The aim of this study was to develop a dendritic cell (DC)–targeted peptide vaccine against melanoma using ligand-modified polymeric nanoparticles (NPs) as carriers for melanoma antigens along with potent immunoadjuvant oligonucleotides (Toll-like receptor ligands – TLRl). The nanoparticulate vaccine demonstrated successful targeting of DCs with high internalization levels through actin-dependent endocytic mechanisms and caused DC maturation.

**EXPERIMENTAL METHODS**

NPs were prepared by the double emulsion-solvent evaporation method, as described before, and their physico-chemical properties were fully characterized.

In vitro tests were performed in two murine cell lines of APCs (immature DC JAWSII and macrophage-like J744.1). BMDCs were also used and obtained by culturing C57Bl/6 mice bone marrow cells in the presence of GM-CSF for 8 days. Internalization of NPs was analyzed by flow cytometry (FC; BD Fortessa) and confocal microscopy (CM; Leica TCS SP5), and characterized through specific inhibitors of the endocytic pathways. Cellular viability studies were performed using both MTT test and AlamarBlue assay.

Dynamic Light Scattering and CM were used to demonstrate the presence of mannose residues at manNP surface through the induction of aggregation by Concanavalin A (Con A)-FITC.

BMDCs were incubated with NPs with different combinations of payloads (OVA, CpG and Poly I:C) in order to evaluate the induction of BMDC activation and maturation through the upregulation of maturation markers (CD40, CD80 and CD86).

**RESULTS AND DISCUSSION**

Polymeric NPs were successfully produced in a size range between 153 – 179 nm with a very low polydispersity index (PdI), despite the presence of antigenic peptides and oligonucleotides. At pH 7.4, NPs presented a slightly negative zeta potential (~ -3 mV) that reverted to neutrality at pH 5.0, which indicates potential to escape from lysosomes and avoid lysosomal degradation. In vitro, NPs showed no significative cytotoxicity in two cell lines of APCs and a primary DC culture (BMDC).

Internalization levels measured by FC and CM demonstrated different patterns of internalization by the two types of cell culture. Both APCs lines and BMDCs internalized NPs in a concentration-dependent manner, also confirmed by CM (Fig. 1A).

The % of positive BMDCs was constant regardless the time of incubation, while for APC lines this value increased with time, only reaching
comparative levels to BMDC internalization 42h after incubation. Moreover, NP internalization by cell lines increased significantly with the functionalization of NPs with mannose, which was not surprisingly observed for BMDCs (Fig. 2).

Figure 3. Man-NPs size distribution in the presence or absence of Con A-FITC. The confocal microscopy image shows the aggregation of man-NPs in the presence of Con A-FITC. Mean±SD; n = 3.

The presence of TLRI demonstrated to be essential in the induction of BMDC maturation by OVA-loaded NPs. Fig. 4 shows a synergistic effect between two different TLRI. Similar results were obtained with CD80.

Figure 4. Upregulation of CD86 by BMDCs after 24h incubation with NPs containing different payloads. Mean±SD, n=3.

CONCLUSION
This study demonstrates promising results for a DC–targeted multifunctional nanoparticulate vaccine against melanoma. This vaccine successfully targeted DCs with high internalization levels through actin- and clathrin-dependent endocytic pathways and caused DC maturation. Intracellular trafficking studies and in vivo studies are currently being performed either to characterize the elicited immune response (C57Bl/6 OT II wt mice), either to evaluate its therapeutic and prophylactic potential on a murine melanoma model.

REFERENCES

ACKNOWLEDGMENTS
This work was supported by Fundação para a Ciência e a Tecnologia (FCT) – Portugal (PTDC/SAU-FAR/119389/2010 and SFRH/BD/64295/2009), and by Fonds de la Recherche Scientifique Médicale - Belgium.