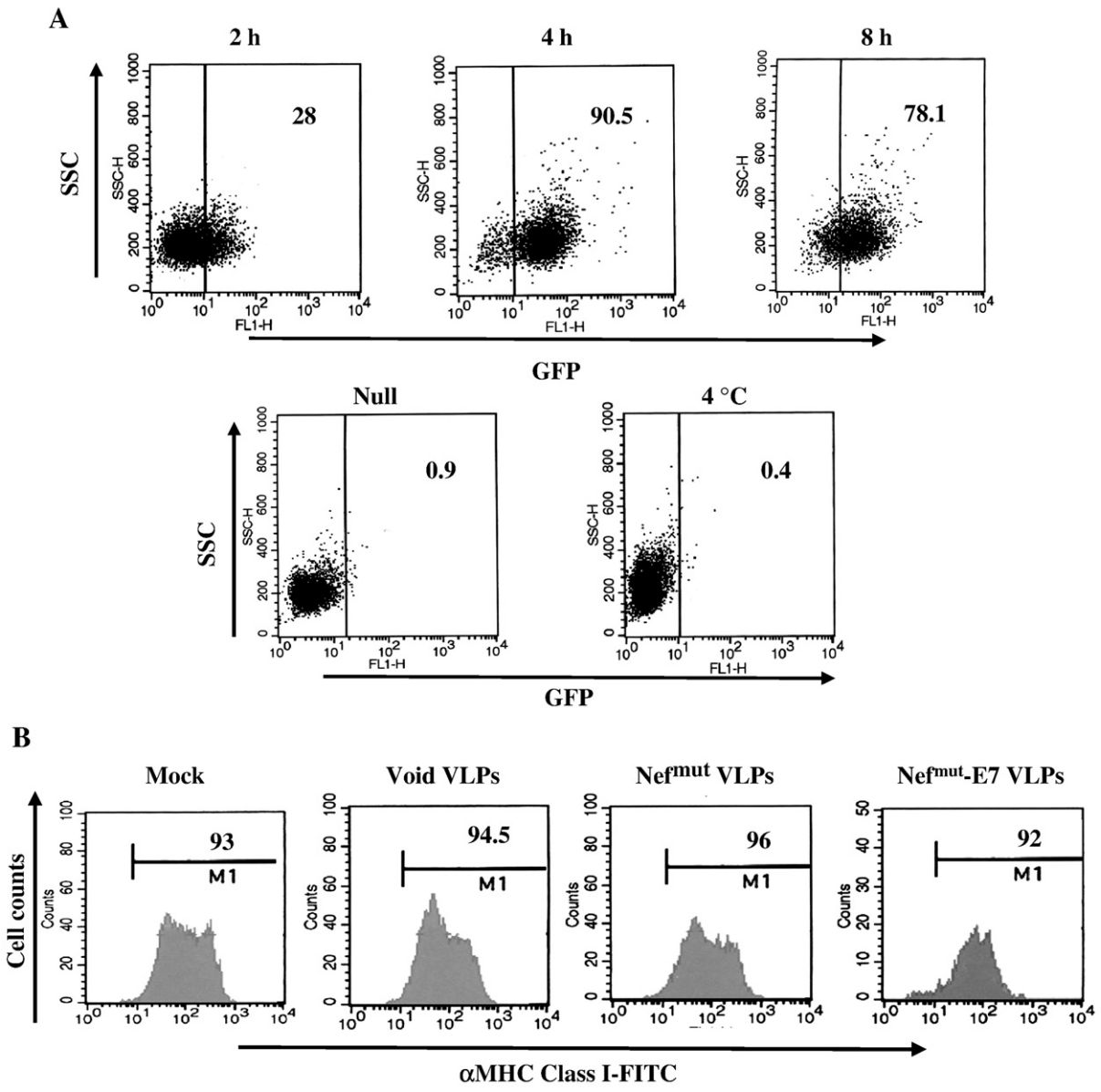

Fig. 4. Expression of cell membrane MHC Class I in iDCs is not affected by the challenge with (VSV-G) Nef^{mut}-based VLPs. (A) Kinetic of internalization of (VSV-G) Nef^{mut}-GFP VLPs in iDCs. FACS analysis of iDCs at different times after challenge with (VSV-G) Nef^{mut}-GFP VLPs (top row) or 4 h after challenge with the receptor-less counterpart (Null VLPs) (bottom row). As additional control, (VSV-G) Nef^{mut}-GFP VLP-treated iDCs were also maintained for 4 h at 4 °C. SSC: side scatter. (B) MHC Class I expression in VLP-challenged iDCs. Human iDCs were mock infected or challenged with 500 ng HIV-1 CAp24 equivalents/10^5 cells of (VSV-G) void, Nef^{mut}, or Nef^{mut}-E7 VLPs, or with an equivalent volume of medium (Mock), and 16 h later FACS analyzed for the expression of MHC Class I. Control of VLP internalization efficiency was carried out in parallel conditions using the same amounts of (VSV-G) Nef^{mut}-GFP VLPs (not shown), and only experiments showing ≥80% of VLP cell internalization were considered. M1 identifies the range of positivity as determined by labeling the cells with control IgG isotype. The percentages of positive cells are indicated in plots of both panels. Results are representative of three independent experiments.

since it is focused on the elimination of HPV-E7 expressing tumor cells (Bourgault et al., 2004; Hung et al., 2007).

Our data open the way towards the exploitation of CD8$^+$ T cell immune response against additional viral and tumor antigens delivered by Nef^{mut}-based VLPs. The most significant advantages of the Nef^{mut} VLP platform over other viral vector-based vaccine strategies can be summarized as follows: (i) VLPs are immunogenic in the absence of genetic material transfer; (ii) in particular, Nef^{mut}-based VLPs include only four viral products besides Nef^{mut}-based fusion protein, that, of major importance, is incorporated at rather high levels and can accommodate quite large proteins (i.e., up to 630 amino acids) without significant impairment of both VLP assembling and cell entry (unpublished results). Conversely, alternative chimeric VLP systems based on assembling of few antigen types (i.e., HBV core VLPs) can accommodate only small epitopes, thus rendering the development of universal vaccines quite complicate; (iii) similarly to retro- and lentiviral vectors, Nef^{mut}-based VLPs can be easily pseudotyped with different receptors. Differently pseudotyped VLPs could be of utility to circumvent the antibody-mediated VLP neutralization occurring after multiple immunizations; and (iv) as an alternative to HIV-1 VLPs, the Nef^{mut}-based-VLP system can be translated into the Moloney Leukemia Virus system without loss of efficiency (Peretti et al., 2005).

Finally, it is also conceivable that co-inoculation of VLPs carrying different immune determinants from the same pathogen (e.g., different antigens from the same virus) would generate a pool of CTLs that could synergize in an immune response difficult to obtain with current vaccine designs. For all these reasons, the Nef^{mut}-based VLP system would be considered a very promising tool for the development of still unexplored vaccine strategies.

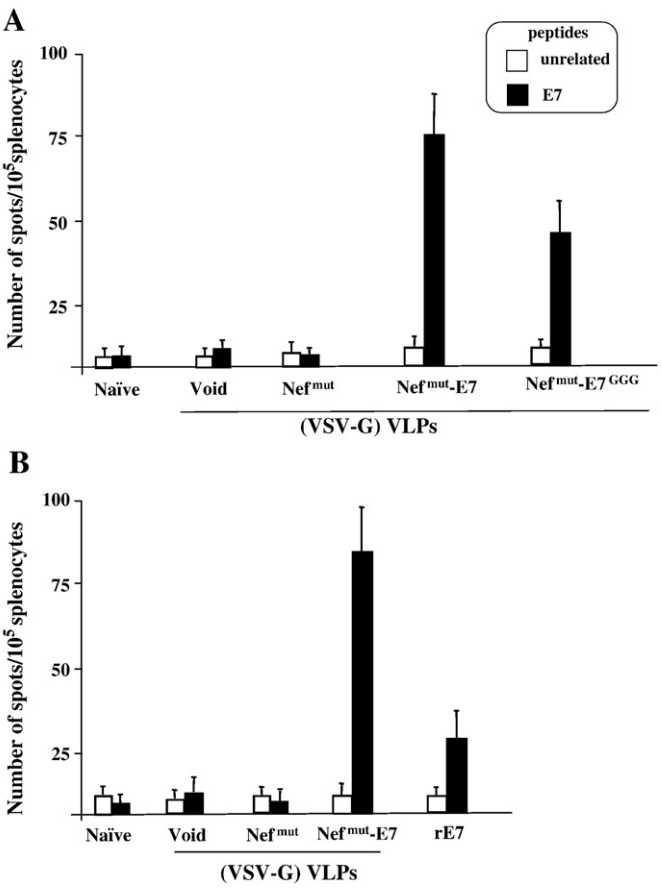

Fig. 5. Cellular immune response in mice inoculated with (VSV-G) Nef^{mut}-based VLPs or recombinant E7. (A) C57 Black/6 mice (at least five per group) were inoculated with empty VLPs (Void) or with VLPs incorporating the indicated Nef^{mut}-based products. As additional control, mice were also inoculated with equivalent volumes of medium (Naive). Splenocytes recovered from mice of each group were pooled, and incubated for 4 days with 5 μg/ml of either control (open bars), or E7 peptides (closed bars). Afterwards, the cell activation extents were evaluated by IFN-γ ELISpot assay carried out in triplicate with 10^5 cells/well. Shown are the mean + SD number of IFN-γ spot-forming cells (SFU)/10^5 cells from four independent experiments. (B) Comparative analysis of the anti-E7 cell immune response in (VSV-G) Nef^{mut}-E7 VLP versus rE7 inoculated mice. C57 Black/6 mice (three per group) were inoculated as described with empty VLPs (Void), VLPs incorporating either Nef^{mut} or the Nef^{mut}-E7 fusion product, or with rE7 protein and adjuvant. As additional control, mice were also inoculated with equivalent volumes of medium (Naive). Splenocytes recovered from mice of each group were pooled, and the cell activation assay was completed as here above described. Shown are the mean + SD number of IFN-γ SFU/10^5 cells from three independent experiments.

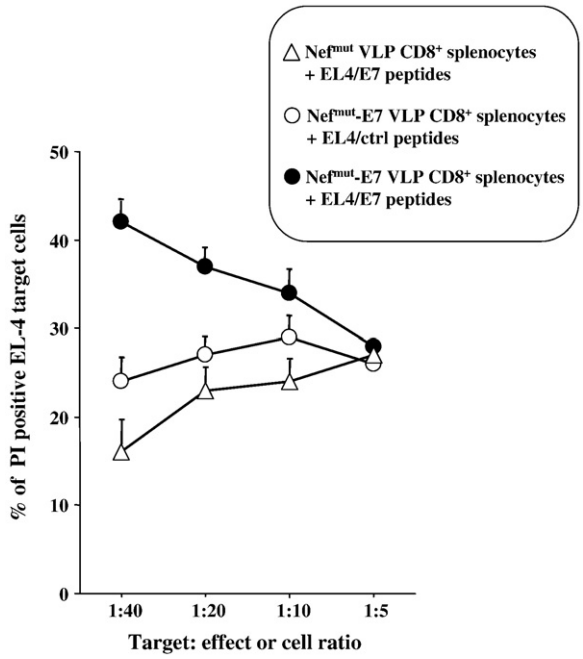

Fig. 6. CTL assay carried out with CD8$^+$ cells from VLP inoculated mice. Splenocytes from mice (five per group) inoculated with either (VSV-G) Nef^{mut} or Nef^{mut}-E7 VLPs were pooled and cultured for 4 days in the presence of E7 peptides. Thereafter, the CD8$^+$ cell fraction was isolated and then co-cultivated for 6 h at the indicated cell ratios with EL-4 cells pre-treated with control or E7 peptides for 16 h. Finally, co-cultures were labeled with FITC-conjugated anti-CD8 mAbs, and the cell mortality in EL-4 cell population was scored by FACS analysis upon propidium iodide (PI) labeling. Shown are the mean + SD values calculated from three independent experiments.

Materials and methods

Cell cultures

For the recovery of HLA-B7-restricted Nef-specific CD8$^+$ T-cell line, peripheral blood mononuclear cells (PBMCs) from previously characterized HIV-1-infected individuals (Cosma et al., 2003) were stained using fluorochrome-linked TPGPGVRYPL/HLA B7 MHC Class I multimers. The cells were then sorted with a Moflo cell sorter (Cytomation, Fort Collins, CO, USA) and expanded in the presence of anti-CD3 mAb (OKT3; Janssen-CILAG, Neuss, Germany), rIL-2 (Chiron, Emeryville, CA), rIL-15 (Sigma, Milwaukee, WI), and irradiated B-LCLs and PBMCs as feeder cells as previously described (Geretti et al., 1996). Similar procedures were carried out for the generation of HLA-Cw7 restricted RRQDILDLWIY-specific CD8$^+$ T-cell line, except that PBMCs from HIV-1 infected patients were stimulated with the specific peptide and antigen-specific response evaluated by the IFN-γ Secretion Assay (Miltenyl Biotech, Bergisch Gladbach, Germany) according to the manufactory protocol. Antigen-specific CD8$^+$ T-cell lines were regularly monitored for their specificity by antigen-dependent intracellular cytokine staining (Kutscher et al., 2008). Human B-LCLs were generated from peripheral blood mononuclear cells purified by standard Ficoll (Biochrom, Berlin, Germany) density centrifugation according to a previously described method (zum Buschenfelde et al., 2001) and cultivated in RPMI, 10% decomplemented fetal calf serum (FCS).

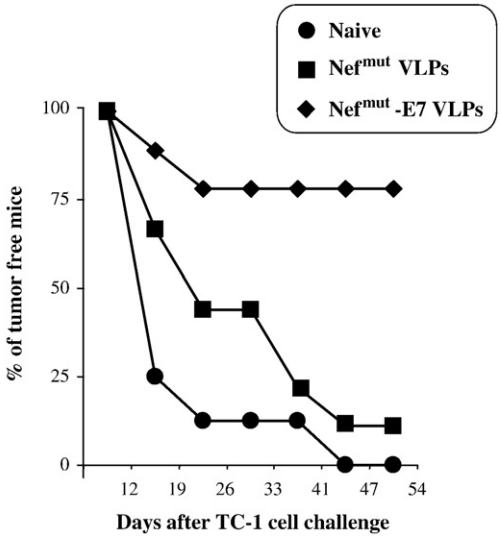

Fig. 7. Anti-tumor activity of Nef^{mut}-based VLPs. C57 Black/6 mice (nine per group) were inoculated three times with (VSV-G) VLPs incorporating the indicated Nef^{mut}-based products. As control, mice were also inoculated with equivalent volumes of medium (Naive). Two weeks after the last inoculation, mice were challenged with 5×10^4 TC-1 cells, and tumor growth was monitored once a week. Results are representative of two independent experiments.

Human embryonic kidney epithelial 293T cells, 293 GPR HIV-1 inducible packaging cells (Sparacio et al., 2001), and TC-1 cells (Lin et al., 1996) were grown in DMEM plus 10% FCS. Human primary iDCs were differentiated from purified monocytes upon 5–8 days culture in RPMI medium supplemented with 20% FCS, 30 ng/ml GM-CSF (Serotec Ltd, Oxford, UK), and 500 U/ml IL-4 (R&D Systems, Oxford, UK). DC phenotype was routinely characterized by FACS analysis as described below. EL-4 cells, a murine thymic lymphoma $CD4^+$ cell line obtained from C57 Black/6 mice upon treatment with 9,10-dimethyl-1,2-benzanthracene (Gorer, 1950), were cultivated in RPMI medium supplemented with 10% of FCS.

VLP production and characterization

The open reading frames coding for GFP and HPV-16 E7 and $E7^{GGG}$ (i.e., a non-tumorigenic E7 allele mutated in the pRb binding site) (Smahel et al., 2001) fused at the C-terminus of Nef^{mut} were recovered by overlapping polymerase chain reaction (PCR)-based protocols and then inserted in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). All constructs were accurately sequenced and selected for the absence of nucleotide mutations. The 293 GPR cells were used as HIV-1 packaging cells for VLP production. In these cells, *gag-pol* genes are expressed under control of an ecdysone-inducible promoter, so that the lentiviral particle production requires cell stimulation with the ecdysone analogue ponasterone A (PonA). VLPs were obtained by co-transfecting immediate–early CMV promoted vectors expressing the respective Nef^{mut}-fused products and the VSV-G protein by Lipofectamine 2000 (Invitrogen). Transfected 293 GPR cells were induced 8 h post-transfection with 5 mM sodium butyrate and 2 μM of PonA. Twenty-four hours later, supernatants were replaced with fresh medium containing the inducers. VLP containing supernatants were finally harvested 24 and 48 h later, clarified, and concentrated by ultracentrifugation on 20% sucrose cushion 100,000×*g*, 2 h at 4 °C. VLP preparations were titrated by measuring HIV-1 CAp24 contents by quantitative ELISA (Innogenetics, Gent, Belgium) and by reverse transcriptase assay.

Western blot analyses of 293T cells transfected with Nef^{mut}-based expressing vectors were performed by lysing cells in PBS, 1% Triton X-100 in the presence of anti-proteolytic agents. Cell lysates were then centrifuged at 6000×*g* for 10 min at 4 °C and supernatants frozen at −80 °C. Aliquots of 30 μg of total cell proteins were separated in 10% SDS–PAGE, thereby undergoing immunoblot analysis. For Western blot analysis of VLPs, 500-ng HIV-1 CAp24 equivalents of VLPs were lysed as described for the cells and then separated in 10% SDS–PAGE. Filters were revealed using the following Ab preparations: sheep anti-Nef antiserum ARP 444; anti-HPV E7 monoclonal antibody from Zymed (S. Francisco, CA); and polyclonal anti-VSV-G protein from Immunology Consultant Laboratories (Newberg, OR). For the semi-quantitative Western blot analysis, serial amounts of rNef preparations obtained and quantified as previously described (Federico et al., 2001) were included.

VLP endocytosis assay

For APC challenge studies, 500-ng HIV-1 CAp24 equivalents of VLPs/10^5 cells were adsorbed on B-LCL by spinoculation at 150×*g* for 30 min at room temperature (r.t.). In the case of iDC challenge, equal VLP amounts were adsorbed through 2 h of incubation at 37 °C in 20 μl of volume. Afterwards, cells were re-fed by adding fresh medium and incubated at 37 °C. Finally, cells were treated for 15 min with trypsin at 37 °C, fixed with 2% v/v formaldehyde in PBS, and FACS analyzed.

Cross-presentation assay

HLA-B7 and -Cw7 B-LCLs were challenged by spinoculation with 500-ng HIV-1 CAp24 equivalents of (VSV-G) Nef^{mut} VLPs/10^4 cells and, after 3 h of incubation, were extensively washed and co-cultured at 1:2 ratio with Nef-specific and HLA-B7 or -Cw7 restricted $CD8^+$ T-cell lines. After overnight incubation, supernatants were harvested, and co-cultures were seeded in fresh medium in the presence of 5 μg/ml brefeldin A (BD Pharmingen, San Diego, CA). Five hours later, co-cultures were FACS analyzed for the expression of both cell membrane CD8 and, after cell permeabilization carried out by Cytoperm-Cytofix (BD Pharmingen), intracellular IFN-γ. The supernatants recovered before brefeldin A treatment were assayed for the presence of IFN-γ by commercial ELISA (BD Pharmingen).

FACS analysis of iDCs

For the detection of iDC cell surface markers, cells were FACS analyzed after 1 h incubation at 4 °C with 1:100 dilutions of the following mAbs: FITC-conjugated anti-CD1a from Dako (Glostrup, Denmark), PE-conjugated anti-CD14 from BD Pharmingen, FITC-conjugated anti-HLA-ABC, clone W6/32 from Sigma-Aldrich.

Mice immunization and detection of IFN-γ producing $CD8^+$ lymphocytes

All studies with animals here described have been approved by the Ethical Committee of the Istituto Superiore di Sanità. C57 Black/6 mice were purchased from Charles River Laboratories (Como, Italy) and were inoculated subcutaneously three times at 2-week intervals with 10 μg HIV-1 CAp24 equivalents of (VSV-G) HIV-1-based VLPs or with 10 μg of rE7 protein prepared as described (Di Bonito et al., 2006) and emulsified in incomplete Freund's adjuvant. Two weeks after the last inoculation, mice were sacrificed and splenocytes put in culture in RPMI 20% FCS in the presence of 5 μg/ml of 8- or 9-mer E7 peptides already identified to efficiently bind the H-2 K^b complex of C57 Black/6 mice (Bauer et al., 1995), i.e., DLYCYEQL (aa 21–28), and RAHYNIVTF (aa 49–57). The H-2 K^b binding HPV E6-specific KLPQLCTEL (aa 18–26) and YDFAFRDL (aa 50–57) (Bauer et al., 1995) were used as control peptides. After 4 days of incubation, IFN -γ ELISpot assay was performed using commercially available reagents (Mabtech AB, Hamburg, Germany). Spot-forming cells were analyzed and counted 16 h later using an ELISpot reader (A.EL.VIS. ELISpot reader and Analysis software GmbH, Hannover, Germany).

ELISA

Sera from inoculated mice were pooled, and two-fold serial dilutions starting from 1:100 were assayed for the presence of anti-Gag, Nef, or E7 Abs. The end-point dilution corresponded to a <0.1 OD absorbance at 450 nm. Each serum was assayed in triplicate, and the mean of the absorbance value was taken as final readout. Recombinant E7 and Nef proteins produced as described (Di Bonito et al., 2006; Federico et al., 2001), and HIV-1 Gag, obtained from NIH AIDS Research and Reference Research Program, were used for the assay. The proteins were adsorbed overnight at 4 °C in carbonate buffer (pH 9.4) into Maxisorp microtiter plates (NUNC, Rochester, NY) at the concentrations of 0.25 μg/well for rE7 and rNef, or 0.1 μg/well for rGag. After a blocking step of 2 h at 37 °C in PBS containing 3% non-fat dry milk (NFDM), plates were incubated at 37 °C for 1 h with 100 μl of serially diluted mouse sera in 1% NFDM-PBS. Specific antigen–antibody complexes were detected by a peroxidase-conjugated goat anti-mouse IgG (GE Healthcare Ltd, UK) using tetramethyl benzidine as substrate. After 30 min at room temperature, the enzymatic reaction was stopped by adding 50 μl of 1 M sulphuric acid/well. Washing steps were done with 200 μl/well of PBS containing 0.05 % Tween-20 in an automatic washer.

CTL assay

Splenocytes from inoculated mice were cultured for 4 days in RPMI 20% in the presence of 5 μg/ml of the above described E7 peptides.

After 4 days, the $CD8^+$ cell fraction was isolated by positive immunomagnetic selection (Miltenyi Biotec., Auburn, CA) and maintained overnight in RMPI 20% in the presence of 10 U/ml of rIL-2. After 16 h, EL-4 cells previously treated overnight with E7 peptides were co-cultured with $CD8^+$ mouse splenocytes at different cell ratios (i.e., from 1:40 to 1:5) in 200 μl of RPMI 20% in U-bottom 96-well plates. After additional 6 h, the co-cultures were labeled with 1:100 diluted FITC-conjugated anti-CD8 mAb (BD Pharmingen), and EL-4 cell mortality was scored by FACS soon after addition of propidium iodide at the final concentration of 10 μg/ml.

Tumor protection assay

C57 Black/6 mice (nine per group) were inoculated following the above reported schedule. Two weeks after the last VLP inoculum, mice were challenged with 5×10^4 TC-1 tumor cells/mouse by subcutaneous injection. Tumor growth was monitored by visual inspection, palpation, and measure of the tumor nodule diameter once a week for at least 3 months.

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