Study of sEV internalization by antigen-presenting cells

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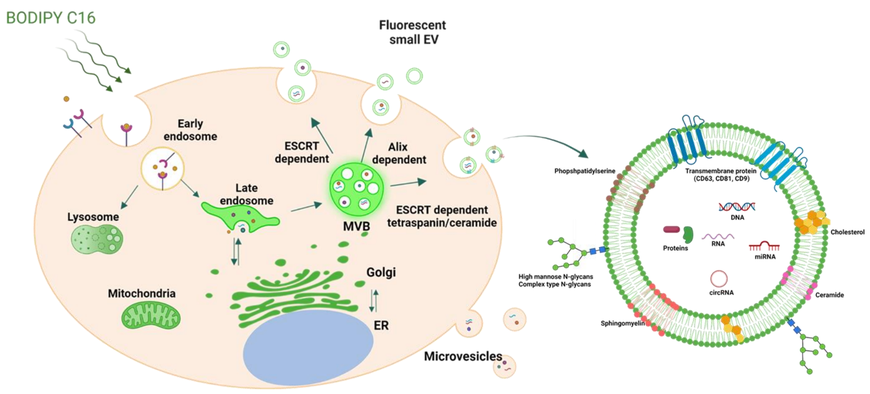
INTRODUCTION

Small extracellular vesicles (sEV) are gaining recognition as important mediators of intercellular communication, playing a crucial role in modulating immune responses. Both normal and tumor cells release sEV, but it is unclear whether they are selectively or non-selectively taken up by recipient cells. Recent studies have shown that sEV of endosomal origin (exosomes) are coated with high mannose glycans on their surface. The glycosylation profiles of both cells and exosomes can be altered in hypoxic conditions, such as those found in the tumor microenvironment. Tumor cells change their metabolism from oxidative phosphorylation to glycolysis, leading to changes in glycosylation patterns. The aim of this study was to investigate the mechanisms of sEV internalization, secreted under normoxic or hypoxic condition, by antigen-presenting cells, specifically immature dendritic cells (iDCs) expressing the mannose receptor (MR) on their surface. We wanted to evaluate the specific sEV uptake mediated by the MR.

METHOD

We developed a methodology to obtain fluorescent exosomes (Bodipy sEV) of endosomal origin by using Bodipy FL C16 (C16), a fluorescent palmitic acid that upon internalization by cells, is converted into phospholipids that are incorporated into the bilayer of secreted vesicles. Bodipy sEV are isolated through differential ultracentrifugation and quantified by flow cytometry (FC). ***(Coscia, C., et al., 2016)***



RESULTS

Characterization of Bodipy sEV

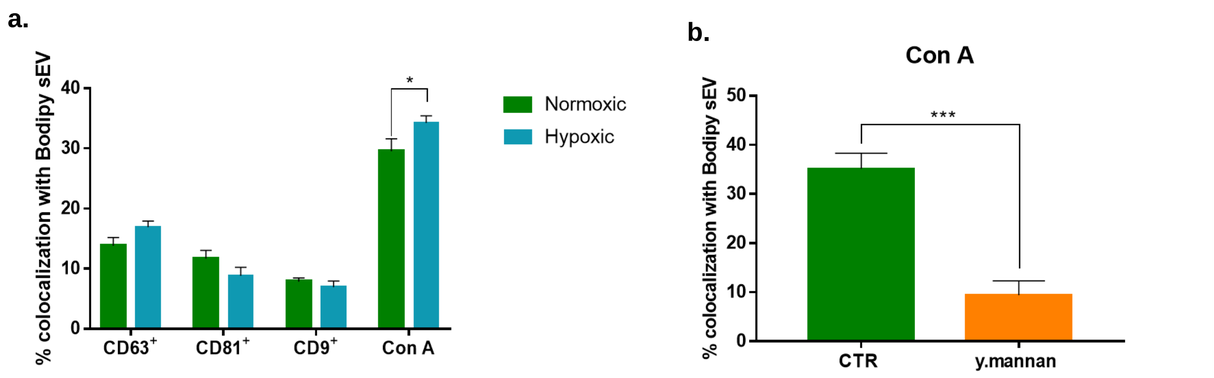


Fig.1 Analysis of protein and glycosylation profile. a) Bodipy normoxic and hypoxic sEV have been incubated with antibodies anti-CD63 BV421, Anti-CD81 APC, anti-CD9 PE and with Con A conjugated with Alexa Fluor 647. Protein analysis shows that hypoxic condition doesn't changes in a significant way the tetraspanins expression. Data are expressed as mean ± SEM (n=8). **b)** Hypoxic sEV present a higher glycosylation than normoxic sEV. Data are expressed as mean ± SEM (n=8).

Bodipy sEV internalization on immature Dendritic Cells

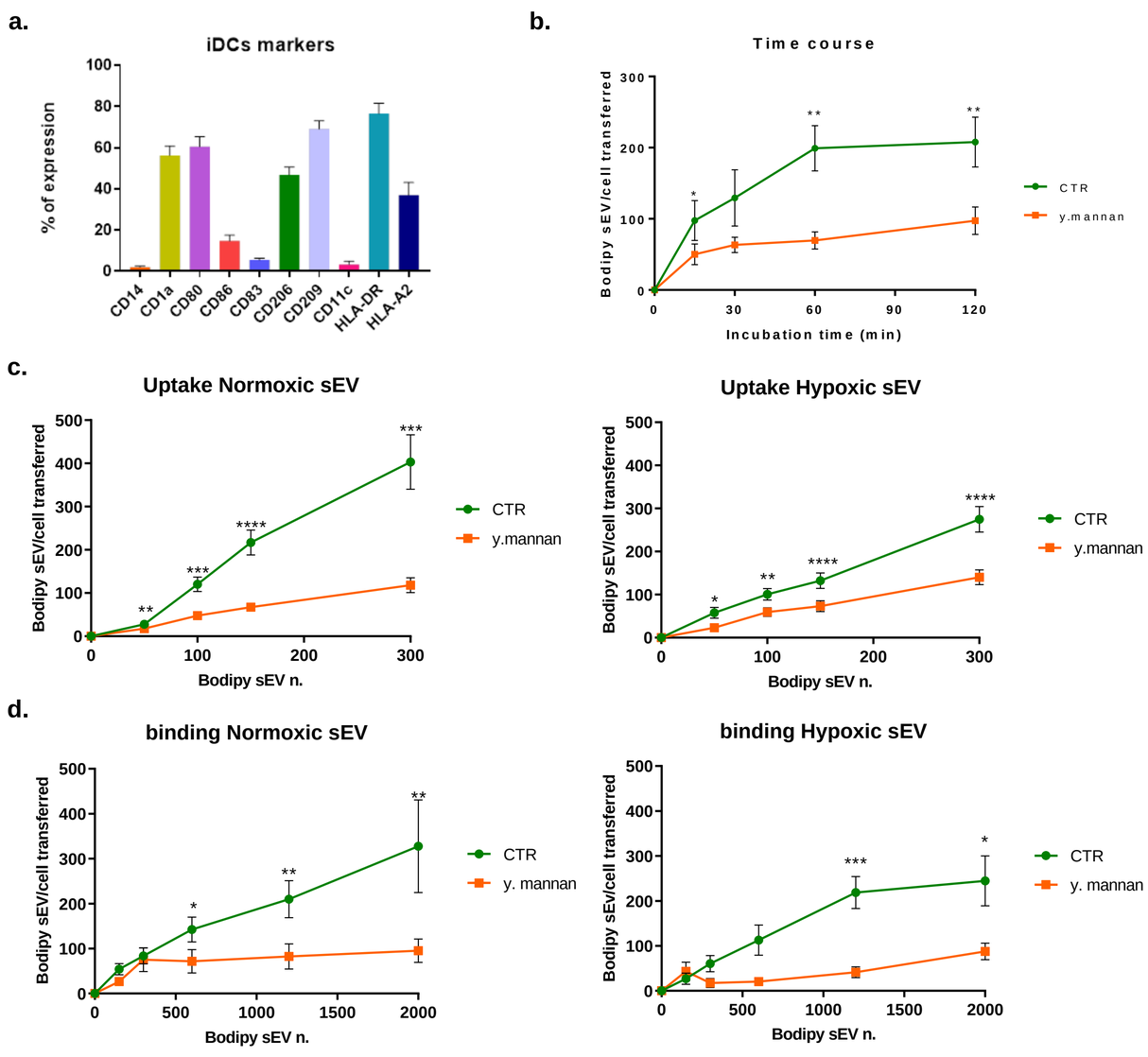


Fig.2 Time course of Bodipy sEV internalization by iDCs. a) After 7 days of incubation with IL-4 and GM-CSF, cells have been analyzed for the expression of principal iDCs markers by FC. Data are expressed as mean ± SEM (n≥10). **b)** A fixed number of iDCs have been incubated with Bodipy sEV for different time points at 37°C. To determine the specificity of uptake by MR/CD206 we used yeast mannan a competitor of MR uptake. Uptake was performed for 30 minutes at 37°C in Hank's Balanced Salt Solution. Cells were then analyzed by FC in the presence of the vital dye TO-PRO3 iodide. Data are expressed as mean ± SEM (n=7). **c)** iDCs have been incubated with normoxic or hypoxic Bodipy sEV +/- y.mannan for 30 minutes at 37°C. FC analysis showed that all sEV incubated with cells have been transferred and in presence of mannan there is an inhibition of 50% of the uptake. Data are expressed as mean ± SEM (n=40). **d)** Both normoxic and hypoxic sEV bind specifically to iDC for 1 hr at 4°C whereas Hypoxic sEV seem to bind more efficiently than normoxic sEV. Data are expressed as mean ± SEM (n=6).

Bodipy sEV internalization is Mannose Receptor dependent

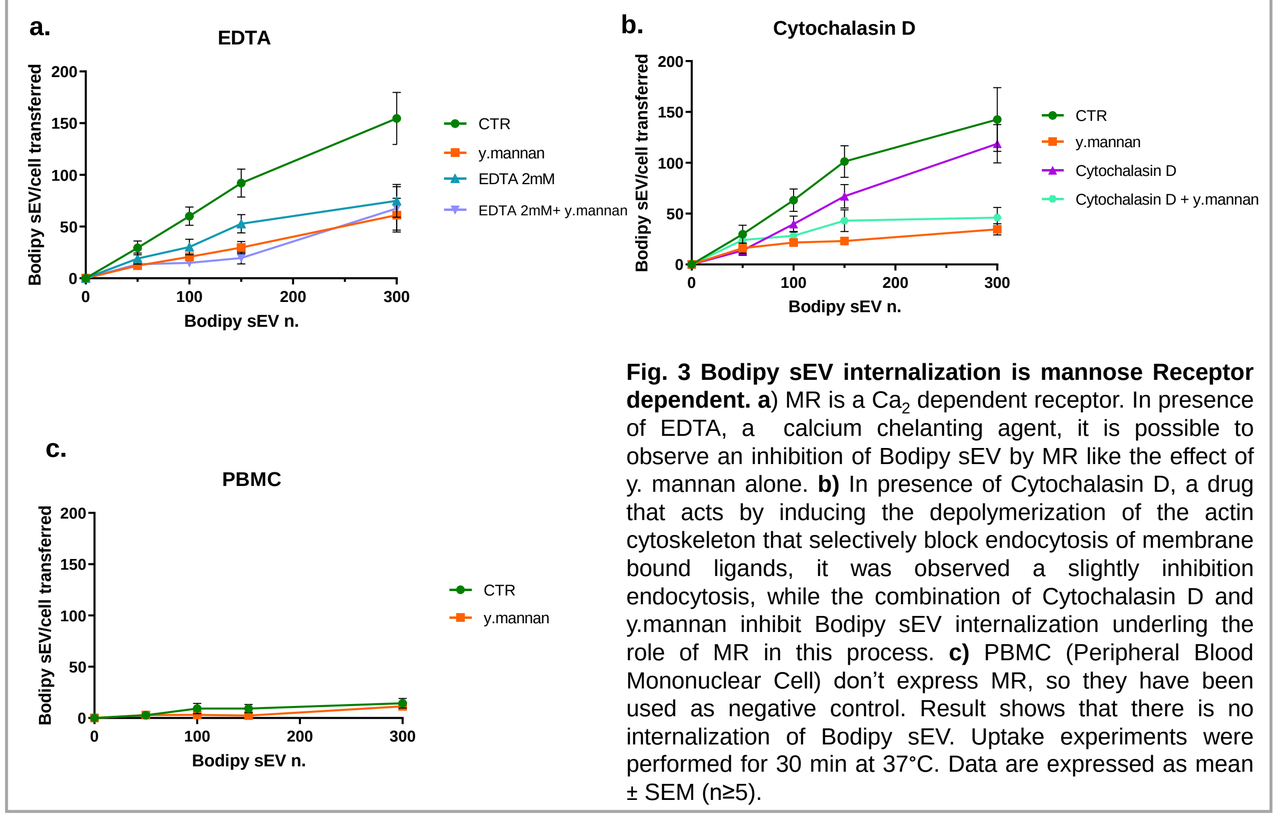


Fig. 3 Bodipy sEV internalization is mannose Receptor dependent. a) MR is a Ca_2 dependent receptor. In presence of EDTA, a calcium chelanting agent, it is possible to observe an inhibition of Bodipy sEV by MR like the effect of y. mannan alone. **b)** In presence of Cytochalasin D, a drug that acts by inducing the depolymerization of the actin cytoskeleton that selectively block endocytosis of membrane bound ligands, it was observed a slightly inhibition endocytosis, while the combination of Cytochalasin D and y.mannan inhibit Bodipy sEV internalization underling the role of MR in this process. **c)** PBMC (Peripheral Blood Mononuclear Cell) don't express MR, so they have been used as negative control. Result shows that there is no internalization of Bodipy sEV. Uptake experiments were performed for 30 min at 37°C. Data are expressed as mean ± SEM (n≥5).

Bodipy sEV induce mature dendritic cells differentiation

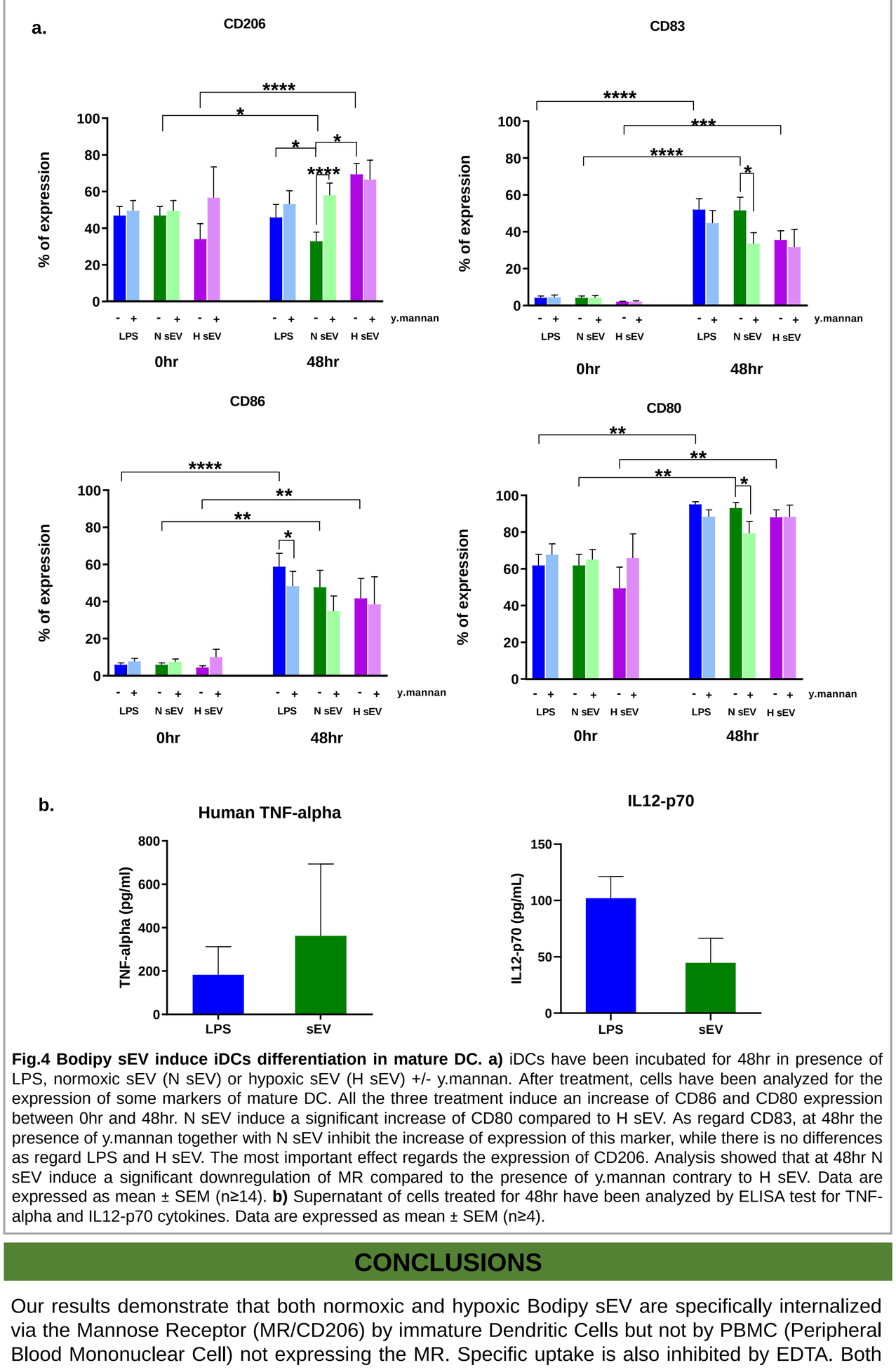


Fig.4 Bodipy sEV induce iDCs differentiation in mature DC. a) iDCs have been incubated for 48hr in presence of LPS, normoxic sEV (N sEV) or hypoxic sEV (H sEV) +/- y.mannan. After treatment, cells have been analyzed for the expression of some markers of mature DC. All the three treatment induce an increase of CD86 and CD80 expression between 0hr and 48hr. N sEV induce a significant increase of CD80 compared to H sEV. As regard CD83, at 48hr the presence of y.mannan together with N sEV inhibit the increase of expression of this marker, while there is no differences as regard LPS and H sEV. The most important effect regards the expression of CD206. Analysis showed that at 48hr N sEV induce a significant downregulation of MR compared to the presence of y.mannan contrary to H sEV. Data are expressed as mean ± SEM (n≥14). **b)** Supernatant of cells treated for 48hr have been analyzed by ELISA test for TNF-alpha and IL12-p70 cytokines. Data are expressed as mean ± SEM (n≥4).

CONCLUSIONS

Our results demonstrate that both normoxic and hypoxic Bodipy sEV are specifically internalized via the Mannose Receptor (MR/CD206) by immature Dendritic Cells but not by PBMC (Peripheral Blood Mononuclear Cell) not expressing the MR. Specific uptake is also inhibited by EDTA. Both normoxic and hypoxic sEV are as effective as LPS in inducing iDCs maturation as evidenced by the increased expression of CD86, CD80 and CD83. On the contrary MR/CD206 seems to be downregulated only by normoxic sEV when internalized by the MR route of uptake. Results show that also the expression of CD83 and CD80, but not CD86, seem to be affected by the MR route of normoxic sEV uptake in that y.mannan interferes with their expression. Preliminary data based on the release of TNF-alpha and IL12-p70 in the supernatant from cells treated with LPS or normoxic sEV would seem to suggest that sEV could have an important role in the immune modulation response. Future experiments will focus on cytokines secretion profiles of sEV treated iDC and miRNA profiles of normoxic and hypoxic sEV.