Targeting and intracellular delivery of neoantigens for cancer immunotherapy

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Declaration of authorship

I, Bárbara Ibarzo Yus, hereby declare that the work presented in this thesis is my own. Where information has derived from other sources, I confirm that this has been indicated in the thesis.

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To my grandmas Ramona and Concha
for being my endless source of inspiration in life.

/ 

A mis abuelas Ramona y Concha,
por ser mi fuente de inspiracion y ejemplo en la vida.
Abstract

The development of efficient anti-tumour vaccines remains a challenge, with only one cancer vaccine approved for their use in the clinic in the last ten years. The inefficiency of cancer vaccines could be explained by the presence of an immunosuppressive environment within tumours, that render anti-tumour responses inactive, as well as a lack of efficient in vivo strategies for antigen and adjuvant targeting and delivery into dendritic cells (DCs). Here, polymer nanoparticles (polymersomes, POs) made of poly(2-(methacryloyloxyethylphosphorylcholine)-poly(2-(diisopropylaminoethyl methacrylate)) (PMPC-PDPA), are proposed as antigen carriers targeting DCs, with potential to act as anti-tumour vaccines. Using POs encapsulating ovalbumin (OVA) as antigen model, it was possible to validate in vitro their uptake by DCs and their potential to trigger antigen-specific CD8 T cell responses, without toxicity. Therapeutic immunisations performed in vivo in animals harbouring the B16-OVA melanoma model highlighted the potential of POs encapsulating OVA to delay tumour growth. This correlated with enhanced CD8 T cell infiltration in the tumour bed, expressing exhaustion-related receptors. Approaches combining PO vaccination and checkpoint inhibitors enhanced anti-tumour responses and delayed tumour growth rates further. In addition, the route of PO administration at the time of immunisation demonstrated influence over disease outcome, likely due to differential targeting of lymphoid organs and DCs. These differences were also present in animals harbouring a colorectal MC38 model following immunisations with an MC-38 derived neoantigen. Altogether, this study proves the possibility to enhance and shape anti-tumour responses utilising POs as cancer vaccine carriers, outperforming conventional and nanoparticle-free cancer vaccines.
Impact statement

The lack of early diagnostics and the absence of efficient treatments for cancer translates into a mortality rate of one every six people worldwide. Conventional interventions such as surgery, chemotherapy and radiotherapy are associated with adverse effects and do not constitute effective cures for every patient. Advancements in the understanding of anti-tumour immune responses have accelerated the development of immunotherapies to tackle cancer. These are aimed at boosting an individual’s immune response, especially CD8 T cells. One of the most successful examples is CAR-T cells, where patients CD8 T cells are extracted and genetically modified to specifically recognise tumour cells. Despite outstanding outcomes in CAR-T cell treated patients with blood malignancies, these are associated with severe toxicity effects including death, and do not pose any benefit against solid malignancies. Moreover, their production is expensive, hindering scale-up of the treatment. This emphasises the need for affordable, reliable, and scalable immunotherapies, that work in solid malignancies and across differing tumours. In this regard, therapeutic cancer vaccines would constitute an ideal alternative to cell-based therapies, being more affordable and feasible to produce, given that they have been on the market for years in context of other diseases. Indeed, the discovery of universal anti-tumour vaccines would constitute a shift in the cancer treatment paradigm without precedents. However, only one vaccine (Sipuleucel-T) has been FDA approved in the last ten years. Their inefficacy is a consequence of numerous factors including the presence of an immunosuppressive environment within the tumours, that render CD8 T cell responses inactive despite efficient immunisations. Therefore, current cancer vaccine strategies entail combinations with other immunotherapies named checkpoint inhibitors (i.e. \(\alpha\)-PD-1 or \(\alpha\)-CTLA-4), which reverse the dysfunctional state of CD8 T cells. These combinations have shown promise in many clinical trials, especially when immunisations are performed with tumour antigens known as neoantigens, whose amino acid sequence is notably different than those of healthy cells. In fact, the development of high-throughput sequencing technologies has accelerated the discovery of neoantigens candidates in both individual patients, allowing the production
of personalised vaccines; or potentially shared across patients, which opens an exciting horizon for the development of universal cancer vaccines.

Nonetheless, a critical step for the development of efficient vaccine immunisations involves their uptake by dendritic cells (DCs). For years, vaccines approaches involving *ex vivo* DC loading with tumour antigen have proved inefficient and expensive. In this regard, the use of nanoparticle-based vaccines can bypass this limitation and provide with enhanced immunisations *in vivo*. A potential nano-vaccine is described in this thesis, based on polymer nanoparticles (polymersomes, POs) entrapping neoantigens. POs can provide with stability and enhanced half-life to antigens or adjuvants entrapped within them in biological fluids. In addition, POs size and features can be tailored to mimic ligands that enhance antigen targeting and intracellular delivery into DCs, as well as improve their drainage to lymphoid organs, where CD8 T cell priming takes place. These considerations may tilt the balance from inefficient immunisations to successful anti-tumour responses, which could ultimately benefit thousands of patients.
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Signal Transduction and Targeted Therapy conference  
Sichuan, China/online due to covid- December 2022

International Symposium on Polymer Therapeutics  
Valencia, Spain- May 2022

British society of Nanomedicine International Conference  
Online due to covid- March 2021

**Poster presentations**

15th Symposium from the Institute of Bioengineering of Catalonia  
Barcelona, Spain- October 2022

Immunoochemistry and Immunobiology International Gordon Research Conference.  
Castelldefels, Spain- June 2022

UCL Chemistry Department PhD student symposium  
London- September 2020

**Workshops**

Eureka Virtual School for translational medicine Program- Cancer Research UK  
*A translational approach to oncology research*. Virtual due to covid- May 2022

ESPERO Entrepreneurial Skills workshop - UCL Innovation & Entreprise:  
*From business idea to business model*. London- Dec 2019
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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>Aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cell therapy</td>
</tr>
<tr>
<td>Adpgk</td>
<td>ADP-dependent glucokinase</td>
</tr>
<tr>
<td>Adpgk-LSP</td>
<td>ADP-dependent glucokinase-long synthetic peptide</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cell cytotoxicity</td>
</tr>
<tr>
<td>ALRs</td>
<td>Absent in Melanoma (AIM2) like receptors</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
</tr>
<tr>
<td>CAR-T</td>
<td>Chimeric antigen receptor T cell</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif chemokine ligands</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule 1</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>CTV</td>
<td>Cell trace violet</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>dLNs</td>
<td>Draining lymph nodes</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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</table>
EP - Electroporation
FAS - Fas Cell Surface Death Receptor
FASL - FAS ligand
FBS - Foetal Bovine Serum
FcR - Fc receptor
FH - Film hydration
FLT3L - FMS-like tyrosine kinase 3 ligand
FOXP3 - Forkhead box protein 3
G-CSF - Granulocyte colony-stimulating factor
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GITR - Glucocorticoid-induced tumor necrosis factor related protein
GZM - Granzyme
GZMB - Granzyme B
HIF-α - Hypoxia inducible factor α
HMGB1 - High mobility group protein B1
HSC - Haematopoietic stem cell
IC - Intracellular
ICI - Immune checkpoint inhibitor
ICOS - Inducible T cell costimulator
IFN - Interferon
Ig - Immunoglobulin
IL - Interleukin
ILC - Innate lymphoid cell
IRF3 - Interferon regulatory factor 3
irAEs - Immune related adverse effect
IS - Immunological synapse
IVIS - In vivo imaging system
I.P. - Intraperitoneal
I.V. - Intravenous
I.M - Intramuscular
LAG-3 - Lymphocyte activation gene-3
Le - Loading efficiency
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LSECTIN</td>
<td>Liver sinusoidal endothelial cell lectin</td>
</tr>
<tr>
<td>LSP</td>
<td>Long synthetic peptide</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>migDC</td>
<td>Migratory dendritic cell</td>
</tr>
<tr>
<td>MPLA</td>
<td>Monophosphoryl lipid A</td>
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<td>MTT</td>
<td>3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>NeoAg</td>
<td>Neoantigen</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer-T cell</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like (NOD-like) receptors</td>
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<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<td>NSCLC</td>
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<td>O/N</td>
<td>Overnight</td>
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<td>PAMP</td>
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<td>Platelet derived growth factor</td>
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<td>PDI</td>
<td>Polydispersity Index</td>
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<td>Programmed Cell Death Protein 1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>POs</td>
<td>Polymersomes</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>Polyinosinic : polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin-streptavidin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Rho</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic Acid-Inducible Gene-I (RIG-I) like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>S.C</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SLO</td>
<td>Secondary lymphoid organ</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour associated antigen</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour Associated Macrophages</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffer Saline</td>
</tr>
<tr>
<td>TCF1</td>
<td>Transcription factor cell factor 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Teff</td>
<td>Effector T cell</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>T_{FH}</td>
<td>Follicular helper T cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocyte</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T cell immunoglobulin and mucin domain-containing protein type 3</td>
</tr>
<tr>
<td>TLO</td>
<td>Tertiary lymphoid organ</td>
</tr>
<tr>
<td>TLR</td>
<td>Tool like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumour specific antigen</td>
</tr>
<tr>
<td>TOX</td>
<td>Thymocyte selection-associated HMG BOX</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform manifold approximation and projection</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vasculature endothelial growth factor</td>
</tr>
</tbody>
</table>
Glossary

- **Antigen**: Foreign molecule capable of being recognised by the immune system. Antigen presentation by antigen presenting cells in MHC complexes is necessary for the generation of adaptive immune responses.

- **Antigen Presenting cell (APC)**: Group of cells including monocytes, macrophages, dendritic cells and B cells, specialised on the processing and presentation of antigenic peptides in association with MHC-II molecules, and with capacity to deliver co-stimulatory molecules to active T cells.

- **Adjuvant**: Molecules co-administered with antigens in vaccines, that mimic danger signals with the aim to activate innate cells including antigen presenting cells, to allow for the initiation of adaptive immune responses.

- **CD8 T cells**: Cytotoxic cells from the adaptive immune system which specifically recognise antigens on the surface of cells and kill them if detected as non-self, through the secretion of cytotoxic molecules.

- **Cross-presentation**: Process by which professional antigen presenting cells present antigens derived from exogenous agents complexed with MHC-I molecules on their surface, necessary to activate CD8 T cells.

- **Co-stimulatory agonist**: Therapies (normally antibodies) that specifically bind co-stimulatory receptors on T cells with the aim to boost their anti-tumour activity.

- **CTLA-4**: Receptor translocated to T cell membrane following activation, which functions as immune checkpoint downregulating T cell activation upon CD80/CD86 binding. Overexpressed on regulatory T cells, in the context of cancer drive tumour tolerance and avoid anti-tumour immunity.
• **Dextramer**: Formed by several MHC-Antigen molecules bound over a fluorescently labelled polymer, these constructs are used for the detection T cell clones expressing TCRs specific the same antigen.

• **Epitope spreading**: Process by which therapeutic cancer vaccines enhance diversification and expansion of CD8 T cell clones with reactivates other than the vaccine epitope, driven by efficient tumour cell killing and release of other tumour-endogenous antigens.

• **Granzyme B (GZMB)**: Cytotoxic molecule secreted by activated CD8 T upon target cell encountering, inducing apoptosis of target cell.

• **Immune checkpoint inhibitor (ICI)**: Monoclonal-based immunotherapy targeting immune checkpoint receptors expressed on T cells, with the aim to the re-invigorate T cell proliferation and effector functions.

• **Neoantigen (NeoAg)**: Tumour specific antigen that arise from non-synonymous mutations in the DNA of cancer cells and that are not present in healthy cells.

• **NeoAg-reactive T cell**: T cell whose TCR specifically recognise a neoantigen epitope.

• **PD-1**: Receptor expressed on the surface of T cells following activation, which acts as a checkpoint molecule downregulating T cell activation upon binding to its ligand, PD-L1. In the tumour context, where dendritic cells and tumour cells express high levels of PD-L1, PD-1 on T cells prevent the development of anti-tumour responses.

• **Regulatory T cell (Treg)**: CD4 T cell subtype which maintains tolerance to healthy cells. In the context of cancer, Tregs mediate tolerance to tumours, allowing their progression.
- **T cell anergy:** Dysfunctional state in which T cells are inactivated due to the absence of co-stimulatory molecules at the time of priming, which renders them in a hyporesponsive state of tolerance.

- **T cell exhaustion:** Dysfunctional state thought to be triggered by continuous T cell activation found in the context of cancer or chronic infections, that result on the inability of T cells to kill cancer or infected cells.

- **T cell receptor (TCR):** Protein complex found on T cells that recognise peptides bound to MHC molecules, mediating T cell activation through the generation of intracellular signalling cascades. Each T cell clone possess a TCR with the exact same antigen specificity.

- **T cell priming:** Process by which a naïve T cells encounter the MHC-antigen they are specific to, for the first time, becoming activated.

- **TOX:** Transcription factor expressed upon persistent TCR signalling on T cells, which triggers chromatin remodelling changes driving T cell exhaustion.
Chapter 1: Background
1.1. Immune system

The immune system is defined by a complex network of cells, tissues, organs, and the molecules they produce that help the body fight infections and other diseases, such as cancer [1].

1.1.1. Lymphatic system and immune cells.

The generation of an efficient immune response relies on the interactions among different types of immune cells in the body. In vertebrates, some immune cell subsets are specialised in the recognition of “non-self” agents (i.e., infectious agents or cancer cells) as dangerous invaders. Meanwhile, others are critical on the effector side of the response, being key for the development of long-lasting immune responses against them [2], [3]. In the body, there are specialised structures that mainly harbour immune cells, which are called lymphoid organs. These are interconnected via lymphatic and blood vessels, forming the lymphatic system [4].

Figure 1. Lymphoid system schematic representation in human (left) and mouse (right). Illustration adapted from BioRender templates.
Lymphoid organs support the development and maturation of immune cells and the interactions among them to elicit immune responses efficiently. Depending on their role, they can be classified as primary lymphoid organs, including bone marrow (BM) and thymus, or secondary lymphoid organs (SLO), comprised of lymph nodes (LN) and spleen [1] Figure 1.

1.1.1.1. Primary lymphoid organs

1.1.1.1.1. Bone marrow: niche for haematopoiesis

The BM constitutes the inside or medullar cavity of some bones, including the hip, femur, sternum, and humerus. This region is packed with cells, among which a very rare cell subset is found, called Haematopoietic Stem Cells (HSC), which have the potential for “self-renewal” and differentiation into all types of immune and haematopoietic cells [5]. This process is termed haematopoiesis (Figure 2) and many other type of cells assist it within the medullar cavity, called stromal cells, which help regulate the proliferation, trafficking, and differentiation of HSCs [6]. HSCs first originate in embryonic tissues [7]. Although their location varies during different stages of development; in adult vertebrates they reside mainly in the BM, except for a small proportion found in the spleen and liver [8], [9]. Whereas in homeostatic conditions HSC remain in a resting state (“quiescence”), in cases of immune demand, such as during an infection, these cells proliferate rapidly and differentiate into myeloid or lymphoid progenitors, which give rise to two very different cell lineages [10].

1.1.1.1.1.1. Myeloid lineage

The myeloid precursors differentiate into several immune cells that are classified into subgroups comprising erythrocytes (red blood cells, RBC); granulocytes, which include neutrophils, eosinophils, basophils and mast cells; and antigen presenting cells (APCs), constituted by monocytes, macrophages and dendritic cells (DCs), (Table 1 for cell markers) [1]. Granulocytes and APCs constitute the innate immune system, which is the first line of defence against pathogens and other agents recognised as dangerous or “non-self”, as explained in 1.2.1. [11]. Following differentiation in the BM, myeloid cells
enter the circulatory system and eventually populate lymphoid and non-lymphoid organs, such as infected tissues or other inflamed environments, including solid tumours [12]. Besides, monocytes can reside in blood and be specialised in blood vessel repair [13], or infiltrate tissues in homeostatic conditions to provide a reservoir of innate immune cells in tissues. In addition, activated monocytes act as precursors of tissue-resident macrophages [14]. However, recent investigations indicate that, in healthy tissues, the majority of resident macrophages arise from embryonic cells, in early stages of life [15].

Figure 2. Haematopoiesis in Bone marrow.
Myeloid and lymphoid cells composing innate and adaptive immune systems, respectively. Illustration created using BioRender templates.

1.1.1.1.2. Lymphoid lineage

Cells from the lymphoid lineage, also called lymphocytes, are constituted broadly by three subgroups: B cells, T cells, and innate lymphoid cells (ILCs), which include the well-defined natural killer (NK) cells (Table 1 for cell markers) [10]. ILCs have recently been recognised as lymphoid-derived and share similarities with innate immune cells by acting as a first line of defence against pathogens and are mainly present in mucosal sites. However, their origins and relationship with other immune cells are yet to be fully understood [16]. B cells and T cells form the adaptive immune system and are
characterised by the expression of receptors of either B cell receptor (BCR) or T cell receptor (TCR), at their surface, respectively. These cells have the potential to recognise and generate immune responses against specific peptide sequences (although some T cell populations can also bind lipids), or antigens, derived from pathogens or other “non-self” agents [17] (further explained in 1.2.2.). This specificity in the response allows for the development of immunological memory, which allows B and T cells to provide with a faster and more efficient response when re-encountering the same antigen or pathogen [18]–[20]. Whereas B cells undergo differentiation in BM throughout all stages in life [21] followed by maturation in the spleen [22], T cells exit the BM in an immature stage and travel to the thymus to complete their development in a process that mainly takes place during the foetal and juvenile stages of life [23].

Table 1. Summary of markers that differentiate cells within innate and adaptive immunity.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCs</td>
<td>MHC-II⁺, CD11c⁺ (*Table 3 for more details)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD11b⁺, MHC-II⁺, CD68⁺, Ly6C⁺</td>
</tr>
<tr>
<td>Macrophages (M1-like)</td>
<td>MHC-II⁺, CD11b⁺, CD68⁺, F4/80⁺</td>
</tr>
<tr>
<td>Macrophages (M2-like)</td>
<td>MHC-II⁺, CD11b⁺, CD68⁺, F4/80⁺, CD206⁺</td>
</tr>
<tr>
<td>NK</td>
<td>CD11b⁺, NK1.1⁺</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>CD11b⁺, Ly6G⁺</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3⁺</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>CD3⁺, CD8⁺</td>
</tr>
<tr>
<td>CD4 Teff</td>
<td>CD3⁺, CD4⁺</td>
</tr>
<tr>
<td>CD4 Treg</td>
<td>CD3⁺, CD4⁺, FOXP3⁺, CD25⁺</td>
</tr>
</tbody>
</table>

1.1.1.2. Thymus and T cell maturation

The thymus was first recognised as a key immunological organ for the maturation of T cells in 1961 [24]. Immature T cells, also called thymocytes, enter the thymus and ultimately mature by generating unique TCRs [25]. Cells in the thymus present surface self-antigens that can react with TCRs expressed in thymocytes with different affinities.
Only thymocytes whose TCRs bind surface peptides with intermediate affinity can become mature in a process called positive selection. Interactions with high or low affinity induce thymocyte death, processes termed negative selection or death by neglect, respectively [23]. Interestingly, 95% of thymocytes die in transit.

1.1.1.2. Secondary lymphoid organs (SLO)

SLOs are finely defined structures where innate and adaptive immune cells interact to initiate immune responses [1]. Although the main SLOs include LN and spleen [26], some physical barriers, such as the mucosal sites of the reproductive, respiratory, and gastrointestinal tracts, and the skin are considered less-organised lymphoid tissues, and termed barrier tissues [1].

![Figure 3. Lymph node structure and the incoming and outcoming flow of lymphatics.](image)

**Figure 3. Lymph node structure and the incoming and outcoming flow of lymphatics.** Illustration adapted from BioRender template.

LN and spleen share key structural features, such as separate zones for B and T cell activity. LNs are the most specialised secondary lymphoid organs and are encapsulated structures packed with T cells, B cells, DCs, and macrophages, connected to blood and lymphatic vessels to allow for a continuous inflow and outflow of immune cells. They are composed of an outer layer (cortex), that includes follicles largely populated by B
1.2. **Immunosurveillance: Anti-tumour immunity**

The concept of immunosurveillance refers to the process by which immune cells search for and recognise infected cells or pathogens. During the 1950s, Lewis Thomas [28] and F. Macfarlane Burnet [29]–[31] suggested that this could also apply to tumours and hypothesised that tumour cells can be identified and attacked by the immune system. However, at the time no evidence supported this hypothesis and only gained acceptance in the early 2000s, when Robert D. Schreiber and colleagues demonstrated that immune-deficient mice could spontaneously develop tumours. This work also suggested that cancer cells can evade immune killing through a mechanism termed “immunoediting” [32]. Since then, there has been significant interest in the field of tumour immunosurveillance and the development of immune-based therapies (immunotherapies and vaccines) to treat cancer patients.

This section reviews the role of the cells forming the innate and adaptive immune systems, emphasising their role in anti-tumour responses. The immune events participating in tumour evasion and immunoediting are described in later sections (section 1.3).

1.2.1. **Innate immune response**

The main functions of the innate immune response are i) to prevent the entry of infectious agents into the body, and ii) to provide a rapid short-term reaction following infection or detection of foreign agents, such as healthy or apoptotic dying cells, including tumour cells. Therefore, the innate immune response comprises i) anatomical barriers against infectious agents, that can be physical (including skin and mucosal and glandular tissues) or chemical (i.e. acidic pH or microbial proteins and peptides present...
within physical barriers) and ii) cells from the innate immune system, specialised in killing pathogens or clearing apoptotic cells [1].

The innate immune system is formed by myeloid-like cells, such as granulocytes and APCs, and lymphoid-like cells, such as ILCs [33]. All these cells express pattern recognition receptors (PRRs), with exception of ILCs, but including NKs. PRRs are proteins specialised in sensing a range of evolutionary conserved structures across pathogens or across damaged tissues or dying cells (including apoptotic tumour cells), termed *pathogen-associated molecular patterns* (PAMPs) [34] or *damage-associated molecular patterns* (DAMPs) [35], respectively. PRRs are located on the outside of the cell membrane, within the inner membrane of intracellular (IC) vesicles (called endosomes) or the cytosol. Each myeloid subset presents a differential expression of PRRs, which can include Toll-like-receptors (TLRs), C-Type Lectin Receptors (CLRs), “nucleotide-binding oligomerisation domain-like” NOD-like receptors (NLRs), “Absent In Melanoma” AIM2-like receptors (ALRs), “Retinoic Acid-Inducible Gene-I” RIG-I-like receptor family (RLR) [36]. Here, the expression of PRRs is presented in the context of APCs activation and a focus on DCs, given their importance in vaccine and anti-cancer immune responses.

### Innate immune system and inflammation

Following tissue disruption (i.e. infection, wound or tumour) and DAMP/PAMP recognition, innate cells mature and mediate the generation of an inflammatory response that helps restore homeostasis and prevent further tissue damage. Inflammatory responses are organised reactions, in which some innate cells are first to detect danger signals (i.e. macrophages), whereas others are recruited in later stages (i.e. neutrophils or monocytes). In addition, if the action of the innate system is not enough, cells from the adaptive system are recruited [37]. This stratified response is achieved due to the release cytokines by immune cells (*Table 2*), following activation. Pro-inflammatory cytokines are a complex network of proteins with various functions, including the generation of chemical gradients that guide immune cells from the BM, blood or SLO to the disrupted tissue, or the activation of other immune cells [38].
Table 2. Cytokines mediating anti-tumour responses in TME.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Type of molecule</th>
<th>Secreted/expressed by</th>
<th>Function in tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GM)-CSF</td>
<td>Cytokine</td>
<td>Macrophages</td>
<td>Promotes monocyte, macrophage and DC proliferation and differentiation. Neutrophil migration and recruitment to tumour.</td>
</tr>
<tr>
<td>FLTL3</td>
<td>Cytokine and growth factor</td>
<td>NKs</td>
<td>DC development.</td>
</tr>
<tr>
<td>IFN-I</td>
<td>Cytokine</td>
<td>pDCs</td>
<td>Promotes DC maturation, T cell and NK cell activation, decreases infiltration of immunosuppressive cells (i.e. Tregs)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Cytokine</td>
<td>T cells, NKs</td>
<td>APC maturation (i.e. MHC II expression). Tumour apoptosis. Decreases tumour vascularisation, inhibiting metastasis. Treg inhibition. (*also contributes to tumour dormancy-immunoediting)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Cytokine</td>
<td>T cells, NK cells and DCs</td>
<td>Promotes CD8 T cell and NK proliferation and cytotoxic ability. (*also promotes Treg proliferation.)</td>
</tr>
<tr>
<td>IL-12</td>
<td>Cytokine</td>
<td>cDC1s, mo-DCs</td>
<td>Promotes NK and T cell proliferation and anti-tumour responses. Decreases Treg pro-tumour activity.</td>
</tr>
</tbody>
</table>

Upon binding to their receptors, cytokines trigger IC signalling cascades that result in the transcription of survival, proliferation or apoptosis genes. Ultimately, inflammation creates an optimal milieu for an efficient immune response that clears the foreign agent. After the danger is neutralised, the immune response is switched off, also by cytokines but anti-inflammatory in nature (i.e., transforming growth factor TGF-β). However, if the response cannot be stopped, chronic inflammation takes place and drives tissue damage due to a prolonged immune response. In this regard, chronic inflammatory processes resemble the immune environment found in some solid tumours [37]. Nonetheless, given that inflammation is a plastic process mediated by a complex network of cytokine interactions, whether inflammation drives tumour rejection or progression remains incompletely understood, as discussed in later section immune-
The main features of innate immune cells will be next described, as well as their role in anti-tumour responses.

1.2.1.1. Granulocytes

The family of granulocytes is comprised of neutrophils, eosinophils, basophils and mast cells, and a key common feature is the presence of enzymatic granules inside cytosolic vesicles. These proteins help eliminate pathogens (especially parasitic infections) by direct cytotoxic activity or indirectly by modulating the activity of other immune cells such as lymphocytes, or by tissue remodelling at the site of infection [1]. Neutrophils are the most abundant granulocyte found in blood and, whereas these cells rapidly infiltrate infected tissues to clear pathogens, their role in tumour clearance is controversial [39]. The presence of cytokines in tumours such as granulocyte colony-stimulating factor (G-CSF), secreted by macrophages, mediate neutrophil migration and tumour recruitment from the BM. Often, high neutrophil levels in the blood of cancer patients is associated with worse outcomes. Nonetheless, neutrophils are highly plastic, and their phenotypes vary from anti-tumour to pro-tumour depending on the cytokine milieu [37].

1.2.1.2. Innate lymphoid cells (ILC): ILC1, ILC2, ILC3 and Natural Killer (NK)

ILCs are composed by NKs (NK1.1+ cells) and three ILCs subgroups, ILC1, ILC2, and ILC3 [40]. As opposed to their lymphoid counterparts (T cells or B cells), ILCs do not express antigen receptors (TCRs or BCRs), however they share many functional features with T cells. ILC1, ILC2 and ILC3 are tissue resident, and secrete cytokines that mediate inflammation and resemble those secreted by some T cells [41]. NK cells and the ILC1 subset share multiple features and can kill tumour cells by secretion of cytokines such as interferon-γ (IFN-γ) or tumour necrosis factor (TNF) type α (TNF-α), which are also released by some T cells (called CD8) for targeted killing. Similarly, NK also resemble CD8 T cells in that they secrete cytotoxic molecules, including perforin and granzymes (GZM) (section 1.2.2.1.2 and glossary.). NK cells also express receptors on their surface (called Fc receptor) that bind antibodies (section 1.2.2.) specific for tumour (and other
pathogenic proteins). Upon binding to NK cells, these antibodies bring together NKS and target cells, promoting the secretion of cytotoxic molecules by NK and targeted cell killing, in a process termed antibody-dependent cell cytotoxicity (ADCC) [42]. In this context, NK cells are known to contribute towards tumour clearance in several types of cancers such as sarcomas, lymphomas or mammary carcinomas [43].

NK stimulation and effector function relies on signalling pathways derived from activating and inhibitory receptors on their membrane. In steady state conditions, NKS recognise MHC-I molecules expressed in the surface of healthy cells, which act as an inhibitory ligand and suppress effector signalling cascades, thus preventing NK-mediated immunity. Frequently, tumour cells downregulate their MHC-I molecules as a mechanism of immune escape. However, this results in a decreased threshold of inhibitory signalling in NKS, that is combined with the upregulation of activating ligands due to cellular stress or DNA damage, thus allowing for the development of effector anti-tumour responses by NKS [43]. The fact that NKS can be activated by tumour cells highlights the critical role of these innate cells in anti-tumour immunity.

### 1.2.1.3. Antigen presenting cells (APCs)

APCs include monocytes, macrophages and DCs, and play a critical role in bridging innate and adaptive immune systems. B cells also fall in the category of APCs, but belong to the adaptive immune system and are described in section 1.2.2. The relevance of APCs lies in their ability to present antigens from pathogens or apoptotic cells (including tumour cells) on their surface to T cells, so that adaptive immune responses can be effectively mounted. Briefly, APCs actively uptake or internalise apoptotic healthy and tumour cells or pathogens into endosomes where they are degraded, and peptides are complexed in major histocompatibility complex molecules (MHC). Whereas all cells in the body (except RBC) express MHC class I (MHC-I) molecules on their surface, where they present self-peptides, only APCs express MHC type II molecules (MHC-II), which allows the presentation of exogenous antigens. Certain DC subsets also present exogenous antigens in MHC-I complexes and can be considered professional APCs. Exogenous antigens complexed with MHC molecules on activated APCs can be recognised by T cells...
via TCRs. While macrophages are more specialised in providing rapid clearance of the antigen within a tissue, DCs are proficient in antigen presentation and have the ability to migrate to the closest LNs draining the tissue (draining LN, dLN), where the T cell activation process takes place mounting adaptive immune responses.

1.2.1.3.1. Monocytes and macrophages

1.2.1.3.1.1. Ontogeny and effector functions

Monocytes from blood (Ly6C<sup>high</sup>) undergo differentiation into macrophages in tissues, losing Ly6C expression and upregulating other markers, including F4/80, or CD68, and MHC-II [44]. In steady state conditions (i.e. in the absence of infection or inflammation), the main function of tissue resident macrophages involves tissue remodelling. Nonetheless, following detection of PAMPs and DAMPs, macrophages polarise, changing function and phenotype and mediate immunological responses, such as efficient clearance of pathogens or dying cells, secretion of cytokines and antigen presentation in MHC-II complexes [45]. However, whether macrophages migrate from tissues to dLN remains uncertain. For instances, skin macrophage migration to skin-dLN has been described with skin UV damage [46], and likewise alveolar macrophages can migrate to lung-dLN with pulmonary gram-positive bacteria infection [47]. Uncertainty also exists regarding whether macrophage migration occurs in the context of tumours.

Tissue macrophages have a high degree of plasticity and can polarise to several functional states, ranging from pro-inflammatory (killing capacity) to anti-inflammatory (heal/growth promotion) depending on the cytokine milieu [15]. Macrophages can be classified as M1-like or M2-like, respectively [14], [48]. These two populations can be differentiated as M2-like macrophages express high levels of the mannose receptor CD206 [49]. In addition, recent discoveries have suggested the potential of monocytes to differentiate into cells with higher antigen presentation potential than macrophages, termed monocyte-derived DCs (Mo-DCs, Ly6C<sup>high</sup> MHC-II<sup>high</sup>), which play an important role in anti-tumour responses [50]. Mo-DCs can migrate to nearby LNs and promote inflammation [51].
Figure 4. Innate anti-tumour response.
NK cells, M1-like TAMs and DCs mediate anti-tumour responses during the early stages of tumour development, through antibody-dependant cellular cytotoxicity, phagocytosis, and T cell activation, respectively. Image created with BioRender (own-design).

1.2.1.3.1.2. Anti-tumour activity of M1- tumour associated macrophages (TAM)

In the context of tumours, macrophages are designated tumour-associated macrophages (TAMs) and normally derive from the differentiation of circulating monocytes. Whereas an abundance of M2-like TAMs is associated with tumour tolerance and progression [52] as described in section 1.3.1.4, M1-like TAMs have been reported to exert anti-tumour responses. Initially they trap, phagocytise and lyse tumour cells. In addition, M1-like macrophages release cytokines (i.e. IL-6, IL-12, TNF-α) that promote T cell responses as well as cytotoxic molecules, such as reactive oxygen
species (ROS) molecules, that kill tumour cells Figure 4 [52], [53]. Thus the presence of M1-macrophages in high proportions is related to better anti-tumour adaptive responses and improved prognosis in a range of tumours (eg small cell lung cancer, non-small cell lung cancer, ovarian cancer, colorectal cancer, oral squamous cell carcinoma or breast cancer)[54].

1.2.1.3.2. Dendritic cells (DCs)

Ontogeny and migration to LN

DCs were first described in mid-1970s by Ralph Steinman, who was later awarded the Nobel Prize in Physiology 2011. These cells were named after the presence of dendrites, which can efficiently sense and take up antigens [55]. In addition to Mo-DCs, two other DC subsets exist in tissues including tumours: conventional DCs (cDCs), which are MHC-II$^{\text{high}}$ CD11c$^{\text{high}}$; and plasmacytoid DCs (pDCs), which express CD11c$^{\text{low}}$ MHC-I$^{\text{low}}$ SIGLEC-H$^+$. In addition, 2 populations of cDCs coexist: cDC1 (XCR1$^+$, CD103$^+$, CD8a$^+$) and cDC2 (CD11b$^+$) [56], [57]. Both pDC and cDC derive from DC precursors in the BM. Nonetheless, lymphoid precursors can also give rise to pDCs. Whereas pDCs are fully differentiated when they leave the BM, cDCs full development is mediated by soluble factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (FLT3L), present in lymphoid and non-lymphoid tissues [58], [59], Table 3. In addition, cells described as cDC3, have recently been identified in human, which seem to share characteristics with cDC2 but might be derived from either cDC or monocyte progenitors [60], whose role is not yet defined.

Upon antigen sensing and activation in peripheral tissues including tumours, DCs (particularly cDCs) have the unique ability to efficiently migrate to nearby dLNs [61]. It is possible to distinguish these migratory cDCs (migDCs, CD11c$^{\text{high}}$MHC-II$^{\text{high}}$) from DCs populating LNs from BM precursors, termed resident cDCs (resDCs, CD11c$^{\text{high}}$MHC-II$^{\text{int}}$) in LNs [62]. Moreover, cDCs are professional antigen presenters to T cells, comprising the most specialised subsets of all APCs in this task. Whereas cDC2 efficiently present antigen to CD4 T cells in MHC-II complexes, cDC1s efficiently present exogenous
antigens in MHC-I molecules, in a process that is known as antigen cross-presentation, which is required for the activation of the CD8 T cells subset [63]. The differences in function across DCs subsets is highly influenced by their differential PRR expression [64].

Presentation of tumour associated antigens (TAAs) and neoantigen (NeoAg)

cDC1s infiltrating tumours cross present antigens from apoptotic tumour cells, known as tumour associated antigens (TAA) and neoantigens (NeoAg) (glossary and section 1.4.4.1), and despite their low numbers within tumour mass they are critical to trigger efficient anti-tumour CD8 T cell responses needed for tumour clearance, Figure 4 [65]. Other DC subsets such as moDCs are essential for antigen presentation and the development of anti-tumour CD8 T cell responses [50]. Despite this, it is apparent that a network of interactions among all DC subsets are necessary for optimal immunosurveillance [66], and that efficient TAA presentation by cDC2, promoting CD4 T cell responses, helps boost anti-tumour CD8 immunity, Table 3 [67].
### Table 3. DC subset classification and role in tumours.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Presence in vivo</th>
<th>Surface markers in mouse</th>
<th>Main functional specialisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDCs</td>
<td>Resident in lymphoid tissues in blood, lung (mouse) and tonsil (human)</td>
<td><strong>CD11c</strong>&lt;sub&gt;low&lt;/sub&gt;, <strong>MHC-II</strong>&lt;sub&gt;low&lt;/sub&gt;, <strong>SIGLEC-H</strong>+, B220&lt;sup&gt;+&lt;/sup&gt;, CD317&lt;sup&gt;+&lt;/sup&gt;, CD172a&lt;sup&gt;+&lt;/sup&gt;, CD209&lt;sup&gt;+&lt;/sup&gt;, CCR2&lt;sub&gt;low&lt;/sub&gt;, CCR9&lt;sup&gt;+&lt;/sup&gt;, CXCR3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Generally poor antigen presentation but can be stimulated to activate CD8 T cells (cross-presentation). Implicated in cancer cell killing</td>
</tr>
<tr>
<td>cDC1</td>
<td>Resident in lymphoid tissues in blood. Migratory subsets in peripheral tissues and LNs</td>
<td><strong>CD11c</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>MHC-II</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>CD103</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>XCR1</strong>&lt;sup&gt;+&lt;/sup&gt;, CD24&lt;sup&gt;+&lt;/sup&gt;, CD8α&lt;sup&gt;+&lt;/sup&gt;, CLEC9A&lt;sup&gt;+&lt;/sup&gt;, DEC205&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Induce CD8 T cell and Th1 cell anti-tumour immunity. Specialised in cross-presentation. High secretion of IL-12 and type I IFNs. Implicated in self-tolerance in the steady state (via cross-presentation)</td>
</tr>
<tr>
<td>cDC2</td>
<td>Resident in lymphoid tissues in blood. Migratory subsets in peripheral tissues and LNs</td>
<td><strong>CD11c</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>MHC-II</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>CD11b</strong>&lt;sup&gt;+&lt;/sup&gt; (high), CD172a&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Provides with CD4 Th responses to support anti-tumour CD8 T cell immunity (context dependent, large repertoire of PRRs and pro-inflammatory and anti-inflammatory cytokines).</td>
</tr>
<tr>
<td>MoDCs</td>
<td>Differentiate from monocytes in peripheral tissues on inflammation. Resident in skin, lung and intestine</td>
<td><strong>CD11c</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>MHC-II</strong>&lt;sup&gt;+&lt;/sup&gt;, <strong>CD11b</strong>&lt;sup&gt;+&lt;/sup&gt;, Ly6C&lt;sup&gt;+&lt;/sup&gt;, <strong>CD64</strong>&lt;sup&gt;+&lt;/sup&gt;, CD206&lt;sup&gt;+&lt;/sup&gt;, CD209&lt;sup&gt;+&lt;/sup&gt;, CD14&lt;sup&gt;+&lt;/sup&gt;, CCR2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mainly generated during inflammation, conditioning their functions: antimicrobial and induction of CD8 T cell, Th1 cell, Th2 cell and Th17 immunity. Cancer: implicated in Treg generation/immunosuppression Regulatory functions in steady state skin</td>
</tr>
</tbody>
</table>
1.2.1.3.2.1. DCs in tolerance and immunity

DCs are specialised in antigen capture, processing, and presentation in MHC complexes. Depending on the context, these antigens can be “self”, from healthy tissues, or derived from pathogens or abnormal tissues, such as tumours [68]. In steady state conditions, DCs in lymphoid and non-lymphoid tissues display an immature phenotype, and despite continuous “self-antigen” presentation in MHC molecules, their T cell activation potential is low [69]. This mechanism contributes to the maintenance of “peripheral tolerance”, a group of immunological mechanisms that prevent the development of auto-immunity towards healthy tissues [70]. On the contrary, upon infection or injury, or cell death (i.e. tumours), PAMP/DAMP are released and interaction with PRRs in DCs takes place, driving a series of IC cascade signalling events which trigger DC maturation [11]. During this process, MHC-II molecules are translocated to the cell membrane from IC vesicles, antigen presentation is enhanced and the expression of co-stimulatory molecules, and those that enhance migration capacities to dLNs, are increased on the surface of DCs.

DCs must provide 3 signals for the activation of naïve T cells, a process termed T cell priming: MHC-Antigen presentation (signal 1), upregulation co-stimulatory molecules (signal 2) and presence of soluble factors such as cytokines (signal 3) [71]. In addition, DC maturation results in antigen uptake cessation which ensures that the presented antigens correlate with the agent driving the infection or inflammation, thereby avoiding the presentation of self-antigens in activated DC which could drive activation of self-reactive T cells [72].

1.2.1.3.2.1.1. Routes of antigen presentation in MHC I and MHC II complexes

APCs internalise particulate materials by macropinocytosis, receptor-mediated endocytosis (clathrin dependent or independent), and phagocytosis [68]. Macropinocytosis is a non-selective process by which large quantities of extracellular material (including proteins, bacteria, viruses or dying cells) are captured via membrane ruffling and folding into pinosomes [73]. During receptor mediated endocytosis, the formation of receptor-ligand complexes and soluble macromolecule recognition drives
the invagination of the cell membrane, that evolve into the generation of vesicle-like membrane structures (endosomes) that are ultimately released in the cytosol [74]. In the case of phagocytosis, larger particulate antigens, senescent cells and pathogens are also engulfed into IC vesicles denominated phagosomes. Both endocytosis and phagocytosis mediated uptake processes follow the binding of exogenous agents to a series of receptors [75], such as mannose receptors [76], FcR [77], complement receptors [78] or scavenger receptors (SRs) [79], which further influence the IC pathway of antigen presentation that the antigen follows [75].

Despite these differences, the internalised antigens undergo proteolysis and degradation driven by endosome or phagosome pH acidification and maturation into lysosomes or phagolysosomes, respectively. The pH in these compartments is between 4 to 4.5, and contain proteases that are active under acidic conditions [80], so that macromolecules or pathogens undergo degradation, and proteins degrade into shorter peptides. In each stage of endosomal and phagosomal maturation, MHC-II molecules are anchored onto the inner side of the membrane, where they form complexes with peptide degradation products. MHC-II-Ag complexes can then recirculate back and fuse with the APC cell membrane, resulting in antigen presentation to CD4 T cells [80].

The mechanisms leading to antigen cross-presentation in MHC-I molecules to CD8 T cells are less clear [81], [82]; two pathways seem to coexist. In the cytosolic pathway, in which antigens in state of partial degradation from endosomes or phagosomes are translocated to the cytosol, where proteasomes further cleave them into 9 to 11 amino acid (aa) residue peptides [82]. MHC-I complexes are assembled in the endoplasmic reticulum (ER), and proteasome-derived peptides in the cytosol access the ER through translocation mediated by the transporter associated with antigen processing (TAP) [83]. Antigens then associate with MHC-I molecules and are recycled back to the cell membrane. It has also been hypothesised that ER-resident proteins, including MHC-I molecules can reach endosomes and phagosomes, in a process that is enhanced by TLR engagement [81]. Simultaneously, TAP might mediate proteasome-derived peptide translocation from the cytosol into endosomes or phagosomes harbouring MHC-I complexes, forming MHC-I-Ag antigens which can then fuse with the cell membrane. The
vacuolar pathway involves recycling of MHC-I molecules from the plasma membrane into endosomes or phagosomes, where they form complexes with peptides derived from degradation within these organelles [84]. It is evident that phagocytised antigens reach the cytosol via translocases located in the endosomal compartments, but the process is not clear. Similarly, it is not fully established whether molecules taken up through endocytosis or pinocytosis are granted access to the cytosol [85]–[87].

In immunosurveillance, cDC1 cells efficiently phagocytise dying cells and cross-present TAA or NeoAg through the cytosolic route [88], [89]. This reinforces the importance of cDC1s in the tumour, despite the fact that they normally constitute a reduced fraction of the total myeloid population within them [90].

1.2.1.3.2.2. PRR expression, cytokine production, and expression of co-stimulatory molecules in DCs

The expression of PRR varies notably among cells in the innate immune system [91], and in the context of cancer, they sense DAMPs derived from necrotic cells. Some of these danger molecules include heat-shock proteins (HSPs), calreticulin, fibrinogen, ATP, endogenous single-stranded and double-stranded (ds) RNA [35], [92]. Nucleic-acid sensing receptors, specifically DC-priming receptors, are critical in myeloid cells in order to trigger adaptive responses. Some examples include endosomal TLR7, 8 and 9, RIG-I and the melanoma differentiation-associated protein 5 (MDA5), as well as cytosolic DNA sensors such as cGAS and STING which induce the production of cytokines, including interferons (IFN), to induce inflammation and to further contribute to antigen presentation processes [93]–[95]. The expression of the endosomal receptor TLR3 is restricted to cDCs and particularly high in cDC1s [96]. Upon dsRNA sensing, TLR3 oligomerises and recruits the adaptor protein “Toll-IL-1 receptor-containing adaptor molecule-1” (TICAM-1, or TRIF), which activates the transcription factors nuclear factor (NF)-κB (NF-κB), interferon regulatory factor 3 (IFR3), and activator protein 1 (AP-1), which induce the production of cytokines (TNF-α, IL-12, IL-6) and type-I IFN [97]. In turn, these molecules contribute to DC activation, driving the expression of co-stimulatory
molecules, enhancing MHC-II expression on the DC surface, and increasing levels of antigen cross-presentation [98], [99].

Co-stimulatory molecules in DCs interact with the counterpart ligands expressed in T cells to induce IC activator pathways. Some of the most important co-stimulatory pathways are dictated by the interaction of CD80, CD86 and CD40 molecules. In the case of CD80 and CD86, these bind CD28 in T cells, which leads to enhanced expression of molecules such as IL-12 in DCs, contributing to T cell activity [100]. CD40 binds CD40-L in T cells also promoting IL-12 production, although this can be unpaired by low CD40-L expression on T cells [101], [102], Figure 5.

**Figure 5. T cell priming.**
Signals required for T cell activation are: (1) antigen recognition, (2) co-stimulation, and (3) cytokine-mediated differentiation and expansion. Image adapted from a BioRender template.

**1.2.2. Adaptive immune response**

The adaptive immune system is composed of lymphoid-derived T and B cell subsets. The presence of membrane bound TCR and BCRs, respectively, allows the recognition of specific antigens, such as NeoAg or TAA in the context of cancer. When tumour cells die, they are taken up by DCs after which these migrate to tumour-dLNs, where they present TAA or NeoAg in MHC complexes and prime T cells whose TCRs are specific for these antigens (here denominated tumour-reactive, or NeoAg-reactive CD8 and CD4 T cells). Upon activation, T cells proliferate, and most daughter clones become effector cells,
which migrate to the tumour site and develop an immunological response against tumour cells. In addition, B cells also take up TAA or NeoAg and become activated with the help of CD4 T cells. The cycle that encompasses DC uptake of dying tumour cells, TAA/NeoAg presentation to T cells in tumour-dLN, and the adaptive anti-tumour responses generated that eliminate cancer cells is termed “the cancer-immunity cycle” [103]. Whereas B cell responses carry out antibody (Ab) mediated responses and can attack cancer cells in blood or the tumour bed, T cells kill tumour cells independently of antibodies. B cell responses are therefore humoral (from the latin humor, meaning “body fluid”), and T cell responses cell-mediated [3].

Figure 6. The cancer-immunity cycle: bridging innate and adaptive anti-tumour responses. DCs in tumour take up tumour associated antigens (TAA) or neoantigens and present them in the context of MHC-I and MHC-II molecules in tumour draining lymph nodes (tumour-dLNs) to CD8 and CD4 T cells, respectively, activating them. CD4 T cells support B cell activation. Following activation, T and B cells migrate to the tumour bed, where they exert anti-tumour responses. Image created with BioRender (own-design).

The importance of the adaptive immune response relies on the generation of immunological memory that provides long-lasting protection (months to years). Upon naïve B and T cell (this is, mature cells that had not encountered antigen) activation, a proportion of clones acquire a memory-like phenotype resulting in a pool of cells that respond rapidly and efficiently to subsequent antigen re-exposure. Memory B and T cells
take residence in spleen or LNs, or persist within the tissues where the first antigen encounter and activation took place [104].

1.2.2.1. T cell responses

T cell precursors in the thymus differentiate into two main T cell subsets, with distinct functions, namely CD8 and CD4 T cells [105]. It is estimated that each individual T cell bears $10^5$ TCR molecules, all of them with the same unique antigen specificity. T cells and their daughter generations that recognise a specific antigen are known as T-cell “clones”. In the human body there are $10^{15}$ to $10^{21}$ different T cell clones, each of them with unique TCRs [106].

1.2.2.1.1. T cell IC signalling during priming

1.2.2.1.1.1. TCR-Ag-MHC recognition

TCRs are heterodimer proteins formed by two chains, $\alpha$ and $\beta$. A small fraction of T cells express different TCRs formed by $\gamma\delta$ chains, which normally reside in mucosal tissues and whose antigen recognition features are different to $\alpha\beta$ TCRs; in this section the focus will be $\alpha\beta$ TCR bearing T cells only. Both chains harbour two well distinguished domains and are anchored to the cell membrane by the carboxyl terminal (Ct) ends. The C-terminus aa sequence of $\alpha\beta$ chains are highly conserved across TCRs, receiving the name of constant (C) domain. On the contrary, the TCR amino terminus (Nt) is extremely variable and termed the variable (V) domain and constitutes the MHC-I-Antigen binding site [107], [108]. The TCR $\alpha$ and $\beta$ chains lack IC domains and cannot provide IC signalling transduction to achieve T cell activation. Thus, TCR is non-covalently paired with three dimer proteins in the cell membrane, that possess IC domains that are specialised in signalling transduction. These proteins ($\delta\epsilon$ pair, $\gamma\epsilon$ pair, $\zeta\zeta$ or $\zeta\eta$) form the CD3 complex [109]. In addition, the TCR non-covalently binds other accessory proteins in the cell membrane. Two of these are CD4 and CD8 molecules, expressed in CD4 and CD8 T cells respectively, and bind conserved regions in MHC-II or MHC-I molecules respectively, that enhance the avidity of TCR – target binding [110].
When DC encounter T cells (through their MHC complexes and TCR, respectively) a series of cytoskeleton remodelling processes drive the formation of the “immunological synapse” (IS), a stable cluster structure in the T cell membrane which encompasses the clustering of TCRs and their CD4 or CD8 coreceptors, co-stimulatory molecules (including but not limited to CD28, or CD40-L), and adhesion molecules [71]. The IC domains in CD3/δ/ε chains sequences contain “Immunoreceptor tyrosine-based activation motifs” (ITAMs) that are key for T cell activation. ITAMs are rich in tyrosine residues, and are phosphorylated intracellularly by a member of the “Protein tyrosine kinase” (PTK) family, known as “Lymphocyte-specific protein tyrosine kinase” (Lck), upon MHC-Ag-TCR interaction [111]. This triggers an IC signalling cascade that modulates the transcription of genes that control activation of CD4 and CD8 T cells, in a process resulting in the activation of transcription factors such as “Nuclear factor of activated T cells” (NFAT), “Nuclear factor kappa light chain enhancer of activated B cells” (NF-kB), or “Activator
protein 1” (AP-1). In turn, the transcription of genes controlling proliferation and pro-survival is triggered [112], Figure 7

1.2.2.1.1.2. Co-stimulation

The interaction between co-stimulatory ligand-receptor pairs between DCs and T cells is needed to achieve efficient activation of naïve T cells (this is, mature T cells awaiting to encounter antigen and be activated). Of note, CD28 receptor engagement in T cells with CD80 and CD86 ligands in DCs triggers IC CD28 recruitment of kinases such as Lck and the θ isoform of “Protein kinase c” (PKC), followed by an IC signalling cascade that results in the activation of the transcription factors AP-1, NF-κB and NFAT, inducing transcription in genes associated with T cell survival [113]. In addition, CD28 signalling lowers the antigen threshold required for naïve T cells to become activated and enhances cell proliferation and the expression of cytokines, especially IL-2; and cytokine receptors, such as IL-2 receptor (also known as CD25) that help in the differentiation and functionality of effector T cells [114], [115]. To control activation and proliferation, T cells induce the expression of the “Cytotoxic T lymphocyte antigen-4” (CTLA-4) receptor, a homologous protein to CD28 that binds CD80/CD86 with a stronger affinity than CD28 [116], downregulating the T cell activation signalling cascade and preventing uncontrolled activation [117], [118], Figure 8.

Although the interactions between CD80/CD86 and CD28/CTLA-4 are paramount for DC-T cell co-stimulation, many other receptor-ligand pairs modulate the activity and differentiation of T cells. These molecules, also including CD28 or CTLA-4, are termed immune checkpoints and can be co-stimulatory or co-inhibitory [119]. Upon ligand binding on APCs, co-stimulatory checkpoints boost the magnitude of the T cell response, including proliferation, survival, and the generation of effector and memory T cells. In addition to CD28, co-stimulatory receptors include “Inducible T cell costimulatory” (ICOS), OX-40, 4-1BB, “Glucocorticoid-induced tumour necrosis factor related protein” (GITR) or CD27, and apart from CD28 and CD27 (which are constitutively expressed in T cells) their expression is triggered following MHC-Ag-TCR interaction and can also be dependent on CD28 co-stimulation. In contrast, co-inhibitory receptors include
“Programmed Cell Death Protein” (PD-1), “T cell immunoglobulin and mucin domain-containing protein” (TIM-3) or “Lymphocyte activation gene-3” (LAG-3), and with ligand binding, restrict the development of effector responses [71], Figure 8. The importance of these receptors in anti-tumour CD8 T cell responses will be discussed in section 1.3.2.3.

![Figure 8. Co-stimulatory and co-inhibitory interactions regulating T cell responses.](image)


1.2.2.1.1.3. Cytokines

The third signal required for efficient CD4 and CD8 T cell activation is provided by a chemokine rich milieu. Type I IFN (which includes IFN-α and IFN-β) and IL-12 produced by mature DCs are crucial for efficient CD8 T cells clonal expansion and development of both effector functions and a pool of memory CD8 T cell clones, by regulating transcription factors needed for these functions (i.e T-bet, eomesodermin or Blimp-1) [120].

MHC-Ag-TCR in the presence of co-stimulatory molecules and a cytokine rich environment provides with efficient T cell priming, leads to the transcription of
proliferation and survival genes that allow T cell clonal expansion (proliferation) of effector T cells, and generation of a pool of memory cells. In addition, efficient priming is needed for the transcription of genes related to T cell effector functions [114], [120], [121]. Upon activation in lymph nodes, T cells migrate to infected or damaged tissues (i.e. tumours) in a process mediated by chemokine gradients [122]. Importantly, CD8 and CD4 T cells exert two different types of immune response (section 1.2.2.1.2/3).

1.2.2.1.2. Anti-tumour CD8 T cell response

Following priming in tumour-dLN by mature cDC1s, tumour-reactive CD8 T cells proliferate and effector cells migrate and infiltrate into tumours. CD8 T cells are denominated CD8 tumour infiltrating lymphocytes (CD8 TILs) and are a main cell in immunosurveillance, as they are able to kill tumour cells expressing TAA or NeoAg in a direct and specific manner, through cytotoxic mechanisms:

In tumours, the first cytotoxic mechanism of CD8 T cells involves the engagement of “Fas Cell Surface Death Receptor” (FAS) apoptotic receptors expressed in the surface of tumour cells. CD8 TILs cells express FAS ligand (FASL), which when bound to FAS receptors activates death domains and signalling mediated by caspases that induce DNA fragmentation and cell death [123]. A second killing mechanism involves the secretion of granules that contain cytotoxic enzymes, such as perforin and GZM. These are synthesised in CD8 T cells following antigen recognition, after which they travel from the Golgi apparatus and lysosomes towards the IS, where they are released in exocytic vesicles. Perforin forms pores in the target cell and endosomal membrane, which allows the transit of ions and other molecules in the cell, triggering osmotic lysis [124]. GZMs are a family of serine proteases that rely on perforin pore formation to access the IC space in the target cells [125]. Once in the cytosol, GZM induce cell death through cleavage of specific proteins. There are 11 families of GZM (A, B, C, D, E, F, G, K, L, M, N) in human and 4 in mice (A, B, H, K and M); GZMA and GZMB are generally the most abundant and reported to have different mechanisms of action [125]. GZMA facilitates an increase in ROS, weakens the nuclear structural integrity and triggers the proteolysis of protein complexes in the endoplasmic reticulum that otherwise would act as DNase
inhibitors, leading to DNA damage and apoptosis. Similarly, GZMB promotes apoptosis following proteolysis and activation of caspases (these are proteins that mediate apoptotic processes) or other related proteins *Figure 9*.

![Anti-tumour CD8 TILs response](image)

**Figure 9. Anti-tumour CD8 T cell responses.**
CD8 tumour infiltrating lymphocytes (TILs) recognise MHC-I molecules (purple) in the surface of tumour cells expressing neoantigens or tumour associated antigens through specific interactions with their T cell receptors (TCRs, blue). This triggers the secretion of cytotoxic enzymes (i.e. granzyme-GZM or perforin-PFN), which kill tumour cells. In addition, secretion of cytokines such as IFN-γ or TNF-α further supports anti-tumour responses. Image created with a BioRender template.

A third mechanism of indirect killing by activated CD8 T cells involve the secretion of cytokines such as IFN-γ or TNF-α, *Figure 9*. Roles for IFN-γ include i) the generation of chemokine gradients that allow T cell migration and motility towards the site of infection or tumours, ii) the upregulation of MHC-I molecules on target cells and iii) enhance effector capabilities, such as enhancing killing through FAS-FASL interaction or increase the expression of IL-12 receptors [126], [127]. TNF-α binds to receptors of the TNF receptor family (TNFRs) that are expressed in the majority of cells, that on binding drives the activation of transcription factors controlling the expression of genes involved in either proliferation and cell survival or apoptosis [128].

1.2.2.1.3. Anti-tumour CD4 T cell responses
During maturation in the thymus, two functionally distinct CD4 T cell subsets are developed. The first one comprises the CD4 T helper (Th) family, also known as effector (Teff) which upon priming, assist the activation of other immune cells, such as innate cells, CD8 T cells [129] or B cell, through the secretion of cytokines. The second subset,
regulatory CD4 T cells (Tregs), is involved in maintaining tolerance against self-antigens that prevents autoimmunity [130]. Both subsets play roles in tumour responses:

1.2.2.1.3.1. CD4 T helper (Th) response in cancer

During immunosurveillance, mature cDC2s loaded with TAA or NeoAg prime naive tumour-reactive CD4 T cells in tumour-dLN. Upon activation, effector CD4 T cells further differentiate into several helper subsets with various functions, in a process that is related with the cytokine milieu and IC signalling and transcription factor activation as a consequence. Accordingly, more than six CD4 T cell fates are identified, including conventional Th 1 (Th1) and Th 2 (Th2) and unconventional Th 17 (Th17) or follicular helper (Tfh), which are the better characterised subsets (Table 4).

Table 4. Differentiation and tumour-related effector functions of CD4 T helper subset.

<table>
<thead>
<tr>
<th>Th subset</th>
<th>Differentiation</th>
<th>Txn factor</th>
<th>Secreted Cytokines</th>
<th>Tumour immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, IFN-γ</td>
<td>T-bet</td>
<td>IFN-γ, TNF-α, IL-2, Macrophage inflammatory protein-1a (MIP1a/CCL3) and Monocyte chemotactic protein-1 (MCP-1 or CCL2).</td>
<td>Anti-tumour: M1-TAM: Enhanced recruitment, differentiation, and phagocytosis. NK: Enhanced recruitment. CD8 Tc: Enhanced priming, proliferation, and acquisition of cytolytic and memory phenotype.</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-2</td>
<td>GATA-3</td>
<td>IL-4, IL-5, IL-9, IL-10, IL-2, IL-13</td>
<td>Anti and pro-tumour: Eosinophil/TAM recruitment.</td>
</tr>
<tr>
<td>Th17</td>
<td>TGF-β, IL-6, IL-23</td>
<td>STAT3 RORgt</td>
<td>IL-17A and IL-17F</td>
<td>Pro-tumour (? unclear): Promotion of angiogenesis and inhibition of anti-tumour CD8 T cell responses</td>
</tr>
<tr>
<td>Tfh (in LNs)</td>
<td>IL-6, IL-21</td>
<td>Bcl-6</td>
<td>IL-21</td>
<td>Anti-tumour responses B cells: Generation of anti-tumour antibodies in TLO CD8 Tc: Enhanced responses?</td>
</tr>
</tbody>
</table>

Th1 and Th2 subsets contribute to anti-tumour immunity by recruitment of CD8 T cells, NK and M1 TAM (Th1) or eosinophils and TAM (Th2) into the tumour bed [130], however
Th2 cells might also contribute to tumour progression [131]. The role of Th17 cells in tumour immunity is not so clear although some studies suggest they have a role in tumour progression via angiogenesis promotion [132]. Tfhs are mainly found in lymph nodes and assist the B cell production of antibodies, which correlates with better anti-tumour responses in solid malignancies [130], [133], [134].

1.2.2.1.3.2. Regulatory CD4 T cells (Tregs)

Tregs are characterised by the expression of the “Forkhead box protein 3” (FOXP3) transcription factor and the IL-2 receptor CD25. Two Treg subset exists: natural Tregs (nTreg) and induced Tregs (iTreg). nTregs are fully differentiated in the thymus and do not rely on the cytokine milieu during priming, whereas iTregs differentiate from naïve CD4 T cell in peripheral tissues, conditioned by TGF-β rich environments (i.e. tumour cells), whose signalling results in FOXP3 transcription [135]. Tregs play an important role in the maintenance of immune tolerance to self-antigens, and alterations in their homeostasis mediate autoimmune diseases. Inversely, Tregs in the context of cancer mediate tolerance against tumour cells, and therefore contribute to disease progression. This is achieved through several mechanisms, including constitutive expression of CTLA-4 receptor; secretion of anti-inflammatory cytokines that reduces activation of effector cells nearby, including CD8 T cells; dominant consumption of IL-2, or induction of apoptosis in other immune cells through Fas-FasL interaction [130], [136].

1.2.2.2. B cell responses

Upon differentiation in BM, B cells undergo maturation in SLO and, similar to T cells, each B cell clone express a BCR with specificity for a certain antigen [137]. BCRs are antibodies, immunoglobulins (Ig), that are produced by B cells. Igs are Y-shaped proteins in which the “stem” constitutes the constant fraction (Fc fraction) and the two short Y arms are comprised of aa chains that are hypervariable among Igs (variable fraction), which provide antigen binding specificity. Each B cell only generates Igs with unique antigen binding sites [138].
B cells recognise soluble antigens that specifically interact with their BCRs, forming BCR-Ag complexes, that are internalised by endocytosis by B cells. In endosomes, the antigen is processed into shorter peptides and further complexed with MHC-II molecules anchored to the endosome’s membrane and then travel to the cell membrane. MHC-II-Ag complexes in B cells are specifically recognised by effector CD4 T cells, initiating immunological synapses, including co-stimulatory receptor-ligand interactions, and cytokines [139]. This process induces B cell proliferation and further maturation into plasma cells, which secrete large amounts of Igs with the same antigen specificity as their BCRs into the extracellular milieu. Following Ab release, plasma cells lack proliferation potential, and although many die, a few clones remain which develop antibodies that possess higher affinity towards the target antigen. In addition, upon CD4 T cell activation, some B cells become memory and can be activated without the help of DCs or T cells, by direct binding of specific BCR antigen from the extracellular media. This enables a faster and more potent antigen-specific Ab response when the antigen is re-encountered [140]. The secreted antibodies travel in the blood and interstitial fluids, where they bind the antigens expressed on the surface of pathogens or altered cells, such as tumour cells; the specific Ab binding is via the variable region, which surround the pathogen or cell, in a process termed opsonisation. The Fc fractions in the surrounding Igs, can be recognised by Fc receptors that are highly expressed in innate immune cells, especially macrophages or NK cells, which activate further and contribute to the pathogen/cell clearance, Figure 10 [141].

B cells are found infiltrating the majority of tumours [142], although were unrecognised as it was assumed that cytotoxic CD8 T cells were the lead drivers of anti-tumour responses. Recent studies indicate that B cells are the main components of immune aggregate structures found within the tumour mass of many types of solid malignancies, termed tertiary lymphoid organs (TLS), and which frequently correlate with improved disease outcomes [143]. Although many mechanisms for tumour rejection are proposed for B cells, including direct antigen presentation to CD4 T cells and presence of high Ig levels produced by plasma cells with tumour cell specificity; this leads to opsonisation and further recruitment of innate immune cells prior to their clearance [144].
1.2.2.3. Natural Killer T cell (NKT) response

NKT cells are lymphocytes at the interface between the innate and adaptive immune system given that they express both surface receptors similar to those of NK cells, and TCRs. In contrast to T cells, NKT specifically bind lipids presented in MHC-I complexes different in structure to those recognised by CD8 T cells. Following lipid antigen recognition and activation, NKT secrete cytokines that activate T helper responses. In addition, they stimulate DC functions [145], [146]. In the context of cancer, NKT cells can be found infiltrating tumours and, although in early stages of tumour development they provide anti-tumour responses, in later stages they can become anergic and promote tumour progression [147].

1.3. Tumour immunoediting: elimination, equilibrium, and escape

Cancer immunoediting refers to the genetic changes that tumour cells undergo over the course of the disease that diminishes their immunogenicity, which is their potential to be detected and eliminated by the immune system. This results in tumour growth and potentially drives metastasis. Tumour immune editing is developed in three phases termed elimination, equilibrium, and escape [148]. During elimination, the innate and
adaptive immune cells cooperate to kill cancer cells. Tumours that are not fully eliminated enter the equilibrium phase, in which net tumour growth is limited but the tumour modifies its immunogenicity following sensing the adaptive response (i.e. PD-L1 expression in tumour cells upon IFN-γ sensing). If cancer cells continue mutating and developing mechanisms to avoid their recognition by the immune system, they enter in the escape phase, where tumour progression is further accelerated due to the accumulation of immunosuppressive molecules secreted by the tumour cells and by other immunosuppressive cells, Figure 11 [149].

Figure 11. Tumour immunoediting.
Throughout disease progression, tumour cells undergo genetic alterations that facilitate evasion from the immune system, facilitating cancer proliferation and eventually, metastasis. 3 phases can be distinguished during immunoediting, which are elimination, equilibrium, and escape. Image created with BioRender (own-design).

Some of these cells are cancer-associated-fibroblast (CAFs), M2-like TAMs, or myeloid derived suppressive cells (MDSC); and together with the cytokine milieu comprise the tumour microenvironment (TME), which plays a key role on tumour evolution.
1.3.1. **Immunosuppressive cells in the tumour microenvironment (TME)**

### 1.3.1.1. **Tumour cells**

Tumours evolve against immune surveillance via range of mechanisms involving genetic and epigenetic changes, metabolic alteration and aberrant signalling pathways [150]. Consequently, tumours evolve to i) reduce the expression of MHC-I molecules as well as TAA and NeoAg, leading to a loss of antigen tumour variants unable to be recognised by CD8 T cells [151] or ii) express ligands (i.e. PD-L1) that bind inhibitor receptors (i.e. PD-1) on T cells [152], facilitating tumour proliferation and escape from the immune system. In addition, a hypoxic environment triggers hypoxia inducible factor (HIF-α) activation in tumour cells [153], a transcription factor that mediates the transcription of pro-angiogenic genes such as vasculature endothelial growth factor (VEGF), PGF (placenta growth factor), ANGPT-2 (angiopoietin-2), CXCL-12 (chemokine C-X-C motif ligand12), involved in tumour vascularisation [154]. VEGF factor promotes secretion of metalloproteases, that modify the extracellular matrix of the tumour, therefore enhancing the probability of tumour cells reaching the bloodstream and thus metastases, **Table 5** [155]. Other genetic changes in tumour cells also result in the secretion of cytokines, which can promote the changes in TAMs phenotype, from TAM-1 to TAM-2 [52].

### 1.3.1.2. **Myeloid derived suppressor cells (MDSC)**

MDSCs are a population of immature myeloid cells that relate directly with cancer progression [156], [157]. Their expansion in tumours is driven by Prostaglandin E2 (PEG2), which diverts the development of DC into MDSCs. Among their immunosuppressive roles, they upregulate the production of ROS, Nitric Oxide (NO), arginine 1 (ARG1) and PGE2, which trigger T cell disfunction [158]. In addition, production of IL-10 further enhances Treg recruitment and differentiation into the tumour bed [159].
1.3.1.3. Cancer-associated fibroblasts (CAFs)

Fibroblasts are key cells for the generation of extracellular matrix and to maintain tissue homeostasis. In solid malignancies CAFs are one of the most abundant stromal cells and secrete growth factors, inflammatory ligands and remodel extracellular matrix proteins that promote cancer cell proliferation, therapy resistance and immune exclusion [160].

Table 5. Molecules mediating tumour progression in TME

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Type of molecule</th>
<th>Secreted/expressed by</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS/NO</td>
<td>Free radicals</td>
<td>M2-TAMs, MDSC, CAFs, tumour cells</td>
<td>Initiate angiogenesis and metastasis. T cell dysfunction</td>
</tr>
<tr>
<td>IL-10</td>
<td>Cytokine</td>
<td>M2-TAMs, Tregs, tumour cells</td>
<td>T cell and NK dysfunction. Hampers DC maturation or antigen presentation</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Cytokine</td>
<td>M2-TAMs, Tregs, CAFs, tumour cells</td>
<td>Treg recruitment and differentiation. DC and NK dysfunction</td>
</tr>
<tr>
<td>CCL2/3/4/5/7/8</td>
<td>Cytokine</td>
<td>Cancer cells, M2-TAMs</td>
<td>Monocyte recruitment and differentiation into M2-TAMs. MDSCA recruitment</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Cytokine</td>
<td>CAFs</td>
<td>Direct tumour growth promotion</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin (lipid mediator)</td>
<td>M2-TAMs, MDSC, CAFs, tumour cells</td>
<td>Increases proliferation and decreases apoptosis of tumour cells</td>
</tr>
<tr>
<td>ARG1</td>
<td>Enzyme</td>
<td>M2-TAMs, MDSC</td>
<td>T cell disfunction</td>
</tr>
<tr>
<td>MMPs</td>
<td>Enzyme</td>
<td>M2-TAMs, CAFs, tumour cells</td>
<td>Extracellular matrix remodelling, eases tumour progression</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Growth Factor</td>
<td>M2-TAM, CAFs, tumour cells</td>
<td>Angiogenesis, tumour progression, NK dysfunction, T cell exhaustion</td>
</tr>
<tr>
<td>HIF-1 α</td>
<td>Transcription factor in response to hypoxia</td>
<td>M2-TAMs</td>
<td>Promotes tumour growth; suppresses T cell functions.</td>
</tr>
</tbody>
</table>
1.3.1.4. M2-like TAMS

Although during early tumour stages TAM are prevalently M1-like, in later stages, the presence of immunosuppressive molecules including PDGF, VEGF or cytokines such as IL-10, C-C-motif chemokine ligands (CCL) 2, 3, 4, 5, 7, 8 (CCL2/3/4/5/7/8) and CXCL12, mediate monocyte recruitment into the tumour and their polarisation to M2-like TAMs [54]. M2-like TAMs sense hypoxia and contribute to VEGF and metalloproteases (MMPs) secretion and consequent angiogenesis and neovascularisation, eventually enhancing tumour proliferation, matrix remodelling [161], [162] and the probability of tumour metastasis. Moreover, M2 TAMs secrete anti-inflammatory cytokines such as IL-10 or TGF-β that trigger immunosuppression of other immune cells, including Treg recruitment. M2 TAMs also present enhanced PD-L1 expression, which is correlated with dysfunction of T cell responses [52], [54]. Overall, M2 macrophages are associated with poor prognosis in a wide range of tumours [163].

Other cytokines including IL-10, TGF-β or PGE2 (Table 5) [164] transform CD4 effector T cells into Tregs, or recruit the latter to the tumour bed, hampering CD8 T cell anti-tumour cytotoxicity, thus easing tolerance and tumour progression. In addition to Treg-induced tolerance, other CD8 T dysfunctional states may occur within the TME (section 1.3.2.1., 1.3.2.2 and 1.3.2.3).

1.3.2. Impaired anti-tumour CD8 T cell responses within tumour microenvironment

The immunosuppressive TME also contributes to the development of inefficient effector tumour-reactive CD8 T cell responses, which is critical for cancer clearance.

1.3.2.1. Tolerance and anergy

The immunosuppressive TME has a direct impact on the priming efficiency of tumour-reactive CD8 T cells, driven through the alteration of cDC1 infiltration or differentiation [77], [165]. For instance, VEGF inhibits FLT3L activity (mainly secreted by NKs), which is critical for cDC1 differentiation [166]. IL-6 and prostanoid secretion also inhibit cDC maturation and survival [167]. Metabolites such as lactic acid impair Mo-DC
differentiation and activation [168]. In addition, oxidised lipids in the TME can be incorporated into cDCs and inhibit the formation of MHC-I-TAA/NeoAg complexes, thus reducing anti-tumour CD8 T cell activation [169]. DCs in the TME also downregulate CD80, CD86 and IL-12 expression upon interaction with TGF-β in the TME, thus unpairing effective anti-CD8 T cell responses and driving tumour tolerance [170], [171]. Other TGF-β mediated immunosuppressive mechanisms in tumour DCs include the downregulation in expression of MHC-I of IFN molecules Figure 12 [172].

**Figure 12. CD8 T cell tolerance and anergy.**
These dysfunctional states are normally found in tumours in the presence of immunosuppressive cells and cytokines, and they prevent effector anti-tumour CD8 T cell cytotoxicity. Image created with BioRender (own-design).

Similar phenotypes are found in DC under the influence of VEGF, which has the potential to abrogate MHC-II, CD40 and CD86 expression [173]. To finish with, tumour- reactive CD8 T cell tolerance to tumour cells can also happen as a result of Tregs presence in the tumour bed, as explained in section 1.2.2.1.3.2.
1.3.2.2. Ignorance

Tumour genetic mutations driving low expression of MHC-I-TAA/NeoAg hampers cancer cell recognition by tumour-reactive CD8 T cells, thus becoming “ignorant” [151]. This can be due to the downregulation of any protein forming the MHC-Ag complexes by tumour cells during the escape phase. In addition, ignorance may arise when the antigen presented by tumour cells cannot be recognised as dangerous by CD8 T cells, due to sequence similarities with the same antigen in healthy cells, or lack of DAMPs or other danger signals that can induce DC maturation and priming of naïve CD8 T cells. The latter situation can occur during early stages of the disease [174].

1.3.2.3. Exhaustion

The term exhaustion was first coined in patients suffering from chronic viral HIV infections, where certain HIV-reactive CD8 T cells clones were hyporesponsive and unable to clear the virus, manifested by decreased CD8 T cell proliferation, cytotoxicity and cytokine production as well as overexpression of inhibitory checkpoint receptors such as PD-1, TIM-3 and LAG-3 [175]. In contrast to anergy, exhausted CD8 T cells are properly activated in a first step, however they undergo epigenetic and transcriptional modifications that result in lower killing efficiency [176]. Chronic infections and cancer are both characterised as long diseases where antigens are permanently exposed to T cells. This suggests that exhaustion is driven by prolonged CD8 T cell activation due to continuous antigen exposure, a proposal reinforced by the observation that exhausted CD8 T cells in chronic infection or tumours are enriched with expression of genes related with TCR signalling [177].

Much attention has been drawn into understanding the transcription mechanism driving the expression of exhaustion-related markers such as PD-1, TIM-3 or LAG-3 receptors in tumour-reactive CD8 T cells. In recent years, the role of the transcription factor thymocyte selection-associated HMG BOX (TOX) has been shown to be critical for the induction of inhibitory checkpoint molecules in exhausted tumour-reactive CD8 T cells [178]. TOX expression is triggered on CD8 T cell activation following Ag-TCR stimulation and consequent NFAT activation, which supports the proposal that exhaustion results
from chronic antigen exposure. In addition, the absence of TOX in cells prevents the generation of exhausted CD8 T cells [178], [179]. TOX is also directly related with early epigenetic events that drive the differentiation of exhausted tumour-reactive CD8 T cells [179], Figure 13.

Figure 13. CD8 T cell exhaustion.
CD8 tumour infiltrating lymphocytes (TILs) can acquire an exhausted phenotype owed to chronic activation due to continuous MHC-I-Ag recognition. This dysfunctional state correlates with enhanced expression of the transcription factor TOX, which enhances inhibitory receptor expression, and decreases proliferative potential following MHC-I-Ag encountering in tumour cells, allowing for tumour progression. Image created with BioRender (own-design).

1.3.2.3.1. Differentiation of CD8 exhausted phenotypes
The differentiation of naïve CD8 T cells upon activation is dependent on the context and duration of the antigen encounter. Differentiation into effector phenotypes is achieved on transcription of genes that regulate proliferation, survival, and cytotoxic mechanisms. Following the expansion phase, effector CD8 T cells undergo contraction and die, although a few clones remain as memory T cells. Thus, naïve, effector, and memory differentiation states, have exclusive transcriptional programmes that shape their phenotype and functional characteristics [180]. In a similar manner, transcriptional
signatures driven by prolonged chronic antigen exposure, might restrict the generation of a pool of memory T cells, and instead these cells might enter an alternative transcriptional program associated with functional unresponsiveness, such as exhaustion [176].

Figure 14. T cell exhaustion.

It is hypothesised that differentiation of naïve CD8 T cells into exhausted phenotypes constitutes a multistep process which takes place over the course of weeks to months of chronic antigen exposure [181]. Recent studies have shown that, in early stages, exhausted CD8 T cells express the “Transcription factor cell factor 1” TCF1, which is a marker for memory T cells and that provides stemness and proliferative potential. Thus, TCF1+ CD8 T cells are termed stem-like. However, in contrast with memory T cells, TCF1+ exhausted T cells also express inhibitory receptors such a PD-1. Importantly, stem-like exhausted CD8 T cells (TCF1+PD-1+) lack TOX expression while retaining their
proliferation potential and give rise to terminal differentiated exhausted CD8 T cells expressing TOX and higher levels of inhibitory receptors, which eventually die [182],

**Figure 14.** The exhaustion state in CD8 stem-like precursors is reversible and whereas terminally differentiated exhausted CD8 T cells in cancer patients are mainly found infiltrating the tumour, stem-like precursors are found in peripheral tissues (i.e. LN or spleen). This pinpoints the role of the TME in the induction of this state and highlights the peripheral tissues as a reservoir for stem exhausted precursors CD8 T cells [183].

1.3.2.3.2. Inhibitory checkpoint receptors in tumour-reactive and exhausted CD8 T cells

Inhibitory checkpoint molecules act as a brake for the activation and effector functions of CD8 T cells upon ligand binding in DCs or tumour cells.

**TIM-3:** One of the 4 members from the “T cell immunoglobulin and mucin domain-containing protein” (TIM) family. This transmembrane protein was first discovered in IFN-γ producing CD4 and CD8 T cells, although it is also expressed in other cells, including CD4 Tregs and myeloid cells [184]. TIM-3 binds to several ligands, including “Galectin 9”, a soluble protein secreted by haematopoietic cells (including APCs) and tumour cells, especially in the presence of IFN-γ; phosphatidylyserine, which is overexpressed in apoptotic cells [185]; the adhesion molecule “Carcinoembryonic antigen-related cell adhesion molecule 1” (CEACAM1), which can be found in tumours, APCs and in activated T cells; the **high mobility group protein B1** (HMGB1) that binds to DNA released from dying cells, acting as a danger signal for TLRs. TIM-3 is expressed on the cell membrane and in the absence of ligand binding, may be recruited into the IS and contribute to T cell activation. However, ligand engagement counteracts the activation of T cells and ultimately, TIM-3 expressing cells become apoptotic [186]. High levels of TIM-3 in solid tumours such as colon, cervical, lung or renal cancer are associated with decreased patient survival [187].

**LAG-3:** “The lymphocyte activation gene-3” is a transmembrane protein and shares structural similarity with CD4 protein together with 20% of aa homology; both genes are located adjacently on chromosome 12, which suggest they evolved by a
duplication event. Similar to PD-1, LAG-3 is expressed on CD8 T cell membranes following antigen stimulation. LAG-3 binds MHC-II in APCs, with higher affinity than CD4, and thus preferentially inhibits CD4 T cells. LAG-3 is also expressed in exhausted tumour-reactive CD8 T cells and on ligand binding, an inhibitory signal that prevents T cell activation is induced, although the mechanism remains unclear [188]. LAG-3 is highly glycosylated and binds lectin proteins including galectin-3, released by tumour cells; or liver sinusoidal endothelial cell lectin (LSECTIN), expressed in liver and tumour cells such as melanoma [189].

**PD-1**: The Programmed Cell Death Protein (PD-1, also known as CD279) is a 55 kDa transmembrane protein and its expression is controlled by transcription factors such as NFAT, NOTCH, TOX and IRF9. Tumour cells overexpress PD-L1 as a mechanism of immunoediting, that is thought to be influenced by sensing of cytokines including IFN-γ [152]. PD-1/PD-L1 interactions counteract the signal 1 (MHC-Ag-TCR) and signal 2 (CD80/CD86-CD28) activation signal transduction, by abrogating cytokine production, promoting cell cycle arrest and decreasing the transcription of cell survival mediators. The direct pathway of PD-1 inhibitory signalling transduction involves the activation of a series of IC phosphatases (SHP1 and SHP2), that terminate with the phosphorylation cascades driving T cell activation following CD28 or TCR interactions. In addition, the PD-L1/PD-1 axis indirectly decreases T cell activation by regulating the expression of proteins involved in the cell cycle, namely cyclin kinases and cyclin-dependent kinases [190]. The discovery of monoclonal antibodies disrupting PD-1 to PD-L1 interactions to restore T cell functionality comprises one of the most revolutionary immunotherapies to date [191].

1.4. **Immunotherapy approaches: Modulating the anti-tumour immune response**

The discovery of tumour immunosurveillance and immune-editing processes opened the possibility to develop immune-related therapies to treat tumours [103]. Despite surgery, chemotherapy and radiotherapy being the principal treatments for cancer,
immunotherapies are showing significant benefit for several types of tumours either in stand-alone or combinatory treatment [192]. Given the key role of tumour-reactive CD8 T cells in tumour immunosurveillance, a large majority of these therapies are directed at strengthening anti-tumour CD8 T cell responses at different levels: from boosting the priming efficiency, the recognition of tumour cells, or their recruitment into the tumour mass, to reversing their dysfunctional state [103]. In this section, immunotherapies, with an emphasis in vaccines and immune checkpoint inhibitors (ICI) are reviewed.

Figure 15. Immunotherapy treatments tackling diverse stages in the cancer-immunity cycle. Adapted from: “Oncology meets immunology: the cancer-immunity cycle”. Daniel S. Chen & Ira Mellan, Cell Immunity, 2013.

1.4.1. Cytokine-based therapy

IFN-α (against hairy cell Leukaemia, melanoma and follicular lymphoma) and recombinant IL-2 (against melanoma and renal cell carcinoma) were the first cytokine-based immunotherapies approved by the FDA during the 1990s and since then other cytokines such as IL-12 and IFN-γ, or TGF-β inhibitors have also been commercialised [193]. Although clinical remission could be achieved, their use also results in the
development of adverse side-effects related with autoimmune disease, which has limited their clinical use [194].

1.4.2. Adoptive T cell transfer (ACT) therapy

ACTs comprise treatments in which tumour-reactive T cells are infused into cancer patients. These cells can be from the same patient (autologous) or from other sources (allogenic, i.e. donor blood or umbilical cord cells). Therapies comprising ACTs are i) Tumour Infiltrating Lymphocytes (TILs), ii) TCR-engineered T cells and iii) Chimeric antigen receptor T (CAR-T) cells [195].

In TILs therapies, T cells are isolated from patient-derived tumour explants obtained from surgery, and later, NeoAg-reactive T cell clones (identified by high-throughput sequencing analysis) are expanded and activated ex vivo, prior to re-infusion. This therapy has achieved tumour remission in some melanoma patients [196], and there is promise in patients with breast cancer [197]. However, its efficacy is largely reliant on the presence of NeoAg-reactive CD8 TILs in the tumour bed at the time of surgery. To overcome this problem, recombinant TCR- and CAR- T cells were developed, which are genetically engineered to specifically target antigens expressed in tumour cells [195]. Recombinant TCR T cells are engineered to express TCRs that specifically recognise NeoAg expressed in the context of MHC-I molecules, however this therapy depends on efficient NeoAg presentation in tumour cells, which might decrease its efficacy [198]. CAR-T cells have been developed to express genetically engineered constructs comprised of an antigen-binding domain (extracellular, Ig variable chain), linked to TCR (CD3) and co-stimulatory-like signalling domains (IC), which allow tumour antigen recognition and T cell activation bypassing the need of MHC-I-TCR synapses. This strategy provides a revolutionary approach for the treatment of blood related malignancies, such as B cell leukaemia and lymphoma (2017 FDA approved drugs: Tisagenlecleucel, Axicabtagene) [199]; it is also showing promising results for the treatment of multiple myeloma [200]. However, its translation into solid malignancies has proved more complicated as these cells infiltrate tumour tissue inefficiently and also due to tumour immunosuppression [201]. In addition, CAR-T cell treated patients
frequently develop immune related adverse effects (irAEs), that range from mild to life-threatening [202].

1.4.3. Immune checkpoint agonists and inhibitors

1.4.3.1. Immune checkpoint inhibitors (ICI)

A range of monoclonal Ab (mAb) have been developed to disrupt the interaction between inhibitory checkpoint molecules in T cells (i.e PD-1, CTLA-4), with their ligands, with the aim to re-invigorate T cell effector functions. *Figure 16.* Ipilimumab was the first ICI approved by the FDA in 2011, which is an α-CTLA-4 Ab for the treatment of melanoma, and may provide up to 3 years of life extension in patients [203]. Proposed mechanism of actions mediating α-CTLA-4 tumour regression involve enhancement of cytotoxic responses of NeoAg-reactive CD8 T cells [204], as well as Treg depletion mediated by ADCC [205], [206]. In 2014, the FDA approved the first α-PD-1 mAbs (*nivolumab* and *pembrolizumab*), for the treatment of refractory and unresectable melanoma [207]. Since then, the use of pembrolizumab has expanded for the treatment of other tumours such as NSCLC and head and neck squamous cell carcinoma, gastric/gastro-oesophageal junction cancer, Hodgkin lymphoma, urothelial carcinoma [195], and the use of nivolumab for head and neck squamous cell carcinoma, urothelial carcinoma, hepatocellular carcinoma, Hodgkin lymphoma and colorectal cancer [195]. Recently, attention is being drawn to the development of antibodies that can result in blockade of other molecules such as TIM-3 [187] or LAG-3 [208].

**α-PD-1: Mechanism of action**

Although ICI have revolutionised cancer treatment, only certain patients respond favourably to these therapies [209]. This is related to the fact that the process of immunosurveillance is not equal in all types of tumours, and in fact, in the absence of any treatment, some tumours are more “immune-inflamed” than others. Immune/T cell infiltrated tumours are denominated “hot tumours” and can be differentiated from “cold tumours”, which present low immune infiltration [210]. Intuitively, the efficiency of checkpoint therapy relies on the presence of exhausted T cells in tumours, whose
effector status might be reinvigorated by checkpoint blockade [211]. Accordingly, ICI therapies are normally efficient in patients harbouring hot tumours, while patients with cold tumours normally present worse ICI outcomes. However, exceptions exist whereby patients harbouring cold tumours respond well to ICI, while poor ICI responses are found in highly infiltrated tumours [212]. Thus, the mechanism by which ICI, such as α-PD-1, mediates anti-tumour responses remains a matter of debate: although α-PD-1 antibodies can revert the functional state of exhausted CD8 T cells in the tumour, allowing them to kill PD-L1+ tumour cells [211], it is also understood that following α-PD-1 therapy, the T cells that mediate anti-tumour responses are de novo recruited into the tumours, from tumour-dLNs [211], [213]–[215]. It is believed that T cells infiltrating the tumour following α-PD-1 therapy are stem precursors (TCF1+TOX-) of exhausted CD8 T cells [216].

Figure 16. α-PD-1 checkpoint blockade.
Diagram describing α-PD-1 and α-CTLA4 therapy disrupting inhibitory signalling. Image created from a BioRender template.

In parallel, it has been shown that cDC1 cells in tumours upregulate the expression of PD-L1 following antigen uptake [217] and that they also require the expression of this receptor for their migration to the tumour-dLN [218]. Interestingly, physical interaction of PD-L1+ DCs with PD-1+ T cells [214] has been reported in tumour-dLNs, and the presence of PD-L1+ DCs is directly related to enhanced responses by α-PD-1 checkpoint
therapy [219], highlighting the critical role of cDCs in tumour-dLN in the responses against this therapy.

1.4.3.1.1. Immune agonists

Similar to immune checkpoint blockade, other agonist mAbs have been developed to target co-stimulatory T cell molecules and enhance activating signalling. Some examples include OX-40, 4-1BB, or ICOs, and are reviewed elsewhere [220], [221].

1.4.4. Therapeutic cancer vaccine

The immunological memory developed by T cells and B cells during tumour immunosurveillance allow for the development of a more efficient effector response to re-exposure of antigens. This facilitates the development of therapeutic cancer vaccines, aimed at boosting pre-existing, or prime de novo anti-tumour adaptive responses in an antigen-specific manner Figure 17 [222]. In addition, tumour killing by CD8 T cells following vaccination might enhance the release of other tumour antigens, which enhances and broadens the repertoire of tumour-specific T cell responses, in a process termed as antigen spreading [223]. Aside from therapeutic vaccines, prophylactic cancer vaccines also exist and have shown great efficacy for the prevention of virus-related cancers, including the Hepatitis B virus or Human papillomavirus [224].

Effective anti-tumour vaccines are composed of i) immunogenic and tumour-specific antigen(s) that can be presented to T cells, ii) appropriate adjuvants (or other PRR stimulators) to induce DC maturation and avoid tolerance and iii) efficient targeting systems to DCs [225]. In addition, the use of combination therapies tackling the immunosuppressive TME should also be considered for enhanced responses (see section 1.4.4.1.2.). Vaccine approaches are classified into two: those that comprise cell-based strategies (i.e. APCs or tumour cells); and those in which antigen and adjuvant are administered in the absence of cells [225].Cell-based immunisations, which date from the 1970s [226], involve ex vivo APC isolation from patients which are further loaded
with protein lysates purified from the patient’s tumour biopsies (autologous vaccine) [227], or from other compatible patients (allogenic vaccine) [228].

Figure 17. Cancer vaccination induces anti-tumour T cell responses that contract into memory pools.

In addition, APCs might be loaded with rationally-selected tumour antigens, which is the case for the only-FDA approved anti-cancer therapeutic vaccine available (Sipuleucel-T), for use in patients with prostate cancer which received approved in 2012 [229]. With this approach, patient’s monocytes are pulsed with an antigen specific for prostate cancer and further stimulated with GM-CSF. However, this vaccine has had minimal clinical impact and it is only useful for patients in early stages of disease [230]. Similarly, in other cell-based strategies, tumour cells obtained from patient’s biopsies are administered in combination with adjuvant molecules into patients, or alternatively, tumour cells can be transduced with adjuvant-like molecules prior to reinfusion into patients. In both cases, the ex vivo DC loading step is bypassed, under the premise that tumour cells will be taken up by DCs and tumour antigens will be presented. An example of this strategy is the vaccine GVAX, that has undergone years of clinical trials with moderate success [231], [232].
Although many cell-based vaccines are still undergoing clinical trials (reviewed in [225]), they comprise expensive approaches, and many impracticalities arise from the tumour sample collection process. More efficient immunisation strategies are based on rationally selected tumour antigens, administered in the form of proteins, peptides, DNA, RNA or viruses, in combination with adjuvants. In the next section, the classification of tumour antigens, the use of adjuvants for cancer vaccine, as well as the DC-targeting strategies and immunotherapy combination strategies to overcome immunosuppression TME is reviewed, with focus on NeoAg based vaccines in nanomedicine.

1.4.4.1. Tumour antigen classification: The neoantigens (NeoAg) era

Tumour antigens with immunogenic potential, that is a likelihood to be recognised as non-self in the presence of co-stimulation, are classified into tumour associated antigens (TAA), and neoantigens (NeoAg).

TAAs refer to either antigens overexpressed in cancer cells compared to healthy cells (typically proteins involved in cell cycle including p53, cyclin B; apoptosis inhibitors such as surviving, or human epidermal grow factor, HER2, in breast cancer); antigens differentially expressed in specific tissues (i.e. melanoma-specific proteins including GP100, Melan-A, also known as MART-1, or tyrosinase); or cancer testis antigens (CT), expressed in prostate cancer and whose expression is absent in healthy tissue [233]. The first TAA was defined in the 1990s [234], and since then, their use as therapeutic vaccines has been widely attempted. However, little success has been achieved in the induction of durable anti-tumour responses, as there is difficulty for the immune system to recognise them as “non-self” and to break tolerance against them [235].

NeoAg are exclusively expressed in cancer cells following non-synonymous somatic mutations in the DNA of tumour cells, that cause changes in the protein sequence [236], which can be point mutations, indels or gene fusions [237], [238]. Consequently, immunisations with NeoAg are more likely to be recognised as “non-self” and break immunotolerance to generate “tumour-reactive” T cell responses. In addition,
immunisations with NeoAg pose less risk than TAA-based vaccines to induce irAEs/off-target killing of healthy tissue [222]. These mutations can be identified at a patient level with the development of bioinformatic tools combined with DNA/RNA sequencing of tumour samples, and the prediction of antigen binding in MHC-I and MHC-II complexes (Figure 18); these have created a revolutionary approach for the development of personalised therapies [239]. During NeoAg vaccine development the mutations that result in NeoAg do not necessarily need to be expressed across all the cells within the tumour. Whereas mutations giving rise to the first phases of tumour development, known as driver or clonal mutations, are likely to be present in all tumour cells, other mutations might arise in later stages of the disease and only in certain cells, and therefore subclonal mutations [240]. Vaccinations with NeoAg that arise in subclonal mutations are less likely to induce a potent tumour regression, thus, NeoAg-based vaccines target several peptides to reduce the probability of immune evasion and enhance tumour eradication [241].

Figure 18. Personalised neoantigen discovery for vaccine development pipeline.

Indeed, evidence indicates that NeoAg that arise from driver mutations can be shared across patients (i.e. mutations in genes coding for proteins involved in preventing DNA damage and regulation during the cell cycle, such as K-Ras or p53), which raises the possibility for “off-the-shelf” tumour vaccine development [242], [243] Nevertheless, practicalities such as cost and duration of manufacture restrict NeoAg use, and an agreed vaccine delivery platform need to be established.
1.4.4.1.1. Pre-defined NeoAgs used in preclinical cancer vaccine (rodent) models: Ovalbumin and MC38 colorectal derived NeoAg

Since the 1900s, the egg-derived protein ovalbumin (OVA, Uniprot: P01012) has been used as an antigen model for the analyses of immune responses and vaccine approaches, as it triggers efficient adaptive responses in the presence of adjuvant [244]. In addition, the repertoire of OVA-derived peptide sequences presented in MHC-I and MHC-II complexes following IC antigen processing, have been extensively studied, and it has been shown that the peptide SIINFEKL binds MHC-I complexes in DCs with high affinity [245]. Several tumour models, including the melanoma B16F10, have been genetically modified to express OVA (B16F10-OVA). In this context, OVA and especially SIINFEKL, act as NeoAg in this type of tumour model.

However, since OVA is an exogenous protein, more physiologically relevant NeoAg tumour models have been studied, for instance mouse colon Adenocarcinoma cells, namely MC38. Yadav and collaborators first sequenced MC38 cells and predicted three NeoAg with MHC-I binding capacity: MUT-1 or Adpgk (ASMTNMELE); MUT-2 or Reps1 (AQLANDVVL); and MUT-3 or Rpl18 (KILTFDRL). Immunisations with a pool of the three NeoAg in combination with adjuvant and ICI triggered specific T cell responses that resulted on MC38 tumour inhibition in mice, validating MUT1, MUT2 and MUT3 peptides as NeoAg [246].

1.4.4.1.2. NeoAgs in immunosurveillance: rational for vaccine and ICI combinatory approaches

Higher mutational burden load in tumours normally associates with the presence of NeoAg and with hot tumours harbouring larger amount of immune infiltrates, especially CD8 TILs with cytotoxic [247], [248], and exhaustion [249], [250] signatures. Accordingly, NeoAg load is a good prognostic indicator in patients, and treatment with ICI therapy results in improved responses [251], as found in patients with melanoma [252] or NSCLC [253]. Moreover, tumours that express higher mutational burden rates are more likely to express larger numbers of NeoAg candidates for vaccine development. Thus, NeoAg immunisations in these patients further activate tumour-reactive CD8 T cells, and
combinatory ICI therapies ultimately optimise anti-tumour responses to a greater extent than ICI or vaccine administered as monotherapy [222]. As an example, complete responses have been found in patients receiving α-PD-1 therapy following vaccination NeoAg based vaccination [254].

1.4.4.2. Vaccine adjuvants

An important component of vaccines are adjuvants, which are synthetic molecules that induce APC maturation mimicking DAMPs and PAMPs, hence binding PRRs. Adjuvants are necessary to induce the expression of co-stimulatory molecules and cytokines in DCs at the moment of T cell priming, and to avoid the development of tolerance against tumour antigens following vaccination [255].

In the context of cancer vaccines, the benefits of TLR-agonists are being widely explored, including NeoAg vaccine clinical trials [256]. Some are TLR4 agonists, such as Monophosphoryl lipid A (MPLA) [257]; TLR7 agonists, including imiquimod [258] or resquimod (R848) [259]; or TLR9 agonists, for instance, CpG oligodeoxynucleotides [260]. The double stranded (ds) RNA analogue, Polyinosinic:polycytidylic acid (Poly(I:C) or pIC), has proved to be an efficient adjuvant for cancer immunisations [261]. Poly(I:C) binds TLR3 receptors found in the endosomal membrane, which are highly expressed in cDC1, thus boosting maturation of these critical subsets for NeoAg cross-presentation [262].

1.4.4.3. NeoAg-based vaccines

As explained above, NeoAg vaccination is normally conducted in combination with checkpoint therapy, and frequently follow surgery, chemotherapy or radiotherapy [222][225]. Personalised vaccine platforms might include synthetic peptides, genetic material (i.e. RNA or DNA), or viral vectors.

Peptide vaccines typically constitute a pool of up to 20 long synthetic peptides (LSP) of 15-30 aa in size, are processed by the proteasome and form complexes with MHC-I, eliciting efficient anti-tumour CD8 T cell responses [263]–[265]. Some LSP-based vaccines are being tested in clinical trials in melanoma (NCT01970358 NCT02897765).
NCSLC (NCT03633110) or renal cancer (NCT02950766) patients, among others, and frequently are administered with the adjuvant Poly(I:C) [222]. For instance, immunisations with the LSP-based personalised vaccine platform (NEO-PV-01) [266] in combination with poly(I:C) and α-PD-1 therapy (Table 6) in patients with NSCLC (n=27), advanced-stage melanoma (n=34), or urothelial carcinoma (n=21) induced T cells reactive to all the NeoAg in all patients, with evidence of epitope spreading responses. In general, this response correlated with increased survival and lack of disease progression for up to 9 months post vaccination in some patients [266]. Similarly, vaccines based on DNA (NCT04015700, NCT04251117, NCT03548467) mRNA (NCT03289962, NCT04267237, NCT03313778) and less frequently, based on viral vectors (NCT03794128, NCT03639714) in clinical trials, code for up to 20 to 50 NeoAgs per patient, and although adjuvants are sometimes co-administered, these vaccines can act as self-adjuvants, bypassing the need of stimulatory agonists [222]. NeoAg RNA vaccines complexed in lipoplexes are gaining wider clinical acceptance due to positive outcomes in trials. As an example, the RNA-lipoplexed and personalised vaccine platform, RO7198457 (Table 6) codes for 20-NeoAgs [267] and is currently being assessed in trials administered in combination with a-PD-L1, in 132 patients with advanced solid malignancies. T cell analysis in blood and tumour samples (including sites of metastasis) revealed the presence of NeoAg specific T cells following vaccination in 77% of the patients, although this did not correlate with enhanced benefit over PD-L1 as a monotherapy. In both examples, patients did not develop serious adverse effects.

It is obvious that challenges remain to be addressed in the field of cancer vaccines. There is a clear need to further improve the activation and expansion of tumour reactive-CD8 T cells, which is highlighted by the lack of correlation between presence of vaccine-induced NeoAg-reactive CD8 T cells and overall benefit in patients. Hence, it is urgent to develop strategies that promote optimal T cell priming, and consequently enhance APC function and targeting. Vaccine carriers that allow for in vivo DC targeting overcome the impracticalities of ex vivo DC antigen loading and activation strategies.
Table 6. Personalised NeoAg based vaccines undergoing clinical trials.

<table>
<thead>
<tr>
<th>Platform</th>
<th>Cancer</th>
<th>Reference</th>
<th>Method</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEO-PV-01</td>
<td>Melanoma</td>
<td>NCT01970358, NCT02897765</td>
<td>20 personalised epitopes + ipilimumab (α-CTLA4) + nivolumab (α-PD-1)</td>
<td>Antigen-specific T cell response elicited; memory-like T cells seen with MPR noted in 14/19 patients; epitope spreading observed.</td>
</tr>
<tr>
<td>NeoVax</td>
<td>Glioblastoma</td>
<td>NCT02287428</td>
<td>20 personalised epitopes + poly-ICLC</td>
<td>Intracranial NeoAg-specific T cells also found in circulation, memory phenotypes.</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>NCT01970358</td>
<td>20 personalised epitopes + poly-ICLC</td>
<td>Neo-epitope-specific T cell reactivity, TCR diversity including non-vaccine NeoAgs, memory phenotypes, tumour infiltration, epitope spreading</td>
</tr>
<tr>
<td>CONSORT</td>
<td>Gastro</td>
<td>NCT03480152</td>
<td>mRNA encoding 20 personalised TIL-reactive neo-epitopes</td>
<td>3/4 patients exhibited neo-epitope-specific T cells but didn’t translate into anti-tumour responses in patients</td>
</tr>
<tr>
<td>RO7198457</td>
<td>Melanoma</td>
<td>NCT03815058</td>
<td>RNA encoding neo-epitopes in a liposomal complex ± pembrolizumab (α-PD-1)</td>
<td>Primary outcome: PFS</td>
</tr>
<tr>
<td></td>
<td>NSCLC</td>
<td>NCT04267237</td>
<td>RNA encoding neo-epitopes in a liposomal complex ± atezolizumab (α-PD-L1)</td>
<td>Primary outcome: DFS</td>
</tr>
<tr>
<td>GAPVAC-101</td>
<td>Glioblastoma</td>
<td>NCT02149225</td>
<td>Shared antigen + neo-epitope dual vaccine + poly-ICLC and GM-CSF</td>
<td>12/13 patients had CD8 cell responses to unmutated peptide; 11/13 patients had CD4 cell induction and TH1 phenotypes against mutated peptide.</td>
</tr>
<tr>
<td>YE-NEO-001</td>
<td>Solid tumours</td>
<td>NCT03552718</td>
<td>Personalised recombinant heat-killed yeast expressing multiple neo-epitopes</td>
<td>Primary outcome: Treatment-emergent adverse effects</td>
</tr>
<tr>
<td>IVAC</td>
<td>Melanoma</td>
<td>NCT02035956</td>
<td>Poly-neo-epitopic-coding RNA vaccine</td>
<td>Immune response against vaccine antigens detected in 13/13 patients; 60% of 125 selected neo-epitopes elicited a T cell response.</td>
</tr>
</tbody>
</table>

MPR- Major pathological response; PFS- Progression Free Survival: The length of time during and after the treatment of a disease, such as cancer, that a patient lives with the disease but it does not get worse; DFS-Disease Free survival: Length of time after primary treatment for a cancer ends that the patient survives without any signs or symptoms of that cancer. Adapted from "Cancer vaccines: the next immunotherapy frontier”. J.Lin et al. Nat. cancer, 2022.
The use of nanoparticles (NPs), for instance lipid-based liposomes or polymer-based POs, present an alternative as they can be functionalised with specific ligands to receptors expressed in DCs, that enhance targeting and IC delivery of encapsulated cargo (i.e. antigen and adjuvants). In addition, the use of NPs can further enhance antigen stability and improve targeting to lymphoid organs, that boosts priming and the elicitation of anti-tumour responses [268].

1.4.4.4. NeoAg targeting and delivery in DCs: From ex vivo DC loading approaches to rational design of in vivo DC targeting strategies.

Tumour antigen delivery into DCs for cancer vaccine development has been attempted for decades using several targeting and delivery strategies, including specific antibodies or nanomedicines [269]. As receptor mediated endocytosis antigen uptake is believed to enhance antigen cross-presentation, TAA/NeoAg targeting towards endocytic receptors in DCs has been widely adopted [270]. Some examples are C-type lectin receptors (CLRs), including DEC-205, Clec9A, and langerin to target cDC1 cells; CLEC4A and CLEC7A for cDC2 targeting; or mannose receptor (CD206) for the targeting of macrophages [65]. Although TAA/NeoAg fused to DC-targeting Ab have shown potential to elicit anti-tumour immune responses preclinically, these have not succeeded in a clinical setting [271]. Only immunisations with the α -DEC-205 Ab fused to the tumour antigen NY-ESO-1, in combination with Poly(I:C) or resiquimod triggered specific CD4 and CD8 T cell responses which correlated with partial clinical responses, without toxicity [272]. In addition to limited translational potential, some drawbacks from Ab mediated DC-targeted vaccines include the inability to deliver more than one antigen and difficulties with recombinant Ab-antigen synthesis [273]. DC targeting approaches based on NPs provide the possibility to bypass some of these limitations and offer an improved alternative to enhance TAA/NeoAg delivery and presentation in DCs in cancer vaccines.
1.4.4.4.1. Nanoparticle-based approaches in cancer vaccine

Nanomedicine is the application of materials at the nanoscale in the field of health and therapy [274]. Among many types of materials [275], NPs composed of amphiphiles have been explored for drug delivery and vaccine components.

**Amphiphile-based NPs**

Amphiphilic molecules have dual hydrophilic and hydrophobic characteristics and assemble in nano-spheres in aqueous conditions, and examples include lipids or synthetic polymer diblocks. The assembly process is controlled by the relative contributions between the hydrophobic blocks aggregating with each other to minimise repulsive forces from the water and the interactions between the hydrophilic blocks with water [276]. These give rise to two types of structure: micelles or vesicles. Micelles are formed by a single monolayer of the assembled amphiphile in which hydrophobic tails bundle together in the inner space of the sphere structure, whereas hydrophilic heads face the external aqueous media (*Figure 19A*).

**Figure 19.** Polymer amphiphile assembly into spherical or cylindrical micelles, or membranes (A), and membrane evolution into vesicles (B).

A. Packing factor defines the generation of micelles or membranes. Adapted from “Block copolymer nanostructures” T.Smart et al, Nanotoday 2008., B. Adapted from “Bottom-Up Evolution of Vesicles from Disks to High-Genus Polymersomes”. C.Contini et al. iScience 2018.

Vesicle-like structures are delimited by an amphiphile bilayer membrane where hydrophobic blocks cluster together in the centre of the bilayer, and the hydrophilic
fractions face the exterior aqueous media, and the inner aqueous core. Ultimately, the formation of vesicles or micelles are determined by intrinsic features of the amphiphiles, such as the area per molecule ($a_0$), the volume ($v$) and the length ($l$) of the hydrophobic block, which can be correlated with each other by the packing factor ($P$) [277], defined as:

$$P = \frac{v}{a_0 l}$$

When: $P < 1/3$, spherical micelles are formed; $1/3 < P < 1/2$, cylindrical micelles form; $1/2 < P < 1$ [277], membranes arise. Then, membranes further evolve into vesicles (Figure 19A and B) [278].

**Liposomes and polymersomes (POs): vaccine carriers targeting lymphoid organs**

Vesicles constituted by lipid or polymer amphiphiles are termed liposomes or polymersomes (POs), respectively. These structures hold higher potential as cargo carriers than those from micelles as vesicles can harbour hydrophobic and hydrophilic molecules within the hydrophobic brush in the membrane or within the aqueous core, respectively [279]. For a vaccine, this allows the encapsulation of diverse antigens (i.e. peptides, protein or genetic material), and adjuvants, providing them with stability [280], [281]. The size of the NPs has a significant influence over LN targeting. 10-100 nm has been reported to be optimal NP size to reach the lymphatic system and SLOs, larger NPs require prior DC uptake in order to enter LNs [282], [283]. Thus, it is possible to tailor POs and liposomes sizes to passively target LNs and enhance antigen and adjuvant delivery in DCs. Moreover, lipid or polymer blocks can be functionalised with one or more targeting moieties binding endocytic receptors in DCs (i.e. mannose receptors or SRs) to further enhance antigen and adjuvant targeting [284], [285]. Despite similarities between liposomes and POs, liposomes can suffer from limited shelf-life or stability and poor loading efficiency [268]. This could be partially explained as the molecular weight of synthetic polymers is greater than lipids. This results in thicker membranes that allow for higher encapsulation of hydrophobic molecules, as well as more entangled membranes that provide POs with higher stability in biological fluids [286].
1.4.4.2. POs based anti-cancer vaccines: Enhancing DC targeting, NeoAg cytosolic delivery and adjuvant effect.

Polymers are chemically diverse and can be tailored to meet requirements for i) improved active DC targeting, ii) antigen IC delivery and, occasionally, iii) mimic the action of adjuvant. A frequently used targeting strategy exploits the mannose receptor for DC targeting by adding mannose moieties to the polymer [287]–[289] (Table 7). Endosomal escape is achieved through pH-sensitive polymers [289], [290] synthesised to assemble in vesicles at pH higher than 6-6.5 which is the pH in late endosomes. Following endocytosis, POs disassemble in these compartments following acidification, which drives antigen escape into the cytosol allowing for proteasome processing and MHC-I complexation [75]. In addition, some polymers can act as self-adjuvants and promote DC maturation as a result of their cationic nature [289], or mimicking peptide crystals that may activate the inflammasome pathway [291], which also mediates DC maturation. Most of the strategies for POs translation into cancer vaccines are based on formulations up to 100 nm in size [268], that enables efficient lymphoid draining Table 7 [285].

A range of POs encapsulating proteins or peptides are being investigated preclinically for the development of cancer vaccines. The use of proteins or peptides vaccines offer a number of advantages over mRNA-based approaches. Proteins and peptides are more stable than mRNA molecules [292], and are more resistant to degradation. This facilitates their manufacturing and administration. Moreover, protein-based vaccines have been extensively studied in medicine for years, and their safety profile is well established.

Among the murine tumour models where POs might be tested as therapeutic vaccines Table 7, the B16 melanoma model transduced to express OVA (B16-OVA) is widely studied owing the strong immunogenicity of OVA protein. POs encapsulating OVA protein or SIINFEKL peptide, might be used as immunisation vectors in mice harbouring B16-OVA.
The first example of a POs/SIINFEKL nano-vaccine with DC-targeting and endosomal escape properties against B16-OVA is based on the pH sensitive poly(ethylene glycol)-block-poly(2-(di-isopropanol amino) ethyl methacrylate) (PEG-b-PDPA) block copolymer, chemically conjugated to the adjuvant 5,6-dimethyl- xanthenone-4-acetic acid (DMXAA) [289]. DMXAA-grafted or ungrafted PEG-b-PDPA diblocks were co-assembled with a cationic polymer (1,2-epoxytetradecane alkylated oligoethyleneamine 800 (OEI-C14)), in order to generate micellar NPs, namely PDPM or PDPE, respectively. The cationic polymer, together with the PDPA block, triggered endosomal escape and cargo release into the cytosol. Some PDPM formulations were next coated with mannose (man-PDPM) ligand to improve DC targeting. All the formulations loaded with SIINFEKL self-assembled in micelles 50 to 70 nm in size. In vivo nano-vaccine accumulation in the mice’s LNs was achieved 24 and 48 h post vaccination, with man-PDPM NPs providing with the highest DCs target. In vitro, PDPM treated BMDCs enhanced the expression of maturation markers, which correlated with enhanced OVA presentation in MHC complexes. DMXAA was critical for this. Coincubation of NPs - pulsed BMDC with OVA-specific CD8 T cell (OT-I) triggered the activation of lymphocytes, with the highest levels of IFN-γ secretion achieved upon Man-PDPM incubation in BMDCs. The potential of SIINFEKL-encapsulating PDPM and PDPE NPs as cancer therapeutic vaccines was assessed in B16-OVA melanoma model. Immunisations with the formulations after tumour inoculation, revealed a reduced tumour growth rate of a 50-80% in PDPE and PDPM immunised groups, respectively. Nonetheless, tumours were completely rejected in 60% of the animals immunised with man-PDPM. These results correlated with highest numbers of SIINFEKL-reactive CD8 T cells in spleens of man-PDPM immunised mice, as well as the highest numbers of mature DC in LN at day 7 post vaccination. Besides, increased numbers of effector CD8 TILs and decreased numbers of CD4+ Tregs in the tumour mass were found in man-PDPM immunised mice, when compared with control groups. The authors also determined that immunisation with 3 boosts of man-PDPM in tumour free animals, elicited a CD8 T cell memory phenotype in the spleen, with a 5.8 fold increase in numbers when compared with OVA free immunisations. This encouraged them to also assess the effect of mannose coated vaccines as prophylactic vaccines in the B16-OVA melanoma model, finding complete tumour suppression in 4 out of 6 animals immunised with the mannose formulations, as
opposed to no survival prolongation or tumour suppression effect found in animals immunised with the naked peptide. The potential of man-PDPM as a therapeutic vaccine in cancer was translated in a 4T1 breast tumour model in mice. For this purpose, man-PDPM formulations were loaded with a 4T1 NeoAg peptide, named M32, and injected into 4T1 tumour-bearing mice. Although M32 loaded man-PDPM particles triggered an extension in animal survival when compared with the control groups, no tumour regression was achieved. However, combination therapy including immunisations of man-PDPM and a-PD-L1 resulted in a decreased tumour growth rate, and clearance of lung metastasis, which was not achieved in groups where mice received this treatment separately. This therefore represents an example of how nanomedicine can contribute to the development of more efficient personalised cancer vaccines [289]

Other studies in B16-OVA models have shown that PO NPs trigger an adjuvant-like effect, in the absence of traditional adjuvants [287], [291]. As an example, a study reported in 2020 described the adjuvant effect of the polymer p(DMAEMA-OGEMA)-b-p(MAVE) linked to naphthalene-conjugated NeoAg through pH sensitive acetal bounds. Although a pH higher than 7.4 drove the amphiphilic polymer-peptide monomers to assemble into nanospheres of around 100 nm, lower pH conditions triggered acetal bond cleavage and peptide release and reassembling. The polymer was linked to OVA_{41-270} (SIINFEKL long peptide). The pH-sensitivity of this polymer-NeoAg vaccine (namely ‘transformable nanovaccines’ or NTV) helped ease the peptide release into the cytosol, which was mediated by the change of pH in the endosomal compartment (following NTV uptake). In addition, this led to the generation of peptide nanosheets, resembling crystals that would disrupt the endosomal membrane and induce DC maturation, acting as adjuvants. After proving the concept of enhanced cytosolic release in DCs in vitro, their ability to induce both antigen presentation and DC maturation was validated. In vivo, dLN targeting was achieved 24 hours after subcutaneous (s.c.) NTV injection. Therapeutic immunisations in animals harbouring B16-OVA tumours, led to a maximum of 37.5% in mice survival, which correlated with higher TIL infiltration with an effector and memory phenotype, and a decrease in the presence of Tregs in NTV treated mice. The same immunisation strategy was then attempted in a cervical cancer model (Papilloma virus), where NTVs were conjugated with the papilloma E6/7 peptide.
Table 7. Polymersome-based therapeutic cancer vaccines preclinically.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>p(DMAEMA-OGEMA)-b-p(MAVE) [291]</th>
<th>PEG-b-PDPA+ OEI-C14 (cationic polymer) [289]</th>
<th>Mannose-PBAE [287]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated Neog/TAA</td>
<td>Long peptides: OVA&lt;sub&gt;241–270&lt;/sub&gt; B16F10 NeoAg E6/E7 (HPV)</td>
<td>SIINFEKL (short peptide) 4T1 (Long peptide NeoAg, breast cancer)</td>
<td>Short peptide Trp-2 (melanoma TAA)</td>
</tr>
<tr>
<td>NP features/size</td>
<td>100 nm vesicles Peptides cleaved to polymer – acetal bound pH sensitive</td>
<td>50-70 nm micelles encapsulating antigen, adjuvant grafted to PEG-b-PDPA</td>
<td>120-140 nm NPs encapsulating peptides and adjuvant</td>
</tr>
<tr>
<td>DC targeting moiety</td>
<td>---</td>
<td>Some coated with mannose: enhanced DC targeting in LN</td>
<td>Mannose</td>
</tr>
<tr>
<td>IC delivery Ag presentat. T cell priming</td>
<td>Endosomal disruption OVA cross-presentation (BMDCs) OT-I proliferation</td>
<td>Endosomal disruption OVA cross-presentation (BMDCs) OT-I proliferation</td>
<td>In vivo Trp2 T cell priming in spleen</td>
</tr>
<tr>
<td>Tumour model</td>
<td>B16-OVA (1M cells) B16F10 (1M cells) Cervical cancer HPV (1M cells)</td>
<td>B16-OVA (1M cells) 4T1 (breast cancer) (number of cells not disclosed)</td>
<td>B16F10 (50K cells)</td>
</tr>
<tr>
<td>Immunisation strategy</td>
<td>3 doses (s.c.) Day 8, 15, 23 5 ug peptide/dose 100 ug PD-L1/dose</td>
<td>2 doses (s.c.) Day 7 and 14 50 ug SIINFEKL/dose 100 ug DMXAA/dose</td>
<td>4 doses (i.d.): Day 9, 13, 17, 21 100ug Trp2/dose 10 ug MPLA /dose) 7 doses a-PD-L1: Day 9-16 (40ug/dose)</td>
</tr>
<tr>
<td>Checkpoint (ICI)</td>
<td>B16-F10: PD-L1</td>
<td>4T1: PD-L1</td>
<td>a-PD-L1</td>
</tr>
<tr>
<td>TILs</td>
<td>Increased TILs (CD8 and CD4), reduced Tregs Enhanced central and effector memory</td>
<td>Increased TILs (CD8 and CD4), reduced Tregs Enhanced central and effector memory</td>
<td>Higher CD8 to CD4 ratio</td>
</tr>
<tr>
<td>Outcome survival</td>
<td>B16-OVA/HPV: 37% survival B16F10-monotherapy: Enhanced survival B16F10 + a-PD-L1: 50% survival</td>
<td>B16-OVA: 80% survival 4T1: No tumour regression, extended survival if combined with a-PD-L1</td>
<td>No tumour regression. 66% tumour reduction when compared to untreated animals.</td>
</tr>
</tbody>
</table>
Table 7 (continuation). Polymersome-based therapeutic cancer vaccines preclinically.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated NeoAg/TAA</td>
<td>Long peptides E6 and E7 (HPV)</td>
<td>Peptides MELAN-A MART-1 (melanoma TAAs, MHC-I and MHC-II binding seq.)</td>
<td>Short peptide SIINFEKL Protein lysate MC38 cells</td>
</tr>
<tr>
<td>NP features/size</td>
<td>420-490 nm vesicles encapsulating peptide (sustained Ag release skin-dLN)</td>
<td>166-181 nm vesicles encapsulating Peptides/adjuvants</td>
<td>31- 50 nm vesicles encapsulating peptides/adjuvants. Adjuvant linked to polymer, pH sensitivity.</td>
</tr>
<tr>
<td>DC targeting moiety</td>
<td>---</td>
<td>Mannose</td>
<td>---</td>
</tr>
<tr>
<td>IC delivery Ag presentat. T cell priming</td>
<td>---</td>
<td>No endosome disruption mechanism</td>
<td>Endosome disruption. Enhanced maturation and antigen present.</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Non self-adjuvant Poly(I:C)</td>
<td>Non self-adjuvant MPLA, CpG, D-α-tocopherol</td>
<td>Non self-adjuvant IMQD</td>
</tr>
<tr>
<td>Tumour model</td>
<td>TC-1 cervical cancer (HPV). 100K cells</td>
<td>RMS cells (300K) (Cherry melanoma cells)</td>
<td>B16-OVA (50K) MC38 (500K)</td>
</tr>
<tr>
<td>Immunisation strategy</td>
<td>2 doses (s.c.) Day 8 and 22 100 ug peptide/dose 50 ug Poly(I:C)/dose</td>
<td>3 doses (s.c.) Day 7, 14 and 21 100 ug total antigen/dose 20 ug MPLA/dose 20 ug CpG/dose</td>
<td>3 doses (s.c.) Day 3, 6 and 9 10 ug SIINFEKL/dose 10 ug IMDQ/dose ug MC38 lysates not disclosed</td>
</tr>
<tr>
<td>Checkpoint (ICI)</td>
<td>---</td>
<td>a-PD-1 a-OX-40 6 doses (200 ug/dose, i.p) Day 7, 14, 21, 24, 27, 30</td>
<td>---</td>
</tr>
<tr>
<td>TILs</td>
<td>TILs not assessed E6-reactive CD8 T cells in blood</td>
<td>Higher CD8/Treg ratio compared to PBS when combined to ICI. No differences with ICI monotherapy</td>
<td>Increased TILs and NK infiltration, higher M1/M2 ratio (compared to PBS)</td>
</tr>
<tr>
<td>Outcome survival</td>
<td>No rejection. Delayed tumour growth</td>
<td>Not reported. (* if prophylactic vaccination, 60% survival in combination with ICI)</td>
<td>50% survival rate (*also prophylactic effect)</td>
</tr>
</tbody>
</table>
Immunisations with NTVs allowed significant tumour growth suppression and survival of 37.5% of the mice. Immunisations with NTVs conjugating NeoAg from B16F10 melanoma in mice harbouring this tumour resulted in prolonged survival; although NTVs could not completely reject the tumours, immunisations in combination with a-PD-1 led to tumour rejection in half of the treated animals. This study not only highlights the potential for the use of polymer NPs as antigen vehicles to the lymph nodes but also provides evidence for their intrinsic potential as adjuvants, and at improving antigen immunogenicity and tumour rejection on their own or in combination with ICIs [291].

Despite promising results, no POs vaccines have entered clinical trials assessment to date. Thus, it is evident that alternative polymer structures need to be assessed to improve outcomes.

1.5. PMPC-PDPA POs as immune-modulating polymers.

The amphiphilic polymer Poly(2-(methacryloyloxy)ethylphosphorylcholine)-co-poly(2-(diisopropylamino)ethylmethacrylate) (PMPC-PDPA), is a good candidate for DC targeting and antigen IC delivery for cancer vaccine. The PMPC block has a hydrophilic nature, whereas the PDPA block is hydrophobic and drives the generation of POs and other assemblies, in aqueous conditions [294]. While the PMPC block mediates POs uptake, the PDPA enhances IC payload delivery [295].

Figure 20. PMPC-PDPA polymersomes (POs) self-assembly.
Image created with BioRender.
1.5.1. PMPC as ligands for APCs: Scavenger receptor mediated endocytosis

The mechanisms of PMPC-PDPA POs cell uptake is mediated through the interaction between the phosphorylcholine (PC) head in the PMPC block with the endocytic receptors known as Scavenger receptors (SR) class B member 1 (SR-B1), or class B member 3 (CD36), as well as the receptor CD81.

SRs are classified into eight families (A to I), according to their function and structure [296], with the SR-B family including the receptors SR-B1, LIMP2 and CD36. They were first discovered in 1979 and were classified as a type of PRR due to their role in taking up oxidized low-density lipoproteins (oxLDL), that can act as DAMPs in disease [297]. In addition to oxLDL, these receptors are specialised in the uptake of cholesterol and high-density lipoproteins for maintaining cell homeostasis [298]. SR-B1 is also involved in pathogen entry into the cell, such as Hepatitis C Virus [299]. CD81 is a protein that belongs to the tetraspanin family, with cholesterol uptake functions [300].

*In vitro* studies performed by Prof. Giuseppe Battaglia’s group that blocked these receptors in fibroblasts, carcinoma cells and macrophages revealed that POs uptake is mediated by contribution of the SR-B1, CD36 and CD81 receptors [301]. Although these are expressed across tissues due to their key role in cholesterol metabolism, levels between cell subtypes vary. In immune cells, APCs express higher levels than other immune cells in *The human protein atlas* (link: CD81, CD36 and SR-B1). In addition, CD36 [302], [303] and SR-B1 [304] are known to be involved on the uptake of apoptotic cells and mediate antigen cross-presentation in DCs, which suggest their potential as immune cell targets for vaccine development strategies. It is also understood that despite their role as PRRs, SRs cannot induce the expression of activator transcription factors and co-stimulatory molecules *per se*, and rather require the simultaneous interaction of other PRR with their agonists, such as TLR-3 [305]. In fact, the lack of a second PRR signal during SR-ligand binding and endocytosis has been associated with anti-inflammatory responses [306].

It is important to note that the use of PMPC-PDPA POs to target SRs bypass the need to functionalise a polymer with ligands, given that PC comprised within the PMPC block
acts as ligand, which stands as a more simple and scalable strategy for the development of cancer vaccines.

### 1.5.2. PDPA mediated intracellular delivery

Following binding to SRs and CD81, PMPC-PDPA P0s undergo cell internalisation mediated by cell receptor endocytosis, which eventually leads to cytoplasmic payload release. This process is driven by the pH sensitivity of the PDPA block, which includes a tertiary amine that remains protonated at pH lower than its pKa (≈ 6-6.3), and whose deprotonation at higher pH drive particle assemble, in a reversible manner. A drop in pH below 6.3 in late endosomes protonates PDPA, leads to P0s disassemble and payload release into the endosome. Consequently, the osmotic pressure rises and pores form within the endosome membrane, allowing payload release into the cytosol (Figure 21).

Accordingly, a number of studies have validated payload encapsulation and IC delivery mediated by PMPC-PDPA P0s, including DNA and RNA [307], antibodies [308] or chemotherapeutic agents for glioblastoma [309]. Of relevance for this project, PMPC-PDPA P0s have proved efficient in targeting macrophages, for the IC delivery of anti-inflammatory against for rheumatoid arthritis [310] or antibiotics against tuberculosis [311], both *in vitro* and *in vivo*.

### 1.6. Hypothesis and aims

The generation of effective polymer-based cancer vaccines would confer advantages over current liposome-based mRNA anti-cancer vaccines: polymersomes are more colloidally stable than liposomes and protein-based vaccines are less prone to degradation, easier to produce, and have a better-studied safety profile than mRNA-based vaccines.

We hypothesise that PMPC-PDPA polymersomes entrapping protein-based neoantigens act as vaccine carriers and enhance the development of anti-tumour CD8 T cell responses. As PMPC is a ligand for scavenger receptors, polymersomes efficiently navigate neoantigens into dendritic cells without the need of polymer functionalisation.
In addition, endosomal escape mediated by PDPA protonation allows for enhanced neoantigen proteasomal processing and cross-presentation Figure 21.

Aims

1. Assess the ability of PMPC-PDPA polymersomes (POs) to target and deliver neoantigens into dendritic cells and prime CD8 T cells in vitro.

2. Understand the potential of POs as therapeutic vaccine carriers in vivo, in the context of melanoma, using OVA as a model of neoantigen.

3. Characterise the immune responses generated by immunisations with POs and explore the potential for combinatory treatment strategies with checkpoint inhibitors.

4. Investigate the contribution of the immunisation routes in the targeting of DCs and lymphoid organs.

5. Translate these findings into a more physiologically relevant tumour vaccine model, using colorectal (MC38) derived neoantigen peptides encapsulated in PMPC-PDPA POs.
Figure 21. Proposed mechanism of action for PMPC-PDPA POs antigen intracellular delivery and cross-presentation in antigen presenting cells. Image created with Biorender (own design).
Chapter 2: Material and Methods
2.1. PMPC-PDPA polymersomes (POs) preparation and OVA and Adpgk-LSP encapsulation

The PMPC \textsubscript{24/25}-PDPA\textsubscript{75/80} polymer used in this thesis was synthesised in-house by Dr. Aroa Duro Castano and Dr. Christina Picken by atom-transfer radical polymerisation, as previously reported [294]. Self-assembly reactions to generate empty POs were mainly performed by solvent switch (SS), although other techniques including pH switch or film hydration (FH) were explored. In addition, OVA (Invivogen) and the MC38 LSP Adpgk (Adpgk-LSP, sequence HLELASMTN\textsubscript{M}ELMSSIVHQ, from genscript) encapsulation was attempted by incorporating them during pH switch and solvent switch self-assembly reactions. Empty POs formulations obtained by SS were further used for OVA protein and Adpgk-LSP encapsulation.

Of note, all self-assembly and purification procedures were performed under sterile conditions, with autoclaved materials and UV-disinfected equipment, inside laminar flow hoods.

2.1.1. Empty POs preparation by film rehydration

To self-assemble PMPC-PDPA POs by FH, 20 mg of the polymer were dissolved in 1 mL of an organic mixture of 2 : 1 (v/v) Chloroform : Methanol in a glass vial, as previously reported [312]. A polymer film was formed following vacuum conditions at 37 °C for 24 - 48 hours, which was rehydrated in 2 mL of PBS pH 7.4 under stirring conditions (260 rpm) to allow POs self-assembly. The samples were left stirring for 4 weeks at room temperature (RT).

2.1.2. Empty POs preparation and encapsulation of Adpgk-LSP by solvent switch

The SS method was used for self-assembly of PMPC-PDPA POs. In brief, 20 mg of polymer was dissolved in 1 mL of a 3: 1(v/v), methanol: tetrahydrofuran (THF) solution in a glass vial [313]. Then, 2.3 mL of PBS (pH 7.4) was injected into the polymer solution at 2 \textmu L/min and 40 °C under stirring conditions (260 rpm) (Figure 22). In addition, the encapsulation of Adpgk-LSP peptide was attempted using this technique. To this end,
250 µg, 500 µg, or 1 mg of the peptide were mixed with the organic phase prior to PBS addition, from a peptide stock at 10 mg/mL in DMSO. To remove the residual organic solvent, all formulations underwent dialysis (3.5kDa membrane) against an excess of PBS (pH 7.4) for 3 days, replacing the PBS 3 to 4 times per day. For further purification from non-encapsulated peptide and from free polymer, POs formulations underwent Size Exclusion Chromatography (SEC) (section 2.2.1).

![Illustration representing a solvent switch self-assembly reaction](image)

**Figure 22. Illustration representing a solvent switch self-assembly reaction**

### 2.1.3. Empty POs preparation and encapsulation of OVA protein and Adpgk-LSP peptide by pH switch method

Empty PMPC-PDPA POs were obtained by pH switch [314] by dissolving 30 mg of polymer in 3 mL of PBS at pH 2. Then, 500 mM sodium hydroxide (NaOH) was injected into the polymer solution at a constant rate of 2 µL/min at RT and under stirring (260 rpm), until reaching pH 7.4. For encapsulation of OVA or Adpgk-LSP, these were added when the pH had reached 5.6, continuing with NaOH addition at the same flow rate until achieving pH 7.4. All samples were kept stirring overnight (O/N) at 4°C and before purification from non-encapsulated protein by SEC [315].

### 2.1.4. OVA and Adpgk-LSP peptide encapsulation by electroporation in empty POs

The encapsulation of OVA or Adpgk-LSP into PMPC-PDPA POs was performed by electroporation (EP) in empty formulations obtained by SS, as previously described by the group [307]. POs obtained at a concentration of 5 mg/mL were used. For OVA encapsulation, POs were incubated in a 400 µl EP cuvette with OVA at a final
concentration of 2 mg/mL (Figure 23) from a protein stock at 25 mg/mL in water. The mixture was subjected to 5 EP pulses at 2500V (2510, Eppendorf electroporator). For Adpgk-LSP encapsulation, 5 mg/mL of empty POs were mixed with the peptide at a final concentration of 375 µg/mL, from a peptide stock at 10 mg/mL in DMSO. The mixture underwent 5 EP pulses at 1200 V, followed by incubation for 1 hour at 4°C and SEC purification.

Figure 23. Encapsulation of protein or peptides into PMPC-PDPA POs by electroporation.

2.2. POs purification

2.2.1. Size Exclusion Chromatography (SEC)

Following POs preparation and OVA or Adpgk-LSP encapsulation, POs were purified by SEC [315] to remove free polymer and nonencapsulated protein or peptide. In brief, glass chromatography columns (53 mL, Sigma-Aldrich) were packed with a stationary phase consisting of Sepharose 4B (Sigma-Aldrich). A maximum volume of 1 mL of POs was run per cycle and column using PBS pH 7.4 as the mobile phase. The eluted fractions containing the POs (turbid fractions) were collected in 96 well plates, in 350-400 µl fractions. The retention time for POs of 40 to 50 nm in size was 15 - 20 mins (post sample addition into the column). In native state, the sizes of OVA (45 kDa) and Adpgk-LSP (2.1 kDa) are smaller than POs (40-50 nm), thus allowing for separation of POs and non-encapsulated cargo by SEC. However, protein and peptides following EP or SS could result in aggregates of unknown sizes, potentially interfering with a correct separation between POs encapsulating peptide and protein, and the nonencapsulated fraction.
Thus, to corroborate that the POs fraction only contained encapsulated OVA or Adpgk-LSP, the retention times of OVA and Adpgk following EP or SS were assessed beforehand, finding these were 25-30 min and 43-46 min, respectively (appendix, Figure 26).

If required, some formulations were manually re-concentrated after SEC, using MicroKros Hollow Fibre Filters (mPES, cut-off 100 kDa column, sterile, spectrum labs).

2.3. **POs characterisation**

Prior to their use *in vitro* and *in vivo* experiments, the average size, polydispersity, and concentration of the POs samples were characterised as follows.

2.3.1. **Dynamic Light Scattering (DLS)**

POs hydrodynamic diameter and polydispersity index (PDI) were analysed using a Malvern Zetasizer Nano set at 20°C. Samples were diluted in PBS pH 7 to a final concentration of 0.2 mg/mL and placed in 400 µL disposable polystyrene cuvettes (Ratiolab). Each sample was equilibrated for 20 seconds and measured in 3 cycles, each consisting of 20 reads. Data were processed using a Dispersion Technology Software (Malvern Instruments).

2.3.2. **Transmission electron microscopy (TEM)**

To characterise the POs morphology, 1mg/mL of polymer sample was diluted in water and 5uL was added on a glow-discharged copper grid (EM Resolutions) for 1 minute to allow adhesion. Afterwards, the excess of sample on grid was removed using a filter paper and then the grid was immersed in a 20 µL drop of 0.5 % phosphotungstic acid (pH 7, in water) for 3 seconds, and the excess of staining was removed using vacuum for 20 seconds. Samples were imaged in a JEM-100CX II by myself or in a Jeol 2100 Transmission electron microscope (TEM), by Gabriel Ing. Further image processing was performed through ImageJ.
2.3.3. Reverse phase high performance liquid chromatography (RP-HPLC)

The concentration of POS and encapsulated OVA and Adpgk-LSP peptide were quantified by RP-HPLC using a Dionex Ultimate 3000 instrument (ThermoFisher) [316]. The samples were run through a C18 reverse-phase analytical column (Phenomenex™ Jupiter C18, 300A, 150 x 4.60 mm, 5 mm), under elution gradient at flow ratio of 1mL/min, consisting of two different phases: phase A) 0.05% V/V trifluoroacetic acid in Milli-Q water (TFA/Milli-Q water); phase B) 0.05% V/V TFA in methanol (TFA/methanol) using a multistep gradient for 30 mins, as described in Figure 24. The gradient started at 20% in phase B, and progressively increased to 40% during the first 5 minutes. From minute 5 to 15, it went up to 45% in phase B, and was 50% after 20 mins. From minute 20 to 23, it increased to 70% and continued rising until 100% from one minute, where it stabilised from minutes 24 and 25. It dropped down to 20% eluent B at minute 27, and it remained at that proportion until minute 30. Prior to RT-HPLC analysis, POs samples were diluted in PBS pH 5.5 to allow for polymer disassembly. The polymer, protein and

![Figure 24](image-url)
peptide absorbances were detected at 220 nm, using a UV-Vis detector. The absorbance curves were analysed with Chromaleon Chromatography Data System (CDS) software.

2.3.4. Protein loading efficiency (Le) calculation

The quantification of protein or peptide molecules entrapped into a PO after a self-assembly reaction and purification can be reported as loading efficiency (Le). The Le number is calculated as previously described by members of the group [307] and can be summarised as follows:

\[ Le = \frac{N_{\text{prot}}}{N_{\text{pos}}} \]  

\( N_{\text{prot}} \) is the total number of protein molecules entrapped within the POs formulation (after encapsulation and SEC purification), and \( N_{\text{pos}} \) is the number of POs in the same formulation after SEC.

\( N_{\text{prot}} \) number is obtained after the mass of encapsulated protein \( (m_{\text{prot}}) \) quantified by HPLC (following self-assembly and purification), and considering its molecular weight \( (M_{\text{wprot}}) \), and the Avogadro number \( (N_A) \):

\[ N_{\text{prot}} = \frac{m_{\text{prot}}}{M_{\text{wprot}}} \cdot N_A \]  

\( N_{\text{pos}} \) is calculated from two parameters. The first one is the total mass of polymer quantified by HPLC mass \( (m_{\text{polymer}}) \) (after self-assembly and purification), and its Mw \( (M_{\text{wpolymer}}) \). The second one is the aggregation number \( (N_{\text{agg}}) \), which refers to the number of polymer chains that constitutes one PO vesicle:

\[ N_{\text{pos}}, i = \frac{N_A \cdot m_{\text{polymer}} \cdot f_i}{\sum_i N_{\text{agg}}, i \cdot f_i} \]
Where $f_i$ refers to the percentage of POs with the same average diameter (as measured by DLS), and $N_{agg}$ can be defined as follows:

$$N_{agg} = N_A \frac{\rho_{PDPA} V_{PDPA}}{MW_{PDPA}} \quad [4]$$

Where $\rho_{PDPA}$ is the density of PDPA block (corresponding to 1.05 g/cm$^3$) and $V_{PDPA}$ is the theoretical membrane volume of hydrophobic PDPA block only. Assuming POs form spheric vesicles, $V_{PDPA}$ can be further calculated using the average size diameter values obtained from DLS data:

$$V_{PDPA} = \frac{4}{3} \pi \left[ (R_i - d)^3 - (R_{lumen} + d)^3 \right] \quad [5]$$

Where $R$ is the radius of the PO vesicle obtained by DLS, $R_{lumen}$ is the radius of the vesicle lumen, $d$ is the theoretical length of one PMPC block and $l$ is the theoretical length of the PDPA block (Figure 25).

![Figure 25. Structure of POs and its membrane.](image)

Given that:

$$R_{lumen} = R - 2d - 2l \quad [6]$$

Equation [5] can be reformulated as:

$$V_{PDPA} = \frac{4}{3} \pi \left[ (R_i - d)^3 - (R_i - d - 2l)^3 \right] \quad [7]$$
2.3.5. Protein retention efficiency (Re) calculation

The retention efficiency (Re) of a molecule (i.e. protein or peptide) into POs is the protein/peptide mass following encapsulation into POs and purification (as quantified by HPLC), relative to the initial protein/peptide mass added into the initial self-assembly or EP reaction.

\[
Re = \frac{\text{final } m_{prot}}{\text{initial } m_{prot}} \times 100
\]

2.4. In vitro experiments

2.4.1. Cell culture

The murine dendritic cell line DC2.4 was obtained from Merck, and the murine melanoma B16-OVA and colorectal MC38 cell lines were provided by Dr. Felipe Galvez-Cancino (UCL Cancer Institute). DC2.4 cells were maintained in Roswell Park Memorial Institute (RPMI 1640) media, whereas for B16-OVA DMEM (Dulbecco’s modified Eagle medium) was used. Both media were supplemented with 10% Fetal Bovine Serum (FBS, ThermoFisher), 100 U/ml penicillin-streptavidin (P/S, Sigma-Aldrich), and glutamine (500mM, Sigma-Aldrich). All cell lines were incubated at 37°C in a 5% CO₂ atmosphere.

Cell lines were split when 80-100% confluence was reached, using Trypsin-EDTA (0.25%) (ThermoFisher) to detach adherent cells before plating them into new dishes and media.

Bone marrow-derived DCs (BMDCs) were differentiated using FMS-like tyrosine kinase 3 ligand (FLT3L), as previously described [317]. Briefly, 6-week-old female C57Bl/6 mice were culled, and the bone marrow within the femurs and tibias was flushed out with PBS (Sigma-Aldrich) using a needle and a syringe. Red blood cells (RBC) were lysed using RBC lysing buffer (Biolegend). Cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL P/S (Sigma-Aldrich), glutamine (500mM, Sigma-Aldrich), 50 µM β-Mercaptoethanol (Gibco), and 100 ng/mL FLT3L (PeproTech), for 10 days. The culture medium was partially replaced on day 5. After day 10, the generation of differentiated DC-like cells was assessed by flow cytometry, as described in section 2.7, Table 10.
2.4.2. Cell viability assays

The potential cell toxicity of POs upon incubation with DC2.4 and BMDC cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay or alamarBlue™ Cell Viability assay, respectively.

2.4.2.1. MTT assay

Briefly, 3x10⁴ DC2.4 cells were seeded in 96-well plates (Corning) in three replicate wells and allowed to adhere in humidified atmosphere at 37°C with 5% CO₂. Cells were then incubated O/N with 1:2 serial dilutions of POs, starting at 1mg/mL (“POs treated” cells, in formula). Negative and positive controls were performed by treating cells with media only or 5% DMSO, respectively. Since the PMPC-PDPA POs formulations were prepared in PBS solution, other cells were incubated with the equivalent volume of PBS in media (“PBS treated cells”), to account for the potential effect of PBS in cell viability. Following O/N incubation, media was carefully removed and cells were washed with PBS and 5mg/mL MTT solution was diluted in media and added to the well to final concentration 0.5 mg/mL MTT (sigma) for 2 hours at 37 °C in 5% CO₂ atmosphere, allowing for the generation of formazan crystals. In order to solubilise the crystals, media was removed and 100 µL of DMSO was added per well. After 5 minutes, the absorbance was measured using a ELX800 plate reader (BioTek) at 570 nm. Results were expressed as percent viability relative to the negative control condition and normalised to wells treated with PBS diluted in media at the same rations than POs samples. Viability was calculated as follows:

\[
\text{Viability (\%) = 100 x } \frac{\text{Abs "POs treated" cells}}{\text{Abs "PBS treated" cells}} \times \frac{\text{Abs "media only" cells}}{\text{Abs "media only" cells}}
\]

2.4.2.2. AlamarBlue™ Cell Viability Assay

To quantitatively measure the viability of FLT3L-derived BMDCs at day 10 after differentiation alamarBlue assay (alamarBlue™ Cell Viability Reagent, ThermoFisher) was performed. A cellular density of 10⁶ cells/mL of BMDCs were seeded in 96-well
plates, in 100 µL of complete RPMI. PMPC-PDPA POs samples were added into the wells in 1:2 serial dilutions, starting at 1 mg/mL, in triplicate and for 24h (for negative and positive controls, cells were treated with just 5% DMSO or media, respectively). After incubation, 100 µL of alamarBlue solution was added to a final dilution of 1X in each well and 4 hours later, absorbance was measured at 590 nm using a ELX800 plate reader (BioTek). Viability was calculated as in 2.4.2.1.

2.4.3. Western blot

For generating cell lysates, 1x10^6 cells were washed with PBS and centrifuged at 400 xg, twice. Cell pellets were then lysed with 100µL of RIPA buffer (Sigma-Aldrich) including protease inhibitors (1:100, Sigma-Aldrich) for 30 minutes at 4°C. Cell lysates were further centrifuged at 17,000 xg, 4 °C for 20 minutes after which supernatants were collected and protein content quantified by Bradford assay following the manufacturer’s protocol (Protein Assay Dye Reagent, Bio Rad).

For western blot analysis, proteins were denatured with 2X Laemmli buffer (Bio-Rad) and β-Mercaptoethanol (Bio-Rad) at 95 °C for 5 minutes prior to sample addition into 12% Bis-Tris acrylamide gel containing 10% Sodium Dodecyl Sulphate (SDS). A total of 20 µg of protein (40 µL maximum per well) was added on gels and run for 30 mins at 80V, followed by 1.5 hours at 120V using a Power Pac source system (Bio-Rad) in 1X Tris-Glycine running buffer. After electrophoresis, proteins were transferred into polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using wet transfer system in tris-glycine buffer with 20% methanol at 50 V for 1 hour, followed by 100 V for 1 hour at 4°C. Subsequently, the PVDF membrane was blocked with 5% non-fat dried milk powder (PanReac, AppliChem) in Tris-Buffer Saline (TBS) for 1 hour at RT. Membranes were then incubated with diluted primary antibodies to a specific working concentration in 1% milk in TBS with 0.1% Tween (TBS-T) O/N at 4°C for detecting the specific scavenger receptors anti-SR-B1 (Novus Biologicals), anti-CD36 (abcam), anti-CD81 (abcam) according with Table 8. Next, the membranes were washed three times with TBS-T, and appropriate diluted secondary antibodies were incubated for 1 hour at RT, and washed again three times with TBS-T. Membranes were imaged on a Licor CXF system (Odyssey) and
analysed in ImageJ software. Membranes were further probed for cyclophilin A (Cell signalling) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell signalling) as a loading control.

Table 8. Primary (top) and secondary (bottom) antibodies used for western blot.

<table>
<thead>
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<th>Primary antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat Number</th>
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<td>Novus Biological</td>
<td>NB400-131SS</td>
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<td>NB400-144SS</td>
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<table>
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<th>Source</th>
<th>Cat Number</th>
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<td>1:15,000</td>
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</tbody>
</table>

2.4.4. Uptake experiments

Analysis of POs cellular uptake in DC2.4 cells were carried out by confocal microscopy. Briefly, 4 x 10⁴ DC2.4 cells were plated per well in a poly-lysine pre-coated glass plate (8-well plate, Ibidi™) and allowed to grow O/N. Cells were then incubated with Rhodamine (Rho)-labelled PMPC-PDPA POs diluted in media for times ranging from 5 minutes to 24 hours. After each timepoint, the cell membrane and nuclei were stained with CellMask™ Deep Red Plasma Membrane Stain (1:2,000, LifeTechnologies) for 8 minutes, and Hoechst Stain (1:2,000, ThermoFisher) for 5 minutes, respectively. Cells were visualised on a Leica TCS SP8 confocal microscope (Hoechst by Diode 405 laser; CellMask™ Deep Red by DPSS 561, and Rho-POs by HeNe 633 lasers). For live-cell imaging, cells were incubated with POs and kept at 37°C in CO₂ atmosphere while imaging for 2 hours. Image analysis was performed using Fiji software (version 2).
POs uptake in BMDCs was analysed by flow cytometry. Cells were incubated with Rho-PMPC-PDPA POs, and following 5 to 24 hours incubation, cells were washed with PBS and resuspended in 2% FBS/PBS. Immediately after, BMDCs were run on a BD LSR Fortessa™ Cell Analyzer (BD Biosciences). Data were analysed with FlowJo software.

2.4.5. Cross presentation experiments

Briefly, 5 x 10^4 BMDCs or 3 x 10^4 DC2.4 cells were plated in 96-well plates (Corning). In some cases, cells were incubated with 10 µg/mL of Poly(I:C) (Invivogen) O/N at 37°C with 5% CO₂. Next, empty POs, P(OVA), or free protein at the same concentration were added to the cells for 6 or 24 hours. PBS and SIINFEKL peptide were used as negative and positive controls, respectively. Later, cells were washed with PBS and stained with anti-H2Kb-SIINFEKL (Biolegend) for assessing cross-presentation by flow cytometry (section 2.7.1, Table 11) using an LSR Fortessa™ cell Analyzer (BD Biosciences) and data were analysed with FlowJo software.

2.4.6. BMDC-OT-I proliferation experiment

In brief, 5 x 10^4 BMDCs were plated in round bottom 96-well plates (Corning). In some cases, cells were activated by O/N incubation with 10 µg/mL of Poly(I:C) (Invivogen) at 37°C with 5% CO₂. Later, equal concentrations of empty POs, P(OVA), or free protein were incubated with BMDCs for 6 hours. PBS and SIINFEKL peptide were used as negative and positive controls, respectively. Towards the end of the 6 hours incubation time, the spleen of an OT-I transgenic mouse (Charles River) was processed (section 2.7.2) and CD8 T cells were purified using magnetic beads according with CD8 Miltenyi biotech purification kit’s instructions. Following purification, OT-I CD8 T cells were labelled with Cell Trace Violet (CTV, ThermoFisher), following manufacturer’s instructions.

After the 6 hours of POs incubation with BMDCs, the media was removed and cells were carefully washed three times with PBS, spinning down at 400 xg for 5 mins between washes. In the same 96-well plate, BMDCs were further co-cultured with 7.5 x 10^4 CTV-
labelled CD8 T cells per well, and the 96-well plates containing the co-cultures were incubated for 4 days at 37°C. Proliferation of CD8 T cells was further assessed by flow cytometry (Section 2.7.1, Table 12) using a Fortessa X20™ (including UV laser) cell Analyzer (BD Biosciences) and analysed with FlowJo software.

2.5. In vivo experiments

All in vivo experiments were carried out following Home Office regulations, under Prof. Sergio A. Quezada licence (PPL: 15899). All experiments were performed in 6- to 8-week-old female wildtype C57BL/6 mice, purchased from Charles Rivers. Animals were kept in UCL animal facilities, meeting husbandry requirements.

2.5.1. Tumour inoculation and therapeutic vaccination

At day 0, mice were injected with 5 x 10⁵ B16-OVA or MC38 tumour cells subcutaneously (s.c.) in 100 µL of PBS, in the right flank, after which animals were randomised in groups. Depending on the experiment, animals were vaccinated in a prime-only (one dose at day 2) or prime-boost (two doses at days 2 and 5) regime. The immunisation route varied among experiments and were performed either s.c or intravenously (i.v). I.v. injections were performed by Dr. Felipe Galvez-Cancino, Dr. Diana L. Matias or Cristobal Costoya.

For each immunisation 1.2 µg of OVA or Adpgk peptide was used, either free or encapsulated in PMPC-PDPA POs (maximum 20mg/kg of mouse), under presence or absence of 100 µg of Poly(I:C). Poly(I:C) pellets were reconstituted at 2mg/ml in nuclease-free water and diluted down with PBS. Vaccines were prepared mixing protein and adjuvant component at maximum volume of 200 µL in PBS. When required, combined therapy with α-PD-1 mAb (Clone RMP1-14, 2B-Scientific Limited) was performed, consisting of 3 Ab doses (200 µg each) administered intraperitoneally (i.p) at days 5, 8 and 11 post tumour inoculation. In specific experiments, blood was withdrawn from the tail of mice a week after receiving the vaccine boost to track OVA-specific humoral (IgGs) or cytotoxic (CD8 T cells) systemic responses by Enzyme Linked
Immunosorbent assay (ELISA, section 2.6, Table 10) or dextramer staining using flow cytometry (section 2.7.2.2., Table 17), respectively.

Tumour growth was monitored by measuring tumour volumes on alternate days. The volume (mm$^3$) was calculated after measuring the tumour diameters (mm) in length (L), width (W), and height (H) with a digital calliper (Lupo) and using the following formula:

$$V = L \times W \times H$$

For survival experiments, mice were sacrificed by CO$_2$ euthanasia when L reached 15 mm in diameter or when two of the diameters were 10 mm, according with the established Home Office humane endpoints. For analysis experiments, mice were culled on day 13, and tumours and lymph nodes were harvested and processed for flow cytometry (section 2.7.2.1).

2.5.2. PMPC- PDPA biodistribution in mice tissues

For biodistribution studies, PMPC- PDPA-Cy5.5 labelled POs were injected s.c. or i.v. in mice (20 mg/Kg). After 6 or 24 hours, animals were anesthetised with 4.5% isoflurane in pure oxygen as carrier gas and blood samples (500uL) were withdraw by cardiac puncture. Next, exsanguination/perfusion using PBS was performed and the main organs (spleen, LN, liver, lungs, blood, gut, stomach, pancreas, kidneys, skin, and heart) were harvested, rinsed with PBS, and the PO’s Cy5.5 fluorescence was qualitatively analysed by in vivo imaging system (IVIs, Perkin Elmer). Exsanguination and perfusion was performed by Dr. Diana L. Matias.

For quantitative analyses, organs were processed and their fluorescence was quantified using a fluorimeter (Spark Multimode microplate reader, Tecan). Briefly, each tissue was weighed and transferred to 2 mL tubes containing ceramic beads for soft tissue disruption, followed by tissue homogenisation buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 10 mM NaF) addition. Tissue disruption and homogenisation was performed in a CK14
machine (Bertin Instruments, VWR, UK), following the Precellys soft tissue lysing kit’s instructions. Afterwards, the fluorescence of Cy5.5 POs within the organ homogenates was measured by fluorimetry (excitation peak at 685nm and an emission peak at 710nm). The amount of polymer per organ was extrapolated using a calibration curve with the Cy5.5 polymer.

2.5.3. PMPC-PDPA POs cell uptake, OVA cross-presentation and activation in vivo

To get an insight into the mechanisms by which POs could act as vaccine carriers, and to understand which cell subsets were targeted, mice were injected with Cy5.5 labelled POs (20 mg/kg). After 6 or 24 hours, blood, secondary lymphoid organs (spleen and skin-dLNs including axillar, inguinal and branchial) and other mucosal tissues (i.e. skin, lung) were harvested and processed before flow cytometry analysis, as explained in section 2.7.2.1. In a similar experiment, Cy5.5-P(OVA) formulations with Poly(I:C) were injected s.c. or i.v., and 6 or 24 hours later OVA cross-presentation and maturation levels (such as CD80, PD-L1) were analysed in APCs within tissues, by flow cytometry (section 2.7.2.2., Table 16). To discriminate immune infiltration within tissues (CD45+ cells), from those immune cells within blood vessels around the tissues, mice were injected via i.v. with 3 µg of labelled anti-CD45.2 (Biolegend) Ab five minutes before being culled.

2.6. ELISA

Anti-OVA IgG in blood 13 days post P(OVA) boost immunisation was quantified by ELISA.

Table 9. Antibodies used for ELISA assay calibration curve and murine serum samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-IgG (kappa light chain)</td>
<td>Goat</td>
<td>1:500</td>
<td>Biorad</td>
<td>105001G</td>
</tr>
<tr>
<td>a-IgG (lambda light chain)</td>
<td>Goat</td>
<td>1:500</td>
<td>Biorad</td>
<td>106001</td>
</tr>
<tr>
<td>IgG</td>
<td>Mouse</td>
<td>1000 to 0.064 ng/mL</td>
<td>Merck</td>
<td>I5381-1MG</td>
</tr>
<tr>
<td>a-IgG-HRP (Heavy chain)</td>
<td>Mouse</td>
<td>1:4,000</td>
<td>Merck</td>
<td>AP503P</td>
</tr>
</tbody>
</table>
Three drops of blood were sampled from the mice tail in Eppendorf tubes and centrifuged down at 800 xg and 4°C. Supernatant containing serum was transferred to a new tube and frozen at -80°C until use. For the ELISA assay, MaxiSorp plates (Nunc) were coated with 50 μL/well of 5 μg/mL OVA final concentration in PBS O/N at 4 °C. Standard curve wells were coated with 200 μL/well of mouse, kappa and lambda light chain antibodies diluted in PBS (1:500, biorad). Afterwards, plates were washed four times with 200 μL/well washing buffer (0.5% Tween-20 in PBS) and blocked for 1 hour with 200 μL/well blocking buffer (0.1% bovine serum albumin, BSA, in PBS) at RT, followed by four washes. Later, 200 μL/well of mice serum samples were added in serial dilutions (1:100, 1:1000, and 1:10,000) for 2 hours at 37°C. In the standard curve wells, 200 μL/well of commercial murine IgG (Merck) was added at 1000 ng/mL starting concentration and was further serially diluted 1 in 5. After 2 hours, 100 μL/well of HRP-conjugated, anti-mouse IgG (merk), was added for 1 hour at RT, diluted 1:4000 in PBS. Following four washes, 50 μL/well of the HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB, abcam) was added for 3 minutes until colour developed. Immediately after, 50 μL of stop solution (abcam) was added to each well and absorbance was read at 450 nm using a ELX800 plate reader (BioTek). Data analysis was performed using Prism8.

2.7. Flow cytometry

2.7.1. In vitro cell staining

For in vitro experiments such as BMDCs POs uptake, OVA cross-presentation, and BMDC-OT-I proliferation, cell suspensions were washed with PBS and plated in 200 μL of FACS buffer (PBS supplemented with 2% FBS) using 96 well plates round bottom (costar). Plates were centrifuged at 720 xg for 2 minutes, and the supernatant was discarded. Then, 50 μL of Fc block Ab was added (1:100, Biolegend) to each well for 10 minutes at 4°C; cells were washed with 200 μL of PBS and incubated with 50 μL of the mix of antibodies (see Table 10, Table 11, Table 12) for 20 minutes, at 4°C. Cells were washed twice with PBS, transferred to FACS tubes (Sarstedt) and immediately acquired in a LSR Fortessa™ or Fortessa X20™ (including UV laser) cell Analyzer (both from BD Biosciences). Data analysis was carried out with FlowJo software.
Table 10. Antibody mix used for characterisation of BMDC maturation.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Cat number</th>
<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
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<tr>
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<td>Biolegend</td>
<td>N418</td>
<td>1/150</td>
</tr>
<tr>
<td>CD11b</td>
<td>BV785</td>
<td>101243</td>
<td>Bio legend</td>
<td>M1/70</td>
<td>1/100</td>
</tr>
<tr>
<td>NK1.1</td>
<td>FITC</td>
<td>108705</td>
<td>Biolegend</td>
<td>S17016D</td>
<td>1/200</td>
</tr>
<tr>
<td>CD3</td>
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<td>53-0031-80</td>
<td>ThermoFisher</td>
<td>145-2C11</td>
<td>1/200</td>
</tr>
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<td>152403</td>
<td>Biolegend</td>
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<td>1/400</td>
</tr>
<tr>
<td>Siglec H</td>
<td>PE</td>
<td>129605</td>
<td>Biolegend</td>
<td>551</td>
<td>1/150</td>
</tr>
<tr>
<td>MHC-II</td>
<td>PE-Cy7</td>
<td>10762</td>
<td>Biolegend</td>
<td>M5/114/15/2</td>
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</tr>
<tr>
<td>XCR1</td>
<td>APC</td>
<td>148205</td>
<td>Biolegend</td>
<td>ZET</td>
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Table 11. Antibody mix used for characterisation of antigen cross-presentation in BMDCs.

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<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
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<td>Biolegend</td>
<td>N418</td>
<td>1/150</td>
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<tr>
<td>CD11b</td>
<td>BV785</td>
<td>101243</td>
<td>Biolegend</td>
<td>M1/70</td>
<td>1/100</td>
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<tr>
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<td>PE-Cy7</td>
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<td>Biolegend</td>
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<td>1/200</td>
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<td>XCR1</td>
<td>APC</td>
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<td>Biolegend</td>
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Table 12. Antibody mix used for characterisation of OT-I proliferation.

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<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
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</thead>
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<td>CD3</td>
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<td>612803</td>
<td>BD</td>
<td>1D3/17A2</td>
<td>1/300</td>
</tr>
<tr>
<td>CD8a</td>
<td>BUV805</td>
<td>612898</td>
<td>BD</td>
<td>53-67</td>
<td>1/300</td>
</tr>
<tr>
<td>CD25</td>
<td>FITC</td>
<td>102005</td>
<td>Biolegend</td>
<td>PC61</td>
<td>1/150</td>
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<tr>
<td>OX-40</td>
<td>PE</td>
<td>119409</td>
<td>Biolegend</td>
<td>OX-86</td>
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<tr>
<td>4-IBB</td>
<td>APC</td>
<td>106109</td>
<td>Biolegend</td>
<td>17B5</td>
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<td>ICOS</td>
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<td>313527</td>
<td>Biolegend</td>
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<td>Viability dye</td>
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<td>65-0865-14</td>
<td>ThermoFis.</td>
<td>-</td>
<td>1/1000</td>
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</table>
2.7.2. **In vivo tissue processing and staining**

To analyse immune populations in either B16-OVA tumours, skin-dLNs, or other tissues (i.e. spleen, skin, liver, lung) following immunisations with POs, mice were culled on the day of the experiment with excess of CO$_2$ and organs were harvested in vials containing RPMI media following dissection. Tissue processing and FACS staining was performed following an optimised protocol from Prof. Sergio Quezada’s group at the UCL Cancer Institute.

2.7.2.1. **Tissue processing and generation of single cell suspension**

Briefly, tissues in RPMI media were disrupted using scissors, and digested with 0.35 µg/mL Liberase (Roche) and 0.23 µg/mL DNAse I (Sigma) for 30 mins at 37°C. Then, a single cell suspension was obtained by filtering the digests through 70 µm strainers (corning). Cells were centrifuged at 400 x g for 5 mins and resuspended in supplemented RPMI. Organs containing large amount of RBCs (i.e., spleen or lungs) were treated with 1mL of RBC lysis buffer (ACK buffer, gibco) for 5 minutes at RT, before spinning down at 400 x g for 5 mins and resuspending in complete media. Tissue harvesting and processing into single cell suspensions was done with the support from Yasmin Morris, Mariela Navarrete Sanchez, and Dr. Felipe Galvez-Cancino.

In the case of tumour analysis, given that the experiments aimed to analyse immune infiltrates, the tumour samples were further enriched in T cells by using a percoll density gradient (GE Healthcare). Briefly, tumours were resuspended in 35 % percoll in PBS, and 3mL of 70% percoll was carefully added at the bottom of the tube, using a glass Pasteur. Then, samples were centrifuged at 700 x g for 25 minutes, and the lymphocyte ring formed between the percoll layers was collected, centrifuged for 5 min at 400 x g and resuspended in RPMI.

2.7.2.2. **FACS staining**

When cell suspensions were ready, 200 µL of each sample was transferred into 96 well plates and washed with PBS before starting with the staining. Cells were blocked with
200 µL of blocking solution (5% rat serum, 5% mouse serum and 5% FBS) for 10 mins at 4°C and centrifuged at 720 xg for 2 minutes. When required, 50 µL dextramer (Immudex) in PBS was added to stain SIINFEKL-reactive CD8 T cells for 15 minutes at RT, for addressing the vaccine specific CD8 T cell response. Next, cells were incubated with 50 µL of Ab mix in PBS (including viability dye) for extracellular receptors for 20 minutes at 4°C (see Table 13, Table 14, Table 15, Table 16, Table 17, for Ab mixes and experiment panels). If no further staining was needed, cells were washed twice with PBS and transferred into FACS tubes for acquisition. Panel 1 and Panel 2 were designed and optimised by Dr. Felipe Galvez-Cancino.

Table 13. Panel 1: Antibody mix used for the staining of T cells infiltrating B16-OVA tumours and in dLNs.

<table>
<thead>
<tr>
<th>Antibody/Dye</th>
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<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
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<td>PK136</td>
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<td>CD4</td>
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<td>BD</td>
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<tr>
<td>CD45</td>
<td>BUV563</td>
<td>565710</td>
<td>BD</td>
<td>30-F11</td>
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</tr>
<tr>
<td>CD11b</td>
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<td>565080</td>
<td>BD</td>
<td>M1/70</td>
<td>1/300</td>
</tr>
<tr>
<td>CD3</td>
<td>BUV737</td>
<td>564380</td>
<td>BD</td>
<td>17A2</td>
<td>1/300</td>
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<td>FJK-16S</td>
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<td>MEL-14</td>
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<td>119721</td>
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<tr>
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<td>102005</td>
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</table>
IC staining of T cells (Table 13) or myeloid populations (Table 14) was performed following instructions from the eBioscience Kit, or BD Biosciences kit. Briefly, 50 µL of IC Ab mix was added to the cells for 20 mins or 2 hours and at 4°C. Then, cells were transferred to microtiter bullet tubes (Biorad) and analysed in a Symphony cytometer (BD Biosciences). FACS data were analysed using FlowJo software, including dimensionality reduction analysis (section 2.8.1).

Table 14. Panel 2: Antibody mix used for the staining of myeloid-like cells infiltrating B16-OVA tumours and in dLNs.

<table>
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<tr>
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<th>Clone</th>
<th>Dilution</th>
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### Table 15. Panel 3: Antibody mix used for POs uptake assays in vivo.

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<td>Invitrogen</td>
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</tr>
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<tr>
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<td>PE</td>
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<td>M5/114/15/2</td>
<td>1/500</td>
</tr>
<tr>
<td>Cy5.5-POs</td>
<td>AF700</td>
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### Table 16. Panel 4: Antibody mix used for cross presentation and activation assays in vivo.

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<td>M5/114/15/2</td>
<td>1/500</td>
</tr>
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<td>Cy5.5-POs</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Viability dye</td>
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<td>65-0865-14</td>
<td>ThermoFisher</td>
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Table 17. Panel 5: Antibody mix used for staining of SIINFEKL-reactive CD8 T cells in blood.

<table>
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<th>Company</th>
<th>Clone</th>
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<td>ThermoFisher</td>
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<td>Immudex</td>
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<td>Viability dye</td>
<td>APC-e780</td>
<td>65-0865-14</td>
<td>ThermoFisher</td>
<td>-</td>
<td>1/1000</td>
</tr>
</tbody>
</table>

2.8. Analysis

2.8.1. Dimensional reduction analysis for flow cytometry data in FlowJo

Given the large number of fluorophores and markers used to analyse single cell suspensions in high dimensional flow cytometry experiments, data analysis is frequently complex. To help with this, several algorithms have been developed in the past years, which allow for the aggrupation of cells with similar phenotype profile and further cluster visualisation in bidimensional maps. These analyses are also denominated “unsupervised”.

Here, single cell datasets from flow cytometry analysis were first partitioned into phenotypically distinct populations using the algorithm “Phenograph” [318] in FlowJo, followed by dimensionality reduction using “Uniform Manifold Approximation and Projection” (UMAP) algorithm and cluster visualisation in two-dimensional maps [319].

2.8.2. Statistical analysis

The results are expressed as mean ± standard deviation (SD), except for tumour growth curves, representing mean ± standard error of mean (SEM). Statistical differences were evaluated by Student’s t-test or one or two-way- ANOVA test followed by Tukey Post-hoc test. In the case of survival experiments, statistical differences were evaluated by Log-Rank (Mantel-Cox) test. Analyses were performed in GraphPad Prism 8. p <0.05 was considered as statistically significant (where ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
2.9. Appendix

Figure 26: Retention time of naked OVA in SEC column following electroporation.
Chapter 3: PMPC-PDPA POs self-assembly and neoantigen encapsulation
3.1 Introduction and aims

The main goal of this PhD project is to translate the use of PMPC-PDPA POs as NeoAg vaccine carriers, exploiting their ability to target SRs expressed in APCs [301], [311] and enhanced IC delivery [320]. Hence, the first step towards this goal is to define the POs self-assembly method and optimise the generation of homogeneous and reproducible POs-based nano-vaccines that efficiently entrap OVA or Adpgk-LSP NeoAg.

Some of the most studied POs vesiculation methodologies rely on amphiphile-water contact, such as FH or SS (Material and methods sections 2.1.1 and 2.1.2). A third method relies on PMPC-PDPA pH-sensitivity, receiving the name of pH-switch (Material and methods sections 2.1.3.). In FH approach, a thin polymer film is rehydrated by an aqueous phase, under stirring conditions. As previously described [278], [312], when this happens the hydrophobic blocks from the copolymer units drive the formation of lamellar structures, that later break into vesicles, in a process that largely depends on the size of the amphiphile [278], [321], [322]. During SS, the driving force for POs vesiculation is the change of solubility of PMPC-PDPA unimers dissolved in an organic phase, by adding an aqueous phase [278]. To finish with, in the pH-switch method the polymer is dissolved at low pH in an aqueous phase, followed by an increase in the pH above the polymer pKa (5.5), that drives PDPA de-protonation and assembly into vesicles [314].

Although cargo encapsulation can be achieved by payload addition into the polymer or solvent phases during self-assembly, protein and peptides are susceptible to changes in solubility and pH, which might drive alterations in their conformational state, such as denaturalisation or aggregation [323]. This can hinder cargo encapsulation into POs and even render them inefficient towards the purpose they are being developed for. Previous group members established EP as an alternative and effective method for protein and peptide encapsulation in POs [307], (Material and methods sections 2.1.4). In the field of molecular biology, EP is routinely used for the IC delivery of exogenous DNA or RNA material. It involves the application of an electrical field in a cuvette coated by a metallic surface, where the cells and genetic material are mixed, which increases
the electrical conductivity and consequently, the cell membrane permeability [324], [325]. In addition, the efficiency of EP is modulated by the voltages of the electrical field [324], [326]. Likewise, these parameters also play a key role in the efficient encapsulation of proteins in POs by EP. In addition, the concentration of protein or number of pulses used during the EP process also has an influence in the encapsulation efficiency outcome [307], [327]. Nonetheless, EP can also trigger protein and peptide aggregation, and therefore there is a need for optimisation of the EP voltages, number of pulses or concentration of protein and polymer, for each protein.

Besides, POs structural features such as morphology and size are relevant aspects to consider for translating their use as nano-vaccines [268]. First, cargo or antigen encapsulation is favoured in polymer vesicles over micelles, as it is possible to entrap molecules in both the vesicle’s polymer brush and lumen. Enhanced entrapping efficiencies are desired as this provides with improved protein target and IC delivery into APCs, and overall improved antigen presentation. Although intuitively this might seem to indicate that bigger polymer aggregate structures would provide with higher encapsulation efficiencies and translate into better immunisation outcomes, it is important to note that, in vivo, antigen access to LNs is key to increase the priming process [328]. In this regard, antigens need to be taken up from the immunisation site’s interstitial space (i.e. skin, when immunisations are performed s.c.) into lymphatic vessels. This process is sensitive to the size, and agents (including nanoparticles) ranging 10-100 nm are most efficiently taken up [282]. Thus, controlling the POs particle size up to 100 nm can further enhance the immunisations when using POs as nano-vaccines.

Thus, this first experimental chapter aims to find the optimal self-assembly methodology for POs generation and NeoAg encapsulation, which can meet optimal features for their use as nano-vaccines. This will be achieved through the following objectives:

1. Optimise the preparation of empty POs by solvent switch, pH-switch and electroporation
2. Define the self-assembly protocols and methodologies that provide optimal OVA and Adpgk-LSP encapsulation into POs, with a special emphasis on electroporation.

3. Characterise the size and morphology of the POs formulations.
3.2. Results

3.2.1. Comparison of self-assembly methods for the production of PMPC-PDPA POs

To understand which technique generates POs in a more reliable way in terms of monodispersity and reproducibility, three different methods were attempted. First, PMPC-PDPA POs were generated by FH. In the experiment, PMPC-PDPA films were rehydrated for 4 weeks in PBS, and then purified by SEC. TEM micrographs (Figure 27A) indicated that the purified formulation was highly polydisperse and was composed of a mixture of structures, ranging from finger-like shapes, tubes, and some vesicles. Next, the generation of POs was attempted by pH-switch. Following SEC purification, samples were analysed by TEM (Figure 27B) showing a mixture between vesicles and micelle structures. Lastly, POs self-assembly was also tried by SS. TEM images acquired after SEC revealed the formation of highly monodisperse POs vesicles (Figure 27C).

DLS analysis were performed to quantify PO size distribution. First, measurements from POs obtained by FH indicated sizes of approximately 120 nm in diameter, by number-weighted peak (Figure 28A and Figure 28D), which correlated with vesicles found in TEM images. Next, data from pH-switch formulations (Figure 28B), demonstrated an average particle diameter of approximately 30 nm (Figure 28D). Finally, the size of the POs obtained by SS was of approximately 43 nm average diameter (Figure 28C and Figure 28D).

Given that the aim was to obtain monodisperse vesicle POs formulations, FH was not used any further. In the case of pH-switch, the presence of a large number of micelles could potentially reduce the encapsulation efficiency of molecules like proteins or peptides within POs when used during EP, thus they were not used to this end. Despite this, pH-switch method was further explored for the encapsulation of protein and peptide during the self-assembly reaction, as explained in the next section. To finish with, SS was the method of choice during the whole project for the generation of empty particles, given the homogeneity of the POs sizes. Appendix 3.4.3, includes a
Figure 27. TEM characterisation of PMPC-PDPA self-assembled structures prepared by different methods following SEC purification. A) Polymer assembles formed by film rehydration. B) Micelles and vesicle images following pH-switch. C) POs vesicles formed by solvent switch. Scale bar in TEM images corresponds to 100 nm.
Figure 28. Characterisation of PMPC-PDPA self-assemblies by DLS. Graphs showing average hydrodynamic diameter and correlograms of formulations prepared by A) Film rehydration B) pH-switch and C) Solvent switch. D) Summary of particles’ average hydrodynamic diameter and PDIs from DLS measurements.
characterisation summary for all these formulations (Section 3.4.3.1. for unlabelled POs and 3.4.3.2 for fluorescently PMPC-PDPA), whose hydrodynamic diameters ranged between 42 to 82 nm.

3.2.2. OVA encapsulation in PMPC-PDPA POs

Following characterisation of empty POs by different self-assembly methods, the encapsulation of OVA in these formulations was addressed.

In the encapsulation of OVA by pH-switch, 1 mg/mL final concentration of protein was added into the polymer phase (at 10 mg/mL) while the solution’s pH was increasing, at the point when the aqueous pH had reached the polymer’s pKₐ (pH 6.4) and the self-assembly process was about to start. After SEC purification, TEM analysis revealed a mixture of structures by pH-switch (Figure 29A). The average hydrodynamic diameters of these polymer structures ranged from 37 nm for micelles and went up to 220 nm for polymer aggregates, which seemed to be less abundant than micelles, as measured by DLS (Figure 29C top and Figure 29D). Due to the presence of these larger amorphous structures, this method was not chosen for the generation of formulations for in vitro or in vivo purposes.

As it was not possible to achieve homogenous POs populations upon OVA addition during the pH-switch self-assembly process, protein encapsulation was attempted after POs had been self-assembled, by EP. To this end, the encapsulation of OVA (at 1 mg/mL) by EP was tried in empty POs (at 5 mg/mL) that had been generated by SS. 5 pulses at 2500V were first attempted. Following SEC purification, TEM analysis (Figure 29B) showed no changes in the POs structures, which remained vesicle-like, and kept monodisperse, resembling the empty POs formulation generated by SS (Figure 27C). DLS analysis indicated that POs after EP presented a size of 50 nm (Figure 29C, bottom and Figure 29D), which was similar to the size of empty POs by SS. Moreover, it was possible to immunodetect the entrapped OVA in POs by western blot (Appendix 3.4.1, Figure 35A.)
Figure 29. Characterisation of POs encapsulating OVA.
TEM images of POs encapsulating OVA by A) pH-switch B) electroporation. C) DLS diagram (right) and correlation functions (left) from formulations prepared by pH-switch (top) and electroporation (bottom). D) Summary of particles’ average hydrodynamic diameter and PDIs from DLS measurements. Scale bar in TEM images corresponds to 100 nm.
3.2.2.1. Deciphering the effect of electroporation pulses in OVA aggregation

Achieving encapsulation of proteins in POs by EP is case-dependant, given that in certain occasions, the application of an electrical field to proteins can cause denaturation and aggregation [329]. Since the purpose of the project is to use these formulations as vaccines, protein aggregation would add an extra parameter that could potentially cause variability across experiments. For this reason, minimising OVA denaturation is essential.

In order to gain insight into the effect of EP in OVA stability, the protein (in the absence of POs) was electroporated at 2 mg/mL, for a different number of pulses, ranging from 0 to 20, with a one-minute recovery time between them and at 4°C. Each pulse was performed at 2500 V, as this was the highest voltage that the electroporator could achieve, and it caused no visual aggregation of the protein. This was in agreement with previous findings that high EP voltages (if no aggregation is detected) correlates with higher encapsulation efficacies [307], [327]. After 5, 10, 15 and 20 pulses of EP, two different experiments were carried out to try to assess the OVA stability. In a first attempt, OVA was run in a non-denaturing Bis:Tris acrylamide gel following EP pulses, after which the gel was stained with Blue Coomassie to detect protein aggregates. As shown in Appendix 3.4.1, Figure 35B, no aggregates were detected with EP pulses. However, Blue Coomassie may not be sensitive enough to detect small amounts of protein, and so potential aggregation was also studied by UV-Vis absorbance (Figure 30). The OVA UV-Vis spectrum in native state revealed two sharp peaks of absorbance, the first one being at a wavelength of 220 nm, derived from the peptide’s bonds contribution, and the second one at 280 nm, from aromatic residues [330](Figure 30A, and Figure 30B). In contrast, after EP pulses, the protein spectrum also showed absorbance in other wavelengths along the UV-Vis spectrum (Figure 30A, and Figure 30B-F). This effect was less obvious after 5 EP pulses (Figure 30A, and Figure 30C), where it was possible to read low absorbance levels at wavelengths from 280 to 400 nm, however the differences increased with the number of EP pulses, and after 20 pulses
the curve reflected absorbance at wavelengths from 220 to 700 nm approximately (Figure 30A and Figure 30C).

**Figure 30.** Characterisation of OVA aggregation upon electroporation by UV-Vis absorbance. UV-Vis spectra of OVA A) In native and electroporated conditions, B) In native state C) after 5 EP pulses D) After 10 EP pulses E) After 15 EP pulses F) After 20 EP pulses. (n=3 independent experiments).
This may indicate the exposure of protein residues that were hidden in the native state, suggesting that the protein might have undergone denaturalisation or aggregation. For this reason, 5 EP pulses at 2500 V appeared to be the condition that could cause less structural changes to the protein, while potentially allowing protein encapsulation within POs.

### 3.2.2.2. Effect of OVA concentration during electroporation in POs encapsulation efficiency.

In addition to the number of EP pulses, the protein concentration during EP into POs was also adjusted to achieve optimal entrapping of OVA (0.5, 1 or 2 mg/mL final concentrations in the EP cuvette). Higher concentrations of protein were not considered as their EP resulted in the formation of aggregates that were visible (data not shown). After performing 5 pulses of EP at 2500V, each formulation was purified by SEC to separate non-encapsulated protein, and re-concentrated using Kros-Flow. Quantification of polymer and OVA mass in the purified formulation was done by uHPLC at 220 nm (retention times as shown in Appendix Fig. 3.4.2, Figure 36A), and OVA entrapment within POs was calculated considering these numbers as well as DLS data, as explained in Material and methods 2.3.4.

The results indicated that the entrapment thresholds increased with higher initial concentrations of OVA (Figure 31A), with a proportional trend. The loading efficiencies (Le) of OVA into POs (Figure 31A, maroon line) were in average 2.46 molecules per PO for the 0.5 mg/mL initial concentration (Figure 31C, left); 6.46 for those formulations prepared at 1mg/mL of OVA during EP (Figure 31C, middle); and went up to 11.9 OVA molecules per PO vesicle (Figure 31C, right), when performing EP at 2 mg/mL initial OVA concentration. The normalisation of encapsulated OVA (µg) per polymer mass (mg), revealed a similar tendency (Figure 31A, black line). POs production efficiency (initial polymer mass/final polymer mass) ranged between 71.5% to 86% (Figure 31B, black line), meaning most of the polymer was being recovered after self-assembly, encapsulation and production and purifications steps. The number of POs, estimated from DLS size distributions (calculations detailed in material and methods 2.3.4)
fluctuated between $10^{13}$ (in EP reaction with OVA at 1 and 2 mg/mL) and $10^{14}$ (in EP reaction with OVA at 0.5 mg/mL), as shown in Figure 31B, blue line. In addition, DLS analysis after purification

A. OVA encapsulation

B. POs production

C. POs encapsulating OVA (EP)

D. Table summarising the size and PDI of the particles by DLS, OVA Le, ratio µg OVA/mg polymer, and % POs production efficiency. Error bars in A) represents standard deviation (SD) (n=3 independent experiments in triplicates). Statistical significance was analysed by one-way-ANOVA (*p <= 0.05)

Figure 31. Characterisation of the effect of OVA concentration in loading efficiency (Le) in POs during electroporation (EP). Following EP, SEC purification and HPLC analysis, the following parameters were calculated A) OVA Le (molecules/PO) in POs (maroon) and ratio µg OVA/mg polymer (black). B) Number of POs (green) and % POs production efficiency black). C) Average number of OVA molecules loaded in vesicles (maroon), according with the average diameter distribution of POs obtained by DLS (green). D) Table summarising the size and PDL of the particles by DLS, OVA Le, ratio µg OVA/mg polymer, and % POs production efficiency. Error bars in A) represents standard deviation (SD) (n=3 independent experiments in triplicates). Statistical significance was analysed by one-way-ANOVA (*p <= 0.05)
showed similar average diameter in number across the formulations which were between 73 to 75 nm (Figure 31C and table Figure 31D), displaying low PDI values (below 0.3), which was in agreement with previous characterisations.

In conclusion, performing 5 pulses of EP at 2500 V and at 2 mg/mL of initial OVA concentration proved to be the more efficient conditions for OVA encapsulation into POs. Hence, this was the method used for the generation of POs encapsulating OVA for the thesis experiments’ involving in vitro and in vivo procedures. A table recapitulating these formulations can be found in Appendix 3.4.3 Figure 37 Figure 38, Figure 39, Figure 40, Figure 41, Figure 42.

3.2.3. *Adpgk*-derived LSP neoantigen (*Adpgk*-LSP) encapsulation in PMPC-PDPA POs

The encapsulation of the LSP NeoAg derived from the Adpgk protein (in short, Adpgk-LSP) expressed in MC38 cells, was assessed by pH-switch, ss, and EP.

In the pH-switch method, a 10 mg/mL polymer solution was mixed with Adpgk-LSP to a final concentration of 0.5 mg/mL. However, peptide aggregates appeared immediately after addition, due to peptide insolubility. As shown in Figure 32A, TEM analysis revealed a mixture of structures, in which, although vesicles were the main population, larger aggregates could also be detected. Diameters in DLS were around 58 nm by number, for vesicles and 225 nm for larger structures, as indicated by intensity measurements (Figure 32A and Figure 32D). Due to peptide insolubility and mixture of polymer structures, this technique was not further optimised.

During the first attempt of peptide encapsulation by SS, 500 µg of Adpgk-LSP was added into 10 mg/mL of PMPC-PDPA dissolved in an organic phase, prior to PBS addition to start the self-assembly process. When finished, no visual aggregates were detected. This sample was further dialysed against an excess of PBS to remove solvent and purified by SEC. Characterisation by TEM (performed by Gabriel Ing Figure 32 B, left), and DLS (Figure
32B, right) revealed the generation a very monodisperse PO formulations, with diameters of 72 nm (number weighted, Figure 32D) and PDI around 0.2.

A.

![POs encapsulating Adpgk by pH switch](image)

B.

![POs encapsulating Adpgk peptide by solvent switch](image)

C.

![POs encapsulating Adpgk peptide by electroporation](image)

D.

<table>
<thead>
<tr>
<th>P(Adpgk) POs by</th>
<th>Number</th>
<th>Intensity</th>
<th>Volume</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH switch</td>
<td>58.37 ± 13.16</td>
<td>225.54 ± 68.03</td>
<td>83.25 ± 16.97</td>
<td>0.262</td>
</tr>
<tr>
<td>Solvent switch</td>
<td>72.3 ± 11.45</td>
<td>130.22 ± 25.82</td>
<td>103.17 ± 24.1</td>
<td>0.202</td>
</tr>
<tr>
<td>Electroporation</td>
<td>54.72 ± 7.78</td>
<td>97.09 ± 19.21</td>
<td>71.71 ± 14.88</td>
<td>0.238</td>
</tr>
</tbody>
</table>

**Figure 32. Characterisation of POs encapsulating Adpgk-LSP.**

TEM images (left), DLS diagrams (middle) and correlograms (right) of POs encapsulating Adpgk-LSP by A) pH-switch B) solvent switch C) Electroporation. D) Summary of particles’ average hydrodynamic diameter and PDIs from DLS measurements. Scale bar in TEM images corresponds to 250 nm.
Peptide encapsulation was assessed by HPLC (Appendix 3.4.2 Figure 36B for retention times), finding no peptide had been entrapped in POs during the self-assembly process (Figure 33A and C, and Figure 33D).

Nonetheless, given the good size and monodispersity of the POs generated by this technique, Adgpk-LSP encapsulation by SS was further attempted by modifying the amount of peptide added at the initial polymer organic phase. To this end, two formulations were prepared, adding either 250 µg or 1 mg of Adpgk-LSP peptide in the polymer organic phase, and after the reaction finished, they were purified and characterised as above. Although aggregates were not large enough to be visualised by eye, both formulations were highly turbid and colloidally instable after self-assembly (and before purification), which might have been caused by the presence of large polymer-only or polymer-peptide assemblies. Accordingly, when performing SEC purification, a big proportion of each formulation was retained at the top of the column.

Following purification, peptide and polymer quantification by HPLC indicated that POs production efficiency ranged between 14 and 40% (Figure 33B and Figure 33D), meaning that most of the polymer was being lost in the self-assembly and/or purification stages, in agreement with the mentioned SEC issues. In addition, no peptide was found in the 250 µg Adpgk-LSP formulation, in contrast with the 1 mg/mL Adpgk-LSP sample, which displayed a peptide loading efficiency of 451 peptide molecules/PO, equivalent to a 3.71 µg peptide/mg polymer ratio (Figure 33A and Figure 33D). DLS sizes in number for this formulation showed the presence of two peaks, one at 77.13 nm, and another one at 29.91 nm. Although the latter peak might have corresponded to micelles, the fact that the formulation was so colloidally instable could indicate that there might have been peptide aggregates in the formulation that could be shown at that size too (Figure 33D). Therefore, the peptide quantified by HPLC could have corresponded to non-encapsulated peptide aggregates, instead of encapsulated peptide into POs. SEC retention times of these aggregates might have been similar to POs, making it impossible to separate one another. Besides, the polydispersity for this formulation was higher than the rest of the formulations (PDI=0.4 as opposed to PDI=0.1) (Figure 33D).
Figure 33. Characterisation of the effect of Adpgk-LSP concentration in loading efficiency (Le) in POs during solvent switch (SS).

Following SS, SEC purification and HPLC, the following parameters were calculated A) Adpgk-LSP Le (molecules/PO) in POs (maroon) and ratio µg OVA/mg polymer (black). B) Number of POS (green) and % POs production efficiency (black). C) Average number of Adpgk-LSP molecules loaded in vesicles (maroon), according with the average diameter distribution of POs obtained by DLS (green). D) Table summarising the size and PDI of the particles by DLS, Adpgk-LSP Le, ratio µg Adpgk-LSP/mg polymer, and % POs production efficiency.
Lastly, encouraged by the successful OVA encapsulation by EP, this technique was attempted for Adpgk-LSP encapsulation. The peptide was added to the EP cuvette at a molarity equivalent to 2mg/mL of OVA (15.25 mM), which corresponds to 375 µg/mL of the peptide, mixed with POs at 5 mg/mL. The EP voltages had to be reduced to 1000V, given that at 2500V aggregation was visible to the naked eye. Following SEC purification, DLS analysis revealed that the POs morphology was of vesicles that were around 54 nm in hydrodynamic diameter in number (Figure 34D), and that it was a monodisperse (PDI=0.11) formulation, as also demonstrated by TEM images (Figure 32C). The quantification of entrapped peptide indicated a loading efficiency of 256.87 molecules per PO (Figure 34G), which corresponded to 3.63 µg peptide/mg polymer ratio. Besides, the POs production efficiency was of 67.5% in average (Figure 34G).

When comparing Adgpk-LSP encapsulation balances, the apparent Loading efficiency was higher by ss (at 1 mg/mL peptide) than by EP (256 molecules vs 451 molecules per PO), as shown in Figure 34B. However, the ratio µg peptide/mg polymer remained similar (3.73 vs 3.63, Figure 34B). With regards the retention efficiency (Re), formulations generated by EP retained a higher percentage of the total added peptide when compared to formulations made by SS (Figure 34D). The formulation generated by EP also showed higher POs production efficiencies values than by SS (Figure 34E), and the same trend was observed when comparing the number of POs (Figure 34F). Given the more efficient Le, retention efficiency, and POs production values achieved by EP as opposed to SS, as well as the monodisperse nature of the formulations, the encapsulation of Adpgk-LSP for the in vivo experiments were performed by EP (Appendix 3.4.4, Figure 43).
Figure 34. Comparison of Adpgk-LSP encapsulation balances between electroporation (EP) and solvent switch (SS).

Following EP or SS, SEC purification and HPLC analysis, the following parameters were calculated:

A) Average number of Adpgk-LSP molecules loaded in vesicles (maroon), according with the average diameter distribution of POs obtained by DLS (green) by EP (right) or SS (left). B) Adpgk-LSP loading efficiency in POs C) µg Adpgk-LSP/mg polymer (black) D) Adpgk-LSP retention efficiency E) % POs production efficiency F) Number of POS G) Table summarising size and PDI of particles by DLS, Adpgk-LSP Le, ratio µg Adpgk-LSP/mg polymer, and % POs production efficiency.
3.3. Discussion and conclusions

The aim of this experimental section was the generation of monodisperse PMPC-PDPA POs formulations entrapping OVA and Adpgk-LSP NeoAgs to be used as anti-tumour nano-vaccines in vitro and in vivo throughout the project.

First, the size and morphology of POs was compared across FH, pH-switch and SS self-assembly methods, following SEC purification. TEM and DLS analysis of PMPC-PDPA formulations upon FH method indicated the presence of a mix of vesicles and tubes, suggesting that the self-assembly transition from tubes to vesicles was not fully achieved during the time of polymer film rehydration. Although purification protocols such as sucrose gradient can be used to separate tubes from vesicles [315], the purification efficiency is very low and large amount of materials are lost [309]. On the other hand, self-assembly by pH-switch and SS resulted in more monodisperse formulations of smaller particle sizes, whereas a mixture of vesicles and micelles ranging from 29 to 176 nm were found in pH-switch generated samples, a homogeneous population of vesicles were found by SS, from 43 to 80 nm. Therefore, the generation of large tubes during FH method, implying lack of control on the self-assembly and therefore, lack of reproducibility, led us to not further explore this technique for OVA or Adpgk-LSP encapsulation, and only pH-switch and SS were put forward to this end.

OVA is water-soluble, thus its entrapment in PMPC-PDPA POs was explored by pH-switch and EP followed by SEC. TEM and DLS analysis indicated that formulations prepared by pH-switch were constituted by a mixture of micelles, vesicles, and large aggregates up to 313 nm in size. This is likely driven by the formation of polymer-OVA coacervates [331] that disrupted the controlled self-assembly of PMPC-PDPA into vesicles/micelles-only that was observed in the previous formulations. Since optimal size of the nanoparticles for their use as nano-vaccines (and subsequent LN targeting) should be under 100 nm [268], [282], [328], POs entrapping OVA by pH-switch were not used for the generation of the nano-vaccines in this work. On the other hand, OVA EP in empty POs formulations generated by SS did not change the morphology of the POs vesicles and remained a monodisperse mixture ranging from 50 to 87 nm, according
with TEM and DLS measurements. However, SEC purified POs electroporated with OVA were around 7nm bigger than their empty counterparts, which might indicate OVA adhesion on the PO surface, as OVA’s diameter is around 4-5 nm. Another hypothesis can be PO expansion driven by OVA encapsulation within the lumen of the POs, although these two options were not further explored, as it was not the main focus for the project. In any case, EP was the method of choice for OVA entrapment in POs for their use as nano-vaccine. Thus, parameters such as concentration, number and voltage of pulses were optimised, to achieve best outcomes. EP of naked OVA followed by UV-Vis analysis indicated OVA structural changes when more than 10 pulses at 2500 V were applied, which was evidenced by an increase in absorption at wavelengths other than 220 (peptide bounds) or 280 (aromatic residues), over OVA native state. In addition, the number of protein molecules entrapped per PO (Le) could be enhanced by increasing the final OVA concentration mixed with POs in the EP cuvette prior to pulsing. Le values oscillated between 2.46, 6.46 or 11.9 upon addition of OVA at final concentrations of 0.5, 1 or 2 mg/mL respectively, which correlated with 2.08, 4.85 or 8.21 µg OVA/mg polymer, respectively. In addition, the majority of the polymer was conserved following EP and purification, with PO production efficiencies (P.E) ranging between 71 to 86%. Overall, this was in correlation with prior studies in the group where it was possible to achieve encapsulation of proteins like IgGs into 200 nm POs with a Le of around 80 molecules per particle upon 10 EP pulses [327]. Although this value is higher than the Le obtained in OVA encapsulation (12 molecules/PO), this could be explained by the difference in diameter of the vesicles in this study, which was 4 times lower.

Next, Adpgk-LSP encapsulation attempt by pH-switch proved to be non-feasible due to peptide precipitation upon addition to the polymer mix during the self-assembly at pH 6, likely due to peptide insolubility. Given the hydrophobicity of the peptide, encapsulation during SS method was next strived by Adpgk-LSP addition at 0.25, 0.5 or 1 mg/mL final concentration (from a 10 mg/mL stock in DMSO) into the organic solvent phase (MetOH:THF 3:1 v/v) dissolving the polymer. Although no peptide precipitation was observed during self-assembly, the formulations had extremely poor colloidal stability and most of the polymer was lost during SEC purification, likely due to generation of polymer-peptide aggregates. However, TEM and DLS from purified
samples revealed formulations were monodisperse and consisted of vesicles oscillating between 47 to 77 nm in size, although in the case of formulations done at 1 mg/ml of Adpgk-LSP two peaks appeared, which were 29 and 77 nm. Polymer and peptide quantification by HPLC revealed that most of the polymer was being lost during SEC, finding production efficiencies that ranged 14, 22 and 40% in 0.25, 0.5 or 1 mg/mL Adpgk-LSP formulations, respectively. Importantly, only the latter formulation seemed to have peptide encapsulated, with a Le=451 which corresponded to 3.72 µg Adpgk-LSP/ mg PMPC-PDPA. However, the formation of peptide-polymer aggregates evidenced by the low production efficiencies, together with the appearance of a 29 nm peak in the DLS analysis of this formulation, arose the possibility of peptide aggregation structures being generated during SS upon change in the solubility following PBS addition. These could have similar retention times than POs vesicles in SEC and thus elute simultaneously, making it impossible to fully separate and distinguish non-encapsulated peptide from those encapsulated in POs. Due to the lack of reliability of this technique, POs encapsulating Adpgk-LSP were not generated by SS. Finally, peptide encapsulation into POs by EP could be achieved, using 375 µg/mL of Adpgk-LSP in the EP cuvette. DLS and TEM analysis revealed monodisperse formulations with vesicles of around 54 nm in size, and HPLC analysis indicated Le was 257 peptide molecules per particle, corresponding to 3.6 µg Adpgk-LSP/ mg PMPC-PDPA and a 67% production efficiency. Thus, EP was the method of choice for the generation of nano-vaccines encapsulating the MC38 NeoAg Adpgk-LSP to be used for in vivo experiments.

To sum up, in this chapter EP was shown to be the most reliable, reproducible, and optimal methodology for the encapsulation of both OVA and Adpgk-LSP peptide. In addition, the size of these formulations, which were under 100 nm made them ideal candidates for their use as nano-vaccines. From now on, POs encapsulating OVA or Adpgk-LSP will be referred as P(OVA) or P(Adpgk-LSP), respectively.
3.4. Appendix

3.4.1. OVA encapsulation in PMPC-PDPA POs

A.

<table>
<thead>
<tr>
<th>EP pulses, free OVA</th>
<th>20x</th>
<th>15x</th>
<th>10x</th>
<th>5x</th>
<th>1x</th>
<th>0x</th>
<th>water</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA 43 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

P(OVA) P(OVA) OVA

OVA 43 kDa -

Figure 35. OVA electroporation in POs.
A) Blue Coomassie staining of free OVA following EP pulses. B) Immunodetection of 29 ng of OVA encapsulated in POS (P(OVA)). 1 µg of free OVA used as positive control.
3.4.2. Retention times of PMPC-PDPA, OVA and Adpgk-LSP peptide in u-HPLC.

A.

Figure 36. Retention times of polymer, OVA and peptide by u-HPLC. Absorbance at 220 nm of A) PMPC-PDPA only (top left), OVA only (top right) or both (bottom), B) PMPC-PDPA only (top left), Adpgk only (top right) or both (bottom).
3.4.3 Empty POs and P(OVA) formulations for *in vitro* and *in vivo* experiments

3.4.3.1 Unlabelled formulations

Figure 37. Characterisation of POs empty and encapsulating OVA by DLS.
Graphs showing average hydrodynamic diameter (left) and correlograms of formulations (right).
Figure 38. Characterisation of POs empty and encapsulating OVA by DLS. Graphs showing average hydrodynamic diameter (left) and correlograms of formulations (right).
Figure 39. Characterisation of POs empty and encapsulating OVA by DLS. Graphs showing average hydrodynamic diameter (left) and correlograms of formulations (right).
**Formulations**  
<table>
<thead>
<tr>
<th><strong>DLS size (nm) ± peak width</strong></th>
<th><strong>HPLC data</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empty and P(OVA)</strong></td>
<td>Number</td>
</tr>
<tr>
<td>Bi.2019.53</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>Bi.2019.54</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Bi.2020.38</td>
<td>73 ± 13.6</td>
</tr>
<tr>
<td>Bi.2020.39</td>
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<tr>
<td>Bi.2020.47</td>
<td>64 ± 17.5</td>
</tr>
<tr>
<td>Bi.2020.51</td>
<td>63.6 ± 8.8</td>
</tr>
<tr>
<td>Bi.2021.03</td>
<td>68 ± 10.2</td>
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<tr>
<td>Bi.2021.16</td>
<td>56 ± 8.4</td>
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<tr>
<td>Bi.2021.18</td>
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<tr>
<td>Bi.2021.32</td>
<td>71 ± 10.5</td>
</tr>
<tr>
<td>Bi.2021.36</td>
<td>70 ± 10.4</td>
</tr>
<tr>
<td>Bi.2022.09</td>
<td>59 ± 7.3</td>
</tr>
<tr>
<td>Bi.2022.09_1</td>
<td>53 ± 8.75</td>
</tr>
<tr>
<td>Bi.2022.12</td>
<td>82 ± 12.3</td>
</tr>
</tbody>
</table>

**Figure 40. Characterisation of POs empty and encapsulating OVA.**
Graphs showing average hydrodynamic diameter (left) and correlograms of formulations (right) from DLS measurements. Table displaying the summary of particles’ average hydrodynamic diameter and PDIs from DLS measurements and HPLC quantifications.
3.4.3.2 Labelled formulations

**Rho labelled Pos**

![Image of Rho labelled Pos](image)

**Cy5.5 labelled Pos**

![Image of Cy5.5 labelled Pos](image)

---

Figure 41. Characterisation of POs labelled with rhodamine (Rho-labelled-POs) or Cy5.5 (Cy5.5-labelled POs). TEM imaging and graphs showing average hydrodynamic diameter and correlograms of formulations. Cy5.5-labelled POs imaging was performed by Gabriel Ing.
**Figure 42.** Characterisation of labelled POs empty and encapsulating OVA.

Graphs showing average hydrodynamic diameter (left) and correlograms (right) from Cy5.5-labelled POs, by DLS. Table displaying the summary of particles’ average hydrodynamic diameter and PDIs from DLS measurements and HPLC quantifications from Rho/Cy5.5-labelled POs.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>DLS size (nm) ± peak width</th>
<th>HPLC data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescent POs</strong></td>
<td><strong>Number</strong></td>
<td><strong>Intensity</strong></td>
</tr>
<tr>
<td>Rho-POs</td>
<td>42 ± 6</td>
<td>77 ± 16.5</td>
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<tr>
<td>BI.2019.26</td>
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<td></td>
</tr>
<tr>
<td>Cy5.5-POs</td>
<td>135±13.9</td>
<td>259 ± 60</td>
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<tr>
<td>Cy5.5-POs</td>
<td>37 ± 5.62</td>
<td>79 ± 17.4</td>
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<tr>
<td>BI.2022.09</td>
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<tr>
<td>Cy5.5-P(OVA)</td>
<td>46 ± 7.2</td>
<td>98 ± 22.1</td>
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</tbody>
</table>
3.4.4 Empty POs and P(Adpgk) formulations for *in vivo* experiments

**Figure 43. Characterisation of labelled encapsulating -LSP.**
Graphs showing average hydrodynamic diameter (left) and correlograms (right), by DLS. Table displaying the average hydrodynamic diameter and PDIs.
Chapter 4: Addressing POs biocompatibility, uptake, antigen intracellular delivery, and CD8 T cell priming, 
\textit{in vitro}
4.1. Introduction and aims

The ultimate use of P(OVA) and P(Adpgk-LSP) formulations as anti-tumour nano-vaccines in vivo is subject to their biocompatibility (this is, their safety and absence of toxicity when used in living systems) and their potential to target and deliver antigen intracellularly in DCs. Previous group members proved efficient PMPC-PDPA POs targeting to macrophages in vitro and in vivo [310], [311], and other cells including cancer cells [286], in a process that involves receptor-mediated endocytosis, through PC interaction with SR-B1, CD36 and CD81 receptors [301]. Nonetheless, DCs are superior to macrophages in antigen presentation and CD8 T cell priming, which is critical for anti-tumour responses [89]. Therefore, POs-DC targeting and IC delivery needed to be addressed in these cells. Following SRs binding and POs internalisation in endosomes, PDPA protonation in acidified late-endosomes enhances cytosolic cargo release [309], [321]. In the case of P(OVA) and P(Adpgk-LSP) vaccines, OVA protein and Adpgk-LSP access to the cytosol is critical for their processing into 8 to 10 aa short peptides by the cytosolic immune-proteasome, to further allow for MHC-I complexation in the ER, followed by presentation on DC cell surface [82]. Activation of antigen-specific CD8 T cells is triggered upon TCRs binding with specific MHC-I-Ag in DCs in the presence of co-stimulatory receptor-ligand interactions. Generally, similar studies using polymer-based nano-vaccines require the co-administration of an adjuvant to provide with DC maturation and expression of co-stimulatory molecules [287], [289], [293]. However, some other studies have reported the ability of certain polymer nano-vaccines to work as “self-adjuvants”, providing with DC maturation in the absence of adjuvant [291]. However, this feature remains to be explored in PMPC-PDPA POs vaccine formulations.

In this chapter, the safety and the potential of P(OVA) vaccines to target, deliver and achieve antigen cross-presentation in DCs is assessed in vitro in DC2.4 cells and in primary BMDC cultures. Priming and CD8 T cell activation analysis was performed using SIINFEKL-reactive CD8 T cells isolated from the spleen of OT-I mice.

In all, the specific aims of this section are:

1. Explore the biocompatibility of PMPC-PDPA POs in DC2.4 cells and BMDCs.
2. Evaluate the expression of SR-B1, CD36 and CD81 in DC2.4 cells and BMDCs.

3. Assess the ability of PMPC-PDPA POs to target DC2.4 cells and BMDCs, and to deliver NeoAgs (OVA) intracellularly, in DC2.4 cells.

4. Understand whether OVA intracellular delivery by POs in DC2.4 and BMDCs drives MHC-I-SIINFEKL cross-presentation, and the potential of PMPC-PDPA POs to act as self-adjuvant to induce the expression of co-stimulatory molecules.

5. Analyse the proliferation and expression of stimulatory immune checkpoints of SIINFEKL-reactive CD8 T cells (OT-Is) following priming by P(OVA)-pulsed BMDC, in the presence or absence of the adjuvant Poly(I:C).
4.2. Results

4.2.1. Viability of DCs upon PMPC-PDPA POs treatment

The biocompatibility of PMPC-PDPA POs was analysed in DC2.4 and in BMDCs by using MTT and MTS assay. To this end, DC2.4 and BMDCs were incubated for 24h with increasing concentrations of empty POs or P(OVA) samples, from 20 µg/mL to a maximum of 1 mg/mL. POs and P(OVA) samples exerted a similar effect on the viability of DCs. Results showed that in the case of DC2.4 cells (Figure 44A), the cellular viability remained above 60% at all concentrations, with values ranging between 75 to 100% upon incubation with lower POs or P(OVA) concentrations (20 to 200 µg/ml) (Figure 44A). BMDCs revealed higher viability values upon POs or P(OVA) incubation, which remained around 90-100% (Figure 44B). Thus, these results indicated that PMPC-PDPA POs were biocompatible for DC2.4 cells and BMDCs. For in vitro studies involving 24 hours of POs incubation, a maximum of 200 µg/mL or 1 mg/mL final concentration was used in DC2.4 or BMDCs, respectively.

Figure 44. Viability of DCs following 24 hours of POs incubation.
A) POs and P(OVA) MTT assay in DC2.4 cells. B) POs and P(OVA) MTS assay in BMDCs. Mean ± SD (n=3 independent experiments, in duplicates)
4.2.2. Expression of Scavenger receptors in DCs

As reported by previous members of the Prof. Battaglia’s group, the endocytosis-mediated cell uptake of PMPC-PDPA POs occurs through the interaction of the PC head from the PMPC block with SR-B1, CD36 and CD81 receptors in a range of cell lines [301]. Thus, the expression of these receptors was assessed in BMDCs and DC2.4 cells. **Figure 45A** and **Figure 45B** finding that DC2.4 cells expressed both CD36 (150 kDa band) and SR-B1 (100 and 75kDa bands), and the expression of these receptors did not vary with the number of cell passages. No CD81 expression could be found in DC2.4 cells.

**Figure 45. Expression of SR-B1, CD36 and CD81 receptors in DCs.**
Western blot analysis of SRB1(100 and 75kDa), CD36 (150 or 25kDa) and CD81(25kDa) levels in A) DC2.4 cells and C) BMDCs. Quantification of receptor density in cells in B) DC2.4 cells (relative to Cyclophilin A) and D) BMDCs (relative to GAPDH). Images representative of n=2 independent experiments, in duplicates.
The expression of SRs and CD81 was also verified in BMDCs (**Figure 45C** and **Figure 45D**). Results showed that all receptors were expressed by BMDCs, although CD81 was differently expressed between BMDCs samples. Interestingly, the band corresponding to CD36 was found at 25 kDa in BMDC lysates (similarly to CD81), as opposed to the 150 kDa band that appeared in DC2.4 lysates (**Figure 45**). This could be due to post-translational modifications at receptor site, such as glycosylation, affecting the molecular weight of the CD36, which in literature is approximately 53kDa [332]. Another potential explanation for the differences in the observed bands’ Mw could be protein aggregation or degradation, in the case of DC2.4 or BMDCs, respectively.

Since PO endocytosis is mediated by SR-B1, CD36 and CD81, the expression of these receptors in DC2.4 cells and BMDCs suggested that POs could be taken up in these cells.

### 4.2.3. PMPC-PDPA POs uptake in DCs

For POs to be used as vaccine antigen carriers, their uptake by DCs is essential. To understand the potential of POs to target DCs, DC2.4 cells were incubated with rhodamine-labelled POs (Rho-POs) from 15 mins to up to 2 hours, after which the cellular uptake was assessed by confocal microscopy. As indicated in **Figure 46F**, POs were internalised by DC2.4, and it was possible to detect them in a cumulative fashion over time from 15 mins after the sample addition. 30 mins after PO incubation, colocalisation of POs (green) with the cell membrane (Red) was detected (yellow spots, merged image). This indicates endocytic-mediated mechanism of POs internalisation, as previously described [333]. Following 2 hours of incubation, most of the Rho signal was accumulated in the cytosol, revealing POs endosomal escape.

Moreover, Rho-POs uptake was also assessed in BMDCs. Qualitative and quantitative analyses were performed by confocal microscopy and FACS, respectively **Figure 47**. Confocal images revealed that Rho-POs were internalised in BMDCs 15 mins after incubation with cells (**Figure 47A**, blue signal), similar to what was observed in DC2.4.
Rho-POs were detected in BMDCs 2 h, 7 h and 24 h after POs incubation. However, BMDCs are semi-adherent in cell culture plates, and they float around, which hampers the imaging of these cells. For this reason, FACS analysis was performed in parallel following 15 mins, 1h, 3h, 6h or 24h of POs incubation. Approximately 75% of the BMDCs had taken up Rho-POs after 15 mins, which increased to 100% following 6 or 24 hours of Rho-POs incubation (Figure 47B). Moreover, Mean Fluorescence Intensity (MFI) analysis from Rho-POs+ cells revealed that a higher number of POs were internalised and accumulated per cell with time (Figure 4.4C), similarly to what was observed in the Rho-POs uptake in DC2.4 (Figure 46).

Figure 46. PMPC-PDPA POs uptake in DC2.4 cells.
Confocal images from Rho-PMPC-PDPA POs uptake at 15 min, 30 min and 2 hours. Blue: Nucleus, Red: Cell membrane, Green: Rho-PMPC-PDPA POs. Blank: DC2.4 cells incubated with media only. Scale bar = 25 μm
Figure 47. PMPC-PDPA POs uptake in BMDCs.
Cells were incubated with POs for 24h and cellular uptake was assessed by confocal microscopy and FACS. A) POs uptake visualised by confocal microscopy (Scale bar = 50 μm). POs uptake was also assessed by FACS: B) plots showing the percentage of Rho-POs+ cells at different timepoints. C) Plots showing mean fluorescence intensity (MFI) in Rho-POs+ cells. Mean ± SD (n=2 independent experiments, in duplicates).
4.2.4. **OVA intracellular delivery by PMPC-PDPA POs**

After proving POs uptake by DCs, the next addressed point was the ability of POs to deliver NeoAgs intracellularly in DCs. This step is crucial for antigen complexing in MHC-I molecules and further antigen presentation to T cells [81]. Confocal microscopy was used for tracking OVA IC delivery in DC2.4. In a first approach, P(OVA) samples were incubated with DC2.4 cells for 4 hours to allow for uptake. After this time, media containing unbound P(OVA) was discarded, cells were washed with PBS, and cell membrane was permeabilised and immuno-stained for OVA. F-actin cytoskeleton was stained with Phalloidin to facilitate the visualisation of cells. Figure 48 shows the IC delivery of OVA (green) in DC2.4 cells by confocal microscopy. To confirm that the protein signal was not caused by unspecific binding of secondary Ab and other proteins expressed intracellularly, untreated DC2.4 cells were permeabilised and stained with the secondary-labelled Ab only (bottom image Figure 48. No signal was found, which implied that no unspecific binding was taking place and that the signal found in treated cells was OVA-specific.

To gain a better understanding of the cytosolic delivery of OVA by POs in DC2.4 cells, P(OVA) uptake was tracked by live imaging. In this experiment, POs and the entrapped OVA were fluorescently labelled with rhodamine and FITC, respectively. Confocal live imaging was performed capturing images from the same well area every 10 minutes, at different Z axes heights. Figure 49A shows a representative video of the first 2 hours after fluorescent P(OVA) incubation (in a single Z plane only), in which POs are shown in red and OVA in green. OVA IC delivery and release from POs was tracked at a single-cell level along 40 to 90 minutes, as represented in Figure 49B. Cell-shaped silhouettes (grey) in 3D were reconstructed from images acquired at different Z planes, and it was possible to identify POs (red) encapsulating OVA (green) within the cells. Similar to what was observed during the timelapse video, OVA-FITC levels also increased intracellularly with time, presumably due to POs disassembly. Thus, these data suggest an efficient IC OVA delivery by POs in DC2.4 cells.
Figure 48. OVA intracellular delivery by PMPC-PDPA POs.
Representative confocal images of DC2.4 cells following 4 hours of incubation with P(OVA) samples. Red: Phalloidin (F-actin staining), Green: OVA, Blue: DAPI (nuclei). Scale bar = 10 μm
Figure 49. Live cell imaging of OVA intracellular delivery by POs in DC2.4 cells.
A) Control picture indicating the cell size. B) 3D image sequence of living DC2.4 cells incubated with Rho-PMPC-PDPA POs encapsulating OVA-FITC for 120 min. Rho-PMPC-PDPA POs: Red. OVA-FITC: Green. Scale bar = 10 μm.
4.2.5. OVA cross-presentation in DC2.4

Antigen uptake and release into the cytosol of DCs are critical steps in antigen cross-presentation. The cytosolic proteasome processes exogenous antigens in the shape of protein or long peptides into shorter 8-10 aa peptides [334]. These are subsequently loaded into MHC-I complexes within the ER and further anchored on the cell membrane of DCs, allowing for CD8 T cell interaction [75]. In addition to MHC-I-antigen recognition, complete CD8 T cell activation relies on the presence of co-stimulatory signalling [335]. These cascades are triggered by the interactions between maturation molecules in the DCs surface (such as CD40, CD80 or CD86) and their ligand counterparts on the surface of CD8 T cells. The lack of co-stimulation leads to anergy and inability of CD8 T cells to kill target cells [71], [336]. To address the effect of P(OVA) in OVA cross-presentation, DC2.4 cells were incubated overnight with P(OVA) samples in the presence or absence of Poly(I:C), as illustrated in Figure 50A. On the following day, OVA cross-presentation and the expression of CD40 and CD86 maturation markers was assessed by FACS. As a control, DC2.4 cells were treated with free OVA at an equivalent concentration to the encapsulated in POS (around 1 µg/mL). Empty POs only were also incubated with DCs to assess its effect on DC maturation.

As indicated in Figure 50B, analysis of OVA cross-presentation levels in DC2.4 cells revealed no significant differences across the groups in the absence of the adjuvant Poly(I:C). In contrast, DC2.4 cells treated with Poly(I:C) and P(OVA) formulations showed a two-fold increase in OVA cross-presentation compared with free OVA or media only. These results are in line with other works, since the presence of adjuvants contributes to DC maturation and increases antigen cross-presentation levels [337]. Besides, these results suggest an improved OVA cross-presentation capacity by P(OVA) samples in the presence of Poly(I:C).

Next, DC maturation was assessed. As shown in Figure 50C, the number of DC2.4 cells expressing CD40 in the absence of Poly (I:C) seemed to be higher in P(OVA) treated cells. This trend was also observed in DC2.4 cells treated with Poly(I:C). Nonetheless, these results were not statistically significant and could not be reproduced. In addition, no changes could be found across groups in the expression levels of CD86, as represented in Figure 50D.
Figure 50. P(OVA) effect in OVA cross-presentation and activation of DC2.4 cells.

Briefly, DC2.4 cells were incubated overnight with empty POs, OVA or P(OVA), in the absence or presence of the poly(I:C). Following incubation cells were stained with anti-CD40, anti-CD80 and anti-H2Kb-SIINFEKL antibodies and FACS analysis were performed A) Experiment schematic. B) Plots representing OVA cross-presentation C) Plots representing CD40 expression. D) Plots representing CD86 expression. n=2 independent experiments. Bars indicate means. Statistical analysis were performed using one-way-ANOVA (*p < 0.05).
### 4.2.6. CD8 T cell priming, activation, and proliferation

To understand whether the antigen cross-presentation levels in DCs achieved with P(OVA) particles could trigger activation of SIINFEKL-reactive CD8 T cells, two different DC – CD8 T cell co-incubation assays were performed.

In a first attempt, B3Z cells were used. These cells are a transgenic SIINFEKL-reactive CD8 T cell hybridoma, which harbours the LacZ gene (coding a β-galactosidase protein). B3Zs are engineered in a way by which, upon TCR interaction with MHC-I-SIINFEKL in DCs, LacZ gene is activated [338]. Thus, when DC2.4-B3Z priming occurs following OVA cross-presentation, β-galactosidase is transcribed and translated. This enzyme can catalyse the degradation of chlorophenol red β-d-galactopyranoside into β-galactose and chlorophenol red. Therefore, it is possible to indirectly detect efficient OVA cross-presentation and B3Z T cell activation through changes in absorbance readout (570 nm).

In the experiment, DC2.4 were incubated with P(OVA) for either 6 or 24 hours in the presence or absence of Poly(I:C). P(OVA) samples were subsequently washed away with PBS, and DCs were co-incubated with B3Z cells for 24h. However, as shown in Appendix Figure 54, it was not possible to detect activation by change of B3Z cell’s colour at any condition. As a positive control, the incubation of SIINFEKL peptide with DC triggered a 3-fold increase in absorbance of B3Z cells after 6 hours. A lack of sensitivity in the experiment readout might be the reason why no activation could be detected upon P(OVA) incubation. As reported by Prof. Nilabh Shastri and his team (who synthesised this hybridoma cell line), the detection of B3Z activation upon H2Kb-SIINFEKL-expressing DC co-incubation relies on the presence of a 3-5% of H2Kb-SIINFEKL+ DCs (upon DCs: B3Z incubation, at a 1: 2 ratio), detected by FACS [338]. However, in this thesis, the highest value of H2Kb-SIINFEKL+ DC2.4 that could be achieved upon P(OVA) incubation was 0.54%. This can indeed be due to the low OVA concentration entrapped in the P(OVA) samples (maximum 15 µg/mL) that were used, in contrast to the OVA concentrations (minimum of 100 µg/mL) used in many positive-outcome experiments described in literature [338], [339].

A more physiologically relevant system to assess T cell priming and activation by OVA-pulsed DCs are primary DCs and CD8 T cells co-cultures. To this end, SIINFEKL-reactive
CD8 T cells were purified from the spleen of OT-I mice [340], and DCs were differentiated from murine bone marrow cell precursors by FLT3L incubation over nine days [317]. Prior to the co-incubation experiment, BMDC lineage was evaluated by FACS (Appendix Figure 55) to guarantee the presence of DC-like cells (CD11c+CD11b+) expressing maturation (MHC-II+) and cross-presentation (XCR1+) markers within the population mix. Of note, P(OVA) cross-presentation experiments were attempted prior to this work, following the same P(OVA) incubation and readout protocol that were used with DC2.4 cells (Appendix Figure 56A for experiment diagram and Figure 56B for gating strategy). However, no cross-presentation or activation differences could be identified among groups (Appendix Figure 57A and Figure 57B). This result might have been due to high background noise in control samples, derived from unspecific binding from the H2Kb-SIINFEKL Ab used for FACS analysis.

As widely described in literature, proliferation is a hallmark of T cell activation [121]. Hence, tracking the number of proliferative CD8 T cell generations following priming allows to understand the efficiency of T cell priming following vaccination. At day nine after differentiation, BMDCs were incubated with POs, P(OVA), or naked OVA at an equivalent concentration for 6 hours in the presence or absence of Poly(I:C). PBS was used as negative control. After this time, samples were washed, and fluorescently labelled OT-I cells (with Cell Trace Violet-CTV) were co-incubated for 72-96 hours, followed by OT-I activation assessment through tracking CTV fluorescence loss in proliferated cells by FACS, as represented in Figure 51A. In addition, the expression of activation receptors, such as CD25, 4-1BB, OX-40, and ICOs, was also assessed in these cells (Appendix Figure 58 for gating strategy), given their key role in the transduction of proliferative and anti-apoptotic signals in T cells, upon DC ligand encountering [220], [341].

The quantification of total CD8 (OT-I) T cells at the end of co-incubation time revealed higher cell numbers triggered by P(OVA) than by naked OVA, POs, or PBS (Figure 51B). Although this trend was irrespective of the use of Poly(I:C), the differences were only significant in the presence of the adjuvant. Besides, the number of CD8 T cells in all treatment groups was two-to-four orders of magnitude higher in Poly(I:C) treated
conditions, which can be due to the fact that BMDCs are primary cell lines and die quicker in the absence of stimulatory molecules. In turn, this might hinder OVA cross-presentation and CD8 T cell proliferation, which would correlate with the absence of proliferating generations that is observed in co-cultures that were not treated with Poly(I:C), except for P(OVA) treated co-cultures (Figure 51C, top and Figure 51D, middle). In contrast, in Poly(I:C) treated samples, OT-I cells from all treatment groups proliferated, and higher cell numbers per proliferating generation were achieved (Figure 51C, bottom and Figure 51D, right). The results also showed a considerable increase in numbers of CD8 T cells with less proliferative profile in co-cultures treated with PBS or empty POs, which gave rise to 4 and 8 proliferating generations, respectively, with decreasing cell numbers at later proliferation stages and up to 100 cells per generation. In contrast, the number of proliferating cells in later generations was up to 10,000 in co-cultures incubated with free OVA, or P(OVA), suggesting an efficient antigen-cross presentation and OT-I priming and activation. Remarkably, P(OVA) treated co-cultures triggered up to 10 times higher numbers of proliferated CD8 T cells (in generations 6 and 7) compared to free OVA, indicating an enhanced priming process (Figure 51C, bottom and Figure 51D, right).

The analysis of activation markers in OT-I cells are represented in Figure 52 and Figure 53. Among them, the frequency of CD25+ OT-I cells in Poly(I:C) untreated cells (Figure 52A, top left) did not vary across groups significantly, although these results suggested a slight increase in the frequency of CD25+ OT-I cells upon P(OVA) treatment. In fact, this trend was the same for co-cultures treated with the adjuvant (Figure 52A, bottom left), although in this case, naked OVA revealed a similar frequency of OT-I cells expressing the IL-2 receptor. This is likely related to the higher proliferation rates observed in Figure 51D, which is associated with CD8 T cell activation and IL-2 metabolism [1]. Interestingly, a decreased CD25 mean fluorescence intensity (MFI, which correlates with the number of receptors expressed per cell) was found in proliferating OT-I cells treated with P(OVA) in Poly(I:C) free conditions, when compared with the rest of the conditions. In contrast, co-cultures treated with P(OVA) in the presence of Poly(I:C) gave rise to an increased percentage of CD25+ OT-I cells, although these differences are not significant when compared with the naked OVA counterpart. Despite higher proportions
**Figure 51. OT-I proliferation triggered upon BMDC P(OVA) pulsing.**

BMDC were treated with PBS, empty POs, OVA or P(OVA) for 6 hours, in the presence or absence of Poly(I:C). Subsequently, cells were washed and co-incubated with CTV-labelled OT-I CD8 T cells, for 72 to 96 hours. After this time, proliferation was assessed by FACS. A) Experiment illustration. B) FACS plot (left) and graphs (right) representing total OT-I numbers after coinoculation. C) FACS plot representing proliferating generations. D) CTV histogram in OT-I cells (left) and quantification of number of cells in proliferating generations (right). Error bars represents SD (n=3 independent experiments in triplicates). P(OVA) significance against OVA only was analysed by one-way-ANOVA (*p < = 0.05 **p<= 0.01)
of CD25+ cells, MFI values indicated a trend in lower receptor density per each CD25+ OT-I cell in P(OVA) treatment compared with naked OVA.

Secondly, the frequencies of ICOS+ OT-I cells in the P(OVA) treated condition were around 30% in both adjuvant-free (Figure 52B, top left) or adjuvant-treated (Figure 52B, bottom left) co-cultures. Interestingly, in the absence of Poly(I:C), these levels were approximately 2 times higher in P(OVA) treated co-cultures than in the rest of the groups, although these differences were not statistically significant (Figure 52B, top left). Of note, the frequency of ICOS+ CD8 T cells in co-cultures treated with naked OVA only reached similar P(OVA) values in the presence of adjuvant (Figure 52B, bottom left). As explained previously, the ICOS receptor is not constitutively expressed in T cells, and its expression might be enhanced by CD80-CD28 or MHC-peptide-TCR interactions (in DCs-Tc) [341]. Therefore, these results seem to suggest that P(OVA) particles are efficient at either cross-presenting antigen and/or induce CD80 activation in DCs, which in turn, might activate CD8 T cells efficiently. This process seems to be independent of the presence or absence of adjuvant, as opposed to naked OVA cocultures, which requires the presence of Poly(I:C) to increase ICOs expression. Despite higher frequencies in ICOS+ CD8 T cells, MFI analysis revealed no differences in receptor density per cell (Figure 9B, middle and left). This result suggests an overall enhanced activation derived from higher levels of proliferating cells expressing the receptor but not from enhanced activation within one single OT-I cell.

As shown in Figure 53C, the expression of 4-1BB receptor in OT-I cells was generally homogeneous among treatment groups in terms of frequencies and MFIs, either in the presence or absence of adjuvant. Nonetheless, a non-statistically significant decrease in the 4-1BB+ frequency of OT-I cells could be observed in P(OVA) treated co-cultures in the absence of adjuvant (Figure 53C, top left). This would imply that following PBS, empty POs, or OVA treatment, a higher proportion of OT-I cells express 4-1BB receptor at the time of readout (72-96 hours following co-incubation), when most of these cells had not undergone proliferation (Figure 51D.). Since the expression of 4-1BB is reported to be enhanced following MHC-TCR signalling, and has an important role in proliferation and the generation of immune memory [221], these results could suggest that the peak in
Figure 52. OT-I activation triggered upon BMDC P(OVA) pulsing.

BMDC were treated with PBS, empty POs, OVA or P(OVA) for 6 hours in the presence or absence of Poly(I:C). Subsequently, cells were washed and co-incubated with CTV-labelled OT-I CD8 T cells, for 72 to 96 hours. Next, activation was assessed by FACS. A) FACS plot and graphs representing the frequencies and MFI of CD25+ OT-I cells in the absence (top) or presence (bottom) of Poly(I:C). B) FACS plot and graphs representing the frequencies and MFI of ICOS+ OT-I cells in the absence (top) or presence (bottom) of Poly(I:C). Error bars represents SD (n=3, independent experiments in triplicates). P(OVA) significance was analysed by one-way-ANOVA (*p <= 0.05)
Figure 53. OT-I activation triggered upon BMDC P(OVA) pulsing.
BMDC were treated with PBS, empty POs, OVA or P(OVA) for 6 hours, in the presence or absence of Poly(I:C). Subsequently, cells were washed and co-incubated with CTV-labelled OT-I CD8 T cells, for 72 to 96 hours. Next, activation was assessed by FACS C) FACS plot and graphs representing the frequencies and MFI of 4-IBB+ OT-I cells in the absence (top) or presence (bottom) of Poly(I:C). D) FACS plot and graphs representing the frequencies and MFI of OX-40+ OT-I cells in the absence (top) or presence (bottom) of Poly(I:C). Error bars represent SD (n=3, independent experiments in triplicates). P(OVA) significance was analysed by one-way-ANOVA (*p ≤ 0.05)
receptor expression could have been earlier than 72-96 hours in P(OVA) treated co-cultures, and that the decreased proportion of 4-1BB+ OT-I cells in this group could be due to a downregulation of the receptor following proliferation.

To finish with, no statistical significance was found in the expression of OX-40 across groups (Figure 53D). As described in literature, the peak expression of this activation marker is seen from 24 to 72 h after T cell priming [342], [343]. Therefore, differences might have been easier to deduce at earlier timepoints.

4.3. Discussion and conclusions

Efficient anti-tumour vaccines must provide with safe, targeted, and enhanced antigen and adjuvant delivery into APCs, specially DCs [344], which proficiently cross-present NeoAgs and mediate tumour-reactive CD8 T cell activation. In this chapter, the safety of PMPC-PDPA POs as NeoAg (OVA) delivery systems in vitro, as well their CD8 T cell activation potential have been addressed.

First, the biocompatibility of PMPC-PDPA POs as delivery systems to DCs has been corroborated in DC2.4 cells and BMDCs, using MTT and MTS assay, which indirectly inform about cell fitness and viability through the reduction of MTT or MTS tetrazolium compounds by cellular enzymes [345]. Thus, viability values can be regarded, in fact, as “cell fitness” indicators. Whereas POs and P(OVA) formulations had no detrimental effect over BMDCs viability, O/N incubation of POs at concentrations higher than 0.6 mg/ml in DC.2.4 resulted on viabilities around 60-70%, with higher values (80-100% viability) at lower concentrations. These results go in line with previous reports in other APC cell lines, such as THP-1, which show minimal toxicity upon PMPC-PDPA POs coincubation at similar doses and time [310].

Next, PO uptake in both cell types was addressed. This has recently been described to be triggered upon PC head (from PMPC block) binding to CD36, SR-B1 and CD81, by receptor mediated endocytosis [301]. Accordingly, it was possible to immunodetect SR-B1 and CD36 receptors in cell lysates from both DC2.4 and BMDCs, and additionally,
CD81 in BMDCs. It is thus likely that these receptors would have mediated POs uptake in DCs. However, additional experiments are required to fully address this point and to further understand the contribution of each receptor in this process in DCs. Further, it was possible to detect PO uptake in DC2.4s and BMDCs following just 15 mins of incubation, and in addition, FACS results suggested that uptake in BMDCs is an ongoing process and that longer POs incubation periods result in higher particles inside a cell. Following 30 mins of uptake, confocal analysis confirmed the colocalisation of POs with IC vesicles, which were likely endosomes. This is in line with PMPC-PDPA PO uptake in other cell lines such as macrophages [310], [311] or glioma cells [309]. Moreover, PMPC-PDPA promoted efficient IC OVA delivery in DC2.4 cells, being possible to immunodetect the protein following 4 hours of P(OVA) co-incubation. Complementary, live imaging analysis it revealed OVA release from POs intracellularly, likely due to PDPA-driven PO disassembly into polymer unimers. Noteworthy, PMPC-PDPA cargo IC delivery has been widely characterised with other molecules such as DNA/RNA [346], drugs [310], [311], other proteins (i.e. antibodies) [308] or dyes [295].

P(OVA) mediated antigen uptake also seemed to allow for OVA processing and complexing in MHC-I-SIINFEKL molecules on the surface of DC2.4 cells, following co-incubation with Poly(I:C). Under this condition, cross-presentation was 2-to-3-fold higher in cells incubated with P(OVA), when compared to those treated with naked OVA, empty POs or PBS controls. Noteworthy, no differences in antigen cross-presentation could be found among groups in the absence of Poly(I:C), which reflects the importance of adjuvants in enhancing antigen presentation [337]. Relevantly, several studies suggest that antigen uptake mediated by receptor mediated endocytosis drives enhanced antigen cross-presentation when compared with other uptake processes, such as pinocytosis [81], [347]. Besides, whereas immature DCs actively uptake antigens through micropinocytosis, this process is stopped upon DC maturation and MHC-II translocation in the cell membrane, to avoid presentation of antigens others than those that they were activated for (i.e. avoid the uptake of self-antigens and trigger autoimmunity). Nonetheless, it has also been reported that, in mature states of DCs, antigen uptake might still happen through receptor mediated endocytosis [270], which
could explain enhanced cross-presentation values in P(OVA) and Poly(I:C) treated cells, owing PO uptake by SR mediated endocytosis.

Maturation markers analysis indicated that, interestingly, the use of Poly(I:C) did not influence the expression of co-stimulatory molecules such as CD86 in any treatment in DC2.4 cells, when compared to cells cultured in the absence of adjuvant. However, CD86 expression was also found in PBS control groups, suggesting that this protein might have been constitutively expressed, rather than induced by PAMP recognition, perhaps due to an intrinsic characteristic of the cell line. Next, CD40 expression seemed to be higher in P(OVA) treated cells, especially in the presence of Poly(I:C), however these results were hard to reproduce and further insight into them would be required.

Finally, there was a correlation between the P(OVA) potential to enhance antigen cross-presentation, and their ability to induce 10 times higher proliferation of SIINFEKL-reactive CD8 T cells (OT-I), when compared with naked OVA. Nonetheless, P(OVA) formulations incubated with BMDCs in the absence of Poly(I:C) could also trigger CD8 T cell proliferation, which is in discrepancy with the previous experiment in which cross-presentation could not be observed in adjuvant-free conditions. In addition, we sought to describe the expression of co-stimulatory markers (CD25, ICOS, 4-1BB and OX-40) that could be differentially expressed in CD8 T cells following PO-mediated or PO-free immunisation strategies. In addition to giving a mechanistical insight into PO priming, these experiments have the potential to inform preliminarily about prospective therapy strategies combining POs-based tumour vaccines with co-stimulatory checkpoint agonists [348]. In adjuvant-free conditions, P(OVA) treated co-cultures appeared to trigger higher frequencies of CD25+/ICOS+ CD8 T cells than the rest of the treatments. CD25 (IL-2 receptor) expression is induced by efficient priming, following T cell activation and IL-2 transcription and secretion. ICOs upregulation is likely linked to efficient cross-presentation and TCR signalling [341], supporting P(OVA) NPs as efficient antigen delivery systems. In the presence of Poly(I:C), where BMDC maturation is expected [349], P(OVA) treated co-cultures followed a similar trend, however, in this adjuvant setup naked OVA treated co-cultures also increased the expression of these receptors, reflecting the need for adjuvant to achieve T cell activation in naked OVA immunisations.
In conclusion, in this chapter the potential of P(OVA) formulations to target and delivery antigen in DCs was corroborated. In addition, P(OVA) formulations drove antigen cross-presentation in DCs, in the presence of the TLR-3 stimulator Poly(I:C), which further translated in efficient proliferation of antigen-reactive CD8 T cells, especially in combination with adjuvant. In addition, enhancement of CD8 T cell activation markers such as CD25 and OX-40 could be found. These results are the first indication supporting the applicability of PMPC-PDPA POs as protein-based antigen carriers for the induction of adaptive responses and thus, for their use as nano-vaccines targeting DCs.
4.4. Appendix

4.4.1. DC2.4-B3Z priming assay

Figure 54. SIINFEKL-reactive CD8 T cell priming.
A) Experiment illustration. Plots representing MHC-I-SIINFEKL recognition by B3Z cells in the absence (left) or presence (right) of poly(I:C), following B) 6 hours or C) 24 hours of sample incubation with DC2.4 cells. Results normalised to PBS.
4.4.2. BMDCs phenotype upon FLT3L differentiation

Figure 55. BMDC phenotype upon FLT3L differentiation.
Gating strategy followed for BMDC lineage differentiation.
4.4.3. OVA cross-presentation in BMDCs

A. OVA cross-presentation experiment in FLT3L-derived BMDCs.

Figure 56. OVA cross-presentation experiment in FLT3L-derived BMDCs. 
A) Experiment diagram and B) Gating strategies followed for cross-presentation and activation experiment following P(OVA) incubation.
Figure 57. OVA cross-presentation experiment in FLT3L-derived BMDCs.
A) Cross-presentation results normalised to PBS B) Activation results normalised to PBS
4.4.4. T cell proliferation assay following BMDCs-OTs coinubcation

![Gating strategy for OT-I proliferation following P(OVA) incubation](image)

Gate: CD3+CD8+

**Figure 58. Gating strategy for OT-I proliferation following P(OVA) incubation**
Chapter 5: Therapeutic effect of subcutaneous P(OVA) immunisations *in vivo*, in the context of B16-OVA melanoma model.
5.1. Introduction and aims

Cytolytic CD8 T cells are essential in tumour rejection, particularly those clones whose TCR recognise tumour cells expressing NeoAg complexed on MHC-I molecules [350]. Interestingly, recent studies indicate that NeoAg-reactive CD8 T cell clones account for just a small proportion of all TILs found in tumours, and it is understood that a large proportion of TILs are specific for virus epitopes, such as Influenza or Epstein-Bar Virus [351]. Indeed, therapeutic vaccinations with NeoAg have proved to prime and expand NeoAg-reactive CD8 T cell clones, which are necessary for tumour clearance [254], [352]–[354]. Nonetheless, the design of in vivo vaccine strategies overlooks that efficient CD8 T cell priming follows IC delivery and cross-presentation of NeoAg in DCs, which are proficient in antigen presentation [89]. Hence, current NeoAg vaccines based on whole protein or peptides [352], [355] are administered in the absence of delivery systems vaccine, that could enhance their stability, DC targeting and antigen presentation which would translate into enhanced immunisations [356], [357]. The use of vaccine nano-carriers increase the bioavailability of proteins or peptide immunogens by protecting them from degradation [358], and by enhancing their delivery and retention in lymphoid organs [282].

Besides, expansion of NeoAg-reactive CD8 T cells in patients is achieved following checkpoint blockade [359], [360] since NeoAg-reactive TILs normally present a dysfunctional exhaustion phenotype [176]. Combination therapies, including vaccine and ICI approaches, improve disease outcomes and can achieve total tumour rejection [222]. This usually correlates with further expansion of pre-existing and de novo-induced NeoAg-reactive CD8 T cells [225]. From a practical perspective, NeoAg-specific TCR clones in blood or other tissue samples are analysed by stainigs with fluorescently labelled MHC multimers. These are macromolecules where 4, 5, or 6 MHC-peptide units (tetramers, pentamers or dextramers, respectively) are linked to a polymer and bind with high affinity to CD8 T cells whose TCR is specific for the antigen [361].

In the previous chapter, P(OVA) formulations were validated in vitro as safe and efficient vehicles for OVA targeting and IC delivery into DCs, which translated into the induction
of SIINFEKL-reactive CD8 T cell responses. In this chapter, the potential of P(OVA) and Poly(I:C) formulations as therapeutic vaccine will be assessed \textit{in vivo} in mice harbouring the subcutaneous B16-OVA melanoma model. Besides, this approach will be compared with free OVA conventional immunisation strategies. To test this therapeutic strategy, the following specific aims will be addressed:

1. Define the P(OVA) immunisation schedules that achieve a therapeutic delayed tumour growth and extended survival in animals harbouring B16-OVA.

2. Characterise the lymphoid and myeloid compartments infiltrating B16-OVA tumours following P(OVA) immunisations, focusing on NeoAg-reactive CD8 T cells.

3. Understand the overall benefit of checkpoint blockade combined with P(OVA) immunisations.

4. Define CD8 T cell activation, cytotoxicity, and exhaustion profiles across treatment groups in tumour infiltrates and LNs.

5. Describe the exhaustion differences found between vaccine-induced NeoAg-reactive CD8 T cell clones versus the general CD8 T cell repertoire.
5.2. Results

5.2.1. P(OVA) dosing optimisation.

5.2.1.1. P(OVA) immunisations in combination with Poly(I:C).

To understand the effect of therapeutic P(OVA) immunisations in the B16-OVA cancer model, B16-OVA tumour cells were s.c. injected in C57Bl/6 mice, and tumour progression and survival was evaluated. First, it was necessary to optimise the number of injected B16-OVA cells and the immunisation schedule. Thus, for the first study in vivo, mice were injected with $1 \times 10^6$ B16-OVA cells, following protocols used in similar studies [289], [291]. Based on the same criteria, immunisations were performed on day 7 in the first instance, when tumour size had reached $\approx 100 \text{ mm}^3$ (Figure 59A and Figure 59B). The maximum amount of OVA entrapped in POs that could be injected per immunisation was limited by the injection volume allowed by the Home Office, where it was 1.2 $\mu$g. The same free OVA dose per immunisation was administered via s.c. injection in all experiments.

First, the effect of P(OVA) therapeutic immunisations on survival enhancement was investigated. To understand the potential self-adjuvant effect of PMPC-PDPA, B16-OVA tumour size progression was followed up over time in mice groups immunised with either free OVA and Poly(I:C); P(OVA) and Poly(I:C); or only P(OVA). Mice injected with empty POs, free OVA and Poly(I:C) were considered as the control groups. 17 days after B16-OVA melanoma inoculation, every animal from all groups had succumbed (Figure 59C), following human endpoint after tumour size reached the maximum established by the Home Office (15 mm in diameter). However, no differences in tumour sizes (Appendix Figure 85A for individual tumour growth curves) or survival rates were found across groups (Figure 59B, C and D).

During tumour growth, the endogenous anti-tumour immune response unfolds following the “immune-editing model”, composed by the phases of elimination, equilibrium, and escape [362].
Figure 59. Anti-tumour effect of P(OVA) therapeutic immunisations in mice harboring B16-OVA tumours, following one vaccine dose.

A) Schematic representation of experiment schedule. C57Bl/6 mice were s.c injected with 1x10^6 B16-OVA cells in the right flank (Day 0). At day 7, one P(OVA) dose containing 1.2 μg of OVA was s.c. injected in the left flank, in the presence or absence of 100 μg of the adjuvant Poly (I:C). Control groups were immunised with an equivalent dose of free OVA and Poly (I:C) or with empty POs. B) Tumour growth curves in time. C) Survival diagram indicating the percentage of survival over time. D) Table showing median survival (in days) for each treatment group. Error bars in B) represent mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel-Cox) test. (5 animals per group, n=1 independent experiment). No statistical differences were found across groups.

In the first phase, the immune system recognises and kills immunogenic tumour cells, reducing tumour burden. During equilibrium, tumour cells that possess mutations which are not recognised by the immune system might survive and proliferate preferentially, leading to tumour resistance. Finally, in the escape phase, the immune system is unable to have control over the selected tumour mutated cells. In addition, the immunosuppressive TME turn-off anti-tumour responses. For this reason, therapeutic interventions with cancer vaccines are more likely to be efficient at the early stages of the tumour immune-editing process, where the tumour sizes are smaller than in the late-stages of the disease. Thus, to mimic an early stage of melanoma, injected B16-OVA cells were reduced to 0.5 x 10^5, and the vaccine administration was performed at day 2.
post tumour inoculation (Figure 60A). In addition to the empty POS, free OVA and Poly(I:C) control groups, a PBS-group was also included.

A.

![Diagram](image)

B.

![Graph](image)

C.

![Graph](image)

D.

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<tr>
<th>Group</th>
<th>Median survival (days)</th>
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<tr>
<td>PBS</td>
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<tr>
<td>Empty POs</td>
<td>14.5</td>
</tr>
<tr>
<td>OVA Poly (I:C)</td>
<td>15</td>
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<td>P(OVA)</td>
<td>12</td>
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<td>P(OVA) Poly (I:C)</td>
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Figure 60. Anti-tumour effect of P(OVA) therapeutic immunisations in mice harboring B16-OVA tumours, following one vaccine dose.

A) Schematic representation of experiment schedule: 0.5x10^6 B16-OVA cells were injected s.c. in the right flank of C57Bl/6 mice. 2 days after tumour inoculation, P(OVA) encapsulating 1.2 μg of OVA was injected s.c. in the left flank, in the presence or absence of 100 μg of the adjuvant Poly (I:C). Control groups were immunised with an equivalent dose of free OVA and Poly (I:C), with empty POs or with PBS. B) Tumour growth curves ± SEM. C) Survival diagram indicating the percentage of survival. Statistical analysis was performed using two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test. 5 animals per group, n=1 independent experiment) (**)p<0.01, *p<0.05).

Following this schedule (Figure 60A), tumour growth was delayed in animals immunised with P(OVA) and Poly (I:C) with respect the rest of the groups (Figure 60B and Appendix Figure 85B for individual tumour growth curves), which also correlated with enhanced survival (Figure 60C), of 17 days (Figure 60D). The differences in survival in this group were statistically significant when compared to animals treated with free OVA Poly(I:C) (mean survival = 15 days) and with P(OVA) without adjuvant (mean survival = 12 days), as reported in Figure 60D. Interestingly, the latter group underwent faster tumour growth.
(Figure 60) meaning that its mean survival time was lower than PBS or empty POs control groups (mean survival=14 and 14.5 days, respectively) (Figure 60C and Figure 60D).

Thus, the data suggested that P(OVA) formulations in combination with Poly(I:C) provided with a moderate anti-cancer therapeutic effect, which outperformed naked OVA and Poly(I:C) treatments. In addition, no therapeutic benefit was provided by groups immunised with P(OVA) in the absence of adjuvant, which indicated a lack of adjuvant-like potential of PMPC-PDPA per se. For this reason, P(OVA) or OVA immunisations were not further explored in the absence of Poly(I:C) in this thesis.

5.2.1.2. P(OVA) immunisations in a prime-boost regime.

Once the B16-OVA model and immunisation schedule had been defined, the possibility of further enhancing survival outcomes was addressed. It is widely understood that immunisations performed in a prime-boost regime result in the development of enhanced antigen-specific adaptive responses due to the activation of T cell and B cell memory pools [363]. The administration of several vaccine doses constitutes a frequently used strategy for the administration of experimental tumour vaccines [364], [365], including GVAX [366] or NeoAg based vaccines [222]. Therefore, a survival and tumour growth experiment were performed including a group of animals immunised in prime-boost schedules with naked OVA or P(OVA). Prime was injected on day 2, and boost was administered on day 5 following B16-OVA inoculation (Figure 61A). The results indicated a statistically significant reduction in B16-OVA growth rate in animals immunised in prime-boost schedules with P(OVA) vaccines over groups that only received a prime vaccine dose (Figure 61B). The trend was the same in mice immunised with free OVA (Appendix Figure 86A for individual tumour growth curves). Similarly, survival was prolonged in animals immunised with 2 doses of free OVA or P(OVA) (Figure 61C and Figure 61D). P(OVA) immunised animals in a prime-boost regimen showed the longest survived rates across all groups, with a median survival of 22 days, 4 more days than animals immunised with free OVA in the same schedule (Figure 61D).

In conclusion, P(OVA) and Poly(I:C) immunisations on a prime-boost schedule provided with prolonged survival and delayed tumour growth over prime-only regimes. Although
the same trend was observed in free OVA and Poly(I:C) immunised animals, the therapeutic effect was worse than in P(OVA) and Poly(I:C) immunised groups.

**Figure 61. Anti-tumour effect of P(OVA) therapeutic immunisations in mice harboring B16-OVA tumours, following prime and boost.**

A) Schematic representation of the experiment schedule: 0.5x10⁶ B16-OVA cells were injected s.c. in the right flank of C57Bl/6 mice and 2 and 5 days later, P(OVA) encapsulating 1.2 μg of OVA were injected s.c. in the left flank, in combination with 100 μg Poly (I:C). Control groups were immunised with an equivalent dose of free OVA and Poly (I:C), empty POs or PBS. B) Tumour growth curves over time. C) Survival diagram indicating the percentage of survival over time. D) Table showing the median survival in days for each treatment group. Error bars in B) represent mean ± SEM. Pooled data from n=4 independent experiments (5 - 8 animals per group and per experiment). Statistical analysis was performed using two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel-Cox) test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).

### 5.2.2. Anti-OVA IgG titres in blood.

Even though the role of IgGs has yet to be fully decrypted in the context of solid tumours [367], interest in this topic has increased in recent years. Several studies correlate the presence of tumour-specific IgG titres in blood with improved survival prognosis in patients [368], [369].
Figure 62. Analysis of anti-OVA IgG titers in blood of mice harboring B16-OVA tumours, following P(OVA) immunisations in a prime-boost regime.
A) Schematic representation of the experiment schedule. 0.5x10^6 B16-OVA cells were s.c. inoculated on the right flank of the mice. After 2 and 5 days, one P(OVA) dose encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence or absence of 100 μg of Poly (I:C). Control groups were immunised with an equivalent dose of free OVA and Poly (I:C), empty POs or PBS. IgG levels in blood were measured at day 7 post vaccine boost. B) IgG titers measured by ELISA. Bars in B) represent mean. Pooled data from n=3 independent experiments (5 - 8 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001).

Motivated by this, the levels of OVA-specific IgGs in blood were analysed in mice harbouring B16-OVA following P(OVA) immunisations in a prime-only or prime-boost schedule, as previously explained. Blood withdrawal from the tail of mice was performed at day 7 post-vaccine boost, and OVA-specific IgG titers were quantified by ELISA (Figure 62A). The results in Figure 62B show a 10-fold increase in OVA-specific IgGs concentration in the blood of mice immunised with P(OVA) when compared to titers achieved in groups immunised with naked OVA, PBS or POs empty controls after one dose. P(OVA) immunisations in a prime-boost regime triggered 100 times higher titers of OVA-specific IgGs than those achieved in prime-boost counterpart groups immunised
with free OVA, PBS or empty POS treated groups. Interestingly, no differences in OVA-specific IgG titers were found between groups vaccinated with free OVA, PBS, POS control groups. The detection of OVA-specific IgG titers in the blood of PBS or empty POS treated animals might indicate the presence of an endogenous OVA-specific IgGs response, responding to OVA expressed within B16-OVA melanoma cells.

Thus, immunisations with OVA encapsulated on PMPC-PDPA POSs (P(OVA)) provides with a 100-fold increase on antigen-specific IgG titers over free OVA immunisations. Whether these IgGs are originated in B cell follicles in LNs, or in TLO within tumours, and whether they contribute towards and anti-tumour response should be further investigated.

5.2.3. B16-OVA TME landscape following P(OVA) prime-boost immunisations

To gain insight in the anti-tumour immunological response driving enhanced survival in animals vaccinated with P(OVA) in a prime-boost schedule, the immune landscape infiltrating B16-OVA tumours was analysed.

To this end, mice harbouring B16-OVA were immunised in a prime-boost regime with P(OVA) or free OVA, using Poly(I:C), as previously described. Control groups were injected with PBS or empty POS (Figure 63A). At day 13, when differences in tumour sizes were evident across groups (Figure 63B), all animals were culled and tumour immune infiltrates (CD45+ cells) were analysed by FACS (Figure 63C, i), with an emphasis on TILs.

The main immune population infiltrating tumours in control groups was myeloid-like (CD45+CD11b+). However, P(OVA) and free OVA vaccinated mice shifted this balance to higher proportion of TILs (CD45+CD3+) over myeloid cells (Figure 63C, ii). These differences were statistically significant between P(OVA) immunised mice and the rest of the groups (Figure 63C, ii). The proportion of myeloid cells in tumours following P(OVA) vaccine were statistically significantly lower compared to the free OVA treated group (Figure 63C, iii). Moreover, this correlated with a statistically significant increase in the numbers of infiltrated T cells (Figure 63C, iv) in P(OVA) groups compared to free OVA-treated mice tumours.
Figure 63. Immune cell infiltration analysis in B16-OVA tumours following P(OVA) therapeutic immunisations in mice.

A) Experiments’ schedule diagram: 0.5x10^6 B16-OVA cells were s.c. inoculated on the right flank of C57Bl/6 mice and 2 and 5 days later, mice were treated s.c with one P(OVA) dose, encapsulating 1.2 μg OVA in the left flank, in the presence or absence of 100 μg of Poly (I:C). Control groups were immunised with an equivalent dose of free OVA and Poly (I:C), empty POs or PBS. TME analysis was performed at day 13 post tumour inoculation. B) Tumour growth progression. C) FACs quantification analysis: i. Plot showing myeloid and T cell population gating strategy; ii. T cell/myeloid rations; iii. Myeloid cells (CD45+CD11b+) frequencies among immune infiltrates; iv. TILs frequencies (CD45+CD3+) among immune infiltrates. Error bars indicate ± SD. Pooled data from 3 independent experiments (n= 4-5 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
Thus, immune infiltrates in B16-OVA tumours from P(OVA) and Poly(I:C) treated groups indicated a higher T cell recruitment at day 13 post tumour inoculation, suggesting improved T cell priming over the rest of the treatments. Since B16-OVA tumours from P(OVA) and Poly(I:C) immunised animals were also the smallest across groups, this suggested that anti-tumour immune responses were being favoured following this treatment. To understand the T cell and myeloid subtypes that were driving tumour rejection, these TILs and myeloid populations were further characterised.

5.2.3.1. TILs landscape

To study the differences on T cell immune surveillance between treatments, cytotoxic CD8 T cells (CD3+CD8+), CD4 Th (CD3+CD4+FOXP3-) and Tregs (CD3+CD4+FOXP3+) were characterised in tumours.

Unsupervised TILs clustering analysis distinguished CD8, CD4 (including CD4 Tregs and CD4 Teff) and double negative (DN) TILs subsets (Figure 64A, left). Remarkably, P(OVA) immunisations triggered increased CD8 T cell and decreased CD4 Teff, and Tregs infiltrates compared to the other treatments (Figure 64A). Supervised quantifications of these populations were also performed following FlowJo gating analysis (Figure 64B and Appendix Figure 87A for gating strategy). These indicated that CD8 TILs frequency in B16-OVA tumours from P(OVA) immunised groups comprised, on average, 40% of the total immune infiltrates, and double the amount of CD8 TILs frequencies found in control mice tumours (Figure 64B, ii), including free OVA immunised mice. Accordingly, the percentage of total CD4 TILs was approximately 6% in P(OVA) immunised mice, which was statistically significantly lower than the CD4 TILs levels achieved from any other treatment group (Figure 64B, iii). No differences were found in levels of DN infiltrates across groups (Figure 64B, iv).
Figure 64. Tumour infiltrating lymphocytes (TILs) analysis in B16-OVA tumours following P(OVA) therapeutic immunisations in mice.

B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Unsupervised clustering analysis was done using Phenograph package followed by UMAP. B) TILs FACs quantification: i. FACs plot showing CD4 and CD8 T cell gates; ii. CD8 TILs frequencies from total immune infiltrates iii. CD4 TILs percentages from total immune infiltrates; iv. DN TILs percentages from total immune infiltrates. Results show mean ± SD. Pooled data from 3 independent experiments (n = 4-5 animals per group and per experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
As explained in chapter 1, CD4 T responses can be effector (T helper), helping towards the development of anti-tumour CD8 T cells or B cells [134], or regulatory (Tregs), which mediate tumour progression by inducing tumour tolerance [370]. Quantification of CD4 T cell subsets (**Figure 65A**) revealed that CD4 Th cells accounted for 4% of the total immune infiltrates in mice immunised with P(OVA), and half amount in free OVA immunised groups (**Figure 65B**). Control groups indicated the same CD4 Th proportions as in P(OVA) immunised groups (**Figure 65B**). On the contrary, tumours from P(OVA) immunised mice had the lowest Treg frequencies of all groups, which comprised 2% of the total immune infiltrates, as opposed to the 6% registered in OVA immunised groups (**Figure 65C**). Comprehensibly, high CD8 TILs to Treg ratios within the TME indicate efficient anti-tumour responses. This result might be explained due to the need for cytotoxic CD8 T cells to clear tumours and the detrimental tolerogenic effect of Tregs. After calculating these values in all groups, the highest numbers (around 25) were found in the TME from P(OVA) immunised groups (**Figure 65E**), which was in sharp contrast with the 5 found within B16-OVA immune infiltrates in free OVA, PBS or POs vaccinated mice. Equally, the same trend was found when analysing CD8 to CD4 Teff ratios, with values of around 15 in P(OVA) immunised groups and lower than 5 in the rest of the groups (**Figure 65D**).

Given that OVA was the vaccine’s immunogen of choice, the presence and expansion of OVA-reactive CD8 T cell clones infiltrating B16-OVA tumours following P(OVA) immunisations was also analysed. These CD8 TIL subsets play a key role in recognising and potentially killing B16-OVA melanoma tumour cells. Although many peptide sequences from OVA can be recognised by different CD8 T cell TCR clones, the most immunogenic peptide is SIINFEKL [371]. Hence, a dextramer (Dext-OVA) that recognises CD8 T cell clones whose TCRs are specific for this peptide (from now on, SIINFEKL-reactive CD8 T cells) was used for the analysis. **Figure 66A** indicates that the frequencies of SIINFEKL-reactive CD8 T cell clones (CD3+CD8+Dext-OVA+) found in the TME of P(OVA) immunised mice were not higher than the rest of the groups, less than 1% of total CD8 TILs (**Figure 66A**, i). However, slightly higher frequencies were observed in OVA immunised mice (between 1 and 2%). Nonetheless, values of SIINFEKL-reactive CD8 T
211 cells per mm³ of tumour volume revealed a trend in higher density of SIINFEKL-reactive CD8 TILs in P(OVA) immunised mice compared to the other groups (Figure 66A, ii).

Figure 65. CD4 Tumour infiltrating lymphocytes (TILs) analysis in B16-OVA tumours following P(OVA) immunisations in mice.
B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Plot showing CD4 Tregs and Teff gating B) CD4 Teff TILs percentages from total immune infiltrates C) CD4 Treg percentages from total immune infiltrates D) CD8/CD4 Teff ration E) CD8/CD4 Treg ratio. Results show mean ± SD. Pooled data from 3 independent experiments (n= 4-5 animals per group and experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).

It is known that chronic tumour antigen exposure and TCR activation might render T cells exhausted, and hinder their proliferative and effector functions [177]. Thus, the expression of inhibitory receptors such as PD-1 and TIM-3 in CD8 TILs (total and SIINFEKL-reactive) in our vaccine model was also analysed. PD-1 expression in UMAP cluster diagrams suggested increased levels of this marker in CD8 TILs in B16-OVA
tumours from P(OVA) immunised mice (Figure 66B). Supervised quantification of PD-1+ CD8 TILs (CD3+CD8+PD1+) and TIM-3+ CD8 TILs (CD3+CD8+PD1+), corroborated this trend (Figure 66C), revealing more than 50% of CD8 TILs in P(OVA) immunised mice expressed both PD-1 and TIM-3. These numbers were statistically higher than the exhaustion levels found in CD8 TILs from free OVA immunised or control groups (Figure 66C). Moreover, the same analyses were performed in SIINFKL-reactive clones, finding the same trend. However, no statistical differences across groups were observed (Figure 66D).

The fact that CD8 TILs in mice immunised with P(OVA) particles, including SIINFEKL-reactive CD8 T cells, expressed inhibitory checkpoint molecules, such as PD-1, suggested T cell exhaustion and the need for a combined vaccination with α-PD-1 checkpoint therapy, to re-invigorate these cells, as explained in section 5.2.4.
Figure 66. Characterisation of OVA reactivity and exhaustion profile from CD8 Tumour infiltrating lymphocytes (TILs) in B16-OVA tumours
B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Representative FACs plot illustrating SIINFEKL-reactive CD8 TILs gating (left) and diagrams representing SIINFEKL-reactive CD8 T cell numbers in frequency (i) or density (ii). B) PD-1 expression in TILs represented by unsupervised clustering analysis using Phenograph package followed by UMAP generation. C) TIM-3 and PD-1 expression in CD8 TILs represented by gating plot (left), histogram (middle), and frequencies (right). D) TIM-3 and PD-1 expression in SIINFEKL-reactive CD8 TILs represented by gating plot (left), histogram (middle), ± SD. Pooled data from 3 independent experiments (n= 4-5 animals per group and experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (**p<0.01)
5.2.3.2. Myeloid landscape in tumour and comparison with dLN myeloid populations.

Myeloid cells are present within the TME and include polynuclear granulocyte-like cells and mononuclear cells (i.e., monocytes, TAMs [44] and DCs [372]). Whereas the presence of some myeloid subsets, such as cDC1, correlates with enhanced antigen cross-presentation and CD8 T cell priming, the role of other myeloid populations remains controversial. Indeed, it is understood that cDC2s are also required for the activation of CD4 Th responses, which enhance CD8 T cell activation [373]. In the case of TAMs, M1-like populations exhibit a pro-inflammatory profile and facilitate anti-tumour responses, that is counterbalanced by the presence of M2-like TAMs, which are associated with anti-inflammatory responses that contribute to tumour progression [54].

To depict the myeloid cells subsets within B16-OVA tumours upon P(OVA) immunisations, parallel analyses were performed on the same tumour samples used for T cell analysis, at the same timepoint (day 13) (Appendix Figure 87B for gating strategy). DC frequencies (MHC-II+ CD68-Ly6C-) from total immune infiltrates were below 5% across groups (Figure 67B, left) and were less abundant than TAMs (MHC-II+CD68+Ly6C-) (Figure 67C, left) or monocytes (MHC-II+ CD68+Ly6C+) (Figure 67D, left), whose frequencies varied between 5% and 10%. Tumours from P(OVA) immunised mice held lower frequencies of myeloid cells than empty POs control group (Figure 67B, left). Analysis of DCs and monocytes in the tumours of P(OVA) immunised animals also suggested lower frequencies than in free OVA immunised groups (Figure 67B and Figure 67D), however TAM frequencies remained similar between both groups (Figure 67C, left).

Special attention was drawn to PD-L1 expression in myeloid populations, given that the interaction of PD-L1 with the PD-1 receptor expressed in CD8 T cells (Figure 66B) would render the latter cells inactive and unable to kill target cells. The analysis in tumours from P(OVA) immunised mice indicated that these harboured the lowest PD-L1+ DCs frequencies (Figure 67B middle and right), and highest PD-L1+ TAMs or PD-L1+ monocytes frequencies among the rest of the groups (Figure 67C and D, middle and right). Quantifications indicated frequencies around ≈30% PD-L1+ TAMs and monocytes
in P(OVA) immunised group, opposed to ≈10% or 20% in tumours from empty POs or free OVA groups, respectively (Figure 67C and D, right).

A.

Figure 67. PD-L1-expressing myeloid cells infiltrating B16-OVA tumours, following P(OVA) therapeutic immunisations in mice.

B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) FACs plots representing gating strategy for monocytes (MHC-II+Ly6C+CD68+), macrophages (MHC-II+Ly6C-CD68+), and DCs (MHC-II+Ly6C-CD68-), as well as PD-L1 expression. (B-C) Bar diagrams representing population frequencies from total immune infiltrates (left); histogram showing PD-L1 expression (middle); frequencies of PD-L1 expressing cells (left) within B) DCs, C) Macrophages, and D) Monocytes. Bar diagrams show mean± SD. Data from 1 experiment (n= 4-5 animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, **p<0.01).
DC and TAMs subsets were further characterised in detail in the tumours of P(OVA) immunised mice and compared to control groups (Figure 68). The cDC1 (CD103+CD11b-), cDC2(CD103-CD11b+), and cDC3 (CD103-CD11b-) DCs analysis suggested a reduced proportion on cDC1, and increased cDC3 frequencies (Figure 68). Noteworthy, “cDC3s” were considered the potential equivalent to the recently discovered cDC3 population in humans. Although they might comprise precursors of cDC1 and cDC2, their exact function is yet to be elucidated [374].

Figure 68. DC and TAM subpopulations analysis in B16-OVA tumours following P(OVA) immunisations in mice.

B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Gating strategy for cDC1 (CD103+CD11b-), cDC2(CD103-CD11b+), and cDC3 (CD103-CD11b-) cells (left), and frequencies (right). B) Gating strategy for M1 (CD68+CD206-) and M2 (CD68+CD206+) TAMs (left) and frequencies (right). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test. No statistical significance was found.
The most abundant population in all treated groups were cDC2s, followed by cDC3s, and finally cDC1s, which constituted 5-15% of the total DC population (Figure 68A). In the case of TAMs, 70-80% of the population was M2-like (CD206+) phenotype in tumours from all different treatment groups (Figure 68B). M1-like TAMs (CD206-) constituted 20-30% of the total TAM population in all groups (Figure 68B).

Thus, the B16-OVA TME from P(OVA) immunised animals harboured high levels of M2-like TAMs, monocytes and DCs, all expressing PD-L1 (Figure 67), which might have enhanced immunosuppression and allowed tumour progression. Therefore, these results also highlighted the need for combinatorial α-PD-1 therapy with P(OVA) vaccine, as explained in section 5.2.4.

5.2.3.2.1. DC trafficking from tumour to LN

DCs in tumours take up debris from dying cells and migrate to dLNs, where they prime tumour-reactive CD8 T cells [65]. Thus, their role is critical for the elicitation of robust anti-tumour responses. migDCs can be distinguished from DC residing in LN (resDCs, which are derived from blood precursors) since they express high levels of MHC-II, whereas resDCs express intermediate levels of MHC-II (Figure 69A).

To further understand the tumour-dLN priming process across treatment groups, the numbers of resDCs and migDCs in dLN, as well as the expression of PD-L1 were analysed by FACS at day 13 post tumour inoculation (Appendix Figure 87C for gating strategy). ResDC frequencies were highest in dLN from P(OVA) immunised mice, followed by free OVA and empty POs immunised animals (Figure 69B, left). An average of 60% of resDC expressed PD-L1, which was similar across groups (Figure 69B, middle and right). MigDC frequencies were highest in dLN of free OVA immunised mice, followed by P(OVA) and empty POs (Figure 69C, left). However, no statistically significant differences were revealed. Frequencies of PD-L1 expressing migDCs were ≈ 95% in all groups (Figure 69C, middle and right).
Figure 69. Quantification and PD-L1 expression of resident and migratory DC in skin draining lymph nodes (dLN), from B16-OVA tumours following P(OVA) immunisations in mice. B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. dLN analysis was performed on day 13 post tumour inoculation. A) FACs plot showing the gating strategy of migratory (CD11c<sup>hi</sup> MHC-II<sup>hi</sup>) and resident (CD11c<sup>hi</sup> MHC-II<sup>int</sup>) DCs. (B-C) Bar diagrams representing population frequencies from total immune infiltrates (left); histogram showing PD-L1 expression (middle); frequencies of PD-L1 expressing cells (right) in B) Resident DCs, C) Migratory DCs. Bar diagrams show mean ± SD. Data from 1 experiment (n= 4-5 animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test. No statistical significance was found.
Interestingly, the levels of PD-L1 expression in migDCs in dLN were 4 to 5 times higher than PD-L1 expression in tumour DCs (Figure 70) across all treatment groups. This finding follows the same reported by recent works where PD-L1 expression has been found to be essential for the migration of DC from tumours to LN [218].

![Figure 70. PD-L1 expression in tumour DCs and migratory DCs in LN, following B16-OVA inoculation and vaccination.](image)

B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. TME and dLN analysis was performed on day 13 post tumour inoculation. Left: FACs plot showing PD-L1 expression in migratory DCs in LN (pink) or in tumour (brown). Right: Frequency of PD-L1 expressing DC in each tissue. The black dashed line in violin diagrams shows the median. Data from 1 experiment (n= 4-5 animals per group). Statistical analysis was performed using two-way ANOVA. (****p<0.0001).

Analysis of cDC1/2/3 in resDCs and migDCs revealed that similar to what was observed within B16-OVA TME (Figure 68A), cDC2 were the most abundant DCs in LN across groups (Figure 71B). LN from P(OVA) immunised mice also revealed lower proportions of migratory and resident cDC1 cells than the rest of their treatment counterparts. Also comparable to the trend observed in tumours (Figure 68A), migratory cDC3s in dLN from P(OVA) immunised mice were higher than in dLN from empty POs control group but was lower than what was found in dLN from OVA immunised mice (Figure 71B, right).
Figure 71. Quantification of cDC1, cDC2, and cDC3 subsets in resident and migratory DC in skin draining lymph nodes (dLN), from B16-OVA tumours following P(OVA) immunisations in mice. B16-OVA bearing mice were immunised in a prime-boost schedule as described in the previous experiment. dLN analysis was performed on day 13 post tumour inoculation. A) Left: Gating strategy migratory (CD11c<sup>hi</sup> MHC-II<sup>hi</sup>) and resident (CD11c<sup>hi</sup> MHC-II<sup>int</sup>) DCs; Right: Gating strategy for cDC1 (CD103<sup>+</sup>CD11b<sup>-</sup>), cDC2 (CD103<sup>-</sup>CD11b<sup>+</sup>) and cDC3 (CD103<sup>-</sup>CD11b<sup>-</sup>) cells (left). B) Cell frequencies of resident (left) and migratory (right) DC subpopulations in LNs. Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test.

5.2.4. **Effect of α-PD-1 therapy in combination with P(OVA) prime-boost immunisations in survival and tumour growth progression.**

CD8 TILs infiltrating B16-OVA tumours expressed PD-1 exhaustion markers, especially in animals vaccinated with P(OVA) (Figure 66B). Besides, it was possible to detect PD-L1 expression in myeloid cells within B16-OVA TME (Figure 67). It is well known that PD-1 interaction with PD-L1 renders CD8 TILs inactive [375]. For this reason, the potential of a combinatory treatment approach, including P(OVA) vaccination in combination with
Figure 72. Anti-tumour effect of α-PD-1 administration in combination with P(OVA) immunisations, in mice harbouring B16-OVA tumours.

0.5x10^6 B16-OVA cells were s.c. inoculated in the right flank of C57Bl/6 mice. After 2 and 5 days, P(OVA) encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence of 100 μg of Poly (I:C). On days 5, 8 and 11, 200μg of α-PD-1 was administered via i.p. Control groups were immunised with an equivalent dose of free OVA and Poly (I:C) or empty POs or PBS and the same of α-PD-1 schedule. A) Schematic representation of the experiment schedule. B) Tumour growth curves over time. C) Survival diagram indicating the percentage of survival over time. D) Table showing the median survival in days for each treatment group. Error bars in B) represent mean ± SEM. Pooled data from 2 experiments (n= 4 – 8 animals per group and experiment). Statistical analysis was performed using two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).

α-PD-1 therapy, was assessed as an anti-tumour treatment in mice harbouring B16-OVA melanoma.

Vaccination with P(OVA) in a prime-boost schedule combined with 3 α-PD-1 injections (Figure 72A) resulted in tumour growth delay (Figure 72B). Moreover, an extended survival from 22 to 26.5 days in the absence or presence of α-PD-1, respectively, was observed (Figure 72C and Table 5.14 Figure 72D). Free OVA or empty POs immunisations combined with α-PD-1 also resulted in enhanced medial survival (23.5 and 17.5 days,
respectively) compared to animals treated with free OVA or empty POs (medial survival of 18 and 14.4 days, respectively).

These results indicated that combinatory α-PD-1 therapy was delaying tumour growth and elongating the survival of all vaccinated treated groups. Particularly, combinatory treatment in P(OVA) and Poly(I:C) immunised animals provided with most positive outcomes. Given that this could be indicating a re-invigoration of exhausted anti-tumour T cell, this was further explored.

5.2.5. Characterisation of anti-B16-OVA T cell responses in tumour and dLN, following prime-boost immunisation in combination with α-PD-1 therapy.

Since α-PD-1 in combination with empty POs, free OVA or P(OVA) vaccine resulted in improved survival and relented B16-OVA progression, the TME of mice from all groups was further characterised, with a special emphasis on TILs.

5.2.5.1. TILs landscape

Following B16-OVA inoculation and combinatory treatments (Figure 73A), animals were sacrificed, and tumours were harvested at day 13 post tumour inoculation. At this time, B16-OVA tumours from P(OVA) + α-PD-1 group were the smallest and only started to be palpable, and a 25% (3/12) of the animals in this group did not show evident tumour growth at this timepoint, hence couldn’t be used for immune infiltration analysis (data not shown). Meanwhile, those control animals treated with empty POs were the biggest ≈300 mm³ in average volume (Figure 73B). FACS analysis of T cell and myeloid subsets revealed that the use of α-PD-1 resulted in higher T cell to myeloid ratios (Figure 73C, ii) in all groups. Nonetheless, these differences were higher in animals that had received P(OVA) immunisations, in which around 30% of the cells were myeloid-like (Figure 73C, iii), and 60% of the infiltrates were T cells (Figure 73C, iv). Free OVA immunisations in combination with α-PD-1 did not result in enhanced TILs recruitment over OVA-only
Figure 73. Immune infiltrate analysis in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations.

0.5x10^6 B16-OVA cells were s.c. inoculated in the right flank of C57Bl/6 mice and 2 and 5 days later, P(OVA) encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence of 100 μg of Poly (I:C). On days 5, 8 and 11, 200 μg of α-PD-1 was administered i.p. Control groups were immunised with an equivalent dose of free OVA and Poly (I:C) or with empty POs or PBS and the same of α-PD-1 schedule. TME analysis was performed on day 13 post tumour inoculation A) Experiment’s schedule schematic representation B) Tumour growth curves until the day of endpoint C) FACS quantification analysis: i. Plot showing myeloid and T cell population gating; ii. T cell/myeloid rations iii. Myeloid cell percentages from total immune infiltrates; iv. TILs percentages from total immune infiltrates. Error bars show mean± SD. Data represents 2 independent experiments (n= 4-6 animals per group and per experiment). Statistical analysis showing P(OVA) + α-PD-1 group comparations to the rest of groups, performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
treated animals (Figure 73B). Administration of α-PD-1 in empty POs treated group increased TILs frequencies in comparison with empty POs only treated mice, from 20% to ≈ 35%. The latter number coincided with TILs frequencies found in tumours from animals immunised with naked OVA (Figure 73C, iv). The increased T cell/myeloid rations observed in P(OVA) and α-PD-1 treated animals correlated with smallest tumour sizes, suggesting a role for α-PD-1 in the reinvigoration of tumour-reactive CD8 T cells following P(OVA) and Poly(I:C) vaccination.

Empty POs, free OVA, or P(OVA) immunisations combined with α-PD-1 resulted in a 5 to 10% increase in the CD8 TILs population as to groups receiving the same treatments in the absence of ICI (Figure 74A, ii). The highest CD8 TILs values were found in tumours from P(OVA) and α-PD-1 treated animals, in which 40% of immune infiltrates were CD8 TILs. A similar trend was found in SIINFEKL-reactive CD8 TILs (Figure 74A, iii). Combinatory treatments of free OVA and P(OVA) with α-PD-1 increased the frequencies of these CD8 T cell clones by up to 2% from total CD8 T cells, in the case of free OVA group, and up to 5% in tumours from P(OVA) treated animals (Figure 74A, iii).

CD4 Teff TILs proportions were very similar between animals receiving α-PD-1 therapy and those just receiving the vaccine. B16-OVA tumours from empty POs and α-PD-1 treated animals harbored a 1% CD4 Teff increase compared to POs monotherapy counterparts. Free OVA and P(OVA) immunised mice in combination with ICI dropped the numbers of CD4Teff by 1-2% over counterparts immunised with free OVA and P(OVA) only (Figure 74B, ii). This trend also held true for Treg populations, for which P(OVA) immunised animals reported the lowest percentages, 2% from total CD45+ cells, with no differences observed between α-PD-1 treated or untreated groups (Figure 74C, iii). CD8/CD4 Teff and CD8/CD4 Treg ratios, highest in P(OVA) treated animals in the previous experiments, did not increase further in that group with combinatory α-PD-1 treatment (Figure 74C). CD8/Treg ratio was slightly lower in animals treated with combination of P(OVA) and α-PD-1 than monotherapy with P(OVA) (Figure 74C, ii), indicating that α-PD-1 did not reduce Treg frequencies any further. Nonetheless, CD8/CD4 Teff and CD8/CD4 Treg values in mice immunised with P(OVA) remained
highest across groups, suggesting the generation of effective and improved anti-tumour responses.

**Figure 74.** Quantification of tumour infiltrating lymphocytes (TILs) in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations.

B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) CD8 TILs: i. Plot showing CD4 and CD8 T cell gating, ii. CD8 TILs frequencies from total immune infiltrates, iii. SIINFEKL-reactive CD8 TILs frequencies from total immune infiltrates. B) CD4 TILs: i. Plot showing CD4 Treg and CD4 Teff, ii. CD4 Teff TILs frequencies from total immune infiltrates, iii. CD4 Treg TILs frequencies from total immune infiltrates. Error bars show mean ± SD. Data represents 2 independent experiments (n= 4-6 animals per group and per experiment). Statistical analysis were performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
Thus, combinatory checkpoint blockade and P(OVA) vaccination enhanced TILs proportions, presumably due to reinvigoration of P(OVA) elicted T cell responses, boosted by α-PD-1 therapy. Accordingly, SIINFEKL-reactive CD8 T cells also followed the same trend. In free OVA immunised counterparts, no differences in the total TILs proportions or SIINFEKL-reactive CD8 T cells were found with or without α-PD-1 treatment, indicating a worse synergistic effect of the combined therapy.

5.2.5.1.1. Tumour reactive CD8 TILs: Total vs SIINFEKL-reactive clones.
B16-OVA bearing animals treated with P(OVA) combined with α-PD-1 achieved prolonged survival, correlating with enhanced total and SIINFEKL-reactive CD8 TILs infiltration. Thus, it was necessary to understand the functional phenotype of these cells.

To this end, cytotoxic (GZMB) and proliferation/reinvigoration (Ki67) markers across groups were analysed by clustering analysis. CD8 T cells, CD4 Teff and CD4 Treg clusters were identified (Figure 75A). As indicated in Figure 75B, a larger amount of CD8 TILs expressing GZMB and Ki67 were present in mice immunised with P(OVA) and α-PD-1, than in animals treated with empty POs, and also the rest of the groups (UMAPs of GZMB and Ki67 from all groups in Appendix Figure 88 and Figure 89, respectively). Quantifications of combinatory treatments indicated that 60% of CD8 TILs cells were GZMB*Ki67* in tumours from P(OVA) and α-PD-1 treated animals. These frequencies were 2 to 3 times higher than in PO or OVA treated mice, respectively. In addition, an increased proportion of GZMB*Ki67*CD8 TILs was found in animals treated with α-PD-1 across groups compared to their vaccine-only treated counterparts (Figure 75C). This could likely be due to the re-invigorating effect of α-PD-1 therapy, by disrupting PD-1 and PD-L1 interactions and thus, reverting the exhaustion state of CD8 TILs. Moreover, the use of α-PD-1 in all treatments also resulted in enhanced proportion of GZMB+ single positive cells (Appendix Figure 90) in CD8 TILs.
Figure 75. Ki67 and GZMB expression in CD8 TILs in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations.

B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Unsupervised UMAP analysis defining T cell populations. B) GZMB (top) and Ki67 (bottom) expression within each T cell population. C) Plot showing gating strategy for GZMB and Ki67 (left) Quantification of GZMB +Ki67+ (right). Error bars in C) show mean± SD. Data represents 2 independent experiments (n= 4-6 animals per group and per experiment). Statistical analysis showing P(OVA) + α-PD-1 group comparisons to the rest of groups, performed using one-way ANOVA followed by Tukey post-hoc test (***p<0.001, **p<0.01)
In summary, combinatory immunisations with P(OVA) and α-PD-1 resulted in enhanced survival and triggered the highest TILs frequencies in B16-OVA tumours, when compared to the rest of the treatments. TILs in this group were mainly CD8+ and presented highly proliferative (Ki67+) and cytotoxic (GZMB+) features, which, according with previous findings in bibliography [250], [376] suggest that recognise tumour NeoAg. Thus, ICI combinatory treatments with P(OVA) gave rise to a more abundant and maybe diverse anti-tumour CD8 T cell response, likely constituted by a pool of CD8 T cell clones whose TCRs recognised a wide range of B16F10 melanoma derived NeoAgs (including OVA), in a highly specific way for each CD8 TIL clone. Nonetheless, it was critical to understand the phenotype of SIINFEKL-reactive CD8 T cells that arose upon P(OVA) immunisations, especially in combination with α-PD-1, since this would provide information about how efficient P(OVA) immunisations are in priming SIINFEKL-reactive CD8 T cells, at the functional level of inducing proliferation and cytotoxicity, and how this phenotype compares to the rest of the treatment groups. Thus, SIINFEKL-reactive CD8 clones were also analysed for proliferation and cytotoxicity.

Plots comparing Ki67 and GZMB expression between global (grey) and SIINFEKL-reactive (maroon) CD8 TILs landscape, across all treatment groups (Figure 76A) and further quantifications (Figure 76B), suggested that the frequencies of proliferative and cytotoxic cells within SIINFEKL-reactive CD8 T cells were higher than in the global CD8 landscape. Moreover, higher frequencies of effector SIINFEKL-reactive CD8 TILs were present in animals immunised with OVA, either naked or P(OVA), over empty POs treated groups, which highlights the importance of priming and boosting endogenous tumour-reactive CD8 responses to further enhance proliferation and cytotoxic features that may lead to tumour killing. Additionally, SIINFEKL-reactive CD8 TILs displayed higher frequencies of Ki67+GZMB+ in animals immunised in combination with α-PD-1, over their vaccine-only counterparts, with highest percentages in P(OVA) + α-PD-1 group.
Figure 76. Ki67 and GZMB expression in global and SIINFEKL-reactive CD8 TILs landscape.
B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Representative overlapping FACs plots showing Ki67 and GZMB expression in total (grey) and in SIINFEKL-reactive (maroon) CD8 TILs. B) Quantification of GZMB +KI67+ population in total (grey) and in SIINFEKL-reactive (maroon) CD8 TILs. Error bars in B) show mean± SD. Data represents 1 or 2 independent experiments, for groups treated without or with α-PD-1, respectively (n= 4-6 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
5.2.5.1.1.1. Exhaustion and activation phenotype of tumour-reactive CD8 T cells.

Tumour-reactive CD8 T cells are known to differentiate into a dysfunctional exhaustion state [176], owing to chronic antigen exposure, which resembles the phenotype of virus specific CD8 T cells in the context of chronic infection [250]. As explained in the previous section (Figure 66), immunisations with P(OVA) or naked OVA resulted in enhanced expression of PD-1 and TIM-3 markers in CD8 TILs. To further characterise the exhaustion phenotype of CD8 T cells elicited by these immunisations, and to explore the contribution of α-PD-1 combinatorial approaches in activation and exhaustion, the expression levels of PD-1, TIM-3 and LAG-3 were investigated. Analyses were performed in proliferative (Ki67+) and cytotoxic populations (GZMB+), given that these would comprehend the functional subset following priming and expansion and exerting cytotoxicity against the tumour (Figure 77A). Interestingly, the frequencies of CD8 TILs expressing exhaustion markers were higher in groups that had been immunised with either naked OVA or P(OVA), over empty POs groups (Figure 77A and Figure 77B).

Figure 77A, i and Figure 77B indicated that frequencies of PD-1+ CD8 TILs, as previously reported, were higher in P(OVA) immunised mice compared to the rest of monotherapy groups, in CD8 TILs. Combinatory α-PD-1 therapy seemed to contribute to the reduction in frequencies of PD-1. Although this might suggest a decrease in exhaustion of CD8 TILs thanks to the use of ICI, lower frequencies of PD-1+ CD8 TILs could have also been due to blockade of the PD-1 receptor by the therapeutic, that could have prevented an efficient staining of the PD-1 receptor by the FACS anti-PD-1 labelled. Figure 77A ii and Figure 77B also indicated that higher frequencies of TIM-3+ CD8 TILs were found in tumours from animals treated in combination ICI therapy, over counterparts’ groups receiving the vaccine as monotherapy. Of note, a correlation could be drawn between higher frequencies of TIM3+ CD8 TILs and longer lifespan rates (Figure 72). Accordingly, the highest percentages of TIM3+ CD8 TILs were found in P(OVA) + α-PD-1 immunised mice, with 70% of TIM3+ CD8 TILs. The co-expression of PD-1 and TIM-3 following α-PD-1 therapy has been identified as a prove of resistance against α-PD-1 in some studies [377], and is a sign of terminal exhaustion of T cells [177]. LAG-3+ CD8 TILs were more abundant in OVA + α-PD-1 immunised mice (= 60% of CD8 T cells), followed by P(OVA) and P(OVA) + α-PD-1 (Figure 77A iii and Figure 77B).
Figure 77. Exhaustion markers in Ki67+ and/or GZMB+ CD8 TILs landscape.
B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation.
A) Representative FACs plots showing Ki67 and GZMB expression in total CD8 TILs and histograms representing expression of i. PD-1; ii.TIM-3; iii. LAG-3. B) Exhaustion markers frequencies from CD8 T cells. Error bars in C) show mean± SD. Data represents 2 independent experiments (n= 4-6 animals per group and per experiment. Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test. No significant differences found between groups within each marker.
Thus, combinatorial vaccine + α-PD-1 therapy enhanced TIM-3 expression, with highest values in mice immunised with P(OVA) and α-PD-1, perhaps indicating terminally exhaustion phenotypes that would explain why tumours in this group eventually escape.

It is understood that several factors may influence the development of an exhaustion phenotype, ranging from chronic tumour antigen exposure to the intensity of CD8 T cell activation derived from the MHC-I-TCR interactions [174]. Hence, proliferative and/or cytotoxic CD8 TILs (GZMB+ and/or Ki67+) were analysed for the expression of three priming and activation markers: CD44, which is upregulated following priming and is a marker for effector and central memory on T cells [378]; CD27 which is induced following MHC-I-TCR signalling, and whose extracellular domain is partially secreted shortly after [379]; and CD25, which is the alpha subunit of the IL-2 receptor complex, that is upregulated following IL-2 secretion upon T cell activation [380]. Among this subpopulation, the frequencies of CD44+ and CD27+ (Figure 78A i and ii, and Figure 78B) cells varied between 60-80% and 40-60%, respectively, and were not statistically significant across groups. Nonetheless, there seemed to be a trend of lower CD27+ frequencies in CD8 TILs from groups treated in combination with α -PD-1, however, there was variability across animals. The percentages of CD25+ CD8 TILs oscillated between 1 and 10%, with lowest frequencies found in tumours from P(OVA) + α -PD-1 immunised mice. In virus models, CD25 expression in antigen-specific CD8 T cells following priming varies across subsets, with those expressing higher levels of CD25 being more effector-like due to IL-2 sensitivity. On the other hand, lower CD25 expression was found in memory-like clones [381].

In conclusion, Ki67/GZMB+ CD8 TILs across groups expressed high proportions of CD44+ and CD27+ populations, indicating priming. No significant differences were found across groups due to variability. The frequencies of CD25+CD8 TILs were higher in P(OVA) + α -PD-1 immunised groups, perhaps suggesting these immunisations develop larger CD8 T cell central memory pools. However, this hypothesis should be further addressed.
Figure 78. Activation and priming markers in Ki67+ and/or GZMB+ CD8 TILs landscape.
B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation.
A) Representative FACs plots showing Ki67 and GZMB expression in total CD8 TILs and histograms representing expression of i. CD44 ii. CD27 iii. CD25. B) Exhaustion markers frequencies from CD8 T cells. Error bars in B) represent mean ± SD. Data represents 1 or 2 independent experiments, for groups treated without or with α-PD-1, respectively (n= 4-6 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test. No significant differences found between groups within each marker.

Furthermore, the exhaustion and activation phenotype of the vaccine and/or tumour-primed SIINFEKL-reactive CD8 TILs was assessed, in cells presenting a functional phenotype (Ki67+ and/or GZMB+). Generally, SIINFEKL-reactive CD8 TIL population presented higher frequencies of exhaustion receptors across groups, when compared
to total functional CD8 TILs (Figure 79A i and ii, and Appendix Figure 91A). This trend was kept in mice treated with P(OVA) + α-PD-1, in which an additional 20% of SIINFEKL-reactive CD8 T cells expressed PD-1 and LAG-3, when compared to global CD8 TIL landscape (Figure 79B). Although no statistically significant differences were found when comparing the expression of each receptor across treatments (Figure 79A ii), TIM-3 expression in SIINFEKL-reactive CD8 TILs seemed to follow a similar trend than in the global CD8 TIL landscape (Figure 79A i), and higher frequencies were found in P(OVA) only and P(OVA) + α-PD-1 immunised mice (Figure 79A i and ii, and Appendix Figure 91A). In addition, a higher frequency of SIINFEKL-reactive CD8 TILs expressed priming and activation markers (CD44, CD27 and CD25) compared to global CD8 TILs landscape (Figure 79 A i and ii). In P(OVA) + α-PD-1immunised mice (Figure 79B), this constituted a 20% increase in average although differences were not statistically significant. Similarly to the results in total functional CD8 TILs, the frequencies of CD44+ SIINFEKL-reactive CD8 TILs did not vary in a statistically significant way among treatments (Appendix Figure 91B).

In all, SIINFEKL-reactive CD8 TILs in tumours from P(OVA) and Poly(I:C) + α-PD-1 treated animals presented higher proportions of exhausted (PD-1+, LAG-3+) and primed (CD44+, CD27+, CD25+) cells than the global CD8 TIL landscape. This likely reflects a strong priming and TCR signalling of SIINFEKL-reactive CD8 TILs following immunisations, suggesting enhanced SIINFEKL cross-presentation in DCs. This primed and exhausted profile matches previously described phenotypes of NeoAg-reactive TILs following α-PD-1 therapy [250].
Figure 79. Exhaustion markers in Ki67+ and/or GZMB+ global vs SIINFEKL-reactive CD8 TILs landscape.

B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Top: Representative FACS plots showing Ki67 and GZMB expression in total CD8 TILs and representative histograms showing expression the exhaustion marker LAG-3. Bottom: Heatmap showing frequencies of exhaustion and priming markers in i) Global CD8 TIL landscape, in grey and ii) SIINFEKL-reactive CD8 TIL landscape, in maroon. B) Bar diagram representing exhaustion and activation frequencies in P(OVA) + α-PD-1 group. Error bars in B) show mean± SD. Data represents 2 independent experiments (n= 4-6 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test.
The expression of inhibitory checkpoint molecules in CD8 T cells is believed to be controlled by the expression of transcription factors such as TOX [179]. Analysis of the expression of TOX in global and SIINFEKL-reactive CD8 TILs, as shown in Figure 80A and B, demonstrated highest frequencies of TOX+ CD8 TILs in P(OVA) + α-PD-1 immunised mice, which values of 50% and 60% in total and SIINFEKL-reactive populations, respectively. Lowest percentages of TOX expressing CD8 TILs were found in control groups (empty POs), which had also reported a reduced expression of exhaustion markers in previous analysis.

To conclude, the increased frequencies of TOX+ CD8 TILs in P(OVA) and α-PD-1 treated animals in global and SIINFEKL-reactive populations also correlated high TIM3+ and PD-1+ proportions, with enhanced proliferation and cytotoxic CD8 TILs. Thus, immunisations with P(OVA) and α-PD-1 treatments seems to enhance priming of CD8 T cell responses, however this ends up driving further T cell exhaustion, perhaps due to continuous MHC-I-SIINFEKL encountering and TCR stimulation. This might explain why tumours growth is notably delayed on the first phases of tumour growth but later escapes, following T cell chronic activation leading to exhaustion.
Figure 80. TOX expression in global vs SIINFEKL-reactive CD8 TILs landscape.
B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. TME analysis was performed on day 13 post tumour inoculation. A) Representative FACs plots showing Ki67 and GZMB expression in total CD8 TILs and representative histograms showing expression of the transcription factor TOX. B) Bar diagram representing frequencies across groups. Error bars in B) show mean ± SD. Data represents 1 or 2 independent experiments, for groups treated without or with α-PD-1, respectively (n= 4-6 animals per group and per experiment). Statistical analysis was performed using one-way ANOVA followed by Tukey post-hoc test.

5.2.5.2. T cell responses in tumour draining LN.
Evidence suggests that priming of tumour-reactive CD8 T cells in tumour dLN is critical for the development of an efficient anti-tumour response [382]. Interestingly, when CD8 T cell egress from LN to tumour is blocked, α-PD-1 therapy turns inefficient, which highlights the importance of de novo priming happening in dLN [383], [384]. For this reason, a more detailed characterisation of anti-tumour CD8 T cells performed within
the tumour dLN, with an emphasis in SIINFEKL-reactive CD8 T cells. Analyses similar to those performed in tumours were done, at day 13 post B16-OVA inoculation.

To start with, CD8 T cell frequencies among total immune cells in dLN (Figure 81A, i) were investigated, finding a gradual yet not statistically significant increase of these cells in dLN of mice immunised with P(OVA), alone or in combination with α-PD-1 (Appendix Figure 93 for frequencies of the rest of T cells). Interestingly, dextramer quantification of SIINFEKL-reactive CD8 T cell clone revealed that immunisations combined with α-PD-1 enhanced OVA-specific CD8 T cells across groups. These values oscillated between 0.5% (OVA + α-PD-1 group) to 1% (POs + α-PD-1 group). This could suggest that α-PD-1 re-invigorated the priming of Neo-Ag specific CD8 T cell responses in the LNs, similar to what was found in the tumours from P(OVA) and α-PD-1 group.

Proliferation and GZMB production in CD8 T cells were further assessed in both total and SIINFEKL-reactive populations (Figure 81B and Figure 81C). Results revealed that up to 20% of the total CD8 T cell populations were undergoing proliferation, regardless of the vaccine treatment (Figure 81C, i). Remarkably, percentages of proliferative Ki67+ cells within SIINFEKL-reactive clones were approximately three times higher, ranging from 75 to 90%. In addition, a very low proportion of CD8 T cells were found to also produce GZMB (Figure 81C, ii), although these frequencies were higher within in SIINFEKL-reactive CD8 T cell clones, especially in dLNs from mice immunised with P(OVA), alone or in combination with α-PD-1, which displayed a 5% of Ki67+GZMB+ cells. The high proliferation rates suggested that priming of SIINFEKL-reactive CD8 T cell clones was ongoing on the dLNs. This priming could be perhaps being triggered by OVA cross-presentation in DCs, derived from the uptake of dying B16-OVA tumour cells.
Figure 81. Ki67 and GZMB expression in global and SIINFEKL-reactive CD8 T cell landscape in dLNs.

B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. dLN analysis was performed on day 13 post tumour inoculation. A) Percentages of CD8 T cells and SIINFEKL-reactive CD8 T cells B) Plots representing GZMB and Ki67 expression in total (grey) and in SIINFEKL-reactive (maroon) CD8 T cell populations. C) Quantification of Ki67+GZMB- (i) and Ki67+GZMB+ (ii) frequencies in total (grey) and in SIINFEKL-reactive (maroon) CD8 T cell populations. Error bars in C) represent mean ± SD. Data represents 1 or 2 independent experiments, for groups treated without or with α-PD-1, respectively (n= 4-6 animals per group and per experiment). Statistical analyses were performed using one-way ANOVA followed by Tukey post-hoc test (**p<0.0001).
Moreover, comparisons in the proliferative and cytotoxic profile of CD8 T cells infiltrating B16-OVA tumours or in dLN of animals treated P(OVA) + α-PD-1 (Figure 82A) were performed. Frequencies of GZMB+Ki67+ CD8 T cells were significantly higher in the tumour, likely owing to tumour recognition and killing (Figure 82B), in both total and SIINFEKL-reactive CD8 T cells. On the contrary, higher levels of Ki67+GZMB- CD8 T cells were found in dLN than in tumours, that could be proliferating following priming (Figure 82C).

The T cell exhaustion and activation phenotype in dLN at the time of priming was also characterised across treatments. The frequencies of TOX+ CD8 T cells were higher within SIINFEKL-reactive CD8 T cells than in global CD8 T cell populations (Figure 83A). Besides, differences between populations were higher within groups that had received combinatorial α-PD-1. Proportions of TOX+ cells in P(OVA) + α-PD-1 treated groups were lower than in the OVA or empty POs counterpart treated groups. In addition, comparisons between dLN and tumour TOX+ CD8 T cells in P(OVA) + α-PD-1 treated animals (Figure 83B), revealed that the exhaustion-related transcription factor is expressed in a much lower proportion of CD8 T cells in LNs than in tumours, in both global and SIINFEKL-reactive CD8 T cell populations.

The reasons why different proportions of TOX+ CD8 T cells are found in LNs across treatments are yet not understood. However, it is interesting to understand that in our vaccine model TOX expression in LNs is much lower than tumours, suggesting CD8 T cells in LN present a much less exhausted phenotype. This is line with literature indicating that LNs act as a reservoir of stem cell T cell precursors, with potential to develop efficient anti-tumour responses [385].

Since the frequencies of proliferative (Ki67+) CD8 T cells in LN were larger in SIINFEKL-reactive clones than in the global landscape (Figure 83), this might imply that this clone is primed in a more efficient manner over other tumour-reactive CD8 T cell populations. Furthermore, given the role of P(OVA) immunisations to prime OVA- and SIINFEKL-reactive CD8 T cells, it was important to further characterise the activation (CD44, CD27 and CD25) and exhaustion-phenotype (PD-1, TIM-3, LAG-3) at the levels of receptors.
Figure 82. Comparison of Ki67 and GZMB expression in global versus SIINFEKL-reactive CD8 T cell landscape, and in B16-OVA tumours vs draining lymph nodes (dLNs) from mice treated with P(OVA) + \( \alpha \)-PD-1.

B16-OVA bearing mice were immunised in a prime-boost schedule + \( \alpha \)-PD-1 as described in the previous experiment. Tumour and dLN analysis was performed on day 13 post tumour inoculation. A) Plots representing GZMB and Ki67 expression in total (grey) and in SIINFEKL-reactive (maroon) CD8 T cell populations in tumour (left) and dLN (right). Quantification of B) Ki67+GZMB+ and C) Ki67+GZMB+ frequencies in total and in SIINFEKL-reactive CD8 T cell populations in tumours (dark blue) or LN (light blue). Bar in B) and C) represent mean. Data represents 2 independent experiments (n= 4-6 animals per experiment). Statistical analyses were performed using one-way ANOVA followed by Tukey post-hoc test (****p<0.0001).
Figure 83. TOX expression in global vs SIINFEKL-reactive CD8 T cells in lymph nodes.
B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. Tumour and dLN analysis were performed on day 13 post tumour inoculation. A) Representative FACs plots showing Ki67 and GZMB expression in global (grey) and SIINFEKL-reactive (maroon) CD8 T cells and bar diagram representing frequencies across groups. B) Representative histograms and bar diagrams showing expression of the transcription factor TOX in tumours (dark blue) and dLN (light blue) in animals treated with P(OVA) + α-PD-1. Bars in B) represent the mean. Data represents 2 independent experiments (n= 4-6 animals per experiment). Statistical analyses were performed using one-way ANOVA followed by Tukey post-hoc test.
Similar to TOX+ CD8 T cell analysis, the dLN results were compared with those obtained in SIINFEKL-reactive CD8 TILs in the tumour (Figure 84 A, B and C). Accordingly, the frequencies of SIINFEKL-reactive CD8 T cells expressing exhaustion receptors in dLN was notably lower than in tumour (Appendix Figure 93A, for frequency values across groups). This was in line with lower TOX expression in SIINFEKL-reactive CD8 T cells in LN and supported a less differentiated and less exhausted profile of these clones in dLN compared to tumours. Combinatory immunisations with α-PD-1 resulted in increased PD-1+ and TIM-3+ SIINFEKL-reactive CD8 T cells in LNs.

Further, all groups treated in combination with α-PD-1 expressed higher frequencies of CD44+ SIINFEKL-reactive CD8 T cells than monotherapy counterparts in LNs. The frequencies were however similar to what was found in tumour, indicating that the vaccine-induced CD8 T cell clone present in both tissues was primed. CD25+ and CD27+ SIINFEKL-reactive CD8 T cells in dLN were lower than in tumours, across groups (Appendix Figure 93B, for frequency values across groups). The reason for this remains unclear. Figure 84C shows the differences frequencies quantifications of these markers between tumour and dLN from P(OVA) + α-PD-1 treated mice, revealing similar trends.
Figure 84. Exhaustion markers expression found in Ki67+ and/or GZMB+ SIINFEKL-reactive CD8 T cells landscape in B16-OVA tumours and dLNs.

B16-OVA bearing mice were immunised in a prime-boost schedule + α-PD-1 as described in the previous experiment. Tumour and dLN analysis were performed on day 13 post tumour inoculation. A) Top: Representative FACs plots showing Ki67 and GZMB expression in SIINFEKL-reactive CD8 T cells and representative plot and histograms showing expression the exhaustion marker TIM-3. B) Heatmap representing frequencies of exhaustion and priming markers in i) tumour and ii) LN, in SIINFEKL-reactive CD8 T cells. C) Quantification of exhaustion and activation frequencies in P(OVA) + α -PD-1 group, in tumours (dark blue) or lymph nodes (light blue). Data represents 2 independent experiments (n= 4-6 animals per experiment). Statistical analyses were performed using one-way ANOVA followed by Tukey post-hoc test.
5.3. Discussion and conclusions

Despite the potential of therapeutic cancer vaccines to prime tumour-reactive cytotoxic T cell responses, they fail to achieve tumour remission when administered as monotherapy [225]. In this chapter, we show the potential of PMPC-PDPA POs encapsulating OVA to act as nano-vaccines in the context of B16-OVA. The vaccine therapeutic effect was improved compared to conventional free-protein immunisation approaches. Since many other aspects remain to be elucidated on the vaccine mechanisms driving efficient anti-tumour responses, we also studied other aspects. Accordingly, we characterised the TME following immunisations to depict differential expression of exhaustion and activation T cell markers across treatments, that could help identify the populations driving improved survivals. In this sense, special attention was put into the phenotype of SIINFEKL-reactive CD8 T cells, as studying this clone could help us understand the efficacy of POs vaccine on T cell priming. To finish with, the mechanism by which P(OVA) vaccine synergized with \( \alpha \)-PD-1 was studied, as well as the role on dLN on this.

5.3.1. On the prime-boost vaccination

5.3.1.1. Survival and tumour progression

s.c. immunisations with P(OVA) and Poly(I:C) provided delayed tumour growth and enhanced survival in B16-OVA harbouring mice. Notably, this effect was enhanced when P(OVA) and Poly (I:C) immunisations were performed with a prime-boost schedule. These achieved a 4-day extended survival (median survival: 22 days) over mice vaccinated with naked OVA and Poly(I:C) at equivalent doses (median survival: 18 days); and 8-day extended survival for PBS (median survival: 14 days) or empty POs (median survival: 14.5 days) treated control groups. Although several polymer-based nano-vaccine studies based on B16-OVA drive tumour remission to a certain degree [290], [291], [289], (summarised in Table 7, introduction), these encapsulate SIINFEKL [289], [290], or a longer peptide from an extended sequence of SIINFEKL (OVA\textsubscript{241–270}) [291], instead of the whole protein. These strategies might result in improved anti-tumour responses since they skew the immune response towards SIINFEKL-reactive CD8 T cells,
which is the most immunogenic OVA-derived peptide [245]. This means that SIINFEKL binds MHC-I with higher affinity than other OVA-derived peptide fragments, thus being more likely to induce stronger CD8 T cell responses specific for this peptide. In addition, the amount of peptide and number of immunisations varies arbitrarily across studies, i.e.: 5 ug/dose, 3 doses (day 8, 15 and 23 post tumour inoculation) [291], 50 ug and 2 doses (day 7 and 14 post tumour inoculation) [289] and 10 ug/dose, 3 doses (day 3, 6 and 9 post tumour inoculation) [289]. Lastly, the number of inoculated B16-OVA cells varies across studies from 50,000 [290] to 1 million [289], [291], as well as the adjuvant type and dose. Thus, comparing the efficiency between different polymer-based is not trivial. However, PMPC-PDPA P(OVA) vaccinations performed in this thesis contain 1.2 ug of OVA per dose, on a prime-boost schedule. This is 4 to 40 times lower in mass than the aforementioned examples, while it should be noted that comparations in moles would further enlarge these differences. Indeed, lack of total remission in my system could be due to a vaccine dosing matter.

5.3.1.2. TME analysis

T cell compartment

Overall, our delayed tumour growth rates indicated that anti-tumour responses were being triggered following P(OVA) and Poly(I:C) vaccination. Accordingly, tumour immune infiltrates were quantified at day 13, when tumour sizes across groups were more evident (i.e. PBS/POs control groups 400 mm³; OVA and Poly(I:C) groups ≈150 mm³; P(OVA) and Poly(I:C) groups ≈ 50 mm³). This provided information about differences between immune landscape in TME that ultimately was resulting in a faster or slower tumour escape, thus allowing to understand the vaccine effect. A 2:1 T cell/myeloid ratio was found in tumour biopsies from P(OVA) and Poly(I:C) treated animals, which was significantly higher than the 1:1 or 1:2 ratios found in OVA Poly(I:C) or PBS/POS treated groups, respectively. Accordingly, TILs in P(OVA) and Poly(I:C) treated animals constituted 50% of all immune cells, while in OVA and negative control counterparts, TILs levels were just ≈35% and ≈20%, respectively. In addition, our group of interest presented 20 times higher CD8/CD4 Teff ratios and CD8/Treg ratios over the rest of the groups, which indicated that TILs were mainly of a cytotoxic nature. Besides, decreased
Treg proportions were found in P(OVA) and Poly(I:C) treated animals. However, this might not necessarily indicate the induction of an immune mechanism actively reducing the cell numbers of this population, and instead, reduced proportions could just be because of the presence of larger CD8 T cells proportions. Strikingly, cytolytic CD8 TILs in P(OVA) and Poly(I:C) vaccinated mice constituted 40% of the total immune infiltrates, which was double of either OVA and Poly(I:C) or PBS/Empty POs treated groups. However, no significant differences were found in the proportions of SIINFEKL-reactive CD8 T cell clones across groups, although there was a trend where highest values were found in OVA or P(OVA) and Poly(I:C) vaccinated groups, where these clones comprised ≈ 1% and 0.75% of total CD8 TILs, respectively. Nonetheless, a higher density of SIINFEKL-reactive CD8 T cells (number of cells/ mm³ of tumour), was found in P(OVA) and Poly(I:C) groups. Thus, immunisations with P(OVA) and Poly(I:C) might have enhanced the priming and proliferation of CD8 T cells over immunisations with the naked OVA, perhaps due to combination of enhanced DC targeting through SRs and PDPA-mediated OVA intercellular delivery. In comparison to other polymer nano vaccines targeting DCs, a Mannose-PLGA system [288] encapsulating melanoma peptide NeoAgs, only achieved 20% of CD8 TILs, and 2:1 CD8/Treg ratios, despite higher immunisation doses (3 injections, 100 ug/dose). Next, a p(DMAEMA-OGEA)-b-p(MAVE) nano-vaccine encapsulating OVA241–270 peptide, which proved to promote antigen endosomal escape, gave rise to 40% of CD8 TILs in B16-OVA tumours, which is 10% less than what was achieved with PMPC-PDPA. This was achieved after 3 doses (5ug/dose) so in total 15ug of antigen, which is 6 times more than our system, however in this example 1M B16-OVA was injected, instead of 0.5M used in our studies [291].

The fact that the sharp increase in global CD8 TILs populations did not directly correlate with higher proportions of SIINFEKL-reactive CD8 T cell clones in tumours from P(OVA) and Poly(I:C) treated mice, might indicate that OVA-derived peptides (other than SIINFEKL) were being favourably complexed in MHC-I molecules following SR-mediated P(OVA) uptake and antigen degradation within the late endosome or cytosolic proteasome [75]. It could also be hypothesised that P(OVA) and Poly(I:C) vaccination might induce antigen spreading [223]. In this process, the vaccine-induced CD8 T cell clones kill tumour cells, which are subsequently taken up by DCs, where a broader
repertoire of tumour antigens (derived from the dying of cancer cells) are presented in MHC-I complexes, thus increasing the spectra of CD8 T cell clones being activated, which may hold anti-tumour cytotoxicity. However, to get a full picture of this hypothesis, further dextramer staining specific for other T cell clones should be performed. In addition, ≈50% of CD8 TILs expressed both PD-1 and TIM-3 exhaustion markers in mice immunised with P(OVA) and Poly(I:C), which was almost thrice as much as the rest of the groups (≈20%). Although not significant, a similar trend was found in SIINFEKL-reactive CD8 T cells. PD-1 and TIM-3 expression follows T cell activation [187], [191], their expression on TILs is also a sign of exhaustion, following TCR overstimulation.

Myeloid compartment
Physiologically, PD-1 interaction with PD-L1 expressed on tumour cells or APCs [152], decreases T cell activation signalling. Myeloid compartment analysis on tumour biopsies in our study revealed PD-L1 expression in DCs, TAMs and monocytes across treatment groups.

To start with, empty POs treated animals presented enhanced TAMs frequencies (≈7% of total than P(OVA)/OVA and Poly(I:C) treated groups). All tumour biopsies across groups were enriched in pro-tumourigenic M2-like TAMs (80%) over anti-tumour M1-like TAMs (20%). This could explain why tumours in POs treated animals progressed faster than the other groups. However, the proportion of PD-L1+ TAMs was significantly higher in P(OVA) and Poly(I:C) treated animals (≈60%) over OVA and Poly(I:C) (≈15%), or empty POs (≈10%) conditions. In addition, these trends were virtually identical in monocyte populations, which act as TAM precursors in tumours [44]. PD-L1+ TAMs have been associated with tumour progression and CD8 T cell dysfunction in other tumour models, such as hepatocellular carcinoma [386], and is associated with poor prognosis in patients with malignancies such as glioblastoma [387]. However, other studies have suggested that PD-L1 expression on TAMs merely protects T cells from being engulfed and destroyed [388]. Thus, enhanced PD-L1 expression in M2-like TAMs could either constitute one of the immune-escape mechanisms that B16-OVA tumours followed after P(OVA) and PD-1 treatment; or could otherwise be protecting anti-tumour CD8 T cells.
Whereas DCs in tumours from P(OVA) and Poly(I:C) immunised groups accounted for less than 2% of all CD45+ cells, POs or OVA immunised animals harboured around 4%. In addition, while cDC2 was the most abundant subset across treatment groups (≈60% from total DCs), cDC1 was the lowest (below 20% from total DCs), which is according to what is described in literature [65]. Noteworthy, “cDC3” population comprised CD11b-CD103- cDCs (MHCII+CD11c+). Although this population does not match, to our knowledge, any described murine cDC population in literature, we hypothesise that these cells might resemble the cDC3 populations recently described in humans [60], which are thought to be in fact cDC1 and cDC2 progenitors [374]. In P(OVA) and Poly(I:C) treated animals, this “cDC3” population was higher in proportion than in the rest of the groups, thus maybe implying increased progenitor percentages. Interestingly, whereas the proportion of cDC1 infiltrating tumours from the P(OVA) and Poly(I:C) group was the lowest (below 10%), empty-POs-treated animals showed highest cDC1 proportions (almost 20%). This exact same trend and values were found also in LNs, showing significant differences. These results were somewhat unexpected, given that cDC1 are key in the elicitation of CD8 T cell responses [372], and are associated with improved disease outcomes [65], which were both found in P(OVA) and Poly(I:C) treated animals. Perhaps, higher cDC1 proportions in P(OVA) and Poly(I:C) treated animals could be found on LNs at the time of priming, which would have driven the already expanded CD8 T cell populations (at day 13). In addition, it could be hypothesised that higher cDC1 proportions in empty POs treated animals might be related with highest tumour volumes found in this group, as a compensation mechanism attempting to increase the numbers of CD8 T cells by enhancing migration of cDC1 subset to LNs. However, this conjecture would require further evaluation. Of note, PD-L1 expression was also analysed in tumour and LN DCs. In tumours from P(OVA) and Poly(I:C) treated animals 15% of DCs were PD-L1+, which was lower than the 25 or 20% values observed in POs or OVA groups, respectively. This would mean that in P(OVA) and Poly(I:C) treated animals, DCs were probably contributing to a lesser extent to T cell inactivation than in other treatment groups. Recent studies have indicated that DCs in the TME upregulate PD-L1 following antigen uptake, and that this might interfere with the elicitation of proper CD8 T cell responses in the tumour [217]. Recent discoveries in a preclinical study by Oh. et al [219] imply that selective PD-L1 depletion in DCs, but not
in TAMs, leads to anti-tumour CD8 T cell responses and correlated with enhanced tumour control. Thus, this indicates that not only PD-L1 expression on tumour cells render CD8 T cell inactive, but that DCs play a key role on the PD-1/PD-L1 regulatory axis too.

In dLN, a ≈60% of resDC and ≈95% of migDCs were PD-L1+, with virtually no differences across treatment groups. The fact that the proportion of PD-L1+ migDCs (in LNs) was 6 times-higher than PD-L1+ DCs in tumours goes in line with studies indicating that DCs require PD-L1 expression for migration from tissues (including tumours) to dLNs [218], with PD-L1 ablation hindering their migration. Although this could suggest that PD-L1 expression in DCs could interrupt CD8 T cell priming processes by interacting with PD-1, certain studies indicate that CD80 and PD-L1 on DCs bind to each other (in the same cell, or “in cis”) [389] to prevent binding with CTLA-4 or PD-1 on T cells, and avoid their inactivation [390].

In conclusion, the enhanced proportions of PD-1+ CD8 TILs and PD-L1+ M2 TAMs in P(OVA) and Poly(I:C) treated groups, in combination with the presence of PD-L1+ DCs in tumours and dLN, served as rationale for assessing the effect of vaccination in combination with α-PD-1 therapy.

5.3.2. Vaccine + α-PD-1 combination

5.3.2.1. Survival and TME general landscape

The co-administration of vaccine and α-PD-1 delayed tumour growth and enhanced survival over vaccine-only counterparts, across groups. Noteworthy, 20% of P(OVA) and + α-PD-1 treated animals survived for 38 to 40 days, which corresponded to a 25% lifespan increase over animals treated with P(OVA) only, or OVA + α-PD-1 groups; and almost a 50% extension over POs + α-PD-1 treated animals. In patients, response to ICI therapies relies on the presence of PD-1+ TILs [211], [251]. Thus, the enhanced effect of α-PD-1 therapy in P(OVA) and Poly (I:C) immunised animals was likely due to the higher PD-1+ CD8 TILs frequencies elicited through P(OVA) immunisations. Mechanistically, α-PD-1 antibodies bind the PD-1 receptor on T cells, blocking their interaction with PD-L1 expressed on DCs or tumour cells, and thus re-invigorating T cell effector functions.
Accordingly, animals treated with combinatorial P(OVA) and + \( \alpha \)-PD-1 harboured an extra \( \approx 10\% \) of TILs over the P(OVA) vaccine only counterpart, constituting the group with highest proportions of TILs. Most remarkably, proportions of SIINFEKL-reactive CD8 T cells underwent a 4-fold increase in P(OVA) and \( \alpha \)-PD-1, over P(OVA)-only immunised groups.

It is understood that NeoAg-reactive CD8 T cell clones are more likely to express exhaustion-related ligands (i.e. PD-1 and TIM-3) [249], [250], [391]. Given that mutational burden rate in tumours (including melanoma) [392] normally correlates with higher proportions of NeoAg, these can be used as a predictor tool for the ICI responses [251]. Accordingly, expansion of NeoAg reactive clones is achieved following ICI [222], [250]. Was SIINFEKL to be considered as a NeoAg model in this study, the expansion of SIINFEKL-reactive CD8 T cells following \( \alpha \)-PD-1 in P(OVA) immunised mice could be explained this way. Interestingly, analysis in dLN from the same animals also indicated a trend in increased SIINFEKL-reactive CD8 T cells following combinatorial \( \alpha \)-PD-1 therapy. Evidence suggests that \( \alpha \)-PD-1 therapies might have their key effect in promoting tumour-reactive CD8 T cell responses at the level of dLN, as evidenced by worse ICI outcomes when blocking T cell egression from the LN to the tumour following ICI [214], [384], [393].

Summing up, extended survival and delayed tumour growth in P(OVA) and \( \alpha \)-PD-1 treated animals correlated with higher TILs frequencies, and with enhancement of SIINFEKL-reactive CD8 T cell responses in tumours over the rest of the groups.

5.3.2.2. GZMB/Ki67 functional CD8 T cell phenotype: tumour and dLN

Tumours:
Next, we proved that \( \alpha \)-PD-1 therapy provided with reinvigoration of vaccine-elicited and exhausted CD8 TILs, as proved by enhanced Ki67+ GZMB+ populations in these cells, especially in P(OVA) immunised animals (60% Ki67+GZMB+ CD8TILs). In groups treated with OVA + \( \alpha \)-PD-1, proportions of Ki67+GZMB+ CD8 TILs were also higher than in vaccine-only counterparts (40% vs \( \approx 20\% \), respectively), although still lower than P(OVA)
treated groups. Increased percentages of GZMB+Ki67+ CD8 TILs directly correlated with delayed tumour growth, evidencing the role of GZMB in tumour killing.

SIINFEKL is one of the most immunogenic peptides from OVA, meaning that it binds MHC-I complexes with high affinity over other peptides. In DCs, this translates into a preferred SIINFEKL cross-presentation over other peptides at the time of vaccination with OVA, and thus, with a high percentage of SIINFEKL-reactive CD8 T cells being primed. On the other hand, in B16-OVA cells the high MHC-I-SIINFEKL binding affinity translates into the presentation of abundant MHC-I-SIINFEKL complexes, which can be easily recognised and killed by SIINFEKL-reactive clones. Similarly, MHC-I-NeoAg complexes in physiological tumours are more easily recognised by the immune system, over other tumour peptides that are not mutated. Thus, in our experiments, SIINFEKL-reactive CD8 TILs represent on the one hand, a hallmark of how efficient our vaccination system is, as well as a model of high affinity NeoAg-reactive CD8 TILs. For this reason, we sought to analyse the functional phenotype of these cells, compared to total CD8 T cells (reactive to not only other OVA peptides, but also for B16 melanoma-derived peptides, that might also include melanoma-derived NeoAg). Accordingly, a trend in higher proportions of GZMB+Ki67+ populations were found in SIINFEKL-reactive over global CD8 TILs, across groups. In this regard, a larger proportion of CD44+ cells were found among SIINFEKL-reactive over global CD8 TILs, although no statistical differences were found among treatment groups. Besides, a higher frequency of SIINFEKL-reactive CD8 TILs were proliferative and cytotoxic in OVA or P(OVA) vaccinated animals, over empty POs treated groups. This remarks the importance of vaccination for priming the activation of tumour-specific CD8 T cells to kill tumours. The effect of 𝛿-PD-1 therapy in proliferation/cytotoxicity reinvigoration on SIINFEKL-reactive CD8 TIL clones followed a similar pattern than in the global landscape, finding that vaccine and 𝛿-PD-1 treatments enhanced Ki67/GZMB+ populations in SIINFEKL-reactive CD8 TILs subsets over vaccine-only groups. Among treatments, P(OVA) and 𝛿-PD-1 showed the highest percentages, which were above 60%.

Thus, 𝛿-PD-1 combination with P(OVA) immunisations drove an increased proportion of CD8 TILs with proliferating (Ki67) and cytotoxic (GZMB) capacities, over the rest of the
groups. In particular, the vaccine-induced SIINFEKL-reactive CD8 T cell clone was further enriched in GZMB/Ki67+ populations.

**LNs:**

Given that dLNs are key for the priming of tumour-reactive T cell responses, the Ki67/GZMB profile of CD8 T cells was analysed in this organ. Remarkably, whereas only 20% of total CD8 T cells were Ki67+ (without changes across groups), 90% of SIINFEKL-reactive clones were proliferating (Ki67+), suggesting this population was still being primed by DCs in LNs. Accordingly, the proportion of CD44+ cells were higher within the SIINFEKL-reactive CD8 T cell clone compared to the percentages found among the global CD8 T cell population. \( \alpha \)-PD-1 treated groups further enhanced CD44 expression, perhaps due to more efficient priming due to \( \alpha \)-PD-1 blocking PD-L1 expressed on DCs [214]. Ki67+GZMB+ in global CD8 T cells populations were minimal, and the \( \alpha \)-PD-1 co-administration did not seem to enhance GZMB expression at the level of global CD8 T cell populations. However, Ki67+GZMB+ proportions in the SIINFEKL-reactive CD8 T clones were higher, and in this case, combinatorial \( \alpha \)-PD-1 treatments seemed to enhance GZMB expression and proliferation over vaccine-only counterparts. In fact, P(OVA) + \( \alpha \)-PD-1 group showed the highest GZMB+Ki67+ proportions in SIINFEKL-reactive CD8 T cell across groups, of around 5%.

Recapitulating, GZMB+Ki67+ CD8 T cell global and SIINFEKL-reactive populations are much higher in tumours than in tumour-dLNs. In addition, both tumour and dLN global and SIINFEKL-reactive CD8 T cell populations expressed similar levels of CD44, indicating T cell priming on both compartments. This goes in line with the 2-step T cell activation process recently described by Prokhnevskia et al [385], in which CD8 anti-tumour T cells present in the LNs are stem-like (TCF1+ [394]), with capacity to be further primed and migrate to the tumours, where they infiltrate them as stem-like. In fact, the presence of TCF1+ CD8 T cells in tumours is associated with enhanced \( \alpha \)-PD-1 responses [395][396]. Within the TME, stem-like activated CD8 T cells are further co-stimulated by DCs, and it is here where they acquire a cytotoxic phenotype (i.e. GZMB expression) that might later evolve into exhaustion. In our experiments, P(OVA) and \( \alpha \)-PD-1 treatment induced a slight increase a 5% of SIINFEKL-reactive CD8 T cells in dLNs expressed GZMB, a higher
proportion over the rest of combinatory treatments, and over monotherapy vaccine with P(OVA) only. Whether the combination of P(OVA) vaccine in combination with \( \alpha \)-PD-1 could be prompting the beginning of the differentiation program of stem-like cells in dLN to an effector phenotype in this set up, would require further investigation. RNAseq analysis in CD8 T cell population from dLNs and tumours could give insight into this.

5.3.2.3. Exhaustion phenotype in tumour and tumour-dLN

Tumours

Although P(OVA) and \( \alpha \)-PD-1 treatment proved to boost global and SIINFEKL-reactive CD8 T cell proliferation and GZMB production over the rest of the treatments, tumours eventually escaped immune control and animals succumbed. This could have been due to many factors, including mutation of tumour cells and downregulating MHC-I/NeoAg expression [397], or the presence of immunosuppressive myeloid cells, such as MDSCD [156] or M2-TAMs [53]. However, we sought to analyse T cell exhaustion, given that our main interest was to understand adaptive responses upon POs-based immunisation. In addition, tumour volumes in P(OVA) + \( \alpha \)-PD-1 animals at the time of analysis were not big enough to provide with enough sample material for more than one type of analysis.

In patients, \( \alpha \)-PD-1 treatment reinvigorate exhausted NeoAg-reactive CD8 TILs inducing GZMB expression [250], [253], which is in agreement with our results. In ICI unresponsive patients, the expression of other exhaustion markers (i.e. TIM-3, LAG-3 and PD-1) might drive this resistance and tumour escape [398]. Analysis of exhaustion markers in GZMB+/Ki67CD8 TILs were thus performed in our B16-OVA tumour model too. Results indicated that the proportion of LAG-3+ in P(OVA) and \( \alpha \)-PD-1 treated animals was \( \approx \)50% and seemed to be lower than LAG3+ CD8 TILs proportions in OVA and \( \alpha \)-PD-1 counterparts. However, it was hard to draw comparative conclusions on these proportions with the rest of the groups, due to variability between the 2 experiments performed for this. Next, \( \approx \)70% of GZMB+/Ki67+ CD8 TILs were TIM3+ in animals treated with OVA/P(OVA) and \( \alpha \)-PD-1, which was \( \approx \)20% extra than in empty POs and \( \alpha \)-PD-1 counterparts. In vaccine-only groups, TIM3+ CD8 TILs proportions were lower than in
their $\alpha$-PD-1 counterparts, and in all cases remained above $\approx 40\%$. In this sense, there is evidence indicating that TIM-3 can be upregulated following $\alpha$-PD-1 therapy as a mechanism of resistance [399]. In addition, PD-1+ TIL was also expressed across treatments. Co-expression of TIM-3+ and PD-1+ normally identifies terminally differentiated exhausted CD8 TILs, which are also identified by the expression of TOX [178]. Accordingly, TOX was expressed across treatment groups, with highest $\approx 50\%$ TOX+ GZMB/Ki67+ CD8 TILs found in P(OVA) + $\alpha$-PD-1 treated groups, which was double than the rest of the treatments. In addition, SIINFEKL-reactive CD8 TILs generally seemed to enhance the expression of PD-1 and LAG-3 exhaustion markers over global CD8 TILs, which might be due to enhanced recognition of MHC-I-SIINFEKL complexes on the tumour cells, over other tumour-reactive CD8 TIL clones recognising tumour antigens that bind MHC-I with less affinity. With this, TCR signaling would also be enhanced, further driving exhaustion. In fact, TOX expression in SIINFEKL-reactive clones was found across therapy groups, with highest values found in P(OVA) and $\alpha$-PD-1 treated groups.

In all, this indicated that $\alpha$-PD-1 therapy could not revert the exhaustion phenotype of CD8 TILs within tumours. One explanation could be the presence of CD8 T cells at a differentiation state close to terminal exhaustion at the time of $\alpha$-PD-1 administration (day 5, day 8, and day 11). In this sense, ICI therapies are administered prior to Neo-Ag vaccine administration in patients, as it is the case for a melanoma vaccine trial, where positive outcomes were achieved [266]. On a pre-clinical MC38 colorectal NeoAg model, combination with $\alpha$-PD-1 performed prior and after the vaccine improved survival outcomes over vaccine followed by $\alpha$-PD-1 therapy, which highlights the relevance in choosing a right schedule [384]. It would be interesting to understand what the TME or tumour-dLNs are like at the time of vaccine or first dose of PD-L1 administration (day 2 and 5, respectively) to rationally design the best schedule.

**LNs:**
Since during $\alpha$-PD-1 therapy, the migration of tumour-reactive stem-like CD8 T cells from LN to the tumour is key for efficient anti-tumour responses [385], exhaustion phenotypes in LNs were also analysed. In P(OVA) and $\alpha$-PD-1 treated mice, TOX was
expressed in less than 10% of global Ki67/GZMB+ CD8 TILs in sharp contrast with the 60% TOX+ CD8 TILs that was found in tumours. Interestingly, it seemed that α-PD-1 treatment in LN slightly enhanced the percentages of TOX+ CD8 TILs over monotherapy counterparts. The same trend was found in SIINFEKL-reactive CD8 T cells in dLNs, in which α-PD-1 also drove higher proportions of TOX+ cells within Ki67+/GZMB CD8 T cells (20% in P(OVA) and α-PD-1 treated groups).

According with the reduced proportions of TOX+ cells in dLN compared to tumours, the proportions of TIM-3+ and PD-1+ global and SIINFEKL-reactive CD8 T cells in dLN were also lower. For instances, within SIINFEKL-reactive CD8 T cells populations in the LNs of P(OVA) and α-PD-1 treated animals, 25% were PD-1+, ≈5% were TIM-3+, and ≈25% were LAG-3+ (compared to 100%, 75% and 75% found in equivalent population in tumours, respectively).

Summing up, it seems that global and SIINFEKL-reactive CD8 T cells in LNs are in a less-exhausted and perhaps more undifferentiated state than SIINFEKL-reactive CD8 T cell tumour counterparts. Although we do not count on data of the stem-like marker TCF-1 in these populations, CD8 T cells LNs show some characteristics of these progenitor T cells, including being highly proliferative and low in exhaustion markers. In addition, the proportion of SIINFEKL-reactive CD8 T cell in the LNs of mice treated with combinatorial vaccine and α-PD-1, were higher over monotherapy counterparts. Perhaps, this would correlate with an enhanced priming mediated by α-PD-1 therapy and thus, a more activated and effector-like phenotype, explaining why the expression of TOX, TIM-3 and PD-1 was slightly higher in these groups, especially in P(OVA) and α-PD-1 treated animals. Despite this, it seems that the contribution of these potential “stem-like” progenitor cells, that were being enhanced in the dLN upon α-PD-1 treatment did not suffice per se, and data from the tumours indicate that probably these cells differentiated to an exhausted phenotype once in the tumour.

5.3.3. General conclusion

In conclusion, this chapter suggests that PMPC-PDPA POs can be used as carriers for NeoAg targeting and IC delivery in DCs: Immunisations with PMPC-PDPA POs entrapping
OVA (P(OVA)) as NeoAg model, in animals harbouring B16-OVA melanoma provide with delayed tumour growth and enhanced survival over animals immunised with free protein. In addition, the therapeutic P(OVA) effect is synergistic when combined with α-PD-1.

Whereas PBS or POs treated animals mounted weaker anti-tumour T cell responses and presented higher TAM-M2 infiltrates, immunisations with P(OVA) and Poly(I:C) improved T cell priming over naked OVA immunisations, as shown by increased proportion of CD8 TILs expressing PD-1 and TIM-3. Overall, this could indicate improved DC targeting by PMPC, enhanced antigen cytosolic delivery (through PDPA protonation in the late endosome) and eventually improved antigen processing. However, since analysis are done at day 13 post tumour inoculation, these experiments do not provide with accurate information of the vaccine priming process, and this will be described in the next chapter. Although TAM proportions on P(OVA) and Poly(I:C) treated groups were lower than in the rest of groups, PD-L1+ frequencies in TAMs were highest following this treatment. This, together with the presence of high proportions of PD-L1+ DC cell in LN provided with the rationale to combine vaccines with α-PD-1. The enhanced survival found in P(OVA) Poly(I:C) and α-PD-1 treated group, correlated with increased TIL infiltrates with cytotoxic (GZMB) and proliferative (Ki67) capacity. These properties were further enhanced in SIINFEKL-reactive CD8 T cells, which underwent a 5-fold expansion compared to P(OVA) and Poly(I:C)-only vaccinated groups. Thus, this highlights the potential of PMPC-PDPA POs in efficiently priming NeoAg-reactive CD8 TILs, which is key for an efficient ICI effect. In addition, tumour-dLN proved to be key for the priming and expansion of SIINFEKL-reactive CD8 T cells, especially in combination with ICI. Despite successful priming, we hypothesise that tumours ultimately escape immune control by promoting terminally differentiated exhaustion phenotypes, as corroborated by the expression of the transcription factor TOX and exhaustion markers including TIM-3, and PD-1 following α-PD-1 therapy. Other possibilities could comprise a high tumour mutation rate [400], MHC-I or OVA-related NeoAg downregulation. Future work should also address the potential effect of changing immunisation and ICI schedules, maybe including α-PD-1 also at the time of vaccine priming and not just during boost.
5.3.4. Limitations of the study

Despite s.c. P(OVA)/Poly(I:C) immunisations outperforming OVA/Poly(I:C) at equivalent protein dosages, a benchmark titration experiment would be required for clinical translational purposes. Assessment of the naked-OVA protein dose required to match anti-tumour therapeutic responses obtained by P(OVA), would provide an understanding of the efficiency of our delivery system over conventional naked protein-based vaccination approaches.
5.4. Appendix

5.4.1. Individual tumour growth curves

Figure 85. Individual B16-OVA tumour growth curves following subcutaneous prime-only immunisations.
Tumour growth curves from C57Bl/6 mice injected with A) $1 \times 10^6$ B16-OVA cells and immunised with empty POs, OVA with Poly(I:C), P(OVA) and P(OVA) with Poly(I:C); B) $0.5 \times 10^6$ B16-OVA cells and immunised with empty PBS, POs, OVA with Poly(I:C), P(OVA) and P(OVA) with Poly(I:C).
Figure 86. Individual B16-OVA tumour growth curves following subcutaneous vaccination in a prime-boost regime, alone or in combination with α-PD-1.
Tumour growth curves from C57Bl/6 mice injected 0.5x10^6 B16-OVA cells and prime-boosted with empty POs, OVA with Poly(I:C), P(OVA) and P(OVA) with Poly(I:C) alone (A) or in combination with α-PD-1 (B).
5.4.2. Gating strategies of T cell and myeloid populations in tumours and dLN

A.

B.

C.

Figure 87. Flow cytometry gating strategies used for the analysis of TME and dLN from B16-OVA tumours
Gating followed for A) T cell analysis in both tumours and dLN B) Myeloid populations in tumour C) Myeloid populations in dLN.
5.4.3. Ki67 and GZMB expression in CD8 TILs

Figure 88. GZMB expression in CD8 TILs in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations. 
A) UMAP analysis of TILs infiltrating B16-OVA in mice B) Visualisation of GZMB expression within TILs.
Figure 89. Ki67 and GZMB expression in CD8 TILs in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations.
A) UMAP analysis of TILs infiltrating B16-OVA in mice B) Visualisation of Ki67 expression within TILs.
Figure 90. Ki67 and GZMB expression in CD8 TILs in B16-OVA tumours following α-PD-1 administration in combination with P(OVA) immunisations.
A) Ki67 and GZMB gating within CD8 TILs. B) Ki67 and GZMB population frequencies represented as parts of whole diagram.
5.4.4. Exhaustion marker frequencies in SIINFEKL-reactive CD8 TILs

A.

![Graph A]

B.

![Graph B]

Figure 91. Exhaustion and activation markers in Ki67+ and/or GZMB+ global and SIINFEKL-reactive CD8 TILs.

0.5x10^6 B16-OVA cells were s.c. inoculated in the right flank of C57Bl/6 mice and 2 and 5 days later, P(OVA) encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence of 100 μg of Poly (I:C). On days 5, 8 and 11, 200μg of α-PD-1 were administered i.p. Control groups were immunised with an equivalent dose of free OVA and Poly (I:C) or with empty POs or PBS and the same of α-PD-1 schedule. TILs analysis were performed on day 13 post tumour inoculation. Exhaustion marker frequencies from frequencies of exhaustion (A) or activation (B) markers from total (top) and SIINFEKL reactive (bottom) CD8 T cells populations.
5.4.5. T cell populations in dLN

Figure 92. Quantification of T cell populations skin draining lymph nodes (dLN), from B16-OVA tumours following P(OVA) immunisations in mice.

0.5x10^6 B16-OVA cells were s.c. inoculated in the right flank of C57Bl/6 mice and 2 and 5 days later, P(OVA) encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence of 100 μg of Poly (I:C). On days 5, 8 and 11, 200μg of α-PD-1 were administered i.p. Control groups were immunized with an equivalent dose of free OVA and Poly (I:C) or with empty POs or PBS and the same of α-PD-1 schedule.
Figure 93. Exhaustion and activation markers in Ki67+ and/or GZMB+ global and SIINFEKL-reactive CD8 T cells draining lymph nodes (dLN).

0.5x10^6 B16-OVA cells were s.c. inoculated in the right flank of C57Bl/6 mice and 2 and 5 days later, P(OVA) encapsulating 1.2 μg OVA was s.c. injected in the left flank, in the presence of 100 μg of Poly (I:C). On days 5, 8 and 11, 200μg of α-PD-1 were administered i.p. Control groups were immunised with an equivalent dose of free OVA and Poly (I:C) or with empty POs or PBS and the same α-PD-1 schedule. dLN analysis were performed on day 13 post tumour inoculation. Exhaustion markers frequencies from Frequencies of exhaustion (A) or activation (B) markers from total (top) and SIINFEKL reactive (bottom) CD8 T cells populations.
Chapter 6: Contribution of immunisation route to POs biodistribution, APC targeting and activation, and anti-tumour therapeutic effect in B16-OVA or MC38 models.
6.1. Introduction and aims

Effective immunisations require antigen and adjuvant delivery into the LNs, where T cells get activated [382]. Licensed protein-based subunit vaccines are administered by s.c. or intramuscular (i.m.) injection, in combination with conventional adjuvants such as alum or water in-oil compounds (i.e. MF59) [401]. These induce the secretion of chemokines recruiting myeloid cells to the injection site, such as neutrophils, monocytes, macrophages or DCs, which take up the antigen and transport them to the vaccine dLN [402]. In addition, immunogens remaining on the extracellular milieu might enter the lymphatic system, or the systemic circulation [403]. Ideally, vaccinations should result in high concentrations of antigen in the LNs. This is since the development of CD8 T cell responses in LN is limited by the amount of antigen that can drain into this organ, given that efficient cross-presentation in DCs is required [404]. Hence, following s.c. or i.m subunit vaccine administration, antigen should be targeted to enter the lymphatic system. However, molecules below 5 nm in diameter, including proteins (5 nm = 40-50 kDa of a globular protein), can easily diffuse through the endothelium membrane of blood vessels, and enter systemic circulation instead of lymphatic system [403]. This is nonetheless inefficient as those immunogens are distributed across organs, reducing LN delivery. Because of this, some NeoAg vaccines are being tested through LN injection in clinical trials [405]. Altogether, this evidences the need to improve platforms for antigen target and delivery onto DCs, especially into LNs. NPs between 5 to 100 nm can efficiently drain to LNs [328] and be decorated with ligands or synthesised with motifs to target endocytosis receptors in DCs, to enhance their uptake and cross-presentation [268].

In addition to DC targeting, recent studies indicate the importance of systemic responses throughout lymphoid organs in the body (i.e spleen or bone marrow) to leverage anti-tumour responses following administration of immunotherapy agents such as checkpoint inhibitors [383]. In the case of vaccination, it has recently been reported that the route of NP-based cancer vaccines can improve the anti-tumour responses, through priming in lymphoid organs other than LNs [406].
After verifying PMPC-PDPA POs potential to elicit anti-tumour responses when acting as NeoAg carriers via s.c. route, this chapter explores their in vivo targeting selectivity for immune cells, especially DCs. In addition, it addresses the influence of the PMPC-PDPA POs administration route on the modulation of anti-tumour responses.

To this end, the following objectives were defined:

1. Study the in vivo biodistribution of PMPC-PDPA POs upon subcutaneous and intravenous administration.

2. Decipher POs targeting and activation of immune cells, with a focus in APCs, in secondary lymphoid and mucosal tissues.

3. Compare the therapeutic effect of intravenous P(OVA) and Poly(I:C) immunisations, as monotherapy or in combination with α-PD-1, over subcutaneous vaccination in B16-OVA tumours

4. Understand whether the therapeutic responses achieved by P(OVA) immunisations in the B16-OVA model can be reproduced in a more physiologically relevant cancer model. For this, progression of MC38 colorectal tumours will be analysed following immunisations with POs encapsulating a long synthetic version of one MC38 described neoantigen, namely Adpgk.
6.2. Results

6.2.1. Biodistribution and APC targeting of s.c. administered POs

To get a better understanding into the mechanism by which P(OVA) immunisations triggered a therapeutic anti-tumour immune response, POs distribution across organs was analysed 24 hours after s.c. administration in C57Bl/6 mice. Further, POs targeting, and APC activation was assessed in the tissues where a higher proportion of POs had been retained, with a main focus on the immunisation site (skin) and LNs.

6.2.1.1. Biodistribution

Cy5.5-POs were s.c. injected in the left flank of C57Bl/6 mice. 24 hours later, animals were perfused with PBS and PFA and heart, lungs, skin, intestine, pancreas, omentum, thymus, genital tract, spleen, skin-dLN, liver, kidneys, stomach and brain were collected for Cy5.5-POs fluorescent visualisation by IVIs. The images (Figure 94) indicated that Cy5.5-POs fluorescent signal was strongest at the injection site (skin) and in skin-dLNs (including axilar, inguinal and braxial LNs), implying higher POs accumulation at those sites.

To further characterise the immune modulation process mediated by POs following its s.c. administration, myeloid and lymphoid populations in skin and skin-dLN were quantified by FACS 24 hours after injection. In addition, APC targeting and activation by POs alone or in combination with Poly(I:C) was analysed. Finally, the potential of P(OVA) and Poly(I:C) formulations to induce antigen cross-presentation in DCs in vivo was addressed.

6.2.1.1.1. Skin: POs targeting and activation in immune cells
6.2.1.1.1.1. Immune landscape upon immunisation

The skin is formed of 3 layers namely epidermis, dermis, and hypodermis. Whereas a high density of APCs is found within the epidermis and dermis, the hypodermis
Figure 94. Biodistribution of Cy5.5-PMPC-PDPA POs 24 hours post subcutaneous administration.

Cy5.5 labelled POs were injected via s.c. administration in the left flank. 24 hours later, animals were perfused with PFA 4% in PBS and PBS, tissues were collected and Cy5.5 fluorescence was visualised in IVIs. Images show fluorescence in heart, lung, skin, intestine, pancreas, omentum, thymus genital tract, spleen, skin-dLN, liver, kidneys, stomach and brain in A) PBS injected mouse B) Cy5.5-POs injected mouse. n= 1 (2-3 animals per group), images are representative of 1 mouse per condition.

constitutes a reservoir for interstitial fluid and it has a loose matrix structure [407].

Injections performed subcutaneously targeting the hypodermis have historically been an attractive for vaccine delivery, since it allows the diffusion of inflammatory cytokines, that may also activate APCs in the upper layers of the skin [408]. It is estimated that immune cells in skin constitute about 7% of total cells, with DCs being 4%, T cells 0.41%, NK cells 0.45%, and the rest, other such as ILCs, neutrophils or macrophages [409]. However, these proportions change following dermal immunisations, due to the development of an inflammatory response. To understand the changes in the immune
populations in skin following s.c. immunisations with POs, phenotyping of skin immune cells (macrophages, DCs, B cells, T cells and NK cells) at the site of POs injection was performed by FACS, 24 hours after administration. In previous studies in models of autoimmune disease, administration of empty PMPC-PDPA POs has induced an anti-inflammatory-like responses [310]. Since this was the first time that PMPC-PDPA POs were being administered as vaccines encapsulating OVA and in combination with Poly(I:C), it was necessary to understand the particular contribution of each component (antigen, adjuvant and polymer) towards the elicitation of a functional immune response. Thus, differences in skin immune cells among animals immunised with either empty POs, empty POs and Poly(I:C), OVA and Poly(I:C), or P(OVA) and Poly(I:C) were assessed. A control group was injected with PBS only.

First, unsupervised dimensional reduction and clustering analysis of total immune cells infiltrating skin were performed. Phenograph and UMAP data treatment defined 15 clusters (Figure 95A) from which it was possible to identify the following groups: 1. Macrophage-like cells (CD11b+, PDL1+, MHCII-), 2. DC-like cells (CD11c+ MHC-II+ CD11b+), 3. B cells (CD19+ MHC-II+) and 4. T cells (CD3+CD11b-). A fifth population expressed a mix of B and myeloid markers (CD19+, CD11c+, CD11b+), which does not correlate with a specific subset and some authors define as “memory B cells” in blood, with migratory capacity [410], [411]. It was not possible to differentiate a population for NK cells, perhaps due to the small frequencies of these cells in skin, and the very low numbers of skin immune cells that could obtained during the tissue processing for FACS staining and acquisition. UMAPs in Appendix Figure 113 show the relative expression of the phenotype markers that helped define the populations. Comparison of UMAP clusters across treatments indicated an increase of macrophage-like cells in skin infiltrates of mice immunised with POs, POs and Poly(I:C), and P(OVA) and Poly(I:C), which was nearly absent in PBS or OVA and Poly(I:C) treated groups (Figure 95A, population 1). Moreover, the data suggested that DCs in PBS-treated animals were more abundant than in the rest of groups (Figure 6.2 Figure 95A, population 2). Relevantly, minimal amounts of B cells could be visualised in the immune landscape diagram from empty POs treated animals, while these cells seemed to be present in higher proportions in the other groups (Figure 6.2 Figure 95A, population 3). A similar trend was found
Figure 95. Immune cells populating skin following subcutaneous injection with POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. After 24 hours, immune populations in injection site were studied by FACS through A) Unsupervised analysis using Phenograph dimensional reduction followed by UMAP clustering B) Supervised quantification of macrophages, DCs, B cells, T cells and NK cells from total immune cells. Results in B) show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (**p<0.01, *p<0.05).
for T cells (Figure 95 A, population 4).

To corroborate the results obtained by UMAP clustering analysis, the frequencies of each immune populations within total immune cells (CD45+, excluding immune cells from adjacent skin capillaries, CD45.2-) were quantified manually from FACS data (gating strategies Figure 115A). Generally, the numbers indicated similar trends than UMAP plots. In the case of macrophage frequencies (Figure 95 B), differences were statistically significantly between empty POs groups (50% of total CD45 cells) and PBS (≈18%), OVA and Poly(I:C) (20%), and P(OVA) and Poly(I:C) (≈30%). Whereas macrophage-like cell numbers seemed to be higher in skin from animals injected with POs, POs and Poly(I:C) and P(OVA) samples, the presence of antigen and Poly(I:C) *per se* did not appear to modify macrophage-like cell frequencies, and OVA and Poly(I:C) treated groups reported similar proportions of these cells than PBS control mice.

B cells proportions varied between ≈8-15%, with lowest values, in average, found in PO treated animals, and highest ones in OVA and Poly(I:C) treated animals, although these differences were not statistically significant.

POs and Poly(I:C) treated animals accounted for the lowest percentages of DCs in skin (≈2%) among treatment groups, whereas PBS control groups had the highest (≈8%), and this difference was statistically significant. A reduction of DC frequencies compared to PBS control group might be an indication of DC migration from skin during the 24 hours that followed the injections and the analysis.

T cell quantification indicated that the highest proportion of these cells were in the skin of POs treated animals (approximately 20% of total immune cells), although these differences were not noticeable in the clustering plots. The rest of the groups presented similar proportions of T cells among them, which were around 10% from total CD45+ cells. Finally, NK cells values were lower than 5% in all groups, with no differences among treatments.

In conclusion, P(OVA) and Poly(I:C) immunisations increased macrophage-like cells proportions compared to PBS and OVA and Poly(I:C) animals. This was PMPC-PDPA POs dependent, as empty POs with or without Poly(I:C) also reported the same trend. In addition, percentages of DCs in the skin of P(OVA) and Poly(I:C) animals were decreased
over PBS controls, although this did not seem to be treatment-specific, as the rest of
groups also reported a decreased proportion of DCs. No changes in T cells, B cells or NK
cells were found in P(OVA) and Poly(I:C) animals with respect to the rest of the groups.
Of note, skin sections from empty POs injected mice showed higher frequencies of T
cells than the PBS control, although this number did not match with what was observed
by UMAP clustering analysis.

6.2.1.1.1.2. POs uptake and activation of Antigen Presenting Cells (APCs)

**CD80 expression in APCs**

It is well known that the activation of APCs, especially DCs, is crucial for efficient T cell
priming and the development of effector T cell responses [1]. For this reason, the
expression of the activation marker CD80 in APCs (DC, macrophage-like cells and B cells)
was analysed across treatment groups, by relative expression visualisation across UMAP
diagrams (**Figure 96A**), and also by further calculation of CD80+ cells across groups,
(**Figure 96B**). Both UMAP plots and supervised quantifications indicated the highest
proportion of CD80+ macrophage-like cells (around 20% from total population) was in
the skin of empty-POs treated mice, which was also coincidental with higher numbers
of macrophages. In P(OVA) or OVA and Poly I:C groups CD80+ macrophage-like cells
were lower, around 5%, similar to the PBS group. However, these differences were not
statistically significant.

Empty POs injection also led to CD80 expression in B cells in the skin, which were in
higher proportion (75%) than the rest of the groups. In all groups receiving Poly(I:C)
CD80+ B cell frequencies were similar and oscillated between 35% to ≈50%, with no
statistical significances among them. This trend was the opposite for DCs, where animals
receiving either POS, OVA or P(OVA) in combination with Poly(I:C) reported ≈50-60% of
CD80+ DCs over the ≈25-30% CD80+ DC population found in PBS or empty POS treated
groups, which goes in line with the maturation effect of the adjuvant Poly(I:C) [412].
Figure 96. CD80 expression in immune cells populating skin following subcutaneous injections with POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune cells populating the injection site were studied by FACS through supervised quantification and unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering. A) Relative CD80 expression across immune cells within treatment groups in UMAP plots B) Quantification of CD80+ frequencies from each cell subset. Results in B) show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (*p<0.05).
Overall, immunisations with P(OVA) and Poly(I:C) triggered the expression of the maturation marker CD80 predominantly in DCs. However, this did not seem to be mediated by the presence of POs, but by the presence of Poly(I:C), since CD80 expression was also enhanced in groups immunised with empty POs or OVA in combination with adjuvant. Interestingly, a trend of lower CD80+ B cell proportions was found in all treatments, that included Poly(I:C), than in PBS control mice, although no statistical significances were found. In addition, empty POs seemed to enhance CD80 expression in B cells and macrophages, but not in DCs. However as this is a first explorative approach the reason why this is happening remains elusive.

**PD-L1 expression in APCs**

As previously explained in this thesis, PD-L1 with PD-1 binding (in APCs/tumour cells with T cells, respectively) renders T cells inactive [190]. Nonetheless, recent works have highlighted the need of PD-L1 expression in DCs populating tissues to migrate to LNs [218]. Thus, PD-L1 expression was also investigated in skin to explore the understanding of APC activation that follows P(OVA) and Poly(I:C) vaccination. Relative PD-L1 expression ([Figure 97A](#)), and PD-L1 frequencies ([Figure 97B](#)) were also quantified. UMAP diagrams indicated highest relative PD-L1 expression in macrophage-like cells in the skin of empty POs treated mice, which was corroborated by quantification analysis, which indicated ≈25% of PD-L1+ macrophages from total CD45+ cells. OVA Poly(I:C) immunised group reported very similar results, and in both cases, the differences were statistically significant with frequencies of PD-L1+ macrophages found in PBS injected mice (<5%). Interestingly, frequencies quantifications suggested that by combining POs or P(OVA) with Poly(I:C) it was possible to modulate the expression of PD-L1 in macrophage-like cells, which was lower than in POs only treated animals. However, these changes in PD-L1 frequencies could also be attributed to variations in the composition of myeloid sub-populations included within the CD11b+ “macrophage-like cells” cluster, among treatment groups. Each of these myeloid populations could express PD-L1 at different levels, skewing the analysis of these data. For this reason, in depth analysis would be required.
Figure 97. PD-L1 expression in immune cells populating skin following subcutaneous injection with POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune cells populating the injection site were studied by FACS through supervised quantification and unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering. A) Relative PD-L1 expression across immune cells within treatment groups in UMAP plots B) Quantification of PD-L1+ frequencies from each APC subset. Results in B) show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (***p<0.01, *p<0.05).
In B cells, quantifications indicated that PD-L1+ proportions were highest in POs treated animals, up to 50%. Interestingly, the proportion of B cells in the skin in this group was the lowest and had nearly disappeared (as reported by UMAPs (Figure 97A), which might indicate migration of B cells from skin to other tissues upon s.c. POs injection. In the rest of the groups PD-L1+ B cells made up 10-15% of total B cells, with lower values found in P(OVA) and Poly(I:C) over OVA and Poly(I:C) treated animals. Lastly, the proportion of PD-L1+ DCs in skin went up to 75%, which were the highest compared to other APC counterparts. These proportions were similar in all groups, except for PBS controls, where only 20% of DCs were PD-L1+ on average.

In conclusion, P(OVA) and Poly(I:C) immunisations triggered the expression of PD-L1 mainly in DCs, with respect to PBS controls. However, the results indicated that the rest of the treatment groups, including empty POs, also triggered a similar increase in terms of relative expression and frequencies of PD-L1+ DCs, which suggested that what drove the increase in PD-L1+ DC frequencies in P(OVA) and Poly(I:C) was likely a combination of the use of PMPC-PDPA POs, OVA and the adjuvant Poly(I:C).

**Cy5.5-POs uptake in APCs and correlation with CD80/PD-L1 expression**

To understand the APC targeting of Cy5.5-POs upon s.c. administration, POs uptake was analysed across skin immune cells in each treatment group (Figure 98A and B). As shown in UMAP plots (Figure 98A), Cy5.5-POs signal could be detected in all animal groups injected with the NPs (Figure 98A), and results indicated that highest relative Cy5.5-POs signal matched the macrophage-like cluster present in skin upon POs, POs Poly(I:C) or P(OVA) and Poly(I:C) injection. In addition, Cy5.5-POs signal was clearly detected in DC populations, although these cells seemed to take up POs with a lower avidity, as less red-coloured dots/cells could be visualised. In fact, the proportion of Cy5.5+ DC (≈50-70%) was higher than the percentages of Cy5.5+ macrophage-like cells (≈30%-60%), or Cy5.5+ B cells (≈25%-60%) (Figure 98B). Moreover, no statistically differences were found in Cy5.5-POs uptake among POs, POs Poly(I:C) or P(OVA) and Poly(I:C) groups, for any APC subsets (Figure 98B).
Figure 98. Uptake of Cy5.5-POs in immune cells populating skin following subcutaneous injection with Cy5.5-POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune cells populating the injection site were studied by FACS through supervised quantification and unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering. A) Relative Cy5.5-POs signal across immune cells within treatment groups in UMAP plots. B) Quantification of Cy5.5+ frequencies from each APC subset. Results in B) show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (no statistical significances were found).
Overall, data suggested that 25-75% of APCs took up POs (Figure 98B). Furthermore, the levels of CD80 and PD-L1 expression varied among total macrophages, B cells and DCs populations, across POs, POs Poly(I:C) or P(OVA) and Poly(I:C) groups (Figure 96 and Figure 97).

To understand the contribution of Cy5.5-POs+ APC populations in CD80 and PD-L1 expression, the frequencies of these markers were calculated in Cy5.5+ or Cy5.5- cells, in macrophages, B cells, or DCs (Figure 99). Interestingly, higher frequencies of CD80+ cells were found among Cy5.5+ macrophages, Cy5.5+ B cells and Cy5.5+ DCs, over their Cy5.5- counterparts (Figure 99A, B, C), suggesting PMPC-PDPA POs might have an influence in activation. In addition, these differences were bigger within B cells than within DCs or macrophages, and CD80 frequencies in Cy5.5+ B cells in animals injected with POs in combination with Poly(I:C) and/or OVA did not change with regards their POs only counterparts (Figure 99B, left). However, lower frequencies of Cy5.5+ macrophages were CD80+ in groups treated with POs or P(OVA) in combination with Poly(I:C) (Figure 99A, left). This trend was the opposite for DCs, which suggested that CD80 expression in these cells is as a result of combination of POs with Poly(I:C) (Figure 99C, left).

PD-L1 expression in macrophages followed a similar trend to CD80 (Figure 99A, right) and PD-L1 values in Cy5.5+ cells were higher than in Cy5.5- cells, when injections were performed in the absence of adjuvant. In the case of B cells, the high PD-L1 values found upon empty POs injection (Figure 97B) seemed to mainly correlate with Cy5.5+ cells, indicating that PMPC-PDPA POs might be modulating the expression of PD-L1 marker in B cells. Remarkably, PD-L1 frequencies dropped drastically within Cy5.5+ B cells in POs/P(OVA) and Poly(I:C) groups (Figure 99B, right). Finally, the combination of POs/P(OVA) with Poly(I:C) did not seem to interfere with the expression of PD-L1 in DCs, and Cy5.5+ DCs in those groups reported higher proportions of PD-L1+ DCs than those found in either Cy5.5+ DCs in POs only treated groups, or in their Cy5.5- DCs counterparts. Moreover, it was worth noting that Cy5.5- DCs in P(OVA) and Poly(I:C)
Figure 99. Contribution of POs uptake in CD80 and PD-L1 expression in APCs in skin, upon subcutaneous injection of Cy5.5-POs labelled POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, the expression of CD80 and PD-L1 was compared in Cy5.5+ or Cy5.5- subsets in A) Macrophages B) B cells C) DCs. Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
immunised group reported the highest proportions of PD-L1+ cells across Cy5.5-populations in the rest of the groups. This could be an indicative of cross-talk between DCs that have taken up the particles and those who did not.

6.2.1.1.2. Skin-draining lymph nodes: POs targeting and activation of APCs.

6.2.1.1.2.1. Immune landscape upon immunisation

After s.c. vaccinations, activated immune cells migrate from the skin to skin-dLNs [413]. DC migration is particularly relevant in the context of immunisations, as activated DCs contribute to the efficient priming, activation and expansion of T cells reactive to the vaccine antigen [414]. Previous results following P(OVA) and Poly(I:C) s.c. immunisations indicated a decrease in the proportion of DCs in skin, which expressed CD80 and PD-L1 (Figure 95, Figure 96, Figure 97), which suggested DC migration from skin to skin-dLN. In addition, prior biodistribution experiments revealed Cy5.5-POs signal in skin-dLN following 24 hours of POs injection Figure 94. Thus, to continue investigating the activation and priming mechanisms of P(OVA) and Poly(I:C) immunisations, skin-dLN immune populations and APC activation were analysed in this group and compared to PBS, empty POs, empty POs and Poly I:C, OVA and Poly(I:C) responses.

Given that B cells and T cells constitute the majority of immune cells in LN [1], visualisation and comparation of myeloid populations following clustering analysis in CD45+ cells proved challenging, and differences in myeloid populations across groups could not be detected (data not shown). For this reason, supervised quantification analyses were performed prior to clustering, as shown in Figure 100A (gating strategies in Appendix Figure 115B). To start with, macrophages in dLNs from PBS control mice constituted a 0.5% of the total CD45+ cells, which was statistically higher than the macrophages frequencies found in dLN of POs and POs and Poly(I:C) immunised mice, ≈ 0.25%. PBS groups reported a 30% of B cells, while in the rest of animals B cells frequencies were higher, approximately 40% of total CD45+ cells. Next, statistically higher migDCs frequencies were found in OVA/P(OVA) and Poly(I:C) immunised mice (≈0.5%) than in PBS or empty POs control groups (0.25%).
Figure 100. Immune cells populating skin-draining lymph nodes following subcutaneous injection with POs.
C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune populations in skin-dLN were studied by FACS through A) Supervised quantification of macrophages, resident DCs (ResDCs) and migratory DCs (migDCs), B cells, T cells and NK cells from total immune cells B) Unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering of CD45+ cells, excluding T cells. Results in A) show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (**p<0.001, **p<0.01, *p<0.05).
Of note, these correlated with findings in skin, where DC populations were lowest in OVA/P(OVA) and Poly(I:C) immunised animals, as shown in the previous section. A similar trend to migDC frequencies was found for resDC. In addition, there were no changes in the percentages of T cells in dLN across groups, and NK cell frequencies were lower in dLN of mice immunised with POS/OVA/P(OVA) with Poly(I:C) (Figure 100A).

To improve cluster diagrams where myeloid populations could better represented so that APC activation markers could be analysed, dimensional reduction analysis were performed in immune cells excluding T cells (CD45+CD3- population), as no differences in T cells had been found by supervised quantifications. Figure 100B show the resulting UMAPs clustering plots, where it was possible to identify: 1. NK cells (CD11b+ NK1.1+), 2. MigDCs (CD11c+MHC-II high), 3. Macrophage-like cells (CD11b+), 4. ResDCs (CD11c+MHC-II int) and 5. B cells (CD19+MHC-II+). Appendix Figure Figure 114 show the relative expression of each phenotype marker.

6.2.1.2.2. Activation of APCs

CD80 expression in APCs

Visualisation of CD80 levels in UMAP graphs (Figure 101A) suggested highest expression in DCs and macrophages, in sharp contrast with B cells, where no signal could be found. Further quantifications (Figure 101B) indicated no statistically significant differences in CD80+ macrophages frequencies among groups, which were around 18% in all cases except for PBS control animals, where CD80 expression remained at background levels in these cells. Remarkably, no CD80 expression was found in B cells in any group, which was in accordance with relative expression studies within clustering analysis. Lastly, higher frequencies of CD80+ migDCs were found in all groups treated with Poly(I:C), with respect to their PBS or empty POs counterparts. In resDCs CD80 expression was significantly higher in P(OVA) and Poly(I:C) than in PBS controls, however empty POs groups showed high CD80 frequencies, similar to P(OVA) and Poly(I:C) groups (=90%).

To conclude, P(OVA) and Poly(I:C) immunisations only enhanced CD80 expression in migDCs and resDCs, in terms of both relative expression and CD80+ cell frequencies.
Figure 101. CD80 expression in immune cells populating skin-draining LN following subcutaneous injection with POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune cells populating the skin-dLN were studied by FACS through supervised quantification and unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering. A) Relative CD80 expression across immune cells within treatment groups in UMAP plots B) Quantification of CD80+ frequencies from each cell subset. Results in B) show mean ± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (****p<0.0001, **p<0.01, *p<0.05).
Nonetheless, this activation did not seem to be treatment specific, and rather Poly(I:C) mediated, since POs and Poly(I:C) or OVA and Poly(I:C) immunised animals triggered CD80 expression in a similar manner.

**PD-L1 expression in APCs**

Visualisation of PD-L1 expression in UMAPs suggested highest levels of this marker in MigDCs than in the rest of populations, and its expression was detectable mainly in groups that had received Poly(I:C) (Figure 102A). Supervised quantification analysis (Figure 102B) corroborated highest PD-L1 frequencies in migDCs (which oscillated between 75%-90%), followed by resDCs (ranging from 30% to 80%), macrophages (up to 30%), and B cells (0-10%). In more detail, proportions of PD-L1+ macrophages were at background levels in dLN from PBS control groups, while PD-L1 frequencies were highest in dLN macrophages from POs only and P(OVA) and Poly(I:C) treated animals. In addition, this increase was statistically significant compared to PD-L1+ expression in macrophages from POs and Poly(I:C) treated animals. Similarly, to what was observed in skin, treatment with POs seemed to trigger an increase in PD-L1 levels in macrophages, although when in combination with Poly(I:C) decrease was observed in this frequency. In the case of B cells, the only statistically significant changes in PD-L1 proportions were found between LNs from PBS and POs and Poly(I:C) treated animals, although a trend in higher frequencies of PD-L1+ B cells was found in LNs from OVA/P(OVA) and Poly(I:C) treated animals. Trends in migDCs and resDCs were similar and higher PD-L1+ proportions were found in POS/OVA/P(OVA) and Poly(I:C) treated groups when compared to POs or PBS control. Nonetheless, PD-L1+ frequencies in PBS controls were lower in resDCs (30%) than in migDCs (75%).

Summing up, skin-dLN anlaysis revealed that s.c. P(OVA) and Poly(I:C) immunisations triggered PD-L1 expression in macrophages, B cells, and mig/resDCs in LN, with respect to PBS injected animals. Nonetheless, no differences in PD-L1 expression could be found between P(OVA) and Poly(I:C) immunised mice with the rest of the groups.
Figure 102. PD-L1 expression in immune cells populating skin-draining lymph nodes following subcutaneous injections with POs.

C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, immune cells populating the skin-dLN were studied by FACS through supervised quantification and unsupervised analysis by Phenograph dimensional reduction followed by UMAP clustering. A) Relative PD-L1 expression across immune cells within treatment groups in UMAP plots B) Quantification of PD-L1+ frequencies from each cell subset. Results in B) show mean ± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (***p<0.001, **p<0.01, *p<0.05).
This suggests that PD-L1 expression might be a result from a combination of the use of POs and also Poly(I:C), although these two vaccine components did not seem to synergise together to further enhance PD-L1 expression, as no differences could be found between groups that only used the Poly(I:C) component (OVA and Poly(I:C) group) or both of them (POs/P(OVA) and Poly(I:C) groups).

6.2.1.2.3. DC subsets and antigen cross-presentation.

cDC1/2/3 subsets among migDCs and resDCs were characterised following each treatment. In addition, OVA cross-presentation was analysed in each subset.

As shown in Figure 103A, cDC2 were present at higher proportions than cDC1 and cDC3 across treatments, in both migDCs and resDCs. In addition, P(OVA) and Poly(I:C) treatment seemed to increase the numbers of cDC1, 2, 3 in migDCs and resDC subsets over the rest of the treatments, although these differences were only significant for cDC2 subpopulations in migDCs and resDC, and for cDC3s in migDCs, and when compared with PBS or POs/POs Poly(I:C) groups. However, no statistically significant differences were found between OVA Poly(I:C) and P(OVA) and Poly(I:C) cDC1/2/3 subsets in any cases.

OVA cross-presentation analysis in DC subsets indicated that, despite the presence of increased frequencies of cDC2 cells, OVA was mainly being cross-presented by cDC1 cells (Figure 103B), which is in line with the literature [372]. Cross-presentation levels were statistically significantly higher in resident and migratory cDC1 from groups immunised OVA and Poly(I:C) and P(OVA) and Poly(I:C) than in the OVA-free counterparts, in which cross-presentation frequencies remained below 5%, and the signal was likely due to background noise Figure 103B.
Figure 103. Quantification of cDC1, cDC2, cDC3 populations and OVA cross-presentation frequencies in skin-draining lymph nodes, following subcutaneous injections with POs. C57Bl/6 mice were s.c. injected with PBS, empty POs, empty POs and Poly(I:C), OVA and Poly(I:C) or P(OVA) and Poly(I:C), at equivalent doses. Following 24 hours, DCs populating the skin-dLN site were quantified by FACS. A) Frequencies of cDC1, cDC2 and cDC3 in migDCs and resDCs B) Quantification of H2Kb-SIINFEKL+ frequencies in each DC subset. Results show mean± SD. Data from n=1 independent experiment (4 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).
6.2.2. Biodistribution of i.v. administered PMPC-PDPA POs

S.c. administration of POs resulted in limited targeting of organs as the spleen. Given its role as SLO, spleen could potentially contribute to increasing the development of immune responses. In addition, recent studies have described enhanced immune priming and therapeutic anti-tumour vaccine responses elicited upon systemic administration, such as intravenous injections [406].

To decipher whether i.v. injections could help in the distribution of POs towards immunological sites, biodistribution of fluorescently labelled Cy.5.5-POs following i.v. injection was also assessed. Indeed, it was possible to visualise a broader distribution of Cy5.5-POs across tissues 24 hours after i.v. injection by IVIs (Figure 104C). Particularly, POs accumulated preferentially in the spleen and liver which are, coincidentally, highly irrigated tissues. It was also possible to detect fluorescence signal in the intestines, LN, kidneys, lymph nodes, pancreas, omentum, thymus and lungs, to a lower extent.

The fluorescent signal visualised by IVIs imaging is calculated relatively to the highest and lowest fluorescence values among specimens during acquisition, meaning that the presence of organs emitting strong fluorescent signal could hamper the detection of fluorescence in other organs that entrap lower amounts of labelled POs. Hence, to accurately quantify the polymer mass infiltrating each tissue, individual fluorescence measurements from each organ were performed in a plate reader, and later, the corresponding mass was extrapolated from fluorescent values obtained from a calibration curve, made by serial dilutions of a known concentration of Cy5-PMPC-PDPA, as explained in material and methods (Section 2.5.2). Polymer mass in each organ, relative to total injected mass (%POs injected dose) and normalised by gram of tissue (%POs injected dose/g of tissue), was plotted across organs, comparing outcomes after s.c. (blue) and i.v. (brown) injections (Figure 105A). The highest value of %ID/g tissue after i.v. POs injection was found in spleen, which was ≈ 1000, followed by liver, where the numbers dropped to ≈250, and skin-dLN, with quantities around 75.
Figure 104. Biodistribution of Cy5.5-PMPC-PDPA POs 24 hours post subcutaneous (s.c.) or intravenous (i.v.) administration.
Cy5.5 labelled POs were injected via i.v or s.c. administration. 24 hours later, animals were perfused with PFA 4% in PBS and PBS, tissues were collected and Cy5.5 fluorescence was visualised in IVIs. Images show fluorescence in heart, lung, skin, intestine, pancreas, omentum, thymus genital tract, spleen, skin-dLN, liver, kidneys, stomach and brain in A) PBS injected mouse B) s.c. Cy5.5-POs injected mouse B) i.v. Cy5.5-POs injected mouse. Images representative of 1 mouse per condition. Data from n=1 independent experiment (2-3 animals per group).
Figure 105. Biodistribution of Cy5.5-PMPC-PDPA POs 24 hours post subcutaneous (s.c.) or intravenous (i.v.) administration.

Cy5.5 labelled POs were injected via i.v. or s.c. administration in the left flank. 24 hours later, animals were perfused with PFA 4% in PBS and PBS, tissues were collected, Cy5.5 fluorescence was visualised in IVIs. The percentage of POs retained in each organ was calculated following tissue fluorescence measurements by microplate fluorometer, and was normalised to tissue weigh A) Graph comparing %Injected dose per gram of tissue across organs and following i.v. or s.c. administration. B) Comparison of tissue fluorescence via IVIs measurements in skin, skin-dLN, spleen, and liver following i.v. or s.c. administration. Data from n=1 independent experiment (2-3 animals per group).
In the omentum, genital tract, thymus, kidney and organs belonging to the digestive tract such as stomach, intestines or pancreas, results were similar and ranged between 20 and 50. Values below 15 were found in lungs, sera and heart, and fluorescence signal was at similar levels to background in brain or skin. On the contrary, “% ID/g tissue” values following s.c. injection were highest in skin, of around 500, followed by those in skin-dLN (=300). Minimal values, up to 5 in average, were found in spleen, liver and sera, and no signal was detected in the rest of the tissues. As intuitively expected, polymer accumulation in skin was remarkably higher upon s.c. injection compared to i.v. (Figure 105A and Figure 105B, top right), and polymer presence in skin-dLN of s.c. mice also seemed higher than in i.v. immunised counterparts (Figure 105A and Figure 105B, top left). Spleen and liver POs targeting was only achieved upon i.v. injection (Figure 105A and Figure 105B, bottom right and left), as this was the case with other tissues like thymus, genital tract, kidney, stomach or omentum (Appendix Figure 116).

6.2.3. POs targeting immune cells s.c. vs i.v. immunisations

After assessing the influence of POs injection route in organ infiltration, POs targeting was investigated at a cell-uptake level, in tissues that reported the highest %ID/g fluorescence values, such as skin, skin-dLN, spleen or liver.

6.2.3.1. POs targeting: immune cells vs tissue cells.

Firstly, the percentage of Cy5.5+ cells from total tissue upon s.c. or i.v. Cy5.5-POs injection was assessed by FACS (Gating strategy Figure 117A). Results indicated that tissues that had reported the highest values of fluorescence after s.c injections (skin) or i.v injections (spleen and LN) by IVIs imaging, also correlated with highest Cy5.5+ proportions (Figure 106A). In more details, 22% of skin cells were Cy5.5+ upon s.c. administration and similar Cy5.5+ cells frequencies were found in liver of i.v. injected mice. In i.v. injected mice, Cy5.5+ frequencies were also high in spleen, around 10%. Remarkably, results suggested that less than 2% of total cells in dLN had taken up Cy.5.5+POs, upon s.c. or i.v. injection. Cy5.5 signal in lungs of mice injected i.v. with POs was present in 5% of the total pulmonary tissue.
Since, during this project, the use of PMPC-PDPA polymersomes was explored as antigen carriers for nano-vaccine purposes, it was relevant to address POs selectivity towards immune cells vs other cells present in the tissue. To this end, the frequencies of Cy5.5+ cells were calculated within the CD45+ (immune cells) or CD45- (tissue cells) populations (gating strategy Appendix Figure 117B and Figure 106B, i). In addition, the amount of POs taken up by a cell (which could act as indicative of cell avidity towards POs), was calculated through mean fluorescence (MFI) of the Cy5.5+ immune or tissue cells. 

*Figure 106B* ii, indicates that upon s.c immunisations, 80% of CD45+ immune cells in the skin were Cy5.5+, as opposed to the 20% found in other skin cells (CD45-). In blood, only up to 2% of CD45+ cells were Cy5.5+, and Cy5.5- cells (red blood cells), did not appear to take up POs. Interestingly, only 1% of CD45+ cells in dLN were Cy.5.5+, possibly being APCs, as explained in section 6.2.3.2, *Figure 107*. MFI values mirrored the trend in CD45+/− frequencies in skin, indicating that not only did a higher proportion of immune cells in skins take up the particles, but also these had internalised more POs than non-immune cells. No changes in avidity seemed to be reported between immune and non-immune cells in the rest of analysed tissues, upon s.c. Cy5.5-POs injection (*Figure 106B* iii).

Analysis of the targeting of POs following i.v. injection indicated that, in skin, dLN, blood, and liver, a higher frequency of CD45+ cells were Cy5.5+, over CD45- cells. Interestingly, although only 2% of total skin cells had been reported to take up particles upon i.v. injection (*Figure 106A*), 40% of these were immune cells (*Figure 106B*, iii). From the tissues that were analysed, the spleen was the only one in which percentages of Cy.5.5+ cells were higher in CD45- cells, with 30% being positive (*Figure 106B*, iv). However, this can be since the spleen is mainly comprised of T cells (≈25%) and B cells (≈50%), which express lower amounts of SRs, whereas APCs comprise only ≈7%.

In addition, POs avidity by immune cells in i.v. immunised animals was higher in blood and skin, whereas the rest of the organs did not show differences in MFI of Cy5.5 between CD45+ and CD45- cells (*Figure 106B*, v).
Figure 106. POs targeting selectivity and uptake in immune (CD45+) vs non-immune (CD45-) cells across organs, following subcutaneous (s.c.) or intravenous (i.v.) administration.

Cy5.5 labelled POs were injected via i.v. or s.c. administration. 6 hours later, single cell suspensions from spleen, liver, skin-dLN, lungs, blood and skin were analysed by FACS. A) Left: FACS plot showing Cy5.5+ gating. Right: Graph showing proportions of Cy5.5+ POs+ cells from total live cells in each tissue, excluding contaminating cells from blood (CD45.2+). B) i. FAS plot showing gating strategy of CD45+ and CD45- cells, excluding contaminating cells from blood (CD45.2+), and Cy5.5+ cells gates within each population. Cy5.5 frequencies (ii, iv) or Cy5.5 MFI
(iii, iv) within immune (CD45+) and non-immune (CD45-) cells, in each organ upon s.c. or i.v. injection. Results show mean± SD. Data from n=1 independent experiment (3 animals per group). Statistical analysis was performed using Two-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001, **p<0.01).

6.2.3.2. POs uptake in immune cells

Motivated by the differential detection of Cy5.5 POs in CD45+ cells across organs, following s.c. or i.v. injections **Figure 106**, in-depth uptake analysis (gating strategies Appendix **Figure 117C**) was performed in immune system cell subsets (DC, macrophages, other myeloids, B cells, T cells and NKS). Similar to what was found in previous experiments, s.c. PO administration resulted in Cy5.5 signal only in CD45+ cells from skin (**Figure 107B**) and skin-dLN (**Figure 107A**). On the other hand, i.v. POs injection resulted in a broad distribution of Cy5.5-POs, targeting immune cells across organs, with lower uptake frequencies found in the lungs and skin.

In skin-dLNs (**Figure 107A**), DCs (including resDC and migDC) and macrophages were the populations showing the highest frequency of Cy5.5+ cells, upon both s.c. (varying from 5 to 10%) and i.v. injection (from 20 to 40%), and minimal uptake was detected in B cells and non-APC cells. In skin, where PMPC-PDPA POs targeted CD45+ in a more selective way than other skin cells (**Figure 106**), all immune cells took up POs, with frequencies ranging from 20 to 80% of Cy5.5+ cells in APCs, and 60% to 80% in T cells and NKS, respectively (**Figure 107B**).

In the spleen (**Figure 107C**), i.v. injection resulted in ≈60% of DCs and macrophages taking up POs, and 50% of other myeloid cells also being Cy5.5+. Uptake in blood from mice receiving an i.v. POs injection, was mainly performed by APCs, including DC, macrophage-like and other myeloid cells (possibly monocytes), with frequencies oscillating between 20 to 80% of Cy5.5+ cells on average (**Figure 107D**). Liver analysis following i.v. PO injection indicated similar immune targeting to that found in skin, in animals s.c. injected with POs, where high uptake frequencies (40-90%) were reported in both APCs and no APC cells (**Figure 107E**). To finish with, macrophages and other myeloid cells in lungs reported lower Cy5.5+ frequencies, up to 20% of Cy5.5+ cells upon i.v. POs injection (**Figure 107F**).
Figure 107. PO cell uptake in CD45+ immune cells across organs, following subcutaneous (s.c.) or intravenous (i.v.) administration.

Cy5.5 labelled POs were injected via i.v. or s.c. administration. 6 hours later, spleen, liver, skin-dLN, lungs, blood and skin were collected, processed, and stained for FACS analysis. Quantifications of Cy5.5-POs+ cell frequencies were calculated within DCs, macrophages, other myeloid cells, B cells, T cells, NK cells from A) Skin-dLNs B) Skin C) Spleen D) Blood E) Liver F) Lung. Bar shows the mean of 3 animals per group from n=1 independent experiment.
6.2.4. Intravenous P(OVA) immunisations

i.v. injection of POs improved APC targeting by POs in lymphoid organs (LN, spleen), blood, lung and liver. Moreover, APC targeting is paramount for efficient priming and elicitation of adaptive immune responses. For these reasons, the therapeutic effect of i.v. immunisations with POs entrapping NeoAgs against solid tumours was further assessed in a B16-OVA model, following the same prime-boost schedule performed during s.c. immunisations (Figure 108A). Tumour measurements (Appendix Figure 119 for individual tumour growth curves) taken over time indicated that no tumour progression or survival benefit was achieved when naked OVA was i.v. administered, compared to s.c. administrations (Figure 108B and C).

Figure 108. Tumour progression and survival of mice harbouring B16-OVA tumours, following i.v. immunisations with P(OVA) in a prime-boost schedule.
A) Schematic representation of tumour and vaccine regime. B) Tumour growth curves over time. C) Survival curves showing percentage of survival over time. D) Table showing median survival per group (in days). Error bars in B) represent mean ± SEM. Pooled data from n=2 (i.v. treated groups) or n=3 (s.c. treated groups) independent experiments (5 to 8 animals per group and per experiment). Statistical analysis was performed used two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test (**p<0.001, *p<0.01, *p<0.05).
Nonetheless, B16-OVA tumour progression was slowed down upon i.v. P(OVA) immunisations, compared to P(OVA) vaccinations performed s.c., and 40% showed a trend in enhanced survival too, although no differences were found upon calculation of median survival values (22 days) (Figure 108D). However, B16-OVA bearing mice immunised i.v. with P(OVA) did show statistically significant improved tumour progression and survival rates than naked OVA-only vaccinated animals, from 17.5 to 22 days (in naked-OVA or P(OVA) immunised animals, respectively). No differences in tumour progression were found in controls PO treated animals, between s.c. or i.v. administration (median survival 14 days), Figure 108D.

6.2.4.1. Intravenous P(OVA) immunisations in combination with α-PD-1

The enhanced APC targeting by POs was found in secondary organs (especially the spleen), might result in enhanced T cell priming by P(OVA) and Poly(I:C) samples. In addition, accumulating scientific evidence highlights the key role of T cells primed in secondary lymphoid organs for the efficacy checkpoint blockade treatments [383]. Hence, the next step addressed was to comprehend whether combinatorial α-PD-1 treatment with i.v. P(OVA) immunisations could further improve the anti-tumour B16-OVA response and thus, tumour growth progression. Animals harbouring B16-OVA tumours were i.v. immunised with empty POs, OVA Poly(I:C), or P(OVA) Poly(I:C) in monotherapy or in combination with α-PD-1 (Figure 109A). Tumour growth and survival were tracked over time, finding no changes in tumour progression or survival between POs groups immunised with or without α-PD-1 (Figure 109B, C), whose median survival was 15 or 14 days (Figure 109D), respectively. Nonetheless, B16-OVA tumour growth progression in OVA or P(OVA) immunised animals in combination with α-PD-1 was slower than in monotherapy-treated counterparts (Figure 109B, C). Relevantly, tumour remission was achieved in 20% of the animals (1 of 5) treated with P(OVA) + α-PD-1 (Figure 109B, C), and animals from this group survived, in median, 11.5 days more than mice administered OVA + α-PD-1 (35 vs 23.5 days, respectively) (Figure 109D).
Figure 109. Tumour progression and survival of mice harbouring B16-OVA tumours, following i.v. immunisations with P(OVA) in a prime-boost schedule in combination with α-PD-1 therapy.

A) Schematic representation of B16-OVA inoculation, vaccine and α-PD-1 regime. B) Tumour growth curves over time. C) Survival curves showing percentage of survival over time. D) Table showing median survival per group (in days). Error bars in B) represent mean ± SEM. Pooled data from n=2 (i.v. treated groups) or n=3 (s.c. treated groups) independent experiments (5 to 8 animals per group and per experiment). Statistical analysis was performed used two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test (**p<0.01, *p<0.05).

6.2.4.2. SIINFEKL-reactive CD8 T cell responses in blood

In vaccine studies, the presence of T cell clones in blood which are reactive to the antigens used in the vaccine, serves as an indicator for the generation of efficient systemic antigen-specific immune responses [383]. To understand whether the improved survival effect obtained through i.v. OVA or P(OVA) immunisations, and with the use of α-PD-1, could correlate with SIINFEKL-reactive CD8 T cell titres in blood, blood was withdrawn from the tail of immunised animals at day 7 post boost (Figure 110A), and dextramer staining was performed by FACS (gating strategies Appendix Figure 118). Remarkably, the levels of SIINFEKL-reactive CD8 T cells in OVA-immunised mice
stayed below 1%, regardless of the immunisation route or the use of α-PD-1, and were similar to those obtained in the blood of empty POS only treated animals (Figure 110B). However, SIINFKEL-reactive CD8 T cell frequencies in P(OVA) immunised mice ranged from ≈2 to 8%, and this increase was also proportional to the survival achieved with each treatment, where mice immunised s.c. with P(OVA) showed the lowest values, and highest ones were achieved in the i.v. P(OVA) + α-PD-1 immunised group.

Figure 110. SIINFEKL-reactive CD8 T cell responses in blood of B16-OVA bearing mice, following s.c. or i.v. immunisations with P(OVA) in monotherapy or combined with α-PD-1.
A) Schematic representation of tumour, vaccine and α-PD-1 regimen. B) Frequencies of SIINFEKL-reactive CD8 T cells, detected by dextramer staining. Data was obtained from n=1 experiment, except for i.v. P(OVA) and Poly(I:C), where n=2 independent experiments were mixed (5 to 6 animals per group and per experiment). Statistical analysis was performed used One-way ANOVA followed by Tukey post-hoc test (****p<0.0001, ***p<0.001).

6.2.5. MC38 colorectal model

The last aim of this PhD project was to assess the therapeutic anti-tumour effect of immunisation with POs entrapping NeoAgs, in a more physiologically relevant model
than B16F10-OVA. To do this, the murine MC38 colorectal model was chosen, as the presence of endogenous CD8 TIL clones against a range of MC38-derived NeoAgs had been characterised in detail in other works [246], [415]. Some of these reactivities were against the NeoAgs derived from the MC38 mutated proteins Adpgk, Reps1, Dpagt and Rpl18, as described in the introduction.

6.2.5.1. Validating MC38 tumour model

Before proceeding to encapsulate the MC38 NeoAgs in PMPC-PDPA POs, it was crucial to validate that the NeoAgs of interest (derived from Adpgk, Reps1, Dpagt and Rpl18 proteins) were conserved within the MC38 tumour cells that were going to be used in these in vivo experiments. This is important as it is the presence of these mutations that may trigger anti-tumour immune responses which could translate into enhanced survivals upon vaccination. Thus, groups of animals harbouring MC38 tumours were immunised with one of the four different MC38 protein derived NeoAgs (which were all 9 aa in size), and Poly(I:C), following the same prime-boost schedule than used previously (Figure 111A). PBS injected animals were used as controls. Analysis of tumour progression and survival of mice suggested that only immunisations with the Adpgk-derived NeoAg provided with delayed tumour growth rates (Figure 111B, and Figure 120 for individual tumour growth rates), and that animals survived, on median, 9 days longer than PBS treated groups (Figure 111C and D). Immunisations with the rest of MC38 NeoAgs, did not result in statistically significant differences in either survival or tumour growth, although mice vaccinated with Reps1 showed a trend towards positive outcomes. Thus, these results indicated that Adpgk-derived NeoAg could act as the best immunogen to use in the POs MC38 vaccine, in combination with Poly(I:C).
Figure 111. Tumour progression and survival of mice harbouring MC38 tumours, following prime-boost s.c. immunisations with MC38 neoantigens.

A) Schematic representation of tumour and vaccine regime. B) Tumour growth curves over time. C) Survival curves representing percentage of survival over time. D) Table showing median survival per group (in days). Error bars in B) represent mean ± SEM. Data from n=1 independent experiment (6 animals per group). Statistical analysis was performed used two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test (*p<0.05).

6.2.5.2. Therapeutic effect of P(Adpgk-LSP) s.c. and i.v. immunisations

Given that immunisations with Adpgk derived peptide seemed to provide the best anti-tumour responses, this was the MC38 NeoAg chosen to be encapsulated in POs. As extensively explained during this thesis, one of the reasons why PMPC-PDPA POs were used as protein carriers for vaccine is their enhanced uptake and cytosolic delivery in APCs. IC protein proteolysis is needed for the generation of peptides ranging from 9 to 11 aa in size [81], as only these sizes can be further intracellularly processed and complexed with MHC-I molecules and be cross-presented on the surface of APCs [75]. However, when peptides that are 9 to 11 aa are administered extracellularly to APCs
(for examples as antigens in vaccines), these do not necessarily require to be intracellularly delivered to form complexes with MHC-I molecules. In fact, it is understood that peptides of this length can directly sit on the groove of MHC-I complexed expressed in the cell surface [235]. Thus, when immunisations are performed with peptides ranging from 9 to 11 aa in size, these can sit on the grooves of MHC-I molecules expressed on the surface of healthy cells (others than APCs), which lack co-stimulatory molecules needed for efficient CD8 T cell activation. This not only decreases the vaccine efficacy but may also drive the development of antigen tolerance [264]. On the contrary, longer peptides, from 20 aa in size, are taken up and intracellularly processed to be cross-presented in MHC-I molecules in APCs, and have proved to be superior at inducing anti-tumour responses [416]. In addition, LSP are much more appealing candidates for encapsulation in POs, as their enhanced DC targeting and IC delivery could further boost their immunogenicity. Since the MC38 NeoAgs used in the previous experiment were 9-11 aa in size [415], a longer peptide derived from the Adpgk MC38 protein was ordered to be synthesised, which contained the 9 aa core NeoAg sequence that included the single aa mutation, as well as a total of 10 flanking aa. These 19-aa (HLELASMTNEMLSSIVHQ) long-synthetic peptide, (Adpgk-LSP), was encapsulated in empty POs by electroporation (P(Adpgk-LSP)), as described in Chapter 3.

Mice harbouring MC38 tumours were immunised in a prime-boost schedule with Adpgk-LSP and Poly(I:C), P(Adpgk-LSP) and Poly(I:C), or with empty POs or PBS as negative controls (Figure 112A). Immunisations were performed i.v. and s.c. to assess whether prior findings in survival differences between immunisation routes (in the B16-OVA model), were also translatable into the MC38 model. Tumour growth curves and survival diagrams (Figure 112B and C, and appendix Figure 121 for individual tumour growth curves) indicated no overall benefit in tumour progression or survival of P(Adpgk-LSP) immunised mice, over naked peptide Adpgk-LSP immunised animals, regardless of the immunisation route. In addition, s.c. P(Adpgk-LSP) immunisations led to a median survival of 17 days, which did not increase survival time over PBS or POs control animals (also survived ≈17-18 days) (Figure 112D). On the other hand, s.c. Adpgk-LSP immunised mice survived, in median, 22 days, which also correlated with slower tumour
progression (Figure 112A). Interestingly, i.v. P(Adpgk-LSP) immunisations seemed to have an anti-tumour effect in the mice, as reflected by delayed growth of tumour mass and enhanced survival (Figure 112B, C), with animals surviving 24 days in median. Remarkably, i.v. naked peptide immunised animals did not show differences in tumour progression or survivals over s.c. immunised counterparts, and the median survival was of 22 days in both groups of mice.

![Figure 112](image)

**Figure 112.** Tumour progression and survival of mice harbouring MC38 tumours, following prime-boost s.c. and i.v. immunisations with the 19 aa long peptide neoantigen derived from the MC38 Adpgk protein (Adpgk-LSP). A) Schematic representation of tumour and vaccine regime. B) Tumour growth curves over time. C) Survival curves showing percentage of survival over time. D) Table showing median survival per group (in days). Error bars in B) represent mean ± SEM. Data from n=1 independent experiment (5-8 animals per group). Statistical analysis was performed used two-way ANOVA followed by Tukey post-hoc test (A) or Log-Rank (Mantel Cox) test (***p<0.001, **p<0.01).

In conclusion, these results suggested that, despite the fact that immunisations with P(Adpgk-LSP) did not provide with any benefit in survival or tumour progression over Adpgk-LSP treated groups, the route of administration did seem to have an impact on the efficacy of immunisations done with P(Adpgk-LSP), similarly to the trend observed
in B16-OVA tumours upon P(OVA) immunisations. Of note, his immunisation route did not seem to be relevant in the case of naked Adpgk-LSP immunised animals in tumour progression outcome, and differences between both animals group were negligible. These results also mirror the trends obtained in B16-OVA tumour growth and survival upon naked OVA s.c. or i.v. immunisations.

6.3. Discussion and conclusions

Conventional immunisation strategies based on subunit protein vaccines are performed through s.c. or i.m. administration routes, in combination with an adjuvant [417]. In both strategies, the presence of adjuvants is critical for generating an inflammatory context at the site of immunisation, which follows recruitment of innate cells, and their migration to LNs to prime adaptive responses [413], [414], [418]. Although studies using polymer-based cancer vaccines administered via s.c. verify NPs draining to LNs [285], [288], [291], these do not address the influence of APC activation and antigen presentation at the site of immunisation. Since our PMPC-PDPA POs-based vaccines led to enhanced anti-tumour immunity, it was paramount to address the biodistribution of POs-based vaccines as well as their effect in recruiting innate cells to the skin and to drain and activate cells in LNs.

6.3.1. On the subcutaneous immunisation mechanism of action: skin-dLN axis

Following 24 hours post injection, labelled PMPC-PDPA POs were found in vaccine dLNs and accumulated at the site of immunisation (skin), with minimal signal found in other organs such as the spleen or liver. This suggested that POs did not enter systemic circulation and instead effectively drained into LNs, which correlates with literature describing that NPs of 5 to 100 nm in size effectively enter lymphatic vessels [403], and suggests that POs can efficiently deliver NeoAg to LNs following s.c. injection. The fact that POs were found in skin at high concentrations may indicate that PMPC-PDPA POs act as antigen depots and provide with prolonged antigen delivery into the LNs. This can be hypothesised to be happening by a) continuous recruitment of innate immune cells
to the site of injection that take up POs to further deliver them into LNs or b) sustained PO draining to LNs. This is further reinforced by the fact that it was possible to visually identify skin thickening at the injection site in mice immunised by P(OVA) and Poly(I:C), but not OVA and Poly(I:C) treated animals, which lasted weeks (data not shown), perhaps correlating with an inflammatory response driving the recruitment of immune cells. Vaccination studies using water-in-oil as an adjuvant during s.c. immunisations, also reported this effect [413]. In fact, immunisation strategies that maintain antigen dosing in time, such as repeated injections or osmotic pumps have been used with the aim to provide continuous antigen uptake to DCs in the lymph nodes [417], and have correlated with enhanced B cell activation [419]. Thus, the hypothesis of a depot-like effect by P(OVA) immunisations would support enhanced anti-OVA IgG titres, as indicated by our results in the previous chapter. However, whether POs act as sustained antigen delivery systems should be further evaluated by addressing biodistribution at timepoints later than 24 hours.

6.3.1.1. Immune populations in skin

To detail the inflammatory process by which P(OVA) and Poly(I:C) vaccines triggered immune activation in skin, macrophage-like cells, B cells, DCs, T cells and NK cells were analysed 24 hours after vaccine injection. Results were compared to other animals immunised with POs and Poly(I:C) or OVA and Poly(I:C), since this would help understand the contribution of the vaccine components (PMPC-PDPA polymer, OVA or Poly(I:C)). No differences were found across NK cells, and although T cells seemed to increase upon PO injection, these should be further analysed as UMAP did not indicate these variations. Some changes were nonetheless found in APCs across treatments:

Macrophages: There was a clear correlation between the injection of POs and POs/P(OVA) and Poly(I:C) formulations and enhanced recruitment of macrophage-like cells in the skin, in a process that seemed to be independent of Poly(I:C). In fact, POs/P(OVA) and Poly(I:C) treated groups recruited fewer macrophages than animals injected with only POs. In any case, P(OVA) and Poly(I:C) treated animals presented higher macrophage proportions than OVA and Poly(I:C) treated groups, maybe suggesting stronger mounting of a immune response [417]. The expression of activation
markers such as CD80 did not significantly vary across groups, although the POs-only group seemed to express the highest CD80+ macrophages proportions (≈10%, compared to ≈5%). Regarding PD-L1+ macrophage-like cells in skin, higher values were found in PO or OVA and Poly(I:C) treated groups (≈25%), however clustering analysis revealed a more diverse macrophage-like population recruited in skin of mice immunised with POs-only, meaning that this population could be contributing to PD-L1 expression. Thus, no conclusions could be drawn into the contribution of POs, OVA or Poly(I:C) intro PD-L1 expression. In addition, 25-50% of macrophage-like cells seemed to have taken up POs, with no statistically differences observed across groups. However, Cy5.5+ macrophages upregulated CD80 and PD-L1 expression compared to macrophage-like cells which had not taken up the NPs in the empty POs injected group. When POs were administered in the presence of Poly(I:C) these differences surprisingly disappeared. Nonetheless, macrophage-like populations in this experiment are just defined as CD11b+, which could certainly include other types of myeloid cells. It could be hypothesised that these might be differentially recruited with or without the use of adjuvant in combination with POs, being these populations responsible for the changes in the expression of activation markers. In fact, UMAP analysis suggest that POs-only group recruit a population of “macrophage-like” subpopulation that is absent in POs/P(OVA) and Poly(I:C) groups UMAPs, which in the CD80/PD-L1 Phenograph analysis correlates with enhanced expression of these markers too. Further analysis with these subpopulations would be needed to understand if the CD80/PD-L1 populations found in POs would correlate with other myeloid cells, i.e. neutrophils or monocytes.

Summing up, P(OVA) and Poly(I:C) immunisations outperformed OVA and Poly(I:C) in the recruitment of macrophage-like cells in skin, which seemed to be in higher frequencies due to the presence of NPs. In addition, no changes in CD80+ or PD-L1+ macrophage-like cells were found between groups. Studies indicate the strong innate immune cell infiltration in the vaccine site, together with antigen uptake correlate with the quality of adaptive responses, supported enhanced priming processes in P(OVA) and Poly(I:C) treated animals [417], [420]
B cells: Although no significant changes in B cell numbers were found among treatments, B cells in the skin of POs-only injected groups were below 5% (of CD45+ cells), which is two to three times lower than in the other groups (10-15%). However, CD80+ and PD-L1+ B cell frequencies were significantly higher in PO-only treated group (75%) over the rest (10%), maybe indicating POs mediated activation and potential B cell migration [410], perhaps to blood or LNs although this should be further investigated. B cells in the skin, contrary to those in LNs, were also targeted by POs, which seemed to trigger CD80 and PD-L1 expression, which was enhanced by 50% or 100%, respectively, in Cy5.5+ cells compared to Cy5.5- populations. POs/P(OVA) and Poly(I:C) treated groups followed the same pattern for CD80, however the presence of Poly(I:C) did revert PD-L1 expression in B cells. This suggests an effect of empty POs in targeting and skewing B cell activation perhaps towards an anti-inflammatory type, that is counterbalanced by the presence of adjuvant. In fact, studies describe a drop in PD-L1+ B cell populations in patients suffering from the auto-immune disease rheumatoid arthritis (RA) [421]. This would go in agreement with previous work from Prof. Battaglia’s group members, indicating that empty POs can revert the inflammatory effect found in this same disease [310]. In any case, further experiments would be required to confirm this hypothesis.

Concerning differences between B cell activation between P(OVA) or OVA and Poly(I:C), no differences could be found in terms of numbers or CD80 expression. PD-L1+ B cells, however, were ≈5% higher in OVA and Poly(I:C) treated mice vs P(OVA) and Poly(I:C) treated groups.

DCs: In skin, frequencies of DCs among total CD45+ cells remained higher in PBS controls than the rest of the groups, suggesting DCs from treated animals had migrated to LNs [422]. In OVA/POs/P(OVA) groups injected with Poly(I:C), percentages of CD80+ DCs were around 50%, approximately double than empty POs or PBS treated groups. This indicated the adjuvant-like effect of Poly(I:C), as previously reported in other vaccine settings [423]. PD-L1+ DC proportion were also enhanced in the same groups to equivalent levels (≈75%). Thus, no differences were found in skin DC numbers or activation, following OVA and Poly(I:C) or P(OVA) and Poly(I:C) immunisations that could explain differences in vaccination outcomes. In addition, similar to B cells or
macrophages, empty POs also increased PD-L1 expression in DCs cells, however, CD80 expression was much lower. This might further implicate a role for PMPC-PDPA POs in the absence of adjuvant or antigen as anti-inflammatory agents.

### 6.3.1.2. Immune populations in LNs

In LNs, the main differences in cell proportions across treatments were found in NK, DCs, and macrophages.

**NK:** the use of Poly(I:C) in combination with POs/OVA/P(OVA) reduced the proportion of NK cells from 1% (in PBS or POs only groups) to 0.5%. However, this did not correlate with any changes in numbers in the skin, thus it is hard to draw any conclusions from the contribution of this cell population at the time of priming.

**Macrophages:** Macrophages in LNs are key for the uptake of antigens travelling in the lymph, and their transfer across LN compartments to help in the antigen presentation to B cells, T cells and even DCs [424]. In our results, the number of macrophage-like cells were decreased in all groups (≈0.3%) compared to PBS controls (≈0.5%). However, to our knowledge macrophages in LNs are resident, and do not migrate to other tissues. Thus, a more detailed analysis including other markers would be necessary to understand the presence and contribution of other CD11b+ cell populations, such as neutrophils, in these numbers [413][425]. All treatment groups saw an increase in CD80+ macrophage populations (all around ≈10%) compared to the PBS control (0%). A similar trend was observed for PD-L1, where PD-L1+ macrophages constituted ≈25-30% in all treated groups, whereas in the PBS control the percentages were below 2%. In addition, 40% of macrophages-like cells in dLNs had taken up POs, indicating either efficient PO draining to the LNs and/or POs being delivered by other myeloid like cells, such as monocytes or neutrophils.

Thus, OVA and Poly(I:C) or P(OVA) and Poly(I:C) groups did also not report differences in macrophage numbers or activation in LNs after 24 hours of injection. Nonetheless these data suggest that POs per se could induce CD80 and PD-L1 expression in
macrophages, and that stimulation Poly(I:C) or the presence of antigen did not further enhance this activation.

**B cells:** In skin, mice treated with empty POs showed reduced numbers of B cells, however a higher proportion of these were PD-L1+. Although this reduction in B cell frequencies was hypothesised to caused by their migration to other tissues, LNs of PO-only treated mice did show enhanced B cell frequencies compared to the rest of the treatments. In addition, B cells in LNs did not uptake POs, indicating that the Cy5.5+ B cells found in skin had not migrated to LNs.

B cells did not express CD80 in LNs and also no PD-L1 differences could be found between OVA Poly(I:C) or P(OVA) and Poly(I:C) treated animals, suggesting differential vaccination outcomes between these two groups in the context of B16-OVA might not have been directly influenced by B cells stimulation at the time of priming. However, differences in B cells activation could be appearing at later timepoints than 24 hours. Physiologically, the lack of CD80 expression is according to literature indicating that CD80 expression in B cells is upregulated upon second antigen encountering [426]. In LNs, B cells reside in B cell follicles where they are activated following lymph-borne antigen encountering or with the help of follicular DCs and CD4 T follicular helper cells (Tfh), which simultaneously are activated by migratory cDC2 cells [1]. Since no PO uptake was found in B cells, the high IgG titres found in mice immunised with P(OVA) and Poly(I:C) must be related to cDC2 and CD4 Tfh mediated B cell activation. Accordingly, enhanced cDC2 values were found in LNs from P(OVA) and Poly(I:C) mice over the rest of the groups, including OVA and Poly(I:C).

**DCs:** Coincidental with the low DC numbers in skin, migDCs in dLN were increased in PO/OVA/P(OVA) groups injected with Poly(I:C), over PBS or PO only animals. 10% of migDCs delivered POs into LNs, and similar values were obtained for ResDCs, suggesting that either POs drain into lymphatic vessels to reach LN, or that POs transfer from migDCs to resDCs. As reviewed by Irvine *et al* [403], the DC antigen uptake in LNs following immunisation strategies in the absence of NPs, and in combination with adjuvants, usually remains below 2%. Next, 75% of migDCs in adjuvant-combined groups were CD80+, compared to 50% found in the LNs of PO or PBS groups, which evidenced
the effect of Poly(I:C) on DC maturation. PD-L1+ migDCs frequencies were just slightly higher in OVA and P(OVA) and Poly(I:C) treated groups (≈80%) compared to PBS/POs groups (75%), which is in line with literature revealing the need for PD-L1 in migDCs to migrate to LNs [218]. Similar trends were found in resDCs across groups, although interestingly, percentages of PD-L1+ resDCs in control (PBS) groups were half (25%) than in the rest of the groups, including PO treated groups. This reinforced the potential of POs to induce PD-L1 expression in DCs, as found in skin. DC analysis revealed that cDC2s were the most abundant subtype in LNs across treatment groups, with P(OVA) and Poly(I:C) groups reporting the highest proportions across groups. Differences were statistically significant with the rest of the groups except for OVA and Poly(I:C) treated groups, although the trend was still in highest proportions. Whereas cDC2 in LNs support the development of CD4 T helper responses to enhance efficient CD8 T cell responses and B responses [67], cDC1s are responsible for direct cross-priming and activation of CD8 T cells [372]. A trend in higher cDC1 frequencies was found in P(OVA) and Poly(I:C) treated groups compared to the rest of the treatments, in both resDCs and migDCs. In addition, cDC1s cross-presented OVA (H2Kb-SIINFEKL) in OVA/P(OVA) treated groups, indicating that OVA was reaching LNs in both groups.

Thus, a trend in higher proportions of migDCs and ResDCs in the P(OVA) and Poly(I:C) group compared to OVA and Poly(I:C) group was found in LNs. These cells expressed high levels of CD80 and PD-L1, which were similar between both treatment groups. Moreover, cDC2 and OVA-cross-presenting cDC1 populations in P(OVA) and Poly(I:C) were slightly more abundant than in OVA and Poly(I:C) treated groups. In all, these small differences in cDC1/2 between OVA and Poly(I:C) and P(OVA) and Poly(I:C) treated groups, might support an enhanced CD8 T cell priming mechanism in the latter group.

6.3.1.3. Conclusions: subcutaneous P(OVA) and Poly(I:C) priming-mechanism of action

In conclusion, s.c. administration of P(OVA) and Poly(I:C) NPs enhanced the recruitment of macrophage-like cells in the skin over OVA and Poly(I:C) treated animals. Macrophages, B cells and DCs mediate P(OVA) uptake in the skin, and the latter migrate
to dLNs. This translates into higher frequencies of migDCs and resDCs cross-presenting antigen compared to LNs from OVA and Poly(I:C) treated animals. No changes in CD80 or PD-L1 expression in APCs were found in LNs between OVA or P(OVA) and Poly(I:C) treatment groups, indicating that Poly(I:C) rather than POs likely control the overall activation of these cells. In this regard, Poly(I:C) induced CD80 expression in APCs, in both lymph nodes and skin, alike other adjuvants used during s.c. immunisations, such as alum, oil-in water or TLR7 stimulators [413], [414], [427].

6.3.2. On the PO immunisation route: differences in targeting and anti-tumour therapeutic effect.

Accumulating evidence suggests that effective anti-tumour immune responses are elicited on a systemic level following immunotherapeutic approaches [383][213], including vaccination [406]. Our results indeed corroborated that i.v. administration of P(OVA) and Poly(I:C) vaccine in mice harbouring B16-OVA melanoma resulted in delayed tumour growth and enhanced survival in 40% of animals, over equivalent s.c. immunisations. Enhanced survival following i.v. over s.c. immunisation was also observed in a NeoAg setting within the MC38 colorectal model, resulting in a 7-day lifespan extension of mice following intravenous P(Adpgk-LSP) immunisations. In contrast, i.v. immunisations with free OVA or free Adpgk-LSP did not enhance survival compared to s.c. counterparts. This could be related to their smaller sizes and their lack of protection by POs or DC-targeting moieties, which altogether would decrease their stability and half-life in blood [357]. In a similar work, vaccination was performed by peptide-adjuvant assemblies (20 nm in size) based on the MC38 NeoAg Reps1, in mice harbouring MC38 tumours. The authors found that i.v. immunisations enhanced the presence of TCF1+ CD8 T cell progenitors in the spleen and blood compared to s.c. immunisations, and that these expanded upon combinatory vaccine + α-PD-1 approaches, resulting in improved anti-tumour responses [406]. Indeed, our study matches these results and show that i.v. P(OVA) vaccination in combination with α-PD-1 further enhanced survival and controlled tumour development totally in 10% of the animals compared to vaccine only groups. Although the contribution of anti-tumour CD8 T cell responses from systemic organs (i.e. spleen or mucosal tissues) has not been
analysed in our studies, SIINFEKL-reactive CD8 T cell clones in blood doubled from 2.5% to 5% in animals immunised with P(OVA) s.c. or i.v., respectively. In addition, combinatorial α-PD-1 treatment in animals vaccinated with P(OVA) s.c. or i.v. further enhanced these values to ≈3% and ≈7.5% respectively. The presence in blood of these vaccine-induced CD8 T cell clones, especially upon i.v. immunisations, might indeed indicate that peripheric organs, perhaps the spleen or other mucosal tissues, act as a reservoir for these cells that would travel to tumours through the blood following their activation by vaccination/immunotherapy. In line with this, biodistribution experiments at 24 hours following i.v. PO injection indicated that the spleen, liver and LNs are the organs retaining highest levels of POs, and at this timepoint POs could also be detected in the blood. Among the targeted immune cells, POs in LNs were mainly taken up by DCs and macrophages, similar to what was found in the spleen and blood. Although s.c. PO injection also drove their uptake into the LN by APCs, no PO signal could be found in the spleen or blood. This suggests that the enhanced APC targeting in peripherical organs following i.v. PO immunisations could drive the priming of adaptive responses. In addition, other mucosal tissues such as the omentum, genital tract or intestines, which are very rich in immune cells also contained POs 24 hours after i.v. injection, which could be all contributing to priming adaptive responses, which could confer with anti-tumour protection in the context of cancer.

6.3.3. Limitations of the study

Despite the differences in biodistribution observed following s.c. or i.v. immunisations with PMPC-PDPA POs for OVA delivery, we lack information on the biodistribution of naked OVA following s.c. or i.v. injections. This would provide a deeper understanding into mechanistical differences in tissue targeting of antigens delivered naked or entrapped into POs. In addition, although i.v. immunisations with P(OVA)/Poly(I:C) resulted in enhanced mice survival compared to OVA/Poly(I:C) vaccinations at equivalent protein dosages, a benchmark titration experiment would inform the i.v. naked-OVA dose needed to match the anti-tumour effect achieved following P(OVA) i.v. administration.
6.4. Appendix

6.4.1. Relative expression of phenotype markers in skin and skin-dLN UMAPs

A. Clustering skin

1. Macrophage-like (CD11b+, MHCII-)
2. DC (cDC2) -like (CD11c+CD11b+ MHCII+)

Also, CD19+

3. B cells (CD19+ MHCII+)
4. T cells (CD3+)
5. ¬ (memory B cells?) (CD19+CD11b+CD11c+)

B. Global particles Global MHC-II Global CD19

Global CD11b Global CD11c Global CD3

Figure 113. UMAP plot showing relative expression of phenotyping markers used for distinguishing myeloid and T cell clusters in skin.
Figure 114 UMAP plots showing relative expression of phenotyping markers used for distinguishing myeloid and T cell populations in skin-dLN.
6.4.2. Gating strategies for skin and skin-dLN immune populations

Figure 115. Gating strategies followed for supervised FACS analysis in A) skin B) skin-dLNs.
6.4.3. IVIs images of Cy5.5-POs in organs

Figure 116. IVIs analysis of Cy5.5-POs fluorescence in organs 24 hours after s.c or i.v. injection.
6.4.4. Gating strategies for PO uptake in skin and skin-dLN immune populations

A.

B.

C.

Figure 117. Gating strategies followed for Cy5.5-POs uptake analysis in skin, skin-dLN, spleen, liver, lung and blood.
A) Gating strategy followed for the calculation of Cy5.5 frequencies in A) total tissue cells B) CD45+ or CD45- cells C) B cells, DCs, NK cells, T cells, macrophages or other myeloid cells.
6.4.5. Gating strategies for SIINFEKL-reactive CD8 T cells in blood

Figure 118. Gating strategies followed for SIINFEKL-reactive CD8 T cells in the blood of B16-OVA harbouring mice.
6.4.6 Individual tumour growth curves

Figure 119. Individual B16-OVA tumour growth curves following s.c. or intravenous vaccination in a prime-boost regime, as A) monotherapy or B) i.v. treatments in combination with α-PD-1.
Figure 120. Individual MC38 tumour growth curves following s.c. prime-boost vaccination with 9 aa length MC38 neoantigens included in Adpgk, Reps1, Dpagt, and Rpl18 proteins.
Figure 121. Individual MC38 tumour growth curves following s.c. or i.v. prime-boost vaccination with 19 aa length MC38 neoantigen coded within the Adpgk protein (Adpgk-LSP)
Chapter 7: General conclusion and future perspectives
7.1. General conclusion

The development of effective cancer vaccines would constitute a turning point in its treatment. However, within the last 10 years, only one cancer vaccine (Sipuleucel-T), has been approved and commercialised. In addition to refining tools for NeoAg discovery, increasing awareness is being raised to the need for improved vaccine delivery technologies that enhance cargo stability and DC targeting and IC delivery in vivo.

The work in this thesis supports PMPC-PDPA POs entrapping subunit protein and peptide NeoAg as safe and efficient nano-vaccines targeting DCs and enhancing cytosolic delivery, translating into improved in vivo anti-tumour therapeutic effects over vaccines based on naked antigens.

Briefly, PMPC-PDPA POs were first assessed in vitro, where efficient priming of antigen-specific CD8 T cells was achieved by P(OVA) with and without Poly(I:C) pulsed BMDCs. Second, preclinical in vivo studies on the murine melanoma B16-OVA model revealed enhanced anti-tumour CD8 TILs responses upon prime-boost s.c. P(OVA)/Poly(I:C) over free OVA/Poly(I:C) immunisations. This correlated with extended survival and delayed tumour growth rates in P(OVA)/Poly(I:C) immunised mice, which was further enhanced in combination with α-PD-1 therapy. Tumours from P(OVA)/Poly(I:C) + α-PD-1 treated animals were infiltrated by highly cytotoxic and proliferative CD8 TILs, presenting higher percentages of antigen-reactive clones, proving efficient vaccine priming.

Third, the s.c. P(OVA)/Poly(I:C) priming mechanism was studied, finding it was mediated trough recruitment of macrophage-like cells in the skin, which together with DCs took up P(OVA) and travelled to skin dLN, where migDCs delivered the POs. In addition, P(OVA) POs seem to diffuse directly to the LNs and target resDCs. Both migDCs and resDCs proportions in dLN from P(OVA)/Poly(I:C) immunised mice were higher than in the rest of the groups, with CD80+ PD-L1+ cDC1 cross-presenting OVA, suggesting potential for driving efficient T cell priming.

Fourth, this thesis unveiled the influence of POs immunisation route on immune response and tumour rejection outcome. Accordingly, P(OVA)/Poly(I:C) therapeutic vaccination via i.v. administration in mice harbouring B16-OVA tumours enhanced
antigen-reactive CD8 T cells in blood and correlated with improved survival and delayed tumour growth over s.c. P(OVA)/Poly(I:C) vaccinations and over i.v. injections with OVA/Poly(I:C). In addition, P(OVA)/Poly(I:C) i.v. immunisations in combination with α-PD-1 therapy controlled tumour growth in 20% of animals, an effect which could not be achieved by i.v. immunisations with free OVA/Poly(I:C) + α-PD-1.

The therapeutic immunisations with P(Adpgk-LSP)/Poly(I:C) in mice harbouring MC38 colorectal model failed to improve survival outcomes over groups immunised with equivalent doses of free Adpgk-LSP peptide and Poly(I:C). However, i.v. immunisations with P(Adpgk-LSP)/Poly(I:C) did enhance mice survival over s.c. immunised counterparts, alike the pattern followed by P(OVA)/Poly(I:C) POs. Finally, POs biodistribution following i.v. injections were found widely across organs, especially spleen, liver, LNs and other mucosal tissues. In addition, POs specifically targeted APCs in spleen, blood and LNs suggesting that in the context of cancer, therapeutic i.v. immunisations would enhance the priming of tumour-reactive CD8 T cells in peripheric organs, contributing to improved vaccine outcomes.

7.2. Future perspectives

Alongside promising results, challenges and observations have arisen across the project that require further attention and investigation, as described in this section.

7.2.1. POs vaccine design: size, dose and co-encapsulation

The first milestone of this thesis was to generate reproducible and homogeneous POs formulations encapsulating OVA and Adpgk-LSP. Self-assembly by FH method resulted on large number of tube-like structures, and therefore this technique was not further explored for payload encapsulation. Next, OVA encapsulation by pH-switch resulted on polymer-protein-like aggregates of sizes larger than 100 nm, which made them unsuitable for the porpoises of s.c. vaccine administration and LN drainage. Adpgk-LSP encapsulation was attempted by SS at three different initial concentrations within the self-assembly reaction. In all cases, POs production efficiency was very low (up to 40%), and a large part of the polymer being lost during the SEC. In addition, after SEC followed
by HPLC quantification only the formulation with highest peptide concentrations indicated presence of peptide. However, two peaks were detected in DLS (29 nm and 70 nm), which raised the question on whether the smallest peak could be peptide aggregate. Finally, in both cases, the most reproducible encapsulation method was EP into empty POs obtained by SS. However, protein or peptide encapsulation efficiency by EP is limited by several factors. First, EP might drive protein or peptide aggregation derived from the electrical field and pulses applied during the process [307], [327]. In addition, POs size, particularly the lumen volume, might limit the number of encapsulated protein/peptide molecules. This limit has a direct repercussion on the amount of peptide or protein that eventually is injected as immunogen during a vaccination, which might directly interfere on the generation of effective immune responses. In fact, as discussed in chapter 5, immunisation with other polymer-based POs use larger amounts of OVA or SIINFEKL and result indeed on improved anti-tumour responses [287]–[290]. Although for s.c. immunisations optimal POs size should be below 100 nm for efficient draining to LNs, this factor might not be as critical in the context i.v. immunisations, as biodistribution in SLO such as spleen, or in other mucosal tissues is dictated by the route of systemic circulation. Thus, it would be worth exploring techniques than can enhance encapsulation efficiencies, even in POs larger than 100 nm. For instances, the P(OVA) formulations obtained by pH-switch which contained bigger polymer-protein aggregates could perhaps be of use as nano-vaccines in the context of i.v. immunisations. Despite this, it should also be considered that previous studies performed by ex-members of Prof.Battaglia’s group indicate that the size of POs can interfere with endocytosis. In this sense, POs over 200-400 nm show worse endocytosis rates compared to smaller POs in vitro, in human dermal fibroblasts [333]. In addition, in vitro studies performed in neutrophiles and epithelial cells showed that tubular structures (≈200 nm in length) also are endocytosed with lower efficiencies than vesicles up to 100 nm in diameter, although still with higher frequencies than vesicles larger than 200 nm in diameter [321]. In all, this evidences that it is possible to modulate POs uptake by modifying their sizes, hence this should be taken in consideration for designing further antigen encapsulation strategies in POs for delivery in DCs.
On another note, long peptide and personalised vaccines currently being explored in clinical trials comprise a pool of up to 20 different NeoAg per patient [254]. In this thesis, therapeutic vaccination experiments performed against the colorectal MC38 model were just based on one NeoAg (Adpgk-LSP). Thus, it could be beneficial to consider co-encapsulation of a pool of peptides in one PO and understand whether this multiple co-delivery would benefit immunisation outcomes. In addition, encapsulation of mRNA coding for multiple NeoAg could be an alternative, similar to the strategies being followed by BIONTECH anti-tumour vaccines based on lipid NPs [428][31]. In fact, both strategies (encapsulation of NeoAg peptide pools and RNA) were explored in parallel to this thesis (data not shown), however this proved challenging. On the one hand, differences on peptide solubilities can easily translate on peptide aggregation at the time of self-assembly (i.e., during SS or EP). On the other hand, RNA size seemed to be an issue for their encapsulation and release by EP in POs obtained by SS.

Another aspect that could be worth exploring is adjuvant encapsulation in the PO, ideally in combination with the antigen, to provide with target and co-delivery into APCs. During i.v. immunisations, the systemic administration of free adjuvant entails a potential risk of disruption of tolerance against self-antigens, and of irAEs development. Despite this, in our i.v. immunisation experiments no evident toxicities/distress signs could be detected in animals a priori.

### 7.2.2. DCs targeting: Including range selectivity in POs

A key requisite to validate PMPC-PDPA POs as vaccine nano-carriers was that they could target and deliver antigen into APCs, specially DCs. Previously in the group, POs uptake had been assessed in macrophages [310], [311] and cancer cells [309], [429], finding that it was mediated by the contribution of SR-B1, CD36 and CD81 receptors [301]. In this study, the potential of PMPC-PDPA POs to target DCs was also verified, in vitro and in vivo. In vitro, both DC2.4 and BMDCs could take up POs after 15 mins of incubation. In vivo, s.c. and i.v. injection of labelled POs efficiently targeted DCs and macrophages in LN, spleen, blood, skin and liver. This uptake was remarkably selective in DCs and macrophages over other immune cells (i.e. T cells, B cells or NK cells) in many cases,
especially in spleen, LNs and blood. Nonetheless, up to 20% of tissue cells (non-immune cells) could also take up POs in organs such as skin or spleen. Although this did not interfere later with the development of efficient priming of anti-tumour T cell responses when POs were used as vaccine nano-carriers, there is room for improving POs-DC targeting. In this sense, it would be valuable to further define the APC populations that take up PMPC-PDPA POs in skin or LNs following s.c. immunisations, perhaps by RNAseq, to find endocytic receptors differentially expressed on these cells over CD45- cells (non-immunological) that also take up POs. This would help define ligands targeting these receptors on APC, that could be conjugated to PMPC-PDPA to skew POs binding towards APC cells. In fact, some students at Prof. Battaglia’s group have been performing in vitro screens to find range selectivity for POs-APC targeting using similar polymer-ligand formulations, which indeed would be of use to translate in vivo in the context of vaccine.

7.2.3. POs IC delivery of antigens and cross-presentation

One of the enabling features of PMPC-PDPA POs is that they can deliver OVA intracellularly. It was possible to track OVA IC delivery in DC.4 following P(OVA) incubation by live imagining and confocal IF. OVA IC delivery by P(OVA) samples correlated with enhanced H2Kb-SIINFEKL+ cells over naked OVA counterparts (all in the presence of Poly(I:C)) meaning POs delivery could improve cross-presentation in DC2.4 cells. Cross-presentation by P(OVA) and Poly(I:C) formulations was also found in dLN DCs in vivo, 24 hours post-vaccination. However, the exact mechanism by which P(OVA) provided with cross-presentation both in vitro and in vivo has not yet been characterised. The better characterised route of antigen cross-presentation involves cytosol delivery and proteasomal processing [75], followed by ER translocation and MHC-I complexation, however this was not corroborated in this study. Some alternative theories suggest the presence of MHC-I molecules anchored in late endosomal compartments, which would bind proteolyzed peptides generated following protein cleavage by endosomal enzymes and acidic conditions, following uptake [81]. Thus, analysis to understand the contribution of each of these pathways in cross-presentation of protein delivered by POs would need further addressing. This could be for instances performed by actively blocking proteasome or TAP at the time of POs incubation and
antigen presentation [430]. Indeed, understanding better this process could have implications in the vaccine design: if the endosomal MHC-I-Ag complexation route was favoured over proteasomal processing, this would bypass the need for proteasomal processing and POs could just be loaded with peptides of 9-11 aa that can straight bind to MHC-I molecules.

7.2.4. Inflammatory response following s.c. POs-mediated immunisations.

7.2.4.1. POs-APC activation in vitro and in vivo:
The increased effect of P(OVA) in SIINFEKL cross-presentation in DC2.4 cells, in vitro, also translated into enhanced proliferation of SIINFEKL-reactive CD8 T cells (OT-I CD8 T cells) in co-cultures, over equivalent naked OVA treatment. Whereas in the presence of Poly(I:C) both OVA and P(OVA) treatments primed CD8 T cells, in Poly(I:C)-free conditions only P(OVA) triggered OT-I proliferation. This could imply that P(OVA) drive antigen cross-presentation and act to some extent as adjuvant per se, inducing BMDC maturation and the expression of co-stimulatory ligands (i.e. CD80, CD86, CD40) needed for efficient T cell priming [71]. However, results from in vitro cross-presentation and activation analysis in DC2.4 in the absence of Poly(I:C) were unconclusive in this regard, due to variability between experiments.

Nonetheless, maturation markers in DCs, macrophages and B cells were assessed in vivo in skin and LN, 24 hours following s.c. immunisations. In skin, empty POs seemed to enhance CD80 expression of macrophage-like cells and B cells over treatments combined with Poly(I:C), including P(OVA) and Poly(I:C). The same trend was observed for PD-L1 in B cells and macrophage-like cells. In DCs, however, CD80 and PD-L1 expression seemed to be mainly driven by the presence of Poly(I:C), with no differences in the proportions of CD80+ or PD-L1+ DCs found between OVA and Poly(I:C) and P(OVA) and Poly(I:C) treated groups. With these results, it could be hypothesised that PMPC-PDPA polymer might have to some extent an adjuvant-like effect in some cells over others. Indeed, it would be interesting to also pursuit investigation in this line. B cells [431], macrophages [432] and DCs [433] express different PRRs patterns, which have been defined evolutionary and are key for the functions of each immune cell. As
reviewed by Weiss et al. [434], some synthetic polymers can innately act as immune-stimulators, through different mechanisms. For instances, the induction of “NACHT, LRR, and PYD domain-containing protein 3” (NLRP3) also known as NLRP3 inflammasome, has been associated with polymer-based nanoparticles. NLRP3 gets activated following potassium efflux that might be driven by a number of agents that act as DAMPs, including ROS, extracellular ATP or lysosome disruption. NLRP3 responses eventually drive a signalling cascade that drive the expression of cytokines and inflammatory cell death [434], and despite being associated with pathologies such as Alzheimer’s, its study is gaining attention in the field of vaccine [435], [436]. Thus, it would be interesting to perhaps study the differential expression of inflammasome between B cells, DCs, and macrophages following POs treatment, to understand their potential as self-adjuvants. On the other hand, it should not be overseen that all the priming in vivo experiments were just performed once, thus before driving these types of analyses it would be interesting to understand the reproducibility of these results. In addition, it would be worth it to understand the differential cytokine production by each cell following different treatments. However, it should be noted that enhanced CD80 expression by empty-POs adjuvant/activator in macrophages and B cells found in these experiments did not enhance anti-tumour therapeutic effect in vivo, in B16-OVA or MC38-bearing animals. Perhaps this is due to the enhanced co-expression of PD-L1 in these cells too, which although controversial, might contribute to tolerant-like immune responses.

7.2.4.2. Skin-dLN axis activation following s.c. immunisations

In our analysis in skin at 24 hours after s.c. immunisations, it was possible to detect a myeloid population, defined as “macrophage-like” being recruited in animals injected with POs (POs, POs and Poly(I:C) or P(OVA) and Poly(I:C)), which was not recruited in mice injected with PBS or OVA and Poly(I:C). In addition, these cells seemed to uptake large amount of the POs. Since this indicates that the presence of PMPC-PDPA POs mediates the recruitment of this population, further experiments should be aimed at defining what these cells are, and what is their contribution in the priming process at the level of dLN. In this sense, DCs played a key role on the P(OVA) uptake in skin and migration to dLN, where they showed a CD80+PD-L1+ phenotype and cDC1 subtypes
were cross-presenting OVA, likely suggesting priming of CD8 T cells could take place. Although in this set of experiments CD8 T cell priming was not analysed, a correct antigen presentation by mature DCs in LNs could indeed explain the presence of enhanced tumour-reactive CD8 T cell responses achieved following P(OVA) immunisations in B16-OVA bearing mice, in parallel experiments. In addition, dLNs of P(OVA) and Poly(I:C) immunised mice counted on the highest proportions of cDC2 populations across treatments, which at the same times were 4 times higher than cDC1s.

cDC2 in LNs contribute to the priming of CD4 Tfh cells, which support B cell co-stimulation in B cell follicles. Simultaneously, follicular DCs in LNs can directly present antigen to B cells, which altogether drive B cell activation, generation of plasma cells and IgG production [1]. In addition, it is understood that extended antigen presentation from follicular DCs to B cells might enhance this process and IgG production [437].

Coincidently, P(OVA) and Poly(I:C) s.c. immunisations in B16-OVA bearing mice drove 100 times higher anti-OVA IgG titters than what was found in OVA and Poly(I:C) vaccinated mice, in blood. Reasons for this were not investigated but it would be critical to understand if this is a result of priming performed with OVA entrapped in POs. In this sense, it would be important to address whether P(OVA) formulations directly target follicular DC by direct draining, or whether migDCs arriving from the skin might have some role on this priming. Evidence suggests that proteins (including vaccine antigens) arriving into the LNs undergo proteolysis, and that this can unpair the development of efficient antigen-specific B cell responses [438]. In contrast, antigens that are captured by follicular DCs remain intact for weeks within LNs [439], providing with enhanced B cell and responses. Using a HIV immunogen, Irvine et al have proved that nano-vaccine strategies targeting these follicular DCs lead to enhance antigen-specific titters in serum, as well as the formation of germinal centers in LNs, which are required for B cell maturation and production [440]. From another angle, sustained antigen release following vaccination is thought to contribute to a continuous follicular DC priming and enhanced IgG responses [417], [441]. In this regard, observations in our vaccination system indicated that POS and P(OVA) and Poly(I:C) injection in the skin drove skin thickening on the injection site, that would persist for weeks. It would need to be investigated whether this sustained inflammation could be in fact controlling antigen release and drainage to the dLNs. This perhaps could be a reason why enhanced IgG
responses are generated over free OVA immunisations, where the protein seems to diffuse in a much faster way or at least, it does not generate skin inflammation. Shedding light in this aspect could indeed have implications in the potential translation of PMPC-PDPA POs as prophylactic vaccines nanocarriers also for the porpoises of infectious diseases.

7.2.5. P(OVA) anti-tumour therapeutic effect and immune response

Finally, B16-OVA bearing mice immunised with P(OVA) and Poly (I:C) in a prime-boost schedule delayed tumour growth and enhanced survival over free OVA and Poly(I:C) immunised groups. However, tumours were eventually escaping, and the full mechanism for this is remains to be deciphered. Tumour escape took place despite double proportion of CD8 TILs being achieved in P(OVA) and Poly (I:C) groups, as well as striking 20 times higher CD8/Treg ratios. We first hypothesised that escape could be due to CD8 TIL exhaustion, as 50% of these co-expressed PD-1 and TIM-3 in Tumour-reactive CD8 TILs, including SIINFEKL-reactive clones. In addition, enhanced proportions of PD-L1-expressing TAMs and Monocytes were found in our group of interest. Combinatory s.c. P(OVA) and Poly (I:C) vaccination and α-PD-1 still provided with further delayed tumour progression, however tumours were finally escaping. Although α-PD-1 treatment did not provide with enhanced total CD8 TIL infiltration in tumours from P(OVA) and Poly (I:C) treated mice, SIINFEKL-reactive CD8 TILs did increase, and overall α-PD-1 reinvigorated CD8 TILs cytotoxic and proliferative capabilities across treatment groups. Nonetheless, α-PD-1 treatment did not reduce the markers such as TIM-3 or LAG-3 in P(OVA) and Poly(I:C) treated animals. Thus, it should be addressed whether combinatory treatments with other ICI such as α-TIM-3 could contribute to improved outcomes in this regard. This combinatory strategy is being used in the clinic in patients with resistance to PD-1 blockade in a number of tumours, with positive outcomes [377]. However, it should be noted that the frequencies of TOX+ CD8 TILs (global and SIINFEKL-reactive) were enhanced following α-PD-1 over P(OVA) and Poly(I:C) vaccine only counterparts. According with literature, this is indicative of a terminal and non-reversible exhaustion differentiation state of CD8 TILs [442], whereby combinatory ICI
approaches might not guarantee reversion to a progenitor-exhausted or effector phenotype, thus still not allowing for tumour control. In this regard, it would be of use to perform more in-depth analysis, perhaps using RNAseq, to confirm the presence of progenitor exhausted CD8 TILs (TCF1+), which are necessary for positive ICI outcomes [395]. As explained in chapter 1 and in chapter 5, the presence of T cell progenitors is not only important in tumours but are also critical in SLO for efficient ICI responses. Accordingly, our results indicated that following ICI, SIINFEKL-reactive CD8 T cells in LNs were 2 to 3 times more abundant over vaccine-only counterparts, across groups. In addition, the expression of TOX, GZMB or exhaustion markers was drastically reduced in CD8 T cells in dLN. Experiments should be performed to analyse the differentiation phenotype (naïve, effector, central memory or stem cell/terminally differentiated exhaustion precursors) of T cells in LN to address how P(OVA) and Poly(I:C) modulate the priming and differentiation of these cells. As explained in chapter 6, enhanced survival and tumour growth outcomes were achieved upon i.v. P(OVA) and Poly(I:C) immunisations, which translated into tumour control in 20% of animals following ICI. This also clearly correlated with enhanced SIINFEKL-reactive CD8 T cells in blood. On the other hand, biodistribution analysis following i.v. POs injections indicated that the majority of POs accumulated within spleen and targeted APCs. Hence, it would be interesting to investigate to which extent APC targeting and T/B cell priming on spleen contributed to the enhanced anti-tumour therapeutic effect found upon P(OVA) and Poly(I:C) i.v. vaccination. This could indeed evidence the potential of nanomedicine to modulate immune responses just by varying parameters such as immunisation route.

To finish with, it would be worth exploring two aspects that for the porpoises of time have not been addressed in this thesis. The first one is the physical distribution of immune cells within the tumour mass. This is important given that CD8 T cells need to access to tumour bed for efficient tumour rejection. However, T cell infiltration inside the tumour bed can be comprised by the extracellular matrix, or even the vasculature surrounding the tumour [443]. Thus, analysing tumour sections by techniques such as multiplex immunofluorescence [444] could help understand whether tumour escape could be due perhaps to T cell “exclusion” from the tumour bed. Second, increasing evidence is pointing at the contribution of tertiary lymphoid organs (TLO) within the
TME towards anti-tumour responses. These are compartments found in solid tumours (although not in all patients) that recapitulate the structure of SLO, with differentiated T and B cell zones. While many questions remain unanswered regarding the dynamics of the formation of these structures, the presence of at least 2 TLO per tumour is associated with better outcomes in patients. Importantly, the presence of B cells infiltrating tumours seem is also associated with increased anti-tumour responses [445]. Vaccination strategies have been associated with increased TLO formation [446] thus, it would be key to characterise these structures in B16-OVA tumours across treatments, and to potentially investigate whether the enhanced IgG titters found in blood in P(OVA) and Poly(I:C) immunised mice are related with their presence.
Todos los altibajos de la marea

Tantas encrucijadas quedan detrás (...)

Ya está en el aire girando mi moneda

Y que sea lo que sea
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