UNIVERSIDADE DE LISBOA

Faculdade de Farmácia


# MULTICOMPONENT PARTICULATES FOR IMMUNE MODULATION

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Orientadores: Prof. Doutora Helena Isabel Fialho Florindo Roque Ferreira
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Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia especialidade em Tecnologia Farmacêutica

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## Abstract

Although immune checkpoint therapy improved the clinical outcomes of melanoma treatment, low response rate, severe side effects, and acquired resistance have been observed in patients. To overcome these limitations, complementary strategies that inhibit tumor immunosuppressive pathways and enhance immunity are urgently needed. We hypothesized that the anticancer activity of the combination of anti-PD-1, for immunosuppression blockade, with the monoclonal anti-OX40 agonist, for T-cell stimulation and expansion, could be improved by cancer vaccination. For this purpose, we designed, synthesized and characterized mannose-grafted poly(lactic-co-glycolic acid) and poly(lactic acid) (PLGA/PLA) nano-vaccines, containing both melan-A/MART-1 peptides and immune potentiators. These nanoparticle-based vaccines can amplify antitumor immune response by increasing tumor-associated antigen recognition, processing, and presentation to effector T cells. The nanoparticles were shown to have a spherical shape with an average diameter of 170 nm, displaying narrow polydispersity index and near-neutral surface charge. Immunization with these nano-vaccines induced splenocyte activation and antigen-specific cytotoxic T-cell activity against melanoma cells *ex vivo*.

Treatment with the combination of the prophylactic nano-vaccines and PD-1/OX40 antibodies *in vivo* led to maximal tumor growth inhibition, with minimal systemic toxicity. Whereas treatment with anti-PD-1/anti-OX40 alone led to 20% survival 42 days following tumor inoculation, the combination approach with the mannose-PLGA/PLA prophylactic nano-vaccine resulted in 100% survival for the same period. Moreover, the latter presented a survival rate of 50% two months following tumor inoculation and recapitulated a T-cell inflamed tumor microenvironment.

On the other hand, the combination of therapeutic mannose-PLGA/PLA nano-vaccines with PD-1/OX40 immune checkpoint therapy failed to show benefit in comparison with PD-1/OX40 immune checkpoint in monotherapy. This result seems to be related to infiltration of MDSC into the tumor microenvironment, over time, which may have overcome the effect of $CD8^+$ T cell stimulation, inhibiting T cell infiltration and cytotoxic activity.

Altogether, these findings reveal important aspects on the synergism of immune checkpoint targeting with polymeric cancer nano-vaccines, as well as the effect on the

modulation of tumor-infiltrating immune cells in melanoma. Therefore, polymeric nano-vaccines emerge as a potential strategy to improve clinical outcomes of melanoma treatment.

**Keywords:** vaccine, melanoma, immune checkpoint, anti-PD-1 & anti-OX40, nanomedicine.

## Resumo

A incidência e mortalidade do melanoma têm vindo a aumentar nas últimas décadas. De acordo com a Organização Mundial de Saúde (OMS), ocorrem mundialmente, a cada ano, mais de 50 000 mortes devido a melanoma. Embora as opções terapêuticas para o tratamento do melanoma em estadio avançado ou metastático sejam limitadas e com prognóstico reduzido, os progressos recentes na área da imunoterapia trouxeram melhorias nos resultados terapêuticos. Estas terapêuticas inovadoras contra o cancro, baseiam-se em respostas anti-tumorais desencadeadas pelo próprio sistema imunitário do doente, através da ativação de uma complexa rede de células imunitárias, tais como os linfócitos T efectores $CD8^+$, células *natural killer* (NK) e linfócitos T auxiliares $CD4^+$. Todavia, um microambiente tumoral tolerante, com prevalência de mecanismos imunossupressores, tais como o aumento da expressão dos recetores proteína 4 associada aos linfócitos T citotóxicos (CTLA-4) e morte celular-programada 1 (PD-1), tem a capacidade de restringir as respostas imunitárias anti-tumorais. O desenvolvimento de anticorpos capazes de bloquear estes recetores, conhecidos como inibidores dos *immune checkpoints*, permitiu um aumento da resposta imunitária anti-tumoral mediada por linfócitos T específicos. A sua utilização na clínica tem revelado resultados extremamente promissores, o que motivou uma extensa investigação focada na descoberta de novos *immune checkpoints* e de novos agentes com a capacidade para modelar a actividade destes. O anticorpo monoclonal anti-OX40, por exemplo, é um agente terapêutico com acção sobre o receptor OX40 (CD134 ou TNFRS4), que é um receptor co-estimulatório da família dos receptores do fator de necrose tumoral (TNF), que tem a capacidade de ativar o sistema imunitário, através do aumento de citocinas pró-inflamatórias e indutoras da proliferação, sobrevivência e tráfego de células T efectoras. Além disso, possui também uma acção inibitória sobre a infiltração de células T $CD4^+$ reguladoras, ao bloquear a sinalização celular de FoxP3, TGF-β e IL-10.

Apesar dos resultados promissores, os moduladores dos *immune checkpoints*, em particular quando usados em regimes de monoterapia, têm sido associados a efeitos adversos graves e a baixas taxas de respostas prolongadas e perduráveis em doentes que apresentam a forma disseminada desta patologia. No caso da monoterapia com o anticorpo monoclonal anti-PD-1, por exemplo, foram obtidas taxas

de respostas, em clínica, entre os 30-40%, em doentes com melanoma em estadio avançado. No que toca aos resultados de ensaios clínicos com o agonista anti-OX40, não obstante os resultados iniciais promissores em tumores primários tradicionalmente tidos como muito imunogénicos, não se conseguiu verificar indução de respostas imunitárias anti-tumorais noutros tumores com menor imunogenicidade. Neste sentido, têm surgido diversas propostas de combinações terapêuticas que envolvem a quimioterapia, radioterapia ou combinação de outros agentes moduladores dos *immune checkpoints* ou estratégias imunitárias. Por exemplo, a utilização de anticorpos estimuladores do sistema imunitário, como é o caso do anti-OX40, juntamente com vacinação anti-tumoral, consiste numa abordagem particularmente promissora, ao amplificar respostas imunitárias mediadas por linfócitos T contra antigénios libertados após a morte imunogénica das células tumorais. Por conseguinte, tendo em conta que a combinação do bloqueio de PD-1 com a estimulação do recetor OX40 demonstrou atividade sinérgica num modelo pré-clínico de cancro dos ovários, este trabalho antecipou que esta combinação poderia não só ser relevante para o tratamento de melanoma, como também poderia ser potenciada por vacinas anti-tumorais que permitissem a entrega de antigénios tumorais a células dendríticas. Com esse objetivo, foram desenvolvidas nano-vacinas anti-tumorais baseadas em nanopartículas biodegradáveis funcionalizadas com manose (man-NP), constituídas por uma matriz polimérica de ácido poliláctico-co-glicólico (PLGA) e ácido polilático (PLA), de forma a promover a interação das partículas com as células dendríticas, através do receptor da manose (MR/CD206). Estas nano-vacinas continham ligandos dos receptores *Toll-like* (TLR), designadamente o CpG ODN 1826 (TCCATGACGTTCCTGACGTT) (CpG) e o monofosforil lípido A (MPLA), com a função de potenciar a resposta imunitária, juntamente com os antigénios peptídicos melan-A/MART-1, associados ao melanoma. As nano-vacinas apresentaram uma forma esférica, com um diâmetro hidrodinâmico médio de 170 nm, um índice de polidispersão estreito e carga à superfície da nanopartícula próxima da neutralidade. A imunização de animais com a nano-vacina induziu a activação dos esplenócitos e potenciou a actividade dos linfócitos T especificamente contra células de melanoma *ex vivo* que expressam estes antigénios associados ao melanoma.

Os estudos pré-clínicos em ratinhos imunizados com a nano-vacinas e subsequentemente tratados com os anticorpos anti-PD-1 e anti-OX40 posteriormente à indução do tumor, resultou numa inibição máxima do crescimento tumoral, com toxicidade sistémica residual. Enquanto o tratamento apenas com os anticorpos anti-PD-1/anti-OX40 originou uma sobrevivência de 20% 42 dias após a inoculação do tumor, a estratégia de combinação destes anticorpos com as nano-vacinas manose-PLGA/PLA profiláticas resultou numa sobrevivência de 100% para o mesmo período. Inclusivamente, esta mesma estratégia permitiu uma sobrevivência de 50% dois meses após a inoculação do tumor, com restabelecimento da infiltração de células T em tumores em estadio não-inflamatório.

Por outro lado, ratinhos com melanoma tratados com a combinação de nano-vacinas manose-PLGA/PLA com os *immune checkpoints* PD-1/OX40, em esquema terapêutico, não apresentaram uma resposta anti-tumoral superior à induzida pelo tratamento com anti-PD-1/anti-OX40. O resultado parece relacionar-se com a infiltração tumoral de células supressoras da linhagem mielóide, induzida pelas nano-vacinas manose-PLGA/PLA. A infiltração destas células, com o tempo, obviou o efeito da estimulação das células T $CD8^+$, inibindo a sua infiltração tumoral e actividade citotóxica.

Em suma, nesta tese é reportado o desenvolvimento de nanoparticulas poliméricas biodegradáveis como nano-vacinas contra o cancro e a sua combinação com moduladores dos *immune checkpoints*, que já são atualmente utilizados na clínica, para ultrapassar as limitações apresentadas na clínica aquando da utilização destes agentes imunoterapêuticos. A utilização de nanopartículas permite a entrega de antigénios tumorais e potenciadores da resposta imunitária directamente às células apresentadoras de antigénios, o que potenciará a indução de uma resposta imunitária anti-tumoral. Embora esta resposta seja restringida, em último caso, por mecanismos de imunossupressão desenvolvidos pelo próprio tumor, demonstrou-se que a combinação das nano-vacinas com a terapia de *immune checkpoints* permite uma potenciação da resposta imunitária anti-tumoral, com aumento da sobrevivência e inibição do crescimento tumoral. Além disso, é revelado o papel determinante das células supressoras da linhagem mielóide no comprometimento da eficácia da imunoterapia. Este resultado constitui um importante avanço nesta área, abrindo

novos caminhos para o desenvolvimento de abordagens terapêuticas alternativas, através da modulação destas células que, até então, têm impedido a obtenção de melhores resultados no imunoterapia do cancro, particularmente na resposta a vacinas anti-tumorais. As nanopartículas, com papel de nano-vacinas, serão certamente fundamentais na elaboração de estratégias inovadoras que permitirão o avanço do tratamento do cancro.

**Palavras-chave**

Nanopartículas, vacinas para cancro, imunoterapia, PD-1, OX40, células apresentadoras de antigénios, melanoma.

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**Obrigado!**

## List of abbreviations

| | |
|---|---|
| **α-GalCer** | Alpha-galactosylceramide |
| **α-PD-1** | Monoclonal antibody against programmed death-1 receptor |
| **α-OX40** | Monoclonal antibody against OX40 receptor |
| **ADCC** | Antibody-dependent cell-mediated cytotoxicity |
| **AFM** | Atomic force microscopy |
| **APC** | Antigen-presenting cells |
| **BCG** | Bacillus Calmette-Guérin |
| **BSA** | Bovine serum albumin |
| **CAR** | Chimeric antigen receptor |
| **CCL** | Chemokine (C-C motif) ligand |
| **CCR** | Chemokine (C-C motif) receptor |
| **CD** | Cluster of differentiation |
| **CDC** | Complement-dependent cytotoxicity |
| **CLR** | C-type lectin receptors |
| **Conc A** | Concanavalin A |
| **CpG** | Cytosine phosphorothioate-guanine motifs |
| **CTL** | Cytotoxic T lymphocytes |
| **CTLA-4** | Cytotoxic T Lymphocyte-associated antigen-4 |
| **CXCL** | Chemokine (C-X-C motif) ligand |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DCM** | Dichloromethane |
| **DC** | Dendritic cells |
| **DCC** | *N,N'*-Dicyclohexylcarbodiimide |
| **DLS** | Dynamic light scattering |
| **DMAP** | Dimethylaminopyridine |
| **DMSO** | Dimethylsulfoxide |
| **DMF** | Dimethylformamide |
| **DNA** | Deoxyribonucleic acid |
| **EE** | Entrapment efficiency |
| **EMA** | European Medicines Agency |
| **EPR** | Enhanced permeability and retention |
| **ER** | Endoplasmic reticulum |

| | |
|---|---|
| **FACS** | Fluorescence-activated cell sorting |
| **FasL** | Fas ligand |
| **FBS** | Fetal bovine serum |
| **$F_c$** | Fragment crystallizable |
| **FDA** | Food and Drug Administration |
| **FITC** | Fluorescein isothiocyanate |
| **GITR** | Glucocorticoid-induced tumor necrosis factor receptor |
| **GM-CSF** | Granulocyte macrophage colony-stimulating factor |
| **H&E** | Haematoxylin and eosin |
| **Her2** | Human Epidermal growth factor Receptor |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **i.d.** | Intradermal |
| **i.l.** | intralymphatic |
| **i.m** | Intramuscular |
| **i.t.** | intratumoral |
| **i.v.** | intravenous |
| **ICH** | International Conference on Harmonization |
| **IFN** | Interferon |
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |
| **intDC** | Interstitial DC |
| **LC** | Langerhans cells or Loading capacity |
| **LDV** | Laser Doppler Velocimetry |
| **LN** | Lymph nodes |
| **LAG** | Lymphocyte-activation gene |
| **LPS** | Lipopolysaccharide |
| **MAA** | Marketing Authorization Applications |
| **mAb** | Monoclonal antibodies |
| **MALT** | Mucosal-associated lymphoid tissue |
| **man-PLGA** | Mannose-grafted poly(lactic-co-glycolic) acid |
| **man-NP** | Mannose-grafted poly(lactic-co-glycolic acid)/poly(lactic acid) nanoparticles |
| **mDC** | Mature dendritic cells |
| **MAPK** | Mitogen-activated protein kinase |
| **MCP** | Monocyte chemoattractant protein |

| | |
|---|---|
| **MDSC** | Myeloid-derived suppressor cells |
| **MEK** | Mitogen-activated protein kinase kinase |
| **MHC** | Major histocompatibility complex |
| **MHCI-ag** | melan-A/MART-1 (26-35(A27L) MHCI-restricted antigen |
| **MHCII-ag** | melan-A/MART-1 (51-73) MHCII-restricted antigen |
| **MIP** | Macrophage inflammatory protein |
| **miRNA** | microRNA |
| **MMM** | Metastatic malignant melanoma |
| **MPLA** | Monophosphoryl lipid A |
| **MR** | Mannose receptor |
| **MUC1** | Human milk mucin |
| **NF-κB** | Nuclear factor-κB |
| **NK** | Natural killer |
| **NKT** | Natural killer T |
| **NHS** | *N*-hydroxysuccinimide |
| **NOD** | Nucleotide oligomerization domain |
| **NLR** | NOD-like receptors |
| **$^{1}$H NMR** | Proton Nuclear Magnetic Resonance |
| **NP** | Nanoparticles or PLA/PLGA NP |
| **ODN** | Oligodeoxynucleotides |
| **PALS** | Phase Analysis Light Scattering |
| **PAMP** | Pathogen-associated molecular patterns |
| **PAT** | Process analytical technologies |
| **PBS** | Phosphate-buffered saline |
| **PCL** | Polycaprolactone |
| **PD-1** | Programmed death-1 |
| **pDC** | Plasmacytoid dendritic cells |
| **PdI** | Polydispersity Index |
| **PEG** | Poly(ethylene glycol) |
| **PEST** | Penicillin - streptomycin |
| **PFA** | Paraformaldehyde |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PLA** | Polylactide/polylactic acid |
| **PLGA** | Poly(lactic-co-glycolide) or poly(lactic-co-glycolic) acid |
| **Poly(I:C)** | Polyinosinic:polycytidylic acid |

| | |
|---|---|
| **PRR** | Pattern recognition receptors |
| **PVA** | Polyvinyl alcohol |
| **R&D** | Research and development |
| **RBC** | Red blood cells |
| **RFU** | Relative Fluorescence Units |
| **RIG** | Retinoic-acid inducible gene |
| **RLR** | Retinoic-acid inducible gene-like receptors |
| **ROS** | Reactive oxygen species |
| **RT** | Room temperature |
| **s.c.** | Subcutaneous |
| **SD** | Standard deviation |
| **SDS** | Sodium dodecyl sulfate |
| **SEM** | Scan Electron Microscopy or Standard error of the mean |
| **siRNA** | Small interfering RNA |
| **T reg** | T regulatory cells |
| **TAA** | Tumor-associated antigens |
| **TACA** | Tumor associated carbohydrate antigens |
| **TAM** | Tumor-associated macrophages |
| **TAP** | Transporter-associated with antigen processing |
| **TCR** | T cell receptor |
| **$T_c$** | T cytotoxic |
| **TEM** | Transmission electron microscopy |
| **TGF** | Transforming growth factor |
| **TIL** | Tumor-infiltrating lymphocytes |
| **Tim-3** | T cell immunoglobulin and mucin domain |
| **TLR** | Toll-like receptors |
| **Th** | T helper |
| **TNF** | Tumor necrosis factor |
| **TPGS** | D-α-tocopheryl poly(ethylene glycol) succinate |
| **UV** | Ultraviolet |
| **VEGF** | Vascular endothelial growth factor |
| **VLP** | Virus-like particles |
| **WGA** | Wheat germ agglutinin |

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### Chapter I

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## Chapter II



## Chapter II

## Aims and outline of the thesis

The main goal of the research described in this thesis was to evaluate whether the combination of PD-1 blockade and OX40 stimulation could be potentiated by dendritic-cell targeted cancer vaccines delivering tumor-associated antigens.

For this purpose, several questions were addressed:

1. Can biodegradable polymeric nanoparticles be produced with optimal physicochemical characteristics for antigen-presenting cell targeting, following the entrapment of multiple tumor-associated antigens and immune potentiators?
2. Can antigen-presenting cells extensively internalize these nanoparticles without affecting their viability?
3. Can these nanoparticles induce antigen-specific immune responses and therefore act as nano-vaccines?
4. What is the immune response profile induced by those nanoparticles under physiological conditions?
5. Can nanoparticles induce a cytotoxic antitumor immune response *in vivo*?
6. Can we induce a synergistic antitumor immune response by combining DC-targeted nanoparticles with PD-1 blockade and OX40 stimulation?
7. Which populations of tumor-infiltrated immune cells are driving the therapeutic response?

The results of the research are presented in chapter III, which is preceded by chapter I, which is a general introduction where several aspects of the field are addressed. Chapter II presents detailed information regarding materials and methods used to prepare nano-vaccine and the evaluation of their efficacy under both *in vitro* and *in vivo* studies. In chapter IV, the results are subject to discussion and in chapter V, the final one, concluding remarks and future perspectives are presented.

## Table of contents

# CHAPTER I

# Chapter I – General introduction

This chapter is adapted from the following published manuscripts:

Scientific publications in peer review international journals:

1. **Conniot J**, Silva JM, Fernandes JG, Silva LC, Gaspar R, Brocchini S, et al. Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking. Front Chem. 2014;2:105. doi: 10.3389/fchem.2014.00105.

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3. Peres C, Matos AI, **Conniot J**, Sainz V, Zupančič E, Silva JM, et al. Poly(lactic acid)-based particulate systems are promising tools for immune modulation. Acta Biomater. 2017;48(Supplement C):41-57; doi: 10.1016/j.actbio.2016.11.012.

4. Silva AL, Peres C, **Conniot J**, Matos AI, Moura L, Carreira B, et al. Nanoparticle impact on innate immune cell pattern-recognition receptors and inflammasomes activation. Seminars in Immunology. 2017. doi: 10.1016/j.smim.2017.09.003.

# Chapter I – General introduction

Cancer is one of the most common diseases afflicting people globally. It is a heterogeneous disease, resulting from a multi-step process, which is characterized by uncontrolled proliferation, invasion and metastasis. It has also been described that tumor cells have the ability to evade cell death (1) and to escape immune system surveillance (2).

Despite significant improvements in diagnosis and therapies, cancer is still the most fatal disease worldwide with 11.5 million deaths being predicted in 2030 (3). Current strategies for cancer treatment include chemotherapy, radiotherapy, immunotherapy and surgery (3). However, many of these approaches are unspecific and are characterized by severe side effects (4). In fact, it has been described that single treatment regimens have limited chances to eliminate cancer cells in a permanent manner due to its heterogeneous nature (5, 6). More effective and specific alternative treatments continue to be needed. For instance, in metastatic melanoma, prognosis is very poor, since current therapies have low levels of efficacy (7). It is believed that the success and the improvement of cancer therapy are dependent on the development of additional strategies to overcome severe side effects, drug resistance and circumvent tumor evasion mechanisms (8-12).

The progress in the development of nanodelivery systems in the last decades has shown the potential of these systems to overcome some of the drawbacks of current cancer therapies. These nano-based platforms can display improved pharmacokinetics and targeting of tissues and cells to enhance efficacy, specificity and lower toxicity (13). Accordingly, nanosystems have been designed to target tissues, cells or intracellular components, allowing development of more specific approaches for cancer treatment.

## 1. Cancer and the immune system

### 1.1. Innate and adaptive immunity to cancer

The immune system is composed of two main branches – innate and adaptive immune responses. The innate immunity is a non-specific first line

defense of our body against antigens. It comprises anatomic, physiologic, phagocytic, and inflammatory barriers, such as skin or macrophages and neutrophils. On the other hand, the adaptive immunity is a highly specific component of the immune system, which is stimulated by a specific antigen challenge to the organism. These two branches of the immune system are connected by phagocytic cells, players of the innate immunity, which have a pivotal role in specific immunity activation, namely dendritic cells (DC) (14, 15).

DC, along with macrophages and B lymphocytes, are described as antigen presenting cells (APC) (14-16). DC are the most powerful “professional” APC, controlling and regulating the immune system. They are present in the majority of mammalian tissues and they are organized in an intricate network throughout the human lymphatic and non-lymphatic tissues, having different functions, depending on their stage of maturation (17-19). Non-activated immature DC capture antigens and induce tolerance in the steady state, whereas mature antigen-loaded DC can prime an antigen-specific immune response. DC can also be categorized in three main subsets – i) Langerhans cells (LC); ii) interstitial DC (intDC) and iii) plasmocytoid DC (pDC). Though all subsets descend from the same precursor cells – CD34+ hematopoietic stem cells, present in the marrow – they are originated from two major distinct pathways (17). LC and intDC derive from the myeloid pathway, are CD11c+ and both produce interleukin-2 (IL-2). LC are found in stratified epithelia, such as skin and upper airways, whilst intDC may be present in all other tissues. Additionally, intDC can secrete IL-10 and elicit naïve B cell differentiation (16). The other parallel pathway originates phenotypically CD11c- pDC with the ability to produce high amounts of type I interferon (IFN) and to modulate T cell differentiation (16).

In tumor immunology, DC are crucial for tumor-associated antigen (TAA) presentation and for stimulating the immune system after DC activation (18). DC patrol the different tissues, processing exogenous and endogenous antigens that are then presented to T lymphocytes, after DC maturation. The maturation process of DC can be induced directly through ‘danger signals’ detected by pattern recognition receptors (PRR) or triggered by the presence of inflammatory mediators, such as TNF-α or IL-1β (19).

Antigen presentation to T lymphocytes by DC occurs through T-cell receptors (TCR) that recognize antigens associated to major histocompatibility complex (MHC) molecules. MHC proteins can be divided in two main groups: MHC class I – expressed on the membrane of the majority of nucleated cells in vertebrates – and MHC class II only present on APC's membrane (19, 20). After the contact of a naïve T cell with an MHC-antigen complex, T cells proliferate and differentiate in both memory T cells and effector T cells. Effector T cells may be categorized in T helper (Th – CD4+) or T cytotoxic ($T_c$ – CD8+) cells (16, 21). The stimulation of $T_c$ cells can lead to the generation of cytotoxic T lymphocytes (CTL) that secrete low levels of cytokines, unlike $T_c$ cells. CTL act as cell-killing agents, controlling and eliminating cells that exhibit any type of antigen, such as infected cells or tumor cells (16). Other innate lymphocytes subsets, such as γδ T cells, natural killer (NK) and natural killer T (NKT) cells, have been reported as being engaged in a complex immunomodulatory network, displaying antitumor activity. Preclinical studies described that NKT cells are involved in antitumor and immune-regulatory mechanisms (22).

The interaction between B cells, T cells and mature DC results in an integrated immune response. DC migration from the tumor site of antigen capture to secondary lymphoid organs extensively widens antigen-specific T-cell responses, promoting effective antitumor immune responses and regression (18).

## 1.2. Cancer immune regulation and evasion mechanisms

### 1.2.1. Cancer immunoediting

Since Paul Ehrlich proposed the immune system as a useful strategy against cancer, in the beginning of the 20$^{th}$ century, the immunosurveillance has been a central point for tumor immunology (23). Based on Ehrlich's studies, Thomas and Burnet suggested that cancer arousal was caused by a lack of efficiency in the immune system or a modification in antigen expression by tumor cells, leading to its evasion (24, 25). In the beginning of this century, the immunosurveillance hypothesis was revised, as several studies have shown that

the immune system may not only destroy tumor cells but also shape their phenotypes, leading to reduced immunogenicity (9, 26, 27) Currently, there is an increasing evidence that tumor cells can be recognized and destroyed by the immune system, as developing tumor cells often co-express tumor antigens and ligands for activating receptors (26). For that reason, it is important to describe which immune components display major roles in tumor rejection. It is also fundamental to clarify the appropriate time and efficient type of action (28).

Immunoediting theory is now used to describe the complex immune responses against tumors. Regarding the immune system and tumor progression, this hypothesis proposes the existence of three main phases:

In the first stage – “elimination” – both innate and adaptive immunities act combined to recognize tumor cells and to destroy them, resembling the immune surveillance theory. APC, such as DC, incorporate and process tumor-associated antigens (TAA). After maturation, APC finally migrate to draining lymph nodes (LN), where they present the MHC-TAA complexes to T cells (29). Once T cells recognize both TAA and additional co-stimulatory signals by APC, they become activated. Activated T cells secrete cytokines, such as IL-2 and IFN-γ, which will induce proliferation and differentiation of antigen-specific CTL. CTL are then able to target and destroy tumor cells (26, 30). If the “elimination” stage is well succeeded, tumor cells are destroyed, constituting an endpoint for the immunoediting process (26, 31).

The next stage – “equilibrium” – is described as a period of tumor latency. When tumor cells survive the “elimination” phase, the new derived tumor cells are continuously “edited” by the adaptive immunity. Tumor cell growth is thus controlled and immunogenicity is shaped. “Equilibrium” is believed to be the longest phase of the entire process. It seems to allow cancer cells to reside in patients’ body even decades before it restarts to grow and become clinically evident (26, 31).

The third phase – “escape” – occurs when tumor cells have developed the ability to evade the mechanisms of recognition of the immune system and/or their elimination. Tumor cells are thought to progress from “equilibrium” phase to “escape” through several mechanisms and/or pathways. For instance, an

alteration in immune system response or even immune system deterioration may be triggered by cancer-induced immunosuppression or a change in tumor cells induced by immunoediting (26, 31).

### 1.2.2. Cancer immune evasion mechanisms

Although it has been described that cellular immunity plays an important role in the tumor prevention, multiple findings revealed that tumors often manage to evade host immune system. Several mechanisms have been reported, such as the reduction or even loss of MHC class I molecules, mostly associated to gene mutations or impairment of MHC class I-dependent antigen processing (31-33). In addition, an antigenic drift in cancer cells has lately been observed and appears to be related with the mutation, loss or down-expression of TAA in tumor cells (33, 34). Similarly, the absence or reduction of the expression of co-stimulatory patterns by tumor cells drives T lymphocytes to a state of anergy. These mechanisms altogether seem to reduce and difficult the detection of cancer cells by CTL and NK cells, which consequently lead to tumor growth (33).

Alterations in apoptotic receptor signaling seem to help tumor cells to evade the immune system. The expression of molecules such as phosphatidylinositol 3-kinase (PI3K), protein kinase B and Fas ligand (FasL) is altered and might be implicated in this process (35, 36).

Tumor eradication is also under influence of the immunosuppressive properties of tumor microenvironment. Inducing and suppressing cytokine imbalance impairs DC activation and maturation, compromising immune cell effector properties and supporting tumor growth. Tumor cells can indeed secrete immunosuppressive molecules, including vascular endothelial growth factor (VEGF), IL-10 and transforming growth factor-β (TGF-β) (37-39). VEGF appears to be responsible for down-regulation NF-κB expression, which interferes in DC maturation and differentiation, limiting the immune response against tumor cells (39). While IL-10 inhibits the process of antigen presentation and expansion of antigen-specific T cells, TGF-β inhibits T-cell activation and proliferation directly (40-42).

Moreover, high levels of indoleamine-2,3-dioxygenase (IDO) have also been found in tumor microenvironment, which leads to the reduction of tryptophan pool levels. The depletion of tryptophan induces T lymphocytes arrest at G1 phase of the cell cycle, with no progression, and also promotes T cell apoptosis and/or tolerance (33). In addition, T cells display receptors on their surface, which are responsible to down-regulate T-cell activation. For instance, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) can down-regulate antitumor immune response (43-45). All these immunosuppressive molecules and referred receptors are very interesting targets to achieve tumor growth inhibition and consist in a useful tool in cancer immunotherapy.

### 1.3. Tumor microenvironment

Tumor progression, after immune evasion mechanisms, has been mostly associated with molecular pathways and infiltrating cells in tumor microenvironment, rather than the ignorance and/or defects in T-cell antitumor activity (22, 46). In fact, both the presence of certain cells and their dynamic interaction with malignant cells have profound effect on tumor progression (47-50).

For many years, it has been described that the density of T cell infiltrates within tumor microenvironment was the most important factor to predict cancer patients' survival (51-53). However, recent findings underline that the role of other cells, such CD103+ DC, is crucial for the recruitment of those T cells (54, 55). Macrophages are also regarded as a critical cell type within tumor infiltrates. Macrophages are regulated by transcription factors, which generates different phenotypes of tumor-associated macrophages (TAM) and a heterogeneous population with distinct polarization status (56). M1 and M2 macrophages have already been characterized, and are engaged with the pathogenesis of inflammatory and tumor diseases (57). Nevertheless, the classification of TAM is quite complex and seems to be dictated by several mediators that result from cellular cross-talk and environmental conditions (58-60). M1 macrophages have

been defined as pro-inflammatory cells after activation by IFN-γ. They elicit Th1 immune cell activity and potentiate the eradication of tumor cells. In contrast, M2 phenotype regulates tissue repair and stimulates Th2 immune responses, displaying pro-tumoral properties (61, 62). M2 TAM can secret several cytokines, such as IL-1, IL-6, IL-10, VEGF, CCL22, and TGF-β, promoting tumor cell proliferation and metastasis (63, 64). As a result, it has been described that the number of M2 macrophages and the overall M2/M1 ratio of TAM are important predictors of survival for some cancers, as melanoma (65, 66), ovarian (67, 68), breast (69) and pancreatic cancers (70). DC are also present in the tumor microenvironment, where they can recognize and capture live and dying tumor cells (46, 71). In fact, the prolonged survival and lower tumor invasiveness is associated with presence of DC in tumors of different stages and grades, as described by Palucka and Banchereau, 2012 (18). However, DC are a heterogeneous population. Depending on environmental factors and molecular pathways, some DC may display antitumor activity, whereas others may have immunosuppressive properties. Tumor-infiltrating $CD8\alpha^+$ DC are particularly efficient in the cross-presentation of antigens via MHCI pathways, thus stimulating the cytotoxic T cell immunity. Also, the infiltration of $CD103^+$ DC has been associated with the inhibition of tumor progression by secretion of chemokines, such as CXCL9 and CXCL10, which are responsible for T cell recruitment into the tumor microenvironment (72, 73). On the other hand, plasmacytoid DC (pDC) are usually related to T cell tolerance (74-76).

Infiltrating CD8+ T-cell activity may be down-regulated by immune system-inhibitory mechanisms in the tumor microenvironment (22). For instance, high infiltration levels of CD4+ FoxP3+ regulatory T (Treg) cells, which are attracted by the chemokine CCL22 via CCR4, have been associated to poor prognosis (77, 78). Additionally, low amounts of tumor-infiltrating T cells were described when higher levels of angiogenic factors were found within the tumor microenvironment. The role of T-cell subsets within tumor microenvironment is extremely complex and takes part in a multidimensional network that depends on several molecular and cellular players (79).

NK, NKT and γδT cells are also engaged in the immunomodulation of tumor microenvironment (47, 80, 81). The antitumor effect of NK-cell antitumor effect has been associated to solid and hematopoietic tumors, while γδT cells and NKT cells have been involved in tumor inhibition. However, they show immunoregulatory functions in certain circumstances that are not yet completely understood (47, 80-82).

Besides the cells within the tumor region, tumor stroma has also been associated with tumor growth. In fact, it includes several different components, such as collagen, endothelial cells, fibroblasts and several macrophage subsets, which contribute for tumor immune evasion (83).

Tumor microenvironment anatomical and physiological features are also distinct from healthy tissues, presenting abnormal architecture of blood vessels and lower pH. Increased angiogenesis guarantees the supply of oxygen and different nutrients to tumor cells. It results from the action of different factors, as pro-angiogenic proteins, extracellular matrix proteins and matrix metalloproteinases (84, 85). Acidic pH is caused by increased production of lactic acid by tumor cells. The higher rate of metabolism and proliferation of tumor cells dictates higher levels of glycolysis to fulfill the needs. Thus, a greater amount of lactic acid is produced, affecting the surrounding areas. Acidosis is further exacerbated by both carbon dioxide, which is the final product of cell respiration, and hypoxic environment in the core of solid tumors (86).

### 1.4. Cancer immunotherapy

Cancer immunotherapy has been explored for some decades. This term is often used to describe treatments based on the modulation of the immune system through ‘active’ or ‘passive’ approaches. The concept of immunotherapy relies on specific immune mechanisms and targets, which could confer greater efficacy and specificity with less toxicity. The overall aim is to improve the presence of antitumor T cells and to silence immune inhibitory pathways, leading to optimal therapeutic approaches.

### 1.4.1. Passive cancer immunotherapy

Passive immunotherapy is based on the administration of *ex vivo* generated immune effector molecules or cells, such as antibodies and CTL, respectively. These molecules or cells can target specific receptors, leading to enhanced efficacy of the treatment and also to less side effects.

#### 1.4.1.1. Monoclonal antibodies (mAb)

The use of mAb has increased in the last decades. Currently, they constitute the major class of cancer immunotherapy to treat solid tumors and lymphomas in clinic. For instance, trastuzumAb has been used to treat HER2+ breast cancer and adenocarcinoma, whereas alemtuzumAb has been applied in chronic lymphocytic leukemia treatment (87). The mechanism of action of mAb is related to their ability to interfere with both growth factor ligands and receptors or pro-apoptotic targets, inducing apoptosis of cancer cells. Besides, mAb may activate components of the immune system through Fc-region-based mechanisms. This leads to antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) responses by macrophages and NK cells (88).

The first generation of mAb used in cancer therapy was of mouse origin. Thus, the half-life was often limited, which resulted in decreased efficacy. Further progresses conducted to the development of chimeric mAb, with enhanced properties, and then humanized mAb. Nowadays, fully human mAb are already available. Several novel mAb for different cancer types are presently under investigation in clinical trials. For example, ganitumAb – for pancreatic cancer – and necitumumAb – for non-small cell lung cancer – are now available in phase III clinical trials (87). Several monoclonal antibodies for immune checkpoint targeting are already in clinic or in clinical trials, and will be discussed in Section 1.5.1.

#### 1.4.1.2. Adoptive T cell therapy

This approach is based on the transfer of mature tumor-reactive T lymphocytes fight against tumor cells. Unlike cancer vaccines, this strategy is

independent from an immune response elicited by an exogenous antigen. Instead, it relies on the delivery of *ex vivo*-expanded cells (6, 89, 90). In a successful reported study, autologous tumor infiltrating lymphocytes (TIL), which are found in tumors and have potent antitumor activity, were harvested, activated *ex vivo* and reinfused into patients. The total remission was reported in more than 20% of treated patients (91). Complementary research has been developed to improve T-cell adoptive therapies. Genetically engineered T cells, such as Chimeric Antigen Receptor (CAR) T cells have already been approved by the FDA for the treatment of acute lymphoblastic leukemia in children in August 2017. Additional strategies are still under study in order to manipulate the properties of the administered T cell population, such as proliferation and migration characteristics (92, 93).

### 1.4.2. Active cancer immunotherapy

Active cancer immunotherapy is also defined as cancer vaccination. It relies on direct stimulation of the patient's immune system in order to induce a response against tumor cells. Unlike vaccination for infectious diseases, which efficiency is based mainly on neutralizing antibodies and B-lymphocyte responses, cancer vaccination depends on the administration of TAA, in order to induce a systemic immune response with the involvement of CTL responses. Cancer vaccines are expected to induce tumor specific immune responses able to either eliminate the malignant cells or keep it under constant restraint, delaying tumor recurrence and prolonging survival. Both prophylactic and therapeutic cancer vaccines have been proposed to enhance a specific immune response to tumor cells, based on DC activity and CTL induction (94).

Several engineered molecules designed to enhance immune-based mechanisms are available, from proteins and peptides, to antibodies and oligonucleotides. They represent promising players in re-shaping the future of immunotherapeutic outcomes. Also, the identification of several defined T-cell TAA, along with fact that specific T cells can mediate the elimination of tumors, has encouraged cancer vaccine development (95-97). However, as cancer vaccine candidates move towards clinical investigation, it becomes clear that

their biological effect depends on the design of a tool able to promote their transport across biological barriers and overcome tumor-related evasion mechanisms. In addition, the identified TAA, as recombinant proteins, are usually weakly immunogenic, requiring multiple administrations and their association with adjuvants. Accordingly, the potential of those bioactive molecules has pointed nanomedicines as an approach to achieve targeted selectivity and safety. These factors are required for their therapeutic *in situ* efficacy and thus clinical efficacy. As reviewed by Silva et al. 2013, an ideal vaccination strategy needs to engage the administration of the most immunogenic TAA together with the most effective adjuvants, including delivery platforms. These elements are essential for priming tumor-specific T cells, inducing tumor-specific antibodies and killing tumor cells by host immune effector mechanisms (98). It has been described that both antigen and adjuvant must act in a concerted way on the same APC, which can be provided by a singular delivery system (88, 99, 100).

#### 1.4.2.1. Cancer vaccines: the immune system

The focus of cancer vaccines is the stimulation of cell-mediated immunity, rather than humoral responses.

To develop an effective vaccine, three components are reported to be essential: antigen, adjuvant, and delivery system (101). Antigens are molecules that can be recognized by the immune system and elicit an adaptive immune response. Adjuvants are responsible for stimulating the innate immune system and enhance the immune response activated by antigens.

The selection of an appropriate TAA is one of the critical steps in the development of cancer vaccines. The choice should rely not only on the presence of that antigen in tumor tissues, but also on its role in tumor growth and metastasis. TAA can be proteins, peptides, or even lipids and carbohydrate-based tumor antigens (102-104). For an effective priming, tumor-associated proteins or peptides should exhibit the following characteristics: (i) contain MHCI-binding peptide sequences, (ii) become available to form complexes with MHCI molecules, after being processed, (iii) be recognized by the T cell repertoire in an

MHC I-restricted manner, and (iv) stimulate the expansion of CTL precursors expressing specific T-cell receptors (104, 105).

The recognition of TAA-MHCI complexes on DC membranes by $CD8^+$ T cells, in the LN (106), triggers the proliferation of $CD8^+$ T cells and subsequent differentiation into CTL. These cells migrate to peripheral tissues, developing contact-mediated cytotoxicity activity. They secrete effector cytokines, such as IFN-γ and TNF-α, leading to local inflammation. Activated $CD8^+$ T cells can also acquire memory. Memory $CD8^+$ T cells can be sustained for long periods without additional stimulation, which allows an improved and earlier immune response in case of tumor relapse or metastasis (107). Besides peptides and proteins, it is now accepted that the recognition of lipid antigens represents an important component of host defense. APC, such as DC, macrophages and B cells, express a class of MHC I-like CD1 molecules, which are engaged with antigen presentation and lipid trafficking and processing (102). If lipid antigens are from external origin, they can achieve endocytic organelles by fusing with the plasma membrane or through a process facilitated by lipoproteins. In the cells, lipid antigens can form complexes with CD1 molecules, which can present them to T cells. Several self and non-self lipid antigens presented by CD1 molecules have already been reported, such as lipopeptides, sphingolipids, diacylglycerolipids, mycolates and osphomycoketides (108). α-galactosylceramide (α-GalCer) is a CD1d antigen extensively studied. It is an extremely potent ligand, able to elicit the secretion of cytokines by NKT cells. These cells will then enhance the activation of DC and up-regulate cross-priming of tumor antigens, constituting a robust adjuvant for CTL responses (109). Tumor-associated carbohydrate antigens (TACA) are also a promising toll for cancer immunotherapy, since glycosylation is known to be a hallmark that defines the stage, direction and progression of cancer cells (110). Targeting TACA broadens the range of antigens that can be recognized by the immune system when fighting cancer. Thus, the risk of developing resistant tumor, due to loss or mutation of peptide or lipid antigens, can be decreased. However, TACA can be also expressed in healthy tissues and are poorly immunogenic, which may lead to tolerance and autoimmunity (103). Regarding TACA's characteristics, they have been applied

in several antitumor cancer strategies, such as glycoprotein- and glycopeptide-based vaccines to facilitate cellular responses. Glycoproteins can be altered to increase their immunogenicity. Human milk mucin (MUC1) is overexpressed on tumor cells, exhibiting a modified glycosylation pattern, which makes it one of the most promising TAA (108). Also, as carbohydrate-containing molecules, such as lipopolysaccharides, they are recognized by toll-like receptor (TLR) and C-type lectins, TACA have been involved in approaches to target these PRR, leading to enhancement of antigen internalization and DC activation.

PRR, mainly toll-like receptor (TLR) family, are important molecules to potentiate the presentation of TAA to $CD8^+$ T cells and increase cancer immunotherapy efficacy. Among TLR ligands, cytosine phosphorothioate-guanine motifs (CpG; TLR9-ligand), double stranded RNA mimic polyinosinic:polycytidylic acid (poly(I:C); TLR3-ligand) and monophosphoryl lipid A (MPLA; TLR-4) have been associated to stronger antitumor immune responses (17, 74, 111).

Besides $CD8^+$ T cells, it has been recently reported the importance of $CD4^+$ Th cells for an effective antitumor immune response. In fact, $CD8^+$ T cells need $CD4^+$ T cells to survive and retain their function. Also, $CD4^+$ Th cells can improve antitumor CTL responses and act as APC for $CD8^+$ T cells (112). Effector $CD4^+$ Th cells can differentiate in Th1 and Th2 cells. The combined action of $CD8^+$ T cells and Th1 cells is initiated by the activation of tumor-specific $CD8^+$ T cells that display antitumor activity directly. Both Th1 $CD4^+$ T cells and $CD8^+$ T cells, in a lower extent, secrete IFN-γ. This cytokine up-regulates MHC class I molecules and antigen-processing machinery, increasing tumor predisposition for recognition by $CD8^+$ T cells. Also, IFN-γ promotes the recruitment of other immune cells, such as NK cells, granulocytes or macrophages, which can play an important role in tumor eradication, by interfering with its stroma (113).

#### 1.4.2.2. Dendritic cells as targets for cancer vaccines

DC represent an outstanding target for cancer vaccines, since they represent pivotal mediators between innate and adaptive immunities. First of all, DC are strategically positioned in peripheral tissues, such as skin and mucosal

areas. They can easily capture pathogens near entry regions and become activated in response to 'danger signals' or pro-inflammatory molecules. Second, after antigen internalization, DC can migrate to LN in a chemokine-driven manner, where they can prime naïve T cells (114, 115). Activated DC express CCR7, a LN-homing chemokine receptor, thereby entering lymphatic vessels towards LN, where CCR7-ligands – CCL19 and CCL21 – are expressed (116). In LN, DC can present antigens on their surface to activate T cells via: i) MHC class I; ii) MHC class II; or iii) lipid antigen-presenting CD1 molecules (117). T-cell activation is also engaged with the up-regulation of co-stimulatory cytokines and chemokines, which act in a paracrine way on T cells (118). For instance, DC-secreted type I IFN is important for innate immune recognition of tumor cells and has been associated to positive prognostics in early stage of the disease (119). In the steady-state, DC and other APC continuously present self-antigens to T cells. This prevents autoimmune responses, by inducing T-cell tolerance to self-antigens. An additional stimulus or "danger signal" needs to be detected by DC when an external antigen is captured, in order to initiate T-cell expansion and differentiation in effector T cells.

The ability of DC to capture antigens by endocytosis is equivalent to macrophages, with similar number of lysosomes. However, DC lysosomes have lower proteolytic activity, which reduces antigen degradation and improves the effectiveness of antigen presentation (120). DC can internalize exogenous antigens in vesicular structures through different mechanisms of endocytosis, which can be either a receptor-dependent or receptor-independent mechanism. Receptor-dependent pathways include clathrin-mediated endocytosis, caveolae-mediated endocytosis, lipid-raft-mediated endocytosis and phagocytosis. On the other hand, receptor-independent mechanisms encompass fluid-phase endocytosis and micropinocytosis (121). After entry in the cytoplasm, the vesicles carrying extracellular antigens undergo several alterations, until they fuse with lysosomes or MHC class II compartments. There, MHC class II molecules are loaded with peptide antigens, forming an MHC class II-antigen complex. This complex will be ferried to the cell surface, becoming available to be recognized by $CD4^+$ T cells. In contrast, endogenous antigens, from the cytosolic origin, are

processed in the proteasome and translocated by the 'transporter associated with antigen processing' (TAP) complex, from the cytoplasm to the endoplasmic reticulum (ER). In the ER, antigens are loaded onto MHC class I molecules and transported to the cell surface, where they will be able to prime $CD8^+$ T cells (122, 123). However, MHC class I may also exhibit extracellular antigens on the cell surface of DC, through a process known as cross-presentation or cross-priming. Cross-presentation seems to rely on several successive steps: i) retrotranslocation of antigens from endosomal compartments into the cytosol; ii) re-importation of peptides into the endosomes via TAP, after proteasomal processing; and iii) peptide-loading of recycled MHC class I molecules in endosomes (123). Cross-presentation is extremely important for cancer vaccination. TAA carried by nano-based cancer vaccines may escape endosomes and reach the cytosol in higher concentrations than those administered in soluble form. MHC class I molecules can thus present those antigens more effectively and for longer periods of time, eliciting the activation of $CD8^+$ T cells. This leads to an effective cellular response, which is fundamental for a successful eradication of tumor cells. T cells are stimulated when MHC-antigen complexes are recognized by TCR. Each T cell expresses a specific TCR. Only T cells expressing a particular TCR, able to recognize a specific MHC-antigen complex, will become effectively activated. MHC class I complexes are recognized by $CD8^+$ T cells, while the MHCII class II trigger by $CD4^+$ T cells (123, 124).

CTL immunity can be performed by DC, using two main strategies – *ex vivo* and *in vivo* targeting. *Ex vivo* DC-based cancer vaccines use isolated CD14+ monocytes or CD34+ DC precursors from a patient. After being isolated, these cells are cultured and differentiated in immature DC (125, 126). The next process is TAA-loading of DC, which consists in adding proteins, peptides or tumor lysates by transfection or through the culture medium. Additional maturation stimuli, such as CD40L or pro-inflammatory cytokines, may be used to ensure DC will be able to induce a strong cellular immune response. Finally, loaded mature DC are administered back into the patient by intravenous (i.v.), subcutaneous (s.c.), intradermal (i.d.), intratumoral (i.t.) or intralymphatic (i.l.) route (127). When

administered to the patient, antigen-loaded DC will bypass the *in vivo* uptake of tumor antigens. DC are already activated and will be able to migrate to secondary LN, wherein they will trigger T cells. However, the half-life of TAA-MHC complexes on DC membrane is relatively short. Plus, only a small amount (3-5%) of DC can migrate to the LN and contact with T cells. These factors may contribute to the low success rate of *ex vivo* DC-based cancer vaccines (127, 128). Additionally, being produced specifically for a particular patient, *ex vivo* cancer vaccines constitute a highly complex, laborious, time-consuming and expensive approach. Also, the vaccine quality might depend on the clinic where it is produced, once there are several variable parameters in the process, such as dose of DC and posology (127). The type of DC stimulated, antigen loading method and DC maturation level are also important aspects to be considered in order to better understand the adjuvant role of DC (129).

*In vivo* DC-based cancer vaccines have been developed to overcome the lack of clinical efficacy of the *ex vivo* strategy. It is extremely important to design alternatives to target antigens directly to DC *in vivo*, which can be achieved using peptide-based vaccines. These are mainly based on MHC class I peptides, which are simple to produce and administer, and guarantee DC activation and expansion for prolonged periods of time (98, 130). However, the cytoplasmic delivery of antigens is limited by low membrane permeability and frequent destruction after intracellular entry, being their immunogenicity considerably lower than the traditional vaccines. Consequently, their association with potent adjuvants, as particulate vaccine delivery systems, or immunomodulatory molecules is being widely investigated (121, 131-134). Another advantage of *in vivo* DC-based vaccines is the fact that clinical intervention is limited to vaccine administration. This saves time in fastidious cycles of blood withdrawal and *in vitro* cell culture. Also, it offers the possibility for off-shelf products, which can be produced at large scale with cost reduction and increased quality.

### 1.5. The case of melanoma

As a highly immunogenic cancer, melanoma stands as a good target for immunotherapy and cancer vaccines (135). Melanoma is defined as a malignant tumor originated from melanocytes, being the most aggressive form among the skin cancers. For the last decades, the incidence of the disease has been constantly rising at a faster rate than any other solid tumor. Global incidence is predicted around 160,000 new cases per year, with 48,000 deaths (136-138). The prognosis of the disease strongly associates with its stage at diagnosis. At early stages, for instance, through direct observation of melanoma features or biopsy, melanoma is potentially treatable. On the other hand, the prognosis for patients with metastatic malignant melanoma (MMM) is reduced, with limited therapy options. MMM has a median survival of only 6 to 9 months and overall 5-year mortality close to 90% (7, 138-140). In advanced stages, melanoma can widely metastasize to certain regions such as lungs, gastrointestinal tract, skin and brain. Brain metastasis occurs in around 10% of patients with melanoma and it is usually correlated with a median survival of less than 4 months. Results from autopsies to patients who died of melanoma report the prevalence of brain metastasis at 55% to 75% (141-143).

The pathogenesis of melanoma is still not fully understood. Even though, it is clear that both genetic and environmental factors play a role in the transformation of melanocytes. In particular, extended UV light exposure and malignant melanoma have been causally related. It has been described that UV radiation has direct mutagenic effects on DNA and promotes reactive oxygen species (ROS) of melanin that provoke DNA damage and apoptosis suppression. Recent data show that mutations in melanoma genes such as STK19, FBXW7, and IDH1 are caused by the UV radiation (144, 145). Additionally, UV light can also provoke malignant changes in the skin by decreasing cutaneous immune defenses and by stimulating the production of growth factors by cellular components of the skin (138, 146).

As reviewed by Meyskens *et al.*, the alterations of antioxidant melanin to its pro-oxidant form are decisive in early pathogenesis of melanoma (146). Melanin is produced by melanocytes and presents specific characteristics.

Among them, complex redox and free-radical properties and interactions must be underlined. Melanin is typically an antioxidant molecule, which can be oxidized by ROS generated by UV light, normal metabolic processes and/or inflammatory responses. Likewise, some particular exposure to certain metal species, such as Cu, Fe, Mn, and Co, or melanin-binding chemicals, as organic amines, pesticides, herbicides and dyes, may enhance melanin pro-oxidant response (147, 148).

The increased intracellular levels of ROS generated by the abovementioned mechanisms can result in DNA damage. The cumulative genetic damages in melanocytes may then lead to cellular abnormalities, which can induce cell proliferation and inhibit normal cascades of apoptosis (146). Thus, the transformed melanocytes will be susceptible to accumulate additional genetic mutations, resulting in typical properties of malignant phenotypes, such as stimulation of angiogenesis, evasion of the immune system response, tumor invasion and metastasis (149).

### 1.5.1. Melanoma therapy

The type of treatment used for melanoma depends on the stage of the disease. It may comprise surgery, for the excision of the lesions, and also immunotherapy, targeted therapy, radiation and chemotherapy (136, 139).

Until 2011 and for several decades, the United States Food and Drug Administration (FDA) had only approved three molecules for the treatment of melanoma – dacarbazine, hydroxyurea and IL-2. Hydroxyurea was first approved in 1967 and dacarbazine in 1975. For many years, the latter has been reported as the most efficacious, being widely applied as single-agent therapy or in combination with other agents (150). As consequence of its extensive usage, dacarbazine has been considered the standard treatment for comparing the efficacy of alternative regimens and/or agents in clinical trials (151, 152). Nevertheless, the results of chemotherapy in metastatic melanoma patients are very limited, due to a strong resistance exhibited by these malignant cells to cytotoxic agents (139, 153, 154).

Since melanoma was reported as capable of inducing a natural immune response, extensive research has been developed to establish immune-based therapies (155). After IFN α-2b, IL-2 became the second FDA-approved exogenous cytokine with antitumor activity against melanoma, in 1998 (156). IL-2 plays a pivotal role in immune regulation and T cell proliferation (157). Even though, until recently, the treatment options for melanoma patients with advanced or metastatic melanoma were not able to improve the overall survival and showed complete responses only in a small group of patients (158).

In the beginning of the decade, the BRAF inhibitors vemurafenib and dabrafenib were approved by the FDA and European Medicines Agency (EMA) for melanoma treatment. Selective tyrosine kinase inhibitors silence the BRAFV600 mutation, which is found in 50% of melanoma patients (159). These drugs inhibit oncogenic events, such as unchecked proliferation and immunity evasion. However, clinical trial data revealed the development of resistance to these BRAF kinase inhibitors within 6 to 7 months. To overcome this resistance, trametinib, an inhibitor of mitogen-activated protein kinase (MAPK) kinase (MEK), was combined with BRAF inhibitors. The combination resulted in both reduced cell signalling and cancer cell proliferation. Following the FDA approval of trametinib as single agent for metastatic melanoma, in 2013, the combination of dabrafenib and trametinib was then approved on January 2014 (160, 161). Also in the first years of this decade, the FDA and the European Commission approved a fully human monoclonal antibody against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). By binding to CTLA-4, this antibody limits the access of its ligands and blocks the co-inhibition of T cells, potentiating T cell antitumor activity. Following the same approach of T cell inhibition blockade, anti-programmed cell death-1 (PD-1) antibodies were approved in 2014 for the treatment of metastatic melanoma. The clinical outcomes of CTLA-4 and PD-1 blockade have been related with the enhancement of specific T-cell-mediated tumor immunity (162, 163). These exciting findings stimulated research and development of new immune checkpoint targeting agents, not only for immunosuppression blockade, but also for triggering the immune system, such as the stimulatory monoclonal

antibodies anti-OX40, anti-Glucocorticoid-induced tumour necrosis factor receptor (GITR) and anti-4-1BB.

Despite the promising results, the majority of the clinical trials involving immune checkpoint modulators revealed cases of resistance to therapy and low percentages of long term overall survival. To achieve better clinical outcomes, several strategies combining different immune checkpoint modulators have been proposed. Additionally, further studies on regulatory pathways of immune responses have led to the identification of novel targets to be used as monotherapy or in combination. For instance, the combination of anti-CTLA-4 and anti-PD-1 induced tumor regression in about 50% of patients with advanced melanoma (164). Among the emerging immune checkpoints, Lymphocyte-activation gene 3 (LAG-3) and T cell immunoglobulin and mucin domain 3 (TIM-3) are currently in clinical trials for the treatment of advanced solid tumors (NCT01968109, NCT02817633, http://www.clinicaltrials.gov). As for co-stimulatory molecules, such as OX40, several clinical trials are ongoing, either in monotherapy or combination schemes (NCT02318394, NCT02205333, and NCT02221960, http://www.clinicaltrials.gov). The combination of anti-OX40 and PD-1blockade has been associated to a significant increase of CD8+ T cells relative to Treg and Myeloid-derived suppressor cells (MDSC) at the tumor site (165).

However, despite the promising results, immune checkpoint therapies have been associated with severe side effects and toxicity in general. The adverse events consist mainly of immune-related conditions, including hepatitis, pancreatitis, colitis, pneumonitis, dermatitis and hypophysitis and usually require administration of immunosuppressive drugs, such as corticosteroids (166). The schedule and the duration of the immune checkpoint therapy have not been clearly established yet and additional clinical data and knowledge on the field will potentially elicit further optimization of these therapies.

Regarding the high immunogenicity of melanoma and the discovery of several shared melanoma-associated antigens, novel antigen-specific therapies are currently in development, such as adoptive T cell therapy and cancer vaccines. Preclinical studies with melanoma cancer vaccines showed tumor

regression and/or prolonged tumor growth stabilization. However, despite the promising results in early clinical trials (135), prospective randomized trials with a melanoma vaccine failed to increase the overall survival compared with Bacillus Calmette-Guérin (BCG) (156). When compared with chemotherapy in randomized phase III studies, melanoma vaccines failed to show consistent results, due to the limited immunogenicity and lack of robust antitumor immune responses (167). As a result, there is still an urgent demand for developing novel and more effective therapeutic solutions for melanoma, especially for the disseminated form of the disease.

## 2. Nanodelivery systems for DC-based vaccines

DC-targeted delivery platforms can increase the uptake of antigens and adjuvants by DC, leading to effective immune responses (**Figure 1A**) (99, 168, 169). Nano-based vaccines allow DC *in vivo* targeting delivery of antigens and additional stimuli (i.e. adjuvants) to the same cell, in a single platform (170). Particulate delivery systems range from micro and nanoparticles, liposomes, to virus-like particles (VLP).

The development of nano-based systems has provided protection strategies for incorporated agents, such as biomolecules – nucleic acids, peptides and proteins – which are generally quickly degraded when administered *in vivo*. Therapeutic agents can be embedded, encapsulated, or even adsorbed or conjugated onto nanosystems, which can be modified and associated to other adjuvants to achieve optimized release profiles (171). Nanocarriers can also potentiate the cytosolic delivery of valuable biomolecules involved in the regulation of gene expression, as small interfering Ribonucleic Acid (siRNA) and microRNA (miRNA), enabling endo-lysosomal escape (172, 173).

The widely recognized versatility of nanotechnology strategies has permitted the accurate design of multifunctional nanocarriers. Several nano-based systems composed by diverse materials with different characteristics have been proposed. They can be classified in polymeric, lipid, metal and inorganic nanocarriers. Among them, it is important to underline liposomes, polymeric

nanoparticles, micelles and dendrimers. In the present work, polymeric nanoparticles and micelles are focused in more detail.

### 2.1. Polymeric nanoparticles (NP) for DC vaccines

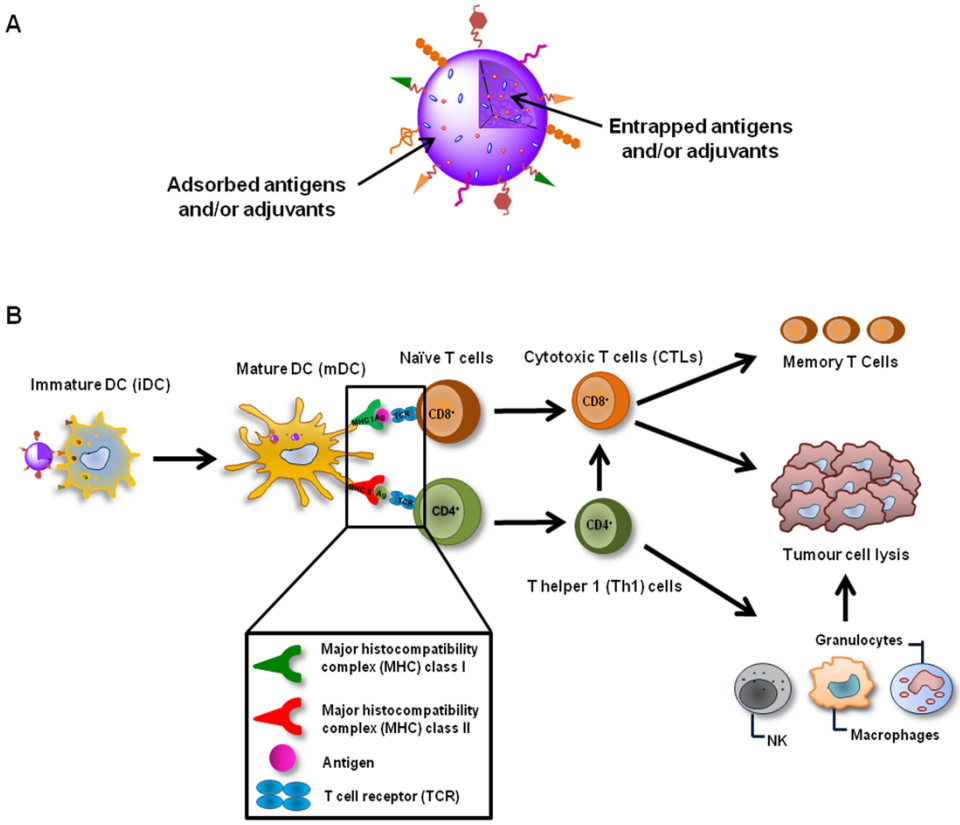


**Figure 1. A.** NP can to deliver several TAA and adjuvants simultaneously, enabling a coordinated activation of DC. NP can also be functionalized in order to actively target DC *in vivo*, increase their cellular internalization and immunogenicity or even target specific intracellular compartments. **B.** NP-based cancer vaccines can be targeted to DC *in vivo* and after their internalization induce the maturation of these cells. TAA and adjuvants are simultaneously released inside the same DC, which guaranties its coordinated activation. TAA are presented through MHC class I and class II molecules to $CD8^+$ and $CD4^+$ naïve T cells, which recognize the processed antigens through TCR. Activated $CD8^+$ T cells differentiate into CTL, which can destroy tumor cells, and memory T cells, that are important to avoid recidivism and metastasis. $CD4^+$ T cells should differentiate in Th1 cells, which will potentiate the action of CTL and will also activate cells of the innate immune system, such as NK cells, granulocytes and macrophages that play a role in the tumor destruction process as well. From Conniot J. et al. (2014) *Front. Chem.* 2:105.

Polymeric micro and nanocarriers are micron and submicron-sized polymeric colloidal particles, respectively. These delivery systems have been widely studied, and several materials have been explored for their production (101, 171, 174-176). The properties of the polymers used have emphasized these delivery platforms as attractive strategies, due to biocompatibility, non-toxicity and biodegradability. For long, several polymers have been approved and used in humans for surgical sutures, bone and dental implants, and other medical devices (177, 178). Since then, many groups have reported their potential use in the formulation of polymeric vaccines. The technological advantages of polymer-based delivery systems make them noteworthy drug carriers for vaccine development (179-183). First of all, polymeric NP are usually highly stable, when compared to liposomes, which have poor storage stability (184, 185). Also, polymeric NP have high entrapment and/or adsorbance capacity. Entrapped vaccine antigens are thus more protected from degradation during the delivery, which may decrease the amount required to enhance an immune response (182, 184). Besides, polymeric NP can easily incorporate both hydrophilic and hydrophobic molecules. Whereas in liposomes, hydrophilic small drugs are difficult to entrap, resulting in low encapsulation efficiency and fast leakage. Additionally, the chemical structure of polymers usually allows remarkable options for modifications, which may open paths for multifunctional-engineered particles. Many polymer properties may be easily modulated to tailor particle size and shape, particle surface, degradation kinetics and mechanical properties. By modulating polymer properties, antigen release rates from drug carriers can also be controlled, in order to achieve the desired level in a specific target (186). This constitutes an interesting alternative to the boosting requirements of the usual vaccination regimens (187).

The transport of polymeric particles through extra and intracellular barriers is facilitated, as they can be tailored down to the nanoscale. As a result, entrapped molecules may be delivered to a specific site. Vaccines based on NP may also be designed to disrupt endosomes after internalization. This is an important feature for antigen cross-presentation, because it elicits both $CD4^+$ and

$CD8^+$ T cell responses, which are essential for the induction of an antigen-specific immune response (**Figure 1B**) (188). Surface modifications on polymeric particles are more easily applied as well. For example, shielding polymers can be attached – as polyethylene glycol (PEG) –, allowing the attachment and variation of targeting ligands for specific tissue and cell-targeting (189, 190).

### 2.1.1. Influence of NP properties in cellular uptake by DC

Several physicochemical properties may influence the uptake of TAA-loaded NP by DC. Size, morphology, surface charge, hydrophobicity and receptor interactions are generally underlined (191, 192).

#### 2.1.1.1. Size

Although the ideal dimensions of NP for APC uptake are still under discussion, it has been reported that particulate size can indeed dictate the DC subset that is targeted. According to Foged and colleagues, NP size should be 0.5 μm or less to be quickly and efficiently incorporated by DC (191). Small size platforms (<200 nm) may drain freely to LN, being thus taken up by LN-resident DC subsets, such as $CD8\alpha^+$, which is an advantage for cancer immunotherapeutic approaches. However, delivery systems larger than 200 nm appear to be taken up by circulating monocytes, which differentiate after particle uptake and migrate to LN afterwards (100). Additionally, NP size also influences cellular uptake mechanisms and the endocytic pathway of NP, dictating their ultimate intracellular fate and overall biological effect. Particulate systems with a larger diameter (>0.5 μm) tend to be assimilated through macropinocytosis and/or phagocytosis by specific cells, as macrophages and Langerhans cells in the skin. Smaller particles usually enter the cell through endocytosis. NP with size <150 nm are generally taken up by cells via classic receptor-mediated endocytosis (clathrin-dependent) or endocytosis caveolae-mediated, if ranging from 50-80 nm (193). These NP, with size equivalent to viruses, are usually able to initiate virus-like immune responses, with activation of CTL and Th1. On the other hand, larger particles normally generate a similar immune response to those induced by bacteria, with Th2 activation and antibody production (194).

#### 2.1.1.2. Morphology

Particle morphology may influence cellular uptake and biodistribution. Non-spherical particles have been highlighted because of their increased blood circulation time, due to reduced phagocytosis by unspecific cells. However, the cellular uptake of those particles is decreased, when compared to spherical NP. According to Gratton *et al.*, rod-shaped NP show the highest uptake performance, followed by spheres, cylinders and finally cubical NP (195).

#### 2.1.1.3. Surface charge

NP surface charge also plays an important role in particle internalization and thus will also determine the nature of the induced immune response. Positively charged molecules/systems will show higher affinity to cell membranes due to their natural negative charge. After cellular uptake, negatively charged or neutral NP tend to localize within lysosomes, whilst positively charged NP showed ability to escape from endo-lysosomal pathway. Cationic NP were found in the perinuclear area and have been effectively taken up by macrophages and DC (196, 197). On the other hand, the interaction of those delivery systems with cell depends on multiple factors and some studies have reported the presence of neutral NP at ER, suggesting their ability to escape degradation at lysossomal/endossomal compartments (198).

### 2.1.2. Polymers for particulate delivery systems

Many polymers may be used to produce micro and nanodelivery systems, from aliphatic polyesters, to poly(amino acids), or even polysaccharides.

Aliphatic polyesters have been extensively used as biomaterials for drug delivery systems. Poly(D,L-lactide-co-glycolide) (PLGA) and polylactide (PLA) are the most well-studied (176, 178, 199). Also, in the last years, intensive investigation has been done under the scope of poly(ε-caprolactone) (PCL) for controlled delivery of peptides and proteins (101). Generally, aliphatic polyesters are biodegradable polymers formed by ester linkages. For many years, they have been used in several medical applications, such as absorbable sutures and body

implants (177, 178). The properties of aliphatic polyesters may vary according to some factors, such as molecular weight, composition of repeat units, crystallinity, flexibility of the chain, and degree of branching. For instance, the biodegradability is strongly influenced by the presence of hydrolysable and/or oxidisable linkages in polymer structure, which depends on parameters as the repeated units, the end groups, chain length and molecular mass (177, 178, 200). The characteristics of these polymers may be modulated through blending and copolymerization, or by alteration of their macromolecular architecture, which leads to new materials with improved properties (178).

#### 2.1.2.1. Poly(lactic acid) (PLA)

In the 1960s, PLA, also known as 2-hydroxypropionic acid, (**Figure 2**) was proposed as a biocompatible, biodegradable, and bioresorbable material (178). Depending on the isomers, PLA properties, such as annealing time and molecular weight, may vary. Regarding poly-L-lactide, it has glass transition temperature between 50–80ºC, melting temperature between 173–178ºC, and crystallinity of 37% (201).

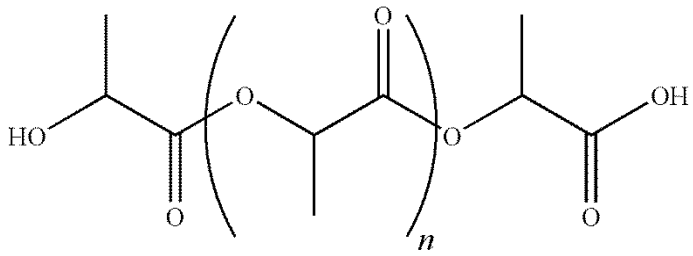


**Figure 2.** Schematic representation of PLA chemical structure.

PLA is a naturally occurring hydroxycarboxylic acid. In the body, it is metabolized in monomers of lactic acid, which is an intermediate product of anaerobic processes. Lactic acid is transformed in glucose, in the liver, and is further used by muscles as source of energy (202).

The FDA approved PLA for several purposes, such as tissue engineering, medical devices, and drug carriers for anticancer drugs, peptides, proteins and vaccines. PLA particles have been produced by many different methods;

however, the most used techniques are the solvent evaporation, solvent displacement, solvent diffusion and salting out (203).

PLA presents both a hydroxyl and a carboxyl end groups, which easily enables chemical modifications to the polymer. Through alkaline hydrolysis, reactive functional groups, such as carboxylic acids (–COOH) and hydroxyls (-OH), can be generated. Carboxylic acids may then be conjugated with molecules containing amine (-$NH_2$) and hydroxyl groups. Before conjugation, carboxylic acid groups are usually first activated, for instance with water-soluble carbodiimides (204, 205).

#### 2.1.2.2. Poly(lactic-co-glycolic acid) (PLGA)

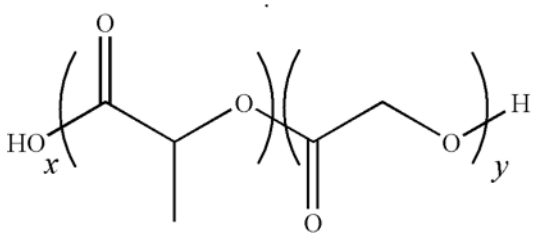


**Figure 3.** Schematic representation of PLGA chemical

Over the last 40 years, PLGA (**Figure 3**) has been one of the most successfully used biomaterials for medical applications, approved by the US FDA and EMA (206, 207). PLGA polymers are considered biocompatible, biodegradable, bioresorbable and “nontoxic” (206). In the body, PLGA is hydrolised in lactic acid (*x*) and glycolic acid (*y*) – the two composing monomers. These two monomers are endogenous and enter Krebs cycle, which leads to minimal systemic toxicity, since they are easily metabolized (174, 207). Lactic and glycolic acids may be present in diverse proportions in PLGA polymers, leading to different polymer degradation rates. This aspect is in fact used to identify different polymer variations. For instance, PLGA 75:25 is composed by 75% lactic acid and 25% glycolic acid. Indeed, having PLGA 50:50 as reference, polymers with higher content of glycolic acid will have faster rate of degradation (208).

Different methods have also been developed to produce PLGA particulate delivery systems. The most commonly used is the emulsification-solvent evaporation technique (207). PLGA-based particles have been intensively studied in order to obtain advanced delivery systems for protein and peptide nanomedicines and vaccines, as reviewed by Mahapatro *et al.* (171).

#### 2.1.2.3. Poly(-ε-caprolactone) (PCL)

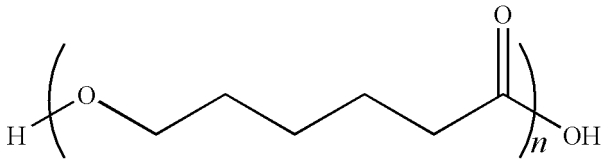


**Figure 4.** Schematic representation of PCL chemical structure.

PCL (**Figure 4**) is also hydrolyzed in physiological conditions, as the previously mentioned aliphatic polyesters. Therefore, PCL is considered to have minimal or no toxicity. When compared to other aliphatic polyesters, PCL has lower rate of degradation, constituting a valuable alternative for the particles designed to have a prolonged/sustained release rate. Solvent evaporation, solvent displacement and nanoprecitipation have been the most used techniques to prepare PCL particulate delivery systems (101, 171).

### 2.1.3. Surface modifications of NP for immune cell targeting

#### 2.1.3.1. Passive targeting

Passive targeting results from the transport of nano-based systems across the abnormal leaky vasculature of tumors, into the tumor interstitium or cells, by their movement within fluids – convection – or by passive diffusion (209, 210). As the architecture of blood vessels and its regulation are compromised, caused by unpaired angiogenesis, nanocarriers tend to accumulate selectively in tumor interstitium due to the 'Enhanced Permeability and Retention (EPR) effect'. The increased size of gaps in endothelial cells creates pores ranging from 10 nm to 1000 nm, which along with the poor lymphatic drainage, contributes to the 'EPR effect' (85). This effect has become very important for the design of targeted-

nanocarriers for cancer therapies. It has been reported that NP levels of accumulation in tumor interstitium are 10-50-fold higher than in normal tissues, leading to improved therapeutic efficacy and less side effects (209, 210).

#### 2.1.3.2. Active targeting

Active-targeted nanosystems are based on the design of nanocarriers with bioactive ligands on their surface or periphery (**Figure 5**). Nano-based systems can thus be delivered to specific sites, by binding recognition ligands to NP surface, which will be recognized by overexpressed molecular patterns of tissues and/or cells. This will promote receptor-mediated endocytosis of NP, influencing their intracellular trafficking and prolonged biological effects (210-216). Consequently, surface modifications represent an outstanding tool for cell targeting. The use of targeting moieties, such as peptides, antibodies and antibody fragments, carbohydrates and even vitamins, enables a specific contact of NP with targeted immune cells. For example, molecules that can direct NP to ligand DEC-205, which is highly expressed by $CD8^+$ DC, represent an interesting strategy. Moreover, recent studies have indicated that triggering CD40 on APC leads to $CD8^+$ T-cell effectors without the need for the common stimulation by MHCII-related helper T cells (217). Mannose receptors at DC are also associated to ligand internalization and further processing and presentation by immune cells, leading to a more extensive immune response (98, 218, 219). Mannose-receptor targeting in APC drive antigens to distinct early endosomes that remain stable over extended periods. These antigens will then be presented exclusively by the cross-presentation pathway to $CD8^+$ T cells, through MHC class I complex (220). For instance, PLGA NP decorated with mannan, a natural polymannose molecule, have higher binding affinity and increased $CD4^+$ and $CD8^+$ T cell responses compared to non-functionalized NP (221).

In general, PRR are useful strategies for immune cell targeting. PRR are cell surface receptors that recognize pathogen-associated molecular patterns (PAMP) and are involved in several stages of the immune response, since its initiation, proliferation and also effector phase (212). Different types of molecules may act as PAMP, known as ‘danger signals’, for instance lipids, lipoproteins,

proteins, carbohydrates and nucleic acids. The recognition of PAMP by PRR triggers immune responses by activating multifactorial signaling pathways. This leads to induction of inflammatory responses mediated by several cytokines and chemokines (212, 222).

Several classes of PRR have been described, such as TLR, retinoic-acid inducible gene (RIG)-like receptors (RLR), nucleotide oligomerization domain (NOD)-like receptors (NLR), DNA receptors (cytosolic sensors for DNA), scavenger receptors, and C-type lectin receptors (CLR) (218, 222, 223). In mammals, the most studied PRR class is the TLR class. TLR are predominantly expressed by APC, as DC, but they are also found in cells of the adaptive immune system, such as in αβT cells, Treg, γδT cells, as well as NKT cells (224). Through TLR activation, both the innate and the adaptive immune systems can be engaged, either by direct activation of TLR with their ligands on T and B cells, or by indirect mechanisms involving TLR-activated DC (98). CLR belong to another class of PRR expressed by APC. This receptor family is characterized by the presence of domains that bind to carbohydrates (225). CLR are specific receptors particularly involved in the internalization of antigens. CLR enable the intracellular uptake and processing of antigens, as well as influence their cytosolic fate and, consequently, their loading on MHC class I and/or II (226). Regarding the involvement of PRR in several strategic immune pathways, the design of nano-based systems for immune cell targeting can be extremely interesting. Not only because a more specific delivery can be achieved, but also because the cellular internalization of the targeted nanosystem can be modulated and potentiated. Additionally, the attachment of PRR ligands on the surface of nanocarriers may boost their immunogenicity, which constitute an outstanding strategy for the development of vaccines, since it allows the incorporation of an antigen and the 'danger signal' in the same platform (98).

#### 2.1.3.3. Surface modifications

Surface modifications of nano-based systems have been used, not only to improve tissue and cell surface targeting, but also to modulate nonspecific distribution and prolong the blood circulation time of nanocarriers. The use of

PEG has been extensively described for this purpose. PEGylation is a widespread strategy to improve the half-life time of nanodelivery systems, through steric stabilization and ‘stealth’ properties (**Figure 5 and 6**). It relies on the introduction of PEG molecules by conjugation, grafting or adsorption onto the surface of nanosystems. The terminal groups of PEG chains also present very suitable moieties to attach functional ligands and develop active-targeted carriers (227). The conjugation of antibody fragments to PEG ends, using disulfide bonds, may consist in an interesting strategy to design platforms for active targeting (228). Alternatively to PEG, D-α-tocopheryl poly(ethylene glycol) succinate (TPGS) has been reported for the development of platforms for immune stimulation, due to its immunogenic properties (**Figure 5**) (229).

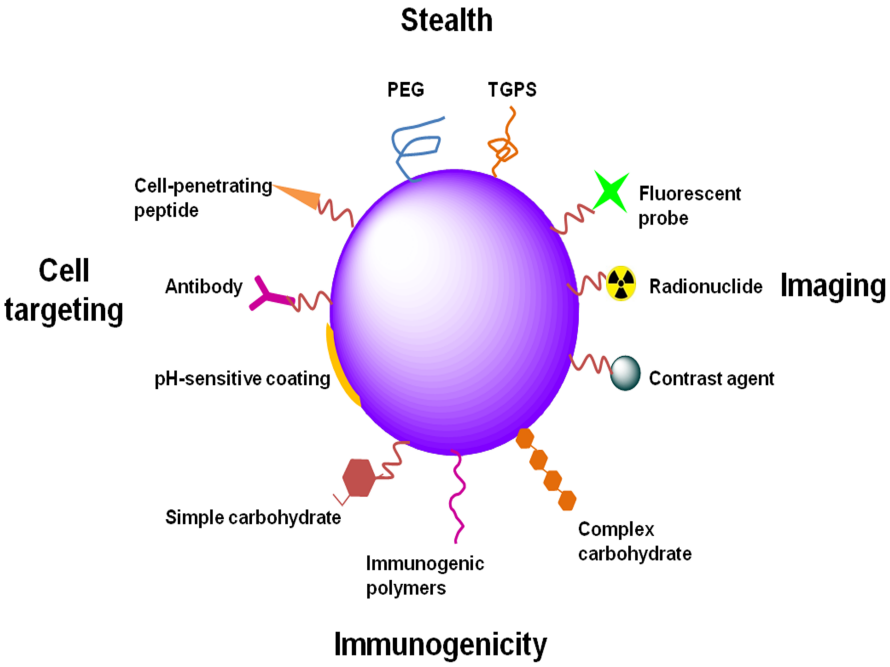


**Figure 5. Examples of Surface modifications of NP.** NP can be functionalized differently in order to attain distinct goals. PEG or TGPS functionalization provide stealth properties to NP, avoiding capture by phagocytic cells and increasing their circulation time. Specific tissue and cell targeting can be achieved through the functionalization of NP with antibodies directed to specific or overexpressed antigens. Cell-penetrating peptides can improve NP internalization. pH-sensitive coatings allow drug release in specific tissues or intracellular compartments in a pH-dependent manner. Functionalization of NP with imaging agents, such as fluorescent probes, radionuclides or contrast agents (e.g. gold or magnetic NP), provide applicability of NP to diagnostic, theranostic or even *in vivo* real-time imaging. The immunogenicity of NP can be increased for immunotherapy or prophylactic vaccination. Different molecules can be used for that propose, such as PAMPs (several carbohydrates, lipids or nucleic acids) or immunogenic polymers (e.g. chitosan, alginate, poloxamers). From Conniot J. et al. (2014) *Front. Che*m. 2:105.

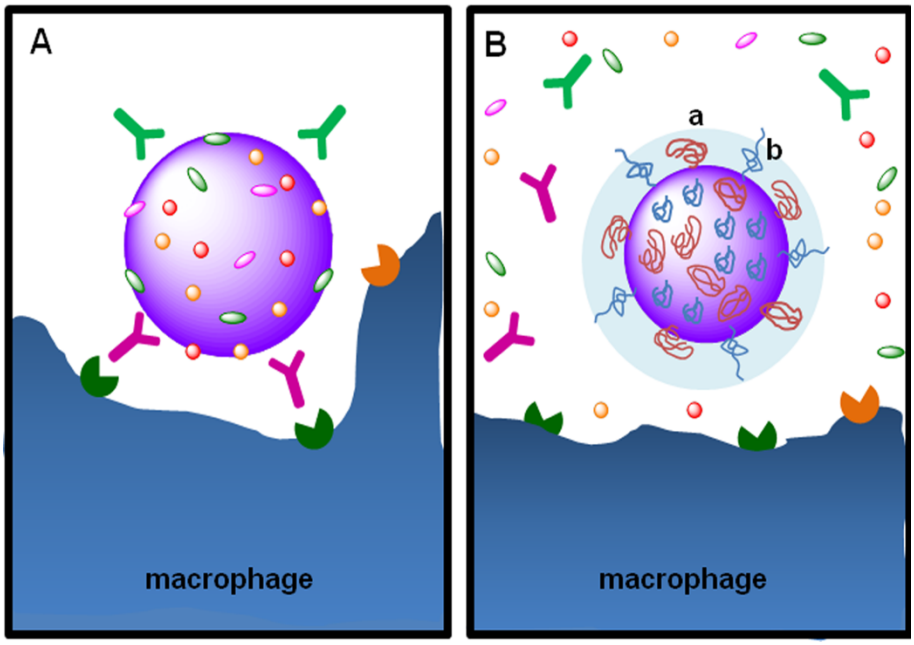


**Figure 6. The stealth effect from NP modification with PEG. A.** Particulate foreign entities in body fluids are promptly covered with opsonins, such as the immunoglobulins IgG and IgA and the complement proteins C3b C4b, in a process called opsonization. Opsonins mark the particulate entity to phagocytosis through their recognition by Fc receptors on phagocytic cells, such as macrophages. **B.** Functionalization of NP with PEG by grafting, conjugation or adsorption – note the "mushroom-like" (a) or "brush-like" (b) configuration of PEG chains – provides steric stabilization and stealth properties, preventing the adsorption of opsonins at the surface of nanoparticles. PEG hydrophilicity attracts water molecules to particle surface avoiding the adsorption of opsonins at NP surface, rendering them "invisible" to phagocytic cells. From Conniot J. et al. (2014) *Front. Che*m. 2:105.

### 2.1.3.4. Strategies for NP surface modification

Several strategies can be used to perform surface modifications on NP. As referred, molecules can be adsorbed onto the surface or attached through conjugation methods. Also, the process of modification can be either implemented before or after the production of NP. The strategy for attaching ligand molecules after production is usually applied when antibodies, proteins and polypeptides are chosen as targeting agents. As some organic solvents are generally used in the preparation of nanosystems, this method is preferred to

avoid denaturation of the secondary structure of ligands. Plus, since they are bulky molecules, they will disturb the hydrophilic/hydrophobic balance which can difficult the method of NP production (215). The drawbacks of this approach are related with subsequent purification of the formulation and its characterization. The processes frequently used for purification, such as centrifugation, filtration and dialysis, may degrade or alter the nanosystems. Additionally, it is generally difficult to distinguish whether the ligand is covalently linked to the surface of the nanocarrier or only adsorbed (215).

Several chemical pathways have been developed to attach ligands onto nanosystems surface, such as the carbodiimide strategy, the Michael addition pathway, the biotin–streptavidin approach and the Copper-catalized ligation method (215, 230). Native terminal groups of some polymers or specific moieties, introduced through chemical modifications, are generally used to apply these schemes of functionalization (e.g. carboxylic acid terminals in aliphatic polyesters and PEG) (230). The most used scheme is based on the carbodiimide chemistry. It relies on the coupling of a molecule containing a terminal amine group with another with another presenting *N*-hydroxysuccinimide (NHS) ester end or an end group that can be easily esterified to NHS moiety (215, 230). Michael addition pathway is based on thiol-maleimide coupling. Maleimide-polymers are used to produce nanocarriers, which surface is then modified by thiol-containing targeting agents (215, 230). However, the presence of native thiol groups in some molecules, as proteins and peptides, is usually low, or absent in some cases, and many are hard to access. To overcome this, disulfide bonds can be reduced in thiol groups or heterobifunctional cross-linking agents may constitute an alternative (215). Using the biotin–streptavidin approach through strong non-covalent biological interaction between biotin and avidin has also been reported. Still, for this strategy, a targeting agent is usually chemically bound to avidin, which is a bulky glycoprotein that may obstruct the interaction ligand-receptor, essential for targeting (230). Copper-catalysed ligation is a highly efficient method, based on a cycloaddition reaction that fits in the ‘click chemistry’ class of reactions. The chemical reaction is developed in mild conditions and with little

or absent byproducts. The major disadvantage of this approach is the difficult elimination of the Cu-based catalyst used for the reaction (215).

## 3. Translational and regulatory aspects

Polymeric NP are promising vaccine delivery systems, with many advantages compared to the currently available strategies. This is a fast-moving field and it is expected to bring a major contribution for vaccines and immunotherapy. However, some obstacles need to be overcome before this happens.

Translational requirements for NP developed for immune modulation should consider: (i) risk-benefit specific assessment and toxicological effects of activating immunomodulatory pathways (231); (ii) evaluation of potential deleterious effects caused by non-intended accumulation of polymers or stronger immune response (232, 233).

As for regulatory aspects, the clinical use of sophisticated complex nanosystems for immune modulation is strongly dependent on extensive assessment, characterization and understanding of crucial properties. Some of these are the ability to overcome physiological barriers, such as cell membranes and uptake mechanisms; intracellular pharmacokinetics, and effect on cell viability and functions, and their clearance from cells, tissues or organs. Moreover, as researchers routinely attach targeting molecules, and tracking and imaging agents to NP, new methods must be developed to characterize not only their physicochemical properties, such as size and size variability, morphology and charge, but also different techniques to assess their performance, as antigen/adjuvants release, metabolism, protein binding, and cellular uptake (234). Depending on size range and physicochemical properties, these NP are known to interact with immune cells and to adsorb plasma proteins. Thus, during the preclinical assessment, biocompatibility and immunotoxicity must be taken in consideration to avoid unpredicted adverse effects (235, 236). An appropriate evaluation of toxicity during the development includes dosage and therapeutic index, administration route and targeted disease environment.

In general, one of the hurdles underlying the regulation of these nanosystems are related to their particular characteristics. Their properties can in fact be easily altered not only by slight changes in raw materials, but also by small alterations in manufacturing processes. And even though these changes might result in limited alterations in the structure, biological properties and biodistribution patterns may be significantly altered (237). Quality control assays and robust methods should be developed to effectively monitor and characterize these small modifications. These methods would further allow relating the modifications with overall physicochemical alterations and consequent effect on biological properties, biocompatibility, and therapeutic effect (238, 239).

The adaptation of manufacture processes has also been an obstacle in the development of these nanosystems. Major questions related to manufacturing processes are arising during extensive efforts to achieve these pharmaceutical innovative nanosystems at production units, regarding their scale-up potential, mostly due to the extensive diversity of properties of new materials (240). It is crucial to identify and control the critical points during each manufacturing process. Applying concepts such as suitable product experimental design, “quality-by-design”, or improved techniques as process analytical technologies (PAT) will ensure an on-line/at-line quality assessment approach. Knowing and anticipating the most critical points of production, facilitates the implementation of automated procedures to resolve problems as they occur in line (240). These innovative conceptions have prompted the introduction and implementation of International Conference on Harmonization (ICH) Q8, Q9 and Q10 as novel pharmaceutical development regulations, aiming to nurture the development of nano-based pharmaceuticals and manufacturing processes. Despite some divergent perspectives on some procedures the FDA and EMA, in general, there have been efforts from regulators and industry from USA, Europe and Japan in order to develop comprehensive regulatory approaches through the ICH guidelines (240, 241). The harmonized assays intend to address aspects known to clearly affect *in vivo* safety and efficacy of NP. Major difficulties are related to the implementation of sensitive assays to detect low concentrations of nanocarriers, to differentiate them from formed aggregates or to distinguish intact

from metabolized forms (242). Alternative imaging techniques, fluorescence or cellular imaging methods have been proposed and explored as strategies to overcome these limitations (243-246). Another main hurdle behind the regulation of nano-based medicinal products is the nature of data that must be provided, before and during the product life cycle, requiring *in vivo* animal or clinical studies. In Europe, for Marketing Authorization Applications (MAA) the regulatory system allows the opportunity of “scientific counselling” from regulators to applicants, since early stages of R&D (240).This contributes to a harmonized development of advanced pharmaceuticals and reduces the impact of major obstacles during the process. Recently in the last years, EMA has created a working group to specifically address issues of quality, safety and efficacy of nano-based pharmaceuticals. Also, this group has prepared documents –"orientation documents" –, referring essential aspects to be considered by applicants in the development of nano-based products. Despite the lack of specific protocols, since 2009-2010 the regulatory organisms from the EU (EMA), USA (the FDA) and Japan (PDMA/MHLW) have worked together in order to achieve common perspectives in the field of nanopharmaceutical development. As for cancer vaccines particularly, although there have been some guidance documents with impact in the field, there is still a long way to go (247). Meanwhile, major pharmaceutical companies have focused on “proof of concept” of these complex systems. This will clarify mechanisms of safety and efficacy of innovative nanotechnology-based products. Additionally, before commercialization of innovative nanomedicines, it will be essential to perform pharmacoeconomic studies to demonstrate both social and economic added value of these novel products when compared with established treatments. Important indicators such as increment in QALYs (quality-adjusted life expectancy years) or costs associated to future consecutive hospitalizations shall be considered in the development of these innovative nanomedicines.

# CHAPTER II

# Chapter II - Materials and Methods

This chapter is adapted from the unpublished original manuscript:

**Dendritic cell-targeted nano-vaccines synergize with anti-PD-1/anti-OX40 immune checkpoint modulators for melanoma therapy**

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(Ready for submission)

## 1. Materials

Poly(L-lactic acid) (PLA, 2000 Da) with an average molecular weight (Mw) of 2000 was purchased from PolySciences, Inc., UK. Tumor-associated peptides MHCI-restricted Melan-A:26-35(L27), ELAGIGILTV, (MHCI-ag), and MHCII-restricted Melan-A:51-73(RR-23), RNGYRALMDKSLHVGTQCALTRR (MHCII-ag) were purchased to GeneCust Europe. CpG ODN 1826 (TCCATGACGTTCCTGACGTT) was purchased from InvivoGen (San Diego, CA, USA). MEM α, nucleosides, no ascorbic acid was purchased from Invitrogen. RPMI 1640, heat inactivated fetal bovine serum (FBS), trypsin EDTA 0.05%, penicillin (10,000 Unit/mL) and streptomycin (10,000 μg/mL), sodium pyruvate (100 mM), GM-CSF recombinant mouse protein (5 ng/mL), HEPES buffer (1 M), ACK lysing buffer, paraformaldehyde (PFA) 4%(v/v) and AlamarBlue® reagent were purchased from ThermoFisher Scientific, Cat.#15140122). Proteome Profiler Mouse XL Cytokine Array Kit was purchased from R&D Systems, Inc (Minneapolis, MN, USA). Matrigel Matrix (Cat. no. 356231) was purchased from BD Biosciences - Discovery Labware, Erembodegem, Europe. IncuCyte® Caspase-3/7 Apoptosis Assay Reagent (Cat No 4440) was acquired from Biological Industries Israel Beit-Haemek Ltd.. PLGA (Resomer 503H; Mw 24 000 – 38 000), mannosamine · HCl, dimtehylformamide (DMF), 4-dimethylaminopyridine (DMAP), *N,N'*-Dicyclohexylcarbodiimide (DCC), methanol, anhydrous sodium sulfate, Concanavalin A, FITC-labeled Concanavalin A from Canavalia ensiformis (Jack bean) Type IV lyophilized powder, Lipid A monophosphoryl from Salmonella enterica serotype minnesota Re 595 (Re mutant) (MPLA), bovine serum albumin, d-α-tocopherol polyethylene glycol 1000 succinate (TPGS), Poly(vinyl alcohol) (PVA) Mw 13000 to 23000 Da, 99% hydrolized, dichloromethane (DCM) and chloroform-d were purchased from Sigma Aldrich. Anti-PD-1 and anti-OX40 monoclonal antibodies were acquired from Bio X cell. Rabbit monoclonal anti-caspase 3 was purchased to Epitomics (CA, USA). Antibodies anti-mouse CD4 (clone: CK1.5), CD8α (clone: 53-6.7) and DAPI were acquired from BioLegend (San Diego, CA, USA). Streptavidin–horseradish peroxidase conjugate was purchased from Histostain,

Life Technologies (CA, USA). Fluorochrome labeled antibodies for flow cytometry were purchased from Miltenyi Biotech.

## 2. Methods

### 2.1. Synthesis and characterization of mannose-PLGA polymer

Mannose-PLGA (man-PLGA) was synthesized from PLGA (Resomer 503H; Mw 24 000 – 38 000). Carboxylic acid terminal groups of PLGA were modified with mannosamine under nitrogen atmosphere in mild conditions (248). Briefly, Mannosamine・HCl (10.8 mg, 0.05 mmol, 3.5 eq.) and 4-dimethylamino pyridine (DMAP) (6.5 mg, 0.05 mmol, 4 eq.) were added to 4 mL of dimethylformamide (DMF). The mixture was stirred for 10 minutes at room temperature to achieve complete dissolution of mannosamine. PLGA (Resomer 503H; Mw 24 000 – 38 000) (400 mg, 0.0133 mmol, 1 eq.) was added to the previous solution and stirred for 10 minutes at room temperature. *N,N'*-Dicyclohexylcarbodiimide (DCC) (5.5 mg, 0.027 mmol, 2 eq.) was added to induce the reaction between PLGA and mannosamine. The reaction was allowed to stir for 48 hours at room temperature under argon atmosphere. The polymer was precipitated with water and recovered by centrifugation. Man-PLGA was dissolved in DCM and anhydrous sodium sulfate was used to remove remaining water. The solution was filtered. DCM was evaporated through rotary evaporation until oil was obtained. Methanol was added to precipitate the polymer and to wash the reaction crude. Methanol was discarded. Man-PLGA was dissolved in DCM again and the procedure was repeated. Finally, man-PLGA was dried under vacuum overnight and weighed after 24 hours (186.7 mg; $\eta$ = 47%). $^{1}$H NMR of man-PLGA was acquired at 300 K using a Bruker Avance NMR spectrometer at 300 MHz. Instrument was equipped with a 5 mm BBO probe including Z-axis pulse field gradients. NMR spectra were processed using Bruker NMR Suite 3.5 and MestReNova 10.0. Chemical shifts are reported relative to the deuterated solvent used ($CDCl_3$ $\delta$ 7.26 pmm).

### 2.2. Synthesis of NP and man-NP

PLGA/PLA nanoparticles (NP) were formulated by the double emulsion-solvent evaporation (w/o/w) method (249). PLGA/PLA (2:8) blend was dissolved in dichloromethane (DCM) at 50 mg/mL. MPLA (100 $\mu$g) was added to DCM polymer solution. A 10% (m/v) PVA aqueous solution (100 $\mu$L) containing CpG at 0.5 mg/mL and melan-A/MART-1 (26-35(A27L) or melan-A/MART-1 (51-73) at 5 mg/mL was added to DCM phase containing polyester polymers previously dissolved (250). For empty NPs, 100 $\mu$L of 10% (m/v) PVA aqueous solution was added. The mixture was emulsified with a microprobe ultrasonic processor for 15 seconds at 20% amplitude. A 2.5% (m/v) TPGS aqueous solution (400 $\mu$L) was added and the second emulsion was formed using the same conditions. The double emulsion was added dropwise into a 0.25% (m/v) PVA aqueous solution and stirred for 1 hour at room temperature. Particle suspension was collected by centrifugation at 20,000*g* for 45 minutes, 4ºC (SORVALL® RC-5B PLUS Superspeed centrifuge). Particles were washed with ultrapure water, collected by centrifugation and finally resuspended in PBS or ultrapure water. Mannose-PLGA/PLA nanoparticles (man-NP) were prepared as previously described with man-PLGA/PLA (2:8) blend instead. Cy5.5-labeled or rhodamine-labeled NP and man-NP were synthesized by adding 0.5 μg Cy5.5-grafted PLGA or Rhodamine-6G-grafted PLGA to the polymer blend. Cy5.5-grafted PLGA and Rhodamine-6G-grafted polymers were synthesized according to the method described in Silva, *et al.* (2014) (250).

### 2.3. Physicochemical characterization of NP and man-NP

#### 2.3.1. Size distribution and ζ-Potential Measurements

Particle size was measured by Dynamic Light Scattering (DLS) with Malvern Nano ZS (Malvern Instruments, UK). Z-average size was determined by cumulative analysis. ζ-Potential of particles was measured by Laser Doppler Velocimetry (LDV) in combination with Phase Analysis Light Scattering (PALS), using the same equipment. Particles were diluted in ultrapure water and

electrophoretic mobility was determined at 25 °C with the Helmholtz-von Smoluchowski model.

### 2.3.2. Particle morphology

The morphology of particles was evaluated by complementary methodologies:

**Atomic Force Microscopy (AFM)**. Particles were diluted at 5 mg/mL in ultrapure water. A drop of sample was placed onto freshly cleaved mica for 20 minutes and dried with pure $N_2$. Samples were analyzed by AFM in tapping mode in air at room temperature, using a Nanoscope IIIa Multimode Atomic Force Microscope (Digital Instruments, Veeco), and etched silicon tips (ca. 300 kHz), at a scan rate of ca. 1.6 Hz.

**Scanning Electron Microscopy (SEM)**. Particles were diluted in trehalose 5% (m/v) and fast frozen at -80ºC for 2 hours. Samples were dried under vacuum, first at -20ºC for 14 hours and then at 20ºC for 2 h. Dried specimens were coated with gold on a Peltier-cold stage sputter-coater and examined using a FEI Quanta 200 FEG ESEM Phillips 500 scanning electron microscope at 5 kV accelerating voltage.

**Transmission Electron Microscopy (TEM)**. Particles were diluted in PBS and placed on a carbon-coated copper grid and dried. Samples were analyzed by Philips CM 120 Bio-Twin TEM.

### 2.3.3. Entrapment efficiency and loading capacity of antigens and immune potentiators

Supernatants collected from centrifugations were used for the indirect quantification of entrapped antigens and immune potentiators. Entrapment efficiency (EE % (m/m), Eq. (1)) and loading capacity (LC $\mu$g/mg, Eq. (2)) of melan-A/MART-1 (26-35(A27L)) and melan-A/MART-1 (51-73) were determined with FAM-labeled melan-A/MART-1 (26-35(A27L)) and melan-A/MART-1 (51-73), respectively. Relative Fluorescence Units (RFU) were measured with SpectraMax M5e plate reader (Molecular Devices, CA, USA) at 498/518 nm,

excitation/emission wavelengths. The amount of CpG in the supernatant was determined by the Oligreen® ssDNA quantitation kit (251). RFU were measured using the fluorometer at 485 nm excitation and 530 nm emission wavelengths.

$$Entrapment\ Efficiency\ (EE\ \%) = \frac{initial\ amount\ of\ agent - amount\ of\ agent\ in\ the\ supernatant}{initial\ amount\ of\ agent} \times 100 \quad (1)$$

$$Loading\ Capacity\ (LC\ \mu g/mg) = \frac{initial\ amount\ of\ agent - amount\ of\ agent\ in\ the\ supernatant}{total\ amount\ of\ polymer} \quad (2)$$

### 2.3.4. Mannose detection on the particles' surface by the Lectin Recognition Assay

Mannose residues at the surface of man-NP were detected by the Lectin Recognition Assay. Man-NP (1 mg/mL) were incubated under gentle stirring at 37ºC for 1 hour with Concanavalin A (0.7 mg/mL) in PBS (pH 7.2) supplemented with 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 3% (m/v) BSA. Non-targeted NP were used as a negative control. Particles were washed twice by centrifugation at 20,000*g* for 45 minutes at 4°C, with supplemented PBS without BSA. The formation of aggregates was assessed by DLS, using a Malvern Nano ZS (Malvern Instruments, UK). The experiment was repeated with FICT-labeled concanavalin A. The formation of aggregates was detected by fluorescence microscopy.

## 2.4. Cell lines

Murine bone marrow dendritic cells (DC) JAWSII cell line (ATCC#CRL-11904) were cultured in MEM-α, nucleosides, with no ascorbic acid, supplemented with 10% (v/v) FBS, 1% PEST (Penicillin10,000 U/mL and -Streptomycin 10,000 μg/mL)), 1% Sodium Pyruvate (Sodium Pyruvate (100 mM)), 5 ng/mL GM-CSF Recombinant Mouse Protein. Ret melanoma cells were obtained from spontaneously occurring skin tumor in ret transgenic mice (252) and engineered to express mCherry reporter gene. Murine Ret melanoma cells were cultured in RMPI-1640 and supplemented with L-glutamine (2 mM),

medium, with 10% FBS, 1% PEST (Penicillin 10,000 U/mL and -Streptomycin 10,000 μg/mL), sodium pyruvate (1 mM) and HEPES (25 mM).

### 2.5. *In vitro* cell viability in the presence of NP or man-NP

Cell viability of JAW SII DC was assessed by AlamarBlue® assay. Briefly, $10^4$ cells were seeded in 96-well plates and incubated overnight. Cells were then treated with different concentrations of particles for 48 hours. AlamarBlue® reagent was added at 10% (v/v) and incubated for 8 hours. Fluorescence measurements were performed at excitation wavelength of 530 nm and emission of 590 nm with a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The 0.5% (v/v) triton X100 solution and cell culture medium were used as positive and negative controls, respectively.

### 2.6. Hemolysis assay

A 2% (m/v) mouse red blood cells (RBC) solution was incubated with serial dilutions of NP and Man-NP for 1 hour at 37°C. The highest particle concentration (20 mg/mL) is the one used for injection in the *in vivo* experiments. Sodium dodecyl sulfate (SDS) was used as a positive control and dextran (Mw 70 kDa) as a negative control. Following centrifugation, the supernatant absorbance was measured at 550 nm using a SpectraMax M5e plate reader (Molecular Devices, CA, USA). The results were expressed as percentage of hemoglobin released by % (m/v) of Triton X100 (100% lysis).

### 2.7. *In vitro* particle internalization by dendritic cells

JAW SII DC ($5x10^4$ cells/well) were seeded in 96-well plates and incubated overnight. Cells were then incubated with rhodamine-grafted NP or man-NP (500 μg/mL) for 4, 12 and 24 hours. Cells were then washed with DPBS and resuspended in flow cytometry buffer. Non-treated cells and non-labeled NP were used as negative controls. The individual fluorescence JAW SII DC was collected

for each sample using LSR Fortessa cytometer (BD Biosciences) and analyzed with FlowJo software version 7.6.5 for Microsoft (TreeStar, San Carlos, CA).

### 2.8. Animal studies

Male C57BL/6 mice (8-12 weeks old) were purchased from Envigo RMS LTD (Jerusalem, Israel) or Charles River (Wilmington, MA, USA), and housed in the animal facility of Tel Aviv University or the Faculty of Pharmacy at University of Lisbon. All animal procedures were completed in compliance with both Tel Aviv University, Sackler School of Medicine and Faculty of Pharmacy, University of Lisbon guidelines. Protocols were reviewed and approved by the institutional animal care and use committee (IACUC) and Direção-Geral de Alimentação e Veterinária (Portugal) and performed in accordance with NIH guidelines. Mice body weight change was monitored 3 times per week. Mice were euthanized according to ethical protocol when showing signs of distress or with rapid weight loss (above 10% within a few days or 20% from the initial weight). For tumor-bearing mice, animals were also euthanized in case the tumor size exceeded 2000 $mm^3$ or if the tumor was necrotic/ulcerative. Mice were perfused intracardially with PBS, immediately after euthanasia, and then with 4% (v/v) paraformaldehyde (PFA). Tumor and major organs were collected for histology.

### 2.8.1. Immunization of animals with tumor-associated antigens

For immunization studies, 8 weeks old C57BL/6 male mice were randomized into 8 groups ($N = 6$), (**Table 1**).

**Table 1. Immunization study design**

| Group | Treatment |
|---|---|
| 1 | PBS |
| 2 | NP (empty) |
| 3 | MHCI-ag (free in solution) |
| 4 | MHCI-ag/MHCII-ag (free in solution) |
| 5 | NP MHCI-ag |
| 6 | NP MHCI-ag/NP MHCII-ag |
| 7 | man-NP MHCI-ag |
| 8 | man-NP MHCI-ag/man-NP MHCII-ag |

*Obs.: Groups from 3 to 8 included the administration of both melanoma-associated peptides (MHCI-ag or MHCI-ag/MHCII-ag) and Toll-like receptor ligands (CpG and MPLA).*

Treatments (100 $\mu$L) were injected in each mouse by hock immunization, via subcutaneous injection proximal to popliteal and axillary LN. Half dose was injected on the right side and the other half on the left side. When the two peptide antigens melan-A/MART-1 (26-35(A27L)) and melan-A/MART-1 (51-73) were administered to the same mouse (groups 4, 6, and 9), 25 $\mu$L of each treatment was administered on each side. Each dose contained 100 $\mu$g of antigen (50 $\mu$g of melan-A/MART-1 (26-35(A27L)) and 50 $\mu$g of melan-A/MART-1 (51-73), when two antigens were used), plus 20 $\mu$g of CpG and 20 $\mu$g of MPLA, either in solution or entrapped in 2 mg of particles (20 mg/mL). To simplify reference, melan-A/MART-1 (26-35(A27L)) MHCI-restricted peptide was designated as MHCI-antigen (MHCI-ag), and melan-A/MART-1 (51-73) MHCII-restricted peptide as MHCII-antigen (MHCII-ag).

### 2.8.2. Tumor antigen-specific proliferation of splenocytes from immunized mice

Splenocytes from whole spleen of treated mice (*N* = 6) were harvested and seeded in sterile 60 mm petri dishes, 10 days after the last immunization. Splenocytes were seeded for 6 days in complete RPMI medium, in the presence of melan-A/MART-1 (26-35(A27L)) or melan-A/MART-1 (26-35(A27L)) + melan-A/MART-1 (51-73) (100 μg/mL) (for groups 3, 6, and 9) and CD28 (2 μg/mL). After incubation, the culture media were collected and used to determine cytokine and chemokine secretion, while splenocytes were co-cultured with melanoma cells to evaluate their cytotoxic activity against melanoma cells.

### 2.8.3. Cytokine and chemokine secretion by splenocytes from immunized mice

A membrane-based sandwich immunoassay was performed according to the manufacturer protocol of Proteome Profiler Mouse Cytokine Array Panel A Kit (catalog number: R&D-ARY0068; R&D Systems, Inc, Minneapolis, USA). Splenocyte's culture media from each group (Table 3) were pooled (*n* = 6) and concentrated in concentration tubes (Amicon®, Millipore, UK). Each sample was applied on a nitrocellulose membrane containing capture antibodies that bind to specific target protein. After overnight incubation at 4ºC, streptavidin-HRP and chemiluminescent reagents were added and incubation steps were performed according to the protocol provided by the analysis kit. Membranes were exposed to X-rays for 10 minutes. Results were detected by a transmission-mode scanner proportionally to the quantity of analyte and determined by protein array analyzer software.

### 2.8.4. *Ex vivo* immune cell killing assay

Immune cell cytotoxic activity was assessed using IncuCyte® ZOOM live-cell instrument. Murine Ret mCherry cells ($6x10^3$ cells/well) were seeded in 96-well tissue culture plates in complete RMPI without phenol red, on the day before

the addition of splenocytes. Pre-stimulated splenocytes were added to Ret mCherry cells in a 1:100 ratio. IncuCyte™ Caspase-3/7 Apoptosis Assay Reagent (Cat No 4440) was diluted in RPMI without phenol red and added to each well to a final concentration of 5 $\mu$M. Data was collected every 2 hours for 12 hours. The 0.5% (v/v) triton X100 solution and cell culture medium were used as positive and negative controls, respectively.

### 2.8.5. Effect of prophylactic nano-vaccines on the therapeutic efficacy of immune checkpoint therapy

Male C57BL/6 mice (8-12 weeks old) were randomized into one of the following groups ($N$ = 4-5), (Table 2.2.).

**Table 2. Combination groups of prophylactic nano-vaccines with immune checkpoint therapy**

| Group | Prophylactic Immunization | Immune checkpoint therapy |
|---|---|---|
| 1 | PBS | - |
| 2 | PBS | anti-PD-1/anti-OX40 |
| 3 | MHCI-ag/MHCII-ag (free in solution) | - |
| 4 | MHCI-ag/MHCII-ag (free in solution) | anti-PD-1/anti-OX40 |
| 5 | NP MHCI-ag/NP MHCII-ag | - |
| 6 | NP MHCI-ag/NP MHCII-ag | anti-PD-1/anti-OX40 |
| 7 | man-NP MHCI-ag/man-NP MHCII-ag | - |
| 8 | man-NPMHCI-ag/man-NP MHCII-ag | anti-PD-1/anti-OX40 |

*Obs.: groups from 3 to 8 included the administration of melanoma-associated peptides (MHCI-ag/MHCII-ag) and Toll-like receptor ligands (CpG and MPLA).*

Animals from each group were immunized on days 28, 21 and 14 before tumor inoculation (**Figure 6A, Chapter III**). On day 14 after the last immunization, 50 $\mu$L of cell suspension containing 4.5x$10^5$ murine Ret melanoma cells mixed with growth-factor reduced matrigel (1:1) were inoculated on the right dorsal region, as reported before (253). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg). The right dorsal area was treated with depilatory cream before the injection.

Nine days following tumor inoculation, the first dose of immune checkpoint antibodies was administered. Anti-PD-1 and anti-OX40 were administered i.p. at 10 mg/kg. Tumor size was measured every 3 days with a caliper. Tumor volume was determined by $X^2 \cdot Y \cdot 0.5$ ($X$ – smaller diameter; $Y$ – larger diameter) and body weight was monitored.

### 2.8.6. Effect of therapeutic nano-vaccines on the therapeutic efficacy of immune checkpoint therapy

On day 0, male C57BL/6 mice (8-12 weeks old) were inoculated with 50 $\mu$L of cell suspension containing $3x10^5$ murine Ret melanoma cells mixed with growth-factor reduced matrigel (1:1) on the right dorsal region as reported before (253). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg). The right dorsal area was treated with depilatory cream before the injection. On day 7 after tumor inoculation, animals were randomized into one of the following groups ($N$ = 6-7), (**Table 3**).

**Table 3. Combination groups of therapeutic nano-vaccines with immune checkpoint therapy**

| Group | Treatment |
|---|---|
| 1 | PBS |
| 2 | anti-PD-1/anti-OX40 |
| 3 | man-NP MHCI-ag/man-NP MHCII-ag |
| 4 | man-NP MHCI-ag/man-NP MHCII-ag + anti-PD-1/anti-OX40 |

Groups 1, 2 and 3 received treatment on day 7, 14 and 21, following tumor inoculation (**Figure 8A, Chapter III**). Group 4 was treated with man-NP MHCI-ag/man-NP MHCII-ag on day 7, 14 and 21 and received anti-PD-1/anti-OX40 3 days later, respectively, on day 10, 17 and 24. Two doses of anti-PD-1/anti-OX40 were further administered on days 27 and 30. Anti-PD-1 and anti-OX40 were administered i.p. at 10 mg/kg. Tumor size was measured 3 days a week with a caliper. Tumor volume was determined by $X^2 \cdot Y \cdot 0.5$ ($X$ – smaller diameter; $Y$ – larger diameter) and body weight was monitored.

### 2.8.7. Immunohistochemistry

Formalin-fixed paraffin embedded (FFPE) samples of melanoma tumors were sectioned at 5 µm thickness, mounted on positively charged glass slides

and dried for 30 minutes at 37°C. After deparaffinization, samples were rehydrated and stained with haematoxylin and eosin (H&E). Immuno-staining was performed with the automated immunohistochemistry (IHC) and *in situ* hybridization staining system Bond RX (Leica Biosystems, Germany). First, sections were submitted to heat-induced epitope retrieval with Epitope Retrieval solution 1 (ER1, AR9961, Leica Biosystems) for 20 minutes. Hydrogen peroxide 3-4% (v/v) (part of DS9263, Leica Biosystems) was used for 12 minutes to block endogenous peroxidase activity. Sections were then incubated with goat blocking serum (Biological Industries, Israel) for 35 minutes. Afterwards, staining was performed using Intense R Detection system (Leica Biosystems) according to the manufacturer's protocol. Primary antibody incubation time was 1 hour. Tumor sections were stained for apoptotic cells with rabbit anti-cleaved caspase 3 antibody (1:50), for $CD4^+$ T cells with rat anti-mouse CD4 antibody (1:100) and for $CD8^+$ T cells with rat anti-mouse CD8$\alpha$ antibody (1:50). Anti-caspase 3, anti-CD4 and anti-CD8 antibodies were applied in 1% BSA at room temperature for 1 hour. A broad spectrum biotinylated secondary antibody was added and incubated for 1 hour. Slides were then incubated with streptavidin–horseradish peroxidase conjugate for 30 minutes. Staining intensities of cleaved caspase 3, CD4 and CD8$\alpha$ from two tumor sections per treatment group were determined using ImageJ® software.

### 2.8.8. Flow cytometric analysis of tumor-infiltrated immune populations

Tumors were isolated from the animals directly after euthanasia. Tumor single-cell suspensions were obtained by mechanical disruption of the tissues and enzymatic digestion for 1 hour at 37ºC. Enzymatic digestion solution was prepared in RMPI medium with 0.5%BSA, 0.1% collagenase type II (LS004177, Worington), 0.1% dispase (LS02109, Worthington) and DNAase (LS002007, Worington). After digestion, the suspension was filtered through a 70 $\mu$m filter (BD Biosciences) to remove the debris. The obtained single-cell suspension was then stained with fluorochrome labeled antibodies and analyzed using a LSR Fortessa (BD Biosciences) and FlowJo software (Tree Star Inc.). Intracellular

staining was performed with the Inside stain kit (Miltenyi Biotec Biotec, Cat.# 130-090-477), according to the manufacturer's protocol.

**Lymphocyte panel:** CD3ε-APC (Miltenyi Biotec, Cat.# 130-109-240, mouse, clone: REA606 – 1:10), CD4-FITC (Miltenyi Biotec, Cat.# 130-109-419, mouse, clone: REA604 – 1:10), CD8a-PE (Miltenyi Biotec, Cat.# 130-109-247, mouse, clone: REA601 – 1:10), CD107b-APC-Cy7 (Miltenyi Biotec, Cat.# 130-106-292, mouse, clone: M3/84 – 1:10), CD279 (PD1)-Pac. Blue (Miltenyi Biotec, Cat.# 130-102-741, mouse, clone: HA2-7B1 – 1:10).

**T reg panel:** CD3ε-APC (Miltenyi Biotec, Cat.# 130-109-240, mouse, clone: REA606 – 1:10), CD4-FITC (Miltenyi Biotec, Cat.# 130-109-419, mouse, clone: REA604 – 1:10), CD25-PE (Miltenyi Biotec, Cat.# 130-109-051, mouse, clone: REA568 – 1:10).
Intracellular staining: FoxP3 -Vio515 (FITC), (Miltenyi Biotec, Cat.# 130-111-681, mouse, clone: REA788, 1:50).

**Cytokine panel:** CD3-PerCP-Cy5.5 (Miltenyi Biotec, Cat.# 130-109-841, mouse, clone: REA641 – 1:10), CD4-Pac. Blue (Miltenyi Biotec, Cat.# 130-110-310, mouse, clone: REA604 – 1:10), CD8a- APC-Cy7 (Miltenyi Biotec, Cat.# 130-109-328, mouse, clone: REA601 – 1:10). Intracellular staining: IL-2-PE (Miltenyi Biotec, Cat.# 130-110-180, mouse, clone: REA665, 1:10), TNF-α-APC (Miltenyi Biotec, Cat.# 130-109-767, mouse, clone: REA636, 1:10), IFN-γ-FITC (Miltenyi Biotec, Cat.# 130-109-768, mouse, clone: REA638,1:10).

**Myeloid Panel:** CD11c-FITC (Miltenyi Biotec, Cat.# 130-102-466, mouse, clone: N418 – 1:10), CD11b-APC-Cy7 (Miltenyi Biotec, Cat.# 130-109-288, mouse, clone: REA592 – 1:10), MHC Class II-Pac. Blue (Miltenyi Biotec, Cat.# 130-102-145, mouse, clone: M5/114.15.2 – 1:10) Gr-1-APC (Miltenyi Biotec Cat.# 130-112-307, mouse, clone: REA810 – 1:50).

### 2.9. Statistical Methods

Data are presented as mean ± standard deviation (SD) for *in vitro* assays and as mean ± standard error of the mean (SEM) for *ex vivo* and *in vivo* assays. Statistical analyses were performed with Student's t-test. Statistical significance in overall survival was determined with log-rank test using SigmaPlot software

(Systat Software Inc.). $P < 0.05$ was considered statistically significant. * $P < 0.05$; ** $P < 0.01$; ** $P < 0.001$.

# CHAPTER III

# Chapter III – Results

This chapter is adapted from the unpublished original manuscript:

## Dendritic cell-targeted nano-vaccines synergize with anti-PD-1/anti-OX40 immune checkpoint modulators for melanoma therapy

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(Ready for submission)

# Chapter III – Results

## 1. Synthesis and physicochemical characterization of PLGA/PLA nanoparticles and mannose-PLGA/PLA nanoparticles

To deliver tumor-associated antigens to DC, two different types of biodegradable polymeric particles were developed, PLGA/PLA nanoparticles (NP) and mannose-PLGA/PLA NP (man-NP) (**Figure 1A**). To prepare man-NP, mannose-grafted PLGA polymer was synthesized by amidation of terminal carboxylic acid groups of PLGA with mannosamine (254). The reaction was confirmed by $^{1}$H NMR. Mannose-PLGA $^{1}$H NMR spectrum (**Figure 2**) was compared with the individual $^{1}$H NMR spectra of mannosamine and PLGA. The multiplet signal between 3.2 and 3.8 ppm indicates the presence of mannose in mannose-PLGA polymer, as previously reported (248). NP and man-NP were prepared by the double emulsion solvent evaporation method with some modifications (249). NP were produced with a polymer blend of PLGA/PLA, whereas a mannose-PLGA/PLA mixture was used to prepare man-NP. The average hydrodynamic diameter of NP and man-NP was equivalent, ranging from 166 ± 5 nm and 181 ± 8 nm, depending on the entrapped molecules (**Table 1**), with low polydispersity index (PdI; 0.13 ± 0.04 to 0.18 ± 0.04). For both non-targeted and DC-targeted formulations, we obtained homogenous populations of spherical-shaped particles with slight roughness on the surface (**Figure 1B, C, D**) and near-neutral surface charge (**Table 1**).

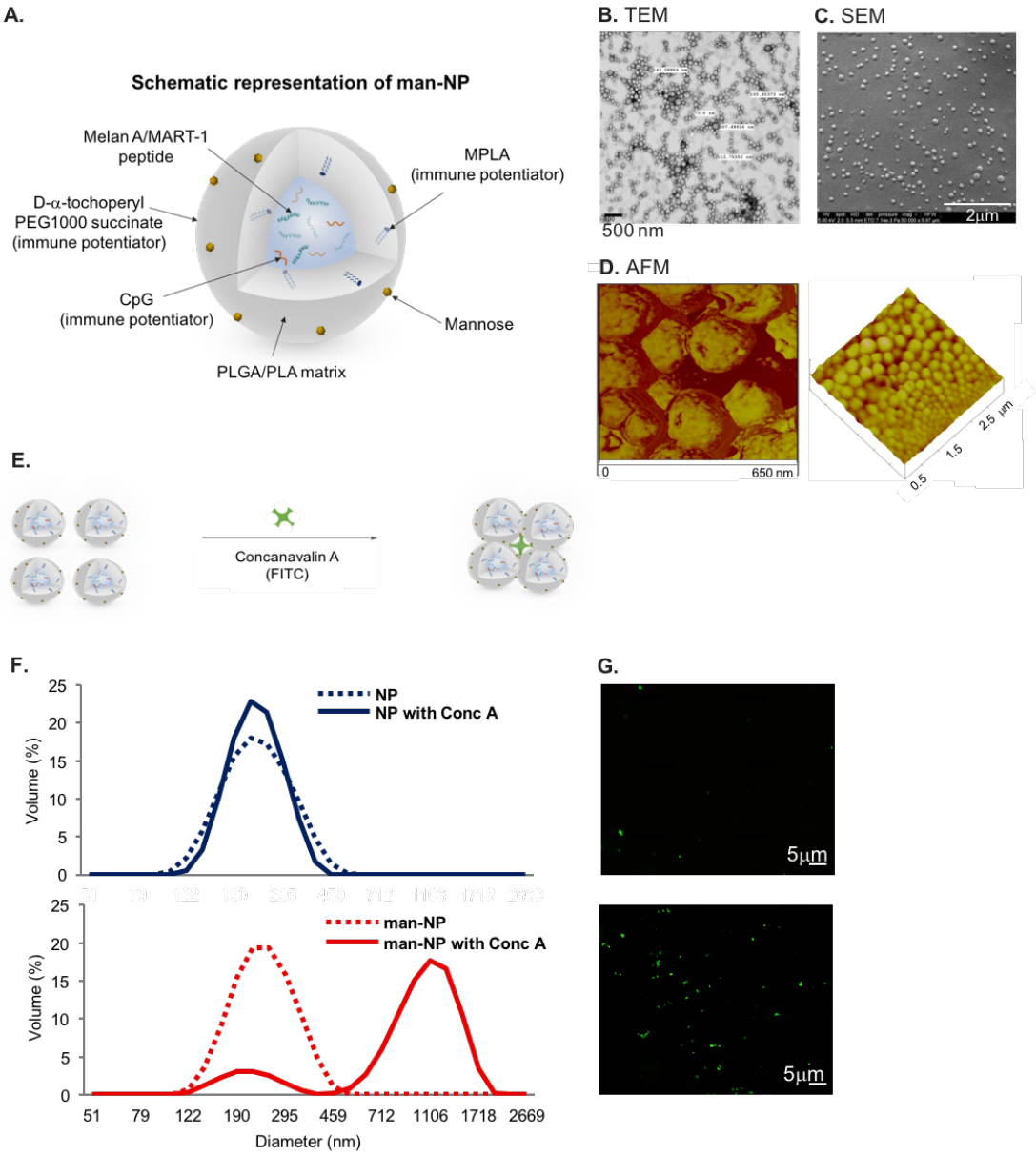


**Figure 1. NP and Man-NP are potential delivery systems for vaccination. A.** Left: Schematic representation of DC-targeted mannose-PLGA/PLA nanoparticles (man-NP). **B.** TEM image of spherical man-NP. **C.** SEM image of spherical man-NP. **D.** AFM images of spherical man-NP, showing narrow size polidispersity. **E.** Scheme of the mannose detection method using binding assay to Concanavalin A (Conc A). **F**. Particle aggregates detected by DLS after incubation of concanavalin A with control - NP (top) and DC-targeted man-NP (bottom). **G.** Fluorescence microscopy pictures of particle aggregates after incubation of FITC-labeled Conc A with NP (top) and targeted man-NP (bottom).

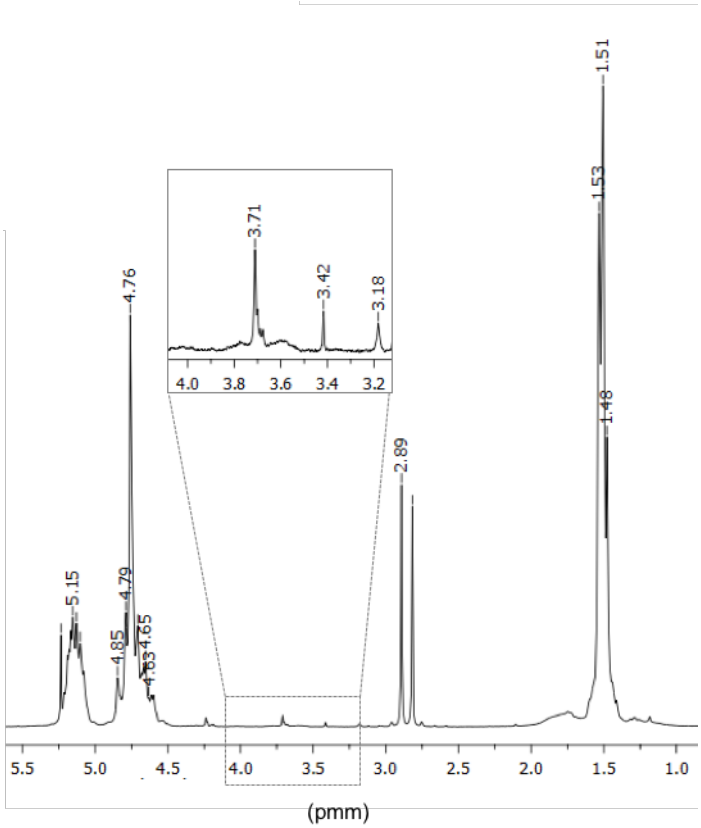


**Figure 2.** $^{1}$H-NMR spectrum of mannose-PLGA in $CDCl_3$.

**Table 1. Physicochemical properties of the particles**

| Particles[d] | Size[a] (nm ± SD[b]) | PdI[c] ± SD[b] | ζ-Potential (mV ± SD[b]) |
|---|---|---|---|
| NP (empty) | 168 ± 10 | 0.15 ± 0.05 | -2.17 ± 0.40 |
| NP MHCI-ag | 178 ± 6 | 0.16 ± 0.03 | -3.11 ±0.50 |
| NP MHCII-ag | 170 ± 5 | 0.18 ± 0.03 | -2.34 ± 0.65 |
| man-NP (empty) | 169 ± 16 | 0.13 ± 0.05 | -2.11 ± 0.40 |
| man-NP MHCI-ag | 181 ± 8 | 0.15 ± 0.04 | -3.02 ± 0.46 |
| man-NP MHCII-ag | 166 ± 5 | 0.18 ± 0.04 | -1.72 ± 0.47 |

[a] Z-average hydrodynamic diameter.

[b] SD, standard deviation.

[c] PdI, polydispersity index.

[d] CpG and MPLA were entrapped in all NP and man-NP, with exception of empty nanoparticulate systems.

For each formulation, we prepared 5 batches ($N = 5$) and measured in triplicate ($n = 3$).

The presence of mannose exposed on the surface of man-NP was confirmed by the Lectin Recognition Assay, using Concanavalin A (Conc A) (**Figure 1E**), and it was assessed by DLS and fluorescence microscopy. Conc A binds to mannose residues accessible on the particles' surface (251, 255). After incubation of man-NP with Conc A, we detected a shift in the hydrodynamic diameter to around 1000 nm, by DLS analysis (**Figure 1F**), which resulted from particle aggregation induced by Conc A. The formation of aggregates (in green) was confirmed by fluorescence microscopy, after incubation of man-NP with FITC-labeled Conc A (**Figure 1G**). For non-targeted NP, formation of aggregates was negligible after incubation with Conc A. We observed only a reduced number of aggregates by fluorescence microscopy and no shift in the hydrodynamic diameter was detected by DLS analysis. These results demonstrate the accessibility of the mannose residues on the surface of man-NP.

Levels of entrapment efficiency (EE) and loading capacity (LC) for MHCI-ag and MHCII-ag were similar in both NP and man-NP (**Table 2**). MHCI-ag and MHCII-ag were entrapped in different nanoparticulate systems, but always in combination with CpG and MPLA.

NP displayed higher loading levels for the short peptide MHCI-ag (EE >97.5 ± 0.2%, LC >48.8 ± 0.1 µg/mg) in comparison to those obtained for the MHCII-restricted peptide MHCII-ag (>74.6 ± 3.5% and >37.3 ± 1.7 µg). The EE of CpG was 81.4 ± 0.8% and 80.8 ± 2.5% in NP and man-NP, respectively, which corresponds to a LC of 8.1 ± 0.1 and 8.1 ± 0.3 $\mu$g/mg of the polymer blend.

**Table 2. Entrapment Efficiency (EE) and Loading Capacity (LC) of antigens in NP and man-NP**

| Particles | EE (% ± SD[a]) | LC (µg/mg ± SD[a]) |
|---|---|---|
| NP MHCI-ag | 99.1 ± 0.1 | 49.6 ± 0.05 |
| NP MHCII-ag | 82.4 ± 0.6 | 41.2 ± 0.3 |
| man-NP MHCI-ag | 97.5 ± 0.2 | 48.8 ± 0.1 |
| man-NP MHCII-ag | 74.6 ± 3.5 | 37.3 ± 1.7 |

[a] SD, standard deviation.

For each formulation, we prepared 4 batches ($N$ = 4) and measured in triplicate ($n$ = 3).

## 2. NP and man-NP do not affect DC viability and membrane integrity

The effect of increasing concentrations (125 $\mu$g/mL - 1000 $\mu$g/mL) of NP and man-NP on JAW SII DC viability was evaluated by Alamar Blue® for 48 h. NP and man-NP did not affect DC viability (>90%) in the concentration range tested (**Figure 3A**), which supports the physiological biocompatibility of both delivery systems. Physiological biocompatibility of NP and man-NP was also assessed *ex vivo* by red blood cells (RBC) lysis determination. Mouse RBC solution (2% m/v) was incubated with sequential dilutions of NP and man-NP. The extent of hemoglobin released from RBC was determined by absorbance measurements. NP and man-NP did not disrupt cell membranes at concentrations up to 20 mg/mL. The negative control (dextran) did not cause cellular lysis, whereas the positive control (sodium dodecyl sulfate; SDS) showed a dose-dependent lytic activity (**Figure 3B**).

## 3. DC-targeted man-NP are efficiently internalized by DC

JAW SII DC were incubated with Cy5.5-labeled NP or man-NP to measure cellular internalization using FACS. NP and man-NP internalization by DC increased with time. DC-targeted man-NP revealed much higher levels ($P$ = 0.001) of uptake by DC, when compared to non-targeted NP (**Figure 3C**). Particle internalization was confirmed by confocal microscopy (**Figure 3D**), supporting the FACS results. NP and man-NP that were produced with d-α-tocopherol polyethylene glycol 1000 succinate (TPGS) as surfactant and immune potentiator displayed internalization levels of about 150% higher compared to the same particles formulated with the surfactant poly(vinyl alcohol) (PVA) instead of TPGS (**Figure 4**).

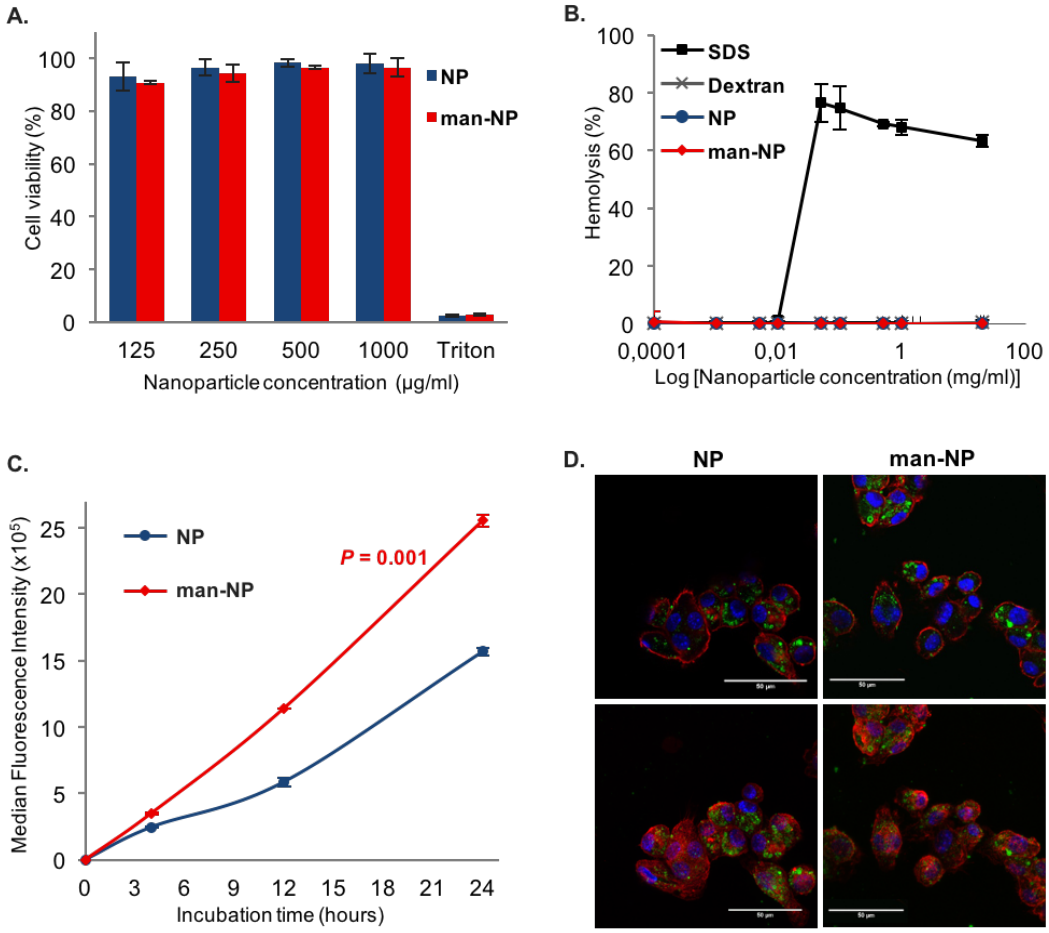


**Figure 3. NP and man-NP are biocompatible and efficiently internalized by DC. A**. Cell viability of DC after incubation of NP and man-NP for 48 hours. **B**. Red blood cells (RBC) lysis assay following 1 hour incubation. Sodium dodecyl sulfate (SDS) was used as a positive control and dextran as a negative control. **C**. Particle internalization by DC determined by FACS. Non-treated cells and non-labeled NP were used as negative controls. Data are presented as mean ± SD, $N$ = 4-6, from three independent experiments performed in triplicate. **D**. Confocal images of DC after 3 hours of incubation with NP (left) and man-NP (right). Z-stacks (top) and projections (bottom). ($N$ = 3; $n$ = 6) Scale bars = 50 µm.

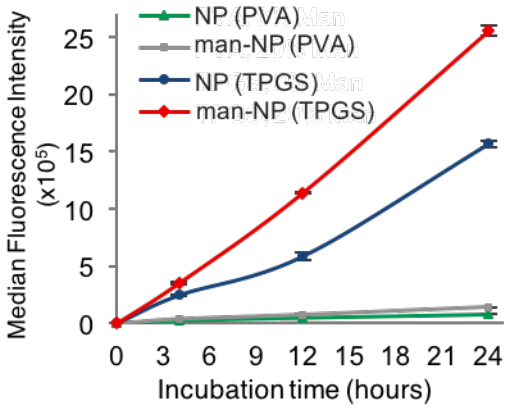


**Figure 4.** Intracellular uptake of NP or man-NP produced with PVA or TPGS after incubation with DC, determined by FACS.

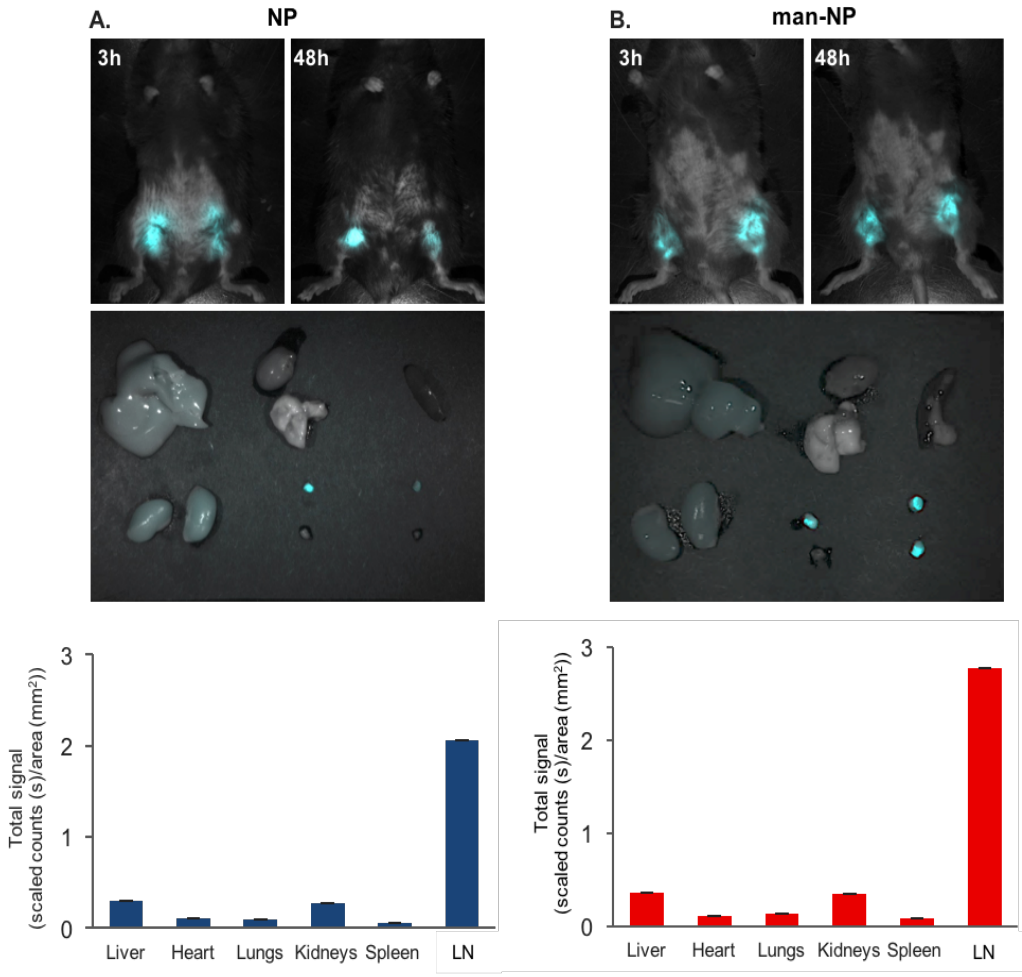


**Figure 5. NP and man-NP remain at the immunization site and accumulate preferentially in the lymph nodes**. Non-invasive intravital fluorescence imaging of C57BL/6 mouse 3 hours and 48 hours following hock immunization (*N* = 4) with NP (**A.**) and man-NP (**B.**). Data represent mean ± SD**.**

## 4. NP and man-NP accumulate preferentially in the lymph nodes

Mice (*N* = 4) received 100 μL of Cy5.5-labeled NP or man-NP in suspension (20 mg/mL) by subcutaneous (s.c.) injection of 50 μL in the right and left hind feet via hock immunization. Cy5.5 fluorescence signal was measured by non-invasive intravital imaging, showing that both NP and man-NP remained at the site of immunization 48 hours following injection. Major organs and popliteal and axillary LN were harvested at 24 and 48 hours after injection, and Cy5.5 fluorescence signal measurements revealed preferential accumulation of NP and man-NP in the LN. Residual accumulation was detected in the liver and kidney, due to the excretion of metabolic derivatives of NP and man-NP (**Figure 5**).

## 5. NP and man-NP vaccines induce splenocyte activation and cytotoxicity against melanoma cells

To determine the ability of our NP and man-NP vaccines to induce melanoma-specific immune responses and characterize Th1 and Th2 cytokine profiles, a 3-time immunization treatment, seven days apart was performed on C57BL/6 mice, according to the schedule in **Figure 6A**.

There was no evidence of considerable body weight change in all groups, attesting for vaccine tolerability and safety (**Figure 6B**).

Splenocytes of immunized mice were harvested 10 days following the last immunization and were re-activated in cell culture for 6 days with anti-CD28 and MHCI-ag alone or in combination with MHCII-ag. The groups immunized with NP and man-NP vaccines showed increased secretion of inflammatory cytokines, such as IFN-γ, a Th1 cytokine, and GM-CSF, when compared to control groups. In addition, the highest levels of these cytokines were obtained for groups immunized with the DC-active targeted man-NP. Indeed, the group immunized with man-NP MHCI-ag demonstrated extensive secretion of IL-2. Secretion of CCL1/TCA-3 and TARC/CCL17 revealed the same trend, suggesting that nanoparticulate vaccines played a major role in priming antigen-specific T cells towards the secretion of inflammatory chemokines (**Figure 6C**).

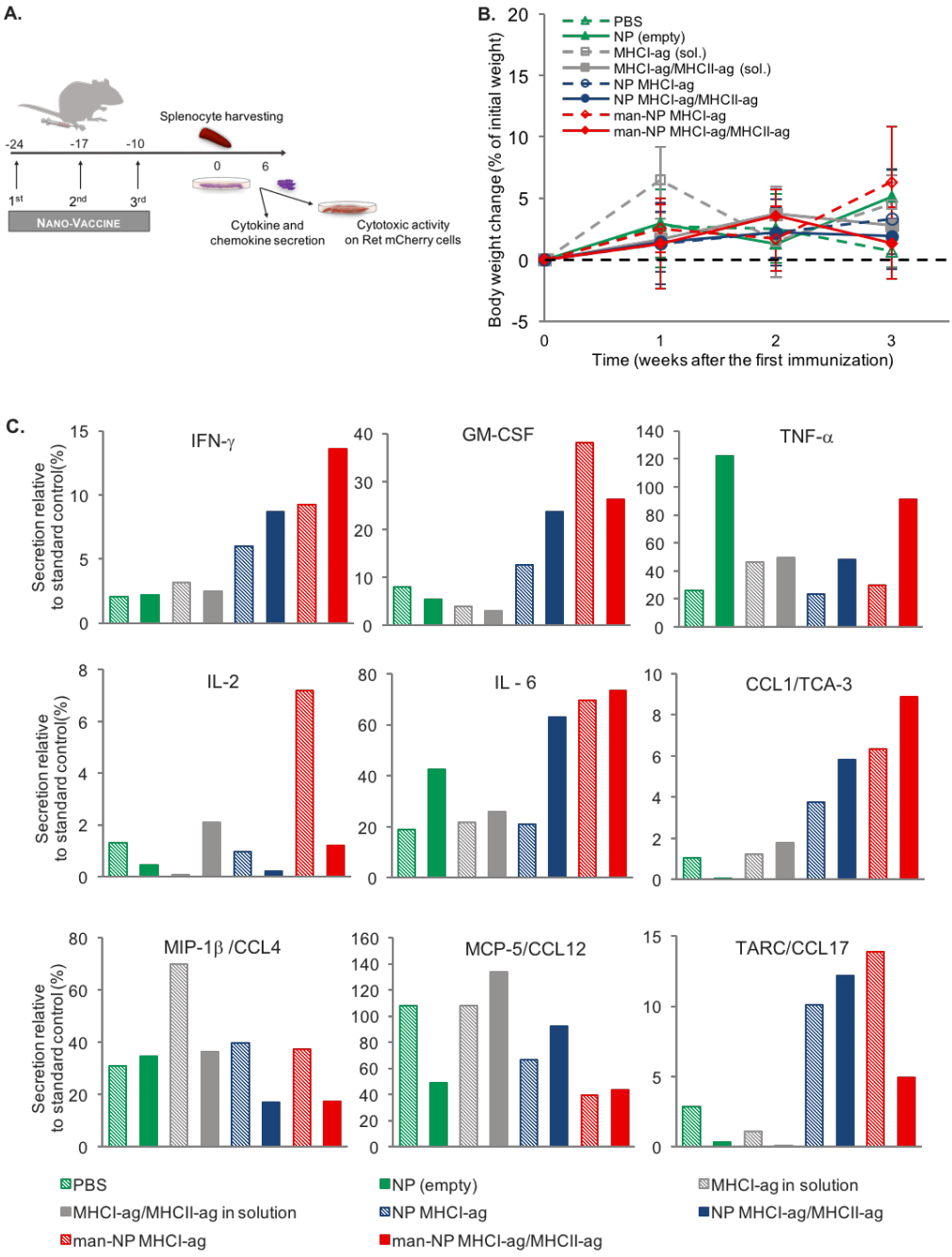


**Figure 6. NP and man-NP vaccines trigger cytokine secretion. A.** Immunization scheme of C57BL/6 mice and *ex vivo* splenocyte cytotoxic activity timeline. **B.** Monitoring body weight change, expressed as percentage of change from the first day of immunization. Data represent mean ± standard error of the mean (SEM). **C.** Secretion of IFN-γ, GM-CSF, TNF-α, IL-2, IL-6, CCL1/TCA-3, MIP-1β, MCP-5/CCL12 and TARC/CCL17 upon re-stimulation of splenocytes in culture.

Analysis of Th2 cytokines revealed that man-NP triggered secretion of IL-6, despite the delivery of MHCI-ag isolated (man-NP MHCI-ag) or in combination with MHCII-ag (man-NP MHCI-ag/man-NP MHCII-ag). On the other hand, regarding non-mannosylated NP, the secretion of IL-6 was only increased upon vaccination with those NP combining both MHCI-ag and MHCII-ag peptides (NP MHCI-ag/MHCII-ag). The administration of NP MHCI-ag/NP MHCII-ag and man-NP MHCI-ag/NP MHCII-ag decreased MIP-1β/CCL4 secretion, whereas DC-targeting either with man-NP MHCI-ag or with the combination man-NP MHCI-ag/man-NP MHCII-ag equally reduced MCP-5/CCL12 levels. These results indicate the role of nanoparticulate vaccines on the modulation of Th2 cytokine and chemokine secretion profile.

Besides the antigen recognition, capture and presentation to T cells, the major goal of a successful immunotherapeutic approach is to trigger antigen-specific cytotoxic CD8+ T cells (CTL) to recognize and destroy target cells. Reactivated splenocytes collected from mice immunized with man-NP MHCI-ag/man-NP MHCII-ag demonstrated the highest *ex vivo* cytotoxicity when co-cultured with mCherry-labeled murine Ret melanoma cells (**Figure 7A**). These data suggest that the immunization with man-NP MHCI-ag or man-NP MHCI-ag/MHCII-ag efficiently drove the immune responses towards antigen cross-presentation and consequent priming of CTL responses. Nevertheless, immunization with man-NP MHCI-ag/MHCII-ag triggered higher CTL activity when compared to man-NP MHCI-ag ($P = 0.014$) and the non-targeted NP co-entrapping both antigens with ($P = 0.02$) or without the immune check points (P= 0.002).

The immunization with DC-targeted man-NP demonstrated positive influence on the induction of CTL activity, with man-NP MHCI-ag/MHCII-ag leading to a dramatically stronger response when compared to NP MHCI-ag/MHCII-ag ($P = 9.6x10^{-6}$).

Interestingly, the antigen-specific killing activity obtained for groups immunized with NP combining both MHCI-ag and MHCII-ag was significantly lower ($P = 0.01$) than the one induced by NP containing only the MHCI-ag short peptide (**Figure 7A**).

Splenocytes from groups immunized with antigen/immune potentiators in solution or empty particles demonstrated cytotoxic activity equivalent to that observed for the splenocytes collected from the PBS-treated group (**Figure 7A**).

This prominent cytotoxic ability of T cells in the spleens of animals immunized with man-NP MHCI-ag/MHCII-ag is clearly evidenced by the increased green fluorescent staining related to caspase 3/7 activation, a marker of apoptosis (**Figure 7B**). These data overall suggest the successful induction of a systemic immune response.

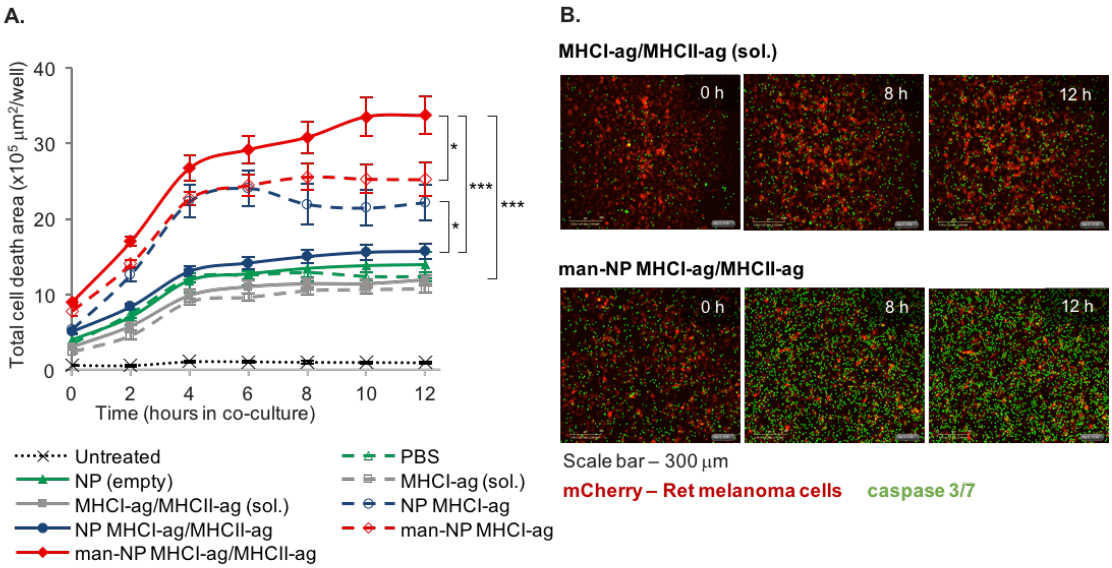


**Figure 7. NP and man-NP vaccines induce splenocyte activation and *ex vivo* cytotoxicity against melanoma cells. A.** Cytotoxic activity of splenocytes harvested from immunized C57BL/6 mice, after incubation with Melan-A/MART-1 and CD28 in solution for 6 days. Data are presented as mean ± SEM ($N = 6$). * $P < 0.05$; *** $P < 0.001$. **B.** Images of mCherry-labeled Ret melanoma cells co-cultured with reactivated splenocytes from the group immunized with MHCI-ag and MHCII-ag/immune potentiators in solution (top) and the group immunized with man-NP MHCI-ag/man-NP MHCII-ag (bottom). Cell death was detected with an apoptosis reagent that couples to activated caspase-3/7 recognition motif and quantifies apoptosis (in green).

## 6. Prophylactic nano-vaccines synergize with anti-PD-1/anti-OX40, restricting melanoma growth and prolonging survival

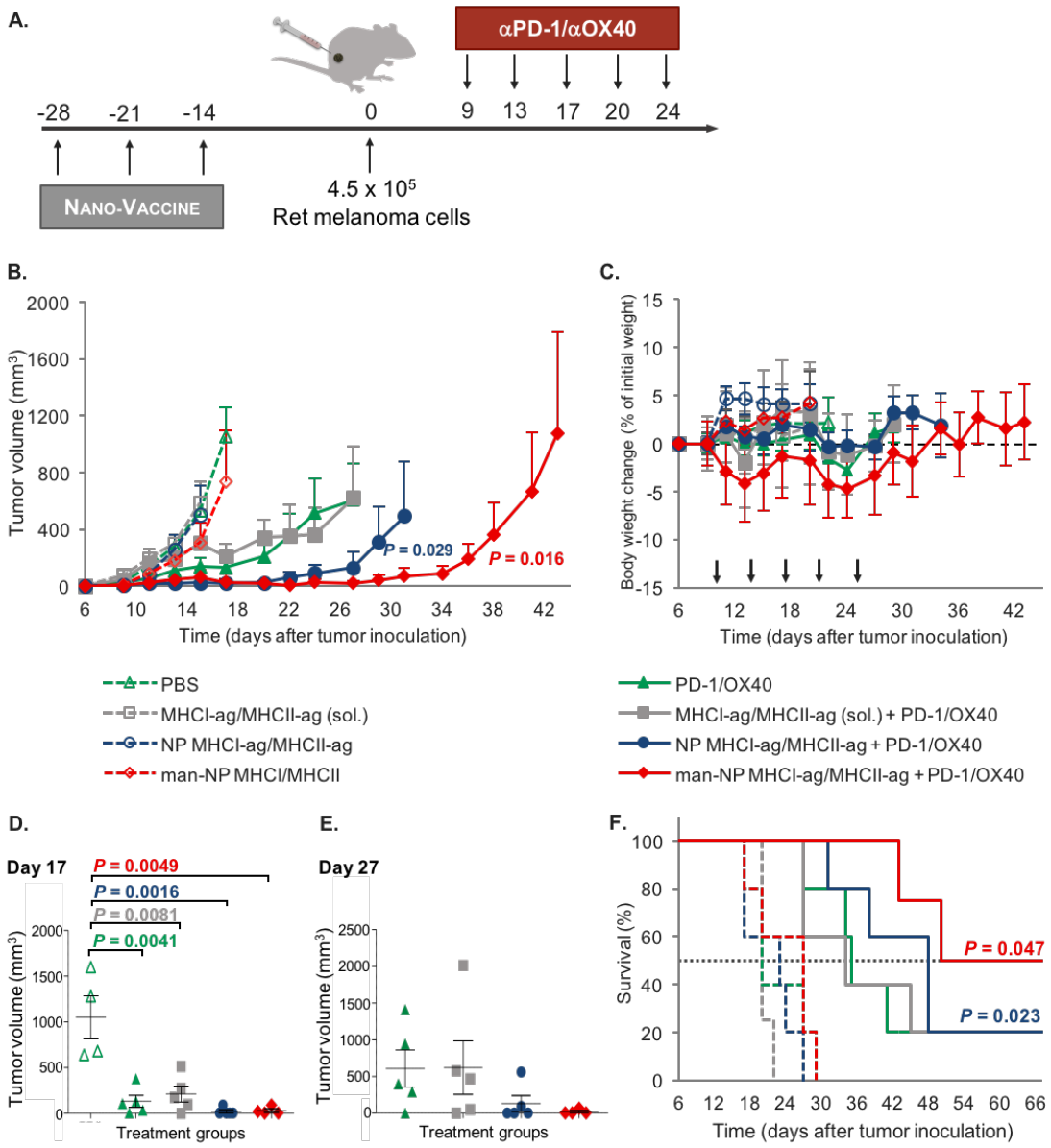


**Figure 8. Antigen/adjuvants-loaded polymeric cancer nano-vaccines have synergistic effect inhibiting melanoma growth and prolonging survival. A.** Timeline of immunization, tumor inoculation and immune checkpoint therapy. **B.** Tumor growth curve. *P* values at day 17 compared to PBS treatment. **C.** Body weight change, expressed as percent change from the day of treatment initiation. **D.** Tumor size at day 17 and **E.** Day 27 following tumor inoculation. Data are presented as mean ± SEM (*N* = 4-5). **F.** Kaplan-Meier overall survival over time graph for mice inoculated with 4.5 x $10^5$ murine Ret melanoma cells (*N* = 4-5).

Given the *in vitro* and *ex vivo* findings, we hypothesized that the immune response elicited by the developed nano-vaccines could provide a synergistic effect with PD-1 blockade and OX40 activation for melanoma therapy. Mice were treated according to the schedule in **Figure 8A**, receiving prophylactic antitumor vaccination before tumor inoculation. When PD-1/OX40 immune checkpoint therapy was combined with man-NP MHCI-ag /man-NP MHCII-ag given prior to tumor challenge, antitumor responses were strongly improved with marked tumor growth inhibition (**Figure 8B, D, E**) and minimal systemic toxicity (**Figure 8C**).

At day 17-post tumor cell inoculation, the tumor volume of all animals treated with anti-PD-1/anti-OX40, and those previously immunized with the nano-vaccine and further injected with those immune-check points, was similar and significantly different from those inoculated with PBS ($P < 0.0016$) (**Figure 8D**). However, at day 27 it was observed an increment in intra-group variability of tumor volume for all those treatment groups, with exception for the animals treated with the combination of man-NP MHCI-ag/ MHCII-ag + anti-PD-1/anti-OX40 (**Figure 8E**).

Of note, the animals treated with anti-PD-1/anti-OX40 and previously immunized with DC-targeted or non-targeted nano-vaccines, showed a more prolonged overall survival. DC-targeted man-NP MHCI-ag /man-NP MHCII-ag + anti-PD-1/anti-OX40 resulted in 100% of survival 42 days following tumor inoculation, whereas anti-PD-1/anti-OX40 alone provided only 20% of survival (**Figure 8F**). The combination of PD-1/OX40 with both MHCI-ag and MHCII-ag free antigens in solution (MHCI-ag/(MHCII-ag (sol.)) led to 40% of survival, for the same period of time. Mice treated with the combination man-NP MHCI-ag /man-NP MHCII-ag plus PD-1 blockade/OX40 stimulation showed survival rate of 50% two months following tumor inoculation (**Figure 8F**).

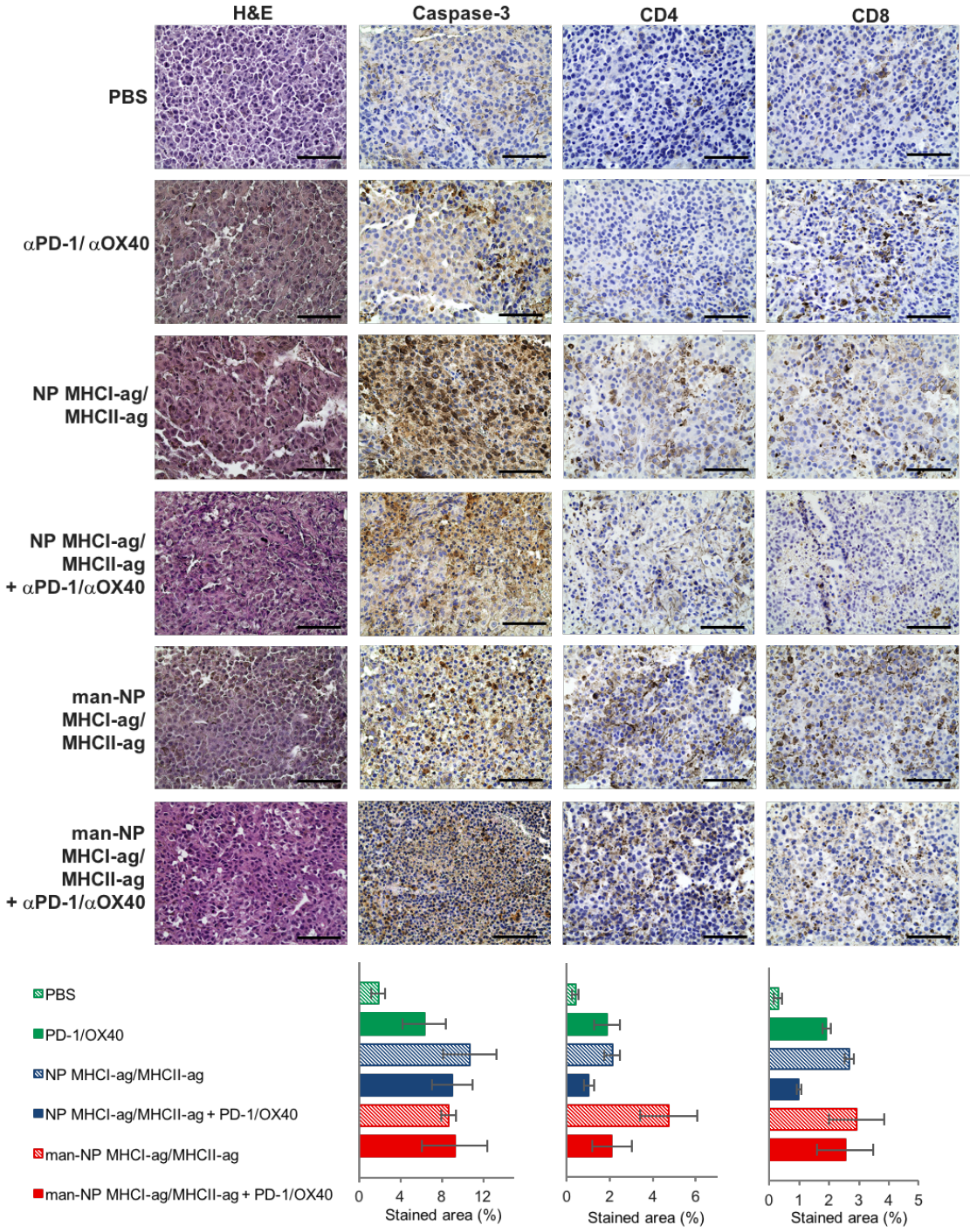


**Figure 9. Caspase-3 activity and TIL in melanoma tumor sections. A.** Images representative of murine Ret melanoma histology sections at endpoint with H&E and immunohistochemical staining for caspase-3, CD4 and CD8, of multiple fields. Scale bars represent 100 μm. **B.** Determination of the percentage of staining. Data are presented as mean ± SD ($N>3$, $n=4$). * $P<0.05$; ** $P<0.01$.

Immunohistochemistry staining revealed high levels of caspase-3 and tumor-infiltrating $CD4^+$ and $CD8^+$ T cells, both in groups treated only with the nano-vaccine and in the groups which further received the anti-PD-1/anti-OX40 combination (**Figure 9**). The tumors of animals treated with the anti-PD-1/anti-OX40 showed higher infiltration levels of $CD4^+$T cells than those that received both antibodies and DC-non-targeted vaccine immunization. On the other hand, higher infiltration of $CD8^+$ T cells was observed within the tumors of animals immunized with DC-targeted NP and further treated with the immune checkpoint therapy than in those that received the non-targeted vaccination combined with antibodies.

## 7. Combination of therapeutic nano-vaccines with anti-PD-1 and anti-OX40 immune checkpoint therapy

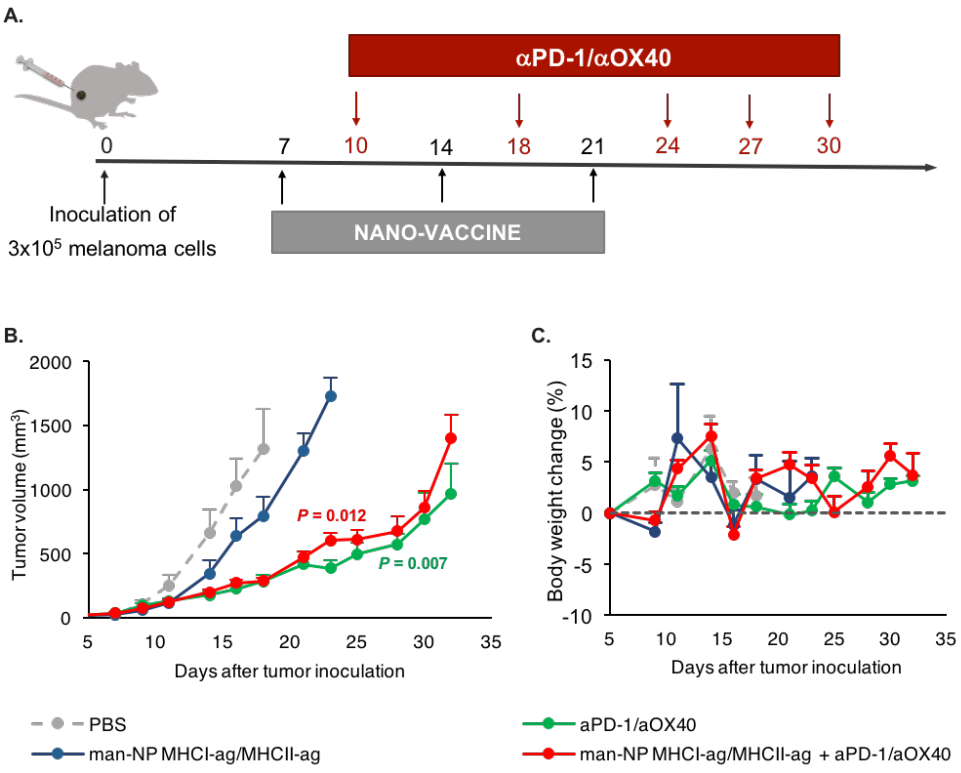


**Figure 10. Combination of nano-vaccines with anti-PD-1 and anti-OX40 immune checkpoint therapy. A.** Timeline of tumor inoculation and treatments. **B.** Tumor growth curve. *P* values correspond to tumor volume at day 18. **C.** Body

weight change, expressed as percent change from the day of treatment initiation. Data are presented as mean ± SEM ($N = 7$).

Motivated by the remarkable synergism observed with the combination of prophylactic man-NP MHCI-ag/man-NP MHCII-ag nano-vaccines with the immune checkpoint therapy with anti-PD-1 and anti-OX40, we hypothesized whether the same effect could be obtained by combining PD-1/OX40 immune checkpoint therapy and the same man-NP MHCI-ag /man-NP MHCII-ag nano-vaccines, administered after tumor inoculation, in a therapeutic scheme. Treatments were performed in accordance with **Figure 10A**. All the groups received the respective treatments on days 7, 14 and 21 after tumor inoculation. The group treated with the combination of nano-vaccine and anti-PD-1/anti-OX40 therapy, received anti-PD-1/anti-OX40 therapy three days after each immunization, on days 10, 17 and 24, accordingly.

In groups treated either with anti-PD-1/anti-OX40 only or the combination man-NP MHCI-ag /man-NP MHCII-ag and anti-PD-1/anti-OX40, the average tumor volume at day 18 was equivalent and significantly lower compared to the group treated with PBS ($P < 0.0012$), with negligible systemic toxicity observed.

Although the average tumor volume of the group treated with anti-PD-1/anti-OX40 was similar to the group treated with the nano-vaccine in combination with anti-PD-1/anti-OX40, at day 18 after tumor inoculation, the levels of T cell infiltration (**Figure 11A**) were superior for tumors isolated from animals that received the combination treatment. Compared to other groups, the combination of nano-vaccine and anti-PD-1/anti-OX40 induced the highest level of $CD8^+$ T cells in tumor infiltrates ($P < 0.001$) (**Figure 11B**). Even though the nano-vaccine alone induced only a slightly increase of T cell enrichment, compared to PBS-treated animals, its combination with PD-1/OX40 immune checkpoint therapy seems to enhance the infiltration of T cells, leading to higher levels of TIL then those quantified in the group treated only with anti-PD-1/anti-OX40. These data support the synergism previously observed. Despite the lower levels of tumor-infiltrating $CD4^+$ T cells tumor infiltrates observed for the treatment with anti-PD-1/anti-OX40 at day 18 (**Figure 11C**), most of those $CD4^+$ T cells

were CD4 effectors (**Figure 11D**), with reduced levels of T regs (**Figure 11E**). In addition, tumor infiltrates of the groups treated with the nano-vaccine only and the combination nano-vaccine and anti-PD-1/anti-OX40 revealed higher levels of T regs compared to animals that received only anti-PD-1/anti-OX40.

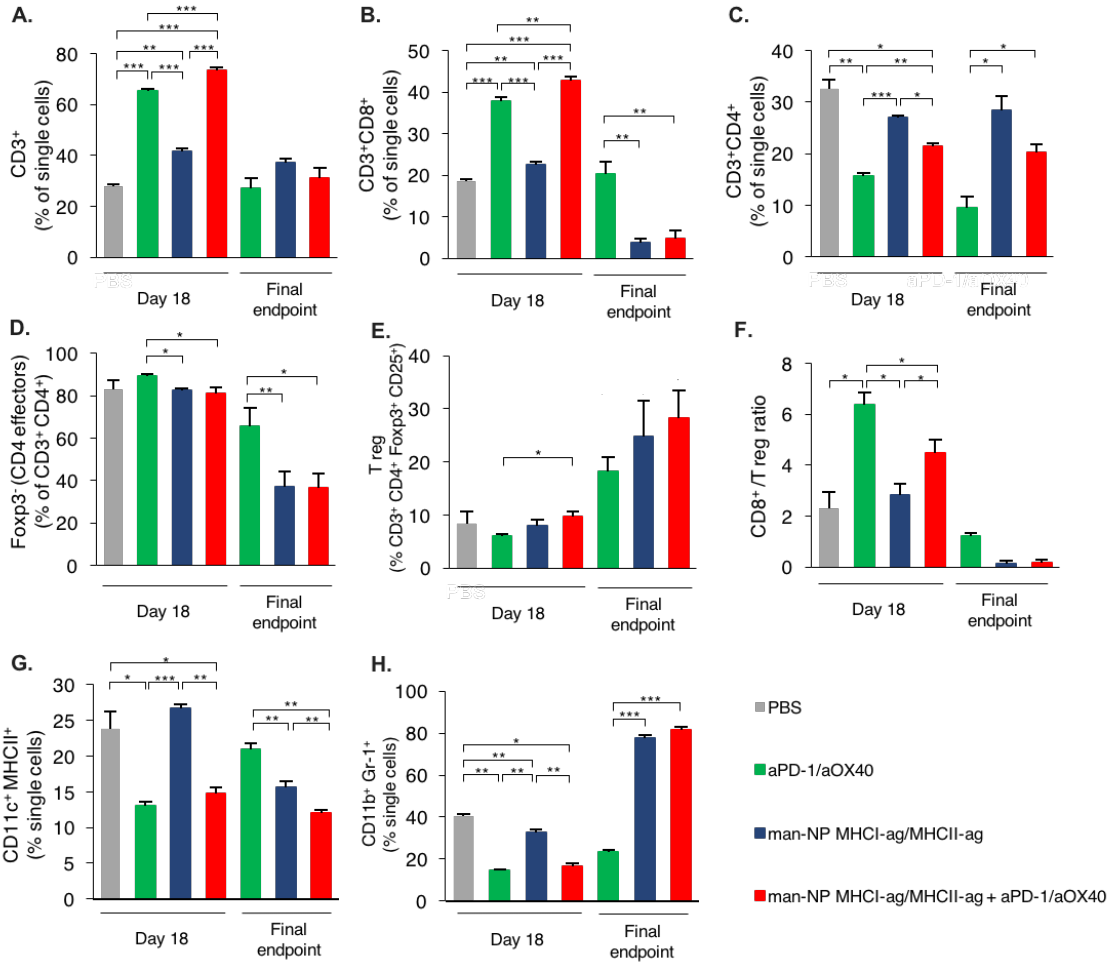


**Figure 11. Low CD8⁺:T reg ratio and high infiltration of Myeloid-derived Suppressor Cells (MDSC) are associated to increased tumor growth. A.** Tumors were isolated on day 18 after tumor cell inoculation (at least 3 mice/group) and when reached the volume for final endpoint. Quantification was performed by flow cytometry. Data are presented as mean ± SD of 3 independent replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Consequently, compared to PBS treated-group, the CD8:T reg ratio was the highest for anti-PD-1/anti-OX40 treatment, followed by the combination nano-vaccine with PD-1/OX40 immune checkpoint therapy (**Figure 11F**). At final

endpoint, even though the levels of T regs were equivalent, the CD8:T reg ratio remained significantly higher for anti-PD-1/anti-OX40 group and it was evident a large decrease on the infiltration of $CD8^+$ T cells for the group treated with the combination scheme. The CD8:T reg ratio of the groups treated only with vaccine only or with the combination of nano-vaccine with the PD-1/OX40 immune checkpoint therapy was seven times lower compared to animals that received only anti-PD-1/anti-OX40 ($P < 0.01$).

Regarding the infiltration of $CD11c^+MHCII^+$ APC, at day 18, levels were higher for groups treated with PBS and nano-vaccine only. APC levels within the tumor tend to decrease with time for groups treated with nano-vaccine only and with the combination nano-vaccine with PD-1/OX40 immune checkpoint therapy (**Figure 11G**). On the other hand, infiltration of $CD11b^+$ $Gr\text{-}1^+$ Myeloid-derived suppressor cells (MDSC) tends to increase, from day 18 to the final endpoint (**Figure 11H**). Remarkably, at the final endpoint, the levels of MDSC were surprisingly elevated for groups that received the nano-vaccine, either alone or in combination with anti-PD-1/anti-OX40 therapy, being approximately four times higher than those quantified in the tumors of animals treated only anti-PD-1/anti-OX40. The extremely elevated levels of MDSC seem to correlate with the marked decrease of $CD8^+$ T cell infiltrates and with the high percentage of T reg at the final endpoint in the two groups treated with the nano-vaccine. This contributes to lower CD8:T reg ratios at final endpoint and, consequently, higher tumor growth rate.

# CHAPTER IV

Discussion

# Chapter IV – Discussion

This chapter is adapted from the unpublished original manuscript:

**Dendritic cell-targeted nano-vaccines synergize with anti-PD-1/anti-OX40 immune checkpoint modulators for melanoma therapy**

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(Ready for submission)

Discussion

# Chapter IV – Discussion

The recent clinical validation of different immune checkpoint modulators for cancer treatment has revitalized this field, but alternative combination schemes are urgently needed to overcome immune checkpoint therapy resistance and increased intensity and frequency of serious side effects (256).

Here, we report the synergism of cancer nano-vaccines with PD-1 blockade and OX40 triggering. Non-functionalized NP (NP) and mannose-grafted NP (man-NP) were designed and synthesized for the *in vivo* delivery of combinations of melanoma antigens and TLR agonists to DC via passive and active targeting, respectively.

Both formulations were biocompatible and yielded particles with similar morphology and physicochemical characteristics in terms of size, PdI, surface charge, EE and LC of both antigens and immune potentiators. When NP and man-NP were injected s.c. by hock immunization, they remained near the local of injection, which may anticipate an extended contact with DC at the periphery, as well as proximity to the popliteal and axillary lymph nodes. This may explain the obtained preferential accumulation of the nano-vaccines in these tissues, which is extremely important for the induction of antigen-specific adaptive immune responses and vaccine efficacy (257). In addition, nano-vaccine mean average diameter lower than 200 nm is suitable for their trafficking through the lymphatic drainage directly to those lymphoid organs (258). Despite the similar biodistribution profiles, levels of particle internalization by DC were significantly superior for functionalized man-NP ($P$ = 0.00067), which is attributed to their increased affinity to mannose receptors at DC surface (225). The presence of mannose residues on the surface of these particles was corroborated by the formation of aggregates in the presence of Conc A. An active targeting of APC was thus anticipated for man-NP, promoting an extensive internalization of their payload to target cells.

We showed that NP and man-NP carrying melanoma-associated antigens and TLR agonists induced splenocyte activation and cytotoxic activity against melanoma cells. The particulate nature of the nano-vaccines played a pivotal role

in the process, as the antigens and immune potentiators induced residual immune response when injected in solution. This effect was expected as the co-delivery of tumor-associated antigens and TLR ligands by a nanoparticulate carrier was previously shown to enhance antigen internalization, processing and subsequent presentation, which is a key step to overcome host tolerance to tumor cells by improving effective T-cell priming and lymphocyte expansion (251).

The TLR synergy has been recently reported (259) and their activation on DC plays a crucial role in the induction of tumor-specific T-cell responses by enhancing the expression of co-stimulatory molecules (e.g. CD40, CD80, CD86) at the surface of those APC (260). This is highly promising for cancer immunotherapy due to the common absence of those co-stimulatory molecules within TME. Therefore, by entrapping both CpG and MPLA TLR agonists within a polymeric matrix, in contrast to their administration in a solution, our nanoparticulate system allowed the multi-targeting TLR agonist co-stimulatory effect due to the simultaneous engagement of TLR9 at the endosomal compartment and TLR4 at cellular membranes, respectively. If successful, the co-delivery to a single DC may thus potentiate the efficient maturation of DC towards balanced Th1- and Th2-type immune responses, which have a crucial role in controlling homeostasis within tumor site, especially regarding events related to tumor inflammation and angiogenesis (261, 262). Therefore, the characterization of the Th1/Th2 cytokine profile induced by the developed nano-vaccines may add further insight into their impact on the modulation of T-cell activity and functionality under steady-state physiological conditions.

Immunization with NP or man-NP vaccines triggered the secretion of a differential cytokine profile. Upon re-stimulation of the splenocytes harvested from those groups, with MHCI-ag or MHCI-ag/MHCII-ag, a significant increase in the secretion of IFN-$\gamma$ and GM-CSF was observed. These molecules are associated with enhanced antigen priming and subsequent presentation by APC (263, 264), as well as cytotoxic T cell activity. IFN-$\gamma$ is extensively produced by CD8+ T cells and it is a predictor of antigen-specific cytotoxic T-cell mediated responses (265). $CD8^+$ T-cell augmented activity in groups immunized with NP or man-NP co-entrapping antigens and immune potentiators is also supported by

the increased secretion of CCL1/TCA-3 and TARC/CCL17. Of note, these chemokines are associated to activated T cells (266, 267). Although the secretion of IL-2 was strongly improved only by man-NP MHCI-ag, TNF-$\alpha$ secretion was clearly augmented in animals immunized with man-NP MHCI-ag/man-NP MHCII-ag. An inclusive assessment of the triad IFN-$\gamma$, IL-2 and TNF-$\alpha$ predicted an improved CTL activity for DC-targeted man-NP MHCI-ag/man-NP MHCII-ag. It was also observed that NP and man-NP had an important role in increasing the secretion of IL-6, which is involved in the recruitment and differentiation of T lymphocytes, as well as in the suppression of T regulatory cells (268, 269). Suppression of these cells is important for eliciting CTL cell activity triggered by our nano-vaccines.

In groups treated with MHCI-ag and MHCII-ag carried by NP or man-NP, the secretion of MIP-1$\beta$/CCL4, a promotor of the activation and proliferation of T reg, was decreased. Similarly, for groups immunized with man-NP MHCI-ag or man-NP MHCI-ag/man-NP MHCII-ag, a reduction of CCL12 secretion was also observed. The inhibition of CCL12 has been correlated with intra-tumoral decrease of T regulatory cells, reduction of tumor angiogenesis and enhanced antitumor efficacy when administered in combination with cancer vaccines in a malignant mesothelioma (270, 271).

Cytokine secretion by splenocytes of immunized animals was clearly associated with the strong CTL activity obtained against melanoma cells *ex vivo*. Animals treated with man-NP co-entrapping both MHCI- and MHCII-restricted melanoma-associated antigens with immune potentiators led to an increased and robust CTL activity against murine Ret melanoma cells *ex vivo*. The engagement of synergistic MHCI and MHCII responses has been associated to sustained $CD8^+$ cytotoxic T-cell responses (272), but the benefit of immunization with MHC class II peptides remains elusive after clinical trials (273, 274). Some clinical trials have reported that the addition of MHC class II peptides did not improve clinical outcome and pointed that an increased $CD4^+CD25^+$ T reg activity or increased apoptosis of $CD8^+$ activated T cells may be responsible for the poor overall efficacy (273, 274). Actually, we obtained a slight enhancement of antitumor efficacy in animals immunized with NP or man-NP carrying both MHCI-ag and

MHCII-ag peptides, when compared to NP or man-NP entrapping MHCI-ag, despite the potent cytotoxic activity observed *ex vivo*. Moreover, all animals immunized with NP or man-NP vaccines showed a modest tumor growth inhibition *in vivo,* which is however coherent with the poor to moderate efficacy and response reported for cancer vaccines due to tumor aggressiveness and immunosuppressive microenvironment (275).

The synergistic superior antitumor effect of prophylactic nano-vaccines with PD-1/OX40 therapy is highlighted by this body of work (**Figure 1**). Notably, the delivery of the MHCI-ag and/or MHCII-ag peptides in NP or man-NP was essential for the induction of a robust response in combination with PD-1/OX40 immune checkpoint therapy, as the administration of those peptides in solution led to a lower efficacy. The active DC-targeting with man-NP MHCI-ag/man-NP MHCII-ag in a prophylactic scheme resulted in the most robust antitumor effect, leading to prolonged survival. It was previously reported a superior antitumor effect obtained with the combination of OX40 with CpG in a melanoma model (276). However, CpG-mediated antitumor immune stimulation required the intra-tumoral administration of this oligonucleotide. Our delivery system constitutes a promising approach for the targeted delivery of this oligonucleotide, as our *ex vivo* and *in vivo* studies support the successful protection of CpG once entrapped within our nano-vaccine polymeric matrix.

We observed that the combination regimen of anti-PD-1/anti-OX40 and nano-vaccine was only associated with minimal reversible systemic toxicity, reflected by slight body weight changes relative to the initial body weight.

Tumors collected from groups treated with the combination of anti-PD-1/anti-OX40 with NP or man-NP entrapping both antigens demonstrated an extensive infiltration by $CD8^+$ T cells, a known good prognosis for immune checkpoint therapy response. In addition, increased caspase-3 levels were obtained in these tumors, correlating with the superior T lymphocyte cytotoxic activity against melanoma cells observed *ex vivo*.

The remarkable results obtained by the combination of the man-NP MHCI-ag/MHCII-ag prophylactic nano-vaccine with PD-1/OX40 immune checkpoint therapy encouraged the design of a novel therapeutic strategy involving the same

nano-vaccine, but in a therapeutic scheme, combined with the pair anti-PD-1/anti-OX40. The use of a therapeutic nano-vaccine would increase the applicability of the treatment strategy and also the chances of its translation to the clinic. Previous studies combining the administration of cellular vaccines with immune checkpoint therapies have indeed led to promising outcomes under clinical settings against several malignant diseases, such as pancreatic cancer (277), colorectal cancer (278), prostate cancer (279) and melanoma (280). Our therapy strategy could offer a clinical alternative to those therapies with autologous DC, which are associated to complex, long and expensive procedures (281).

In the beginning of the treatment, it is clear that the combination of man-NP MHCI-ag/MHCII-ag with PD-1/OX40 immune checkpoint therapy increased the infiltration of $CD8^+$ T cells, which are important for the generation of a cytotoxic immune response against melanoma tumor cells. However, with time, levels of $CD8^+$ T cell within the tumor tend to decrease, with increasing infiltration of T reg. This led to a very low CD8:T reg ratio, indicating an immunosuppressive tumor microenvironment, which limits the expected antitumor immune response. In parallel, MDSC infiltrated significantly in the tumor microenvironment, which has been associated to decreased infiltration of $CD8^+$ T cells and hampered cytotoxic activity (282, 283). Man-NP MHCI-ag/MHCII-ag therapeutic nano-vaccines seem to induce infiltration of MDSC over time, which may have hindered the early effect of $CD8^+$ T cell stimulation, and proliferation, inhibiting T cell infiltration and cytotoxic activity. Due to this imbalance, we can anticipate that, favoring an immunosuppression within the tumor microenvironment, the combination of man-NP MHCI-ag/MHCII-ag with PD-1/OX40 immune checkpoint therapy failed to show benefit in comparison with PD-1/OX40 immune checkpoint in monotherapy.

Nevertheless, altogether, these findings bring new and important insights about the synergism of immune checkpoint targeting with polymeric nanoparticle-based cancer vaccines, as well as the effect on the modulation of immune cell infiltrates within the tumor microenvironment in melanoma. Therefore, polymeric nano-vaccines gain more and more space as promising tools and potential off-

the-shelf products to enhance immune checkpoint clinical outcomes in melanoma therapy.

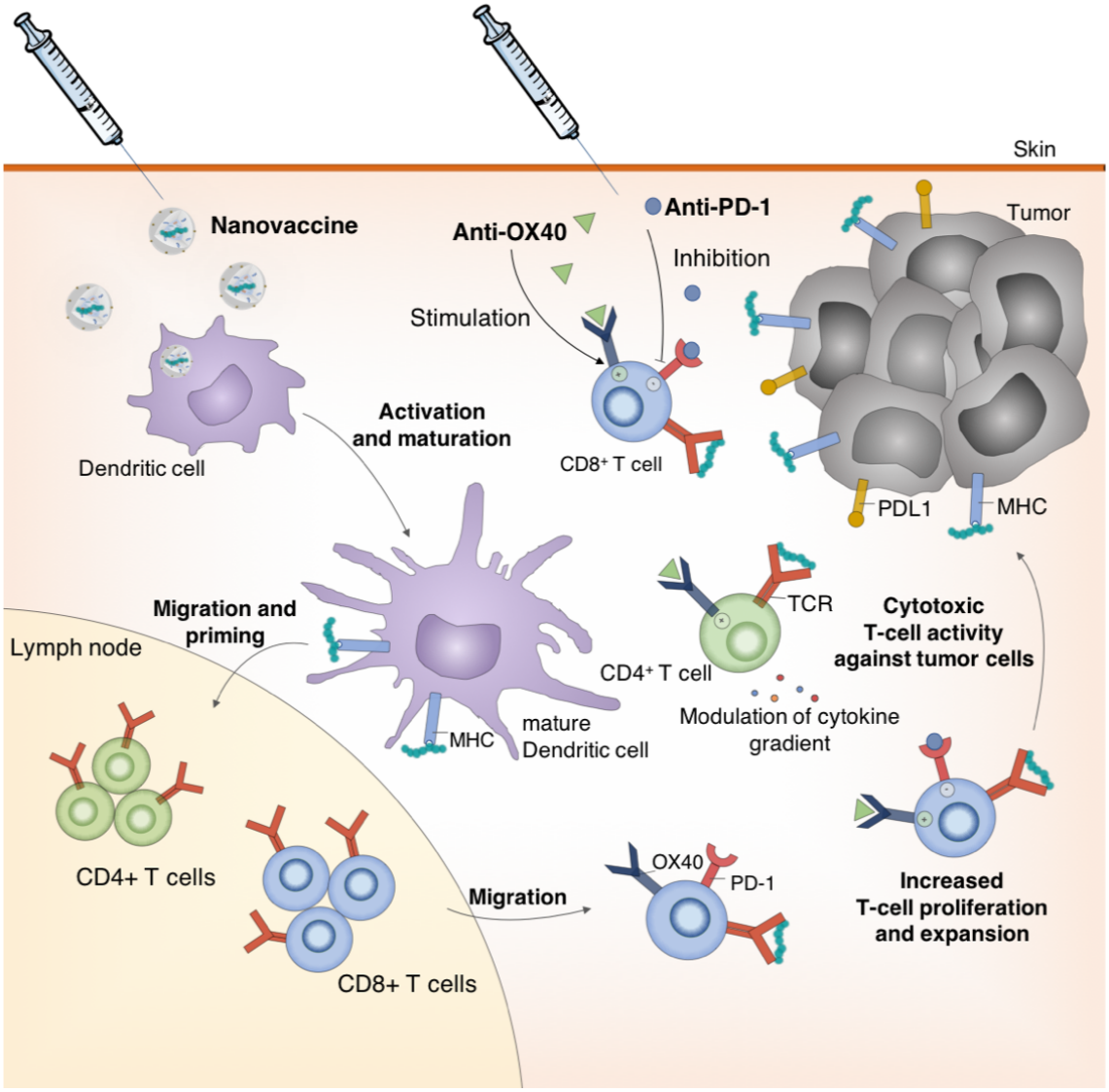


**Figure 1.** Proposed model for the strategy combining nano-vaccines with anti-PD-1/anti-OX40 immune checkpoint therapy.

# CHAPTER V

# Chapter V – Conclusions and Future Perspectives

The findings described in this thesis support the role of biodegradable aliphatic polyester-based NP, as promising cancer vaccine candidates to synergize with immune checkpoint therapy and improve melanoma therapeutic outcomes. On the basis of this synergism, it is the ability of these polymeric NP to target DC and subsequently prime antigen-specific T cell responses, by delivering both tumor antigens and immune potentiators within a single platform. The groundbreaking findings in the cancer immunology, in recent years, have helped to unveil the intricate network of mechanisms behind the immune modulation of cancer progression. These findings, together with the astonishing fast pace of this research field, have boosted great development of immunotherapy and seeded hope for cancer treatment, in particular for melanoma, which had very limited therapeutic options for several decades. However, despite the early promising results of these innovative immunotherapies, known as immune checkpoint modulators, main clinical trials have reported therapy resistance and low percentages of long term overall survival. This strongly points out that preclinical model systems are frequently poorly predictive of clinical challenges, with promising preclinical outcomes often not translating into patient survival benefits. This may be attributed, not only to the differences between slow-growing clinical disease and the fast-growing transplantable tumors, regularly used in murine models, but also to the fact that murine models fail to reflect the high heterogeneity and related inflammatory environment of human malignancies.

In response to poor clinical outcomes of immune checkpoint modulators, in the majority of the patients, alternative strategies, combining different immune checkpoint agents, have been proposed and further research has been developed for the identification of novel targets. Antitumor vaccines also emerged as interesting tools for the improvement of those therapeutic outcomes, once they can increase tumor-associated antigen recognition, processing and presentation to effector T cells. Cancer vaccines may be the key to favorably shift the equilibrium between tumor-induced immunosuppression and antitumor immunity,

overcoming therapy resistance. Additionally, antitumor therapeutic vaccines may be an ideal player in the prevention of micrometastases and tumor relapse through the activation of long-term immune mechanisms and consequent reduction of circulating cancer cells. In clinic, however, the use of antitumor therapeutic vaccine in monotherapy regimens has generally resulted in unclear outcomes and limited applications. The effect of cancer vaccines on tumor growth inhibition is modest and not always significant, but the reasons have always been ambiguous. Successful antitumor immunotherapeutic strategies should benefit from immune checkpoint targeting to revert immunosuppression and tolerance, opening pathway for the intervention of a strong antigen-specific immune response stimulated by cancer vaccination, which would be supported by limiting the activity of immunosuppressive cells. Moreover, the findings described in this thesis highlight that tumor infiltrated MDSC can be one of the major players, within the tumor microenvironment, responsible for the failure of cancer vaccines in restricting tumor growth and point new directions for improved immunotherapies. In fact, our studies also evidence that the depletion of MDSC may potentiate immunotherapy approaches, such as immune checkpoint modulators and cancer vaccination (284). Immunotherapeutic strategies could also profit from combination with targeted therapies, such as BRAF inhibitors and MEK inhibitors, which have shown to favor activity of immunotherapy (285).

These promising therapeutic strategies can definitely take advantage of polymeric NP as delivery systems, not only for cancer vaccination, but also as versatile platforms for the delivery of other agents for combination schemes. In fact, the progresses in the field of particulate delivery systems have emphasized appealing features of NP as tools for antitumor immunomodulatory approaches. For instance, by enabling the entrapment of different entities, such as tumor-associated antigens and immunostimulatory molecules, NP can help overcoming the down-regulation of MHC molecules that occurs in many cancer cells and that limits therapy efficacy. Also, due to the vast diversity of components present within the tumor microenvironment of solid tumors, the delivery of agents to modulate the immunoregulatory complex network through a unique platform constitutes an attractive strategy.

Particulate vaccines, in particular those based on aliphatic polyesters, have shown promising results and advantages over the currently available alternatives. The vast experience with aliphatic polyesters in biomedical applications has long demonstrated their safety and versatility for functionalization and modifications. However, before they can be translated to clinic, there are still several issues to be addressed. These concerns are related not only with the development of the vaccine, such as the selection of the most relevant tumor antigens and the optimal production method for these particulate systems, but also with aspects regarding unexpected biodistribution of the NP in the cellular and subcellular compartments and release profile. The regulatory entities have looked particulate cancer vaccines from two perspectives, considering the materials used in the production or the cancer vaccine. It is a fact that a number of polymeric delivery systems have already reached the clinic. However, in each specific case, a full assessment needs to be performed according to the application and its impact on overall clinical risk-benefit assessment. Regulatory agencies have issued guidelines to provide assistance for the development of therapeutic cancer vaccine, including several recommendations and considerations for these products. In 2016, EMA published the “Guideline on evaluation of anticancer medicinal products in man”, which contains a section concerning immune modulating compounds including tumor vaccines. Together with the “Clinical Considerations for Therapeutic Cancer Vaccines”, issued by the FDA in 2011, it is believed to have a major impact in the near future, in the translation of cancer therapeutic vaccines to clinic.

In this thesis, the successful design and development of biodegradable polymeric NP as anticancer nano-vaccines is reported. The combination of these nano-vaccines with immune checkpoint modulators already used in clinic shed light on the potential of these strategic approaches to overcome some of the limitations of immunotherapy. The use of NP enables the target of TAA and immune stimulators to APC, inducing antitumor immunity. Although this immunity is overcome by immunosuppressive mechanisms, it is shown the combination with immune checkpoint therapy potentiated antitumor responses, leading to

prolonged survival and tumor growth restriction. Besides, it is shown the significant value of NP for delivery of TAA and immune potentiators for eliciting antitumor immunity, in particular those functionalized with mannose. Also, the relevance of combining man-NP MHC class I- or class II-restricted antigens together with both immune potentiators is revealed, supporting the importance of activating both $CD4^+$ and $CD8^+$ T cell-mediated responses for improved efficacy. The determinant role of MDSC cells for restriction of immunotherapy efficacy is also revealed. This provides valuable insights into development of novel strategies that can remove brakes that, so far, have constrained better results in cancer immunotherapy. Particulate delivery systems, as nano-vaccines, will certainly be fundamental players in innovate strategies for remarkable achievements in cancer treatment.

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