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Immunogen design and delivery: a new method based on exosomes

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Chapter 1. Introduction

Vaccines

The first effective vaccine was developed in the early 19th century in England; even before that date It was known that the treatment with material coming from infected people could confer immunity against some diseases (e.g. smallpox). Most of these vaccines have been identified in empirical manner; the idea underlying the work of the first scientists was that a small amount of infected material could protect a subject in the same way as a small quantity of poison would have (Stanley Plotkin, 2014). Later in 19th century Pasteur and his colleagues, among others, understood that pathogens could be inactivated using mild heat or chemical treatment and by the 20th century the use of serial replication in abnormal hosts was also used to achieve attenuation.

A crucial passage for vaccine development was the establishment of cell cultures and their use to cultivate viruses. The first vaccine formulated with a preparation of inactivated viruses was the one against Influenza. (Francis T Jr., Magil TP, 1936) and polio vaccine followed after twenty years.

Vaccines act in two different ways: some trigger a humoral, antibody based, response, others stimulate T cell mediated immunity. The majority of therapeutic vaccines, those that are intended to eradicate an established disease, fall into the second category.

Humoral vaccines

Whatever its composition and source are, a good humoral vaccine must induce a good and durable antibody production. Antibodies, or, better, Immunoglobulins (Ig) are glycoproteins composed of one or more units, every unit is formed by four polypeptide chains: two identical heavy chains (usually abbreviated as H) and two identical light chains (L). The amino terminal ends of this polypeptides contain domains showing a considerable variability in amino acid composition and are thus called variable (V) regions to distinguish them from the more constant (C) regions. Each L chain consists of one constant domain (CL) and one variable domain (VL). The H chains, instead, have one variable domain (VH), and three constant domains, called CH1, CH2 and CH3. Each heavy chain has a molecular

weight (about 50kDa), that is about twice the one of a light chain, this gives a resulting total molecular weight of 150 kDa. The variable regions of heavy and light chains contain the antigen-binding sites of the immunoglobulin. Each Ig, thus, can bind 2 molecules of antigen. Another domain involved in the antigen-antibody interaction is the hinge region which is the area of the heavy chain between the first and second constant domain and is held together by disulfide bonds. Not all the immunoglobulins contain a hinge domain, however, when present, it confers flexibility to the molecule and allows the distance between the two antigen-binding sites to vary.

As mentioned before, not all the immunoglobulins are the same, there are five primary classes of immunoglobulins, called IgG, IgM, IgA, IgD and IgE. The classes are distinguished by the type of heavy chain found in the molecule. IgGs have gamma-chains, IgMs have mu chains, IgAs have alpha chains, IgEs have epsilon chains and IgDs have delta chains. The differences in heavy chain polypeptides reflect in the different ways the Igs work, in fact they take part in different types of immune responses and at particular stages of the response. The polypeptide protein sequences responsible for these differences are found primarily in the Fc fragment. There are only two main types of light chains: kappa (κ) and lambda (λ).

Different vaccines have defined levels of serum antibody (as measured by ELISA, hemagglutination, or neutralization assays) that serve as correlates of protective immunity (Plotkin, 2010). A series of experiments performed in rhesus macaques treated with different doses of the yellow fever vaccine demonstrated that protection against the disease occurred in animals with neutralizing antibody titers of ≥0.7 LNI (log neutralizing index) (Mason et al., 1973). For measles, the titer considered to be effective falls between 120–200 mIU/mL

The mechanisms underlying long term antibody responses remain controversial (Amanna and Slifka, 2010; Radbruch et al., 2006). The primary source of serum antibody are terminally differentiated plasma cells (PC). An antibody secreting cell can produce huge amounts of Ig (up to 20,000 immunoglobulins per second)(Conrad and Ingraham, 1974), Which is a crucial feature to block viruses at the site of infection or prevent systemic spread; moreover, pre-existing antibodies play a clear role in vaccine-mediated defense against infections. At the mucosal surfaces, where many pathogens gain entry to the host, IgAs are the primary defense against the incoming threat. A mucosal IgA is generated by local PCs and released as a dimer (dIgA). DIgA molecules bind the polymeric immunoglobulin receptor (pIgR) located at the basolateral surface of mucosal epithelial cells. This complex is

then transported through the epithelial cell and cleaved to form a mature secretory IgA (SIgA) which is excreted into the mucosa. Some vaccines, such as those targeting cholera and typhoid fever, have been developed specifically to promote SIgA responses through oral administration, but immunity appears to wane shortly after vaccination (Holmgren and Czerkinsky, 2005). Parenterally administered vaccines, instead, often elicit long-lasting serum IgG responses, but much lower SIgA production. IgG concentration is maintained at approximately 10 mg/mL in the circulation and is important in controlling systemic viral infections (Amanna and Slifka, 2009). Anyway, IgG may also play an important role at mucosal sites, through the process of transudation. While the active transfer of SIgA across epithelial barriers is well described, the mechanism for IgG transudation is less clear and is usually reported as passive diffusion, though active transport has been described (Spiekermann et Al., 2002) IgG transudation from serum is thought to have a significant protective role against several viral pathogens. Respiratory syncytial virus (RSV), is one of them: RSV is a pathogen that can severely affect infants and children and no vaccines against this virus are available to date (Blanco et Al., 2010). However, one of the most effective strategies for preventing RSV affections is the parenteral administration of a highly neutralizing monoclonal IgG (Empey, Peebles, and Kolls, 2010; Krilov et Al., 2009). The use of parenterally administered IgGs for the neutralization of a respiratory pathogen indicates that systemic IgGs can act at mucosal surfaces. The efficacy of vaccines against human papillomavirus (HPV), a pandemic infectious agent, may be ascribed primarily to the transudation of serum IgGs at the cervical mucosa (Schwarz and Leo, 2008).

There are two theories concerning plasma cell survival (and antibody maintenance). According to one theory, long-lived antibody responses are memory B cell (MBC)-dependent and MBC are the cells that cyclically undergo proliferation resulting in the appearance of antibody-producing daughter cells which home to the BM, replenish declining PC numbers and produce new antibodies (Bernasconi, Traggiai, and Lanzavecchia, 2002; Traggiai, Puzone, and Lanzavecchia, 2003). An opposite theory is that long-lived antibody responses do not depend on MBC and plasma cells can be intrinsically long-lived and produce antibody for years, or decades, without the need for a turnover (Amanna and Slifka, 2010; Elgueta, de Vries, and Noelle, 2010; Radbruch et Al., 2006; Slifka and Ahmed, 1998). This scenarios seems to be confirmed by Tritium incorporation experiments in rats indicating that plasma cells (or their immediate precursors) can be

long-lived. Moreover several studies have demonstrated that antibody responses are not effectively impaired by memory cell depletion (Ahuja et Al., 2008); another study in human subjects vaccinated against tetanus, and treated with anti-CD20 antibodies, indicates that antibody responses do not decrease for at least one year after depletion of peripheral CD20+ B cells (Cambridge et Al., 2003).

T cell vaccines

T cell inducing vaccines have acquired great popularity lately. They are vaccines designed to induce the formation of specific CD4+ or CD8+ T-cells that directly contribute to pathogen clearance through cell-mediated effector mechanisms, rather than only helping B cells to produce protective antibodies; it is a common belief that effector cell mediated responses are needed to fight some classes of pathogens and even more to hinder neoplastic cells (Gilbert SC, 2012).

T cell progenitors originates in bone marrow, from hematopoietic stem cells; lymphoid progenitors translocate to the thymus and expand by cell division to generate immature thymocytes. (Schwarz BA, Bhandoola A, 2006). Initially thymocytes express neither CD4 nor CD8 co-receptors, and are classed as double-negative (CD4−CD8−) cells. As they advance through the stages of their development, they become double-positive thymocytes (CD4+CD8+), and finally single-positive thymocytes; these cells are released to peripheral tissues. The distinctive sign of T cells is their T-cell receptor (TCR) which is involved in the recognition of antigens loaded on class I or class II major histocompatibility complex (MHC) molecules displayed on all cells, whit few exceptions. CD8+ lymphocytes interact with class I MHC (MHC- I) while CD4+ bind MHC-II. The TCR is formed by two peptide chains: α and β, only the cells that express a functional TCR are selected for survival. The first chain which is selected is the β: early thymocytes express an invariant α-chain but rearranges the β-chain locus; when a β-chain that successfully pairs with the invariant α-chain is expressed, the rearrangements stop and the thymocyte starts to proliferate. Next the α-chain is selected. At this stage thymocytes express both CD4 and CD8 and progress to the double positive stage: positive selection selects the cells that can interact with either class I or class II MHC. The process take place in the thymic cortex, where the cells come in contact with antigens expressed by thymic cortical epithelial cells

on MHC molecules present on their surface. Only those thymocytes that have successfully rearranged their TCRα locus and recognize a peptide-MHC complex with the appropriate affinity will receive a survival signal; too strong and too weak interactions lead to cell death. Double-positive cells that interact well with MHC class II molecules will downregulate their CD8 co-receptors and become CD4+ cells, whereas the ones that bind better MHC class I molecules will turn into CD8+ cells.

Thymocytes that have survived the first steps of selection migrate to the medulla in the thymus; there they are again presented with a self-antigen presented on the MHC-I complex of medullary thymic epithelial cells (Hinterberger M et Al., 2010). These cells express self-antigens, i.e. normal antigens present on all kinds of cells on their MHC class I peptides; moreover some thymic epithelial cells are phagocitated by local dendritic cells so that self antigens can be presented also on MHC class II molecules; this allows for selection of both CD8+ and CD4+ thymocytes, since the latter interact with MHC class II molecules present on antigen presenting cells (APCs). Thymocytes that efficiently bind self-antigens receive an apoptotic signal that leads to their death.

Mature Lymphocytes are naïve cells that need to be activated to become fully functional. Activation of T cells is triggered by Antigen presenting cells by the engagement of the T-cell receptor and a co-stimulatory molecule (like CD28, or ICOS) respectively by a peptide loaded on the MHC complex and a co-stimulatory molecules on the APC. The primary, TCR mediated, signaling requires CD3 proteins (CD3ε,γ and ζ) that are part of the TCR complex, and when phosphorylated, serve as the basis for the formation of a complete signaling complex; this leads to an activation cascade involving proteins like LAT and, eventually, PLC-γ. The activation of PLC-γ triggers the production of interleukin-2 (IL-2) in two different ways: one involves the transcription factor NF-κB and the other NFAT. The phosphorylation of CD28 is essential for the formation of the TCR signaling complex, that's why both the primary and the co-stimulatory signals are essential for the start of an effective immune response. In the absence of co-stimulation, T-cell receptor signaling alone leads to T-cell anergy. In addition, optimal CD8+ T cell response needs also CD4+ cells support; they are, in fact, useful for the first activation of naïve CD8 T cells, and help memory CD8+ T cells after an acute infection. (Janssen Edith M et Al., 2003) Co-stimulatory receptors on APCs are induced by a relatively small number of stimuli, usually coming from pathogens, or dead cells, (i.e. heat shock proteins or necrotic bodies); Typical costimulatory receptors are CD80 and CD 86, which together form the complex called B7 as mentioned,

they interact with receptors on T-cells, namely CD28, which is costitutively expressed; other receptors (for instance OX40 and ICOS) are expressed on T-cells following activation, but only after CD28 engagement. The presence of two distinct activation signals prevents T-cells from responding to self-antigens that aren't usually presented in the context of a proper co-stimulation. Activated T-cells show distinctive surface markers such as CD69, CD71 and CTLA-4; the latter avoids over activation, since it competes with CD28 for binding to the B7 proteins.The interaction between APCs and Lymphocytes occurs in the spleen and in the lymph nodes, where mature APCs migrate after having loaded the antigens. T-cells that specifically interact with an APC remain in the lymphatic organs for few days, next they enter the efferent lymphatic vessels and leave the lymph nodes.

As said before, T- cells bind antigens loaded on class I MHC; these peptides originate from degradation of proteins present in the cytoplasm, and thus coming from intracellular pathogens like viruses, or belonging to the cell itself (e.g. neoplastic cell) In the presentation process, these proteins are mainly degraded into small peptides by cytosolic proteases in the proteasome, then, peptides, bound to heat shock proteins, are conveyed to the endoplasmic reticulum (ER) and eventually to the transporter associated with antigen processing (TAP) which moves the cytosolic peptides to the ER lumen using an ATP-dependent mechanism. The ER chaperones involved in MHC-I assembly are, among others, tapasin, calnexin and calreticulin. Peptides are loaded between the α1 and α2 domains of the MHC class I molecule, next the MHC-peptide complex is transported to the cell surface by exocytic vesicles. An alternative way of loading antigens on class I MHC is cross-presentation; this mechanism is of remarkable importance, since allows uninfected, normal, APC to present antigens coming from extracellular space. Cross-presentation, therefore, serves as an alternative to the classic presentation of extracellular antigens on class II MHC. The mechanism of cross-presentation is not fully understood; it happens when proteins acquired by the cell from the outside, mainly in phagosomes, are transportated to the cytosol, cleaved by the proteasome complex and the resulting peptides are moved by TAP transporter to the endoplasmic reticulum, or back into an endosome, where they associate with MHC-I (Hari A et Al., 2014). An alternative model implies the action of endo-lysosomal proteases degrading both vesicles and associated proteins, with the resulting peptides being then loaded into Class I MHC at vesicular levels.

Many different strategies have proven suitable for the induction of T-cell responses. Some of the early vaccines contained attenuated pathogens (for instance, the one against

tubercolosis) while the newest trends today are probably retroviral vectors and more generally DNA based vaccines.

The most promising immunogen, or immunogens, are obviously those that have been found to generate a T-cell mediated protective response, either in humans or animals, highly conserved antigens are usually more promising, but it is possible to combine conserved regions from different proteins to produce a synthetic immunogen (Letourneau S et Al., 2007). As mentioned, It is preferable to include more than one antigen in the vaccine to reduce the insurgence of immune escape. T-cell epitopes may also been identified by bionformatic prediction, or by using a library of peptides spanning the complete antigen sequence.

Cancer vaccines

The first evidences that tumors can induce immune responses date back in the middle of the past century; the idea that cancer can be prevented, or cured, by our immune system is today widely accepted and the discovery of virus induced cancers and tumor-associated antigens (TAA) has boosted the research in the field.

Cancer cells display a variety of proteins that can theoretically function as immunogens: some are non mutated proteins against which T-cell tolerance is incomplete because they are expressed in very limited quantity or only in some tissues, others are peptides present only in malignant cells and can be either non-self antigens or neoantigens. The former are peptides belonging to pathogens that cause cancer (i.e. HPV, HBV), and are usually common between different individuals; this fact obviously makes developing a vaccine easier. Neoantigens derive from proteins that are created by cancer-specific DNA alterations and thus have a unique peptide sequence (Schumacher T. N. & Schreiber R. D., 2015). Identifying these highly variable peptides is becoming easier after the creation of new generation sequencing platforms that allow large scale studies of DNA and RNA of cancer cells This could lead to the development of personalized anti-cancer therapies. Weather a peptide is unique or commonly found in different individuals, to be a good immunogen it must be efficiently displayed on MHC molecules and presented to lymphocytes.

So far, there are only two prophylactic vaccines against cancer, one against some forms of liver tumor and another against cervical cancer. Both malignancies are virus related and

the vaccines induce the formation of antibodies to prevent infections. Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death in humans (Chang MH, 2014); the association of hepatitis B virus (HBV) infection and liver cancer is well documented in epidemiological studies. Patients with chronic hepatitis B have increased risk of hepatocelluar carcinoma, the virus is not directly toxic for hepatocytes but its interaction with the host immune system creates the opportunity for HBV DNA to integrate into the host genome. The HBV infected patients with the highest risk of developing HCC are those that show persistent high viral replication; virus genotype is also important with G and D being the most related to HCC onset; other risk factors are; host age, smoking, mother-to-infant transmission, alcohol abuse.

Anti HBV vaccine is a preparations of hepatitis B virus surface antigen (HBsAg); the newest reagents contain mammalian cell-derived recombinant proteins and some of them include, in addition to the S antigen, peptides from the pre-S2 region while others contain antigens from the pre-S1 and pre-S2 regions (Liliane C Meireles, Rui Tato Marinho, and Pierre Van Damme, 2015). Long-term follow-up studies of newborn vaccination showed that antibodies titers drastically decrease in 15%-50% of vaccinees and this decline is proportional to the antibody titer initially acquired (Vitaliti G et Al., 2013). Anyway, protection against HBV is usually maintained in vaccinees despite a decrease in anti-HBs antibody titer; the exact reason isn't fully understood, but it is probably due to the priming of memory cells.

Cervical cancer is another common malignancy, being the fourth most common cancer affecting women worldwide. HPV is believed to be the cause of the vast majority of these tumors. In many HPV-positive lesions that evolve into cancer, the viral DNA genome is integrated into the host's genome. This process often coincide with the loss of many early (E1, E2, E4, and E5) and late (L1 and L2) genes and this renders prophylactic vaccines useless against HPV-associated cancers. In fact, none of the available vaccines induce clearance of the infection or disease in women already HPV positive at the time of vaccination, since they are composed of recombinant virus like particles (VLPs) bearing the L1 epitope of many different viral types (16 an 18 among others). Antibody titers after vaccination remain high for at least 5/10 years but this doesn't apply to all viral types. (Olsson S.E., et Al., 2009).The oncoproteins E6 and E7 are required for the initiation and progression of HPV associated cancers and are commonly found in transformed cells. For these reasons they are considered the principal source of antigens for the development of a

therapeutic, T-cell inducing vaccine. Several attempts in this direction have been made, the types of therapeutic vaccines created and tested in trials include live vectors, nucleic acids, proteins or peptides, and also cell-based vaccines. As said, most of these preparations target the proteins E6 and E7 with the aim to deliver antigens derived from this polypeptides to APCs in order to activate CD8+ cytotoxic T-cells or CD4+helper T-cells. E7 is the most immunogenic and immunologically better characterized protein, thus most of the therapeutic anti HPV vaccines are based on this polypeptide.

Several viral vectors have been used to deliver HPV E6 and E7 antigens including vaccinia viruses, adenoviruses, adeno-associated viruses, lentiviruses, and alphaviruses, (Cassetti MC et Al., 2004), (Gomez-Gutierrez JG et Al., 2007). TA-HPV is a vaccine prepared with live recombinant vaccinia virus that encodes E6 and E7 of both HPV type 16 and HPV18, it was used in a clinical trial in eight patients with advanced stage cervical cancer; after 21months of follow up the vaccine had induced an HPV-specific cytotoxic T-cell response in three patients and two of them were tumor free (Borysiewicz LK et Al., 1996). In an additional study carried out with TA-HPV 8 out of 29 patients developed HPV specific serological responses, but cytotoxic T cell response didn't last long. A common drawback of viral vectors, anyway, is their intrinsic immunogenicity, which often leads to the insurgence of antiviral immune responses, especially after secondary challenge. (Kaufmann AM et Al., 2002).

E6 and E7 peptides are other good candidates for anti-HPV vaccine design, they are safe and easy to produce but they are poorly immunogenic and require lipids or other adjuvants, such as Toll-like receptor ligands, chemokines and cytokines. Moreover, peptides are MHC specific, and immunogenic epitopes needs to be identified for each patients. One possible solution to overcome this problem are overlapping long-peptide vaccines. A phase II study investigated the efficacy of a synthetic long peptides vaccine in triggering a long-term protection in patients with low-grade abnormalities of the cervix (de Vos van Steenwijk PJ et Al., 2014). In this study the patients received HPV16-peptides vaccination or placebo, a booster was administrated one year later. 97 % of vaccinated patients showed a significant HPV16-specific immune response, lasting up to one year. Full length proteins can be also used to vaccinate against HPV; this approach do not suffer from the MHC specificity issue of short peptides since full length proteins obviously contain all human leukocyte antigen (HLA) epitopes. Protein-based vaccines, however, are generally little immunogenic and the

peptides derived from protein cleavage are usually presented through the MHC class II pathway which activates the production of antibodies instead of generating a CTL response.

DNA vaccines

DNA vaccines are developed to deliver high amount of antigens to APCs, namely dendritic cells (Torrieri-Dramard L, et Al. 2011). Activated dendritic cells induce upregulation of both CD4+ and CD8+ T-cell responses, since CD4+ T-cells are crucial to promote strong effector and cellular memory responses. Direct loading of dendritic cells with the HLA-binding epitopes is possible but challenging, since cells must be acquired from patients, cultured in vitro to undergo priming and then re-injected into the patient. The procedure is long, complicated and also expensive. A fairly efficient level of antigen delivery to APCs is reached through intramuscular injection of DNA, but other DNA delivery methods such as nasal delivery (Bolhassani A, et Al., 2011), particle mediated gene guns (Haynes J.R, et Al., 1996), mucosal delivery and liposomal coating are used.

A DNA plasmid for vaccinal use must have several features: a strong promoter, an antibiotic selection marker and a poly-A sequence to stabilize the mRNA transcript. The most common promoter for human DNA vaccines is human CMV promoter that usually induces high gene expression in a wide array of tissues and at the same time does not affect downstream read through (Manthorpe M, et Al., 1993). Chimeric promoters containing CMV and SV4 sequences have been also successfully used. As well known, the addition of an intron in the vector backbone can positively affect the expression of the target gene and so does the inclusion of a kozak sequence immediately upstream of the gene. Reasons for plasmid instability are, instead, the presence of palindrome sequences, direct or inverted repeats.

In the last decades, viral vectors, especially adenoviral ones, have emerged as a promising gene delivery tool for a variety of applications. This vectors have many advantages: they are often non-pathogenic for their natural hosts and they can efficiently infect a variety of both proliferating and quiescent cell types, such as epithelial cells, fibroblasts, hepatocytes, etc. The generation of an immune response against the vector, anyway, is one of the major drawbacks of viral DNA vaccines. For instance, intravenous inoculation of adenoviral vectors results in the direct interaction of viral capsid proteins with innate immune sensors and is characterized by the early induction of proinflammatory

cytokines. Subsequently, the development of adaptive immune response is often observed, due to the expression of some viral genes and the generation of specific CD8+ T-Cells. Subsequently the host will also develop an Ad-specific humoral immune response (Segura MM, et Al; 2008). The reaction against the vector carrying the antigen results in a lowered expression of the same antigen and repeated treatments often lead to cytotoxicity and acute adverse reactions in general. Intramuscular administration seems to be safer, since the production of antibodies against viral products does not seem to dramatically lower the expression of the transgene and the following vaccinations are better tolerated.

Covalent Modification of viral capsid is one of the possible strategies to overcome vector toxicity. PEGylation (covalent coupling with polyethilene glicol, or PEG) is commonly used; thousands of PEG molecole can be linked to a single viral particle, to modify capsid proteins, hexons, fibers and pentons, which are also targeted by neutralizing antibodies produced by the host (Croyle MA, et Al; 2000). This approach reduces host's adaptive immune response and positively affects transgene expression.

Exosomes

Exosomes are vesicles with a diameter of about 50/100 nm which are secreted by cells into the external space or culture medium. They are produced by practically every kind of cell and they have been found in numerous biological fluids, such as serum, urine and plasma (Caby M.P. et Al., 2005). The cellular pathways involved in the biosynthesis of these vesicles are, therefore, the endo- and exocytotic pathways, let's see them briefly.

As widely known, all cells depend for their survival on the internalization, sorting and possible expulsion of numerous molecules; some of them, due to their size or nature, can pass through the cell membrane passively or in cell-mediated way, while others pass the plasma membrane inside vesicles. Internalization of external, or membrane bound molecules occurs through the invagination of the plasma membrane itself and the formation of endocytic vesicles, which usually fuse together to form a primary endosome in the proximity of the cell membrane. (Luzio et Al., 2009) Inside the vesicles and in the primary endosome the first processing of the endocyted material occurs (i.e. the separation of a ligand from its activated receptor). During its maturation process inside the cell, the primary endosome can evolve into a tardive endosome and, finally, fuse with a lysosome; event that leads to the degradation of endosomal content and membrane turnover. Late endosomes are also known

as multivesicular bodies (MVBs), since they contain a great number of smaller vesicles in their lumen. These small particles form upon budding and detachment of small portions of MVB membrane and thus they retain its lipidic and proteic profile. This process involves particular membrane domains enriched in cholesterol and sfingolipids called lipid rafts and also tetraspanins, 4 transmembrane helices proteins that show high affinity for lipid rafts (Hemler, 2003).

MVBs can face different destinies:

- As said, fusion with a lysosome and consequent degradation.
- Retention in the cell to serve as storage site for several molecules (i.e. MHC molecules in immature dendritic cells (Kleijmeer M. et Al., 2001).
- Fusion with cell membrane leading to the release of the inner vesicles that are therefore called exosomes (Raposo G. et Al., 1996).

Exosomes contain numerous molecules that are exclusive signature of the mother cell, such as MHC II molecules of APCs (Raposo G. et Al., 1996, Zitvogel L. et Al., 1998) or the GluR2/3 subunity of the glutamate receptor in the case of neural cell derived exosomes, or adhesion molecules (integrins, CAMs, etc.) (Fevrier B. & Raposo, 2004).

Other molecules are, instead, found in all exosomes.

- Molecular chaperones (HSP70 e HSP90) (Mathew A. et Al., 1995).
- tetraspanins (CD63, CD9, CD37, CD53, CD81, CD82) (Thèry C. et Al., 1999), (Escola J.M. et Al., 1998).
- The subunities of the trimeric G protein.
- Raft and glycolipid associated proteins (flotillin, CD55, CD59, GM1, GM3), (Rabesandratana H. et Al., 1998).
- Components of the endosomal sorting complexes required for transport (ESCRT), Tsg 101, Alix (Fevrier B. & Raposo, 2004).
- Proteins involved in cellular transport and fusion events (Rab7, Rab2, annexins).

Some of these components (Tsg 101, Alix, GTPases belonging to the Rab family) are for sure involved in the biogenesys and secretion of exosomes, while the functional meaning of other molecules is still to be clarified (Wubbolts R. et Al., 2003).

The ESCRT proteic complex is responsible for the recruitment of membrane proteins inside the MVB. It assembles on monoubiquinated proteins and is disassembled before the

packaging of esosomal proteins inside the vesicles is complete. The ESCRT is composed by 4 subunits numbered from 0 to 3. ESCRT-0 owns several ubiquitin binding domains and it's probably the first protein to bind the target protein (cargo); it also has clathrin affinity domains which preferentially anchor this subunit to the membrane of the endosomal vesicles where it finds its targets.

ESCRT-0 binds the other three subunits of the complex; ESCRT-3, in particular is involved in the shaping and excision of the intraluminal vesicle where the cargo protein will be housed. A subunit of ESCRT-3, Vps24, is the ATPase responsible for the assembly of the ESCRT complex and if its function is precluded, the normal protein trafficking inside the MVB is impaired. A protein which typically interacts with the ESCRT is the transferrin receptor (TfR), even if it recruits the complex through a non-canonical ubiquitin-dependent mechanism (Geminard C. et Al., 2004).

ESCRT is also involved in the budding of retroviral particles, a process that is severely impaired by the deplection of some interactors of the complex (Morita et Al. 2011).

Not all the proteins, anyway, rely on ubiquitination signals to be shifted to the MVBs; some seem to be recruited because of their particular affinity for membrane rafts or tetraspanins; in some cases (transferrin receptor, acetylcholinesterase) the association of different molecules together seems to foster the secretion into exosomes instead of degradation and turnover (Wubbolts R. et Al., 2003). There are also proteins (one is the melanosomal protein Pmel17) that are shifted to the intraluminal vesicles in a way which seems to be independet from ubiquitination and ESCRT functions (Theos A.C. et Al., 2006).

Cytosolic proteins don't seem to be incorporated into the intraluminal vesicles thanks to a particular molecular machinery, but probably they are casually enclosed inside the particles with portions of the cytosol during the MVB membrane budding or maybe because of a temporary association with that membrane. An example is the HSP 70 which interacts with the already cited transferrin receptor and is essential for its recruitment into the exosomes (Geminard C. et Al., 2004).

As mentioned, the GTPases of the Rab family play a crucial role in the process of MVB fusion and the consequent exosome release These small cytosolic proteins can associate to membranes when a geranylgeranyl pyrophosphate group is attached to one (or two) amino acid residues at the C-terminus of the protein. A and b isoforms of Rab2 are important for the correct localization and maturation of MVBs at the cell periphery, in absence of such proteins, in He-La cells, MVBs are retained in perinuclear position and their volume results increased. Moreover, silencing of Rab27 effectors leads to a diminished

exosome release in culture medium (Ostrowski et Al. 2010). In neurons, exosomal secretion seems to positively correlate to potassium induced membrane depolarization, (Faure J. et Al., 2006) and to the increase of intracellular calcium concentration (Mobius W. et Al., 2003).

Another important molecule impacting exosome secretion is the sphingolipid ceramide; when the enzyme that synthesize it (the neutral sphingomyelinase) is downmodulated the release of exosomes is impaired (Trajkovic et Al. 2008). Judging from these and other evidences, it is clear that the lipidic part of MVBs plays a pivotal role in the trafficking of these vesicles. As said, MVBs and exosomes are enriched in cholesterol and they can contain the major part of the cholesterol of the entire endosomal compartment; this seems to be important for the recruitment of MVBs to the cell membrane in alternative to its degradation (Mobius W. et Al., 2003).

Cancer cells are known to release higher amounts of exosomes compared to their non-transformed counterparts (Pap et Al., 2011). This vesicles contain tumor promoting RNAs (miRNAs among others) and proteins (Taylor D D, et Al.,) that can be delivered to other cells; this vesicle-mediate transfer is today considered a novel type of communication between cells. Different sources have reported that cancer-derived exosomes contribute to tumor angiogenesis, they spread growth promoting proteins, such as mutant KRAS, epidermal growth factor receptor (EGFR), and SRC family kinases, and they can also play a role in the development of metastatic niches, facilitating the invasion of circulating tumoral cells (Miller I V, Grunewald T G, 2005). Breast cancer exosomes contain miRNAs associated with the RNA-induced silencing complex (RISC) and Dicer. These exosomes, were found able to transfer their miRNAs content in recipient, non tumorigenic, epithelial cells, which became capable of generating tumors in a dicer-dependent manner.

Exosomes can promote both adaptive and innate immune responses (Zhang et Al. 2014). It is known that B lymphocytes release exosomes containing MHC-II, and these vesicles can present peptide–MHC-II complexes to T-cells and activate them (Raposo et Al., 1996). Also dendritic cells produce their exosomes and they have the capacity to stimulate T-cell responses (Zitvogel et Al., 1998;). These features have generated a noticeable interest around exosomes and prompted their use in immunology and vaccination studies; the overall immunogenicity of non engineered vesicles, anyway, appears to be quite limited.

Exosomes have been also found able to activate innate immunity, for instance as the result of association with molecules inducing secretion of pro-inflammatory cytokines (Xie Y.F et Al, 2010). Macrophages infected with some types of mycobacteria produce exosomes

containing microbial molecules that may bind innate immune receptors and eventually regulate antigen-presenting cells and other cells of the immune system.

Not only eukaryotic vesicles deserve to be studied, pathogen derived exosomes are the subject of numerous papers that demonstrate how those vesicles can regulate host defense and immune responses (Silverman et Al., 2010). In particular, exosomes may spread pathogen's molecules, thereby expanding host responses. This applies to parasites as well, for instance to protozoa of the genus *Leishmania* (Silverman et Al., 2010).

Exosomes can be modified in many ways to improve their natural features or to obtain vesicles with new useful properties. Typical modifications include the addition of homing peptides or ligands on their surface, which confers tropism or, at least, improves the affinity of the vesicles for a particular tissue/cell, significantly decreasing off-target effects, and increasing therapeutic efficacy (Hartman et Al., 2011)

Tissue specific molecules are usually linked to one of the transmembrane proteins that are enriched in exosomes; many groups have used Lactadherin, replacing its EGF domain (which is not required for inclusion in the vesicles) with any targeting peptides. This means that Lactadherin C1 and C2 domains, are sufficient to obtain an efficient display on the external surface of the vesicles (S. Zeelenberg et Al., 2008).

Another protein widely used for exosome targeting is LAMP-2b. LAMP-2b contains a signal peptide, a luminal N-terminus, a transmembrane domain, and then its C-terminus. The insertion of a protein sequence between amino acids 39 and 40 of Lamp-2 allows an efficient inclusion in exosomes (El-Andaloussi et Al., 2012). The fusion to LAMP-2b, obtained as mentioned above, of rabies viral glycoprotein (RVG) (that specifically binds to the acetylcholine receptor) resulted in its efficient display on the surface of exosomes. The RVG engineered vesicles were found able to target mouse neurons, microglia, oligodendrocytes in the brain when injected in the bloodstream (Alvarez-Erviti et Al., 2011).

Whether they are targeted or not, exosomes can be loaded with a variety of different molecules: drugs, proteins, nucleic acids, etc. Electroporation is one of the most popular methods used to this purpose: an electrical field applied to a suspension of exosomes generates pores into the lipid bilayer, facilitating the internalization of the molecules present in solution. Possible drawbacks of this procedure are the formation of aggregates of the cargo molecule (for instance of short interfering RNAs, siRNAs) or the aggregation of the exosomal particles themselves. On the other hand, electroporation parameters can be easily

controlled and this fact is highly valuable for clinical applications. Chemical-based transfection using commercial reagents has also been used to load exosomes with RNA (T.A. Shtam et Al., 2013),. The efficiency of siRNA loading into exosomes was lower when compared to electroporation, and the lipid micelles generated by the transfection reagent (loaded or not with RNA) may still be present in the final exosome preparation. This complicates the evaluation of this method and discourages its clinical application.

Many micro RNA (miRNA) have been found in exosomes and many others have been artificially loaded into them. It can be done in different manners including, again, the simple transfection of a plasmid encoding the precursor miRNA and electroporation of purified vesicles together with synthetic miRNAs. This method has been used to efficiently knock down the expression of GAPDH in mouse brain with a specific siRNA (Alvarez-Erviti et Al., 2011).

Exosomes loaded with miRNA-150 have been used to decrease endothelial cell migration and to mediate suppression of effector T-cells. Exosomes derived from multipotent mesenchymal stromal cells (MSC), enriched in miR-133b, increased the level of neurite outgrowth in cultured neurons, suggesting a potential use of these MSC exosomes in brain ischemia treatment (H. Xin et Al, 2012). In addition, miR-214 transferred into exosomes to hepatic stellate cells decreased the expression of CCN2, a gene known to be important in regulating liver fibrosis (L. Chen et Al., 2013)

Drugs, mainly chemotherapeutics, have been successfully loaded into exosomes, suggesting a potential use in cancer therapies. Vesicles loaded with doxorubicin inhibited the growth of breast and colon adenocarcinoma xenograft tumors in vivo (Y. Tian et Al., 2013). Moreover, when dendritic cell exosomes loaded with doxorubicin were targeted to the tumor tissue, drug's efficacy was greatly enhanced. This associated with significantly less adverse effects, especially in the heart, which is normally damaged by doxorubicin, implying that exosome mediated delivery might decrease the toxicity of chemotherapeutic drugs. Electroporation was the method used to load doxorubicin into exosomes and the results obtained demonstrate that the chemical is not affected by the current used.

HIV Nef^{mut} based exosomes

Nef (negative factor) is a 27 kDa protein of the human immunodeficiency virus (HIV). It is an early viral product, being the first protein synthesized after infection. It lacks enzymatic activity and it's expressed at levels comparable to those of structural proteins.

Nef acts as a scaffold protein; it is anchored to the inner face of the plasma membrane thanks to its miristoyl group, located at the N-terminus; another domain involved in membrane anchoring is in the alpha elix loop 1 and consists of basic residues. Nef is involved in the assembly of a complex of kinases and adaptor proteins called NAKC, for Nef-associated kinase complex that stimulate viral replication by transcriptional derepression (Lee JH et Al., 2010). In particular, Nef recruits to the cell membrane Eed, a protein of the Polycomb Group, which is a nuclear factor and a transcription repressor. The translocation of Eed boosts transcription, including Tat-dependent HIV transcription; Nef is therefore required for optimal Tat function. The same phenomenon is induced by the stimulation of integrin α4β1 through coated fibronectin or VCAM (Witte V et Al., 2004), suggesting that Eed might be recruited predominantly via an integrin-mediated pathway. Thus HIV exploits a cellular mechanism to its advantage. Other components of the Nef-associated kinase complex are hnRNPK, Lck, PKC and PI3K and the activation of these proteins leads to the phosphorylation of Erk1/2. The function of the complex is not entirely known, but in the last few years a group of researchers found that Eed bound neutral sphingomyelinase (nSMase2) and mediated its activation (Philipp S et Al., 2010). This enzyme is responsible for the synthesis of ceramide, a sphingolipid which stimulates the formation of exosomes and other secreted vesicles. The involvement of HIV Nef in extravesicles secretion in vitro and in vivo had been already described by several authors (Muratori et Al., 2009; Lenassi et Al., 2010). Moreover this viral protein is often found associated to lipid raft microdomains wich are abundant in exosomes

A biologically inactive mutant form of Nef (called Nef^{mut}) is incorporated in exosomes at notably high levels (Lattanzi L, Federico M, 2012). This mutant shows several mutations, including the substitution of valine 153 with a leucine and that of glutamic acid 177 with a glycine; in addition, it has a palmitoylation site formed by the presence of a cysteine instead of a glycine at the position 3. This feature has been found to increase the incorporation of Nef^{mut} into exosomes even when other peptides or proteins are fused to its C-terminus. Nef^{mut} can thus serve as an exosome anchoring protein.

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Chapter 2. Aims

This thesis, and more importantly, the experimental work it is based on, originate from the observation that a virus (HIV) hijacks cellular mechanisms to replicate and bud from the infected cell. Being an enveloped virus, HIV strongly depends on host's machinery for vesicle formation and protein cargo sorting.

The HIV protein Nef is known to interact with the membranes of several cellular organelles, inducing exocytosis (Muratori et Al., 2009) and has been found in the plasma of infected patients. This extracellular Nef is believed to negatively affect the survival of $CD4^+$ T-cells by triggering activation induced apoptosis and potentially contributing to the insurgence of AIDS. Nef has been found also inside exosomes secreted by infected or transfected cells (Lenassi et Al., 2010).

A mutant form of HIV Nef (called Nef ^{mut}), that contains a N-terminal palmitoylation site, is incorporated at higher levels in exosomes, compared to its wild type homologous; notably this feature is retained also when foreign proteins are linked to its C-terminus (Lattanzi L, Federico M, 2012). This finding suggested that linking an antigen to Nef^{mut} could result in the production of exosomes bearing that antigen and possibly capable of delivering it to cells such as APCs, which are known to uptake exosomes, to induce an immune response. Remarkably, the retention of the chimeric antigen inside the vesicle would help it to escape degradation. The antigen chosen to be incorporated into exosomes is HPV E7, an oncogenic protein that binds retinoblastoma protein (pRb) and triggers the beginning of an S phase in infected or transformed cells. E7 is normally expressed in all HPV associated cervical cancers and precancerous lesions and the anti HPV vaccines available today don't confer immunity against this protein, since they contain VLP composed of structural L proteins. Many attempts have been made to produce an anti E7 T-cell inducing vaccine, but a definitive results has not been achieved.

Thus we asked if we could use Nef^{mut}-E7 exosomes to induce antigen cross-presentation and CD8+ T cell activation in vitro and in vivo and if these responses were strong enough to protect mice inoculated with E7 positive tumorigenic cells. Exosomes from transfected HEK 293 cells have been used for these experiments. In this context we also tested the efficacy of the adjuvant ISCOMATRIX ™.

Finally we tried to set up a simple DNA vaccination protocol to induce the formation of endogenous Nef^{mut}-E7 exosomes. Namely, we inoculated mice intramuscularly with a plasmid encoding the chimeric antigen, hoping to transfect muscle cells, which are good

exosome producers. This strategy could overcome the typical problems encountered when working with small vesicles: time consuming procedures and poor reproducibility.

Chapter 3

HPV-E7 Delivered by Engineered Exosomes Elicits a Protective CD8+ T Cell-Mediated Immune Response

In this article we used a platform for in vivo immunogen delivery based on Nef engineered nanovesicles (exosomes) that we already described.

Nef^{mut} is a mutant form of the HIV protein Nef. It is efficiently incorporated in exosomes even when a foreign protein is fused to its C-terminus. The chimeric protein is retained into the vesicles and can be transferred to a target cell.

We already demonstrated that Nef^{mut} engineered exosomes can veicolate antigens to APCs for cross-presentation to CD8+ T-lymphocytes, here we show that vesicles produced in vitro bearing the HPV protein E7 can elicit a CD8+ T-lymphocyte mediated immune response after intraperitoneal inoculation in mice. Their effectiveness is comparable to that of HIV based VLPs but they only contain one, inactive, viral protein. Two inoculations with Nef^{mut}-E7 exosomes were sufficient to effectively slow the growth of E7 positive tumorigenic cells infused in mice after immunization and to a lesser extent they functioned also as therapeutic vaccine.

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Article

HPV-E7 Delivered by Engineered Exosomes Elicits a Protective CD8$^+$ T Cell-Mediated Immune Response

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Abstract: We developed an innovative strategy to induce a cytotoxic T cell (CTL) immune response against protein antigens of choice. It relies on the production of exosomes, *i.e.*, nanovesicles spontaneously released by all cell types. We engineered the upload of huge amounts of protein antigens upon fusion with an anchoring protein (*i.e.*, HIV-1 Nef^{mut}), which is an inactive protein incorporating in exosomes at high levels also when fused with foreign proteins. We compared the immunogenicity of engineered exosomes uploading human papillomavirus (HPV)-E7 with that of lentiviral virus-like particles (VLPs) incorporating equivalent amounts of the same antigen. These exosomes, whose limiting membrane was decorated with VSV-G, *i.e.*, an envelope protein inducing pH-dependent endosomal fusion, proved to be as immunogenic as the cognate VLPs. It is noteworthy that the immunogenicity of the engineered exosomes remained unaltered in the absence of VSV-G. Most important,

we provide evidence that the inoculation in mouse of exosomes uploading HPV-E7 induces production of anti-HPV E7 CTLs, blocks the growth of syngeneic tumor cells inoculated after immunization, and controls the development of tumor cells inoculated before the exosome challenge. These results represent the proof-of-concept about both feasibility and efficacy of the Nef^{mut}-based exosome platform for the induction of $CD8^+$ T cell immunity.

Keywords: exosomes; CTL immunity; HIV-1 Nef; HPV-E7; antigen cross-presentation

1. Introduction

Eliciting a strong and broad cytotoxic T cell (CTL) immune response is expected to be of therapeutic relevance for treatment of several pathologies. We tried to establish a novel way to produce $CD8^+$ T cell immunogens against protein antigens of choice. Our strategy was based on the use of engineered exosomes as immunogen carriers. Exosomes are vesicles of 50–100 nanometers forming intracellularly upon inward invagination of endosome membranes [1]. The intraluminal vesicles (ILVs) that are formed in this way become part of multivesicular bodies (MVBs). They are intracellular organelles consisting of a limiting membrane enclosing ILVs. MVBs can traffic either to lysosomes for degradation or to plasma membrane. In the latter case, MVBs release their vesicular contents in the extracellular milieu upon fusion with plasma membrane. Vesicles released by this mechanism are defined exosomes. Exosomes are part of the intercellular communication network [2]. They incorporate messenger RNAs, microRNAs, and proteins that are functional in target cells [3,4].

Exosomes are nanoparticles with a low intrinsic immunogenic profile. Their immunogenicity is essentially related to the amounts and quality of antigens they incorporate. Exosomes became the focus of many investigations aimed at testing their efficacy as anti-tumor immunostimulatory agents, and in some cases they reached approval for clinical trials [5–7]. Exosomes spontaneously uploading tumor antigens have been found to induce activation of specific anti-tumor T cell immunity [8,9]. These antigens are mainly *trans*-membrane proteins like gp100, TRP-1, Her2/neu, and CEA. Exosome-associated proteins can also affect the immune system in a non-antigen-specific way as in the case of immunosuppressive effects that are the consequence of either induction of apoptosis in T cells through the Fas-ligand pathway [10], differentiation of T_{regs} [11], or a decrease of cell cytotoxicity of natural killer cells [12]. On the other hand, exosomes can display unspecific immune-activation properties, most commonly as the result of association with molecular determinants inducing secretion of pro-inflammatory cytokines from target cells [13].

Tumor antigen-bearing exosomes have been tested in a number of clinical trials carried out on late-stage tumor patients. These trials demonstrated both feasibility and good tolerance to exosomes as cell-free vaccines in tumor patients. However, their therapeutic efficacy appeared quite limited. These results posed the need for new methods to increase the overall immunogenicity of therapeutic exosomes. This issue has been faced by engineering desired antigens to increase their association with exosomes. In this regard, two strategies have been described thus far. The first one exploits the binding of C1C2 domains of lactadherin to exosome lipids [14,15]. The other relies on coating exosomes with

Staphylococcus aureus enterotoxin A tailed with a highly hydrophobic *trans*-membrane domain [16]. Both techniques result in a modification of the external contents of exosomes.

The budding of human immunodeficiency virus (HIV) and related lenti- and retroviruses is preceded by the interaction with a number of cell factors also involved in exosome biogenesis, *i.e.*, Alix, Tsg101, and several other components of the endosomal sorting complex required for transport (ESCRT) [17]. HIV budding occurs at lipid rafts, *i.e.*, cell membrane microdomains enriched in cholesterol, phospholipids with saturated side chains, and sphingolipids. Also exosomal membranes contain lipid-raft microdomains [18]. The convergence of exosome and HIV biogenesis implies the possibility that viral products incorporate in exosomes. This was already proven for both Gag and Nef HIV-1 proteins. Nef associates with exosomes by anchoring its *N*-terminal myristoylation to lipid raft microdomains [19].

HIV-1 Nef is a 27 kDa protein lacking enzymatic activities [20]. It is the first HIV product synthesized in infected cells, thereby being expressed at levels comparable to those of HIV structural proteins. After synthesis at free ribosomes, Nef reaches both intracellular and plasma membranes to which it tightly interacts through both its *N*-terminal myristoylation and a stretch of basic amino acids located in alpha helix loop 1. Nef acts as a scaffold/adaptor element in triggering activation of signal transducing molecules. In most cases, this occurs upon Nef association with lipid raft microdomains. The fact that exosome membranes also are enriched in lipid raft microdomains explains why Nef can incorporate in both exosomes and HIV viral particles.

We previously identified a $^{V}153\ ^{L\ E}177^{G}$ Nef mutant incorporating at quite high levels in HIV-1 virions, HIV-1-based virus-like particles (VLPs) [21], and exosomes [22]. The incorporation efficiency of this mutant further increases by adding an *N*-terminal palmitoylation site through $^{G}3^{C}$ mutation, expectedly leading to improved association with lipid rafts. This Nef mutant (referred to as Nef^{mut}) is defective for basically all Nef functions [23], and its efficiency of incorporation in nanovesicles does not change significantly when foreign proteins are fused at its *C*-terminus [22].

Here, we provide evidence about both feasibility and efficacy of the Nef^{mut}-based exosome system as a vehicle for inducing $CD8^{+}$ T cell immunity. This strategy is expected to overcome the already proven limited CTL immunogenicity of exosomes, meanwhile maintaining their overall low basal immunogenicity and high biosafety profile. All these features, together with the demonstrated flexibility in terms of incorporation of foreign antigens and ease of production, make Nef^{mut}-based exosomes a convenient candidate for a novel CTL vaccine platform.

2. Materials and Methods

2.1. Molecular Constructs

Both Nef^{mut} and Nef^{mut}/HPV-E7 expressing vectors have been already described [24]. The Nef^{mut}/MART-1 expressing vector has been recovered upon PCR amplification of MART-1 cDNA, and cloned in the previously described pTarget-Nef^{mut} shuttle vector [24]. The resulting molecular construct was checked for the absence of mutations. VSV-G was expressed by an IE-CMV-promoted vector.

2.2. Cell Cultures

HEK293T, 293/GPR37 [25], and TC-1 cells [26] were grown in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal calf serum (FCS). Human monocytes were separated from peripheral blood mononuclear cells (PBMCs) using anti-CD14 microbeads (Miltenyi, Bergish Gladbach, Germany), and differentiated to iDCs upon 4–5 days of culture in Roswell Park Memorial Institute medium (RPMI) medium supplemented with 20% FCS, 30 ng/mL GM-CSF (Serotec Ltd., Kidlington, UK), and 500 units/mL IL-4 (R & D Systems, Minneapolis, MN, USA). The iDC phenotype was routinely characterized by fluorescence-activated cell sorting (FACS) analysis for the expression of CD11c and the absence of CD14 cell membrane markers. Both isolation and expansion of the $CD8^+$ T cell clone specific for MART-1 have been previously described [27]. MART-1-specific $CD8^+$ T cells recognize the HLA-A.02-restricted $AAGIGILTV_{27–35}$ amino acid sequence. They were cultivated in RPMI plus 10% AB human serum (Gibco, Life Technologies, Monza, Italy), and regularly monitored for its specificity. Both mouse splenocytes and EL-4 cells, *i.e.*, murine thymic lymphoma $CD4^+$ T cells originally obtained from C57 Bl/6 mice upon treatment with 9,10-dimethyl-1,2-benzanthracene [28], were cultivated in RPMI medium supplemented with 10% FCS.

2.3. VLP and Exosome Production

Lentiviral VLP preparations were obtained from supernatants of transfected 293/GPR37 cells. In these cells, HIV-1 *gag-pol* genes are expressed under control of an ecdysone-inducible promoter, so that the lentiviral particle production requires cell stimulation with the ecdysone analog ponasterone A. VLPs were obtained by transfecting Nef^{mut}-based vectors in the presence or not of the vector expressing VSV-G by Lipofectamine 2000 (Invitrogen, Life Technologies, Monza, Italy). Transfected 293/GPR37 cells were induced 8 h post transfection with 5 mM sodium butyrate and 2 μM of ponasterone A. Twenty-four hours later, supernatants were replaced with fresh medium containing the inducers. VLP-containing supernatants were finally harvested both 24 and 48 h later, clarified, and concentrated by ultracentrifugation on 20% sucrose cushion at 100,000 × *g*, 2.5 h, 4 °C. VLP preparations were titrated in terms of HIV-1 CAp24 contents by quantitative ELISA (Innogenetics, Gent, Belgium).

To produce exosomes, HEK293T cells were transfected with vectors expressing the Nef^{mut}-based fusion proteins. The cell cultures were washed 24 h later, reseeded in complete medium in the presence of exosome-deprived FCS, and the supernatants were harvested from 48 to 72 h after transfection. Exosomes were recovered through previously described methods [29]. In detail, supernatants were centrifuged at 500 × *g* for 10 min. Then, supernatants underwent differential centrifugations consisting in a first ultracentrifugation at 10,000 × *g* for 30 min. Supernatants were then harvested, filtered with 0.22 μM pore size, and ultracentrifuged at 70,000 × *g* for l h. Pelleted vesicles were resuspended in 1 × PBS, and ultracentrifuged again at 70,000 × *g* for 1 h. Afterwards, pellets were resuspended in 1:100 of the initial volume of 1 × PBS. The amounts of recovered exosomes were evaluated by measuring the activity of acetylcholinesterase (AchE), *i.e.*, a classical exosome marker [30], through the Amplex Red kit (Molecular Probes, Life Technologies, Monza, Italy).

2.4. VLP and Exosome Characterization

Both VLP and exosome preparations were characterized by Western blot and FACS analyses. Through a previously described FACS-based assay for binding with cholera toxin, subunit B (CTX-B, Sigma-Aldrich, St. Louis, MO, USA) [22], we established that, in terms of number of nanovesicles, 1 μg HIV-1 CAp24 equivalent of VLPs equals 200 μU of AchE activity of exosomes [22,31]. Considering that an HIV-1 particle contains about 5,000 CAp24 molecules [32], one can calculate that 1 ng CAp24 of HIV-1 contains about 10^7 vesicles. By consequence, we estimated the presence of about 10^{10} vesicles in both 1 μg CAp24 of HIV-1-based VLPs and 200 μU of AchE activity of exosomes. Equivalent amounts of nanovesicles were lysed in PBS, 1% Triton X-100 in the presence of anti-proteolytic agents, and then separated in 10% SDS-PAGE. Filters were revealed using the following Ab preparations: sheep anti-Nef antiserum ARP 444 (a generous gift of M. Harris, University of Leeds, Leeds, UK), anti-HPV E7 mAb from Zymed (Thermo Fisher, Waltham, MA, USA), and polyclonal anti-VSV-G protein from Immunology Consultant Laboratories (Portland, OR, USA). For semi-quantitative Western blot analysis, serial amounts of recombinant Nef obtained and quantified as previously described [33], were included as reference samples.

For the FACS analysis of nanovesicles, samples were incubated with 5 μL of surfactant-free white aldehyde/sulfate latex beads (Invitrogen, Life Technologies, Monza, Italy) overnight at r.t. on a rotating plate. Then, beads were incubated at 37 °C for 2 h with 1:50 diluted FITC-conjugated CTX-B. Therefore, bead–VLP complexes were treated with Cytoperm-Cytofix solution (BD Pharmingen, S. Diego, CA, USA) for 20 min at 4 °C, and finally labelled with 1:50 dilution of PE-conjugated KC57 anti-CAp24 mAb (Beckman-Coulter, Milano, Italy) 1 h at 4 °C. Bead-exosome complexes were labelled with PE-conjugated anti-CD63 mAb (BD Pharmingen) for 1 h at 4 °C. Finally, beads were washed, resuspended in 1× PBS-2% *v*/*v* formaldehyde, and FACS analyzed.

2.5. Mice Immunization and Detection of IFN-γ Producing $CD8^+$ T Lymphocytes

All studies with animals here described have been approved by the Ethical Committee of the Istituto Superiore di Sanità, Rome, Italy (protocol n. 555/SA/2012) according to Legislative Decree 116/92 which has implemented in Italy the European Directive 86/609/EEC on laboratory animal protection. Animals used in our research have been housed and treated according to the guidelines inserted in the aforementioned Legislative Decree. C57 Bl/6 mice were purchased from Charles River Laboratories (Calco, Italy), and inoculated subcutaneously (s.c.) 3 times at 2-week intervals with nanovesicles carrying equivalent amounts of antigens. Two weeks after the last inoculation, mice were sacrificed, and splenocytes put in culture 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 Bl/6 mice [34], *i.e.*, DLYCYEQL (aa 21–28), and RAHYNIVTF (aa 49–57). H-2 K^b binding HPV E6-specific KLPQLCTEL (aa. 18-26) and YDFAFRDL (aa 50–57) peptides [34] were used as control. After 4 days of incubation, IFN-γ Elispot assay was performed in triplicate conditions using commercially available reagents (Mabtech AB, Nacka Strand, Sweden). Spot-forming cells were analyzed and counted 16 h later using an Elispot reader (A.EL.VIS. Elispot reader and Analysis software GmbH Version 6.0, Hannover, Germany).

2.6. Cross-Presentation Assay

HLA-A.02 iDCs were challenged with equivalent amounts of exosomes (*i.e.*, 50 μU of AchE activity/10^5 cells) associating or not with VSV-G. After 4 h of incubation, the iDCs were extensively washed and co-cultured in triplicate conditions at 1:2 ratio with the MART-1-specific HLA-A.02-restricted $CD8^+$ T cells in an IFN-γ Elispot microwell plate. After overnight incubation, IFN-γ Elispot assay was performed using commercially available reagents, and spot-forming cells were analyzed using an Elispot reader.

2.7. Detection of Anti-E7 Abs

Sera from inoculated mice were pooled, and two-fold serial dilutions starting from 1:100 were assayed for the presence of anti-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 produced as described [35] was used for the assay. The protein was adsorbed overnight at 4 °C in carbonate buffer (pH 9.4) into Maxisorp microtiter plates (NUNC) at the concentrations of 0.25 μg/well. After a blocking step of 2 h of 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., Hatfield, 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.

2.8. CTL Assay

Splenocytes from inoculated mice were cultured for 4 days in RPMI 20% FCS in the presence of 5 μg/mL of the above-described E7 or control peptides. After 4 days, the $CD8^+$ cell fraction was isolated by positive immunomagnetic selection (Miltenyi Biotec.), and maintained overnight in RMPI 20% FCS in the presence of 10 U/mL of recombinant IL-2. After 16 h, EL-4 cells, previously labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) and treated overnight with either E7 or control peptides, were co-cultured with $CD8^+$ mouse splenocytes at different cell ratios (*i.e.*, from 1:100 to 1:2) in 200 μL of RPMI 20% in U-bottom 96-well plates. After additional 6 h, EL-4 cell mortality was scored by FACS analysis soon after the addition of 7-AAD at a final concentration of 1 μg/mL.

2.9. Anti-Tumor Effects of Nef^{mut}/E7 Exosomes

C57 Bl/6 mice (5 per group) were inoculated following the previously reported schedule. Two weeks after the last exosome inoculum, mice were challenged with 10^5 TC-1 tumor cells/mouse by s.c. injection. Tumor growth was monitored by visual inspection, palpation, and measurement of tumor nodule diameter.

Anti-tumor activity of Nef^{mut}/E7 exosomes was also evaluated upon inoculation of mice already challenged with 2×10^5 TC-1 cells. Exosome inoculations were performed 6, 11, and 19 days after tumor cell challenge only in mice which developed palpable tumors before the first immunization. At the end of the observation time, tumors were explanted and weighted.

2.10. Statistical Analysis

When appropriate, data are presented as mean + standard deviation (SD). In some instances, the paired Student's *t*-test was used and confirmed using the non-parametric Wilcoxon rank sum test. $p < 0.05$ was considered significant.

3. Results

3.1. Similar CD8$^+$ T Cell Immune Responses Elicited by HPV-E7 Uploaded in Either Nef^{mut}-Based Lentiviral VLPs or Exosomes

Retro- and lentiviral VLPs are flexible vehicles for foreign immunogens. However, major hindrances regarding safety and ease of production strongly limit their potential application in clinic. The identification of the Nef^{mut} allele having an extraordinary ability to incorporate into both lentiviral-basedVLPs and exosomes even when fused with heterologous proteins opened the possibility to compare the two nanovesicle types in terms of efficiency of immunogen delivery. To this end, preparations of lentiviral VLPs and exosomes incorporating either Nef^{mut} alone or the product of its fusion with HPV-E7 protein were obtained and characterized. Both nanoparticle preparations were decorated with the G protein from vesicular stomatitis virus (VSV-G) to improve the delivery of nanoparticle contents in the cytoplasm of APC, thus favoring cross-presentation. Figure 1A shows the Western blot analysis of 500 ng of CAp24 of VLPs and equivalent amounts of exosomes, *i.e.*, 100 μU of AchE activity. We estimated that the samples comprised about 5×10^9 nanovesicles. As already shown for alternative Nef^{mut}-based fusion products [22], Nef^{mut}/E7 also incorporates in VLPs and exosomes with comparable efficiencies.

The nanoparticle preparations were identified by FACS analysis upon binding with aldehyde-sulfate latex beads in terms of contents of both monosialotetrahexosylganglioside (GM1) and HIV-1 CAp24in VLPs, and both GM1 and CD63 in exosomes (Figure 1B).

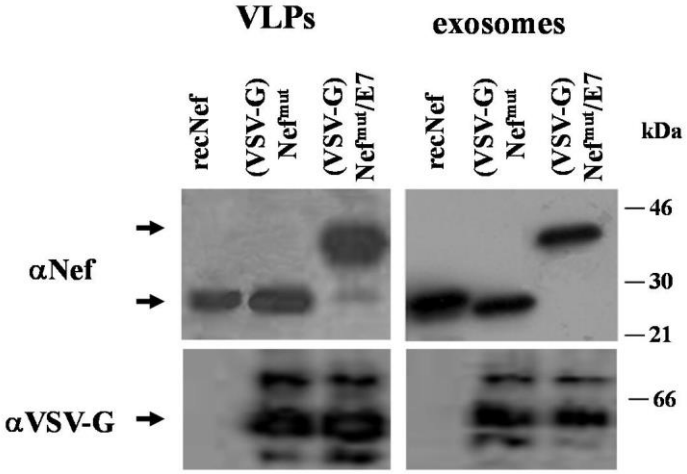

Figure 1. *Cont.*

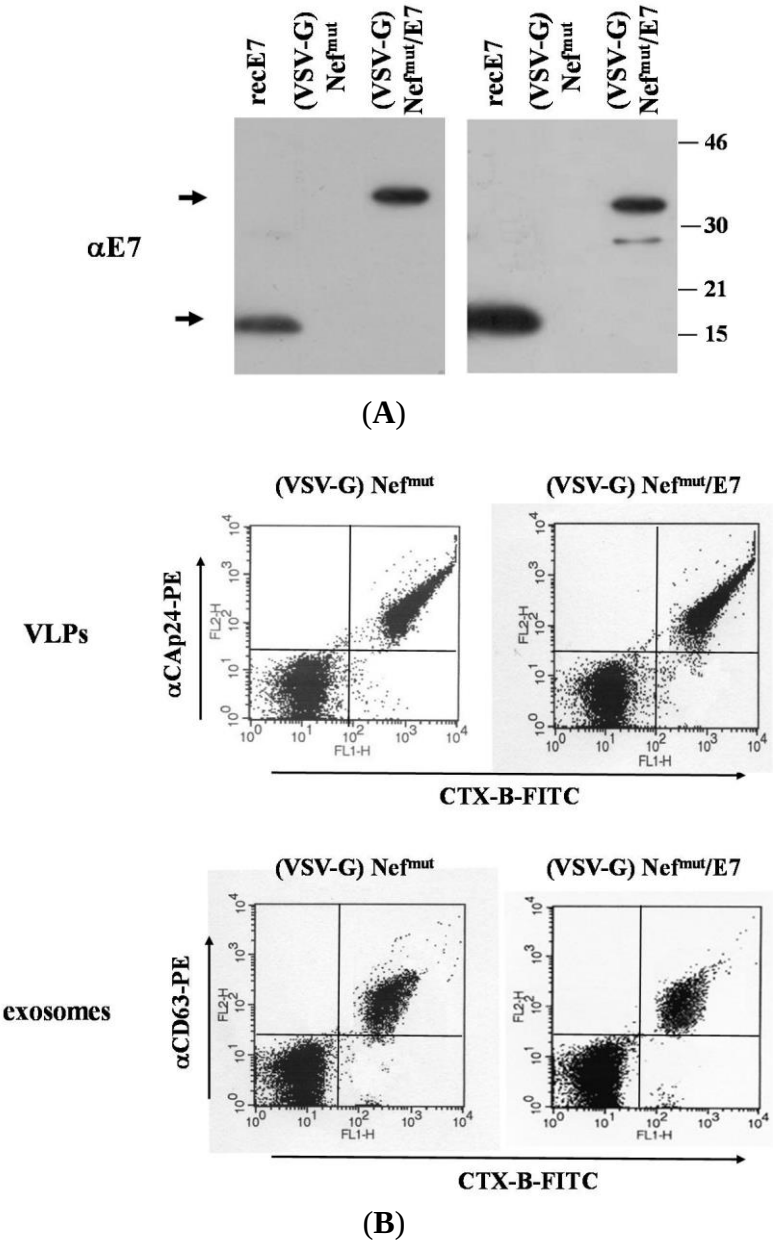

Figure 1. Molecular characterization of VLP and exosome preparations. (**A**) Western blot analysis of (VSV-G) VLPs and exosomes incorporating either Nef^{mut} or Nef^{mut}/E7. In the left panels, the contents of both (VSV-G) Nef^{mut} and (VSV-G) Nef^{mut}/E7 VLPs as revealed by anti-Nef, anti-VSV-G, and anti-HPV-E7 Abs are shown. The same Abs were used to detect the contents of (VSV-G) Nef^{mut} and (VSV-G) Nef^{mut}/E7 exosomes (right panels). As control, 100 ng of either recombinant Nef or E7 were loaded. Arrows indicate the relevant protein products. Molecular markers are given in kDa. Results are representative of five independent experiments; (**B**) FACS analysis of both VLPs and exosomes incorporating either Nef^{mut}, or Nef^{mut}/E7. Equivalent amounts of VLPs and exosomes were bound to surfactant-free white aldehyde/sulfate latex beads, and then assayed for the contents of GM1 and HIV-1 CAp24 (VLPs), or GM1 and CD63 (exosomes). Quadrants were set on the basis of the fluorescence of beads alone labeled with the respective ligands. Results shown in both panels are representative of four assays carried out on two different VLP and exosome preparations.

C57 Bl/6 mice were inoculated subcutaneously (s.c.) three times with volumes of VSV-G pseudotyped VLPs or exosomes containing 650 ng of immunogen, as determined by semi-quantitative Western blot analysis carried out using recombinant Nef as reference standard (not shown). Two weeks after the last inoculation, mouse splenocytes were isolated and cultured for 4 days in the presence or absence of either unrelated or HPV-E7-specific nonamers to selectively stimulate $CD8^+$ T lymphocytes. Afterwards, 10^5 cells were seeded in IFN-γ Elispot wells, and the number of spot-forming units (SFU) was scored 16 h later. Data reported in Figure 2 show that, in terms of induction of $CD8^+$ T cell response, E7 uploaded in exosomes was as immunogenic as that delivered by VLPs. Of note, no anti-HPV E7 antibodies were found in sera from mice inoculated with either VLPs or exosomes (not shown).

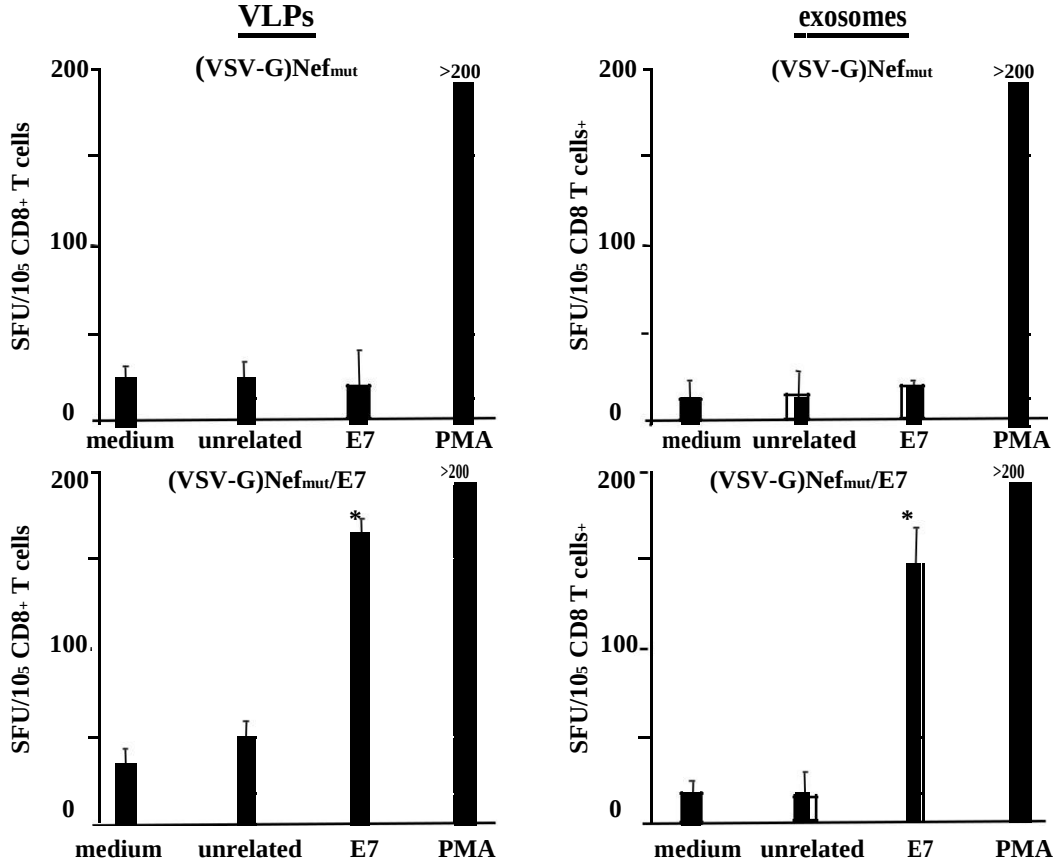

Figure 2. $CD8^+$ T cell immune response in mice inoculated with (VSV-G)Nef^{mut}-based VLPs or exosomes. C57 Bl/6 mice (five per group) were inoculated three times with VLPs or exosomes incorporating Nef^{mut}/E7. As control, mice were also inoculated with equivalent amounts of both nanovesicle types incorporating Nef^{mut} alone. Splenocytes recovered from mice were incubated with or without 5 μg/mL of either unrelated or E7-specific peptides. Afterwards, cell activation extents were evaluated by IFN-γ Elispot assay carried out in triplicate with 10^5 cells/well. As control, untreated cells were also incubated with 5 ng/mL of PMA and 500 ng/mL of ionomycin. Shown are the mean + SD number of IFN-γ spot-forming cells (SFU)/10^5 cells. The results are representative of two independent experiments. ***** $p < 0.05$.

These results support the idea that Nef^{mut}-based exosomes can be considered antigen carriers as efficient as Nef^{mut}-based VLPs.

3.2. The Association of VSV-G to Exosomes Is Dispensable for Eliciting an Optimal Immune Response in Mice

The association of the fusogenic VSV-G envelope protein to exosomes was expected to favor presentation of cargo on Class I MHC. Consistently, we previously observed increased cross-presentation of foreign antigens uploaded in Nef^{mut}-based exosomes when B-LCLs were challenged with VSV-G exosomes compared with non-pseudotyped exosomes [22]. However, inclusion of VSV-G or, expectedly, alternative pH-dependent fusogenic envelope proteins represents a major limitation in terms of scalable production of exosomes. Hence, we were interested in investigating the immunogenicity of Nef^{mut}-based exosomes in the absence of envelope proteins.

To this aim, we first reproduced the *in vitro* assays for cross-presentation of exosome-associated foreign antigen, however using monocyte-derived immature dendritic cells (iDCs) as APC instead of the previously tested B-LCLs [22]. This approach was pursued since iDCs represent an APC system more realistically recapitulating the events occurring upon *in vivo* exosome inoculation. The antigen cross-presentation assay was carried out by challenging HLA-A.02 iDCs with exosomes uploading either Nef^{mut} alone or the Nef^{mut}/MART-1 fusion product. MART-1 (also known as Melan-A) is a human melanome-related tumor-associated antigen protein [36]. Its epitope at aa 27–35 represents an immunodominant domain restricted to HLA-A.02 Class I MHC [37]. Preparations of exosomes uploading Nef^{mut}/MART-1 pseudotyped or not with VSV-G were characterized by Western blot analysis (Figure 3A), and in terms of both GM1 and CD63 contents (Figure 3B). As control, (VSV-G) exosomes uploading Nef^{mut} alone were used.

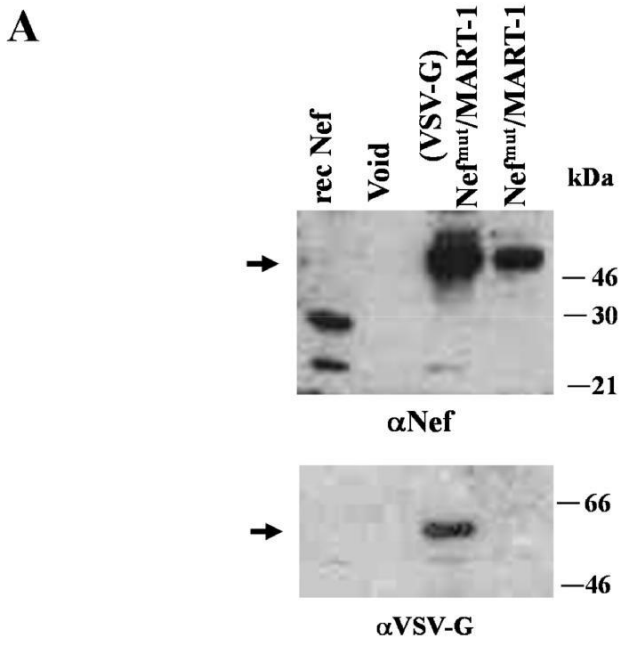

Figure 3. *Cont.*

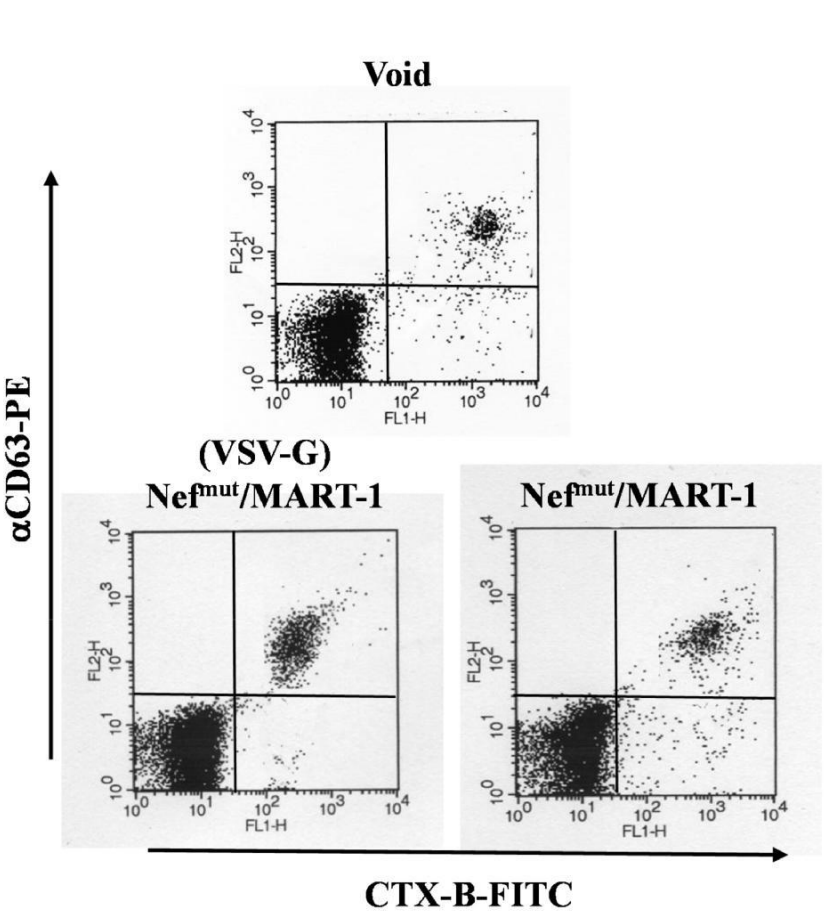

Figure 3. Molecular characterization of exosome preparations uploading Nef^{mut}/MART-1. (**A**) Western blot analysis of 100 μU of AchE activity of exosomes associating Nef^{mut}/MART-1 and pseudotyped or not with VSV-G. As control, exosomes from mock-transfected cells (void) and 100 ng of recombinant Nef were loaded. Shown are the results obtained upon incubation with either anti-Nef or anti-VSV-G Abs. The arrows indicate the relevant protein products. Molecular markers are given in kDa. Results are representative of two independent experiments; (**B**) FACS analysis of exosomes uploading Nef^{mut}/MART-1 pseudotyped or not with VSV-G. Equivalent amounts of exosomes were bound to surfactant-free white aldehyde/sulfate latex beads, and then assayed for the contents of GM1 and CD63. Quadrants were set on the basis of the fluorescence of beads alone labeled with CTX-B and anti-CD63 mAb. Results shown in both panels are representative of three assays carried out on two different exosome preparations.

Equivalent amounts of either (VSV-G)Nef^{mut}, (VSV-G)Nef^{mut}/MART-1, or Nef^{mut}/MART-1 exosomes were used to challenge iDCs. After 4 h, the cells were put in co-culture for 16 h in an IFN-γ Elispot 96-well plate with a CD8^{+} T cell clone recognizing the 27–35 epitope of MART-1. Figure 4 shows the increase of IFN-γ production in co-cultures comprising iDCs challenged with Nef^{mut}/MART-1 exosomes, irrespective of the presence of VSV-G, compared with control conditions, *i.e.*, iDCs challenged with exosomes either from mock-transfected cells (Void), or uploading Nef^{mut} alone.

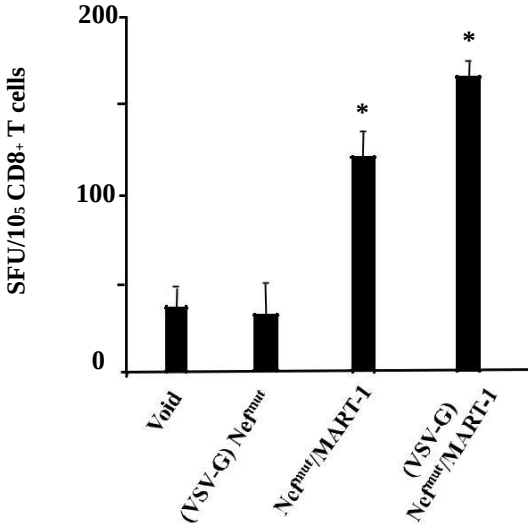

Figure 4. Cross-presentation of MART-1 delivered by exosomes. HLA-A.02 iDCs were challenged with 100 μU of AchE activity of exosomes associating Nef^{mut}/MART-1 in the presence or not of VSV-G. As control, equivalent amounts of (VSV-G) Nef^{mut} exosomes or exosomes from mock-transfected cells (void) were used. After 4 h, the cells were put in co-culture overnight with a MART-1-specific, HLA-A.02-restricted CD8$^+$ T cell line in IFN-γ Elispot microwells. Shown are the mean + SD number of SFU/10^5 cells calculated from five independent experiments. ***** $p < 0.05$.

This result prompted us to compare the immunogenicity of exosomes associating or not VSV-G. Exosomes uploading Nef^{mut}/HPV-E7 in the presence or not of VSV-G were prepared and characterized in terms of both antigen contents (Figure 5A) and membrane markers (Figure 5B).

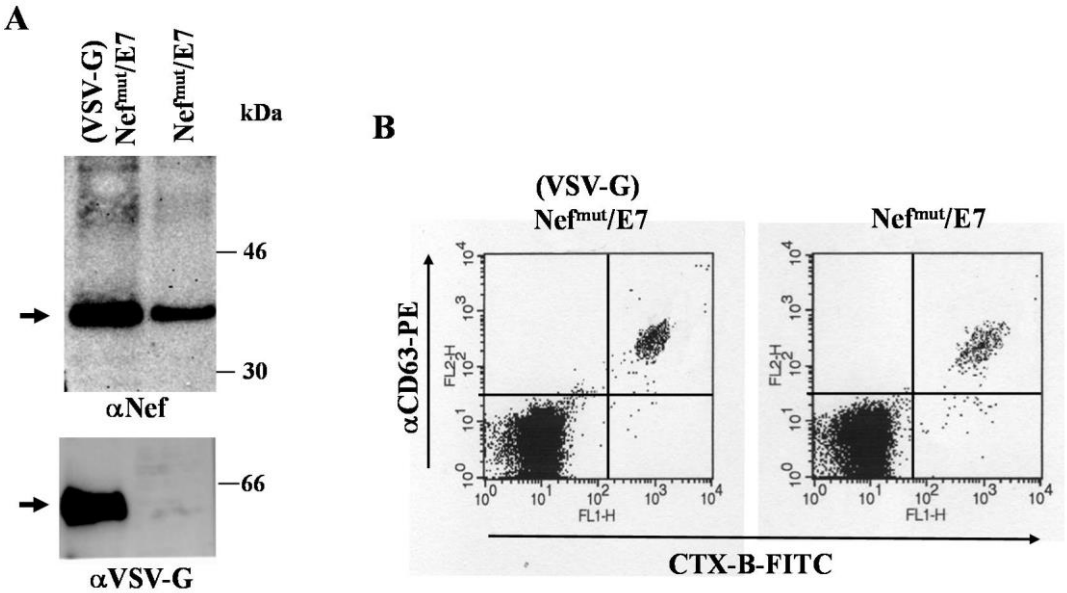

Figure 5. Molecular characterization of exosome preparations uploading Nef^{mut}/E7 in the presence or not of VSV-G. (**A**) Western blot analysis of 100 μU of AchE activity of exosomes associating Nef^{mut}/E7 in the presence or not of VSV-G. Shown are the results obtained upon incubation with either anti-Nef or anti-VSV-G Abs. Arrows indicate the relevant protein

products. Molecular markers are given in kDa; (**B**) FACS analysis of exosomes uploading Nef^{mut}/E7 in the presence or not of VSV-G. Exosomes were bound to surfactant-free white aldehyde/sulfate latex beads, and then assayed for the contents of GM1 and CD63. Quadrants were set on the basis of the fluorescence of beads alone labeled with CTX-B and anti-CD63 mAb. Results shown in both panels are representative of the assays performed on four different exosome preparations.

Afterwards, C57 Bl/6 mice were inoculated s.c. three times with exosomes carrying equivalent amounts of Nef^{mut}/E7 in the presence or not of VSV-G. Two weeks after the last inoculation, mouse splenocytes were isolated and cultured for 4 days in the presence or absence of either unrelated or E7-specific peptides. Then, 10^5 cells were seeded in IFN-γ ELISPOT wells, and the number of SFU was scored 16 h later. Data reported in Figure 6 show that E7 carried by exosomes without VSV-G was as immunogenic as that delivered by VSV-G pseudotyped exosomes.

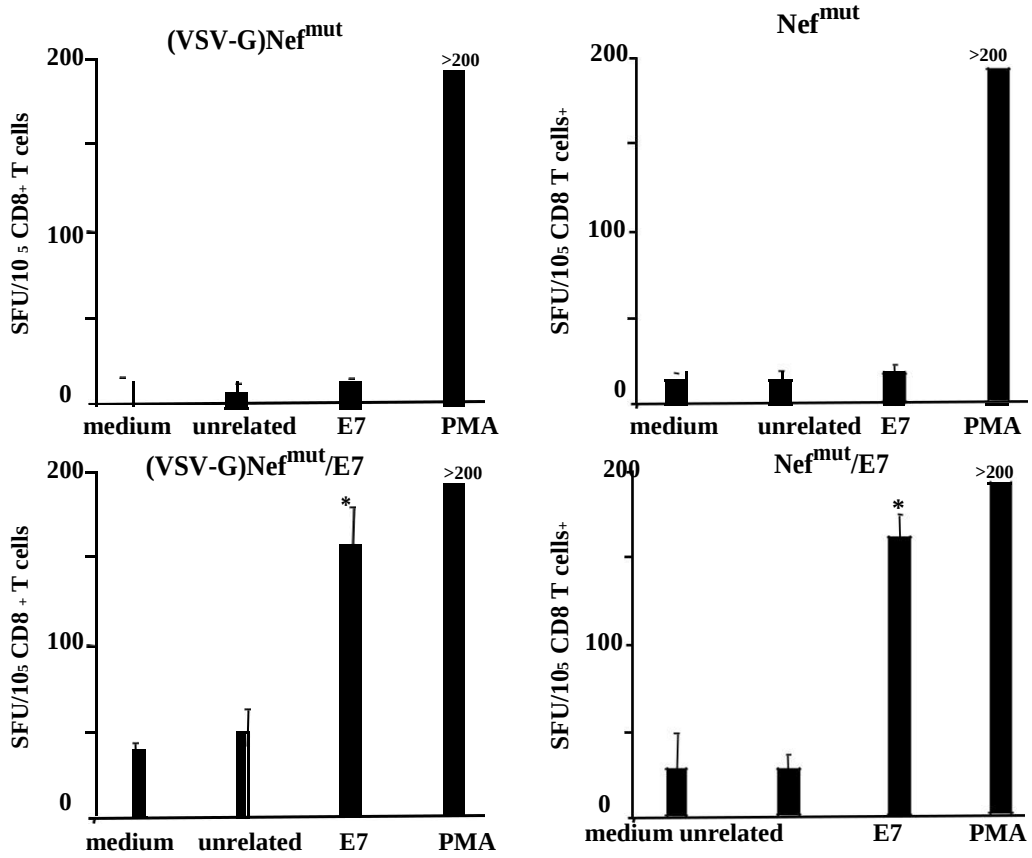

Figure 6. $CD8^+$ T cell immune response in mice inoculated with Nef^{mut}/E7 exosomes in the presence or not of VSV-G. C57 Bl/6 mice (5 per group) were inoculated three times with exosomes uploading either Nef^{mut} or Nef^{mut}/E7, with or without VSV-G. Two weeks after the last inoculation, splenocytes were recovered and incubated 4 days with or without 5 μg/mL of either unrelated or the above-quoted E7 peptides. Afterwards, cell activation extents were evaluated by IFN-γ Elispot assay carried out in triplicate with 10^5 cells/well. As control, untreated cells were also incubated with 5 ng/mL of PMA and 500 ng/mL of ionomycin. Shown are the mean + SD number of SFU/10^5 cells. Results are representative of two independent experiments. ***** $p < 0.05$.

Consistently, similar anti-E7 CTL activity was detected in splenocytes inoculated with exosomes incorporating Nef^{mut}/E7 pseudotyped or not with VSV-G (Figure 7).

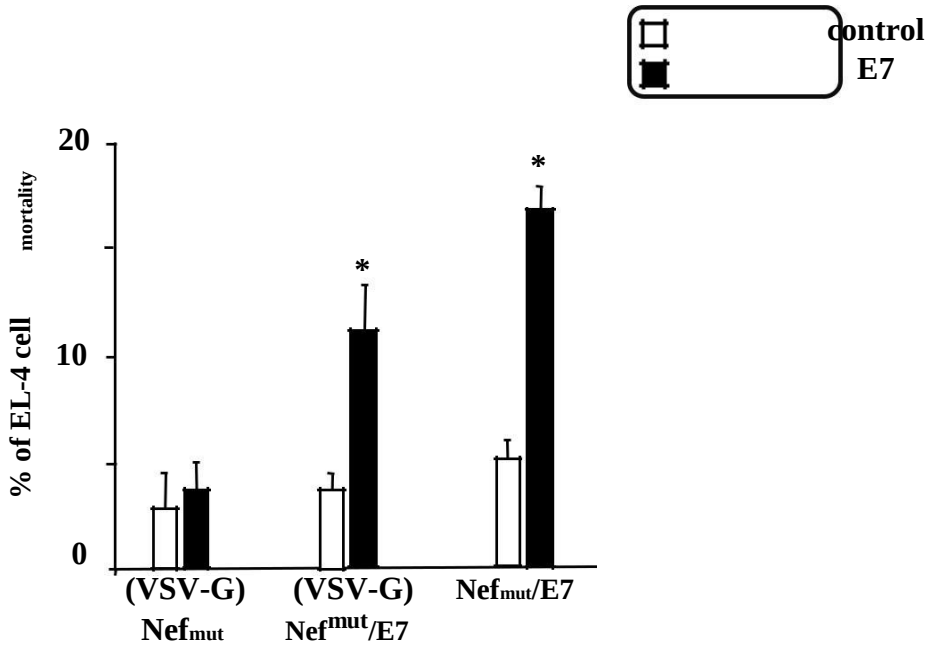

Figure 7. CTL assay carried out with CD8$^+$ cells from mice inoculated with exosomes uploading Nef^{mut}/E7 in the presence or not of VSV-G. Splenocytes from mice inoculated with either (VSV-G) Nef^{mut}, (VSV-G)Nef^{mut}/E7, or Nef^{mut}/E7 exosomes were pooled and cultured for 4 days in the presence of either control peptides or the previously described E7 peptides. The CD8$^+$ cell fraction was isolated and then co-cultivated for 6 h at different cell ratios (*i.e.*, from 100:1 to 2:1) with EL-4 cells previously labeled with CFSE and pre-treated with either unrelated or E7 peptides for 16 h. Finally, the cell mortality within the EL-4 cell population was scored by FACS analysis upon 7-AAD labeling. Shown are the results obtained with co-cultures carried out at a 20:1 cell ratio, and are representative of two independent experiments. ***** $p < 0.05$.

3.3. Anti-Tumor Effects of Nef^{mut}-Based Engineered Exosomes

Next, we investigated the potency of the immune response evoked by Nef^{mut}-based exosomes in terms of anti-tumor activity. To this end, we used the well-characterized experimental tumor system consisting in the implantation in C57 Bl/6 mice of syngeneic TC-1 tumor cells which express HPV-E7 [26]. We evaluated the anti-tumor effects of the exosomes in both preventive and therapeutic settings.

To investigate whether the immunity elicited by the Nef^{mut}-based exosomes can counteract the growth of implanted TC-1 cells (preventive immunization), mice were inoculated three times with Nef^{mut}/E7 exosomes, and, a week later, with 10^5 TC-1 cells. As control, mice were inoculated with either vehicle, equivalent amounts of exosomes uploading Nef^{mut} alone, or amounts of recombinant E7 protein equivalent to those associated with each exosome inoculum. The growth of tumor cells was followed for 41 days. Tumor cells grew most efficiently in mice receiving recombinant E7 protein.Their sacrifice was anticipated in view of the rapid decay of their health conditions. Conversely, mice receiving Nef^{mut}/E7

exosomes appeared efficiently protected by tumor cell challenge (Figure 8), indicating a strong anti-tumor efficiency of Nef^{mut}/E7 exosomes when the immunogen was provided before tumor cell implantation.

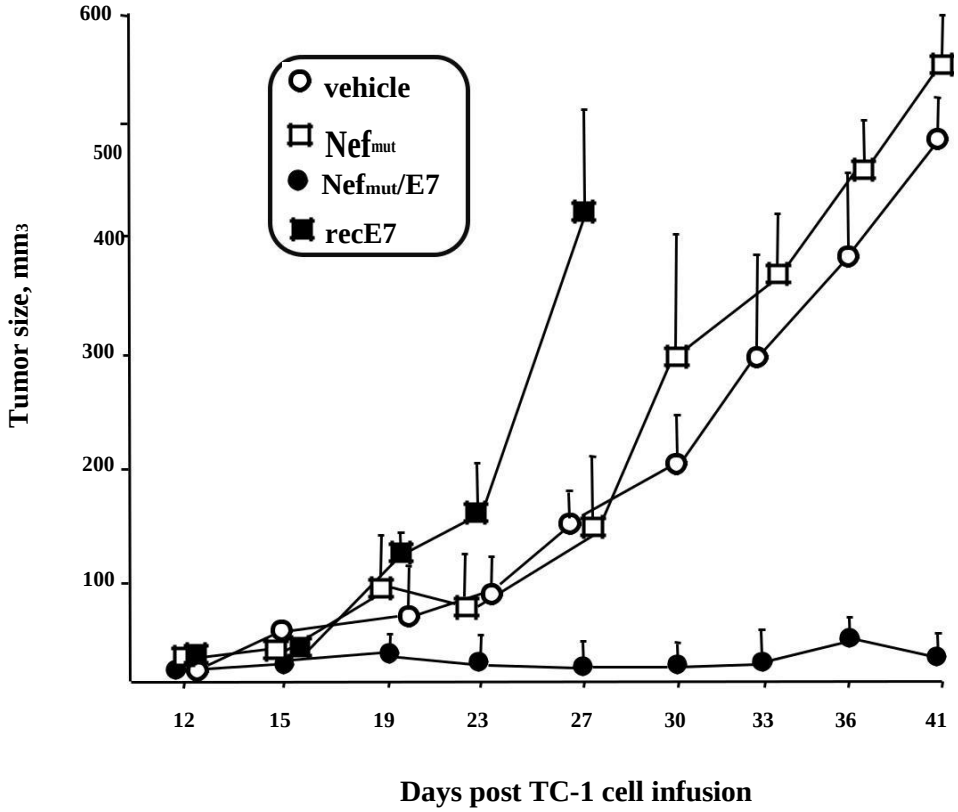

Figure 8. Growth of TC-1 tumor cells implanted in mice after inoculation of exosomes incorporating Nef^{mut}/E7. C57 Bl/6 mice (5 for group) were inoculated three times at two-week intervals with exosomes incorporating Nef^{mut}/E7 or, as control, with exosomes uploading Nef^{mut} alone. Mice were also inoculated with amounts of purified recombinant (rec) HPV-E7 protein equivalent to those associated with the exosome inocula. Two weeks after the last inoculation, mice were challenged with 10^5 TC-1 cells, and tumor growth was monitored over time. Mice inoculated with recombinant E7 have been sacrificed at day 27 due to their heavily compromised health. Tumor size was calculated as (width × 2) × (length/2). Shown are the mean sizes + SD of tumors developed in mice within the different groups.

To assay possible effects on already implanted tumor cells (therapeutic immunization), the engineered exosomes were applied in mice previously inoculated with 2×10^5 TC-1 cells, and which developed a tumor mass detectable by palpation, *i.e.*, of about 2 mm of diameter within 6 days. The exosome inoculations were repeated three times. As reported in Figure 9, tumors grew slower in mice treated with Nef^{mut}/E7 exosomes than in control conditions, *i.e.*, mice inoculated with vehicle or exosomes uploading Nef^{mut} alone.

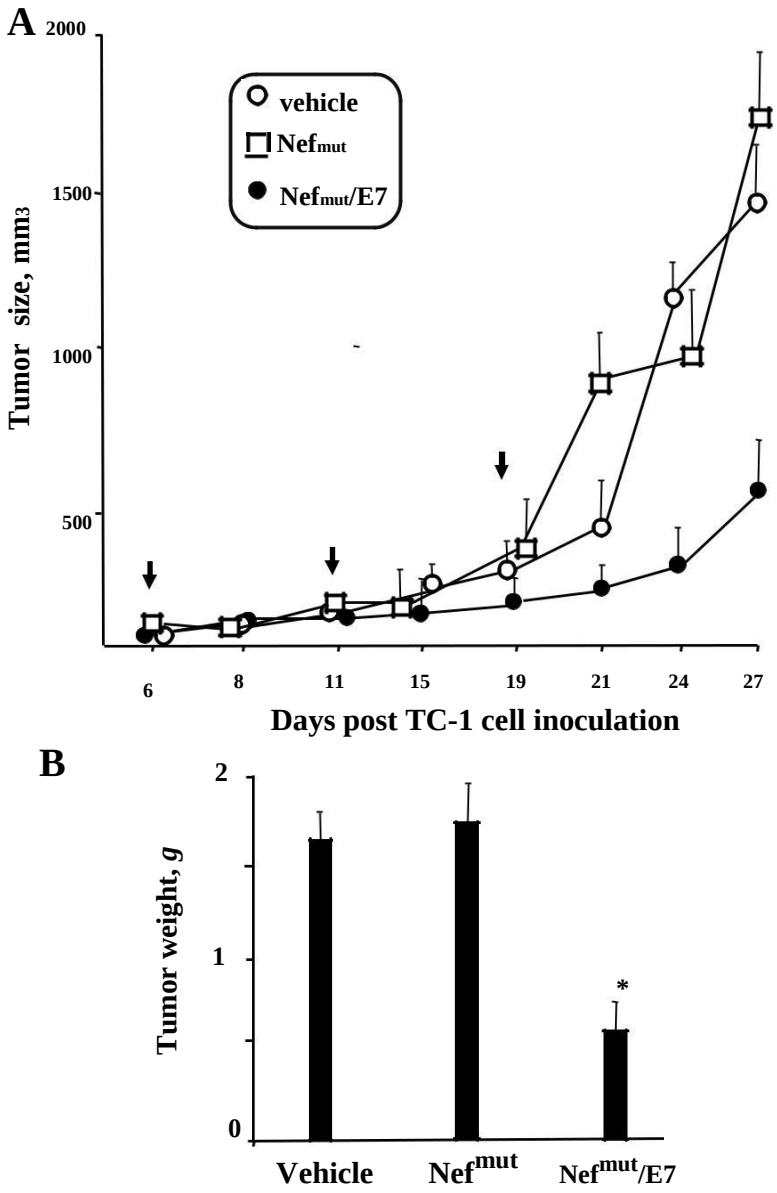

Figure 9. Anti-tumor effect of exosomes uploading Nef^{mut}/E7 in mice implanted with TC-1 tumor cells. C57 Bl/6 mice (3 for group) were challenged with 2×10^5 TC-1 cells and starting from 6 days later, when tumor mass was detectable by palpation, were inoculated 3 times with equivalent amounts of exosomes incorporating Nef^{mut}/E7 or, as control, Nef^{mut} exosomes, or equal volumes of vehicle. (**A**) Determination of the tumor size over time calculatedas (width × 2) × (length/2). Shown are the mean sizes + SD of tumors developed in mice within the different groups. The times of exosome inoculation are indicated by arrows; (**B**) Measure of tumor weight. At the time of sacrifice, tumors were explanted and weighed. Shown are the mean weights + SD of tumors developed in mice within the different groups. Results shown in both panels are representative of two independent experiments.

We concluded that the inoculation in mice of engineered exosomes incorporating E7 elicits a $CD8^+$ T cell response efficiently counteracting the tumor cell growth.

4. Discussion

We aimed at establishing a novel way to produce $CD8^+$ T cell immunogens against full protein antigens of choice. Our strategy was based on the use of engineered exosomes as immunogen carriers. Foreign antigens are uploaded in exosomes upon fusion at the *C*-terminus of a functionally defective HIV-1 Nef protein incorporating in exosomes at extremely high levels. In this paper, we report data supporting the proof-of-concept that, in this configuration, the foreign antigen is cross-presented and elicits a strong CTL response correlating with effective anti-tumor activity in both preventive and therapeutic settings.

Nanovesicles similar to exosomes can be released also through direct extrusion of plasma membrane [38]. Current protocols of purification cannot distinguish between endosome-produced nanovesicles and vesicles with similar size but extruding from cell membranes. The detection of tetraspannins, which associate with exosomes but not with plasma membrane nanovesicles, is considered an appropriate tool to identify true exosomes. On the other hand, GM1, *i.e.*, a structural component of lipid rafts which strongly binds the subunit B of cholera toxin (CTX-B), associates with both exosomes and nanovesicles arising from plasma membrane. The proportion of non-exosomal nanovesicles present in our exosome preparations can be deduced by the CD63/CTX-B double FACS analysis we reported in Figure 1B where the small populations of CTX-B+/CD63− vesicles likely account for the presence of plasma membrane nanovesicles which, however, appeared quantitatively negligible.

The immunogenicity of the foreign antigen incorporated in exosomes has been compared with that of the same antigen incorporated in HIV-1-based VLPs since: (i) VLPs are widely considered quite effective immunogens [39]; and (ii) both mechanisms and efficiency of Nef^{mut} uploading in VLPs and exosomes are basically similar. We previously observed that foreign antigens carried by Nef^{mut}-based VLPs and exosomes are cross-presented with a similar efficiency [22]. Consistently, we here report that heterologous antigens incorporated in VLPs and exosomes elicit comparable $CD8^+$ T cell-specific immunity.

Antigen cross-presentation in DCs relies on two non-mutually exclusive mechanisms [40]. In the first one, referred to as "cytosolic," the antigen transits from the endosomal compartment to cytosol. In the case of endocytosed vesicles, this passage can be greatly favored by pH-dependent envelope proteins like VSV-G. In cytosol, the antigen is degraded by proteasome and the resulting peptides are loaded on Class I MHC upon TAP-mediated translocation into endoplasmatic reticulum. The second mechanism is defined "vacuolar," and includes the action of endolysosomal proteases which degrade both vesicles and associated proteins taken up by endocytosis. The resulting peptides are loaded into Class I MHC recycling at vesicular levels. We assumed that the results we obtained with exosomes deprived of VSV-G were the consequence of vacuolar cross-presentation activity of APCs ingesting the exosomes.

Through the efficacy assays we carried out in mice we obtained the proof-of-concept that the $CD8^+$ T cell immunity elicited by Nef^{mut}/E7 exosomes correlates with the apparent destruction of target cells, which occurs in the absence of production of specific antibodies. On the contrary, inoculation of equivalent amounts of recombinant E7 had no effects in terms of $CD8^+$ T cell-specific response, in the presence however of a well-detectable induction of specific antibodies. Hence, the association with exosomes strongly influences the type of adaptive immune response elicited against the antigen. This immune response can be potent enough to control the growth of tumor cells inoculated in mice before exosome immunization. In this case, the magnitude of the anti-tumor effect appeared comparable to that recently

described in mice vaccinated with vaccinia virus-based vectors expressing E7 fused with calreticulin, *i.e.*, a protein strongly favoring Class I association of antigenic peptides [41].

Exosomes have been already a matter of consideration in human clinical trials. The major restriction in their use as vaccines has been identified in their overall limited immunogenicity. Since this aspect mainly depends on the amount of immunogen uploaded in exosomes, finding a reliable method to overload exosomes with the antigen(s) of choice would be a powerful way to strengthen their immune potency. With these premises, engineering exosomes exploiting the unique efficiency of exosome uploading of Nef^{mut} represents an original strategy whose potentialities in terms of both immunogenicity and anti-tumor efficacy have been disclosed by the results presented here.

At present, the identification, production, and marketing of CTL-based vaccines are quite limited although there is a wide consensus about their potential usefulness against chronic infections and tumor diseases. Most commonly, efficient CTL immune responses can be achieved when the immunogen is either associated with or expressed by inactivated viral particles, viral vectors, or virus-like particles. In these cases, the presence of viral nucleic acids and proteins can represent a risk factor. Conversely, the use of exosomes virtually guarantees the absence of potentially dangerous viral material. In other instances, the combination of recombinant proteins with particulate adjuvants has been found effective in eliciting CTL immunity. Considering that in Nef^{mut}-based exosomes the antigen resides inside the nanovesicle, neutralization by pre-existing or induced immunity, as can occur for both recombinant proteins and viral vaccines, is not expected to take place even in the case of repeated inoculations. All these features, together with the demonstrated flexibility in terms of incorporation of foreign antigens and ease of production, make Nef^{mut}-based exosomes a convenient candidate for novel CTL vaccines.

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Author Contributions

Paola di Bonito. designed both immunogenicity and efficacy experiments, and performed ELISA assays on mouse sera; Barbara Ridolfi made Elispot assays form mouse splenocytes; Sandra Columba-Cabezas. established and carried out cell cultures from spleens of the inoculated mice; Antonello Giovannelli. is the technician responsible for mouse manipulations and housing; ChiaraChiozzini. produced and characterized the VLP preparations; Francesco Manfredi produced and characterized the exosomes for the *in vivo* experiments; Simona Anticoli performed the cytotoxicity assays; ClaudiaArenaccio produced and characterized the MART-1 exosomes, and performed the cross-presentation assays; MaurizioFederico planned the experiments and wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 4

The CD8+ T Cell-Mediated Immunity Induced by HPV-E6 Uploaded in Engineered Exosomes Is Improved by ISCOMATRIX™ Adjuvant

In the previous paper (Di Bonito et Al., 2015) we described an efficient antigen delivery platform based on Nef^{mut} exosomes and used it to immunize mice against HPV E7. The antigen loaded vesicles proved capable of slowing the proliferation of tumorigenic cells bearing E7 infused after the immunization. A therapeutic vaccination, gave also positive but less sharp results. Thus we looked for new protocols aimed at increasing the CD8+ T cell specific response to the antigen, assuming that an optimized CD8+ T cell immune response would correlate with a more effective anti tumor activity in the therapeutic setting. This time we used HPV-E6 as a model of tumor associated antigen and we found that the *in vitro* co-administration of engineered exosomes and ISCOMATRIX™ adjuvant induced a stronger antigen cross-presentation in both B-lymphoblastoid cell lines and monocyte-derived immature dendritic cells. The mice inoculated with the adjuvant and exosomes together developed a stronger anti-E6 response, suggesting a possible future application of ISCOMATRIX™ to vesicle based vaccination.

vaccines

MDPI

Article

The CD8+ T Cell-Mediated Immunity Induced by HPV-E6 Uploaded in Engineered Exosomes Is Improved by ISCOMATRIX™ Adjuvant

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Abstract: We recently described the induction of an efficient CD8+ T cell-mediated immune response against a tumor-associated antigen (TAA) uploaded in engineered exosomes used as an immunogen delivery tool. This immune response cleared tumor cells inoculated after immunization, and controlled the growth of tumors implanted before immunization. We looked for new protocols aimed at increasing the CD8+ T cell specific response to the antigen uploaded in engineered exosomes, assuming that an optimized CD8+ T cell immune response would correlate with a more effective depletion of tumor cells in the therapeutic setting. By considering HPV-E6 as a model of TAA, we found that the in vitro co-administration of engineered exosomes and ISCOMATRIX™ adjuvant, i.e., an adjuvant composed of purified ISCOPREP™ saponin, cholesterol, and phospholipids, led to a stronger antigen cross-presentation in both B- lymphoblastoid cell lines (and monocyte-derived immature dendritic cells compared with that induced by the exosomes alone. Consistently, the co-inoculation in mice of ISCOMATRIX™ adjuvant and engineered exosomes induced a significant increase of TAA-specific CD8+ T cells compared to mice immunized with the exosomes alone. This result holds promise for effective usage of exosomes as well as alternative nanovesicles in anti-tumor therapeutic approaches.

Keywords: adjuvant; exosomes; Nef; HPV-E6; CD8+ T immune response

1. Introduction

Exosomes are vesicles of 50–100 nanometers released by basically all cell types. They are part of the intercellular communication network [1], and are generated by invagination of endosome membranes leading to the formation of intraluminal vesicles which then become part of multivesicular bodies [2].They can traffic to the plasma membrane, thereby releasing their vesicular contents upon membrane fusion. Exosomes have a low intrinsic immunogenic profile, their immunogenicity being related to both amounts and quality of uploaded antigens, and, in some cases, they have been tested in clinical trials [3–5]. Exosomes spontaneously uploading tumor antigens, mainly *trans*-membrane

proteins like gp100,TRP-1, Her2/neu and carcinoembryonic antigen, induced activation of specific anti-tumor T cell immunity [6,7].

Despite the good tolerance of exosomes as cell-free vaccines, their therapeutic efficacy appeared quite limited, posing the need of new methods to increase their immunogenicity. Attempts to address this issue have been performed by engineering desired antigens to increase their association with the external side of exosome membranes [8,9].

The convergence of exosome and HIV biogenesis at the level of the endosomal sorting complex required for transport (ESCRT) [10] implies the possibility that viral products incorporate in exosomes, as already proven for both Gag [11] and Nef [12,13] HIV-1 proteins. HIV-1 Nef lacks enzymatic activities, yet acts as a scaffold/adaptor element [14]. We identified a Nef mutant (referred to as Nef^{mut}) incorporating in HIV-1 virions, virus like particles (VLPs) [15], and exosomes [16] at quite high levels. This Nef mutant is defective for basically all Nef functions, and its efficiency of incorporation in nanovesicles does not change significantly when fused with large foreign proteins [16]. Manipulating Nef^{mut} allows incorporation into exosomes of high amounts of antigens of choice which are protected from external neutralization/degradation factors.

ISCOMATRIX™ (CSL Behring LLC, King of Prussia, PA, USA) adjuvant is an immunostimulating complex formed by ISCOPREP™ (Gronberg Adv. Byra KB, Stockholm, Sweden), saponin extracted from *Quillaia saponaria*, phospholipids, and cholesterol [17]. Its immune adjuvanticity has already been demonstrated, in particular regarding the induction of both $CD4^+$ and $CD8^+$ T cell immunity directed to soluble protein antigens [18]. We have recently shown that the inoculation in the mice of exosomes engineered to upload high amounts of the TAA HPV-E7 elicits an effective $CD8^+$ T cell response [19]. Here, we originally show that ISCOMATRIX™ adjuvant strongly improves both $CD8^+$ T-related antigenicity and immunogenicity of proteins delivered by engineered exosomes.

2. Materials and Methods

2.1. Cell Cultures and Adjuvant

For the experiment, 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% heat-inactivated Fetal Calf Serum(FCS). The isolation of the Human leukocyte antigen (HLA)-B7 Nef-specific $CD8^+$ T cell clone was already described [20]. It recognizes the amino acid sequence TPGPGVRYPL (aa 128–137). Mart-1-specific $CD8^+$ T cells recognize the HLA-A.02-restricted $AAGIGILTV_{27–35}$ amino acid sequence. Human monocytes were separated from peripheral blood mononuclear cell (PBMCs) of HLA-A.02 healthy donors using anti-CD14 microbeads (Miltenyi Biotec GmbH, Teterow, Germany), and differentiated to immature iDCs upon 4–5 days of culture in Roswell Park Memorial Institute (RPMI) medium supplemented with 20% FCS, 30 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (AbD Serotec, Bio-Rad Laboratories, s.r.l., Milan Italy), and 500 units/mL IL-4 (R&D Systems, Minneapolis, MN, US). The iDC phenotype was characterized by Fluorescence-activated cell sorting (FACS) analysis for the expression of CD11c and the absence of CD14 cell membrane markers. Both isolation and expansion of the $CD8^+$ T cell clone specific for Mart-1 have been previously described [21]. Mouse splenocytes were cultivated in RPMI medium supplemented with 10% FCS. ISCOMATRIX™ adjuvant (CSL Behring LLC, King of Prussia, PA, USA) was prepared as previously described [17], and is composed of ISCOPREP™ saponin, cholesterol, and phospholipids. The undiluted preparation contained 115 ISCO™ Units/mL.

2.2. Production, Purification, and Quantification of Exosomes

Exosomes were produced by transiently transfecting 293T cells with vectors expressing Nef^{mut}-based fusion proteins. The cells were seeded in the presence of exosome-deprived FCS, and supernatants harvested 48–72 h after transfection. Efficiency of transfection was routinely evaluated by intracellular Fluorescence-activated cell sorting (FACS) analysis as previously reported [22] using the anti-Nef MATG mAb kindly provided by Olivier Schwartz, Pasteur Institute, Paris, France. Exosomes

were recovered by differential centrifugations as previously described [23]. The amounts of exosomes were evaluated by measuring the activity of acetylcholinesterase (AchE), i.e., a classical exosome marker [24], through the Amplex Red kit (Molecular Probes, Termo Fischer Scientific, Waltham, MA, USA). The AchE activity was measured as mU/mL, where 1 mU is defined as the amount of enzyme hydrolyzing 1 pmole of acetylcholine to choline and acetate per minute at pH 8.0 at 37 °C.

2.3. Fluorescence-Activated Cell Sorting(FACS) Analysis of Bead-Exosome Complexes

Samples were incubated with 5 μL of surfactant-free white aldehyde/sulfate latex beads (Termo Fischer Scientific, Waltham, MA, USA) overnight at room temperature (r.t.) on a rotating plate. For the assays carried out with Nef^{mut}/Green Fluorescent protein (GFP) exosomes in the presence of ISCOMATRIX™ adjuvant, beads and exosomes were incubated in the presence of different concentrations of the adjuvant for 1 to 3 h before FACS analysis. For the characterization of different exosome preparations, bead-exosome complexes were labeled with phycoerythrin (PE)-conjugated anti-CD63 mAb (BD Biosciences Milan, Italy) for 1 h at 4 °C. Finally, beads were washed, resuspended in 1× PBS-2% v/v formaldehyde, and FACS analyzed.

2.4. Detection of Exosome Cell Internalization

B-lymphoblastoid cell lines (BLCLs) were pre-treated with 100 nM bafilomycin A1 (Sigma-Aldrich, Milan, Italy) for 2 h in the presence or not of ISCOMATRIX™ adjuvant, and then challenged by spinoculation with fluorescent exosomes previously incubated in the presence or not of ISCOMATRIX™ adjuvant. After 2 h of incubation at either 4 or 37 °C in the presence of ISCOMATRIX™ adjuvant and/or bafilomycin A1, cells were treated for 15 min with trypsin, fixed with 2% v/v formaldehyde in 1× PBS, and FACS analyzed.

2.5. Western Blot Analysis

The equivalent of 200 μU of exosomes were lysed in PBS, 1% Triton X-100 (Sigma-Aldrich , Milan, Italy) in the presence of anti-proteolytic agents, and then separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were revealed using sheep anti-Nef antiserum ARP 444, a generous gift of Mark Harris, University of Leeds, Leeds, UK, polyclonal anti-vesicular stomatitis virus G glycoprotein (VSV-G) Abs (Immunological Consultant Laboratories, Newberg, OR, USA), and anti-intercellular adhesion molecule (ICAM)-1 mAb 15.2 (Santa Cruz Biotechnology Inc., Heidelberg, Germany).

2.6. Cross-Presentation Assay

HLA-B7 B-LCLs were challenged by spinoculation with Nef^{mut}–based exosomes in the presence or not of different concentrations of ISCOMATRIX™ adjuvant. Five hours later, the cells were extensively washed and then co-cultivated in triplicate at 1:2 ratio with Class I major histocompatibility complex (MHC)-matched Nef-$CD8^+$ T cells in Elispot multiwell plates pre-coated with the D1K mAb against human interferon (IFN)-γ (Mabtech, Nacka Strand Sweden) in RPMI plus 10% of AB human serum (Gibco, Termo Fischer Scientific, Waltham, MA, USA) for 16 h. Thereafter, co-cultures were removed, the Elispot assay was completed, and the spot-forming cells were analyzed and counted using an Elispot reader (Amplimedical Bioline A-EL-VIS GmbH, Turin, Italy). Cross-presentation assays using Nef^{mut}/Mart-1 exosomes were performed basically in the same way, except that HLA-A.02 immature dendritic cells were used as antigen presenting cells (APCs), and the above described Mart-1 specific $CD8^+$ T cell clone was used as effector cells.

2.7. Immunogenicity Assay

All studies with animals here described have been approved by the Ethical Committee of the Istituto Superiore di Sanità, Rome, Italy (protocol No. 555/SA/2012) according to Legislative Decree

116/92, which has implemented in Italy the European Directive 86/609/EEC on laboratory animal protection. Animals used in our research have been housed and treated according to the guidelines inserted in here above mentioned Legislative Decree. Eight week-old female C57 Bl/6 mice (3 per group in two independent experiments) were purchased from Charles River Laboratories Italia, (Calco, Italy) and inoculated s.c. 3 times at 2 week intervals with a total of 100 µL comprising Nef^{mut}/E6 exosomes in the presence or not of ISCOMATRIX™ adjuvant. Two weeks after the last inoculation, mice were sacrificed, and splenocytes put in culture in the presence of 5 µg/mL of 8- or 9-mer HPV-E6 peptides already identified to efficiently bind the H-2 K^b complex of C57 Bl/6 mice [25], i.e., KLPQLCTEL (aa. 18–26) and YDFAFRDL (aa 50–57). H-2 K^b binding HPV E7-specific peptides DLYCYEQL (aa 21–28), and RAHYNIVTF (aa 49–57) [25] were used as control peptides. IFN-γ Elispot assays were performed after overnight incubation in Elispot microwells. All IFN-γ Elispot assays were performed in triplicate conditions using commercially available reagents (Mabtech AB, Nacka Strand Sweden), and spots counted using an Elispot reader.

2.8. Detection of Anti-HPV-E6 Abs

Sera from inoculated mice were pooled, and two-fold serial dilutions starting from 1:100 were assayed for the presence of anti-HPV-E6 Abs as previously reported [19] using recombinant HPV-E6 produced as described [26].

2.9. Cytotoxic T Lymphocyte (CTL) Assay

$CD8^+$ T cells were isolated from splenocytes of exosome-inoculated mice by positive immunomagnetic selection (Miltenyi Biotec., Teterow, Germany). They were put in co-culture with EL-4 cells previously labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Termo Fischer Scientific, Waltham, MA, USA) and treated overnight with either E6 or unrelated peptides. The co-cultures were run for 6 h in 200 µL of RPMI 20% FCS in U-bottom 96 well plates at 20:1 effector/target cell ratio. Afterwards, EL-4 cell mortality was scored by FACS analysis soon after addition of 7-AAD at a final concentration of 1 µg/mL.

2.10. Statistical Analysis

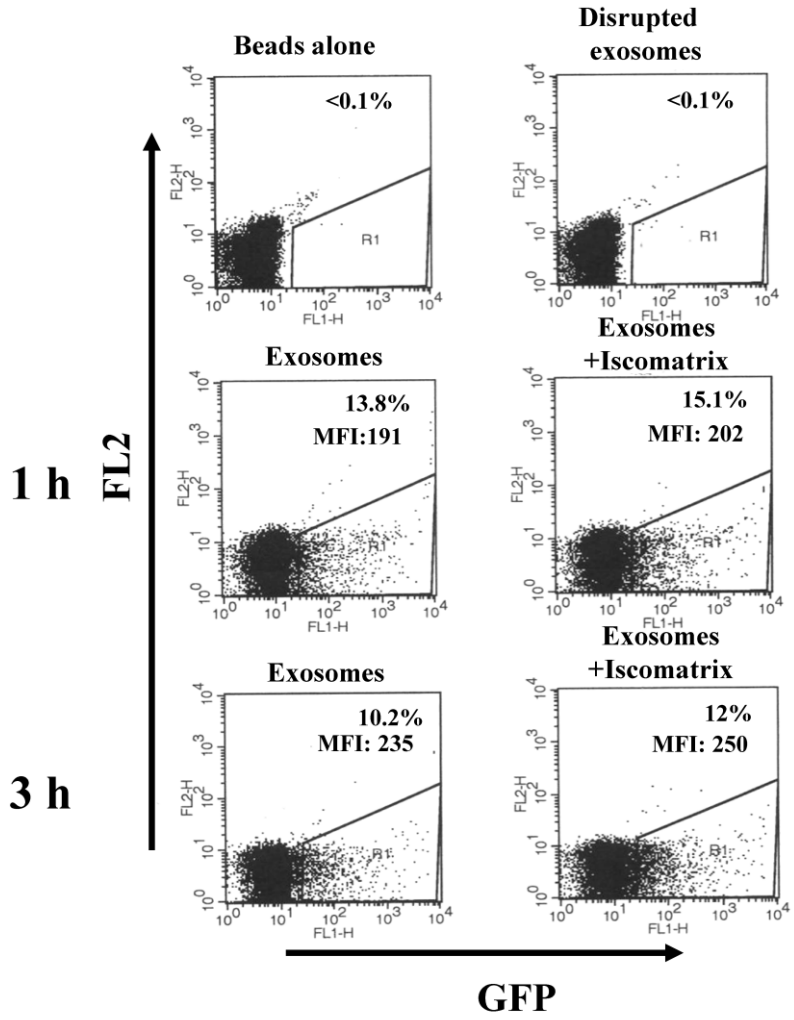
When appropriate, data are presented as mean + standard deviation (SD). In some instances, the paired Student's *t*-test was used and confirmed using the non-parametric Wilcoxon rank sum test. $p < 0.05$ was considered significant.

3. Results

3.1. ISCOMATRIX™ Adjuvant Does Not Affect Both Structure and Cell Entry Efficiency of Exosomes

Attempting to improve the potency of the antigen-specific CTL response that we previously observed in mice inoculated with Nef^{mut}-based exosomes, we looked for adjuvants already proven to increase the $CD8^+$ T lymphocyte response, and whose molecular composition was expected to not impact the exosome structure. ISCOMATRIX™ adjuvant appeared as a valuable candidate. However, preliminary experiments aimed at identifying possible structural and/or functional alterations of exosomes upon interaction with ISCOMATRIX™ adjuvant were mandatory. Structural alterations were evaluated using fluorescent exosomes whose GFP contents were measured by FACS analysis after binding to aldheyde-sulphate beads. In this system, we assumed that relevant damages in the exosome structure couple with a loss of the exosome-associated fluorescence. In detail, GFP-labeled exosomes were recovered by transiently transfecting 293T cells with a vector expressing the Nef^{mut}/GFP fusion protein as previously described [16]. A total of 1 mU of these exosomes were bound to the beads, and then incubated with either 5.75, 11.5, or 23 ISCO™ U/mL of ISCOMATRIX™ adjuvant for 1 to 3 h. As a control, the same amount of fluorescent exosomes was disrupted by heating at 95 °C for 10 min in water before the incubation with the beads. Figure 1 shows the results obtained with the highest

concentration of the adjuvant. Clearly, no differences in the beads-associated fluorescence appeared between control and ISCOMATRIX^{TM} adjuvant -treated exosomes, and similar results were obtained with lower adjuvant concentrations (not shown). This result strongly suggested that the incubation with ISCOMATRIX^{TM} adjuvant does not induce degradation of exosomes.



	% of positivity		MFI	
	Exosomes	Exosomes+Iscomatrix	Exosomes	Exosomes+Iscomatrix
1h	12.5±3	13.8±2	194.6±19	199.1±9
3h	10.1±1	12.3±3	226±15	259±16

Figure 1. The treatment with ISCOMATRIX^{TM} adjuvant does not affect the exosome-associated fluorescence. Fluorescent exosomes were incubated in the absence or presence of 23 ISCO^{TM} U/mL of the adjuvant at 37 °C with aldehyde sulphate beads in a rotating plate. After 1 and 3 h, samples were washed, and analyzed by FACS. As a control, equivalent amounts of fluorescent exosomes were heated at 95 °C for 10 min before incubation with beads. Results representative of three independent experiments with duplicates are shown. In each plot, both percentages and, where relevant, mean fluorescence intensity (MFI) of fluorescent beads are indicated. At the bottom, mean values ± SD of both percentages and MFI of fluorescent beads from the three experiments are reported. Iscomatrix: ISCOMATRIX^{TM} adjuvant.

Next, we looked at possible influences of ISCOMATRIX^{TM} adjuvant on cell entry efficiency of exosomes. To this end, fluorescent exosomes were incubated for two hours with complete medium

either alone or supplemented with 23 ISCO^{TM} U/mL in a total of 50 µL. Meanwhile, cells were pre-treated for 2 h with the adjuvant and/or bafilomycin A1, the latter required to hamper rapid intracellular degradation of incoming exosomes. Afterwards, B-LCLs were challenged with exosomes by spinoculation carried out either at 4 or 37 °C. Cells were then re-plated at the two different temperatures, and after 2 h treated with trypsin, and finally FACS analyzed. As shown in Figure 2, fluorescence levels in B-LCLs did not change significantly when exosomes were pre-treated with ISCOMATRIX^{TM} adjuvant, indicating that the adjuvant does not affect the cell entry efficiency of exosomes.

From this set of experiments, we concluded that both structure and cell entry of exosomes are not influenced by the interaction with ISCOMATRIX^{TM} adjuvant.

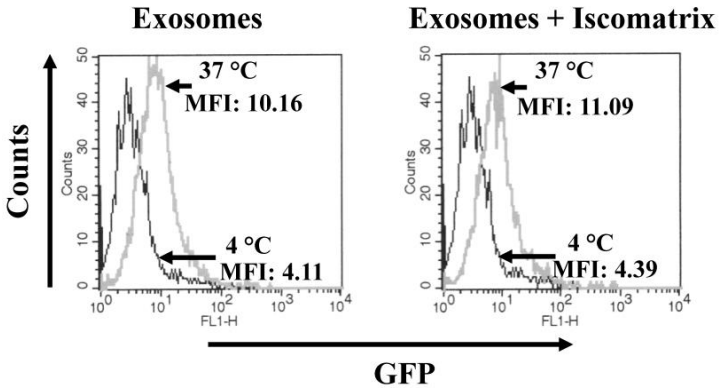


Figure 2. Unchanged cell internalization of exosomes in the presence of ISCOMATRIX^{TM} adjuvant. A total of 2 × 10^5 B-LCLs were pre-treated for 2 h with bafilomycin A1 in the presence or not of the ISCOMATRIX^{TM} adjuvant, and then challenged with 500 µU of fluorescent exosomes pre-incubated or not with the adjuvant. After spinoculation, the cells were incubated for an additional 2 h at either 4 or 37 °C in the presence of bafilomycin A1. Finally, cells were fixed and FACS analyzed. Fluorescence intensity of cells incubated at 4 °C was similar to that of untreated cells. Shown are the results representative of two independent experiments carried out with duplicates. Mean fluorescence intensity (MFI) of the samples are also indicated. Iscomatrix: ISCOMATRIX^{TM} adjuvant.

3.2. ISCOMATRIX^{TM} Adjuvant Increases Cross-Presentation of Antigens Uploaded in Engineered Exosomes

For exogenous antigens, cross-presentation is on the basis of the CD8$^+$ T cell immune adaptive response. Thus, we first tested the efficiency of the exosome-ISCOMATRIX^{TM} adjuvant combination in an in vitro cross-presentation assay assuming that the extents of cross-presentation of antigens uploaded in engineered exosomes detectable in vitro predicts the potency of the immune response in vivo. The effects of ISCOMATRIX^{TM} adjuvant on the cross-presentation of antigens associated with engineered exosomes were evaluated in two already established in vitro systems based on challenging B-LCLs and iDCs with exosomes uploading Nef^{mut} alone [16] and fused with Mart-1 (i.e., a human melanoma-associated antigen also known as Melan-A) [27], respectively. We previously reported that, in this system, the antigens delivered by exosomes are cross-presented poorly unless the exosomes associate with a pH-dependent envelope protein (e.g., the G protein from vesicular stomatitis virus) [16].

Exosomes uploading Nef^{mut} were produced by transient transfection in 293T cells as previously described [16], and characterized for both Nef^{mut} incorporation and CD63 membrane expression (Figure 3A). HLA-B7 B-LCLs were challenged with Nef^{mut}-based exosomes alone or in combination with either 5.75, 11.5, or 23 ISCO^{TM} U/mL of ISCOMATRIX^{TM} adjuvant. Notably, no effects on growth/viability of both B-LCLs and Nef-specific CD8$^+$ T lymphocytes were assessed after 24 h of cultivation with these doses of the adjuvant (Figure 3B, left). Exosome challenge was carried out by spinoculating cells in a total of 50 µL, followed by a 5 h incubation in the presence or not of the indicated doses of ISCOMATRIX^{TM} adjuvant (Figure 3B, right), and thereafter co-cultivated for 24 h

with HLA-matched Nef-specific CD8$^+$ T lymphocytes in IFN-γ Elispot microwells. As shown in Figure 3B, the presence of at least 11.5 ISCO^{TM} U/mL of the adjuvant significantly increased the cross-presentation of Nef^{mut} delivered by exosomes.

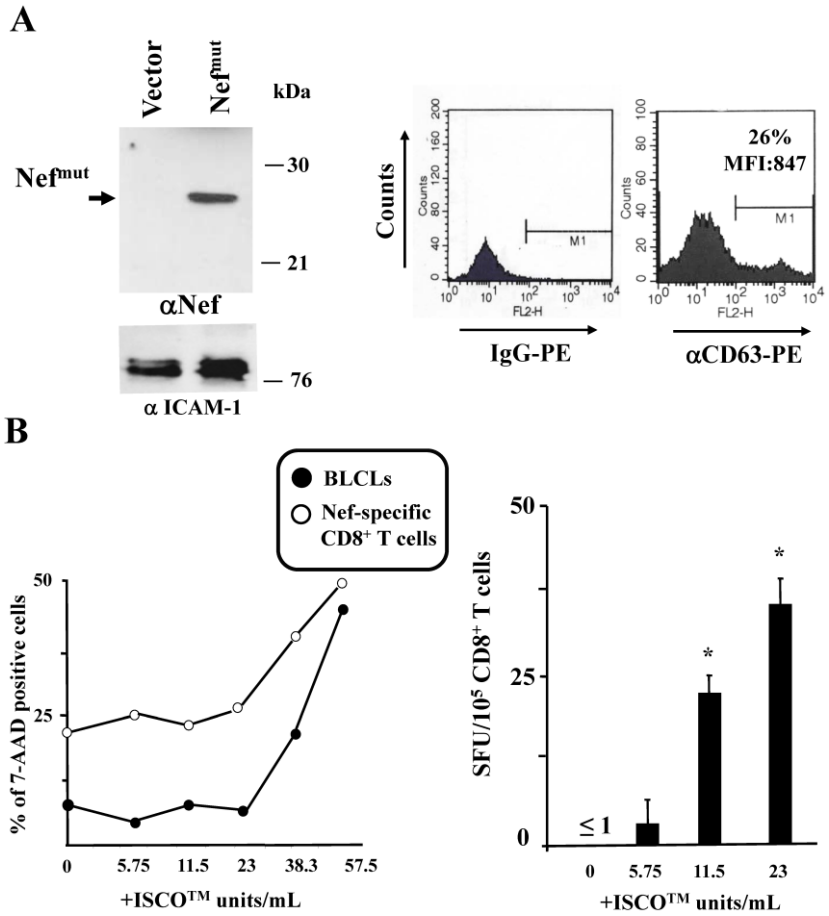


Figure 3. ISCOMATRIX^{TM} adjuvant increases the cross-presentation of antigens delivered by engineered exosomes in B-lymphoblastoid cell lines (LCLs). (**A**) molecular characterization of exosome preparations uploading Nef^{mut}. On the left is the Western blot analysis of 200 μU of exosomes uploading Nef^{mut}. As a control, equivalent amounts of exosomes from cells transfected with the empty vector were loaded. Arrow signs indicate the relevant protein product. Exosome preparations were also probed for the presence of Intercellular adhesion molecule (ICAM)-1, i.e., an exosome marker. Molecular markers are given in kilodaltons (kDa). On the right, fluorescence-activated cell sorting (FACS) analysis for the presence of CD63 in exosome membrane uploading Nef^{mu} is shown. M1 marks the range of fluorescence positivity as determined by the analysis of equivalent amounts of exosomes after incubation with isotype-specific IgGs (**left** histogram). Both percentages and MFI of fluorescent beads are indicated. Results shown in both panels are representative of three assays carried out on two exosome preparations; (**B**) on the left is cell viability of both B-LCLs and Nef-specific CD8$^+$ T cells treated for 24 h with the indicated concentrations of ISCOMATRIX^{TM} adjuvant as evaluated by FACS analysis after 7-AAD labeling. Shown are mean values from two independent experiments with duplicates. On the right, data from cross-presentation analysis of Nef^{mut} delivered by exosomes in B-LCLs are presented. A total of 10^5 cells were challenged with 100 μU of exosomes and then cultivated for 5 h in the presence of the indicated doses of ISCOMATRIX^{TM} adjuvant. Thereafter, the cells were put in co-culture overnight with a Nef-specific, HLA-B7 restricted CD8$^+$ T cell line in IFN-γ Elispot microwells. Shown are the mean + SD number of SFU/10^5 cells calculated from five independent experiments. * $p < 0.05$.

The cross-presentation assay was reproduced using monocyte-derived iDCs and exosomes uploading Nef^{mut}/Mart-1, whose molecular characterization is shown in Figure 4A. When these exosomes were used to challenge HLA A.02 iDCs, whose viability appeared not influenced up until 23 ISCO^{TM} U/mL (Figure 4B, left), the increase of Mart-1 cross-presentation appeared from 5.75 ISCO^{TM} U/mL of ISCOMATRIX^{TM} adjuvant (Figure 4B, right). According to what was already reported [28], ISCOMATRIX^{TM} adjuvant had no detectable effects on activation/maturation of in vitro differentiated iDCs, independently from the exosome treatment (not shown).

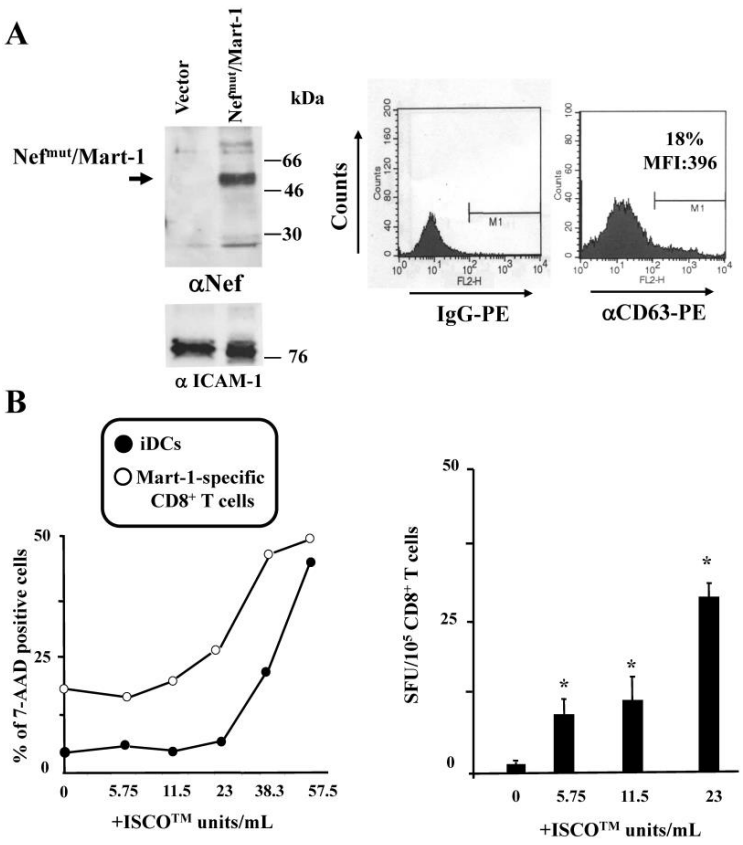


Figure 4. ISCOMATRIX^{TM} adjuvant increases the cross-presentation of antigens delivered by engineered exosomes in iDCs. (**A**) molecular characterization of exosome preparations uploading Nef^{mu}/Mart-1. On the left is the Western blot analysis of 200 μU of exosomes associating with Nef^{mut}. As a control, equivalent amounts of exosomes from cells transfected with the empty vector were loaded. Arrow signs indicate the relevant protein product. Exosome preparations were also probed for the presence of ICAM-1. Molecular markers are given in kDa. On the right, the FACS analysis for presence of CD63 in exosome membrane is shown. M1 marks the range of fluorescence positivity as determined by the analysis of equivalent amounts of exosomes after incubation with isotype-specific IgGs (**left** histogram). Both percentages and MFI of fluorescent beads are indicated. Results shown in both panels are representative of two assays carried out on two exosome preparations; (**B**) on the left is cell viability of both iDCs and Mart-1-specific CD8$^+$ T cells treated for 24 h with the indicated concentrations of ISCOMATRIX^{TM} adjuvant as evaluated by FACS analysis after 7-AAD labeling. Mean values from two independent experiments with duplicates are shown. On the right, results from cross-presentation analysis of Mart-1delivered by Nef^{mut}/Mart-1 exosomes in HLA-A.02 iDCs are presented. A total of 10^5 cells were challenged with 100 μU of the exosomes and then cultivated for 5 h in the presence of the indicated doses of ISCOMATRIX^{TM} adjuvant. Thereafter, the cells were put in co-culture overnight with a Mart-1-specific, HLA-A.02 restricted CD8$^+$ T cell line in IFN-γ Elispot microwells. The mean + SD number of SFU/10^5 cells calculated from three independent experiments are shown. * $p < 0.05$.

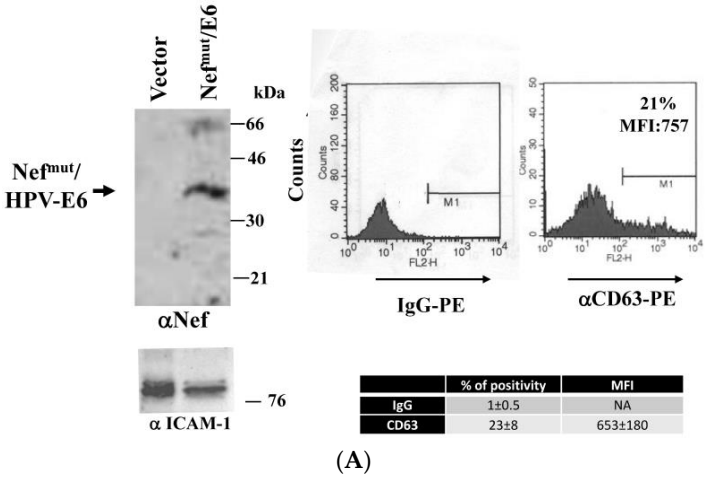
Of note, incubation of both B-LCLs and iDCs with the adjuvant in the presence of exosomes from cells transfected with empty vector did not induce activation of the $CD8^+$ T cells (not shown).

These results indicated together that the treatment with ISCOMATRIX^{TM} adjuvant significantly increases the cross-presentation of antigens uploaded in Nef^{mut}-based engineered exosomes.

3.3. ISCOMATRIX^{TM} Adjuvant and Exosome Co-Administration in Mice Increases the Pool of $CD8^+$ T Lymphocytes Specific for the Antigen Uploaded in Engineered Exosomes

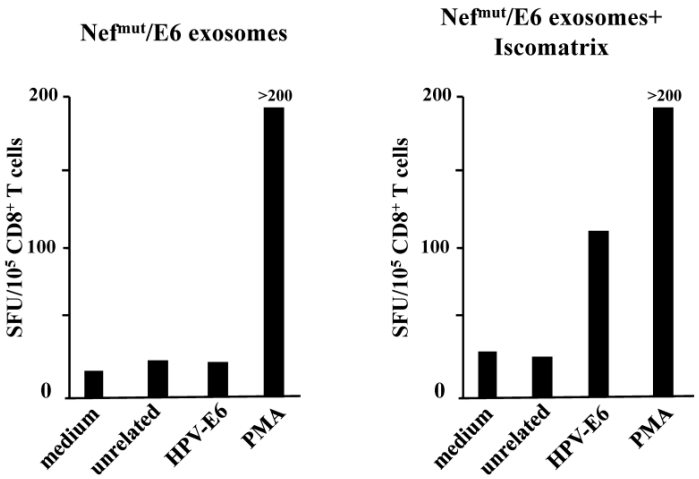
The aim of the present study was the identification of a method to improve the $CD8^+$ T cell immunity induced by the inoculation of Nef^{mut}-based exosomes. Hence, we next were interested in assessing whether the ISCOMATRIX^{TM} adjuvant-dependent improvement of cross-presentation activity that we observed in vitro was associated with increased immunogenicity in animals. To this aim, exosomes engineered with the Nef^{mut}/HPV-E6 fusion protein were purified from the supernatants of 293T transfected cells and characterized in terms of contents of both the fusion product and CD63 tetraspanin (Figure 5A). C57Bl/6 mice were inoculated subcutaneously (s.c.) three times with two week intervals with 2.1 mU of Nef^{mut}/E6 exosomes in the presence or not of 3.8 ISCO^{TM} Units of ISCOMATRIX^{TM} adjuvant, i.e., the highest dose well tolerated by s.c. injected mice. Two weeks after the last immunization, mice were sacrificed, and splenocytes assayed for the presence of HPV-E6-specific $CD8^+$ T lymphocytes. By IFN-γ Elispot assay, we detected HPV-E6-specific $CD8^+$ T lymphocytes in splenocytes from mice inoculated with Nef^{mut}/E6 exosomes plus ISCOMATRIX^{TM} adjuvant, but not from mice inoculated with the exosomes alone (Figure 5B). The latter result was consistent with the previously reported evidence that the $CD8^+$ T cell response against HPV-E7 delivered by engineered exosomes was detectable only after a 5-day culture of splenocytes in the presence of specific peptides [19]. The increased number of E6-specific $CD8^+$ T cells within splenocytes from mice co-inoculated with ISCOMATRIX^{TM} adjuvant correlated with the appearance of a E6-specific CTL activity, as shown by the results we obtained through a CTL assay based on the co-culture with syngeneic EL-4 cells pre-treated with E6 peptides (Figure 5C). Of note, no anti-HPV-E6 antibodies were found in sera from mice inoculated with Nef^{mut}/E6 incorporating exosomes whatever the co-inoculation of ISCOMATRIX^{TM} adjuvant (Figure 5D).

From these data, we concluded that ISCOMATRIX^{TM} adjuvant is instrumental in increasing the $CD8^+$ T cell immune response against exosome-associated antigens.



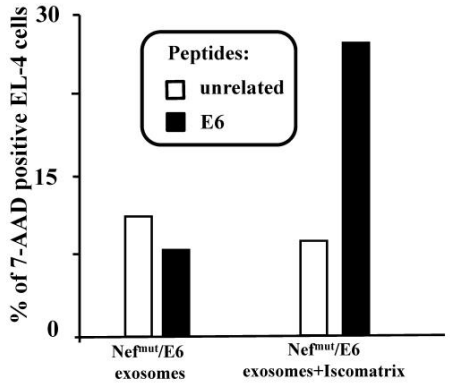
(**A**)

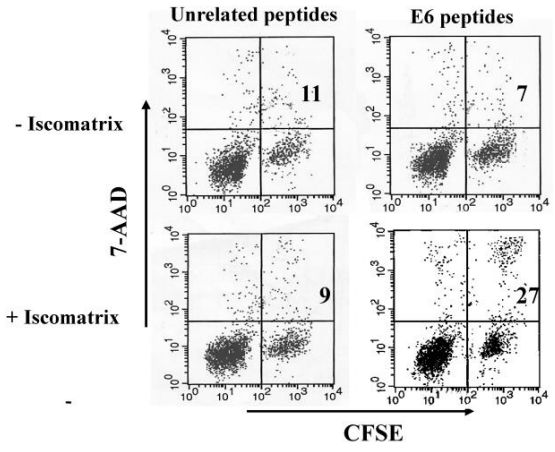
Figure 5. *Cont.*



		Experiment I		Experiment II	
mouse	Peptides	- ISCOMATRIX	+ ISCOMATRIX	-ISCOMATRIX	+ ISCOMATRIX
A	nil	19	27	13	18
	unrelated	21	25	18	23
	E6	17	113	9	109
B	nil	22	26	19	27
	unrelated	24	21	28	19
	E6	16	134	24	107
C	nil	20	37	24	28
	unrelated	25	34	30	21
	E6	29	99	20	117

(**B**)





(**C**)

Figure 5. *Cont.*

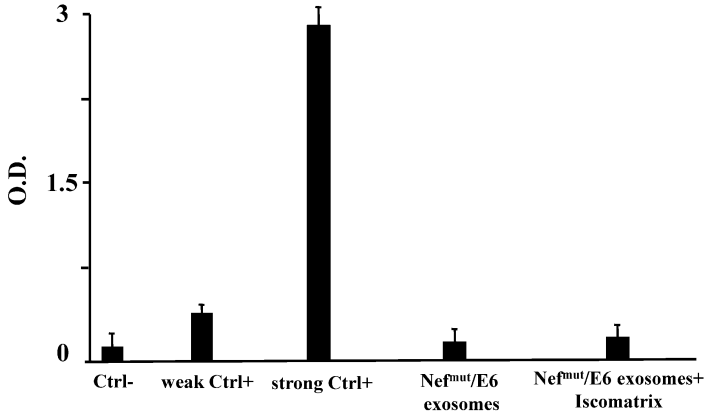


Figure 5. The co-inoculation in mice of ISCOMATRIX^{TM} adjuvant and engineered exosomes increases the number of HPV-E6 specific CD8$^+$ T lymphocytes. (**A**) molecular characterization of exosome preparations uploading Nef^{mu}/HPV-E6. On the left is the Western blot analysis of 200 μU of exosomes associating Nef^{mut}/E6. As a control, equivalent amounts of exosomes from cells transfected with the empty vector were loaded. Arrow signs indicate the relevant protein product. Exosome preparations were also probed for the presence of ICAM-1. Molecular markers are given in kDa. On the right, the FACS analysis for the presence of CD63 in exosome membrane is shown. M1 marks the range of fluorescence positivity as determined by the analysis of equivalent amounts of exosomes after incubation with isotype-specific IgGs (**left** histogram). Results shown in both panels are representative of five assays carried out on three exosome preparations. At the bottom right, mean values ±SD of both percentages of positivity and MFI from the five assays are also reported; (**B**) CD8$^+$ T cell immune response in mice inoculated with Nef^{mut}/E6 exosomes in the presence or not of ISCOMATRIX^{TM} adjuvant. C57 Bl/6 mice (three per group) were inoculated s.c. at the lower right flank three times with exosomes uploading Nef^{mut}/E6 in the presence or not of the adjuvant. Two weeks after the last inoculation, splenocytes were isolated and 10^5 cells were incubated overnight with or without 5 μg/mL of either unrelated or HPV-E6-specific peptides in IFN-γ Elispot microwells in triplicate conditions. As a control, untreated cells were incubated with 10 ng/mL of phorbol-12-myristate-13-acetate (PMA) and 500 ng/mL of ionomycin. Afterwards, cell activation extents were evaluated by IFN-γ Elispot assay carried out in triplicate with 10^5 cells/well. Cultures of splenocytes from each inoculated mouse were carried out separately. The mean of SFU/10^5 cells calculated on the basis of data reported at the bottom which were obtained in two independent immunization experiments are shown. Iscomatrix: ISCOMATRIX^{TM} adjuvant; (**C**) Cytotoxic T lymphocyte (CTL) assay carried out with CD8$^+$ T cells isolated from splenocytes of mice inoculated with exosomes uploading Nef^{mut}/E6 in the presence or not of ISCOMATRIX^{TM} adjuvant. CD8$^+$ T lymphocytes from pooled splenocyte cultures were co-cultivated for 6 h at 20:1 effector/target cell ratio with EL (European lymphoblast)-4 cells previously labeled with Carboxyfluorescein succinimidyl ester (CFSE), and treated with either unrelated or E6 peptides for 16 h. Finally, the EL-4 cell mortality levels were scored by FACS analysis upon 7-AAD labeling. At the **top**, are the results obtained using pooled splenocytes from a representative of two independent immunization experiments. At the **bottom**, the dot-plot FACS analysis of the co-cultures is reported. Cells were gated on the basis of their apparently unaffected morphology, and the percentages of double-positive over the total of CFSE-labelled cells are reported; (**D**) anti-E6 antibody detection in plasma from mice inoculated with the Nef^{mut}/E6 exosomes in the presence or not of ISCOMATRIX^{TM} adjuvant. Plasma were tested in an in-house Elisa assay upon 1:10 dilution. As internal standards, 1:10 diluted plasma from mock-inoculated mice was used as negative control (Ctrl−), whereas both 1:1000 (weak Ctrl+) and 1:100 (strong Ctrl+) dilutions of plasma from mice injected with 10 μg of recombinant E6 protein plus adjuvant were used as positive controls. Shown are the mean absorbance values +SD of triplicates of plasma samples from each inoculated mouse.

4. Discussion

Nef^{mut}-based exosomes represent an original tool for the induction of a Class I MHC-unrestricted CTL immune response against antigens of choice. The basically unvaried efficiency of exosome incorporation of Nef^{mut} when fused at its C-terminus with an heterologous antigen guarantees the great flexibility of this immunogen platform. Considering that the inoculation of Nef^{mut}-based exosomes does not induce specific Abs, leading exclusively to antigen-specific CD8$^+$ T lymphocyte immunity, adjuvants already characterized for their ability to increase this arm of the adaptive immunity could represent useful tools to strengthen the immunogenicity of Nef^{mut}-based exosomes.

Exosomes engineered to upload HPV-E7 fused with the Nef^{mut} exosome-anchoring protein have been recently shown to efficiently elicit a CD8$^+$ T-specific adaptive immune response against E7 [19].This immune response blocked the growth of tumor cells implanted after immunization, appearing however only partly efficient in the therapeutic setting. Here, we present a simple method to increase the immunogenicity of the Nef^{mut}-based exosomes useful for possible therapeutic applications. The increase of antigen-specific CTL immunity that we documented in splenocytes from ISCOMATRIX™ adjuvant-co-inoculated mice is expected to be associated with increased survival of animals challenged with HPV E6-expressing tumor cells, as we already demonstrated for HPV E7- engineered exosomes [19]. To the best of our knowledge, for the first time, the activity of a saponin-based adjuvant favoring the immunogenicity of a nanovesicle-delivered antigen has been demonstrated.

Cross-presentation in DCs relies on two non-mutually exclusive mechanisms [29]. In the first one, referred to as "cytosolic", the antigen transits from the endosomal compartment to the cytosol. In the case of endocytosed vesicles, this passage can be greatly favored by pH-dependent envelope fusion proteins. In cytosol, the antigen is degraded by proteasome, and resulting peptides are loaded on Class I MHC upon Transporter associated with antigen processing (TAP)-mediated translocation into endoplasmatic reticulum. The second mechanism, defined as "vacuolar", is based on the action of endo-lysosomal proteases degrading both vesicles and associated proteins, whose resulting peptides are loaded into Class I MHC recycling at vesicular levels. We assumed that the CD8$^+$ T-related immunogenicity that we observed using exosomes produced in the absence of foreign envelope proteins was a consequence of the vacuolar cross-presentation activity in APCs ingesting the exosomes.

We selected an ISCOMATRIX™ adjuvant mainly based on our in-depth understanding of its mechanism of action. ISCOMATRIX™ adjuvant induces integrated responses including antibody and cellular immune responses to various types of soluble antigens [17]. The particulate nature of ISCOMATRIX™ adjuvant (40–50 nm) contributes to some of its properties. ISCOMATRIX™ adjuvant is efficiently endocytosed by APCs where it exerts its immunomodulatory activities. Generation of high frequency antigen-specific CD8$^+$ T cell responses is a reproducible feature of ISCOMATRIX™ adjuvant vaccines in both animal models and in humans [28,30,31]. We have already demonstrated that ISCOMATRIX™ adjuvant induces prolonged antigen cross-presentation persisting in vivo up to seven days after priming, which, together with efficient Ag delivery, provides a mechanistic explanation for the strong CD8$^+$ T cell responses induced by ISCOMATRIX™ adjuvant vaccines [32]. In the same study, it was also demonstrated that ISCOMATRIX™ adjuvant vaccines are more efficient at inducing CTL responses in vivo than other adjuvants such as aluminum hydroxide, incomplete Freund's adjuvant, CpG, lipopolysaccharides (LPS) or Pam3Cys. Wilson and colleagues further reported that cells (at both draining lymph nodes and injection site) that directly encounter/take up ISCOMATRIX™ adjuvant likely undergo metabolic cell stress, which triggers multiple "danger" signaling pathways [33]. All of these effects could represent an advantage compared to using other adjuvants which either trigger only one particular pathway, or having undefined mechanism of action.

It was already shown that ISCOMATRIX^{TM} adjuvant significantly increases the cross-presentation of soluble antigens [17]. Although the exact underlying mechanisms remains to be fully elucidated, it has been reported that in vivo it: (i) induces DC activation; (ii) facilitates antigen cross-presentation in a specific subset of DCs, i.e., the CD8α^+; and (iii) generates a pro-inflammatory milieu in the

inoculation site, with increased production of both IL-1β and IL-6 [29]. An inflammatory milieu leading to activation of professional APCs is expected to favor adaptive immune responses. However, whether and how inflammatory factors are relevant for cross-presentation of exosome-associated antigens remains to be established.

ISCOMATRIX™ adjuvant has also been proven to boost the antibody response against soluble antigens [17]. In our hands, consistently with what previously reported for HPV E7-uploaded engineered exosomes [19], no antibody response against HPV-E6 has been detected in mice challenged with exosomes incorporating Nef^{mut}/E6 also in the presence of ISCOMATRIX™ adjuvant. This evidence was strongly suggestive of a basically exclusive presentation of HPV-E6 peptides in Class I MHC also in the presence of the adjuvant.

Anti-tumor experimental vaccine strategies described so far are based on the use of either peptides, recombinant proteins, DNA, viral vectors, or VLPs. Some intrinsic limitations reduce the possibility of effective transfer to clinics for many of these approaches. The use of the engineered exosomes in anti-tumor vaccine strategies described here presents a number of advantages including: (i) a quite low basal immunogenicity; (ii) the incorporation of the whole tumor-associated antigen, which implies a Class I MHC-unrestricted use; (iii) the lack of genetic material, and a minimal presence of non-human antigens; (iv) a manufacturing simpler than that required for viral vectors and VLPs. The promising results that we obtained justify further investigations on both immunogenicity and efficacy of exosomes engineered with additional antigens of therapeutic significance.

5. Conclusions

Overall, our results indicate that the molecular composition of ISCOMATRIX™ adjuvant is perfectly compatible with the structural integrity of exosomal nanovesicles. ISCOMATRIX™ adjuvant increases cross-presentation of exosome-associated antigens in vitro, and consistently improves the $CD8^+$ T cell response against the foreign antigen incorporated in engineered exosomes. Hence, besides soluble antigens, ISCOMATRIX™ adjuvant is also useful for increasing the immunogenicity of antigens incorporated in nanovesicles. This result could be of utility for both current and future immunotherapies based on nanovesicle-associated antigens.

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Author Contributions: F.M prepared and characterized exosomes; P.d.B. carried out experiments in mice; B.R. performed in vitro Elispot assays; S.A. carried out exosome-bead binding experiments; C.A. carried out exosome internalization experiments; C.C. performed ex vivo Elispot assays, A.B.M. provided the adjuvant and designed the experiments; and M.F. supervised the experiments and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 5

Antitumor HPV E7-specific CTL activity elicited by *in vivo* engineered exosomes produced through DNA inoculation

We proved that exosomes engineered *in vitro* to deliver high amounts of HPV E7 fused to the exosome-anchoring protein Nef^{mut} elicit an efficient anti-E7 cytotoxic T-cell immune response. Despite the encouraging results, we realized that exosome production was hampered by intrinsic issues: limited reproducibility and time consuming procedures among others, which would be even less desirable in the case of a clinical translation of the platform. Cost of production and storage are also to be taken into account. To overcome these hurdles, we designed a DNA immunization strategy relying on intramuscular inoculation of a plasmid expressing the Nef^{mut}-E7 chimeric protein. Our idea was to induce the production, primarily by muscle cells, of endogenous antigen containing exosomes to achieve high, or at least stable levels of circulating vesicles in plasma.

The production of engineered exosomes was confirmed by FACS analysis of plasma derived vesicles coming from animals treated with Nef^{mut}-GFP expressing DNA. Next, we observed that mice treated with Nef^{mut}-E7 construct developed a CD8+ T-cell immune response against both Nef and E7. No detectable specific CD8+ T-cell responses were found in animals inoculated with DNA expressing either the wild-type Nef isoform or E7 alone; this last treatment, as expected, only induced the formation of antibodies. Nef^{mut}-E7 treated animals that developed a cellular immune response were able to control the growth of E7 positive tumors and the results were clearer than those we obtained inoculating engineered exosomes. Moreover, the vesicles collected from treated mice were immunogenic when used to vaccinate naïve animals.

The overall results obtained with Nef^{mut} DNA constructs are encouraging, also considering that the protocol we used is quite simple and several improvements may be implemented.

Antitumor HPV E7-specific CTL activity elicited by in vivo engineered exosomes produced through DNA inoculation

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Abstract: We recently proved that exosomes engineered in vitro to deliver high amounts of HPV E7 upon fusion with the Nef_{mut} exosome-anchoring protein elicit an efficient anti-E7 cyto-toxic T lymphocyte immune response. However, in view of a potential clinic application of this finding, our exosome-based immunization strategy was faced with possible technical difficulties including industrial manufacturing, cost of production, and storage. To overcome these hurdles, we designed an as yet unproven exosome-based immunization strategy relying on delivery by intramuscular inoculation of a DNA vector expressing Nef_{mut} fused with HPV E7. In this way, we predicted that the expression of the Nef_{mut}/E7 vector in muscle cells would result in a continuous source of endogenous (ie, produced by the inoculated host) engineered exosomes able to induce an E7-specific immune response. To assess this hypothesis, we first demonstrated that the injection of a Nef_{mut}/green fluorescent protein-expressing vector led to the release of fluorescent exosomes, as detected in plasma of inoculated mice. Then, we observed that mice inoculated intramuscularly with a vector expressing Nef_{mut}/E7 developed a $CD8^+$ T-cell immune response against both Nef and E7. Conversely, no $CD8^+$ T-cell responses were detected upon injection of vectors express-ing either the wild-type Nef isoform of E7 alone, most likely a consequence of their inefficient exosome incorporation. The production of immunogenic exosomes in the DNA-injected mice was formally demonstrated by the E7-specific $CD8^+$ T-cell immune response we detected in mice inoculated with exosomes isolated from plasma of mice inoculated with the Nef_{mut}/E7 vector. Finally, we provide evidence that the injection of Nef $_{mut}$/E7 DNA led to the generation of effective antigen-specific cytotoxic T lymphocytes whose activity was likely part of the potent, therapeutic antitumor effect we observed in mice implanted with TC-1 tumor cells. In summary, we established a novel method to generate immunogenic exosomes in vivo by the intramuscular inoculation of DNA vectors expressing the exosome-anchoring protein Nef_{mut} and its derivatives.

Keywords: nanovesicles, cytotoxic T lymphocytes, HIV-1 Nef, DNA vectors

Introduction

Exosomes are vesicles ranging 50–150 nm and are released constitutively by all cell types.[1] They are generated by inward invagination of endosome membranes. These intraluminal vesicles form the multivesicular bodies (MVBs) which can traffic to the plasma membrane to which they fuse, thereby releasing their vesicular contents in the extracellular milieu. Exosomes are part of the intercellular communication network. They incorporate messenger RNAs, microRNAs, DNA, and proteins which can be functional in target cells. Nanovesicles showing both physical and biochemical features resembling exosomes, but those generated through direct extrusion of plasma membrane have also been described in muscle cells.[2–4]

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The exosome immunogenicity basically relies on the amounts and quality of antigens they incorporate. Exosomes were tested as antitumor immunostimulatory agents, and clinical trials demonstrated that they are well tolerated as cell-free vaccines.[5,6] However, their therapeutic efficacy appeared quite limited, posing the need of new methods to increase their immunogenicity. This issue has been addressed through in vitro methods devoted to engineer foreign antigens to increase their display on the exosome membrane.[7,8]

Exosome biogenesis and HIV budding share the functions of a number of cell proteins including Alix, Tsg101, and several components of the endosomal sorting complex required for transport.[9] The convergence of exosome and HIV biogenesis implies the possibility that viral products incorporate in exosomes. Such is the case for HIV-1 Nef, which associates with exosomes through anchoring its N-terminal myristoylation to lipid raft microdomains at the endosome membranes.[10] Nef is a 27 kilodalton (kDa) protein lacking enzymatic activities. However, it can act as a scaffold/adaptor element to trigger activation of signal transducing molecules.[11]

We previously identified a $_{G}3_{C}$ $_{V}153_{L}$ $_{E}177_{G}$ Nef mutant incorporating in exosomes at quite high levels.[12] This Nef mutant (referred to as Nef_{mut}) is defective for basically all Nef functions. Its efficiency of incorporation in nanovesicles does not change significantly when fused at its C-terminus with foreign proteins. Manipulating Nef_{mut} allows the incorporation of high amounts of antigens of choice into exosomes, which thus remain protected from external neutralization/degradation factors. We recently reported that the inoculation in mice of exosomes carrying HPV E7 fused with Nef_{mut} induces an effective E7-specific $CD8^+$ T cytotoxic lymphocyte (CTL) response.[13] This result demonstrates that, through the Nef_{mut}-based engineering strategy, the already proven poor CTL immunogenicity of exosomes can be overcome. However, this strategy would face possible difficulties in view of potential clinical applications, including in areas of standardization of industrial manufacturing, high cost-effectiveness, and storage of the immunogen. For these reasons, we conceived a still unproven, exosome-based vaccine strategy relying on delivery of DNA vectors expressing Nef_{mut}-based fusion proteins into host animal by intramuscular (IM) inoculation. This strategy relies on the very recent observation that muscle cells also, both proliferating and differentiated, constitutively release exosome-like vesicles. Considering that Nef_{mut} and derivatives thereof associate with exosomes with high efficiency, we predicted that the expression of Nef_{mut}-based vectors in muscle cells would be sufficient to create a continuous source of engineered, immunogenic exosomes.

Here, we demonstrate that IM inoculation of mice with a DNA vector expressing Nef_{mut}/E7 elicits a potent CTL immune response, thereby blocking the growth of already implanted tumor cells. We provide evidence that the produc-tion of endogenously engineered exosomes was the basis of the observed antitumor effect.

Materials and methods

Molecular constructs and cell cultures

All molecular constructs were based on IE-CMV-promoted vectors. Vectors expressing Nef_{mut}, Nef_{mut}/green fluores-cent protein (GFP),[12] Nef_{G2A}/GFP,[14] wild-type (wt) Nef,[15] and HPV $E7_{16}$ have already been described. 293T, murine muscle C_2C_{12} (both obtained from American Type Culture Collection, ATCC), and HPV E7-expressing TC-1 tumor cells (a kind gift from Dr Wu, John Hopkins Medical Institutes, Baltimore, MD, USA) were grown in Dulbecco's Modified Eagle's Medium plus 10% heat-inactivated fetal calf serum (FCS). Transfection assays were carried out by the Lipofectamine 2000-based method (Invitrogen, Thero Fisher Scientific, Waltham, MA, USA), which in the case of C_2C_{12} cells was modified by adding liposomes to freshly trypsinized cells. Both EL-4 cells[17] (obtained from ATCC) and mouse splenocytes were cultivated in Roswell Park Memorial Institute 1640 (RPMI) medium plus 10% FCS.

Exosome isolation, detection, and characterization

Exosomes were isolated from cell supernatants through differential centrifugations as previously described.[18] The recovery of exosomes from the plasma of inoculated mice was carried out in a similar way except that samples were fivefold diluted before starting centrifugations. The amounts of recovered exosomes were evaluated by measuring the activity of acetylcholinesterase (AchE), ie, a classical exosome marker,[19] through the use of the Amplex Red kit (Molecular Probes, Thermo Fisher) following the manufacturer's recommendations. The AchE activity was measured in mU/mL, where 1 mU is defined as the amount of enzyme which hydrolyzes 1 pmole of acetylcholine to choline and acetate per minute at pH 8.0 at 37°C.

Fluorescent exosomes from transfected cell cultures were either directly detected by fluorescence-activated cell sorting (FACS, Gallios, Beckman Coulter, Brea, CA, USA), or, in the case of exosomes isolated from plasma, analyzed upon

binding with aldehyde/sulfate latex beads (Molecular Probes, Thermo Fisher). To this end, samples were incubated with 5 µL of beads overnight at room temperature on a rotating plate, washed, and incubated for 1 hour at 4°C with either anti-CD63 mAb MX-49.129.5 from Abcam (Cambridge, UK) or a control isotype. The incubation was then repeated using secondary PE-conjugated Abs. Finally, beads were resuspended in 1× PBS-2% v/v formaldehyde and analyzed with FACS.

Western blot analyses of both cell lysates and exosomes were carried out as described previously.[12] Filters were revealed using 1:1,000 diluted sheep anti-Nef antiserum ARP 444 (a generous gift of M Harris, University of Leeds, Leeds, UK), 1:250 diluted anti-β actin AC-74 mAb from Sigma (Milan, Italy), and 1:100 diluted anti-Alix H-270 polyclonal Abs from Santa Cruz (Heidelberg, Germany).

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

All studies with animals described here have been approved by the Ethical Committee of the Istituto Superiore di Sanità, Rome, Italy (protocol number 555/SA/2012) according to Legislative Decree 116/92, which has implemented in Italy the European Directive 86/609/EEC on laboratory animal protection. Animals used in our research have been housed and treated according to the guidelines mentioned in the Legislative Decree. C57 Bl/6 mice were purchased from Charles River Laboratories Italia (Calco, Italy) and inoculated IM two times at 10 day intervals with 50 µg in each quadriceps with plasmid DNA purified with endotoxin-free Qiagen kit (Hilden, Germany). Mice were also inoculated subcutaneously (SC) three times at ten day intervals with exosomes (6 mU AchE equivalents) purified from plasma of DNA injected mice, and sacrificed 10 days after the last immunization. To detect both E7- and Nef-specific $CD8^+$ T-cell immune responses, splenocytes were put in IFN-γ Elispot microwells (Millipore, Billerica, MA) in the presence of 5 µg/mL of either HPV E7 or HIV-1 Nef 8- or 9-mer peptides binding the H-2 K_b complex of C57 Bl/6 mice, ie, DLYCYEQL (aa 21–28) and RAHYNIVTF (aa 49–57) for E7,[20] and TAATNADCA (aa 48–56) for Nef.[21] H-2 K_b binding HPV E6-specific KLPQLCTEL (aa 18–26) and YDFAFRDL (aa 50–57) peptides[20] were used as unre-lated peptides. After overnight incubation, IFN-γ Elispot plates were developed (Mabtech AB, Nacka Strand, Sweden) and spot-forming units were counted using an Elispot reader (A.EL.VIS. Elispot reader and Analysis software GmbH, Turin, Italy).

Fluorescence microscope analysis

Slices (7 µm) from quadriceps of inoculated mice were prepared using a cryostat (Leika CM 3050, Wetzlar, Germany) and placed to slides. The slices were then incubated with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, UK) together with an antifade mounting medium. Finally, coverslips were mounted on the slides, which were then observed with a Zeiss Axioskop 2 Plus (Oberkochen, Germany) fluorescence microscope.

CTL assay

$CD8^+$ T-cells were isolated from splenocytes by positive immunomagnetic selection (Miltenyi Biotec Gmbh, Teterow, Germany). They were put in coculture for 6 hours in RPMI 10% FCS with EL-4 cells previously labeled with carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen, Thermo Fisher), following the manufacturer's recommendation, and treated overnight with either E7 or unrelated peptides. The cocultures were run at different cell ratios (ie, 20:1, 10:1, and 5:1 effector/target cells) in 200 µL of RPMI 20% in U-bottom 96 well plates. Afterward, EL-4 cell mortality was scored by FACS analysis soon after addition of 7-AAD at final concentration of 1 µg/mL.

Detection of anti-E7 and anti-Nef antibodies in plasma

Plasma from inoculated mice were pooled, and twofold serial dilutions starting from 1:10 were assayed for the presence of both E7- and Nef-specific Abs as previously described.[22] The end-point dilution corresponded to a ,0.1 OD absorbance at 450 nm. Each plasma sample was assayed in triplicate, and the mean of the absorbance values was taken as final readout.

Antitumor effects of Nef_{mut}/E7-expressing vector

The antitumor activity was evaluated in mice previously challenged with $2\times10_5$ TC-1 cells. DNA inoculations were performed 4 and 11 days after tumor cell challenge following the above reported protocol, and only in mice that developed palpable tumors. Tumor growth was monitored by visual inspection, palpation, and measurement of tumor nodule diameter calculated as (length × $width_2$)/2. At the end of the observation time, tumors were explanted and weighted.

Statistical analysis

When appropriate, data are presented as mean + standard deviation. In some instances, the paired Student's *t*-test was

used and confirmed using the nonparametric Wilcoxon rank sum test. *P*,0.05 was considered significant.

Results

Detection of engineered exosomes released by DNA-transfected murine muscle cells

We preliminarily investigated whether, as already assessed in different human cell types, the expression of a Nef_{mut}-expressing DNA vector in murine muscle cells was sufficient to generate engineered exosomes. For the sake of clarity, exosome-like nanovesicles released by murine muscle cells are here defined as exosomes, although their biogenesis somehow differs from that of MVB-generated exosomes.[2] Murine muscle C_2C_{12} and, as control, human 293T cells were transfected with vector expressing GFP fused at the C-terminus of either Nef_{mut} or a Nef isotype inefficiently associating with exosomes (ie, Nef_{G2A}).[23] Transfected cells were monitored for the respective efficiency of transfection (Figure 1A), which in muscle cells appeared to be over 50%. The supernatants were collected, and exosomes isolated through differential centrifugation. Exosome preparations were then titrated in terms of AchE activity. The two cell types produced apparently similar levels of AchE-positive

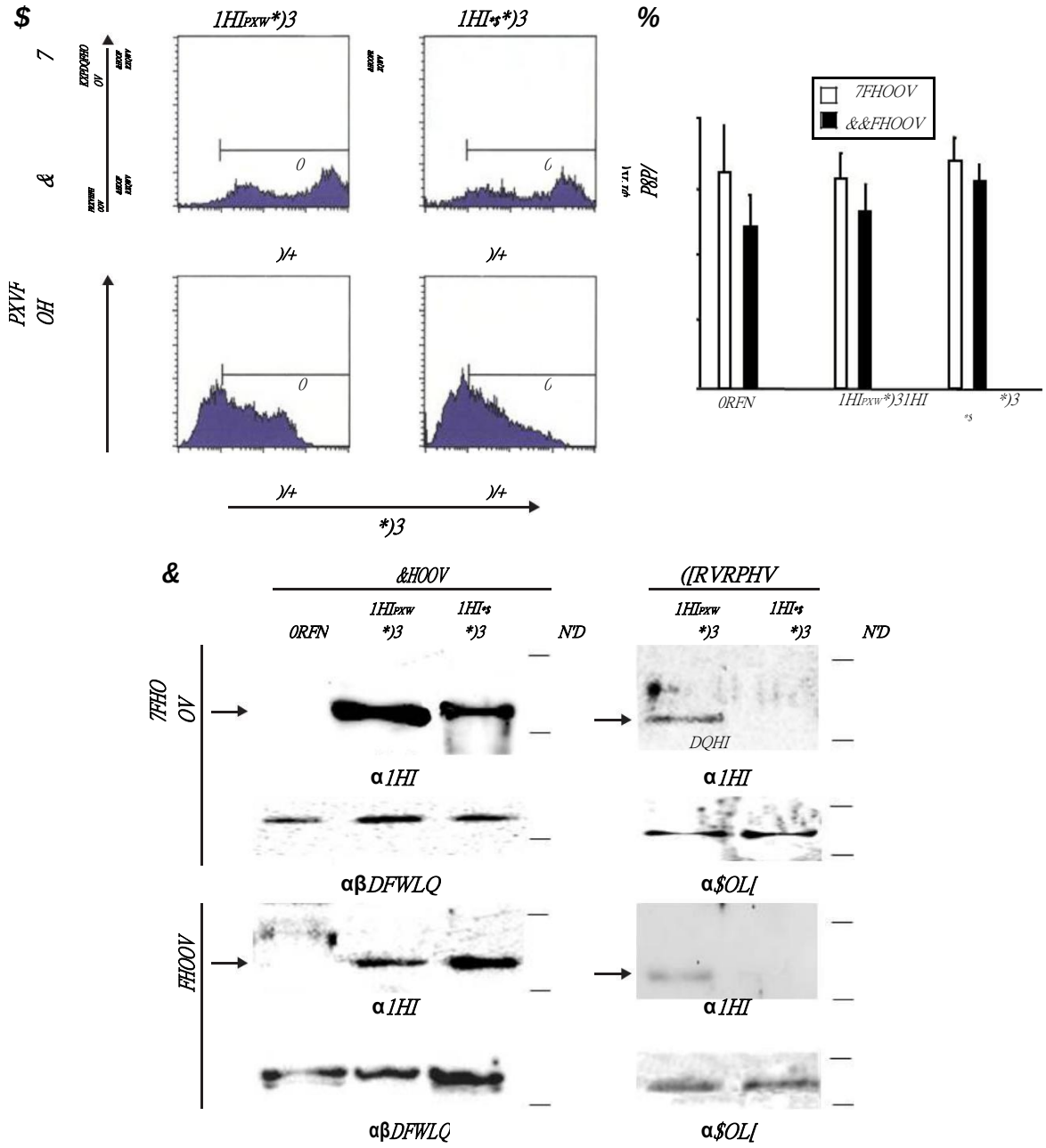

Figure 1 (*Continued*)

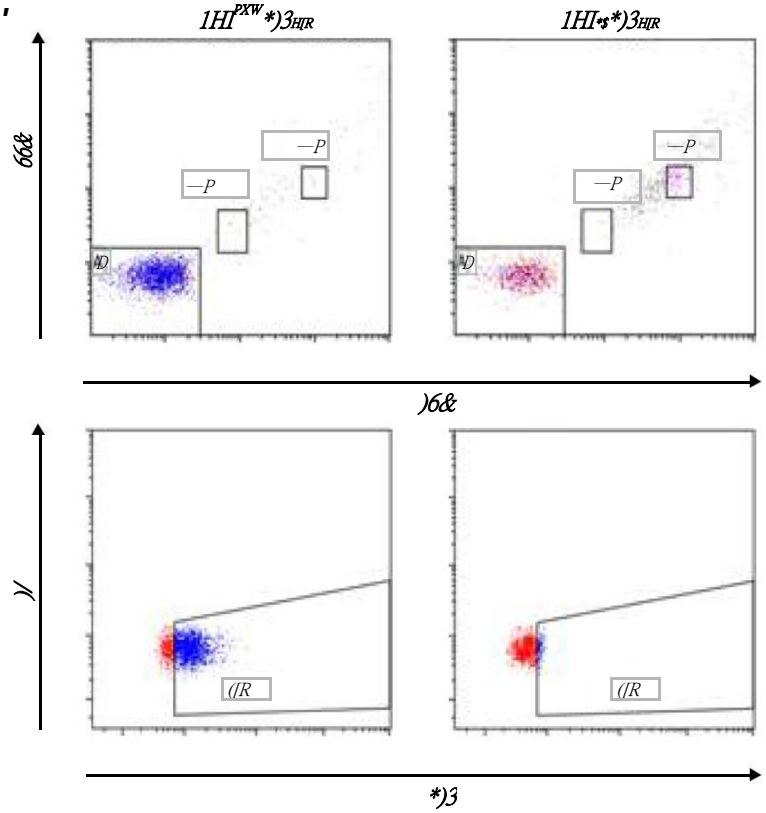

Figure 1 Detection of engineered exosomes in supernatants of transfected murine muscle cells.

Notes: (**A**) FACS analysis of both human 293T and murine C_2C_{12} muscle cells 2 days after transfection with either Nef^{mut}/GFP- or Nef_{G2A}/GFP-expressing vectors. M1 marks the range of positivity as established by the analysis of mock-transfected cells. Percentages of positive cells are reported. (**B**) Quantification in terms of AchE activity of exosome preparations recovered by differential centrifugations of supernatants from the same number (ie, $5\times10_6$) of both 293T and C_2C_{12} transfected cells. (**C**) Western blot analysis of exosomes from both 293T and C_2C_{12} transfected cells. Nef-based products were detected in both cell lysates and exosomes, while β-actin and Alix served as markers for cell lysates and exosomes, respectively. Arrows mark the relevant protein products. Molecular markers are given in kDa. (**D**) FACS analysis of exosomes from C_2C_{12} transfected cells. Ten mU of exosomes from C_2C_{12} cells transfected with either Nef^{mut}/GFP- or Nef_{G2A}/GFP-expressing vectors were analyzed in terms of both FSC and SSC (top panels), as well as GFP fluorescence (bottom panels). Quadrants indicate the dimension of the detected particulate (top panels, a: 0.1 μm) and the range of positivity as calculated by the analysis of exosomes from mock-transfected cells (bottom panels). Results are representative of two independent experiments.

Abbreviations: AchE, acetylcholineesterase; exo, exosomes; FACS, fluorescence-activated cell sorting; FL2, fluorescence channel 2; FSC, forward scatter; GFP, green fluorescent protein; SSC, side scatter.

nanovesicles whatever the transfection conditions (Figure 1B). The western blot analysis of 100 ∝U equivalent of exosomes showed the presence of Nef-derived molecules in exosomes from both 293T and C_2C_{12} cells transfected with Nef^{mut}/GFP but not Nef_{G2A}/GFP vectors (Figure 1C). The FACS analysis confirmed the association of fluorescence with the nanovesicles we recovered from C_2C_{12} cells transfected with Nef_{mut}/GFP (Figure 1D).

In sum, we have proven that exosome-like nanovesicles released by murine muscle cells can be engineered by Nef_{mut}-derivatives as previously proven in epithelial-like, human 293T cells.

Nef_{mut}-derived products can be detected in exosomes from plasma of DNA-inoculated mice

To assess whether engineered exosomes can be generated in vivo, 50 ∝g of either Nef^{mut}/GFP, Nef_{G2A}/GFP, or empty vector were inoculated in each quadriceps of C57 Bl/6 mice. To assess the expression of injected vectors, 3 days later, slices of muscle tissues obtained from the zones of inoculation were analyzed for the expression of GFP-related products. Consistently with what was already described for wt Nef,[24] Nef_{mut} accumulated at the plasma membrane as well as in an intracellular punctate pattern. Differently, the Nef_{G2A} mutant localized preferentially in the cytoplasm, as a consequence of the lack of N-terminal myristoylation (Figure 2A). Three and nine days after inoculation, plasma from the inoculated mice was recovered and exosomes were isolated by differential centrifugations. Exosome preparations were titrated in terms of AchE activity, equivalent amounts of them were bound to white aldehyde/sulfate latex beads, and finally labeled for the CD63 exosome marker. By FACS analysis of the exosome– bead complexes, we detected fluorescent exosomes among those isolated only from the plasma of mice injected with Nef_{mut}/GFP vector (Figure 2B).

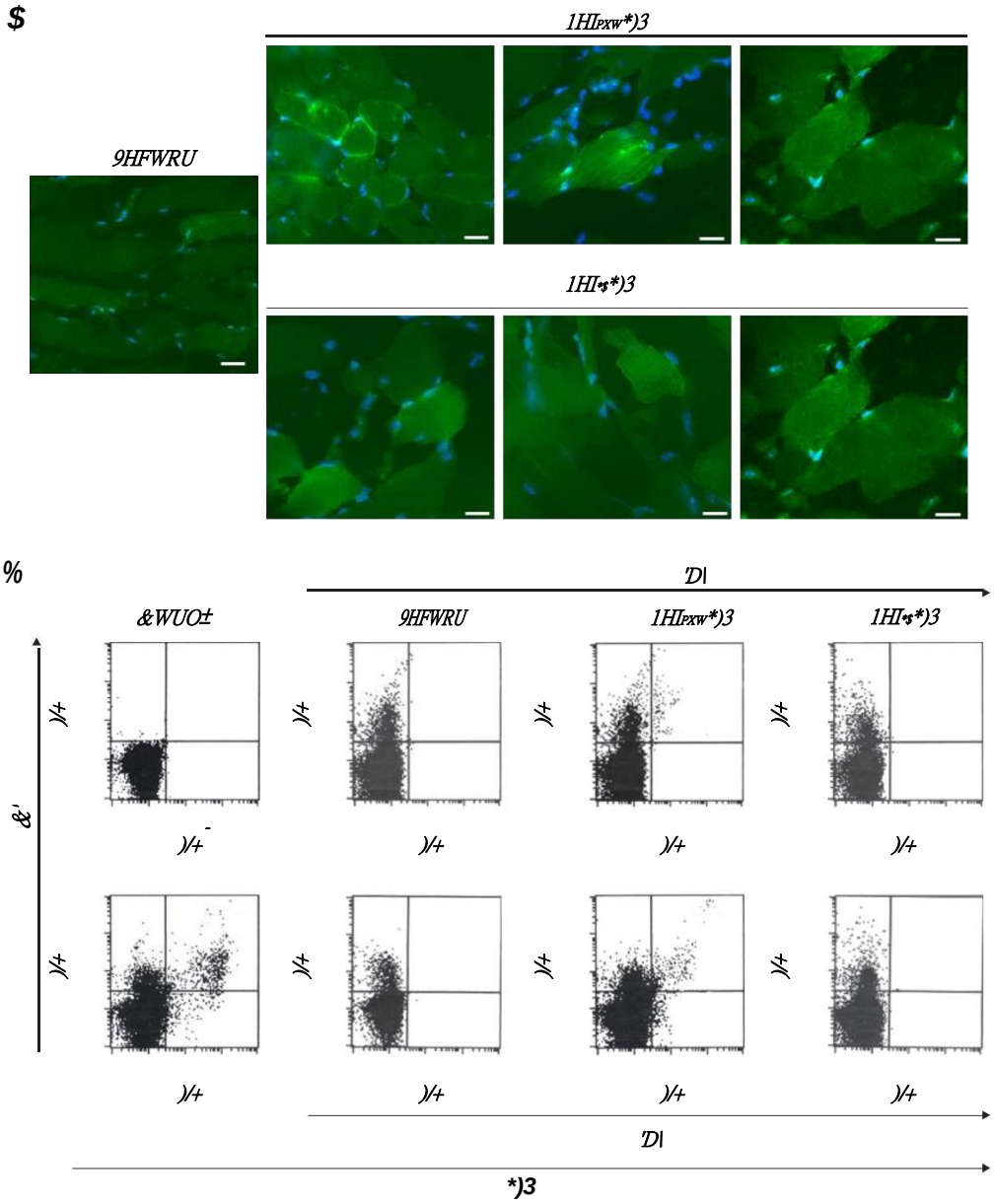

Figure 2 Detection of fluorescent nanovesicles in plasma from mice inoculated with a Nef_{mut}/GFP-expressing DNA vector.
Notes: (**A**) Analysis of the expression of GFP-related products in muscle tissues from mice inoculated with the indicated DNA vectors. Samples from different injected mice are represented by each picture. Scale bars: 50 ∝M. (**B**) FACS analysis of exosomes from plasma of C57 Bl/6 mice inoculated IM with DNA vectors expressing the indicated products. Three and nine days after injection, exosomes were isolated from the pool of plasma of three injected mice by differential centrifugations. Then, equivalent amounts (ie, 1 mU) of exosomes were bound to surfactant-free white aldehyde/sulfate latex beads, labeled with anti-CD63 mAb, and finally assayed for their fluorescence. As control, 10 ∝U of exosomes isolated from the supernatants of C_2C_{12} cells transiently transfected with Nef_{mut}/GFP vector were used (Ctrl+). Quadrants were set on the basis of the fluorescence of beads incubated with anti-CD63 mAb (Ctrl–). Percentages of positive events are indicated. Results are representative of two assays.
Abbreviations: FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IM, intramuscular.

These results indicated that the inoculation in mice of vectors expressing Nef_{mut} derivatives can lead to the genera-tion of engineered exosomes.

HPV E7-specific CTL response induced by IM inoculation of Nef_{mut}/E7-expressing DNA vector

Next, we evaluated the immunogenicity of DNA vectors expressing Nef_{mut} derivatives. C57 Bl/6 mice (six per group) were inoculated IM in each quadriceps with 50 μg of vectors expressing either Nef_{mut}/E7 or E7 alone or empty vector. Since we previously noticed that E7 associates with exosomes at undetectable levels (not shown), the inclusion of a vector expressing E7 alone was instrumental to evaluate the benefit of the Nef_{mut} fusion in terms of $CD8^+$ T-cell immunogenicity. The inoculations were repeated 10 days later, and after additional 10 days mice were sacrificed. Then, the spleno-cytes were isolated and cultured overnight in IFN-γ Elispot

microwells in the presence of either unrelated, Nef-, or E7-specific nonamers. The $CD8^+$ T-cell activation observed in cultures with unrelated peptides remained at background levels as in the case of splenocytes cultured in the absence of peptides (not shown). On the other hand, peptide-specific cell activation was clearly detectable in splenocytes from mice inoculated with Nef_{mut}/E7-expressing vector (Figure 3A). Conversely, no $CD8^+$ T-cell response was observed in cultures of splenocytes from mice receiving either E7-expressing or empty vector, whatever the peptide used. Notably, anti-E7 antibodies were detected only in plasma from mice inoculated with the vector expressing E7 alone (Figure 3B).

To evaluate whether the $CD8^+$ T-cell response coupled with an E7-specific CTL activity, $CD8^+$ T-cells were isolated from pools of splenocytes and then put in coculture for 6 hours at different cell ratios with CFSE-labeled EL-4 cells pretreated overnight with either unrelated or E7 nonam-ers. Afterward, the cocultures were labeled with 7-AAD and percentages of dead target cells were scored by FACS analysis. The results reported in Figure 3C show a clear increase of target cell mortality in both 20:1 and 10:1 cocultures comprising $CD8^+$ T lymphocytes from mice inoculated with the Nef_{mut}/E7-expressing vector and EL-4 pretreated with E7-specific nonamers. This result demonstrates the presence of E7-specific CTLs among the $CD8^+$ T lymphocytes isolated from mice inoculated with the Nef_{mut}/E7-expressing vector.

Taken together, these data indicated that IM inoculation of a vector expressing a heterologous antigen fused with Nef_{mut} leads to the induction of a strong antigen-specific CTL response in the absence of antibody production.

Lack of immunogenicity of a DNA vector expressing the wt Nef isoform

To support the idea that high levels of Nef_{mut} incorporation in exosomes were mandatory to elicit the antigen-specific $CD8^+$ T-cell response, we reproduced the immunogenicity experiments by inoculating mice with vectors expressing the wt isoform of Nef, which is known to incorporate in exosomes at much lower extents compared to Nef_{mut}.[12] To this end, C57 Bl/6 mice (four per group) were injected IM in each quadriceps with 50 μg of a vector expressing either wt Nef or Nef_{mut} or with the empty vector. The inoculations were repeated 10 days later, and after an additional 10 days the mice were sacrificed. Splenocytes were then isolated and cultured overnight in IFN-γ Elispot microwells in the presence of either unrelated or Nef nonamers. As shown in Figure 4, mice inoculated with the vector expressing wt Nef, differently to those receiving the Nef_{mut} vector, failed to develop a detectable Nef-specific $CD8^+$ T cell response.

This result indicates that the levels of antigen incorporation into exosomes are critical for the immune response, meanwhile suggesting that the functions of wt Nef were not per se involved in the antigen-specific $CD8^+$ T-cell activation.

Immunogenicity of exosomes isolated from plasma of mice immunized with Nef_{mut}/E7 DNA

To enforce the hypothesis that the $CD8^+$ T-cell immune response we detected upon inoculation of Nef_{mut}-expressing vectors relied on production of engineered exosomes, we next tested whether exosomes purified from plasma of DNA-inoculated mice were immunogenic in recipient-naïve mice. For this, eight mice were inoculated with vectors express-ing E7, Nef_{mut}/E7, or empty vector following the schedule mentioned earlier. Eight days after the last immunization, peripheral blood mononuclear cells were recovered through retro-orbital bleeding and checked for the E7-specific $CD8^+$ T-cell response. As already observed, the injection of the Nef_{mut}/E7-expressing vector, but not that expressing E7 alone, gave rise to a well detectable E7-specific $CD8^+$ T-cell response (Figure 5A). Plasma from the different mouse groups were pooled and exosomes were isolated by differential centrifugation. Afterward, exosomes were titrated in terms of AchE activity, and 6 mU AchE equivalents were injected SC in syngeneic mice three times at 10 day intervals. Finally, mice were sacrificed, and splenocytes tested for the E7-specific $CD8^+$ T-cell responses, which were noticed only in splenocyte cultures from mice inoculated with exo-somes purified from mice injected with the Nef_{mut}/E7 DNA vector (Figure 5B).

These results indicate that the IM injection of DNA expressing Nef_{mut}/E7 leads to the production of immunogenic exosomes, hence further supporting the idea that the production of endogenously engineered exosomes was on the basis of the strong E7-specific $CD8^+$ T-cell immune response observed in the DNA-injected mice.

Antitumor therapeutic effect of IM inoculation of Nef_{mut}/E7-expressing DNA vector

Finally, we evaluated the potency of the $CD8^+$ T-cell immune response evoked by injection of Nef_{mut}/E7 expressing vector in terms of antitumor effect. To this end, we set up therapeutic

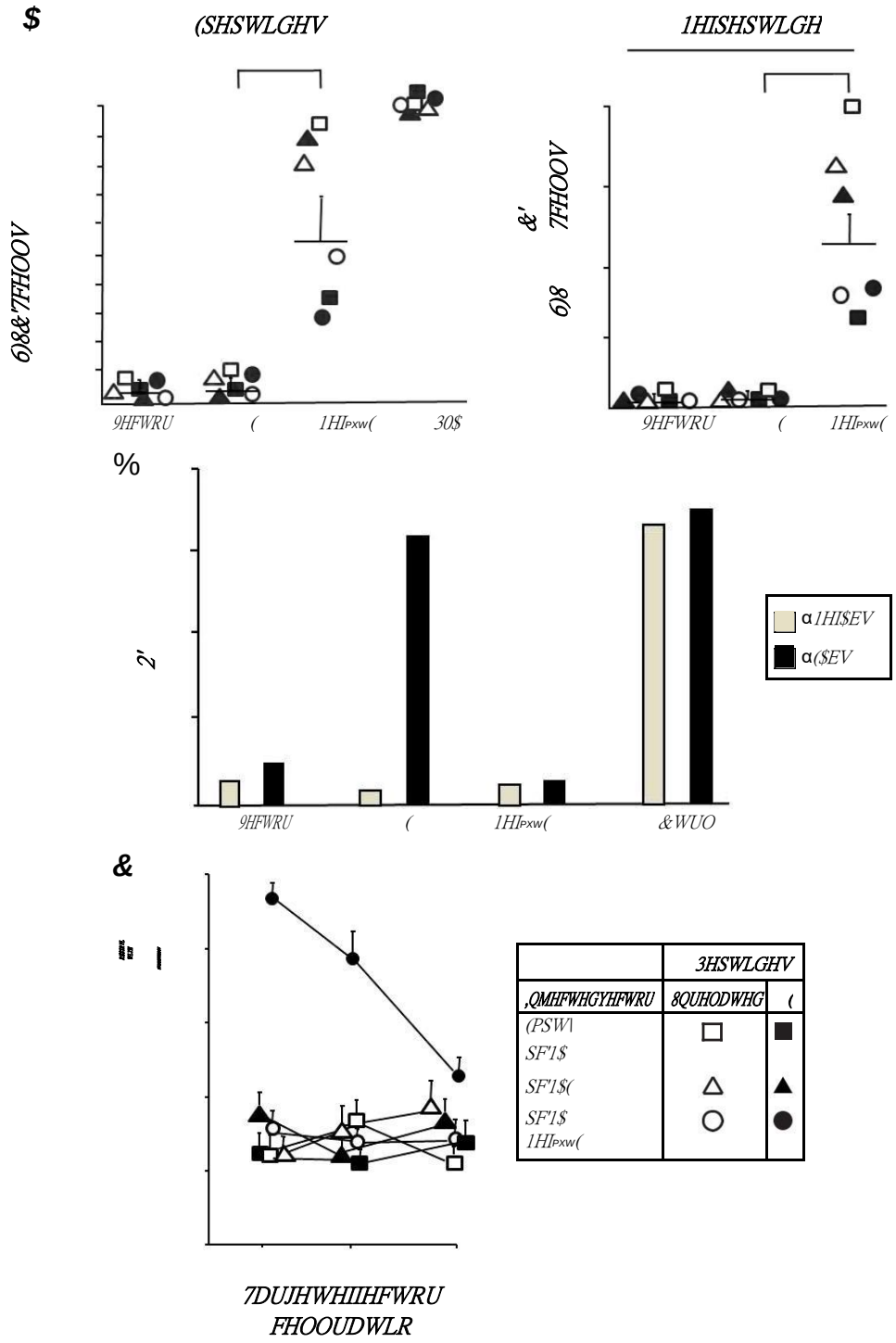

Figure 3 The inoculation of Nef_{mut}/E7 DNA vector induces an E7-specific $CD8^+$ T-cell immune response in the absence of antibody production.

Notes: (**A**) $CD8^+$ T-cell immune response in mice inoculated with DNA vectors expressing either E7, Nef_{mut}/E7, or empty vector. C57 Bl/6 mice (six per group) were inoculated two times with the different DNA vectors. At the time of sacrifice, 10_5 splenocytes were incubated overnight with or without 5 μg/mL of either unrelated (not shown) E7- or Nef-specific nonamers in triplicate IFN-γ Elispot microwells. As control, cells were incubated with 5 ng/mL of PMA plus 500 ng/mL of ionomycin. Shown are the number of IFN-γ SFU/10_5. Intergroup mean + SD of SFU are also reported. The results are representative of three independent experiments. *P,0.05. (**B**) Anti-Nef and anti-E7 antibody detection in 1:10 diluted plasma from mice inoculated with the indicated DNA vectors. As internal positive control standard (Ctrl+), 1:10,000 dilutions of plasma from mice injected with 10 μg of either recombinant E7 or Nef proteins plus adjuvant were used. Shown are the mean absorbance values of triplicates of plasma pooled from six mice per group. (**C**) CTL assay carried out with $CD8^+$ T-cells from mice inoculated with the indicated vectors. $CD8^+$ T-cells were isolated from pooled splenocytes, cultured in duplicate for 6 hours at 20:1, 10:1, and 5:1 cell ratios with EL-4 cells previously labeled with CFSE, and treated with either unrelated or E7 peptides for 16 hours. Six hours later, EL-4 cell mortality was scored by FACS analysis upon 7-AAD labeling. Shown are the mean values + SD as calculated from four independent experiments. *P,0.05.

Abbreviations: CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorting; OD, optical density; PMA, phorbol 12-myristate 13-acetate; SFU, spot-forming unit.

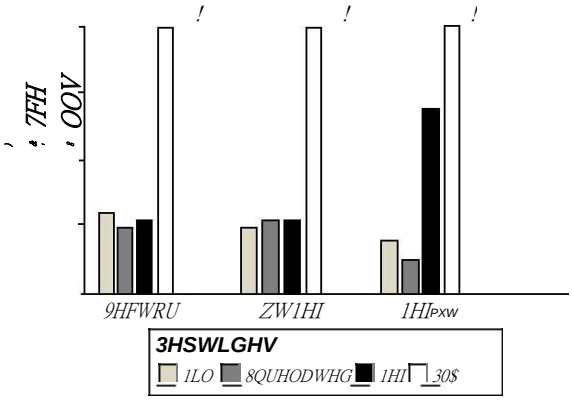

Figure 4 Injection in mice of wt Nef-expressing DNA vector fails to elicit Nef-specific $CD8^+$ T-cell immune response.
Notes: C57 Bl/6 mice (four per group) were inoculated intramuscularly two times with DNA vectors expressing wt Nef, Nef_{mut}, or empty vector, and sacrificed 10 days after the last immunization. Splenocytes were then cultured overnight in triplicate in IFN-γ Elispot microwells in the absence or presence of either unrelated or Nef-specific nonamers. As control, cells were incubated with 5 ng/mL of PMA and 500 ng/mL of ionomycin. Shown are the mean of SFU/10_5 cells from a representative of two independent experiments.
Abbreviations: PMA, phorbol 12-myristate 13-acetate; SFU, spot-forming unit; wt, wild type.

immunization assays on C57 Bl/6 mice inoculated SC with $2\times10_5$ TC-1 cells. Mice developing a tumor mass detect-able by palpation, ie, of about 2 mm diameter, were then inoculated with 50 μg/quadriceps of vectors expressing either empty vector, Nef_{mut} (four mice per each group) or Nef_{mut}/E7 (six mice) at days 4 and 11 after cell implantation. As control, 4 tumor-implanted mice were injected with the vehicle alone. At day 21, retro-orbital bleeding was carried out on mice injected with Nef_{mut}- or Nef_{mut}/E7-expressing vectors to assess the induction of E7-specific $CD8^+$ T-cell immune response (Figure 6A). The growth of tumors was monitored over 30 days, and thereafter mice were sacrificed, tumors explanted, and weighted. Figure 6B shows that, whereas the injection of control DNA vectors did not influence the growth of implanted tumor cells, their expansion was severely impaired in mice inoculated with Nef_{mut}/E7 vector, with tumor cells apparently being cleared in three mice, as confirmed by tumor weights (Figure 6C).

Hence, the inoculation of Nef_{mut}/E7-expressing DNA vector elicited a $CD8^+$ T-cell immune response also in the presence of tumor cells. Most importantly, this immune response was both strong and rapid enough to inhibit the tumor growth and, in some instances, clear the implanted tumor cells.

Taken together, these results represent a relevant milestone toward possible therapeutic applications of immu-nization strategies based on Nef $_{mut}$-based endogenously engineered exosomes.

Discussion

We here describe a simple exosome-mediated strategy to induce unrestricted CTL immunity through IM DNA inoculation. Muscle cells constitutively release exosome-like

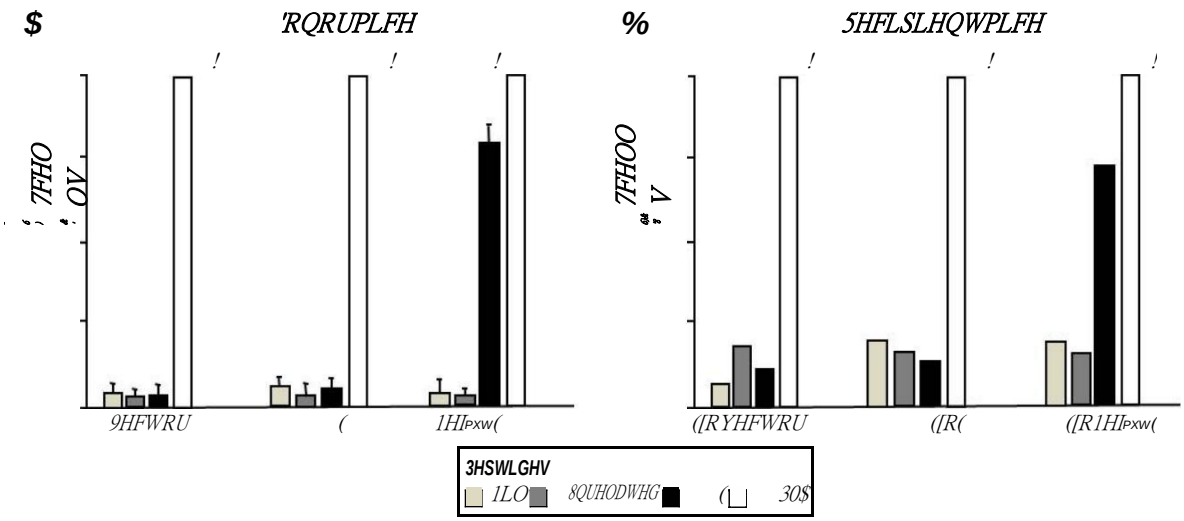

Figure 5 E7-specific $CD8^+$ T-cell immunity induced in mice by injection of exosomes from mice inoculated with Nef_{mut}/E7 DNA vector.
Notes: Donor C57 Bl/6 mice (8 per group) were inoculated two times with the indicated DNA vectors, and 10 days after the last inoculation PBMCs were recovered from retro-orbital bleeding and tested in IFN-γ Elispot assay for the presence of E7-specific $CD8^+$ T-cells. In panel **A** the mean + SD of SFU/10_5 cells as calculated from a representative of 2 experiments are shown. Two days later, the mice were sacrificed and exosomes were isolated from plasma by differential centrifugation. Equivalent amounts of these exosomes were then inoculated in syngeneic mice (3 per group) three times. Ten days after the last inoculation splenocytes were tested by IFN-γ Elispot assay carried out in triplicate. In panel **B** the means of SFU/10_5 cells as calculated from two independent experiments are shown. PMA: cells incubated with 5 ng/mL PMA and 500 ng/mL ionomycin.
Abbreviations: PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; SD, standard deviation; SFU, spot-forming unit.

nanovesicles through extrusion of plasma membrane with a biogenesis at least in part different from that of MVB-generated exosomes. Thus, considering that Nef_{mut}-based in vitro engineered exosomes we previously described were generated in epithelial-like human cells, investigat-ing whether and how efficiently the ectopic expression of Nef_{mut} leads to the release of engineered exosomes in murine cells was mandatory. Data we obtained with C_2C_{12} cells supported the idea that Nef_{mut} accumulates into exosome-like nanovesicles released by murine muscle cells at levels similar to those observed in human cells. Our investigations did not include terminally differentiated muscle cells, ie, the cell type most likely targeted by IM inoculation in mice, since we assumed that the cell differentiation does not significantly impact the mechanisms underlying intracellular vesicle traf-ficking. Since we expected that the majority of muscle cells targeted by IM DNA inoculation was differentiated, the detection of engineered exosomes in plasma from inoculated mice supported the idea that terminally differentiated muscle cells indeed release engineered exosomes in vivo. We cannot exclude that at least part of injected DNA could target other cell types by means, for instance, of the diffusion of DNA in draining lymph nodes, where it can be captured and internal-ized by dendritic cells (DCs). However, we believe that DCs contribute limitedly to the overall production of engineered exosomes since SC inoculation of Nef_{mut}/E7-expressing DNA, which is expected to preferentially target DCs, gave rise to a quite weak E7-specific $CD8^+$ T-cell activation (not shown).

The immune response detected in DNA-inoculated mice appeared much stronger than what we previously observed in mice injected with in vitro produced engineered exosomes.[13] A direct comparative experiments cannot be run in view of the different nature of the two immunogens. However, we noticed that the DNA inoculation elicited an E7-specific $CD8^+$ T-cell activation strong enough to be clearly detected without the in vitro, peptide-directed stimulation/expansion of specific $CD8^+$ T lymphocytes which, on the contrary, was required to detect the immune response in mice inoculated with in vitro produced exosomes.[13] This apparently stronger immunogenicity was likely a consequence of the continuous release from DNA-recipient muscle cells of immunogenic exosomes ready to be internalized by both local and distal antigen-presenting cells. Differently, it is known that both in vitro and ex vivo produced exosomes have a quite short half-life upon injection, ie, about 2 minutes, with a prompt accumulation in liver and spleen, and only residual amounts detectable after 4 hours.[25–28] The detection of fluorescent exosomes in plasma of mice inoculated with Nef_{mut}/GFP-expressing vector represented a key finding supporting the idea that engineered exosomes can indeed be produced upon DNA injection. The lack of Nef-specific $CD8^+$ T-immune response in mice injected with wt Nef highlighted the importance for the immune response of the efficient associa-tion with exosomes, meanwhile excluding the involvement of other Nef functions. Similarly, no E7-specific $CD8^+$ T-cell immune response was detectable in mice injected with vector expressing E7 alone, which per se associates

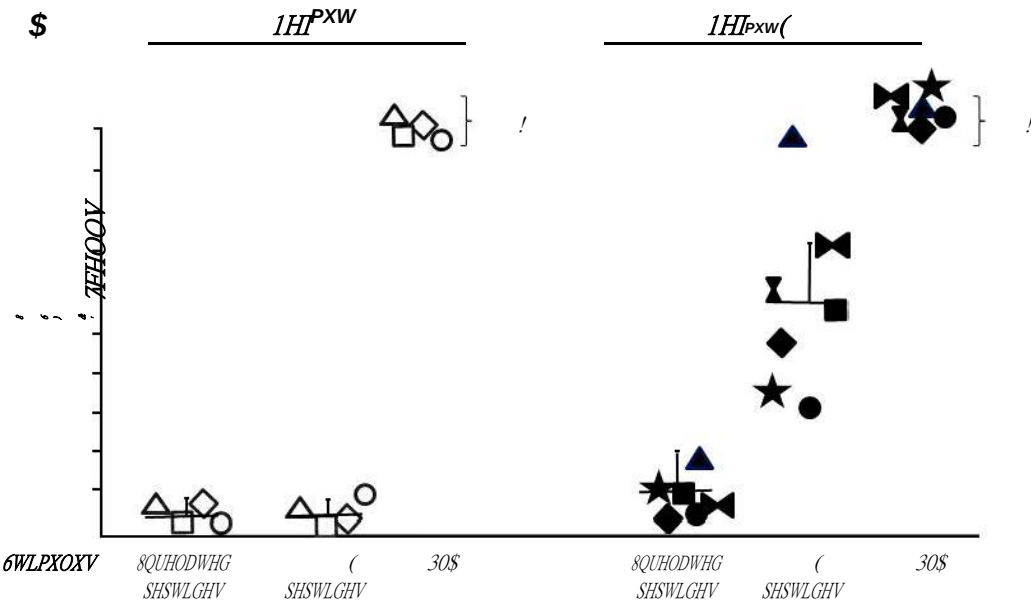

Figure 6 (*Continued*)

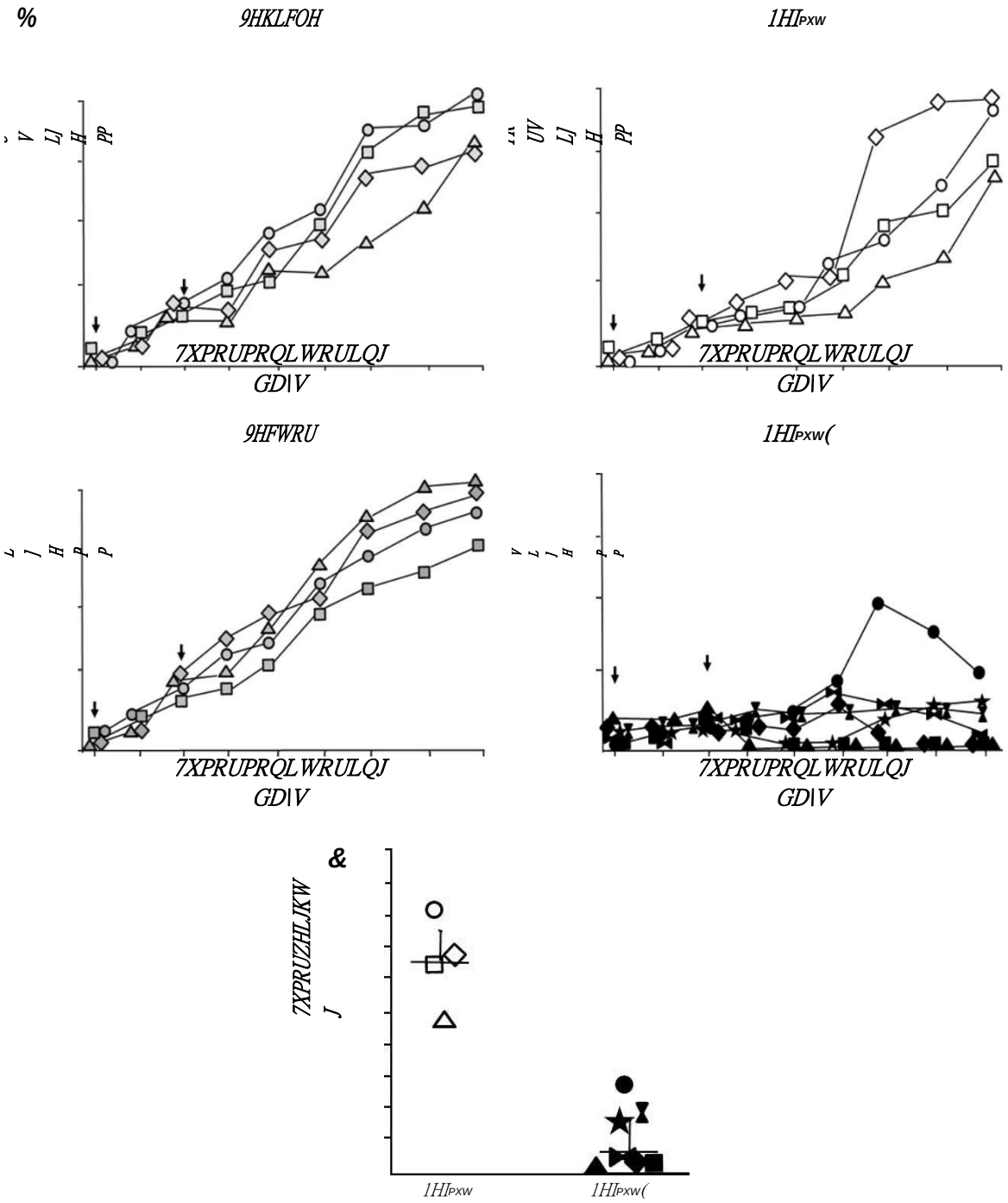

Figure 6 Antitumor therapeutic effect induced by IM inoculation of Nef_{mut}/E7 DNA vector.

Notes: C57 Bl/6 mice were challenged with $2\times10_5$ TC-1 cells, and 4 days later (arrows), when tumor masses became detectable by palpation, inoculated with either Nef_{mut}/E7 vector (seven mice) Nef_{mut} vector, empty vector, or vehicle (four mice per group). The DNA inoculations were repeated at day 11 (arrows) after tumor cell implantation, and the growth of tumor mass was followed over time. (**A**) E7-specific $CD8^+$ T-cell response as detected by IFN-γ Elispot assay was carried out with PBMCs recovered from retro-orbital bleeding 7 days after the last immunization and cultivated for 16 hours in the presence of either unrelated or E7 peptides. As control, PBMCs were incubated with 5 ng/mL of PMA and 500 ng/mL of ionomycin. Shown are the number of SFU/10_5 cells from triplicate wells seeded with splenocytes from mice inoculated with either Nef_{mut} or Nef_{mut}/E7 vectors. (**B**) Determination of the tumor size during the 30-day observation time. (**C**) Weight of tumors from mice injected with either Nef_{mut} or Nef_{mut}/E7 DNA vectors at the time of sacrifice. Shown are the results of one representative of two independent experiments.

Abbreviations: IM, intramuscular; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; SFU, spot-forming unit.

with exosomes poorly, in the presence, however, of a well detectable anti-E7 antibody production. This result further supports the idea that high levels of antigen incorporation into exosomes are mandatory to elicit a strong antigen-specific CTL activity.

Most important, the key role of endogenously engineered exosomes in the antigen-specific immune response was demonstrated by the evidence that exosomes isolated from plasma of DNA-injected mice were immunogenic when inoculated in naïve recipient mice. On the other hand, the

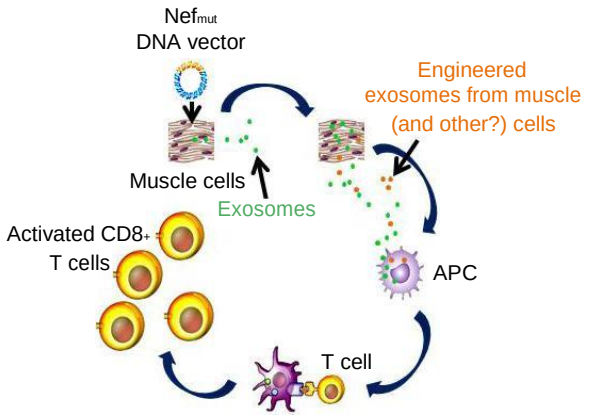

Figure 7 Mechanism of CTL activation induced by inoculation of Nef$_{mut}$-based DNA vectors.

Notes: Muscle (and possibly other) cells expressing the injected DNA vector release both unmodified and engineered exosomes. These latter, once internalized by APCs induce priming/activation of CD8$^+$ T-lymphocytes specific for the antigens uploaded in engineered exosomes.

Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte.

DNA-induced immune response appeared fast and strong enough to efficiently counteract the growth of syngeneic tumor cells implanted before the immunizations. The mechanism underlying the CD8$^+$ T-cell activation induced by inoculation of Nef$_{mut}$-based DNA vectors are depicted in Figure 7. Muscle cells (and, possibly, other cell types) expressing the injected DNA vector release engineered exosomes. These are expected to be internalized by antigen-presenting cells. The internalization of Nef$_{mut}$-based exosomes leads to cross-presentation of the associated antigens and, by consequence, priming of antigen-specific CD8$^+$ T-cells. Boosting of the immune response can be generated, besides the second round of DNA injection, by the continuous release of immunogenic exosomes from DNA recipient cells.

Conclusion

The data reported here allow us to propose a novel strategy to induce CTL immunity based on endogenously engineered exosomes. The major advantages of our vaccine platform are the cost-effectiveness of immunogens, ease of storage, potent immunogenicity, and significant intrinsic flexibility in terms of choice of the immunogen.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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Chapter 6. Concluding remarks

T cell inducing vaccines are powerful tools against viruses and cancer; they can be particularly useful to fight intracellular pathogens or when the eradication of an already established disease is needed (Gilbert SC, 2012). This differentiates T-cell inducing vaccines from classic humoral ones.

HPV infection and HPV related tumors are insidious threats for human health; cervix cancer is undoubtedly one of the most common among women and, even if prophylactic vaccines against HPV infection are now available, there are no effective therapeutic options for infected women. Several attempts to induce a CTL mediated immune response against HPV have been made (Cassetti MC et Al., 2004), (Gomez-Gutierrez JG et Al., 2007); researchers usually focus on two viral proteins, E6 and E7, which are commonly found in infected cells, but in many cases resulted to be poorly immunogenic.

We decided to use a protein delivery platform we already tested *in vitro* (Lattanzi L, Federico M, 2012), to immunize mice with HPV E7. The viral antigen was linked to the protein Nef ^{mut} of HIV, a mutant form which is effectively shuttled to exosomes and released inside them. The vesicles were supposed to deliver their cargo to antigen presenting cells for cross-presentation to lymphocytes. Our goal was to induce a prophylactic and a therapeutic vaccination sufficient to block the proliferation of E7 positive tumorigenic cells. To this aim we designed a plasmid carrying the fusion protein and used it to transfect HEK 293 cells, which are our "vesicle producers". Next we harvested our engineered exosomes with an ultracentrifuge and characterized them by FACS analysis: we tested them for the presence of GM1, a ganglioside which binds the subunit B of cholera toxin (CTX-B) and is a component of lipid rafts and we also identified the CD63 positive vesicles, which are those that can be properly defined exosomes. In our experiments we compared exosomes and HIV virus-like particles (VLPs) in terms of antigen delivery efficacy and immunogenicity; VLPs are, in fact, known to be good immunogen carriers and are widely used in vaccination studies. This comparison is quite straightforward since we already demonstrated that Nef^{mut} based antigens are incorporated in exosomes and VLPs at comparable levels, and in the experiments here reported the two vesicles both performed well. Moreover *in vivo* antigen release isn't apparently affected by the presence or absence of the G protein from vesicular stomatitis virus (VSV-G); this suggests that antigens loaded into exosomes are cross-presented through the "vacuolar" mechanism, that implies the action of endolysosomal

proteases which degrade both exosomes and associated proteins leading to peptide loading inside a vesicular compartment.

The results obtained in ELIspot assays of mouse splenocytes, in therms of CD8+ T cell activation an cytotoxic activity, suggest that our engineered antigen was able to generate a T-cell-mediated immune response that correlates with the remarkable anti-tumor effect observed. As expected, the administration of E7 alone didn't induce any cellular immune response and the effect on tumor growth and survival were poor despite the presence of anti-E7 antibodies in mouse plasma. The same antibodies were absent in animals treated with exosomes containing Nef ^{mut} -E7 and, considering that the viral protein resides inside the nanovesicles, neutralization by pre-existing or induced immunity is not expected to take place even in the case of repeated inoculations.

In a second set of experiments we tried to use the adjuvant ISCOMATRIX™ to investigate on a possible positive effect on antigen cross-presentation and specific CD8+ cell activation. In particular we used for *in vitro* and *in vivo* experiments the adjuvant in combination with Nef^{mut}-HPV-E6 loaded exosomes The results obtained show that ISCOMATRIX™ does not affect exosome integrity, as shown by FACS analysis of beads-bound fluorescent vesicles; instead it increases; cross-presentation of exosome-associated antigens *in vitro* as obtained using B-LCL and immature dendritic cells, and consistently improves the *in vivo* CD8+ T cell response against the chosen antigen. This suggests a possible use of the adjuvant also in combination with proteins and peptides incorporated in nanovesicles.

Having achieved these encouraging results, we asked if we could skip the problematic steps of working with exosomes produced *in vitro*. Such vesicles, in fact, usually derive from cell lines that have very little in common with the system they are applied to, in addition their production is time consuming and the preparations often differ one from another in terms of integrity and even composition. Such issues would be even more concerning in a clinical context.

The strategy to simplify our system was inspired by the increasing number of evidences suggesting that differentiated muscle cells secrete exosomes (or exosome-like vesicles). Thus we tried to set up a simple DNA vaccination protocol to target muscular tissue and induce the formation of endogenous Nef^{mut}-E7 exosomes in mice. First we asked if our exosome producing strategy was efficiently translated using a muscle cell line. We transfected murine C_2C_{12} cells with the Nef^{mut}-E7 construct and were able to recover antigen

containing exosomal vesicles, thus we proceeded to *in vivo* experiments and treated mice intramuscularly with DNA encoding the Nef^{mut}-linked immunogen, Nef^{mut} alone and a plasmid for the expression of E7 alone. A specific anti-E7 CD8+ T-cell activation was clearly detected without the *in vitro* peptide-directed stimulation/expansion of specific CD8+ T lymphocytes, (which was required to detect cell activation in mice inoculated with *in vitro* produced exosomes). This may be due to the fact that engineered exosomes were constitutively produced by mouse muscle fibers (and maybe also by other cell types), leading to an increased antigen uptake by APCs and thus overcoming the issue represented by the short clearance time of exosomes *in vivo*. The recovery of fluorescent exosomes in the plasma of mice inoculated with a Nef^{mut}-GFP expressing plasmid can be considered the proof that a noticeable quantity of vesicles is actually produced by the animals'muscles. Another strong indication pointing in that direction comes from the finding that vesicles isolated from treated mice are immunogenic when reinfused in naïve animals. Consistently with our model, the expression of wild type Nef (which is not efficiently shuttled to exosomes) doesn't lead to the activation of an anti-Nef immune response. Lastly, mice inoculated with the DNA encoding E7 alone didn't develop cellular immunity, but only produced antibodies against the protein.

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