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Strong CD8+ T cell antigenicity and immunogenicity of large foreign proteins incorporated in HIV-1 VLPs able to induce a Nef-dependent activation/maturation of dendritic cells

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ABSTRACT

Virus-like particles (VLPs) are excellent tools for vaccines against pathogens and tumors. They can accommodate foreign polypeptides whose incorporation efficiency and immunogenicity however decrease strongly with the increase of their size. We recently described the CD8+ T cell immune response against a small foreign antigen (i.e., the 98 amino acid long human papilloma virus E7 protein) incorporated in human immunodeficiency virus (HIV)-1 based VLPs as product of fusion with an HIV-1 Nef mutant (Nef^{mut}). Here, we extended our previous investigations by testing the antigenic/immunogenic properties of Nef^{mut}-based VLPs incorporating much larger heterologous products, i.e., human hepatitis C virus (HCV) NS3 and influenza virus NP proteins, which are composed of 630 and 498 amino acids, respectively. We observed a remarkable cross-presentation of HCV NS3 in dendritic cells challenged with Nef^{mut}–NS3 VLPs, as detected using a NS3 specific CD8+ T cell clone as well as PBMCs from HCV infected patients. On the other hand, when injected in mice, Nef^{mut}–NP VLPs elicited strong anti-NP CD8+ T cell and CTL immune responses. In addition, we revealed the ability of Nef^{mut} incorporated in VLPs to activate and mature primary human immature dendritic cells (iDCs). This phenomenon correlated with the activation of Src tyrosine kinase-related intracellular signaling, and can be transmitted from VLP-challenged to bystander iDCs. Overall, these results prove that Nef^{mut}-based VLPs represent a rather flexible platform for the design of innovative CD8+ T cell vaccines.

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1. Introduction

Virus-like particles (VLPs) are genomeless virions produced by the assembling of one or more viral structural proteins [1]. They can be generated by engineering the genomes of many virus species, including gamma-retroviruses [2] and lentiviruses [3]. VLPs mimic the overall structure of virus particles, meanwhile having the ability to bind and enter cells using appropriate receptors. Commercialized VLP-based vaccines have been successful in protecting humans from the infection by both human hepatitis B [4] and human papilloma viruses (HPV) [5], and are currently explored for their potential to challenge other infectious diseases and cancer. Noteworthy, VLPs can be also engineered to incorporate a variety of peptides, proteins and, at least in the case of retro- and lentiviral VLPs, viral envelope glycoproteins (chimeric VLPs), thus potentially representing an extraordinarily flexible tool for new vaccine strategies. Unfortunately, however, in most instances the incorporation of foreign antigens into the VLP structural proteins originated heavy problems in terms of correct assembling of the viral particle.

In infectious diseases, the spread of infecting viruses in many cases can be counteracted by virus-specific antibodies through the so-called "neutralization" phenomenon [6,7]. In some instances, however, this is not sufficient, and the control and clearance of viral infections would benefit from the induction of alternative immune mechanisms. In this regard, specific cytotoxic T lymphocytes (CTLs) can be crucial in light of their ability to recognize and

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kill virus-infected cells. While different VLP types proved suitable to efficiently accommodate peptides, major obstacles remain for the insertion of large polypeptides and proteins addressed to elicit HLA unrestricted CTL immune responses. These responses have been produced through attenuated viruses [8], recombinant viral vectors [9–12], and recombinant DNA [13–16]. However, serious safety concerns, basically regarding the delivery of exogenous genomic material, limit their use in humans.

In an effort to circumvent these limitations, we developed a vaccine platform technology where foreign antigens are efficiently incorporated into lentiviral VLPs. This relies on the ability of an HIV-1 Nef mutant (Nef^{mut}) to be incorporated into human immunodeficiency virus (HIV)-1 and gamma-retroviral virions about 100-fold more abundantly than the wild-type protein, meanwhile acting as carrier molecule upon C-terminal fusion with full-length heterologous proteins [17,18]. Pseudotyping VLPs with envelope glycoproteins supporting pH-dependent cell entry, e.g., that from the Vesicular Stomatitis virus (VSV-G), improves the overall efficiency of delivery into antigen presenting cells (APCs) meanwhile favoring the activation of $CD8^+$ T cell immune response through mechanisms of cross-presentation and cross-priming [19]. This is expected to be consequence of the VLP cell entry through endocytosis, fusion in endosomes, and delivery of VLP products into cytoplasm. Here, these can interact with the proteasome, thereby undergoing degradation and association with Class I MHC molecules.

We recently provided evidence that the incorporation in (VSV-G) Nef^{mut}-based VLPs allows a small size foreign antigen (i.e., the 98 amino acid long E7 oncoprotein from HPV) to be efficiently cross-presented, thus eliciting a strong CTL-based immune response capable of blocking the growth of HPV-related tumors in mice [20]. Since in many instances the size of the heterologous product affects its incorporation efficiency and, as a consequence, its immunogenicity [21,22], we were interested in assaying the flexibility of the Nef^{mut}–VLP system in terms of incorporation, antigenicity and $CD8^+$ T cell immunogenicity of large foreign antigens. Here, we provide evidence that heterologous antigens of up to 630 amino acids (aa) incorporated in Nef^{mut}-based VLPs are efficiently cross-presented and induce a potent $CD8^+$ T cell immune response in mice. The strong antigenicity of the foreign products correlated with the ability of Nef^{mut} incorporated in VLPs to induce activation/maturation of primary human immature dendritic cells (iDCs) through the activation of Src tyrosine kinase-related signaling pathways.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney epithelial 293T cells and derivatives thereof were grown in DMEM plus 10% of decomplemented fetal calf serum (FCS). Human primary iDCs were differentiated from purified peripheral blood monocytes upon 4–5-day culture in RPMI medium supplemented with 20% FCS, 30 ng/ml GM-CSF (Serotec Ltd, Oxford, UK), and 500 units/ml IL-4 (R&D Systems, Oxford, UK). The iDC 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 [23], were cultivated in RPMI medium supplemented with 10% of FCS. The $CD8^+$ T cell clone specific for the HLA-A2 restricted hepatitis C virus (HCV) NS3 peptide KLVALGINAV [24] was kindly provided by V. Barnaba, University "La Sapienza", Rome, Italy. The cell clone was cultured for 24 h after thawing in RPMI medium supplemented with 10% of FCS before setting the cross-presentation assay. PD98050 and Ly294002 were from Sigma–Aldrich. U0126, PP2 and PP3 were from Calbiochem (San Diego CA). Recombinant NS3 protein genotype 1b was from ProSpec, Rehovot, Israel.

2.2. VLP production and characterization

Nef^{mut} is the $^{G}3^{C}$ mutant of the HIV-1 Nef7 allele we previously described and characterized [17]. The procedures for the production of VLPs incorporating Nef^{mut} fused at its COOH terminus with the enhanced green-fluorescent protein (GFP) have been already described [25]. The open reading frames coding for the HCV NS3, genotype 1b, and for the nucleoprotein (NP) of influenza virus, strain PR8, were fused at the C-terminus of Nef^{mut} by overlapping polymerase chain reaction (PCR)-based protocols, and then inserted in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). In detail, the HCV-NS3 sequence was obtained upon RNA extraction (QIAmp viral RNA extraction kit, Qiagen, Hilden, Germany) and RT-PCR amplification (FastStart High Fidelity PCR System, Roche Diagnostics, Mannheim, Germany) of the NS3 region (aa 1027–1658) from a HCV positive human serum. The nucleotide sequence of the clone was determined and classified the HCV isolate as genotype 1b. The NP sequence was amplified from the plasmid pCAGGS-NP, a gift from M. Salvatore (Weill Cornell Medical College, New York, NY). The sequences of the recombinant plasmids were confirmed using the GenomeLab DTCS Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA). Sequencing reactions were run on an automated DNA sequencer (Beckman Coulter).

Phoenix *gp* cells were used as source of MLV VLPs as we previously described [17], while 293 *gag-pol-rev* (GPR) cells were used as HIV-1 packaging cells for VLP production [26]. In the latter 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 vectors expressing the respective Nef^{mut}-fused products and the VSV-G protein under control of the immediate-early cytomegalovirus promoter. Transfections were carried out by Lipofectamine 2000 (Invitrogen)-based method. 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).

Western blot analyses of Nef^{mut}-based VLPs were performed by lysing 500 ng HIV-1 CAp24 equivalents of the VLPs in PBS, 1% Triton-X100 in the presence of anti-proteolytic agents. VLP products were then separated in 10% SDS–PAGE, transferred on filters which were finally revealed using either a pool of HIV-1 positive human sera, the sheep anti-Nef antiserum ARP 444, or the anti-VSV-G polyclonal Abs from Immunology Consultant Laboratories (Newberg, OR).

2.3. Cross-presentation assay

Immature DCs from HLA-A2 donors were challenged 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 an HCV NS3-specific HLA-A2 restricted $CD8^+$ T cell line [24]. After overnight incubation, supernatants were harvested and assayed for the presence of interferon (IFN)-γ by ELISA (BD Pharmingen).

2.4. HCV NS3 antigen presentation assay

The blood from six different HCV positive donors was collected at the Azienda Ospedaleria S. Camillo-Forlanini and at the ASL

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