Engineered Extracellular Vesicles for biogenesis and immunomodulation studies

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INTRODUCTION

Small Extracellular Vesicles (sEV), as naturally occurring vesicles, have a low intrinsic immunogenic profile, thus showing great therapeutic potential. Over the past few years, several engineering strategies have been devised to manipulate tumor-derived sEVs in order to induce cellular and innate immunity. To study exosomes role among immune system, we transfected cells with a DNA vector expressing a non-functional mutant of the HIV-1 Nef protein containing a N-terminal palmitolation domain as EV-anchoring protein fused at its C-terminus with a reporter gene i.e. green fluorescent protein (GFP). Here we studied two mutants of Nef protein, Nef^{G3C} and Nef^{mut}. The first one presents a mutation that converts the glycine in third position with a cysteine thus changing the myristoylation site to a palmitoilation site. Nef^{mut} presents two additional mutations: valine in position 153 is converted to leucine and finally glutamic acid in position 177 is converted to glycine which would result in the abrogation of the functional cellular activity of the nef protein. In our laboratory we developed an innovative methodology to metabolically label sEV using a lauric acid conjugated with a fluorophore that emit fluorescence in the red wavelength Bodipy 558/568 C12 (C12). This fatty acid, once internalized by cells, is metabolized into phospholipids that will be part of sEV membrane. Here we propose an original approach that combine the labeling techniques to generate sEV that present double fluorescence, one derived from the florescent fatty acid C12 and the other derived from the reporter gene fused with the Nef protein.

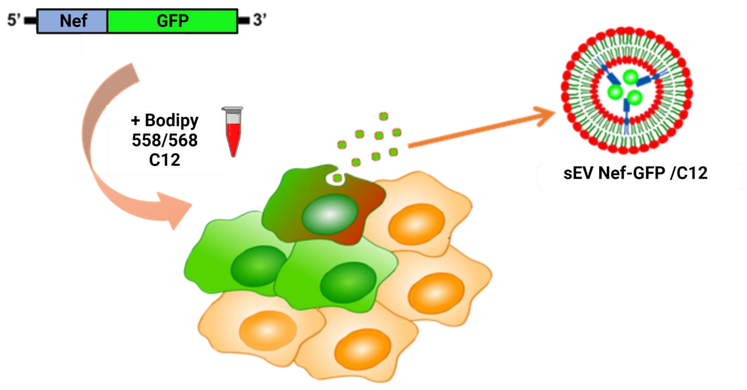

Figure 1. Schematic rapresentation of Hek293T cells transfected with Nef-GFP plasmid and labeled with Bodipy 558/568 C12. Cells release double fluorescent sEV Nef-GFP^{+}/C12^{+}

RESULTS

Distribution of Nef^{G3C}-GFP and C12 in Hek293T cells

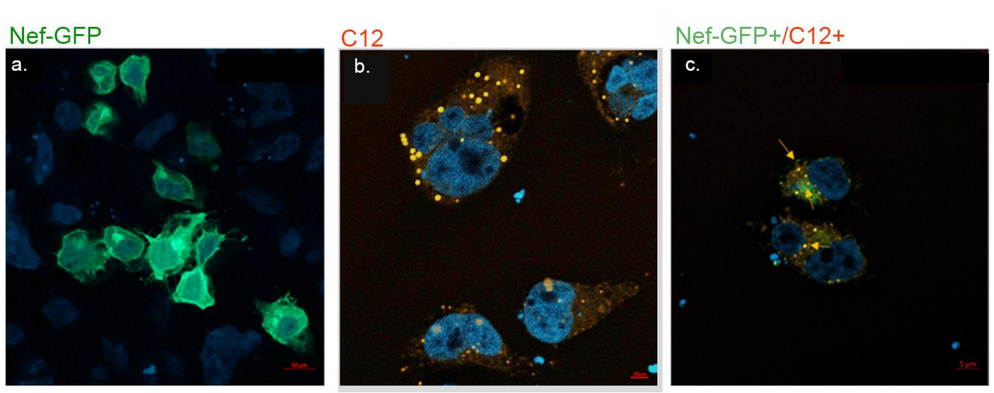

Figure 2. Confocal microscopy images of HEK293T cells: a) Cells have been transfected with 0,25mg of Nef-GFP vector for 16hrs; not all cells acquired the plasmid. b) Cells labeled for 2hrs with 7μM BODIPY 558/568 C12. c) Cells transfected with Nef-GFP and labeled with BODIPY 558/568 C12, it is possible to observe a colocalization (yellow arrows). (scale bare 10μm)

Characterization of Nef^{G3C}-GFP^{+}/C12^{+} sEV

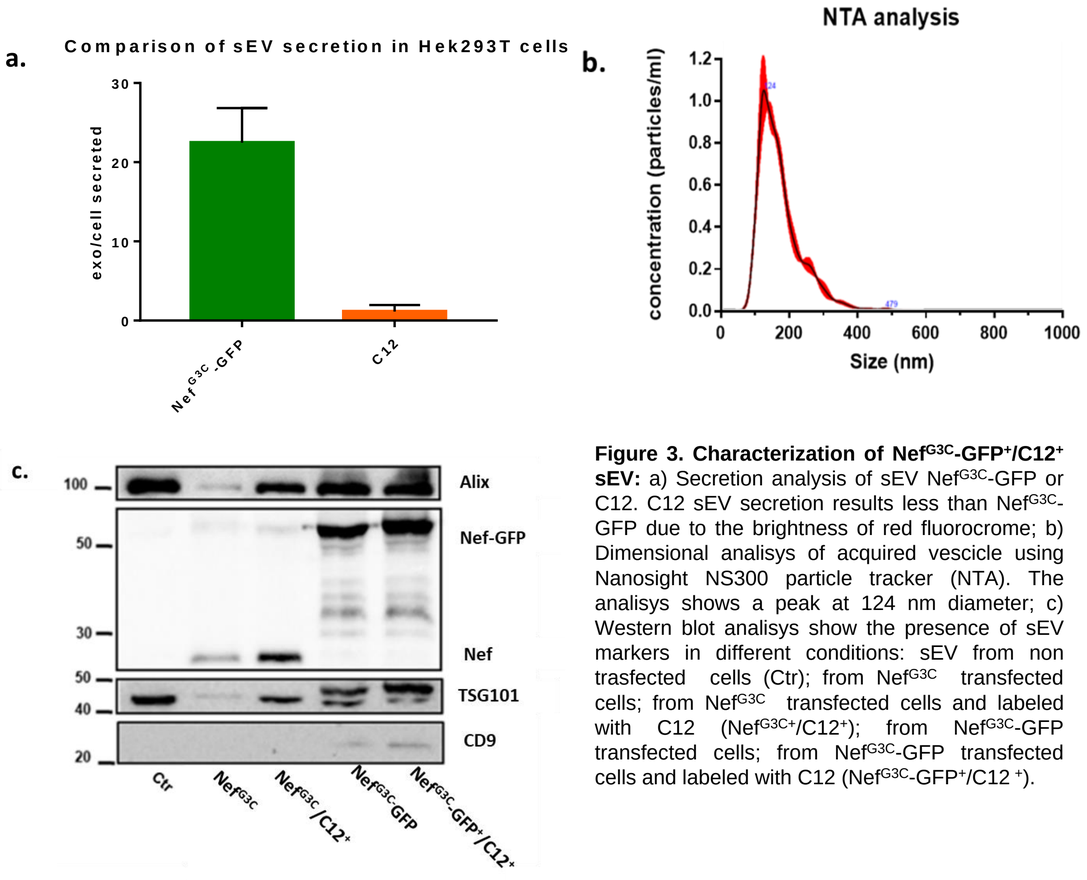

Figure 3. Characterization of Nef^{G3C}-GFP^{+}/C12^{+} sEV: a) Secretion analysis of sEV Nef^{G3C}-GFP or C12. C12 sEV secretion results less than Nef^{G3C}-GFP due to the brightness of red fluorocrome; b) Dimensional analisys of acquired vescicle using Nanosight NS300 particle tracker (NTA). The analisys shows a peak at 124 nm diameter; c) Western blot analisys show the presence of sEV markers in different conditions: sEV from non trasfected cells (Ctr); from Nef^{G3C} transfected cells; from Nef^{G3C} transfected cells and labeled with C12 (Nef^{G3C+}/C12^{+}); from Nef^{G3C}-GFP transfected cells; from Nef^{G3C}-GFP transfected cells and labeled with C12 (Nef^{G3C}-GFP^{+}/C12^{+}).

RESULTS

Separation of Nef^{G3C}-GFP^{+}/C12^{+} sEV on a density gradient

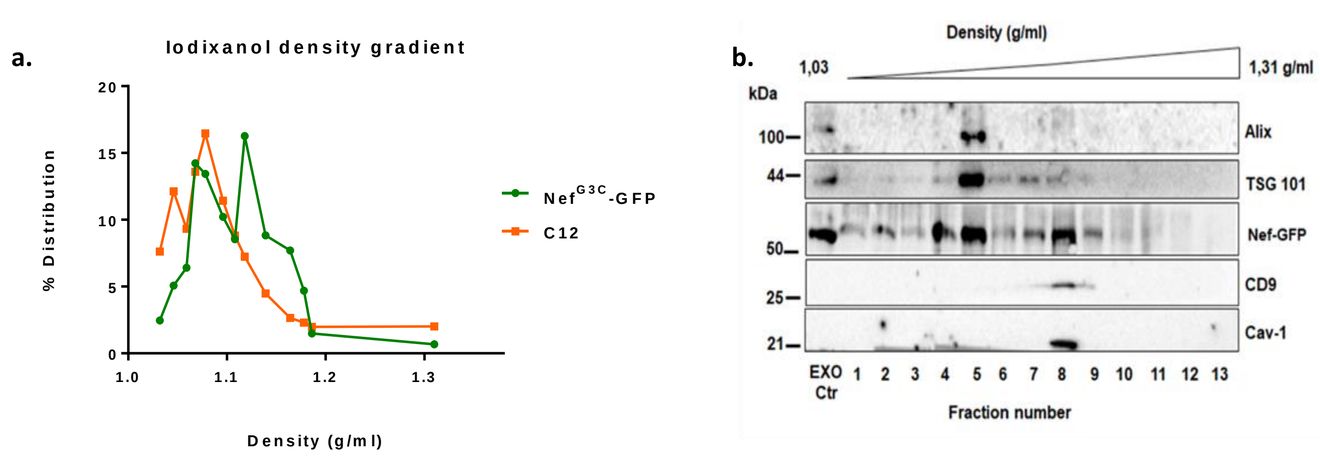

Figure 4. Iodixanol density gradient of sEV labelled with Nef^{G3C}-GFP and C12: a) sEV pellets purified by Hek293T after o/n transfection with the DNA vector and subsequently pulsed for 5 hrs with 7uM C12 and 24 h of chase were loaded from below in an iodixanol gradient and ultracentrifuged for 16 hrs. Collected fractions were analyzed on a flow cytometer to quantify the Nef^{G3C}-GFP and C12 sEV. Here it is possible to observe that sEV containing Nef^{G3C}-GFP protein are distributed in two distinct peaks while C12-labelled sEV show only one peak. The first peak, common to the two fluorophores, has a density value of 1.068-1.078 g / ml, concordant with the density of exo described in literature; b) sEV fractions obtained by iodixanol gradient (5% -35%) were analyzed by Western Blot. As a control there is a sample of sEV not subjected to gradient, normalized by the flow cytometer count for the Nef^{G3C}-GFP protein, with a number equal to the number of sEV counted at the first peak.

Sorting of Nef^{G3C}-GFP and C12 sEV

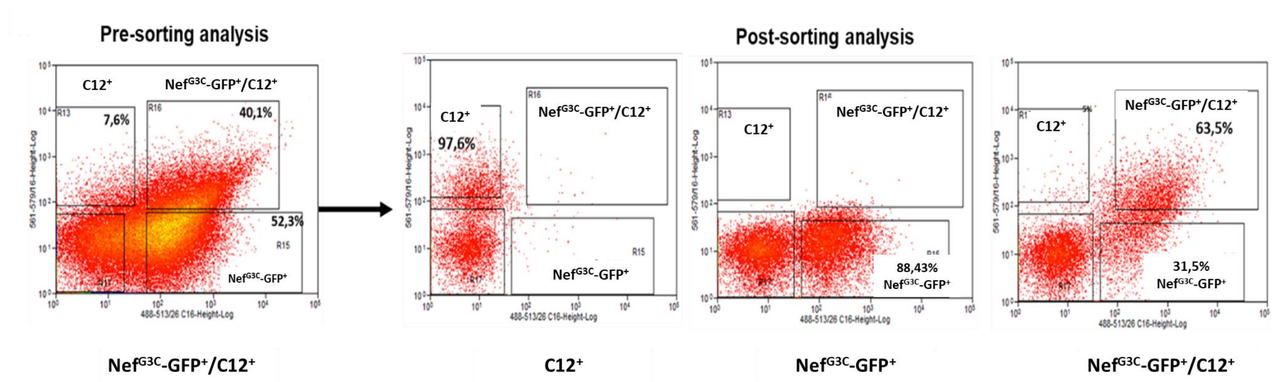

Figure 5. FACS sorting of sEV labelled with Nef^{G3C}-GFP and C12: sEV were sorted by FACS sorter based on fluorescence, respectively green in the case of Nef^{G3C}-GFP (485 nm of excitation and 528 nm of emission) and red in the case of C12 (558 nm of excitation and 568 nm of emission). Starting from a sample of double labelled sEV, it is possible to observe three populations, one positive for both fluorescent molecules and two single positive ones. After the separation it is possible to detect that sEV populations presenting only the lipid probe or the fluorescent protein have been separated correctly, while double labelled sEV are less efficiently separated and present a contamination by single labelled Nef^{G3C}-GFP sEV (31,5% of the total sorted sEV) since it is difficult to draw a well separated gate as showed in the pre-sorting analyses.

Comparison of sEV Nef^{G3C}-GFP and Nef^{mut}-GFP secretion and distribution

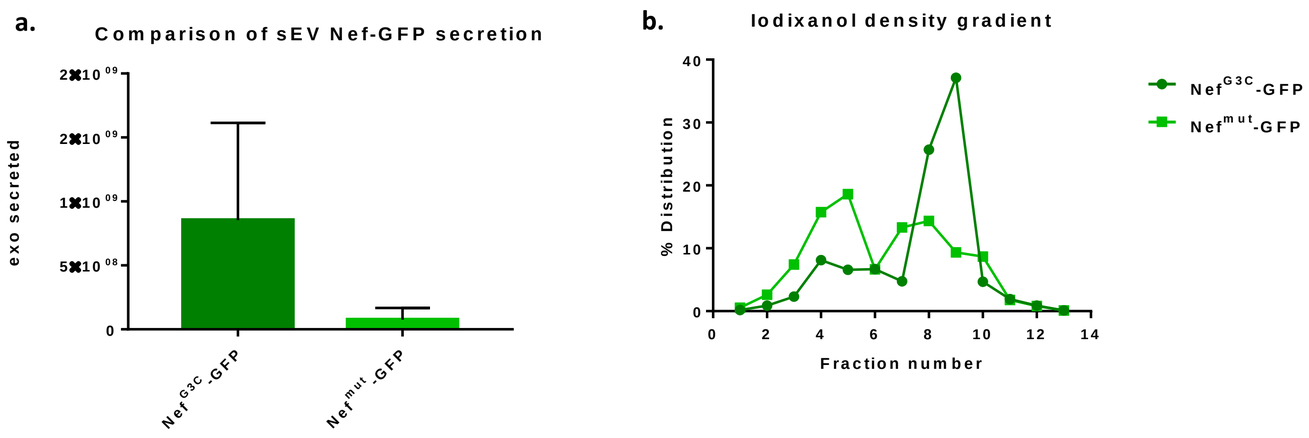

Figure 6. Comparison of sEV Nef^{G3C}-GFP and Nef^{mut}-GFP: a) Hek293T cells have been transfected o/n with both mutant of HIV-1 Nef protein, Nef^{G3C} and Nef^{mut}. The number of sEV have been quantified by flow cytometry. It is possible to observe that there is a consistent difference in terms of fluorescent sEV secreted; b) Nef^{G3C} and Nef^{mut} sEV have been separated on a iodixanol density gradient (5% -35%) and each fraction have been analyzed by flow cytometry. As Nef^{G3C}, also Nef^{mut} sEV are distributed into two peaks, but most of them present an higher density. From these results it is possible to observe that Nef mutants are presents in different populations of sEV.

CONCLUSIONS

Our results suggest that, thanks to this innovative methodology, we are able to obtain sEV that present two fluoresences, one derived from the fatty acid C12 and one derived from the Nef-GFP fusion protein. Confocal images showed that Nef^{G3C}-GFP is distributed in the whole cell, while the lipid C12 is present in specific cellular compartment, in particular at the perinuclear level. Colocalization of the two probes was also observed. Fluorescent sEV have been quantified by Flow Cytometry and NTA and analyzed for the presence of principal sEV markers by Western blot. Density gradient of sEV derived from cells transfected with Nef^{G3C}-GFP and pulsed with C12 showed that there are two types of sEV population at lower and higher density and this was confirmed by Western Blot analysis.

The comparison of Nef^{G3C}-GFP and Nef^{mut}-GFP vesicles showed that Nef^{G3C} induced a major secretion of vesicles respect to Nef^{mut}.

Further analysis will show if these two different populations of sEV display different behaviour in terms of efficiency of transfer to recipient cells and ultimately stimulation of the immune system.