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PLASMIDS ENCODING VIRAL STRUCTURAL PROTEINS TO ENHANCE CANCER DNA VACCINE POTENCY

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Foreword

Cancer is one of the leading causes of death worldwide. In the last decades, cancer immunotherapy has emerged as a new cornerstone in cancer treatment. At the core of this approach lies the fact that cancer patients can produce T lymphocytes that recognize and eliminate cancer cells bearing tumour-specific antigens. Several vaccines strategies have been developed. Particularly, DNA vaccines delivered by electroporation are gaining momentum and are currently under deep (pre)clinical investigation. However, their immunogenicity should be improved to render them clinically applicable.

To enhance cancer DNA vaccine efficacy, an innovative strategy consists in the use of plasmid encoding viral structural proteins. We hypothesize that these plasmids could improve vaccine potency by stimulating innate and adaptive immunity to strengthen the immune responses to the vaccine antigen and the anti-tumour activity. Particularly, this work focusses on two approaches: (i) the co-delivery of a plasmid coding for the HIV-1 gag protein, pGag, with a plasmid encoding the antigen expressed by tumour cells and (ii) the delivery of pTOP, a plasmid that encode a modified VSV-G in which a specific T epitope is inserted. To evaluate the ability of the vaccine candidates to induce potent cellular responses and anti-cancer activity, a melanoma model is chosen and the ovalbumin (OVA) is selected as model antigen.

First, we present pGag as a genetic adjuvant for cancer DNA vaccines and highlight its ability to promote a Th1 response against the vaccine antigen. This strategy takes advantage of both the ability of DNA electroporation to stimulate innate immunity and of the immunomodulatory effects induced by pGag. The administration of pGag with the anti-OVA DNA vaccine (pOVA) induces a shift of the anti-OVA immune response towards a Th1 polarisation. In addition, pGag co-

delivery with either an anti-OVA or an anti-gp100 DNA vaccine increases protection against tumour challenge as compared to the delivery of the DNA vaccine alone. Therapeutic immunization is also improved by combining pOVA and pGag. Finally, we demonstrate that the effects of pGag on the immune response are not mediated by the *in vivo* formation of HIV-Gag virus-like particles.

In the second part of the thesis, we demonstrate the ability of pTOP-based DNA vaccines to induce the proliferation of epitope-specific CD8+ and CD4+ T cells. The vaccine efficacy and cytotoxic T-cell response are improved by co-administering pTOP vectors containing both MHC class I and MHC class II epitopes as compared to administration of the MHC class I epitope alone. A weak therapeutic anti-tumour efficacy can be observed by delivering the two epitopes inserted in pTOP, whereas the administration of pOVA has no effect. These results indicate the ability of the plasmids encoding the VSV-G protein with defined T-epitope insertions to induce specific T-cell responses and elicit antigen-bearing tumour cell recognition and destruction.

This thesis demonstrates the potential of plasmids encoding viral structural proteins to enhance cancer DNA vaccine potency. This work is only a first step in this innovative field. We hope that this research will open new opportunities in the tumour DNA vaccination field and make a positive contribution to cancer immunotherapy.

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Abbreviation list

Δφ	Induced transmembrane voltage
AMEP	Antiangiogenic metargidin peptide
APCs	Antigen presenting cells
AP1	Activator protein 1
cGAMP	Cyclic guanosine monophosphate
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CO	Codon optimization
CTL	Cytotoxic T lymphocyte
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CpG	Cytosine-phosphate-guanosine
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DLNs	Draining lymph nodes
ECT	Electrochemotherapy
EP	Electroporation
GET	Gene electrotransfer
GM-CSF	Granulocyte-macrophage colony stimulating factor
hIL-12	Human Interleukin-12
HV	High voltage
HIV-1	Human immunodeficiency virus type 1
HIV-1 Gag	Gag protein of HIV-1
HPV	Human papilloma virus
ICD	Immunogenic cell death
ID	Intradermal

iDC	Immature dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IRF	Interferon regulatory factor
IT	Intratumoral
LV	Low voltage
Mab	Monoclonal antibody
mDC	Mature dendritic cell
MEA	Multi-electrode array
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MYD88	Myeloid differentiation primary-response protein 88
NK-κB	Nuclear factor-κB
NTIRE	Nonthermal irreversible electroporation
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
pGag	Plasmid encoding HIV-1 Gag
pGag*	Plasmid encoding a mutated HIV-1 Gag protein that cannot form VLP
pGP100	Plasmid encoding gp100
pOVA	Plasmid encoding ovalbumin
PRR	Pattern recognition receptor
PSA	Prostate-specific antigen

pTOP	Plasmid encoding a modified VSV-G containing a T cell epitope
pVSVG	Plasmid encoding VSV-G
ROS	Reactive oxygen species
STING	Stimulator of interferon gene molecule
SV40	Simian virus 40
TA	Tumour antigen
TAA	Tumor-associated antigen
T_{CM}	T central memory cells
T_{EM}	T effector memory cells
TIL	Tumour infiltrating lymphocyte
TBK1	TANK binding kinase 1
TCR	T-cell receptor
Th	CD4+ T helper cell
Th1	T helper 1
TLR	Toll-like receptor
TSA	Tumour-specific antigen
VLP	Virus-like particle
VSV-G	Vesicular stomatitis virus glycoprotein

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Chapter 1 - Introduction

1. Cancer facts and hallmarks

Cancer stands for a group of related diseases characterized by the rapid proliferation and spread of abnormal cells. There are more than 100 types of cancer, often named for the organs or tissues where the cancers started or the type of cell that formed them. Cancer can be induced by external factors, such as tobacco, infectious organisms, alcohol consumption and unhealthy diet for instance. Internal factors such as inherited genetic mutations, immune or hormonal condition can also lead to abnormal cell formation (Cancer Facts and Figures 2016, American Cancer Society, US 2016). These cells divide without stopping and form growth called tumours. Malignant tumours can form metastasis in other tissues and lead to death if the spread is not controlled.

Cancer is one of the leading causes of death worldwide. In 2016, cancer was the second leading cause of death in the United States (US) with 1 685 210 new cancer cases and 595 690 cancer deaths expected[1]. In Belgium, the Belgian Cancer Registry recorded 65 487 new cancer cases in 2013 and 26 923 cancer deaths in 2012 (Cancer burden in Belgium 2004-2013, Belgian Cancer Registry, Brussels 2015). The number of new cancer cases is expected to increase in the coming years, mainly because of the ageing and growth of the population. Over one in three men and one in four women are diagnosed with cancer before their 75th birthday. The occurrence of cancer increases with age, as 77% of male and 66% of female cancers are reported among adults older than 60 years (http://www.kankerregister.org/).

The ethiology of cancer is complex, as there are many forms of cancer and many factors that can induce this disease. To better rationalizing how normal cells can

evolve progressively to malignant, Hanahan and Weinberg have proposed 10 hallmarks of cancer as an organizing principle[2,3]. The hallmarks of cancer are biological capabilities acquired during the multistep development of cancer that enable tumour growth and metastatic dissemination (Figure 1). They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability, inflammation, reprogramming of energy metabolism and evading immune destruction. Targeting these capabilities is essential to develop new and efficient cancer therapeutics.

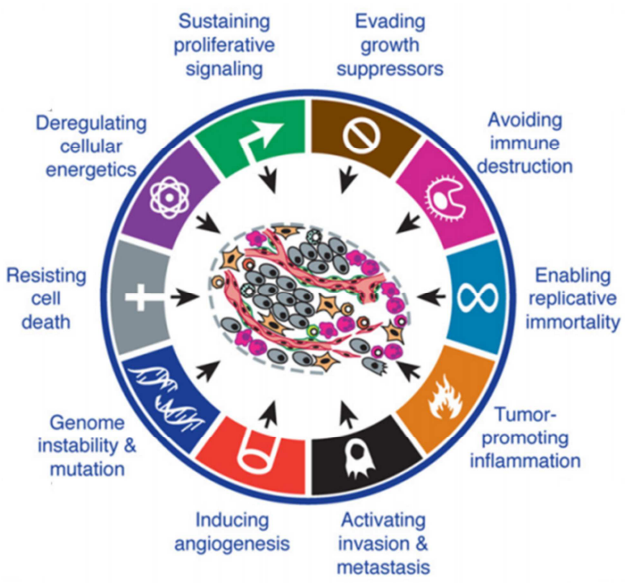

Figure 1 – The 10 hallmarks of cancer (adapted from Hanahan et al., Cell, 2011)

Particularly, one of these hallmarks suggests a role played by the immune system in eradicating cancer cells. This phenomenon has been widely studied these last decades and led to the development of cancer immunotherapy, a strategy that involves the deliberate use of the adaptive immune system to combat tumours[4].

2. Cancer immunotherapy

2.1. Historical background

The idea that the immune system could inhibit tumour growth was firstly postulated by William B. Coley in 1891, after his demonstration that tumour regression occurred in patient after local delivery of *Streptococci* or a mixture of *Streptococcus pyogenes* and *Serratia marcescens* (Coley's toxins)[5]. Few years later, in 1909, Ehrlich proposed that the immune system could scan for and eradicate nascent transformed cells[6], a process that was later named immune surveillance. The enthusiasm for tumour immunology was launched for good in the 1970s, thanks to the work of Thierry Boon and colleagues who showed that specific immunity to spontaneous tumours could be induced by vaccinating mice with mutagenized tumour cells[7,8]. They further demonstrated that cytotoxic T lymphocytes (CTLs) could recognize and destroy cancer cells bearing tumour antigens[9,10]. Today, cancer immunotherapy is considered as a key pillar in cancer treatment, as evidenced by Science's selection of cancer immunotherapy as breakthrough of the Year in 2013[4].

2.2. Cancer immuno-editing and immunity cycle

The path to demonstrate the immune system ability to seek out and destroy tumours has been long and controversial. Indeed, immune surveillance requires the contribution of various actors of the immune system in a delicate balance. It was shown that continued deletion of cancer cells expressing T-cell targets exerts a selection pressure on tumour cells and enable cancer to evolve and avoid the immune system attacks[11]. This phenomenon is due to intricate relationship between a tumour and the immune system and it is summarized by a dynamic three-phase process (elimination, equilibrium, and escape) called immuno-editing[12,13]. During the elimination phase, which can be seen as an updated

version of cancer immune surveillance, the immunogenic transformed tumour cells are destroyed. Several dangers signals activate innate immune cells releasing pro-inflammatory cytokines and facilitating the development of tumour-specific adaptive immunity. However, tumor cells that manage to survive immune destruction may then enter the equilibrium phase, where the immune system maintains them in a functional state of dormancy. Due to constant immune pressure, these tumour cell variants evolve to resist immune recognition. This selective pressure promotes the outgrowth of cell variants that have acquired the most immune-evasive mechanisms. This can eventually lead to the escape phase and the appearance of clinically apparent and poorly-immunogenic tumours in an immunosuppressive microenvironment[13]. Thus, the immune system plays a dual role in cancer, by suppressing cancer cells and promoting at the same time tumour outgrowth[14].

To understand the mechanisms that regulate the delicate balance between the two functions of the immune system, it is essential to understand the tumour and immune components that drive anti-cancer immunity. Chen and Mellman proposed a model called the Cancer-Immunity Cycle[15], in which the generation of an anti-tumour immune response is a cyclic process divided into 7 major steps (Figure 2). At first, tumour antigens are released and captured by antigen presenting cells (APCs), mainly dendritic cells (DCs). Then, DCs process the antigens and migrate towards the draining lymph nodes (DLNs), where they present the antigens to T cells in order to prime effector T-cell responses against the tumour antigens. The effector T cells then migrate to the tumour site, where they can infiltrate the tumour. There, T cells can specifically bind to cancer cells through the interaction between their T-cell receptor (TCR) and their cognate antigen, and kill their target cancer cell. Killing of the cancer cell releases additional tumour antigens, starting the cycle again.

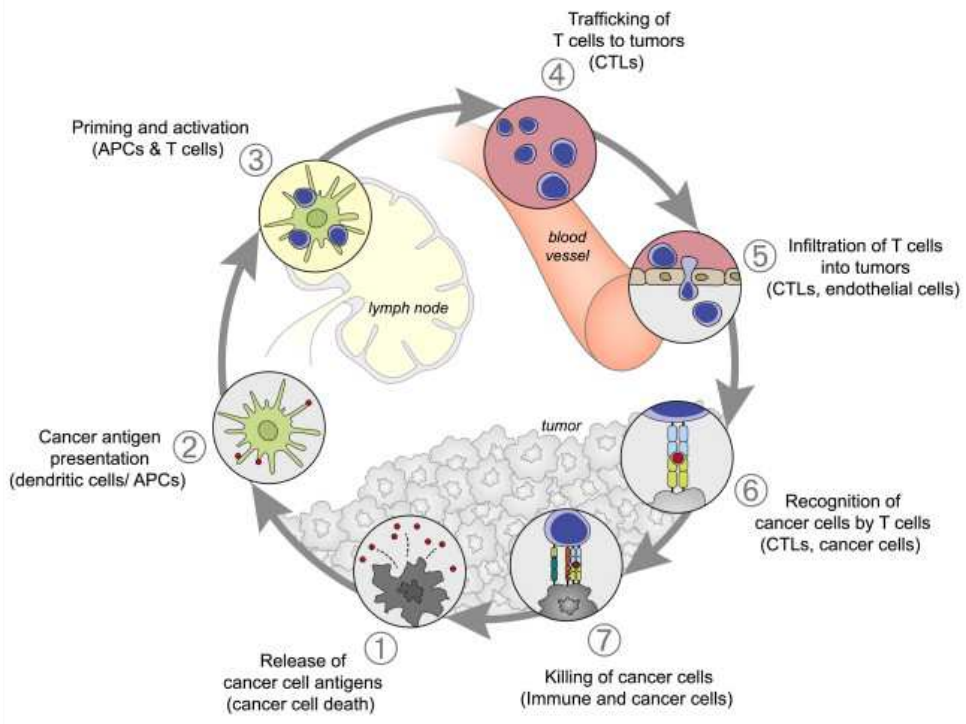

Figure 2 – The Cancer-Immunity Cycle (Chen et al., Cell, 2013)

Each step of the cycle requires the coordination of numerous factors, both stimulatory (that amplify T-cells responses) and inhibitory (to allow regulatory feedbacks)[15]. Tumours that escape immune-mediated elimination have developed strategies that disrupt this cycle by pathologically exploiting immune regulatory-mechanisms to promote resistance[16]. The mechanisms involved in cancer immune evasion have been reviewed by van der Brug et al[17]. Firstly, tumour cells can modify their properties to avoid immune recognition. They can lower the amount of antigens exposed at their surface by down-regulating the expression of MHC class I molecules, altering the antigen processing machinery or losing the expression of certain antigens. Secondly, they can also resist to lysis by immune cells if they shed alarm proteins (for example, to avoid recognition by NK cells), increase their resistance to cell death (by expressing inhibitors of apoptosis

proteins for instance) or express ligands for inhibitory receptors (such as PDL-1). Finally, an immunosuppressive tumour microenvironment can be induced, characterized by a deprivation of soluble nutrients (for instance by secreting indoleamine 2,3-dioxygenase (IDO), a tryptophan degrading enzyme), the expression of immunosuppressive soluble factors (IL-10, TGF-β, VEGF and galectins) and infiltration with suppressive cells comprising regulatory T cells (Tregs), tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) among others.

The aim of cancer immunotherapy is to initiate or re-initiate a sustainable cycle, and restore the immune system's ability to eliminate tumour cells. Therefore, the different factors that come into play in the Cancer-Immunity Cycle are potential immunotherapeutic targets. They involve both tumour and immune components, and they can be handled to lower the immunosuppression or enhance tumour cell recognition and elimination.

2.3. Immunotherapeutic strategies: the case of melanoma

This section briefly describes the main immunotherapeutic strategies explored. Melanoma is taken as an example as it has been widely studied in the context of immunotherapy and it is a cancer for which many immune-based treatments are currently approved by the FDA and many more are under clinical development. Melanoma is a cancer of the melanocytes, the cells that produce the pigments in the skin[18]. It is the most serious form of skin cancer. In Belgium, it accounts for 10 % of all skin cancers and it's the 10th most frequent cancer in males and the 5th in females (http://www.kankerregister.org/). The number of detected cancer is increasing worldwide, due to a change in sun-exposure habits, increasing awareness and screening measures. Melanoma is treated efficiently if discovered early, but it can grow and spread very quickly if left untreated.

Stage as determined by the pathologist is the most important prognostic factor. There are 5 stages that describe melanoma. Stage 0 refers to melanoma in situ that is very unlikely to spread to other parts of the body. At stage I, the melanoma is still only in the epidermis and very thin. It goes to stage II when it is thicker, extending through the different layers of the skin. At stage III, melanoma has spread through the lymphatic system. Finally, in the fourth stage, it spreads through the bloodstream to other parts of the body, such as distant locations on the skin or soft tissue, distant lymph nodes, or other organs like the lungs, liver, brain, bones, or gastrointestinal tract (https://www.melanoma.org /). In Belgium, while a stage I disease has a 5-year survival rate of 99.0% in males and 99.8% in females, the presence in lymph nodes (stage III) reduces these percentages to 55.4% and 68.6%, respectively (http://www.kankerregister.org/).

The treatment options for melanoma vary with the stage of the disease, the age, the overall health, the presence of genetic changes, the rate of melanoma growth and the patient's preferences. The major treatments consist in surgery and, to a lower extent, radiotherapy. In the advanced disease stages or with unresectable melanoma, other treatments are required including immunotherapy, targeted therapy or chemotherapy (http://www.cancer.net/). The immunotherapeutic strategies include monoclonal antibodies (Mab)[19], oncolytic viruses[20], cytokine and adjuvant therapies[21], adoptive cell therapy (ACT)[22] and cancer vaccines.

Checkpoint inhibitors - Taking the brakes off the T cells

T-cell stimulatory antibodies aim at blocking the pathways that allow cancer cells to hide from immune attacks. The most famous are the "checkpoint inhibitors" that target inhibitory checkpoint proteins responsible for holding T-cells at a bay. Normally, these inhibitory proteins protect the body from unwarranted attacks

and autoimmunity, but they can also limit the immune system's ability to detect and fight tumours[23].

Four checkpoint inhibitors treatments were approved by the FDA for melanoma patients. In 2011, Ipilumimab (Yervoy®) arrived on the market. This Mab target CTLA-4 on T-cell surface, a protein that inhibits T cell priming and activation[24]. In 2014, two other Mab that target the programmed death-1 (PD-1) receptor on T cells (Pembrolizumab (Keytruda®) and Nivolumab (Opdivo®)) were approved[25]. This receptor causes inhibition and apoptosis of T cells upon binding with its ligand PDL-1 on tumour cells. A treatment combining Ipilimumab and Nivolumab (Ipilimumab® + Nivolumab®) also received approval in September 2015[26]. Many other checkpoint inhibitors are currently evaluated in clinical trials (http://www.cancerresearch.org/). These drugs are administered in patients with unresectable stage III and stage IV melanoma, and Yervoy® can also eventually be used as adjuvant therapy following surgery for stage III melanoma. They are able to shrink melanoma in about 10 to 15% of patients for Yervoy®, 25 to 45% of patients for the PD-1 antibodies and up to 58% with the combination[26]. However, severe immune-related adverse events are associated with these strategies..

Oncolytic viruses - Initiating anti-tumour immunity

In the late 2015, the FDA approved the use of talimogene laherpavec T-VEC (Imlygic®) for the treatment of unresectable stage III and IV melanoma. This genetically engineered herpes simplex virus targets cancer cells and replicates within them while also causing the cells to produce granulocyte-macrophage colony-stimulating factor (GM-CSF)[27]. Oncolytic viruses may promote anti-tumour responses through a dual mechanism of action that is dependent on selective tumour cell killing and the induction of systemic anti-tumour immunity[20].

Indeed, dying cancer cells can behave as a therapeutic vaccine, releasing danger signals and tumour antigens. This phenomenon, known as immunogenic cell death (ICD), depends on the initiating stimulus and involves changes in the composition of the cell surface as well as the release of soluble mediators, occurring in a defined temporal sequence[28,29]. It is believed that oncolytic viruses can induce ICD[30], as well as other techniques that aim at killing cancer cells such as chemotherapy, radiation therapy and other local ablative techniques including electrochemotherapy[31,32]. However, this systemic effect has not been evidenced so far with Imlygic®. This treatment is therefore usually offered to patients with injectable tumours and limited metastatic disease.

Cytokines and adjuvant therapies – Optimizing the environment

Among the FDA approved strategies, the first immunotherapies relied on the use of cytokines (IL-2 and interferon (IFN)-α) and date back to the end of the nineties. Currently, there are 2 adjuvant therapies approved for stage II and stage III melanoma patient that have been treated by surgery and have high risk of recurrences: IFN-α (Intron A®) and pegylated IFN-α (Sylatron®). However, they are associated with substantial side effects and are not automatically recommended. The use of IL-2 (Aldesleukin® or Proleukin®), approved for the treatment of stage III and IV unresectable melanoma, is also associated with significant side effects and induces response rates lower than 10% (http://www.cancer.net/). It should be noted that other adjuvants are under clinical investigation, some of which aiming at improving T cell priming and functions and other aiming at inhibiting the immunosuppression in the tumour microenvironment[32].

Adoptive cell therapy – Engineering a T cell army

Immunotherapy using autologous T cells has emerged to be a powerful treatment option for patients with metastatic melanoma. These include the adoptive

transfer of autologous tumor-infiltrating lymphocytes (TIL), T cells transduced with high-affinity T-cell receptors (TCR) against major melanoma antigens, and T cells transduced with chimeric antigen receptors (CAR) composed of hybrid immunoglobulin light chains with endo-domains of T-cell signaling molecules[22].

This approach is attractive as it avoids the need for immunization, and may also force the revolution of the Cancer-Immunity Cycle by overwhelming the system with stimulatory immune factors through the infusion of the modified T cells[15]. However, there are concerns about potential toxicity. Regarding the use of TIL expanded *in vitro,* it requires intensive care hospitalization and can be risky[9,33]. It is not known whether the infusion of large number of monospecific engineered T cells will cause resistance or will induce off-target side effects. The three approaches are currently under clinical investigation but no ACT has been approved as standard of care for the treatment of metastatic melanoma so far.

Finally, the use of **vaccines** has been also widely investigated in an attempt to activate cancer antigen-specific T cells. There is no therapeutic vaccine approved so far to treat advanced melanoma in humans ((http://www.cancerresearch.org/). Several strategies are evaluated in preclinical and clinical trials[34]. The tumour vaccination strategy is detailed in the next section.

Melanoma is often taken as an example for immune-oncologists[35], but the strategies developed here are also pursued for several other cancer types. From the preventive vaccine for cervical cancer to the first therapy proven to extend the lives of patients with metastatic melanoma, immunology has already led to major treatment breakthroughs for a number of cancers. Every cancer type is unique, though, and immunology and immunotherapy are impacting each cancer in different ways (http://www.cancerresearch.org/).

3. Tumour vaccination

3.1. Rationale

Vaccination is the administration of antigenic material (a vaccine) to stimulate an adaptive immunity against the antigen (a molecule that is recognized as foreign by the immune system). Both the innate and adaptive immune systems are necessary to provide an effective immune response to an immunization. Upon vaccination the body must detect the antigen and the threat that is associated with it[28]. This detection is accomplished by the innate immune system.

The innate immune system is the first-line defense against pathogenic agents. It is able to detect invading pathogens through a limited set of germ-line encoded receptors. These pattern-recognition receptors (PRRs) recognize conserved molecular structures such as flagellin, peptidoglycans or nucleic acid that are expressed by distinctive set of pathogens, the pathogens associated molecular patterns (PAMPs)[36]. They can also recognize damage-associated molecular pattern (DAMPs), such as reactive oxygen species (ROS), ATP or apoptotic/necrotic cells[37]. Innate immunity includes several protective measures[38]: (i) anatomical and physiological barriers, (ii) inflammation, (iii) the activation of the complement pathways and (iv) the activation of the adaptive immune system through a process known as antigen presentation. Activation of the adaptive immunity is the goal of vaccination strategies as it is highly specific and responsible for immunological memory.

The adaptive immune responses are highly sophisticated and pathogen-specific. It involves the interaction of antigens with their antigen-specific receptors expressed on lymphocytes[39]. The adaptive immune system is composed of two arms: the humoral and the cellular immunity. The humoral immunity is composed of B cells and antibodies and functions mainly against extracellular pathogenic

agents and toxins. The cellular arm is mediated by the action of T cells, both helper (CD4+) and cytotoxic (CD8+) T cells. Helper cells are divided in two main subsets, Th1 and Th2. Th1 cells help promote cell-mediated immunity and Th2 cells help promote antibody-mediated immunity. The main function of cytotoxic T cells (CTLs) is to eradicate infected or transformed cells.

3.2. Tumour antigens

In tumours, some proteins are antigenic because they are for instance mutated or expressed at a different level than in normal tissue. Table 1 summarizes the different categories of tumour antigens recognized by T cells[40,41].

Table 1– Different classes of tumour antigens recognized by T lymphocytes

Name	Characteristics	Particularities
Differentiation antigens	Tissue-specific expression in tumors and the corresponding healthy tissue.	Low tumour specificity
MAGE-type antigens	Expressed in cancer and germline tissues	Tumour-specific
Mutational antigens	Results from somatic mutations or rearrangement of a gene-coding sequence	Tumour- specific
Overexpressed antigens	Overexpression in tumour, but low expression in many normal tissues	Low tumour specificity
Viral antigens	Expressed in tumour with a viral etiology	Tumour-specific

The tumour specificity of the antigen is important to take into consideration. The probability of attack of normal tissues by the antitumoural T cells increases for the antigens of low tumoural specificity. On the contrary, T-cell responses that are elicited against tumour-specific antigens ought to leave normal tissues completely unharmed. However, the risk exists of generating T cells that crossreact with antigens that are present on normal cells, as it is never possible to completely

exclude that a small subgroup of cells located in one organ will eventually show a substantial degree of expression[9].

Melanoma antigens were among the first cancer antigens to be identified and classified, with further studies showing that many of these are also expressed by other tumour types[35]. The first human tumour-specific antigen, encoded by a gene of the cancer-germline-gene MAGE family, was identified in melanoma[42]. Currently, more than 60 cancer-germline genes have been identified. These are substantially expressed in a large range of tumours, making them attractive for immunotherapy[9]. The mutational antigens, another class of tumour specific antigens, were also firstly identified in melanoma[43,44]. Several techniques are now under development to allow rapid identification of antigens that are encoded by tumor-specific mutated genes, the neoantigens[45,46]. These antigens appear promising immunotherapeutic targets[47,48]. Ubiquitous antigens have also been found in melanoma (for instance the ephrin type-A receptor3 (EphA3), a tyrosine kinase receptor overexpressed in melanoma, lung carcinoma and sarcoma)[34]. However, the safety issue is an obvious limitation for applying vaccines against this group of antigens. Finally, spontaneous T cell responses to differentiation antigens (such as Melan-A, tyrosinase and gp100) have been well documented only in patients with melanoma, with T cells recognizing tumour cells and normal melanocytes. It is not known why tolerance is incomplete against these melanocytic antigens and why most patients make spontaneous T-cell responses against them[9]. As a consequence, some patients develop a melanoma-associated vitiligo, a weak side effect accompanied by a favourable prognosis[49,50].

Different methods have been used to predict tumour antigens. A genetic approach based on screening of cDNA library derived from tumours was used to discover the first antigenic peptides recognized by CTL clones[51]. The antigenic peptides can also be identified following their elution from MHC class I molecules

and their direct sequencing by mass spectroscopy. However, this biochemical approach is technically very demanding[9,40]. Nowadays, tumour antigenic peptides are often identified using the reverse immunology method, which consists in selecting peptides with adequate HLA-binding affinities determined by computer-generated algorithms[52,53]. Even if precise identification of tumour antigens is critical to design most cancer vaccines, some immunization strategies bypass the need of precise antigen identification (by using for instance tumour cells or their transcriptome[54,55]).

3.3. Tumour specific T-cell responses induced by vaccines

Cancer vaccines aim at inducing *de novo* properly polarized tumour-specific T-cell responses. The activation of CD8+ CTLs is the main objective of vaccination strategies, as these cells can kill cancer cells after binding of their TCR to MHC-peptide complex on the tumour cells. The lysis mechanism involves the activation of the death-receptor pathway (Fas, TRAIL and TNF) and the granule exocytosis pathways (action of perforins and granzymes)[56]. These effector cells require the help of $CD4^+$ T-helper 1 (Th1) for optimal and sustained cytotoxic activity, as well as for the induction and maintenance of $CD8^+$ T-cell memory (Figure 3).

To obtain the desired T-cell responses against the tumour antigen delivered by the vaccine, the most important immunological players are the dendritic cells. DCs are innate immune cells that develop in the bone marrow and migrate to tissues in an immature form (iDC). They are able to take up the vaccine antigens from their environment and when they are exposed to danger signals, they undergo maturation and they migrate to lymph nodes. There, the mature DC (mDC) can activate the naive T cells by presenting at their surface specific antigenic peptides (called epitopes) recognized by T cells when they are bound to major histocompatibility complex (MHC) molecules. MHC class I and class II molecules

present intracellular peptides to CD8+ T cells and exogenous peptides to CD4+ T cells, respectively[57].

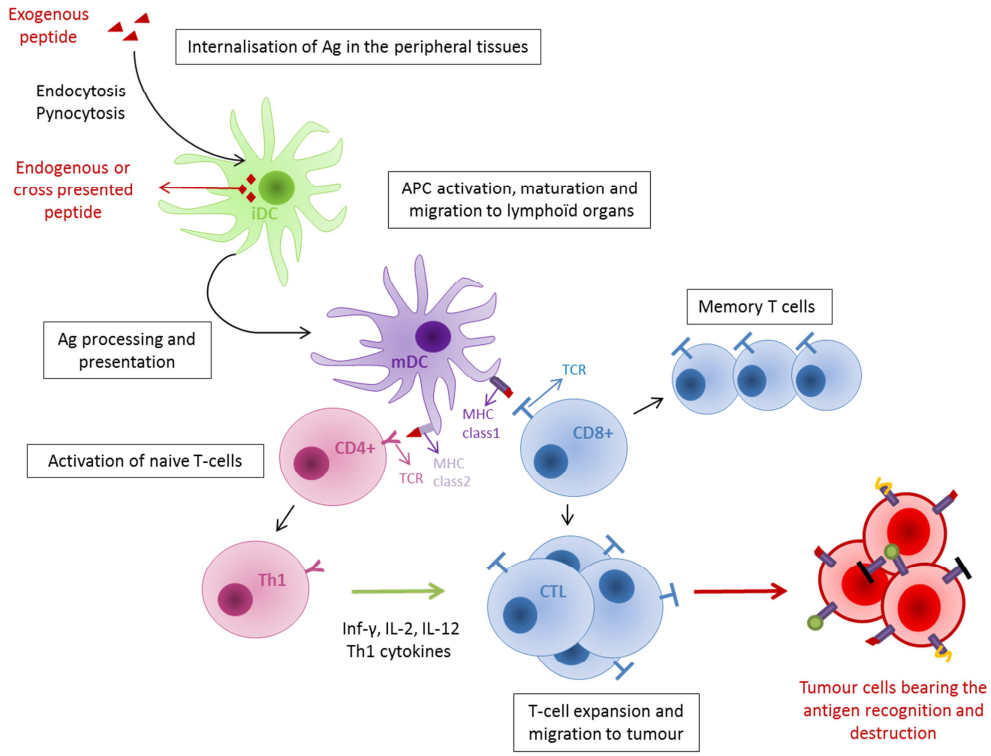

Figure 3 –Tumour specific T-cell responses activation by vaccines

Then, three signals provided by DCs are necessary for expansion and differentiation of naive T cells into effector and memory T cells[58]. These signals consist in (i) an antigenic stimulation through the interaction with the TCR, (ii) the expression of co-stimulatory molecules to enhance T-cell proliferation and (iii) the secretion of cytokines[17]. DCs will also drive functional polarization of CD4+ T cells (i.e., Th1, Th2, Th17 or T-regulatory for instance) and stimulate the production of homing receptors that will direct T cells to migrate through specific tissues[59].

3.4. Tumour vaccine strategies

Different vaccination strategies have been used to deliver tumour antigens to the patient. They include tumour cells and derivates thereof, DCs loaded with tumour antigens, proteins or peptides and genetic vaccines comprising viral recombinant vectors and nucleic-acid-based vaccines.

Tumour cell vaccine consists of autologous or allogenic lysed or killed tumour cells[36]. A great advantage of those preparations is that they contain numerous tumour antigens, allowing both CD8+ and CD4+ T-cell activation[60]. There is no need to identify and purify these antigens to initiate antitumour specific immune responses[55]. In addition, immunization against multiple epitopes diminishes the chance of tumour escape as compared to single epitope vaccines. However, the main limitations of this strategy concern the standardization, quality control and large scale production.

Reflecting their central role in immune response priming, the use of DCs is promising for vaccination. DCs based vaccines are produced by isolating dendritic precursor cells from the patient and loading them with tumour antigens. DCs loading can be achieved by using synthetic peptides, proteins, whole tumour cell lysate or plasmid DNA (pDNA) or messenger RNA (mRNA) encoding the antigen[58]. These vaccines have a good efficacy profile but they suffer from a labor-intensive and expensive production.

MHC-restricted peptides are also attractive for tumour vaccination, as they are easy to produce. However, they require precise identification of the epitopes and are only applicable in patients that have the right MHC-molecule[61]. In addition, they are often poorly immunogenic and can lead to immunological tolerance. It was shown that these short peptides could be directly loaded on MHC molecules on DCs and lead to suboptimal T cell priming[62]. The use of synthetic long peptides

(SLPs) containing multiples epitopes or full length proteins are more likely to be effective than peptides mixtures[63]. They have to be taken up and processed by APCs and they have more chances to circumvent immune escape.

Finally, the use of genetic vaccines holds great promises for cancer vaccination, because the intracellular synthesis of the target antigen induces robust T-cell responses. They can be based on recombinant viruses, pDNA or mRNA. Viral vectored vaccines possess a high efficiency of gene delivery and induce potent immune response[64]. However, they also induce an immune response against the viral vector, making further boosts less effective[65]. In addition, issues concerning large-scale production, stability and strict safety requirements are limiting their use as vaccine vectors[66]. Nucleic acids are a format of choice to deliver antigenic information to the immune system. They can be easily designed and produced *in vitro*. Because mRNA was considered too instable for *in vivo* use, pDNA was the first and most extensively studied nucleic acid vector. Over the last decade, the interest in mRNA increased thanks to an enhanced stability of in vitro transcribed mRNA[67]. mRNA vaccines appear safer as compared to pDNA and easier to deliver as they do not need to enter cell nucleus to be transcribed and they are not likely to integrate the host genome. Innate immune sensing also varies with the type of nucleic acid used for immunization. The next section details the basic principles as well as the advantages and drawbacks of pDNA immunization.

4. DNA vaccines

4.1. Principle

DNA vaccines consist of one or several antigen-encoding genes cloned into a bacterial plasmid. The interest in the DNA vaccination field is important, with several clinical trials launched every year and a large panel of diseases covered

(clinicaltrials.gov). Figure 4 summarizes the principle of DNA vaccination, going from the DNA plasmid production to the immune response induction.

Plasmids are amplified by bacteria and purified before being administered to the patient. For guarantying both optimal production in bacteria and strong expression in eukaryotic cells, the plasmid backbone requires: (i) an origin of replication that allows large copy numbers in bacteria; (ii) a selection marker[68] such as an antibiotic resistance gene to ensure stable inheritance of plasmids during bacterial growth; (iii) a promoter for optimal expression in mammalian cells (common promoters are derived from the cytomegalovirus (CMV) and simian virus 40 (SV40)); (iv) a polyadenylation sequence to stabilize the mRNA transcripts and protect them against the action of nucleases[69,70]. Then, pDNA needs to enter into cells and to cross the nuclear membrane for being expressed by the host cellular machinery. The delivery method is a key parameter to consider in the development of DNA vaccines for observing optimal cell transfection and antigen expression. The most common routes of DNA injection are intramuscular (IM) or intradermal (ID)[71]. Muscles are seen as a protein factory as myocytes can produce extensive quantities of antigens for months. On the opposite, cutaneous keratinocytes express relatively low quantities of antigens for shorter time. However, cutaneous delivery is less invasive and the skin is richer in antigen presenting cells (APCs)[72]. The amount of pDNA used will depend on the site of administration, the type of antigen and the delivery method[73,74]. Following delivery, DNA vaccines induce a wide immune response[75,76], with the activation of innate immunity (see section 6.2) and both humoral and cellular arms of adaptive immunity.

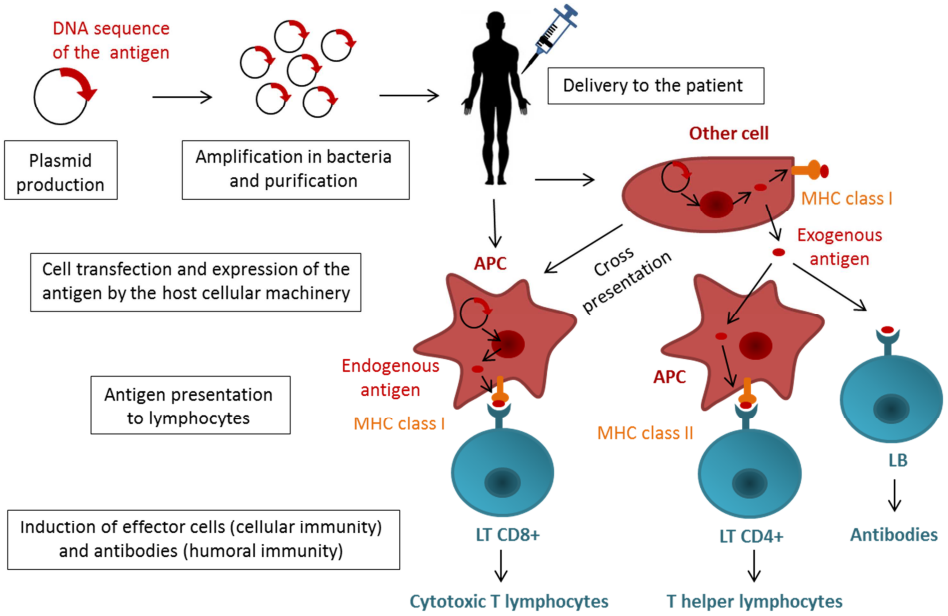

Figure 4 – From DNA plasmid production to patient's immune response

Upon delivery, the pDNA can transfect either APCs or other cells present at the delivery site. The expressed antigenic protein is cleaved by the proteasome into peptides, which are loaded on MHC class I molecules and presented to CD8+ T cells. Transfected APCs migrate in DLNs to promote the proliferation of CTLs. Other will activate different immune pathways. First, non-secreted antigen can be presented by APCs on MHC class I molecules thanks to the cross-presentation mechanism that exploits the ability of certain APCs to take up, process and present antigens expressed by other cells. Secondly, if the antigen is secreted and end up in the extracellular matrix, it can be captured by specific high affinity immunoglobulins expressed on the surface of B cells in the DLNs and trigger humoral immunity. The shed antigen can also be endocytosed by APCs that sample the environment. The exogenous antigen is loaded on MHC class II molecules, thus activating CD4+ T cells and further inducing T helper cell proliferation.

4.2. Advantages and drawbacks

The use of pDNA for immunization has many advantages over other types of cancer vaccines in terms of effectiveness, safety and cost. First, production is fast and plasmids can be easily purified in large quantities with a stability profile that permits long-term storage. They are well tolerated and safe, and can be applied in both prophylactic and therapeutic approaches. Moreover, DNA vaccines allow the expression of antigens in their natural conformation and with their appropriate post-translational modifications[77]. Transcribing and translating the full length protein also eliminates the need to limit patients of a defined HLA type to be eligible to receive the vaccine[61]. From an immunological point of view, an important advantage of DNA vaccines is their ability to stimulate a broad immune response with a good immunological memory. Plasmid encoding the full length gene of interest provides several potential epitopes to stimulate both cytotoxic and helper T cells as well as an antibody response. In addition, insertion of the antigen coding sequence in a bacterial expression vector provides the vaccine with a 'built-in adjuvant' (see section 6.2). Finally, they can easily be engineered to encode alternative forms of the wild type antigen with higher biological potency[61].

Due to these numerous benefits, DNA vaccines first sparked the interested of the scientific community. However, some drawbacks restricted their applicability. First of all, their use raised concerns about the risk on genome integration by insertion mutagenesis. However, it has been shown that no relevant integration into host genomic DNA occurred even after multiple administrations[78] and that integration occurs at slower rates than a spontaneous mutation[79]. Another issue is the lack of control of the expression profiles. Plasmids can persist at the injection site for many months and they can be found far from the original site, probably carried by transfected lymphocytes or macrophages[80]. Despite these concerns, the safety profile is rather good and the risk-benefit balance appears often

favorable for both therapeutic and preventive vaccination approaches. Indeed, it appeared from clinical trials that the side effects associated to DNA vaccine were similar to placebo[81-83]. Currently, two veterinary DNA vaccines have been licensed. Oncept™ (Merial) is commercialized for the treatment of melanoma in dogs and ApexR-IHN (Novartis) for prophylactic vaccination of salmon against infectious hematopoietic necrosis virus[84,85].

The major issue about DNA vaccination has been highlighted by the first clinical trials performed in the nineties. They showed that even if the DNA vaccines were safe and well tolerated, they proved to be poorly immunogenic[86]. Indeed, despite the promising results in small animal models, the efficacy was limited in humans[75,87,88]. The Achilles heel of DNA vaccines was their poor immunogenicity. The induced antibody titers were very low or nonexistent, $CD8^+$ T-cell responses were sporadic, and $CD4^+$ T-cell responses were of low frequency in humans[86].

Effort has been made to understand the different performance of the DNA vaccine in mouse and large animal models and humans[88]. There might be several reasons explaining this phenomenon. First of all the vaccine dose was maybe not appropriate. In addition, the delivery method was thought to be suboptimal to promote efficient uptake of the plasmids by cells[86]. Differences in the immune system of the species could also be responsible for the lower immunogenicity observed in humans[89]. For instance, TLR-9 is involved in pDNA sensing and immunogenicity. However, the unmethylated CpG motifs recognized by human and mouse TLR-9 are different, and the expression of this receptor is not restricted to the same cell types in both species[89,90].

4.3. Improving cancer DNA vaccine efficacy

Much effort has been done to improve the efficacy of DNA vaccines, in an attempt to overcome the current limitation in the clinic. To enhance the immunogenicity of these vaccines, many parameters can be optimized, including the vaccine vector composition, the formulation, the use of adjuvants, the delivery method, the prime-boost strategy (i.e. the consecutive immunization strategy, involving specific delivery timing and eventually the delivery of other types of vaccines)[91] and the combination with other treatments[92].

First, the vaccine vector design and the choice of the encoded antigen are critical to optimize protein expression and to obtain therapeutic efficacy. It is possible to act on synonymous codon replacement to improve the mRNA stability and its translation kinetic[93,94], resulting in enhanced protein expression levels [95]. The use of more efficient[96,97] or tissue specific promoters[98-101] to enhance or control the encoded protein expression is also important, as well as the introduction of immunostimulatory motifs in the plasmid sequence[102]. The delivery approach is another key parameter. The delivery system should ensure optimal transfection efficiency. Three main methods exist: physical methods (gene gun, electroporation, sonoporation,…), chemical vectors (liposomes, nanoparticles,…) and biological vectors (bacterial and viral vectors)[66,86]. Also, the delivery site and the prime-boost strategy should be carefully chosen as they can greatly influence treatment outcome[103-105]. Currently, one of the most successful delivery methods consists in electroporation (EP). EP has come to the forefront of DNA vaccine research these last years, with 45% of all clinical trials concerning DNA vaccination using EP[106,107]. This technique will be discussed in the next section. Finally, the use of adjuvant is critical for enhancing immunogenicity and eliciting long lasting immune memory. The different mechanisms and strategies used to adjuvant DNA vaccines will be discussed in the 6th section of the introduction.

5. Clinical potential of electroporation for gene therapy and DNA vaccine delivery

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5.1. Electroporation

Electroporation allows efficient delivery of DNA into cells and tissues, thereby improving the expression of therapeutic or immunogenic proteins that are encoded by plasmid DNA. This simple and versatile method holds a great potential and could address unmet medical needs such as the prevention or treatment of many cancers or infectious diseases. In this section, we explore the electroporation mechanism and the parameters affecting its efficacy. An analysis of past and current clinical trials focused on DNA electroporation is presented. The pathologies addressed, the protocol used, the treatment outcome and the tolerability are highlighted. In addition, several of the possible optimization strategies for improving patient compliance and therapeutic efficacy are discussed such as plasmid design, choice of appropriate delivery site and electrodes as well as pulse parameters. The growing number of clinical trials and the results already available underline the strong potential of DNA electroporation which combines both safety and efficiency. Nevertheless, it remains critical to further increase

fundamental knowledge to refine future strategies, to develop concerted and common DNA electroporation protocols and to continue exploring new electroporation-based therapeutic options.

5.2. Electroporation for gene delivery - gene electrotransfer

In vivo transfection of a DNA plasmid makes targeted tissues capable of producing a specific protein. pDNA can encode either a therapeutic protein or an antigen and is thus a promising tool to treat a wide range of diseases. To make this gene transfer efficient, the delivery method is critical. Indeed, since the phospholipid bilayer of the plasma membrane has a hydrophilic exterior and a hydrophobic interior, any polar molecules, such as DNA, are unable to freely pass through the membrane. Many methods have been developed to increase transfection efficiency [108]. Among them, electroporation takes advantage of the relatively weak nature of the phospholipid bilayer's hydrophobic/hydrophilic interactions. EP is based on application of controlled electric pulses to cells or tissues, which increase the cell membrane permeability and allow polar molecules to pass through. EP is a highly attractive method and has been applied in several medical applications, namely for electrochemotherapy (ECT), for gene electrotransfer (GET), for nonthermal irreversible EP (NTIRE) and for transdermal drug delivery [109-111].

Basic mechanism of electroporation

Among the various theories that have been proposed to explain the effect of EP at molecular level, the consensual explanation consists in the formation of aqueous pores in cell membrane. Basically, some water molecules can enter into the lipid bilayer, leading to membrane rearrangement to orient the polar heads of phospholipids toward water. Consequently, nanopores are formed in cell membrane [110]. This event occurs spontaneously, but it is neither frequent nor

thermodynamically stable [112]. During the EP process, exposure of cell membrane to an electric field polarizes membrane and thus reduces the energy required for water penetration in the bilayer. Therefore the probability and stability of pore formation is improved [110]. However, a direct visualization of pores after EP is still challenging and the term electropermeabilization is sometime preferred to prevent any misuse by describing not fully elucidated molecular events [113,114].

Gene electrotransfer procedure and parameters affecting transfection efficiency

GET requires pDNA injection into the targeted tissue before pulse application. The application of electric pulses has two complementary effects promoting both permeabilization and electrophoresis of the negatively charged pDNA, necessary for its transfer across the cell membrane [108,115]. EP devices are pulse generators that deliver current through electrodes. Potential difference (V) between electrodes generates an electric field (V/cm) around the cells that causes an induced transmembrane voltage ($\Delta\varphi$). For isolated and regularly shaped cells, a first-order approximation of key parameters is given by the Schwan equation [116]:

$$\Delta\varphi = \frac{3}{2} ER \cos\sigma \, (1 - e^{-\frac{t}{\tau}})$$

where E is the strength of the electric field, R is the radius of the cell, σ is the polar angle measured from the center of the cell with respect to the direction of the field, t is the time elapsed since the onset of the field and τ is the time constant of the membrane charging, that varies with medium conductivity. For irregularly shaped cells or cells that are not in suspension, $\Delta\varphi$ has to be determined experimentally or by numerical analysis [117,118].

The electric field strength, the number and duration of the applied electric pulses are key factors to control membrane permeabilization. At low range or low amplitude of electric pulses, no molecular transport by EP occurs. More intense or longer electric pulses lead to reversible EP that creates the temporary and limited molecular pathways required for GET. Irreversible EP is observed at higher electric

field leading to permanent permeabilization of the membrane and cell death initiation. Finally, thermal damage and molecule release occurs in sufficiently strong fields [110,119]. Beside the amplitude of electric pulses, the efficiency and safety of GET depends on different parameters, such as cell radius, medium conductivity or osmotic pressure. Some of the parameters are difficult to control while some others are easy to adjust. First, the electric field strength needs to be adapted to observe an appropriate $\Delta\varphi$. Another key parameter is the electrode design as it influences the strength, homogeneity, shape and total energy distribution from the electric field to the targeted cells [120].

Advantages and drawbacks

EP not only increases the number of transfected cells and enhances the magnitude of gene expression compared to the injection of pDNA alone, but also allows the co-transfection of several plasmids [121,122]. Moreover, the versatility of GET merits to be highlighted: it can be used with nearly all cell types and at all stages of the cell cycle [123]. It is effective for pDNA delivery to several tissues, explaining its attractiveness for *in vivo* applications [122,124]. Finally, unlike viral vectors for gene delivery, GET does not induce unwanted specific immunity against viral proteins and lowers the risk of integration into the host genome or environmental spread [66,125]. GET is of particular interest for the development of vaccines. First, pDNA can lead to stimulation of both the humoral and the cellular arms of the adaptive immune system. Interestingly, pDNA acts as an adjuvant, triggering inflammatory signal upon cytosolic recognition and transfer to innate immune system, thanks to the stimulator of interferon gene molecule (STING) [126]. GET increases both the intensity and the duration of the immune responses induced by DNA vaccines. This is not only due to a higher antigen expression but also to the infiltration of inflammatory cells with cytokine release at the EP site owing to a moderate tissue injury [127,128]. In situ EP could also lead to the

production of ROS, ATP release and vascular effects[32]. In several preclinical tumor models, antitumor effects occur after intratumoral EP of pDNA[129].

Nevertheless GET has also some drawbacks. First, it can cause strong cell damages or even cell death if the electric pulses are not appropriate. Second, cell specificities that influence the $\Delta\varphi$ must be taken into account and care must be paid to the surrounding tissue to avoid unwanted damages. In addition, the mass transport of material inside and outside the cell through the existing pores caused by EP is non-specific. This can potentially lead to ionic imbalance [130]. EP requires the empirical optimization of many parameters in order to be able to target the right cells. Moreover, specialized equipment is necessary, with the choice of an appropriate generator and electrodes [120,131,132]. Last but not least, it is crucial to adapt the procedure for reaching effective gene expression level and duration in order to trigger adequate clinical effect [115].

5.3. Past and current clinical trials

Overview

There is a long clinical experience about clinical use of EP, in a context of ECT. Combining injected chemotherapy and EP of tumors to render them more susceptible to the treatment is a method used now in many hospitals to treat cancer patients. More recently, several clinical trials have been using GET against several infectious diseases and cancer. Up to now, 64 gene therapies have been recorded (www.clinicaltrials.gov) and this number is increasing continuously [133]. Most of them are currently in phase one and only few have been completed (Figure 5A). DNA vaccination is encountering the largest success for GET, as it accounts for 84% of the trials. The remaining 16% consists in gene therapy trials (Figure 5B). The pathologies addressed are many forms of cancer and infectious diseases (Figure 5C).

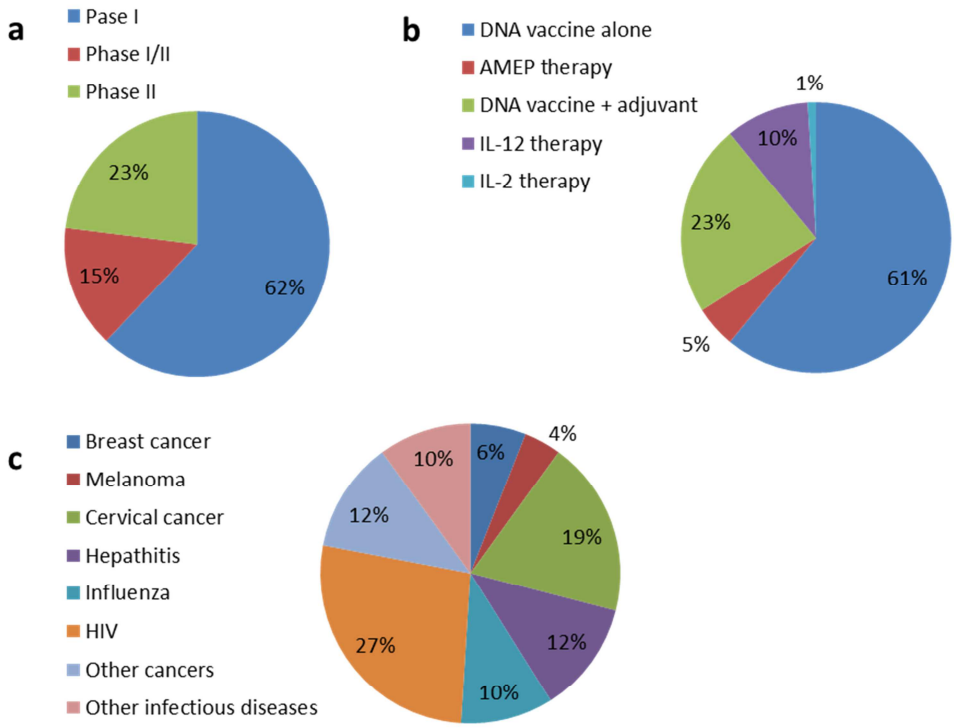

Figure 5 - Clinical trials using GET.

(**A**) Distribution of the clinical phases reached by the ongoing trials. (**B**) Distribution of the type of trials using EP for gene delivery: DNA vaccine alone, combined with adjuvants or gene therapy. (**C**) Distribution of the pathologies addressed in the GET trials.

Tolerability and safety of GET

During GET, the direct nerve stimulation and the intense muscle contraction can potentially induce a local acute pain. Noticeably, pain level depends on the injection site, type of electrodes and pulse parameters. The most important parameters to reduce patient discomfort are the amplitude and duration of the electric field. Similarly, waveform, rate and number of pulses globally influence patient compliance [134]. In most clinical studies, the adverse events associated to GET are mild and they do not request the use of a local anesthetic [135-137]. Several

studies have been evaluating the safety and tolerability of different devices and their use as a gene delivery technique. In all these studies the common patient reaction is a few minutes mild pain. GET is well tolerated whatever the delivery site (IM, ID or intratumoral (IT)) with no systemic toxicity or clinically relevant side effects (Table 2).

DNA vaccine against cancer

In clinical trials using plasmid EP, the most common strategy against cancer is the use of pDNA that encodes tumor antigens overexpressed in a particular cancer type. In some cases, the plasmid coding for the antigen is used in combination with an adjuvant, typically encoding human IL-12 (hIL-12). pDNA vaccines can also be combined with other therapies (radiotherapy, surgical removal, chemotherapy or endocrine therapy) to mutually enhance their potency. Most of the current GET clinical trials against cancer use an IM injection of pDNA followed by EP (Table 3). The cost-effectiveness and the safety profile of the pDNA vaccines allow repeated low-dose administrations (in the order of mg) for a long-term protection [138]. Most of the studies are still in progress and only few results are available. In a phase I clinical trial, pDNA coding for the melanoma tyrosinase (Tyr), a melanocytic differentiation antigen expressed by several melanoma specimens, able to induce $CD8^+$ T -cell response, was tested. At the maximal dose (1.5 mg), 40% of patients developed Tyr-reactive CD8+ T-cell responses and 14% of those that received all the 5 doses had an increase in Tyr reactive $CD8^+$ IFN-γ^+ T cells [139]. It was also shown that a plasmid encoding human papilloma virus (HPV) E6 and E7 viral oncoproteins which promote p53 and retinoblastoma degradation decreased the possibility of cervical cancer progression. pDNA was electrotransfered IM, using Trigrid Delivery System in both deltoid muscles. The level of antibodies against DNA in the blood of patients was below the detection limit. Furthermore, 22% of patients developed an IFN-γ response after a single immunization and it reaches

55% after the second vaccination. High levels of IFN-γ were observed even at week 24, suggesting the vaccine induction of E6/E7- specific memory T cells. After the second immunization half of the patients showed HPV and lesions clearance at week 12 and other 22% reached the same result after week 36 [140]. Encouraging phase 1 safety, tolerability, and immunogenicity results were reported for a therapeutic HPV16/18 candidate vaccine, VGX-3100, delivered by *in vivo* EP in women previously treated for high-grade cervical dysplasia**.** The vaccine was well tolerated and induces antigen-specific humoral and Th1-biased cellular immune responses. Strong CTL response, an important determinant for successful immunotherapy, was observed upon DNA vaccination [141]. More recently, VGX-3100 vaccine was involved in a randomized, double-blind, placebo-controlled phase 2b trial. The results indicate clinical efficacy against cervical intraepithelial neoplasia (CIN) 2/3 following vaccination by EP and the trafficking and tissue infiltration of antigen specific CD8+ T cells in the targeted lesions. VGX-3100 is the first therapeutic vaccine to show efficacy against CIN2/3 associated with HPV-16 and HPV-18 and could present a new non-surgical therapeutic treatment [142]. In a clinical study against prostate cancer, pDNA coding for rhesus macaque prostate-specific antigen (PSA) was delivered ID using a DermaVax EP system. 20% of the patients showed an increased response to self PSA. After 1 year of follow-up, only 13% of the subjects developed metastatic disease. This was the first clinical trial in which EP was combined with ID injection for tumor treatment and the anti-PSA antibodies were less efficiently produced than after IM DNA vaccination [135].

5.4. Possible strategies to optimize gene electrotransfer

Several parameters need to be considered to set up an efficient GET. Figure 6 summarizes the different actors that are involved in GET process, and states how they can be handled to impact the treatment outcome.

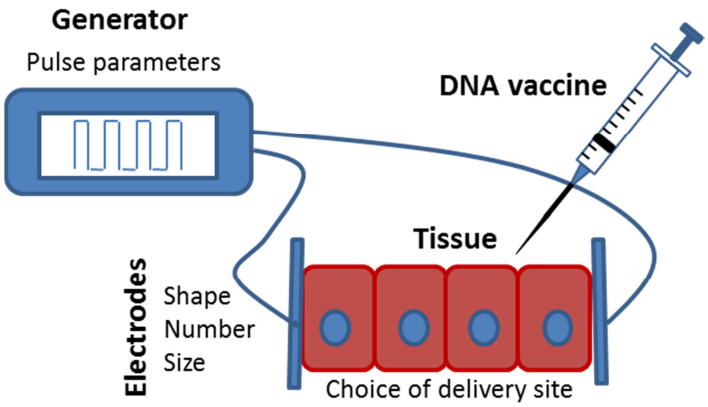

Figure 6 - Schematic representation of EP procedure for GET and description of the protocol parameters that can be handled to optimize treatment outcome.

Choice of the delivery site

The most usual delivery sites are muscle and skin. Delivery to muscle requires a lower electric field and allows a long duration in gene expression and a delivery of a large amount of liquid; however, muscle contraction can be painful. Skin is more accessible and contains many dendritic cells. These characteristics consent a less invasiveness, thanks to a reduced needle depth, a better tolerable delivery and a great immune response [137]. In preclinical model, the choice of the delivery site for GET influenced not only the magnitude but also the type of immune response [71].

Improvement of electrodes

In order to bring gene delivery into broader clinical applications, the delivery needs to be compliant with the patients, i.e. non-invasive and not inducing discomfort. To this end, both the design of electrodes and the choice of electrical parameters are very important. The electrodes used for GET can be divided in two groups: the non-invasive and the invasive electrodes [115,143]. The invasive electrodes consist of different needle configurations that are penetrated to the skin, muscle or tumour [115,122,143-145] (Figure 7). Well covered electric field achieved

by pulse delivery in between the pairs of electrodes and large electrotransfered tissue area results in high transfection efficiency. The commercially available electrodes used in EP clinical trials belong to this category and are additionally modified for more feasible clinical use.

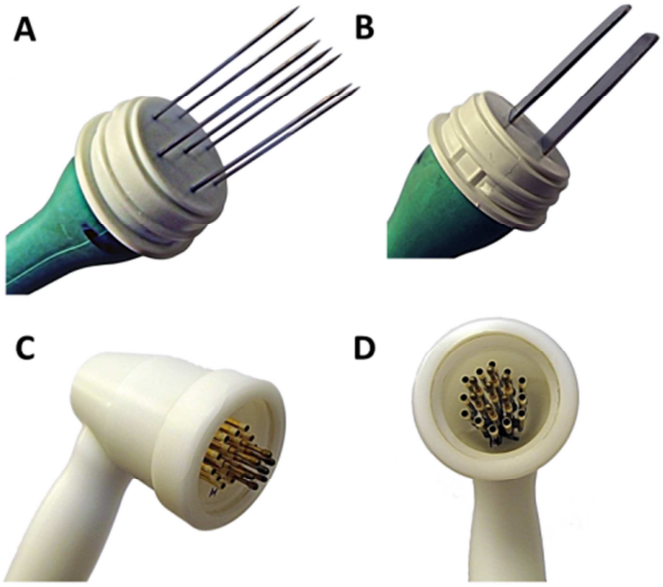

Figure 7 - Different types of electrodes.

(**A**) needle electrode; (**B**) plate electrode; (**C**) and (**D**) multi-electrode array.

However, the invasiveness and discomfort by GET with such electrodes initiated the studies with less invasive electrodes. One of the minimally invasive approaches is the utilization of microneedles, which provide less painful delivery of DNA and achieve sufficient electric field distribution for effective GET [146]. Further advancements have been made using non-penetrating electrodes such as calliper and plate electrodes. The depth of penetration of electric field is rather small [147], basically in the skinfold, which is in certain situations not convenient [147], and would not be optimal for use in humans. Furthermore, these electrodes require high voltages to enhance delivery and therefore can cause tissue damage

[148]. To this end other types of non-invasive electrodes are being explored. Such non-invasive electrodes, the multi-electrode array (MEA) were designed by several groups and have proved to be effective for the delivery of pDNA to the skin [101,148,149] and have promising expectations in cancer gene therapy [148]. With the MEA, the applied voltage was minimized by maintaining a short electrode distance. This diminished or eliminated the muscle twitching and pain associated with the application of the pulsed electric field [148]. The design of novel electrodes therefore leads to less adverse side effects than other EP delivery systems with efficient gene delivery and high immune response and proposes promising clinical applications.

Optimization of pulse parameters

A large variety of pulses has been used for GET. The electric pulses chosen for gene transfection have been either short high voltage (HV) pulses alone [101,150], low voltage (LV) pulses alone [151] or a combination of one or several HV and LV pulses [99,152-154]. The border between HV and LV is not strictly defined but in general the HV pulses (> 400 V/cm) are typically short (50-100 µs) whereas the LV pulses (< 400 V/cm) are longer, typically in milliseconds (10-400 ms) [143]. The optimal pulse conditions reported are not the same but could depend on the type of transfected tissue, type of electrode and also type of injected DNA. To ensure efficient GET electrical parameters need to be adjusted for transfection of different tissues, due to differences in tissue structure, cell size, and DNA and electric field distribution. Typically, muscles are easier to transfect compared to skin or tumours, what requires lower field strengths to reach the efficient gene delivery into muscle cells [154]. Beside the duration and amplitude of electric pulses also the number of pulses and pulse polarity vary between the studies. To minimize tissue damages the tendency is to decrease the voltage and pulse length and to reduce the number of pulses and change the pulse polarity[155,156]. Therefore, to find the best compromise

between the potential damages caused by electric pulses and the efficiency and specificity of gene delivery, the electrical parameters need to be carefully selected for each targeted tissue in order to reach the expression for optimal therapeutic effectiveness.

5.5. Advantages and limitations

Although the use of genes is attractive and opens a promising way to combat many diseases, their delivery in patient's cells remains challenging. EP appears as a safe and effective transfection technique, presenting many advantages over other strategies. EP requires precise and specific protocol optimization in order to obtain the best treatment outcome while avoiding side effects. The last 10 years have shown a growing number of clinical trials based on DNA EP. They consist mainly in DNA vaccines against infectious diseases and cancers. Compared to the other methods of gene delivery, EP benefits from excellent transfection efficiency. After EP, the transgene is locally and transiently expressed. However, systemic effects can be observed such as immune response against an encoded antigen or secretion of a therapeutic protein that can be distributed systemically. Consequently, EP has been used for treating a wide range of diseases and many dynamic research groups focus on the improvement of the current approaches, from the optimization of plasmids to the improvement of electrodes and devices. If patients were initially worried about the use of electrical pulses for GET, it appears from all human studies that EP is well tolerated and only mild and transient side effects have been observed.

EP holds the potential of treating or preventing many diseases such as cancer. Nevertheless, EP is a complex process and the ultimate goal would be development of an approach for effective, controlled and painless gene delivery to various tissues. Providing a way to facilitate the setting up of optimal protocols is a global challenge. First, further refinement of the electrodes and electrical

parameters and adjustment to the clinical situation are required. To help physicians in their clinical practice, it could be of interest to develop monitoring tools. For instance, imaging methods can be useful to establish a treatment planning that models electrode position and determine the number, amplitude, duration and frequency of the electric pulses to obtain the desired effect on the targeted tissue [110,157]. In addition, it is important to increase the current knowledge on how GET parameters (such as electric field, electrode design and delivery site) influence clinical outcome. For instance, studies must be carried out to better understand the basic mechanism of membrane modification at molecular, cellular and tissue level. In particular, the effect of EP procedure itself and the effect of GET on the early inflammatory events and innate immune activation would merit a deep investigation. But, a better understanding is not the only key to success. As illustrated in this review, all the parameters are interconnected and the results of the clinical studies are somewhat difficult to compare. Therefore, a way to organize the data is needed as a platform for the EP technology. This would facilitate adoption of common protocols and would lead to an integrative approach for gene delivery. Apart from its use as a delivery method for DNA vaccines or therapies, it has been recently demonstrated that EP holds also a potential for inducing immunogenic cell death (ICD). Indeed, in certain circumstances depending on the initiating stimulus, dying tumor cells behave as a cancer vaccine and elicit a cytotoxic immune response. It involves the exposure of damage-associated molecular patterns (DAMPs) that are recognized by innate immune cells, which subsequently prime effector T cell response against dead-cell associated antigens. It has been shown that ECT was able to induce ICD [31]. We believe that GET of certain pDNA could similarly trigger ICD and thus further extend the range of GET clinical applications in the future.

In conclusion, the multiplication of clinical trials and the results already available demonstrate the strong potential of GET. Nevertheless, it remains critical to further increase fundamental knowledge about EP to refine future strategies and to continue exploring new EP-based therapeutic options. Finally, long term observation is needed to confirm the safety of GET.

Table 2- EP devices used in clinical trials.

The name, the delivery route, the common side effects and their duration are described.

Name of the device	Route of delivery	Common side effects	Common duration	Ref
CELLECTRA®	- IM : 5 needles, 18 mm depth - ID : 3 needles, 3 mm depth	- Injection pain (IM > ID) - Involuntary muscle contraction after ID EP - Local grade 1 erythema	- Few minutes (< 10) - One day in one patient - One day	[137]
Easy Vax™	- ID : 80 needles, 600 µm depth	- Burning sensation - Tingling	- Few seconds - Few minutes (< 5)	[134]
MedPulser	- IM : 4 needles, 1.5 cm depth - IT (no more information available)	- IM injection pain - Transient local reaction	- One minute - Few minutes	[136]
Trigrid	- IM : 4 needles - ID (no more information available)	- Mild-moderate local pain - Local reaction	- Few minutes - One day	[139,140,158]
Dermavax™	- ID : 4-4-2 or 4-6-2 parallel row needle electrodes, 2 mm depth	- Momentary muscle fasciculation - Minor grade 1 skin reaction	Brief but not further specified	[135]
OncoSec	- IT - IM (no more information available)	- Transient grade 1 pain - Grade 1 injection site reaction	Brief but not further specified	
Cliniporator	- IT : 2 rows of 4 linear needles, 4 mm between needle arrays	- Treatment was performed under local anesthesia - Mild local toxicity: erythema around the treated lesion - Contraction of the underlying muscle - Good tolerability	- Transient - Brief	[159]

Table 3- Clinical trials using **DNA vaccines against cancer**.

A description of the vaccine, the year and the current trial phase are provided. Information regarding the use of adjuvant, the delivery site, the dose, the EP device and additional key parameters are presented. Combinations with other treatments are mentioned: chemotherapy (C), radiotherapy (R), chemoradiotherapy (CR), surgery (S) and endocrine therapy (E). The clinicaltrials.gov identifier of the study and the principal investigator's name (when available) are provided.

Biological	Phase	Year	Adjuvant	Additional treatments	Delivery site	Dose	Electroporation	Additional information
DNA VACCINES AGAINST HPV-INDUCED CANCERS (10 clinical trials)								
VGX-3100 = pDNA encoding E6 and E7 oncogenes (responsible of cellular transformation) of HPV types 16 and 18	I	2008	/	/	IM	0.6, 2 or 6mg 3 doses over 3 months	CELLECTRA	- NCT00685412, Chu, Parker, Sunyecz, Morales et al.
	I	2010	/	/	IM	6mg 1 dose	CELLECTRA	- NCT01188850, Parker, Sunyecz, Morales et al. - Adult females who have been previously immunized with three doses of VGX-3100
	II	2011	/	/	IM	1ml 3 doses over 3 months	CELLECTRA	- NCT01304524, Trimble, Parker, Valenzuela et al.
	I/II	2014	plasmid encoding hIL-12	R, CR	IM	6mg of VGX-3100 and 1mg adjuvant 1 dose	CELLECTRA	- NCT02172911, Gelder et al.
	I/IIa	2014	plasmid encoding hIL-12	S, CR	IM	6mg of VGX-3100 and 1mg adjuvant 4 doses	CELLECTRA	- NCT02163057, Yang et al.
GX-188E = DNA plasmid encoding E6 and E7 proteins of HPV16 and HPV18 fused to extracellular domain of Flt3L and the signal sequence of tissue plasminogen activator (tpa)	I	2012	/	/	IM	1, 2 and 4 mg; The highest dose, 4 mg of GX-188E was split into 2+2 mg	Trigrid	- NCT01634503, Kim et al. - Results published in [140]

						1 dose		
	I	2014	/	/				- NCT02100085, Kim et al. - Follow-up of patients who completed the phase I GX-188E trial
	II	2014	/	/	IM	1 or 4 mg 3 doses over 3 months	Trigrid	- NCT02139267, Park, Kim, Lee, Cho et al.
	II	2015	/	/	IM	1 or 4mg 3 doses	Not mentioned	- NCT02411019, Park, Kim, Lee, Cho et al.
pNGVL4a-CRT/E7 (detox) = pNGVL4a vector coding for CTR (calreticulin) and E7 (detox), a mutated form of E7 genes	I	2011	/	C	IM	0.5, 1, 2 or 4mg/dose 3 doses over 43 days	Trigrid	- NCT01493154, Califano et al.
DNA VACCINES AGAINST MELANOMA (2 clinical trials)								
pINGmuTyr = DNA vaccine encoding the melanosomal antigen tyrosinase (Xenogeneic Tyrosinase)	I	2007	/	R, CR, C	IM	0.2, 0.5 or 1.5 mg 5 doses every 3 weeks	Trigrid	- NCT00471133, Wolchok et al. - Results published in [139]
SCIB1 = Plasmid DNA encoding a human antibody molecule engineered to express T cell epitopes derived from the TRP2 and gp100 and two helper T cell epitopes	I/II	2010	/	/	IM	0.4, 2.0, 4.0 and 8.0 mg 5 doses over 6 months	Trigrid	- NCT01138410, Patel, Lorigan, Mulatero, Ottensmeier, Pandha et al. - Part 1 of the study will escalate through 0.4, 2.0, 4.0 and 8.0 mg dose level cohorts - In part 2, the 4.0 and 8.0 mg doses will be administered
DNA VACCINES AGAINST BREAST CANCER (3 clinical trials)								
Personalized polyepitope DNA vaccine	I	2015	/	/	IM	4 mg 3 doses over 57 days	Trigrid	- NCT02348320, Gillanders et al.
Mammaglobin-A DNA Vaccine	I	2014	/	E	IM (2 sites)	2X3 doses over 84 days	Not mentioned	- NCT02204098, Gillanders et al.
INO-1400 = Immunotherapy designed to target a gene known as the human	I	2014	plasmid encoding hIL-12	/	IM	2 or 8mg of INO-1400 with or without	Not mentioned	- NCT02327468, Vonderheide et al. - Also tested for lung and pancreatic cancers

telomerase reverse transcriptase (hTERT)						0.5mg or 2mg adjuvant 4 doses over 3 months		
DNA VACCINES AGAINST OTHER TYPE OF CANCER (6 clinical trials)								
CEA DNA = Carcinoembryonic antigen (overexpressed in a variety of cancer cell types) fused to a tetanus toxoid T helper epitope	I/II	2010	GM-CSF	C	ID	400µg 2 doses over 3 months	DermaVax	- NCT01064375, Liljefors et al. - Pathology: colorectal cancer
pVAXrcPSAv53l = DNA encoding rhPSA (Rhesus Prostate Specific Antigen), 89% homologous to human PSA	I/II	2009	/	/	ID	50 or 150 or 400 or 1000 or 1600µg 5 doses, 4 weeks apart	DermaVax	- NCT00859729, Yachnin et al. - Pathology: prostate cancer - Results published in [135]
pDOM-WT1-37 = First domain of fragment C (FrC) of tetanus toxin (pDOM) fused to the human Wilms' Tumor gene-1-derived MHC class I-binding epitope	II	2011	/	/	IM	1mg 6 times at 4 weekly intervals	Not mentioned	- NCT01334060 - Pathology: leukemia - Responders may continue vaccination 3 monthly to maximum of 24 months
INVAC-1 = DNA vaccine encoding human telomerase reverse transcriptase (hTERT)	I	2014	/	/	ID	100, 400 or 800 µg 4 weeks X 3 cycles	Not mentioned	- NCT02301754, Culine et al. - Pathology: solid tumors
INO-3106 = Vaccine against HPV6	I	2014	plasmid encoding hIL-12	/	No mention	3 mg or 6 mg INO-3106 alone or in combination with 1 mg adjuvant 4 doses over 9 weeks	Not mentioned	- NCT02241369, Yang et al. - Pathology: aerodigestive malignancies (e.g. squamous cells carcinoma)
V934/V935 hTERT = Cancer vaccine directed against human telomerase reverse transcriptase (hTERT)	I	2008	/	/	IM	2X10^9 or 2X10^11 vector/mL every 2 weeks	Not mentioned	- NCT00753415 - Pathology: solid tumors

6. Adjuvanting cancer DNA vaccines

6.1. Adjuvants: the bridge between innate and adaptive immune system

Adjuvants are defined as substances added to the vaccines to enhance the immunogenicity of an antigen[17]. To elicit robust, specific and long-lasting immune responses, adjuvants can employ one or more of the following mechanisms[160]: i) sustained release of the antigen, (ii) up-regulation of cytokines or chemokines, iii) immune cells recruitment to the vaccine injection site, iv) increased antigen uptake and presentation by APCs and v) the activation, maturation and migration of APCs. This last mechanism is critical as it defines the T-cell polarization and the development of immunity or tolerance[58]. Indeed, DCs are the master regulators of the immune response, making the bridge between the innate and the adaptive immune systems. The type of insult (and thus the danger signals) that prompts DCs maturation affects the signals that DCs address to T cells, and thus whether and how a T cell will respond to an antigen[161].

The delivery system can act as an adjuvant, as it can for instance target vaccines to APCs, concentrate and display the antigen in repetitive patterns, release the antigen in a controlled and sustained manner and help co-localize antigen and immune potentiators. It can also mimic key features of pathogens such as their size, shape and surface molecule organization[162]. For instance, nanoparticle formulations are promising for reaching those goals[163,164]. Electroporation also has adjuvant properties, as explained in section 5.2. Moreover, elements in the vaccine formulation can activate innate immunity directly (for example, cytokines) or through pathogen PRRs recognizing PAMPs (such as microbial components)[165]. Such strategies are discussed in the next sections.

6.2. The intrinsic adjuvant properties of DNA vaccines

The plasmid DNA used for DNA vaccination possesses built-in adjuvant properties that help in the induction of sustainable cellular response. Indeed, it may contain Cytosine-phosphate-guanosine (CpG) motifs, which are PAMPs recognized by the toll-like receptor (TLR)-9 in the endosomal compartment of immune cells. Through the adaptor protein myeloid differentiation primary-response protein 88 (MYD88), the TLR-9 pathway promotes the activation of the transcription factors activator protein 1 (AP1) and the nuclear factor-κB (NF-κB). These induce the expression of pro-inflammatory cytokines. The IFN-regulatory factor 7 (IRF7) is also activated, leading to the expression of type I IFNs[166]. The benefits of CpG insertion in the plasmid backbone to enhance DNA vaccine efficacy have been demonstrated in several studies[102,167].

However, TLR-9 induced innate immune response is not the only responsible for immunogenicity of DNA vaccines, since TLR9 knockout mice can still respond to DNA[168-170]. In fact, it was demonstrated that multiple DNA sensors coexist and play a crucial role in DNA vaccine immunogenicity, as they promote the induction of type I IFNs through the stimulator of IFN genes (STING)- TANK binding kinase 1 (TBK1) signaling pathway[171,172]. In addition, STING engagement may also lead to NF-κB activation and expression of pro-inflammatory cytokines. The mechanisms involved in pDNA sensing are reviewed elsewhere[166]. It has to be noted that the receptors involved in pDNA sensing depend on the type of transfected cells[129].

The pathways involved in DNA vaccine immunogenicity lead to the expression of type I IFN and pro-inflammatory cytokines. These cytokines are required for an effective activation of Th1 and CD8+ T cells[17]. Particularly, type I IFNs are essential for generating CTL and Th1 responses following DNA vaccine administration[166,173]. They regulate T-cell responses by modulating DCs function, promoting cross-presentation or through direct signaling in T cells. Their major role in instigating

anti-tumour T cell immunity is well established[174]. However, type I IFNs are also known to induce the expression of IFN-stimulated genes. The encoded proteins restrain pathogens by several mechanisms, including the inhibition of viral transcription, translation and replication and the degradation of viral nucleic acids[175]. This antiviral program could therefore interfere with pDNA expression.

6.3. Conventional adjuvants

Traditional adjuvants include killed bacteria, bacterial components, aluminum salts, polymers, lipids, polysaccharide, lipopolysaccharides, oil emulsions and cytokines among others[176]. Many conventional adjuvants have been tested in the context of DNA vaccination. Alum has been one of the most studied compounds, as it is found in many licensed human vaccines[177]. The effects of alum on the innate immune system are mainly an increased local tissue inflammation[178-180] and an enhanced antigen presentation by APCs[180,181]. Even if the adjuvant effect of alum was promising in small rodent models[182,183], the results were disappointing in larger animal species[184]. It has been supplanted by more promising strategies. For instance, Advax™ (Vaxine Pty Ltd, Australia) is a delta inulin polysaccharide)[185] known to activate the complement and enhance antigen presentation[186]. It reinforced the mucosal and systemic immunity in a preclinical study using a HIV DNA vaccine[185]. Zymosan (a yeast cell wall)[187] and chitosan (a polymer coming from crustacean shell)[188] could enhance antigen presentation through complement activation[187,189] and acted as ligand of PRRs[189,190]. They were both able to enhance the humoral and cellular immune response to DNA vaccines against viral proteins in preclinical studies[187,188]. The cationic-lipid-based adjuvant Vaxfectin® (Vical, USA)[191], known to enrich antigen presentation, apoptosis, TLR pathways and enhance cell infiltration at the vaccine delivery site[192], enhanced the humoral response and protective immunity in preclinical viral challenge[191] but its benefit in clinical trials is not yet established[193,194].

Overall, mixed results indicate the modest capacity of conventional adjuvant to stimulate immunity with DNA vaccines. Their use has mainly been evaluated with DNA vaccine against viral proteins and their efficacy in clinical trial has not been demonstrated so far. Their limited efficacy could be explained by inappropriate delivery timing. Indeed, most of the time, these adjuvants are mixed with the antigen before administration to benefit from simultaneous interaction with immune cells. However, in the case of DNA vaccines, the antigen has to be expressed by the host cellular machinery after plasmid administration. Since many adjuvants wok immediately after injection, their effect might be lower at the time the antigen is present[84]. Current DNA vaccine researches focus on developing efficient genetic adjuvants.

6.4. Genetic adjuvants

A potent strategy to enhance DNA vaccines immunogenicity is the use of genetic adjuvants, which are plasmids encoding immunomodulatory proteins. They have been widely used in preclinical trials involving DNA vaccines[195,92,106,84]. Advantages of using genetic adjuvants are their low cost and simplicity of administration. They avoid the need of expensive and unstable recombinant cytokines and potentially toxic conventional adjuvants[196], and can also benefit from an adjuvant effect due to pDNA sensing. The adjuvant and the antigen encoding genes can be delivered either on separate individual plasmids, as a bi or polycistronic vector or as a chimeric protein construct. This strategy allows coordinated delivery of the antigens and adjuvants encoding sequences[106].

The immunoregulatory proteins encoded by genetic adjuvants can act at different levels during immune response priming (Figure 8), and aim either at stimulating immune effectors or inhibiting immunosuppressive mechanisms[32]. Firstly, plasmids encoding proteins capable of recruiting, activating and/or enhancing

APCs activity can be used. GM-CSF and proinflammatory cytokines and chemokines (among which IL-1 tumour necrosis factor (TNF)-α, IL-6, IL-8, CC chemokine ligand (CCL) 1 and 2) encoding plasmids may increase potency of immune response by coordinating the movement and functionality of APCs at the inoculation site[106]. Secondly, the encoded adjuvants can modulate innate immune system by acting on the PRR pathways to trigger IFN and pro-inflammatory cytokines production. The most common strategy consists in delivering plasmids encoding intermediates of these signaling pathways such as MyB88, IRF7 or cytosolic DNA sensor. This category of genetic adjuvant has been widely reviewed by Kobiyma et al[197].

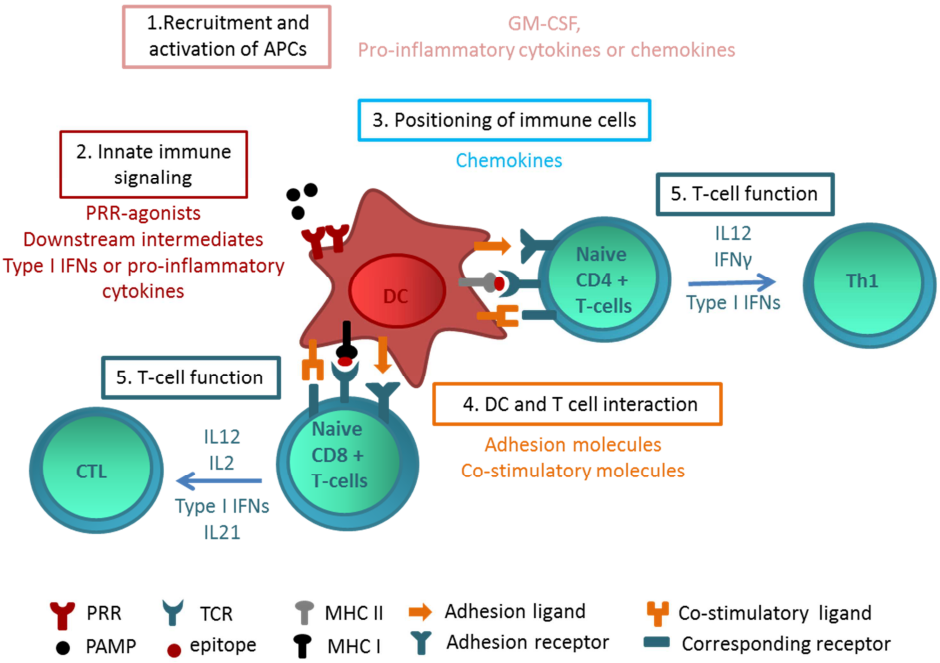

Figure 8 – Potential levels of action of genetic adjuvants for cancer DNA vaccines.

A third role of the adjuvant may be to optimize the migration and positioning of immune cells, by delivering for instance pDNA coding for CCL19 and CCL21, two chemokines that are responsible for T cells and DC homing to lymph nodes[198,92].

Next to that, some genetic adjuvant can act directly on the interaction between DCs and T cells. Plasmids encoding adhesion proteins such as LFA-3 and ICAM-1 can improve the junction between cells. The delivery of plasmids encoding co-stimulatory molecules such as CD80, CD86, and CD40 enhanced T-cell activation. The effector function of T cells can also be upregulated by blocking interactions between their PD-1 receptor and its ligands PD-L1 or PD-L2. Plasmid-encoded soluble PD-1, when used in combination with HPV DNA vaccines, was found to increase antigen-specific $CD8^+$ T-cell responses compared to the vaccine alone in mice[199]. Last but not least, many genetic adjuvants encoding cytokines have been used in an attempt to regulate T-cell functions. In the case of cancer vaccine development, the use of cytokines that induce Th1 and CTL responses are ideal targets (IFN-γ, IL-2, IL-12, IL-15, IL-18 for instance)[32].

Currently, the most common genetic adjuvants are plasmid coding for cytokines and only these molecules have been involved in cancer DNA vaccines clinical trials[200]. Particularly, plasmids encoding hIL-12 are often chosen as adjuvant. This cytokine is known to enhance Th-1 biased cellular immunity[201]. Another strategy consists in the use of GM-CSF encoding plasmid. This pro-inflammatory cytokine helps in the recruitment, activation and enhancement of activity of APCs[202]. However, the genetic adjuvant field is under intense wave of research. Several promising strategies have been considered in preclinical studies and hold a huge potential for future clinical investigations. For instance, type-1 IFN or Th-1 inducing cytokines such as IL-2 and IFN-γ are promising. In these approaches, the timing of cytokine plasmid delivery relative to the antigenic plasmid delivery is a key parameter to consider, as it can lead to different types of immunity[195].

6.5. The use of plasmid encoding viral structural proteins as genetic adjuvant for cancer DNA vaccine

A strategy that has not been extensively studied so far is the use of plasmid encoding viral structural proteins to enhance the efficacy of cancer DNA vaccines. Viruses are not foreigners to the immunotherapy field. They have been used for years either as therapeutic agent or as delivery system. For instance, the use of oncolytic viruses is a highly promising approach for cancer therapy[30]. Viral vectors have also been used to deliver tumour antigens and other immunostimulatory agents[203]. By mimicking natural infection, they can efficiently deliver genes and induce potent immune response[64,24].

Viruses are very important immunological players. Innate and adaptive immunity to viruses has been widely studied and has helped in the understanding of the functioning of the immune system[37]. The initiation of the immune response to an invading microorganism like a virus requires that the host senses the organism and its constituents. Pathogen recognition consists in the interaction of PPRs and PAMPs. Nucleic acids motifs are the main virus-derived PAMPs to be recognized by the innate immune system. TLRs 3, 7/8 and 9 as well as several other cytosolic receptors are involved in this sensing[166]. The sensing of viral infection is also mediated by the TLR 2 and TLR 4 expressed on cell surface. TLR-2 can detect core proteins and TLR-4 is involved in envelope proteins sensing[204]. Several other types of membrane bound, cytoplasmic or secreted PRRs can also be involved in virus sensing[37]. The main consequence of PRR engagement in response to viral infection is the induction of IFN and other inflammatory cytokines that play an important role in orchestrating the adaptive immune response. Another consequence of PRR activation is the induction of autophagy [37]. Autophagy is a catabolic recycling pathway that contributes to viral antigen processing and presentation to CD4+ and CD8+ T cells[205]. The virus contains several foreign

antigens that can stimulate both cellular and humoral arms of the adaptive immune system, and the cytotoxic response is often very strongly induced by viral infections[206].

The ability of viruses to induce strong humoral and cellular immunity has been used to develop several viral-vector based vaccines strategies[206]. Among the developed strategies, virus-like-particles (VLPs) have caught researchers' interest. To date, there are already four VLP vaccines licensed for human use, and many others are in clinical trials[207]. VLPs are self-assembling multiprotein structures that resemble viable virus particles in conformation but lack the viral genome. Consequently, they are non-infectious and non-replicative, but retain the ability to penetrate cells[208]. These properties make VLPs attractive for vaccine design. Their virus-like appearance (repetitive antigenic surface structure and their size) efficiently stimulates the immune system as seen with native viruses but without infectivity risks[209]. They are efficiently taken up and processed by DCs, which is followed by antigen processing and presentation by both MHC class I and II molecules[208]. VLPs are also attractive as delivery system. They can be loaded with foreign nucleic acids, proteins or drugs. Their receptor-mediated transducing properties allow delivery of the foreign compounds very specifically[208]. Finally, these particles are versatile in terms of composition and easy to engineer[210]. It is possible to decorate them with foreign molecules by genetic manipulations, non-covalent interactions, physical methods or chemical coupling. This can be used to modify the labelling, targeting and immunomodulatory properties of these particles[208]. VLPs can be effective vaccines against analogous viruses from which they are derived but can also be used to present foreign epitopes to the immune system[211].

VLPs are produced by transfecting genes encoding viral structural proteins of interest into host cells (Figure 9). Viral structural proteins are components of the

mature assembled virus particles. They may include nucleocapsid core proteins, enzymes packaged within the virus particle, and membrane components (https://www.ncbi.nlm.nih.gov/). The viral envelope is obtained from the host cell's plasma membrane embedded with viral glycoproteins that play a critical role in virus-to-cell fusion. The expressed proteins self-assemble within the cell and then particles bud, as during viral infection[208], and the released VLPs are purified[208]. Several expression systems exist, but mammalian cells are very convenient because they can carry out the post-translational modifications that are required for proper protein folding.

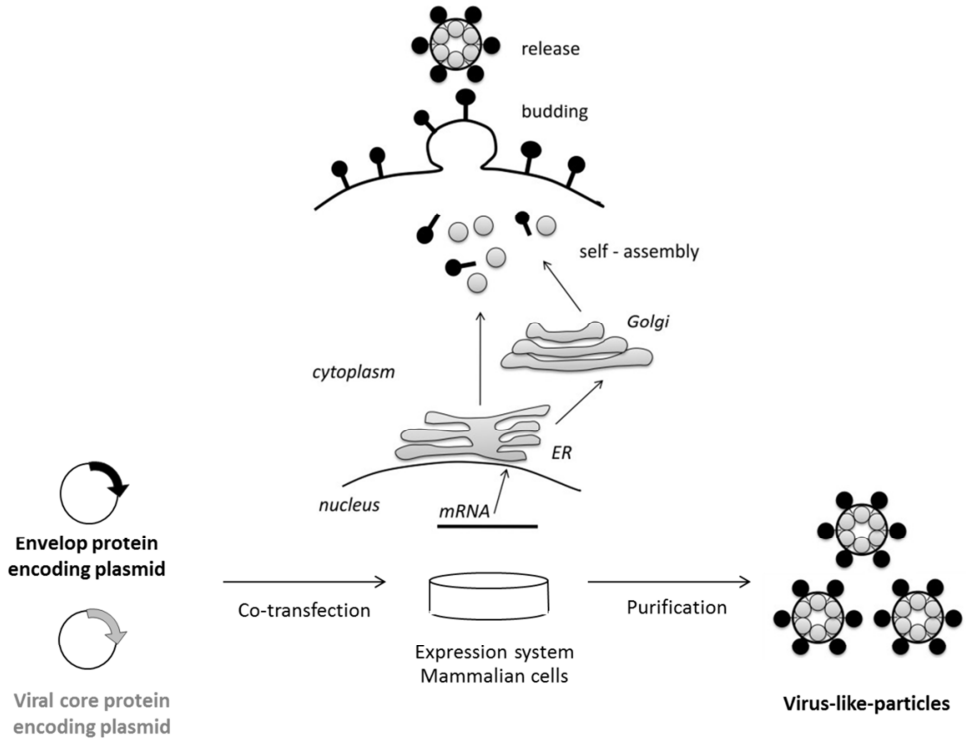

Figure 9 – Schematic representation of virus-like-particles production (adapted from Naskalska et al., Polish Journal of Microbiology, 2015)

The main disadvantage related to VLPs is their time consuming *in vitro* production. Therefore, some groups started to assess whether *in vivo* transfection

of plasmids encoding VLPs could combine the immunogenicity of VLPs and the ease of production of plasmid DNA. Marsac et al. showed that co-administration of a plasmid encoding the viral core protein HIV-1 Gag and a plasmid encoding the vesicular stomatitis virus glycoprotein G envelope improved the Gag-specific CD8+ T cell response in mice[212]. Bellier et al. designed a DNA vaccine based on murine leukemia virus-like particles to induce cellular immune responses to the lymphocytic choriomeningitis virus in mice[213]. The same research group also developed a VLP-based DNA vaccine approach able to induce strong T-cell-mediated hepatitis C immune responses[214,215]. The advantage of this approach was also suggested by Vandermeulen et al. They showed that the gp160 specific immune response was enhanced following administration of a plasmid encoding gp160 envelope together with a plasmid coding for viral core proteins[209].

All these studies highlighted the benefit of using viral structural protein encoding plasmids to strengthen the immunogenicity of DNA vaccines. This was evidenced mainly in vaccines against infectious diseases. However, this approach could be useful to enhance cancer DNA vaccines efficacy. Plasmids encoding viral structural proteins could act as genetic adjuvant for different reasons. Firstly, there might be an innate immune sensing of the viral protein gene sequences. The expressed proteins can also be sensed by PRRs and activate innate immunity. Secondly, the viral structural proteins probably contain several T cell epitopes that may induce cellular responses. The induction of a potent helper response could support the cytotoxic activity of the anti-tumour CTLs. Finally, an adjuvant effect can be related to protein's function. The formation of VLPs or the fusogenic protein properties could improve DC activation and T-cell priming by several mechanisms.

Scientific aims

Cancer immunotherapy is emerging as a very promising strategy to fight cancer. Since the discovery of tumour antigens recognized by T cells, efforts have been made to develop vaccines to treat or prevent cancer. Particularly, DNA vaccines raised high expectations due to their numerous advantages and their ability to elicit a broad immune response, with the induction of both helper and cytotoxic T cells. However, they suffered from low immunogenicity in humans and efforts have been done to enhance their efficacy. Advances in delivery technologies have revived interest in DNA vaccination. One of the most successful has been the use of electroporation. This approach has been validated commercially and holds great promises for gene delivery applications. Studies have also been performed to enhance the immunostimulatory properties of DNA vaccines. These works mainly focus on optimizing the intrinsic adjuvant properties of the pDNA or assessing the use conventional and genetic adjuvants.

A strategy that could be promising to enhance DNA vaccine immunogenicity is the use of plasmid encoding viral structural proteins. Viruses are strong inducers of innate and adaptive immune responses. Hence, they have already been included in many vaccination strategies including the use of virus-like-particles produced *in vitro*. The *in vivo* delivery of plasmid encoding viral structural proteins was shown to strengthen the immunogenicity of DNA vaccines against infectious diseases. In this work, we hypothesize that this approach could enhance cancer DNA vaccines efficacy by acting as a genetic adjuvant able to: (i) stimulate innate immunity via PRR sensing of the viral gene sequence or the expressed protein, (ii) induce strong helper responses thanks to processing of viral T-cell epitopes and/or (iii) enhance DCs maturation and T-cell priming thanks to the protein's function.

In this thesis, we evaluate the use of plasmids encoding viral structural proteins to enhance cancer DNA vaccine efficacy. We check the hypothesis that this strategy would help in inducing potent cellular immune responses to the vaccine antigen and strengthen the anti-tumour activity (Figure 10).

We choose as model two plasmids encoding viral structural proteins that have been used previously to create VLPs displaying tumour antigens. One of them encodes the Gag viral core protein of the human immunodeficiency virus type 1 (HIV-1). This plasmid is a candidate of choice as the Gag protein was shown to possess immunoregulatory properties[209], related to its ability to form VLPs[216,217] or due to its sensing by PRRs[218-220]. Plasmids encoding this protein have been used in clinical trials in the context of HIV vaccination and they were shown to be safe and immunogenic. The second plasmid codes for the viral envelop G glycoprotein of the vesicular stomatitis virus (VSV-G), known to possess permissive sites in which it is possible to insert several amino acids without modifying protein function[221]. VSV-G is often used for the pseudotyping of viral particles because of its high cellular tropism. We assume this plasmid may act as an adjuvant as it was shown previously to induce innate immunity[222-224] and it may be involved in antigen processing pathways modulation[225-227].

To evaluate the anti-cancer potential of the vaccine candidates, a melanoma model is chosen because melanoma is often taken as an example for immune-oncologists[35]. B16F10-OVA melanoma cells that constitutively express ovalbumin (OVA) are used. To characterize the immune response, the OVA model antigen is selected. It is one of the best characterized antigens, in which T-cell epitopes have been mapped. It is often introduced into tumour cell lines to study cancer immunology[228].

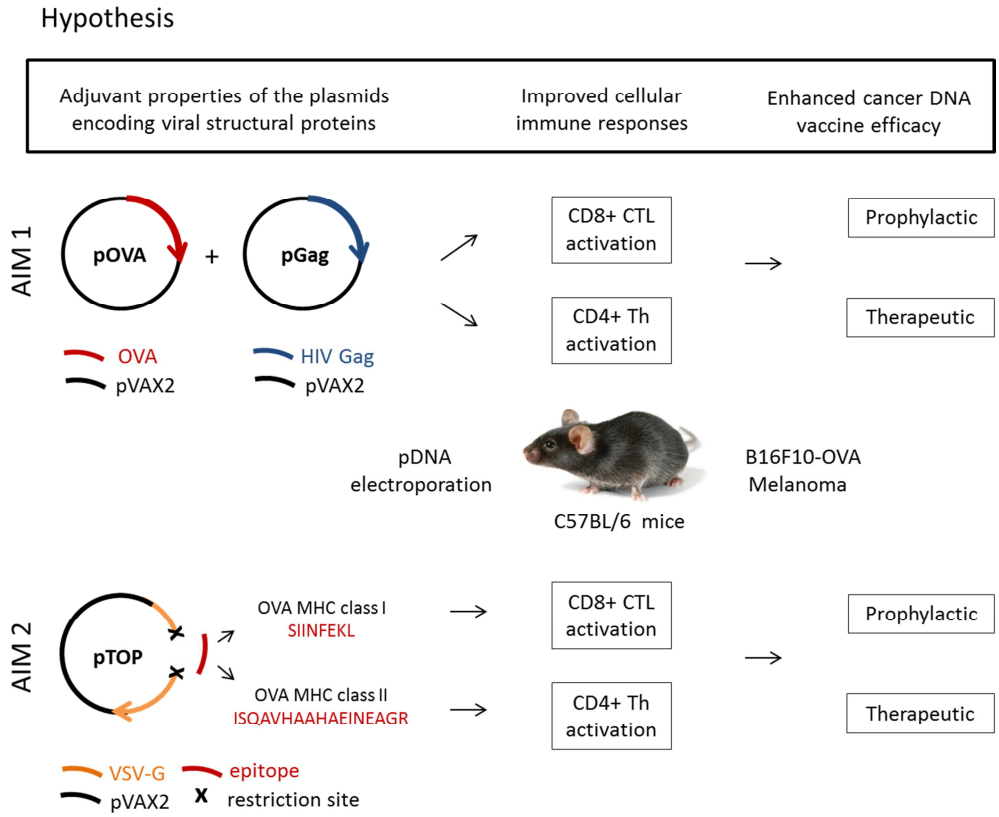

Figure 10- Scientific aims of the thesis

A first aim consists in co-administering **a plasmid encoding HIV-1 Gag (pGag) with a plasmid encoding an antigen expressed by tumour cells** and analyze whether this would enhance the vaccine efficacy and the cellular responses to the vaccine antigen. Therefore, we first test whether cell transfection with this viral core protein encoding plasmid led to subsequent VLP formation. The effects of pGag delivery on the immune response to the vaccine antigen as well as on the protective efficacy against tumour challenge are assessed. We also evaluate whether the immunomodulatory effects are mediated by the formation of VLPs and the use of electroporation as delivery method.

The second objective is the evaluation of the ability of **plasmids encoding a modified VSV-G containing T-cell epitope (pTOP)** to induce potent T cells that specifically recognize and destroy cells expressing the vaccine antigen. This strategy can serve as an efficient epitope-based cancer DNA vaccine, offering high specificity through the induction of an anti-epitope T-cell response and eliciting enhanced immunogenicity due to the encoded viral protein. First, the ability of these modified VSV-G encoding plasmids to activate specific and potent T-cell responses is assessed. Second, we study the prophylactic efficacy of the vaccines and investigate the cytotoxic T-cell response induced by the vaccines, with a particular focus on epitope combination. Finally, the therapeutic potential of the pTOP-based DNA vaccines is analysed.

Chapter 2 - Coadministration of a Plasmid Encoding HIV-1 Gag Enhances the Efficacy of Cancer DNA Vaccines

Adapted from: Lambricht, L. *et al.* Coadministration of a Plasmid Encoding HIV-1 Gag Enhances the Efficacy of Cancer DNA Vaccines. *Molecular Therapy*, 24 9, 1686–1696. doi:10.1038/mt.2016.122 (2016).

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Abstract

DNA vaccination holds great promise for the prevention and treatment of cancer and infectious diseases. However, the clinical ability of DNA vaccines is still controversial due to the limited immune response initially observed in humans. We hypothesized that electroporation of a plasmid encoding the HIV-1 Gag viral capsid protein would enhance cancer DNA vaccine potency. DNA electroporation used to deliver plasmids *in vivo,* induced type I interferons, thereby supporting the activation of innate immunity. The co-administration of ovalbumin and HIV-1 Gag encoding plasmids modulated the adaptive immune response. This strategy favoured antigen-specific Th1 immunity, delayed B16F10-OVA tumour growth and improved mouse survival in both prophylactic and therapeutic vaccination approaches. Similarly, a prophylactic DNA immunization against the melanoma associated antigen gp100 was enhanced by the co-delivery of the HIV-1 Gag plasmid. The adjuvant effect was not driven by the formation of HIV-1 Gag virus-like particles. This work highlights the ability of both electroporation and the HIV-1 Gag plasmid to stimulate innate immunity for enhancing cancer DNA vaccine immunogenicity and demonstrates interesting tracks for the design of new translational genetic adjuvants to overcome the current limitations of DNA vaccines in humans.

1. Introduction

Harnessing the power of the immune system to treat or prevent diseases via vaccine administration is one of the most successful public health interventions. DNA vaccines consist of a recombinant antigen encoded by engineered DNA plasmids and are expressed *in vivo* to solicit immunity against pathogens or cancer cells[105]. DNA vaccines are promising for cancer immunotherapy as they induce a broad immune response[76], including both a T helper type 1 response and cytotoxic T lymphocytes, the most potent effectors against cancer cells[229,230]. They also prime CD4+ T cells, thereby supporting the activity of CTLs and memory T cells[231]. Currently, two DNA vaccines are licensed for veterinary use. Oncept™ (Merial) is commercialized for the treatment of melanoma in dogs and Apex®-IHN (Novartis) for prophylactic vaccination of salmon against infectious hematopoietic necrosis virus. Despite the commercial use of DNA vaccine in veterinary medicine DNA vaccination is still under investigation for use in humans because the immunogenicity is often lower than other conventional vaccines.

In recent years, several advancements have been made to improve DNA vaccine immunogenicity in humans, leading to an increasing number of clinical trials[66]. Particularly, plasmid delivery by *in vivo* electroporation and the use of genetically encoded immune adjuvants have enhanced DNA vaccine efficacy[106]. First, EP is the most commonly used and the most powerful non-viral delivery method for DNA vaccines[66,200]. This method was used to improve DNA vaccine efficacy in large animals[106,232]. EP efficiency is not only attributed to an enhanced gene transfer[106] but also to the induction of a transient tissue inflammation and the subsequent local recruitment of immune cells[105,128]. Second, genetic adjuvants, i.e., plasmid vectors encoding immunomodulatory molecules, aim at enhancing the immunogenicity of antigens *in vivo*. They stimulate the innate immune system to trigger appropriate DC maturation and thereby a robust, specific and long lasting

adaptive immune response. Indeed, the type of insult that drives DC maturation affects whether and how a T cell will respond to an antigen, as T-cell priming, expansion, function and localisation are controlled by sequential signals provided by DCs[161]. These genetic adjuvants include cytokines, chemokines or immune stimulatory molecules, such as TLR agonists [106,195,197,233]. Most of these adjuvants are used in preclinical studies, and even though they are promising, only a few of them have been evaluated in clinical trials until now. Therefore, there is still a critical need of clinically applicable genetic adjuvants.

Plasmids encoding HIV-1 Gag (pGag) have been widely studied during the past decades in the context of HIV vaccine development. In clinical trials, their safety and immunogenicity have been demonstrated[234-236]. The HIV-1 Gag proteins are able to self-assemble leading to virus-like particle formation[237] (see appendix 2) and these capsid proteins may constitute a general PAMP for innate sensing[238]. Moreover, combining a gp160-encoding DNA vaccine with pGag enhanced the immune response to the gp160 envelop antigen of HIV-1[209], thereby suggesting the pGag's adjuvant potential.

In this study, we hypothesize that the *in vivo* electroporation of pGag could enhance cancer DNA vaccine immunogenicity. First, we tested whether cell transfection with pGag led to subsequent VLP formation. Then, we analysed whether EP induced innate immunity *in vivo.* The effects of pGag on the immune response against a model antigen, the ovalbumin, and a tumour associated antigen (gp100) were evaluated in B16F10-OVA melanoma. Finally, we evaluated whether the *in vivo* pGag immunomodulatory effects were mediated by the formation of VLPs.

2. Materials and Methods

Plasmids

Full length and codon-optimised gene sequences (Appendix 1) of HIV-1 Gag, ovalbumin and human gp100 were designed using GeneOptimizer and obtained by standard gene synthesis from GeneArt® (Thermo Fisher Scientific, Waltham, MA, US). These sequences were subcloned in the pVAX2 vector as previously described[239]. A mutant version of the HIV-1 gag encoding plasmid was obtained by introducing three point mutations (alanine mutations at the residues $G_{2,}$ W_{316} and M_{317}) in the gene sequence using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, US) and following manufacturer's protocol. The primers used were (Primer Name - Primer Sequence) g35c - 5'-ccgccaccatggcagctagagcctc-3' for G2A mutation and t976g_g977c_a979g_t980c - 5'-caccagcagtgtctctgtcgccgcgttcttcacttcctggctg-3' for W316A and M317A mutations. The mutated sequence was checked by Sanger DNA sequencing (Beckman Coulter Genomics, Danvers, MA, US). The plasmids were prepared using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Plasmid dilutions were performed in Dulbecco's Phosphate Buffered Saline (1×) (PBS) (Life Technologies, Carlsbad, CA, US). The quality of the purified plasmid was assessed by the ratio of optical densities (260 nm/280 nm) and by 0.5% agarose gel *electrophoresis*. DNA concentration was determined by optical density at 260 nm. The plasmids were stored at -20 °C.

Cell culture

B16F10-OVA, a melanoma cell line from C57BL/6 mice that stably expresses ovalbumin, was cultured in MEM medium supplemented with GlutaMAX with 10% FBS, 100 µg/ml streptomycin, and 100U/ml penicillin (Life Technologies, Carlsbad, CA, US).

HEK 293 T cells were grown in complete DMEM medium supplemented with GlutaMAX (with 10% FBS, 2 mM L-Glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin) (Life Technologies, Carlsbad, CA, US).

VLP production

In total, 3×10^6 HEK 293 T cells were cultured for 2 days in T75 flasks (Sigma-Aldrich, St. Louis, MO, US) and then transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA, US). 17 µg of pGag and 58 µl of lipofectamine were used for lipofection. The medium was replaced 24 h after transfection, and the supernatants were collected at days 2, 3 and 4 post-transfection. Cellular debris was removed by low-speed centrifugation and by filtration through a 0.45 µm filter. Filtered supernatants were then ultracentrifuged (50,000 g, 2.5 h, 4°C) through 20% sucrose. Purified VLPs were suspended in 50 µL PBS, stored at 4°C overnight and placed at -80 °C for long-term storage. The total protein content of each preparation was determined by a Micro BCA assay according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, US).

VLP characterisation

Particle size distribution was determined by dynamic light scattering DLS using a NanoSizer ZS (Malvern Instruments, Malvern, UK). Purified samples were diluted 80-times in PBS, and the measurement was performed in triplicate. The data were analysed using the Dispersion Technology Software 5.00. For particle imaging by transmission electron microscopy, the samples were adsorbed onto 400 mesh formvar-coated EM grids (Ted Pella, Redding, CA, US) for 10 min and then washed three times with distilled water. Excess liquid was removed using filter paper, and the negative contrast was obtained by applying 2% phosphotungstic acid (Sigma-Aldrich, St. Louis, MO, US) at pH 7 for 2 minutes. The grids were air dried for 10 min and then examined in a LEO 922 electron microscope with a CCD camera.

Western blot analyses were performed on 10 µg of protein from the purified VLP samples. The proteins were denatured in 2x Laemmli Sample Buffer for 7 min at 95°C. After loading the samples on a Mini-PROTEAN® TGX™ Precast Gels 4-20% (Bio-Rad, Hercules, CA, US), electrophoresis was performed at 300 V for 15 min using a 1% Tris-glycin – 0.1% SDS running buffer (Sigma-Aldrich, St. Louis, MO, US). The proteins were then transferred to a nitrocellulose membrane using the trans-Blot® Turbo™ Transfer System following the manufacturer's instruction (Bio-Rad, Hercules, CA, US). After blocking, primary rabbit polyclonal antibodies to HIV-1 Gag and ovalbumin (Fitzgerald, Acton, MA, US) were added, all diluted 1:2000, for an hour. Biotinylated anti-rabbit secondary antibodies (Vector Laboratories, Cambridgeshire, UK) at 1:1000 were added for 1 hour and followed by streptavidin-HRP for 20 min. Visualization was performed with a 50:50 ECL Western Blotting Substrate and SuperSignal™ West Femto Maximum Sensitivity Substrate solution (Thermo Scientific, MA, US).

Animals

Six-week-old C57BL/6 female mice were obtained from Janvier Labs (Le Genest Saint Isle, FR) and housed in a minimal disease facility with ad libitum access to food and water. For the immunization studies, the mice were 7 weeks old at the beginning of the experiments. For tumour implantation and electroporation, the mice were anaesthetised by intraperitoneal (IP) injection of 150 µl of a solution of 10 mg/ml ketamine and 1 mg/ml xylazine. Heterozygous luciferase reporter mice (IFN-β+/$\Delta\beta$-luc) with a BALB/c background were bred with ad libitum access to food and water. Male and female mice were aged 12 weeks at the beginning of the experiments. For imaging, the mice were anesthetised by isoflurane (Abbott Animal Health; Medini N.V.). The ethical committee for Animal Care and Use of the Medical Sector of the Université Catholique de Louvain approved our experimental protocols (UCL/MD/2011/007).

Type 1 IFN induction: in vivo bioluminescence imaging

Heterozygous luciferase reporter mice were anesthetised by intra-peritoneal injection of 150 µl of a solution of 10 mg/ml ketamine and 1 mg/ml xylazine. After removing the hair using a rodent shaver (AgnTho's, Lidingö, Sweden), the left tibial cranial muscle was injected with 30 µl of PBS or 1 µg of an empty plasmid (the empty pVAX2 vector) diluted in 30 µl PBS. This injection was followed or not by electroporation. For EP, a conductive gel was applied on the leg to ensure electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy) and eight 20 ms and 200 V/cm electrical pulses were delivered through 4 mm spaced plate electrodes by a Cliniporator system with a frequency of 2 Hz (Cliniporator, IGEA, Carpi, Italy). For *in vivo* bioluminescence imaging, the mice were injected i.p. with 150 mg/kg of VivoGlo™ luciferin (Promega, Fitchburg, Wisconsin, US) in PBS and monitored using an IVIS Lumina II imaging system. Photon flux was quantified using the Living Image 4.4 software (all from Perkin Elmer, Waltham, MA, US).

Mouse immunization

C57BL/6 mice were shaved, and their left tibial cranial muscle was injected with a plasmid DNA solution. The solutions were composed of 1 µg or 50 µg of antigenic OVA or GP100 plasmid alone or with 1 µg HIV-1 Gag encoding plasmid. 1 µg of a plasmid coding for a mutated form of HIV-1 Gag was also used for immunization, combined or not with 1 µg of pOVA. The naive mice were left untreated. A conductive gel was applied, and electroporation was performed as previously described. The plasmids were administered three times, every week or every two weeks for therapeutic and prophylactic DNA immunization, respectively.

Tumour implantation and tumour growth measurement

In total, 1×10^5 B16F10-OVA cells diluted in 100 µl PBS were injected subcutaneously into the right flank of each C57BL/6 mouse two days before the

first plasmid administration or two weeks after the last administration for therapeutic and prophylactic DNA immunization studies, respectively. The tumour size was measured three times a week with an electronic digital calliper. Tumour volume was calculated as the length × width × height (in mm^3). The mice were sacrificed when the volume of the tumour reached 1500 mm^3 or when they were in poor condition and expected to die shortly.

Immunoglobulin titres

Ten days after the last plasmid administration, blood samples were collected and sera were isolated by centrifugation at 3700 x g for 15 min at 4°C and stored at -20°C until use. OVA-specific IgG, IgG1 and IGg2a levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described[240]. Briefly, ovalbumin was coated on Nunc Maxisorp plates. Several sera dilutions were made, and the detection of anti-OVA antibodies was performed using peroxidase-labelled rat anti-mouse immunoglobulin G, G1 and G2a (LO-IMEX, Brussels, BE). IgG titres were defined as the logarithm of the inverse of the sera dilution corresponding to an absorbance equal to the blank value plus 10 times the standard deviation.

Antigen specific IFN-γ levels

Mice were immunized and sacrificed two weeks after the third vaccine administration. The spleens were removed aseptically, and splenocytes were isolated as previously described[164]. Then, 96-well plates were seeded with 1×10^6 cells in 100 µl of media per well. The cells were stimulated by adding 100 µl of 100 µg/ml of ovalbumin solution (Sigma-Aldrich, St. Louis, MO, US). The plates were incubated at 37°C in a humidified incubator at 5% CO2. The supernatants were collected after 24, 48 and 72 h of re-stimulation, and cytokine production was assessed using DuoSet® ELISA Development kits for IFN-γ (R&D Systems,

Abingdon, UK) according to the manufacturer's instructions. The concentrations, expressed in pg/ml, were determined by reference to cytokine standard curves.

In vivo cytotoxicity assay

Splenocytes from female C57BL/6 mice were pulsed with 1 µg/ml of MHC-I OVA peptide or MHC-I gp100 peptide (Anaspec inc, USA) before labelling with 5 µM (hi) or 0,5 µM (low) CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen, Merelbeke, Belgium), respectively. Labelled cells were mixed at a 1:1 ratio, and a total of 1 x 10^7 cells were adoptively transferred into the immunized mice. Two days after transfer, the spleens of the host mice were isolated and analysed by flow cytometry after staining with α-F4/80 (BD Biosciences, San Diego, CA, USA) to exclude auto-fluorescent macrophages. The percentage antigen-specific killing was determined using the following formula: 100 – 100* ((% $CFSE^{hi}$ cells / % $CFSE^{low}$cells)$^{immunized\ mice}$ /(% $CFSE^{hi}$ cells / % $CFSE^{low}$ cells)$^{non\text{-}immunized\ mice}$[241].

Statistical analysis

The results are presented as the means ± SEM. The differences in means between groups were analysed for significance using appropriate tests (GraphPad Prism Software). Non-parametric tests were used to study type-I interferon induction, antibody titres, INF-γ concentration and OVA-peptide-pulsed-target cells specific killing as 4 to 6 mice per group were used in those experiments. Survival curves were compared via Mantel-Cox (log-rank) test. Finally, repeated measures ANOVA was applied for B16F10-ova tumour growth analysis, assuming Gaussian distribution of the data for that tumour model.

3. Results

Cell transfection with a plasmid coding for HIV-1 Gag leads to virus-like particle formation *in vitro*

To assess whether cell transfection with the pGag vector encoding the full length and unmodified HIV-1 Gag protein wouldlead to the expression of HIV-1 Gag proteins and subsequent VLP formation, this plasmid was transfected by lipofection in HEK 293T cells *in vitro.* Particles isolated from the cell supernatant were characterized. After negative staining of the samples, spherical particles were observed under a transmission electron microscope (Figure 11a). Dynamic light scattering analyses showed a uniform population of particles with a size of approximately 180 ± 1 nm and a polydispersity index of 0.06 ± 0.03 (mean ± SD, n=3) (Figure 11b). Finally, particle composition was assessed by western blotting. The HIV-1 Gag protein was detected in the purified samples (Figure 11c).

Plasmid DNA electroporation induces type I IFN responses *in vivo*

Type I IFNs are antiviral cytokines endowed with many biological effects, including anti-tumour activity[242]. The effect of EP on the induction of type I IFNs was assessed using IFN-β reporter mice in which a luciferase gene is placed under the control of the IFN-β promoter. Via *in vivo* bioluminescence imaging, the induction of IFN-β can be localized and quantified. EP applied after an injection of PBS did not significantly stimulate IFN-β induction. The injection of an empty vector without EP induced a low type I IFN induction 24 hours after the injection, but only the combination of EP and an empty pDNA vector strongly induced the IFN-β promoter (Figure 12a,b). The luciferase expression levels increased drastically during the first six hours then remained stable and started decreasing after 48 hours. IFN-β induction remained localized at the injection site (Figure 12b).

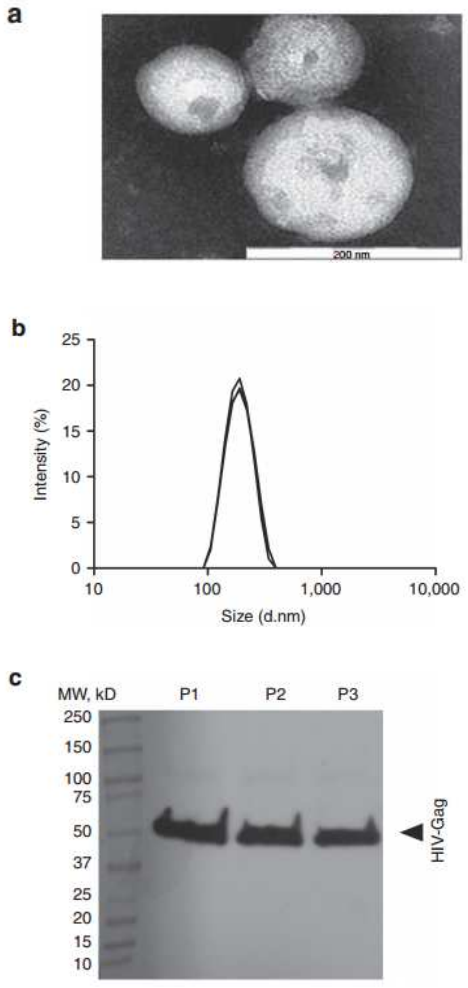

Figure 11 - The characterisation of HIV-1 Gag virus-like particles.
VLPs purified from the supernatants of HEK 293T cells transfected with HIV-1 Gag plasmid. (**a**) Particles observed by transmission electron microscopy after purification and negative staining of samples. This image is representative of data from three replicate experiments. Bare scale: 200 nm. (**b**) Individual measurement of particle size by DLS (n=3). Particle size is calculated from the translational diffusion coefficient using the Stokes–Einstein equation. (**c**) Western blot analysis of three replicate productions of VLPs purified by ultracentrifugation (P1, P2, P3) with rabbit polyclonal antibodies to HIV-1 Gag as primary antibodies and polyclonal goat anti-rabbit immunoglobulins labelled with biotin as secondary antibodies. The HIV-1 Gag protein was detected at approximately 56 kDa.

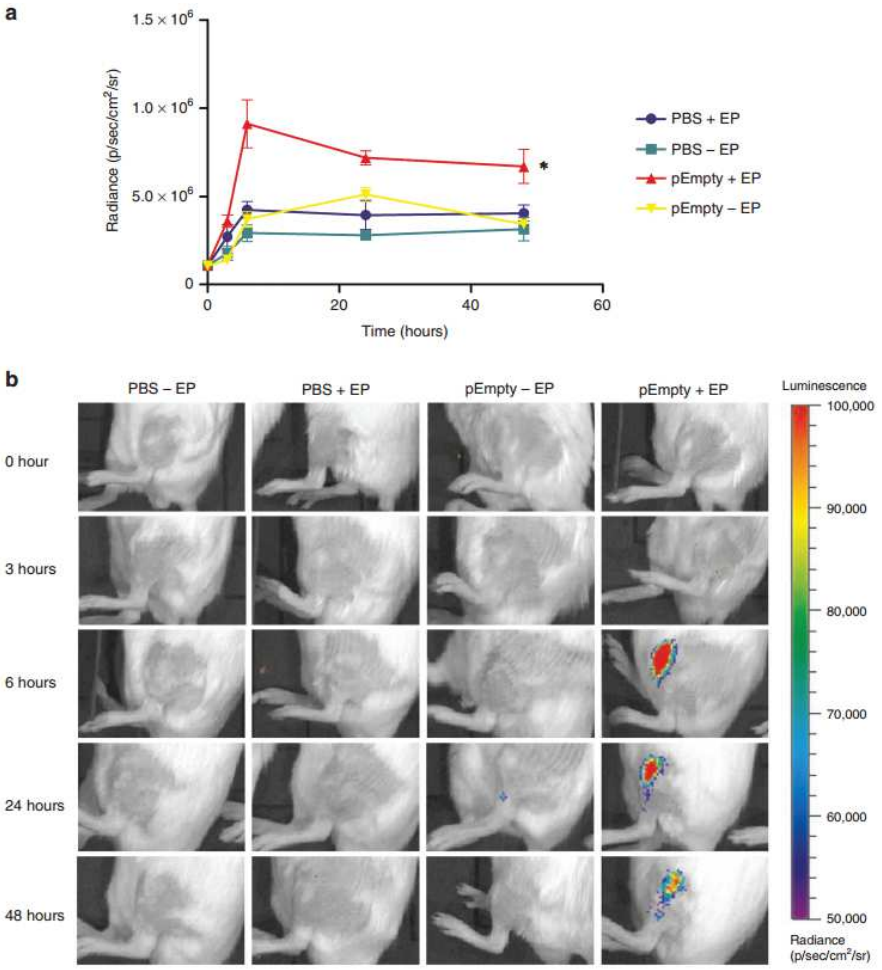

Figure 12 - **The effect of plasmid electroporation on type 1 IFN expression.**
Groups of naive heterozygous luciferase reporter mice (IFN-β+/Δβ-luc) were treated with PBS or an empty plasmid (pEmpty) before applying eight 20ms and 200 V/cm electric pulses with plate electrodes (+EP) or not (-EP). Type 1 IFN expression was quantified by *in vivo* bioluminescence imaging following intraperitoneal injection of luciferin. (**a**) Radiance was observed at 0, 3, 6, 24 and 48 hours following treatment. The results are presented as the mean ± SEM (n=4). The asterisks indicate significant differences compared to the control group PBS without EP (*P<0.05) (Friedman test and Dunn's post-hoc test). (**b**) Representative imaging of the mice as a function of treatment and time. Colour presentation indicates the intensity of bioluminescence, as shown in the bar.

Co-delivery of the HIV-1 Gag plasmid promotes the Th1 polarisation of the immune response

To investigate the ability of the HIV-1 Gag plasmid to modulate the immune response, the ovalbumin model antigen was initially chosen because it allows for both immune response characterisation and tumour growth studies. Taking into account the high immunogenicity of OVA, mice were immunized with a low dose (1 µg) of OVA encoding plasmid (pOVA). The same amount of pGag was co-delivered with pOVA or not to determine if it would impact the immune response *in vivo*. After priming, blood samples analysis indicated that the humoral response remained very low while it slightly increased after the first and second boost (Figure 13a). The delivery of pOVA alone led to higher total anti-ovalbumin antibody titres compared to mice where pGag was co-delivered (Figure 13a). Analysis of immunoglobulins G1 and G2a, associated with a polarization of the T helper response towards Th2 and Th1 respectively, showed that these lower antibody titres correlated with a shift of anti-ovalbumin antibody isoforms towards IgG2a (Figure 13b). In mice immunized with the pOVA and pGag mix, the mean IgG2a/IgG1 ratio was 540 times higher than in mice immunized with pOVA alone. In addition, two weeks after the last boost, IFN-γ levels were analysed at different time points following restimulation of splenocytes with OVA. Significantly higher IFN-γ concentrations were observed in the groups where the antigenic plasmid was combined with pGag (Figure 13c). Finally, the co-delivery of pGag tended to improve the cell-mediated acquired immune defence against cells expressing the vaccine antigen. Indeed, higher killing rate (p-value: 0,09) of OVA-expressing target cells was observed in mice immunized with pGag and pOVA as compared to mice that received the DNA vaccine alone (Figure 13d).

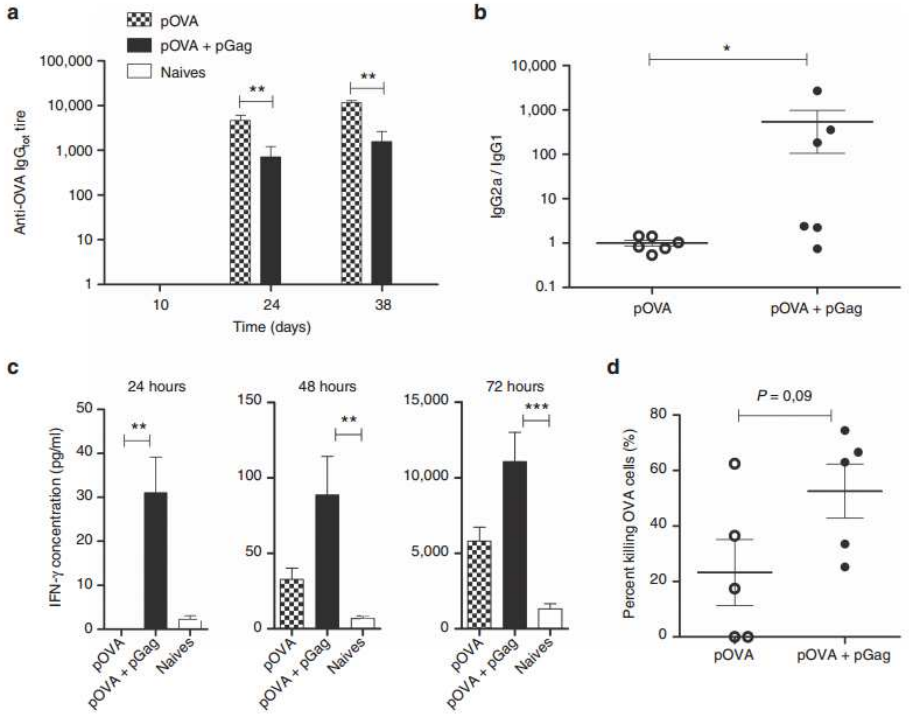

Figure 13 - **The effect of pGag co-delivery during anti-OVA immunization on the immune response.**

C57BL/6 mice were immunized in a regimen of one prime and 2 boosts at a 2-week interval with the antigenic OVA plasmid combined or not with the HIV-1 Gag plasmid. (**a**) OVA-specific total IgG titres were measured by ELISAs in the sera of mice collected at experimental days 10, 24 and 38 (10 days after each vaccine delivery, considering day 0 as the priming day). The error bars indicate mean ± SEM (n=6). The asterisks indicate significant differences between groups (**P<0.01) (Mann-Whitney U test and Bonferroni tests). (**b**) Antibody isotypes were analysed in sera of mice randomly collected 10 days after the last boost. Individual IgG1 and IgG2a titres were measured by ELISAs and each mouse was characterized by an IgG2a/IgG1 ratio (*P<0,05) (Mann-Whitney U test). (c) To analyse OVA-specific IFN-γ levels, mice were immunized and sacrificed one week after the last vaccine administration. IFN-γ concentrations measured in the supernatant of mice splenocytes that had been restimulated with OVA protein 24, 48 and 72 hours before. The errors bars indicate mean ± SEM (n=6). The asterisks indicate significant differences between groups (**P<0.01, ***P<0.001) (Kruskal-Wallis and Dunn's post-hoc test). (**d**) The percentage of antigen specific killing was analysed by in vivo cytotoxic assay. Immunized mice were adoptively transferred with two populations of labelled splenocytes: MHC-I OVA peptide-pulsed-target cells and a MHC-I irrelevant-peptide-pulsed cells. Two days after transfer, the specific killing of target cells was obtained by comparing the relative decrease of the two populations. Percentages of OVA target cell killing were compared using the Mann-Whitney U test.

Co-delivery of the HIV-1 Gag plasmid during prophylactic anti-OVA DNA immunization delayed B16F10-OVA melanoma tumour growth

To explore if co-delivery of pGag would effectively impact cancer DNA vaccine efficiency, mice were prophylactically immunized with 1 µg pOVA combined or not with 1 µg pGag. After challenge, tumour growth and mouse survival were followed (Figure 14a). The median survival in naive mice was 27 days, and no naive mice were alive after 56 days. Survival was significantly better in mice immunized with pOVA. The median survival time reached 54 days with a final survival ratio of 1/6 (Figure 14b). Importantly, the survival was far better in mice immunized with the combination of pOVA and pGag. In that case, the median survival was 83 days and half of the mice were still alive at the end of the experiment. The tumour volume as a function of time profile shows that vaccination strongly delayed tumour growth (Figure 14c). Tumour volumes were significantly lower in vaccinated mice compared to naives, and the size was smaller in mice immunized with the two plasmids, with significant differences observed from day 26 to 70. No progression of the implanted tumour was observed in mice immunized with pOVA combined with pGag for more than two weeks longer than mice immunized with the pOVA DNA vaccine alone. The mean tumour size reached 500 mm^3 23 days later in the group immunized with the two plasmids, compared to mice immunized with pOVA alone.

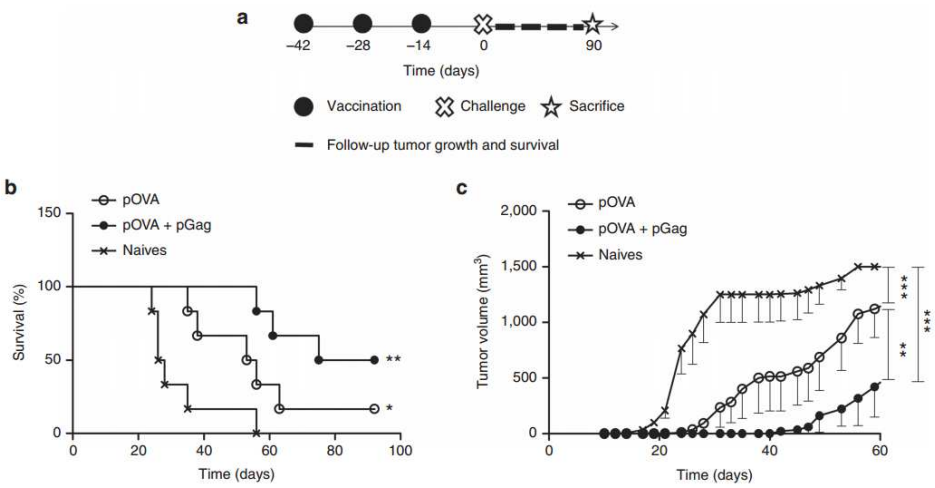

Figure 14 - The effect of pGag co-delivery during prophylactic anti-OVA immunization on the anti-tumour activity.

(**a**) Experimental plan. C57BL/6 mice were immunized in a regimen of one prime and 2 boosts at a 2-week interval with the antigenic OVA plasmid combined or not with pGag. Two weeks after the last vaccination, they were challenged with B16F10-OVA cells. Tumour growth and mouse survival were assessed for three months. (**b**) Survival rates monitoring after challenge. The asterisks indicate significant differences compared with naive mice (*P<0.05, **P<0.01) (Comparison of survival curves, Mantel-Cox test). (**c**) Tumour growth follow-up after challenge in mice immunized with pOVA and pOVA combined with pGag. The errors bars represent mean-SEM (n=6). The asterisks show significant differences between groups (**P<0.01, ***P<0.001) (ANOVA, Dunnett's post-hoc test).

Co-delivery of the HIV-1 Gag plasmid during prophylactic anti-gp100 DNA immunization delayed B16F10-OVA melanoma tumour growth

Next, the ability of pGag to improve DNA vaccine effectiveness in the case of less immunogenic tumour-associated antigen (TAA) was assessed. Prophylactic immunization with 50 µg of a plasmid coding for the less immunogenic gp100 melanoma antigen (pGP100) combined or not with 1 µg pGag was performed before challenging the mice (Figure 15a). Tumour growth was slower when pGag was co-delivered with pGP100 (Figure 15c). At day 14 after challenge, the implanted tumour was still not visible in 4 out of the 6 mice when the pGP100 vaccine was co-delivered with pGag, while only 2 out of 6 mice didn't display progression of the tumour implanted when mice were immunized with pGP100 alone. All tumours were growing in naive mice at that time. Additionally, three weeks after challenge the mean tumour volume in the pGP100 and pGag immunized mice were lower (555 mm^3 ± 301) (mean ± SEM, n=6) than in the pGP100 immunized (1083 mm^3 ± 270; p=0.2) and naïve (1339 mm^3 ± 161; p=0.04) mice. Survival curves confirmed the positive effect of pGag (Figure 15b), as a significant improvement in survival was visible following pGag co-delivery, whereas no significant difference was observed in the case of the gp100-encoding plasmid delivered alone.

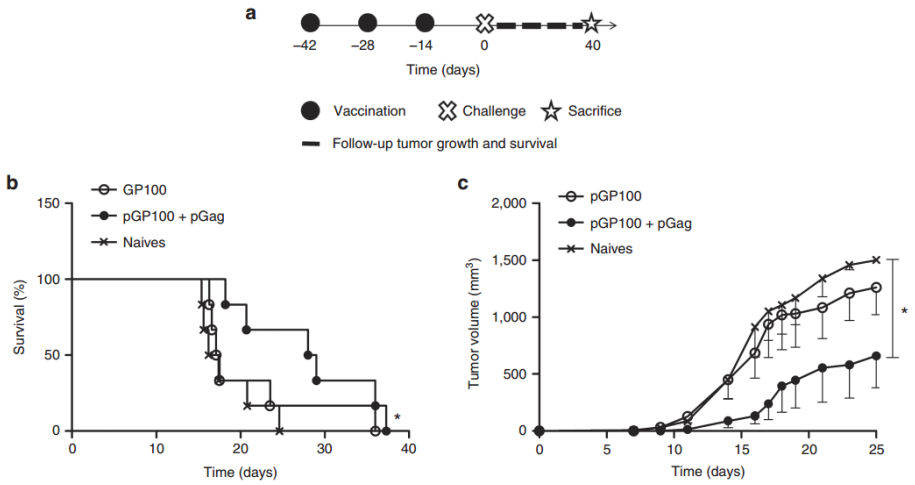

Figure 15 - The effect of pGag co-delivery during prophylactic anti-GP100 immunization on the anti-tumour activity.

(**a**) Experimental plan. C57BL/6 mice were immunized in a regimen of one prime and 2 boosts at a 2-week interval with the antigenic GP100 plasmid combined or not with pGag. Two weeks after the last vaccination, they were challenged with B16F10-OVA cells and tumour growth and mouse survival were monitored. (**b**) Survival rates monitoring after challenge. The asterisks indicate significant differences compared with naive mice (*P<0.05) (Comparison of survival curves, Mantel-Cox test). (**c**) Tumour growth follow-up after challenge in mice immunized with pGP100 and pGP100 combined with pGag. The errors bars represent mean-SEM (n=6). The asterisks show significant differences between groups (*P<0.05) (ANOVA, Dunnett's post-hoc test).

Co-delivery of the HIV-1 Gag plasmid during therapeutic anti-OVA DNA immunization delayed B16F10-OVA melanoma tumour growth

Finally, the therapeutic potential of a DNA vaccine combined or not with pGag was evaluated. To evaluate such a therapeutic effect, the pOVA DNA vaccine was selected. The low immunogenicity of the pGP100 plasmid and the rapid growth of B16F10-OVA tumours predicted a poor efficiency of a gp100 therapeutic DNA vaccine. As therapeutic immunization consists of vaccine administration after the disease onset, the treatment started two days after the implantation of B16F10-OVA tumours (Figure 16a) and consisted of 1 µg of pOVA with or without 1 µg pGag. The mix of pOVA and pGag significantly delayed tumour growth (Figure 16c). Indeed, it took 17 days for tumours to reach 500 mm^3 in mice immunized

with pOVA, and 24 days when pGag was added. At day 21, the mean tumour volume (±SEM) was 217 ± 68 mm³, 703 ± 228 mm³ and 989 ± 208 mm³ with 90%, 56% and 40% of tumours that were smaller than 500 mm³ for mice treated with the combination of pOVA and pGag, with pOVA alone and for non-immunized mice, respectively. Mouse survival was also significantly increased when pGag was co-delivered (Figure 16b). These results demonstrate that therapeutic DNA immunization with pOVA co-delivered with pGag, but not with pOVA alone, promoted tumour growth delay.

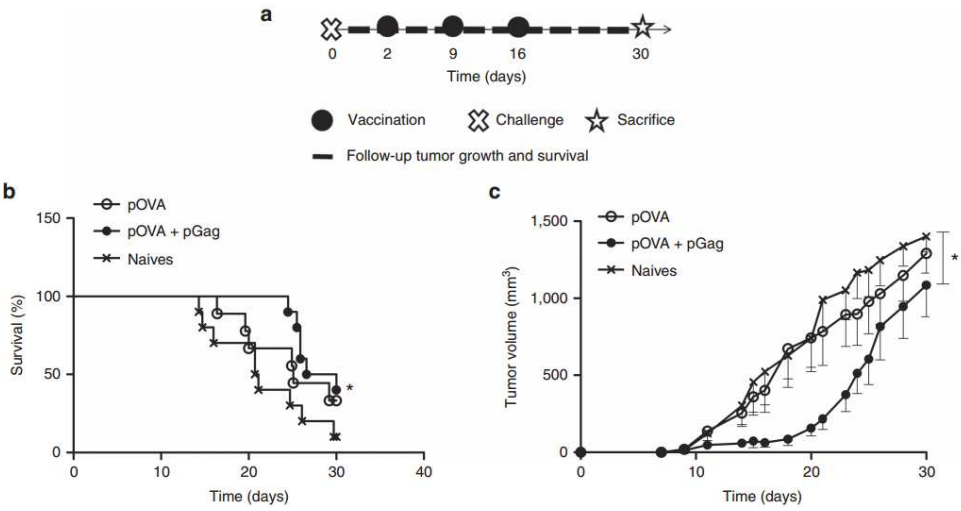

Figure 16 - The effect of pGag co-delivery during therapeutic anti-OVA immunization on the anti-tumour activity.

(**a**) Experimental plan. C57BL/6 mice were challenged with B16F10-OVA cells. Two days later, they were immunized in a regimen of one prime and 2 boosts at a 1-week interval with the antigenic OVA plasmid combined or not with HIV-1 Gag plasmid. Tumour growth and mouse survival were assessed after challenge. (**b**) Survival rates monitoring after challenge. The asterisks indicate significant differences compared with naive mice (*P<0.05) (Comparison of survival curves, Mantel-Cox test). (**c**) Tumour growth follow-up after challenge in mice immunized with pOVA and pOVA combined with pGag. The errors bars represent mean-SEM (n=10). The asterisks show significant differences between groups (*P<0.05) (ANOVA, Dunnett's post-hoc test).

The formation of HIV-1 Gag virus-like-particles is not required for adjuvant immunomodulatory effect.

To assess whether the adjuvant effect of pGag could be mediated by the formation of VLPs, a plasmid encoding a mutated version of HIV-1 Gag (pGag*) was constructed. pGag* encoded a protein very close in sequence to the native Gag protein, but 3 specific points mutations make it neither able to bind to the plasma membrane, nor to self-assemble properly in VLP[243]. First, a prophylactic immunization experiment followed by a tumour challenge (Figure 15a) demonstrated that pGag and pGag*, when combined to pOVA, had the same effect on tumour growth (Figure 18a-c). The same conclusion was drawn from the therapeutic immunization experiments as the combination of pOVA with pGag or pGag* were equally efficient (Figure 17a-c). This suggests that the adjuvant role of pGag on the vaccine-antigen specific immune response is probably not mediated by the formation of HIV-1 Gag VLPs in vivo. Interestingly, electroporation of pGag or pGag* alone had a delaying impact on tumour growth during therapeutic (Figure 17a,c) but not prophylactic (Figure 18a,c) immunizations. This result further supports the role of Gag encoding plasmid as a stimulator of the innate immune system.

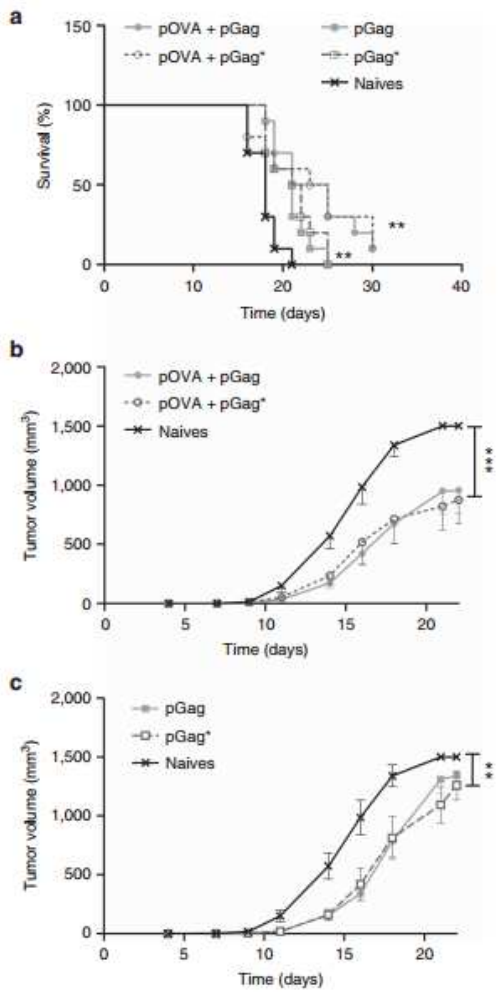

Figure 17 - **Comparison of the effect of pGag and pGag* during therapeutic anti-OVA immunization on the anti-tumour activity.**

(**a**) Survival rate monitoring after challenge. The asterisks indicate significant differences compared with naive mice (**P<0,01) (Comparison of survival curves, Mantel-Cox test). (**b**) and (**c**) Tumour growth follow-up after challenge in mice immunized respectively with pOVA combined with pGag or pGag* and mice immunized with pGag or pGag* alone. The error bars represent mean-SEM (n=10). The asterisks show significant differences between groups (**P<0,01, ***P<0,001) (ANOVA, Dunnett's post-hoc test).

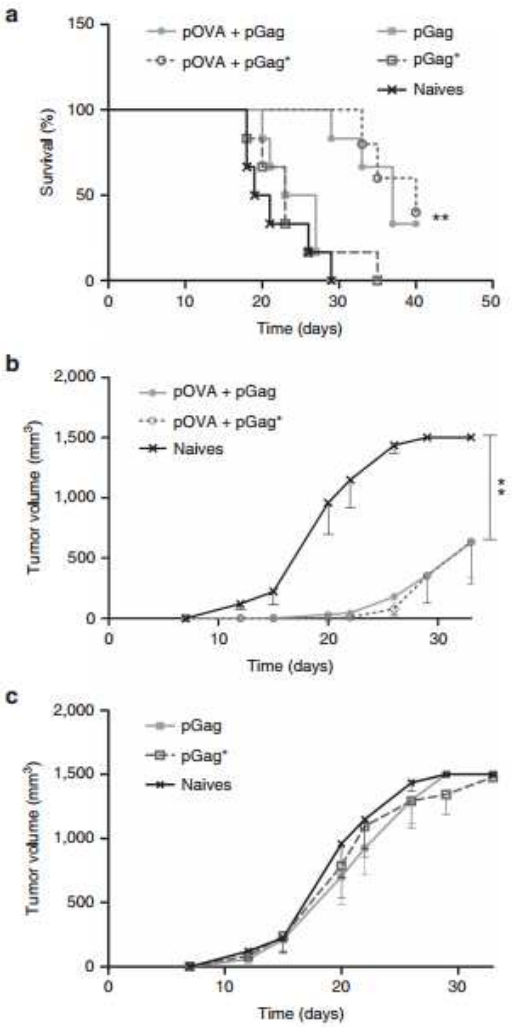

Figure 18 - Comparison of the effect of pGag and pGag* during prophylactic anti-OVA immunization on the anti-tumour activity.

(**a**) Survival rate monitoring after challenge. The asterisks indicate significant differences compared with naive mice (**P<0,01) (Comparison of survival curves, Mantel-Cox test). (**b**) and (**c**) Tumour growth follow-up after challenge in mice immunized respectively with pOVA combined with pGag or pGag* and mice immunized with pGag or pGag* alone. The error bars represent mean-SEM (n=6). The asterisks show significant differences between groups (**P<0,01) (ANOVA, Dunnett's post-hoc test).

4. Discussion

Despite promising results in animal models, the clinical efficacy of DNA vaccines remains to be proven due to the weakness of the immune response observed in humans. There is therefore a critical need to find new strategies to enhance DNA vaccine immunogenicity and to have them train the immune system to generate potent immune responses. As EP and the use of genetic adjuvants were shown previously to enhance DNA vaccine efficacy, we investigated whether the electroporation of a plasmid encoding the HIV-1 Gag protein could increase cancer DNA vaccine immunogenicity. We showed that co-delivery of pGag by EP enhanced the potency of the immune response against the vaccine antigens, suggesting a strong adjuvant effect of this strategy.

Firstly, we showed that cell transfection with pGag led to the production of VLPs by the cellular machinery. This result was expected as HIV-1 Gag proteins are responsible for viral capsid assembly[237]. These particles are similar in size, composition and structure to intact infectious virus (Figure 11). Nevertheless, they do not present any infectivity risk as they are devoid of the viral genome[244]. Several studies have been highlighting the immunomodulatory properties of HIV-1 Gag proteins. On the one hand, HIV-1 Gag VLPs were shown to improve DC maturation and T-cell priming. These particles efficiently promoted maturation of DCs, increasing the expression of MHC- I and II and co-stimulatory molecules such as CD80 and CD86. They also induced the NK cells in the mice, which are known to play a crucial role in the anti-tumour response[216]. Another study demonstrated that HIV-1 VLPs can propagate innate immune signalling by packaging cGAMP, a messenger that activates antiviral signalling pathways in response to cytosolic sensing of DNA, and delivering it to uninfected target cells[217]. However, our data suggest that the immune modulation is not mediated by HIV-1 Gag VLPs formation. On the other hand, the host may sense the invading viral protein via

PRR recognition of PAMPs. The HIV-1 capsid can be detected via cyclophilin A and TRIM5 receptors, activating innate immune signalling pathways and subsequent DC maturation[218,219]. It was also found that the p17 and p24 domains of the HIV-1 Gag protein serve as PAMPs for TLR2 heterodimers[220], significantly increasing the innate immune activation. Agonists of TLR2 and TLR3 are promising adjuvants for vaccine immunotherapy of cancer, to safely enhance anti-tumour immunity[245].

Secondly, we demonstrated that EP as a delivery method used for DNA vaccination induced innate immunity. Indeed, EP of a DNA plasmid (pDNA) led to the induction of type I IFNs *in vivo,* whereas EP without pDNA or injection of pDNA without EP did not strongly induce the IFN-β promoter (Figure 12). An explanation of this phenomenon may be an increase in DNA plasmid transfection[106] and thus a higher sensing of aberrant cytosolic DNA. In most cases, the recognition of cytosolic DNA results in the induction of the innate immune response through the STING-TBK1 signalling cascade, and thus the subsequent expression of type I IFNs[197]. These cytokines play a crucial role in the induction of a protective anti-tumour immune response[242]. Adjuvant properties of EP had already been highlighted previously in a series of studies, showing its capacity to trigger a 100-fold increase of antigen expression [106] but also to recruit DCs to the vaccinated area and induce local tissue inflammation[105,128]. Here, we reported a direct induction of type I IFNs by EP *in vivo*, which is shown for the first time to our knowledge. This result supports the crucial role of using EP in DNA vaccination protocols to further enhance the immune response against DNA vaccine-encoded antigens.

Then, the potential of pGag to act as an adjuvant in the context of DNA vaccine electroporation was investigated. Initial evidence of pGag's adjuvant potential came from the analysis of the adaptive immune response triggered by the anti-OVA DNA vaccination. The co-delivery of pGag led to a clear shift of the anti-OVA

immune response towards a Th1 polarisation. This finding was characterized both by a decrease in the total anti-OVA IgG titres and an increase in the IgG2a/IgG1 ratio, as well as an increase in antigen-specific IFN-γ levels in the presence of pGag and an improved induction of antigen-specific CTLs (Figure 13). This regulation of the Th1/Th2 homeostasis driven by pGag favoured the cellular immunity and is therefore more likely to induce efficient elimination of cancerous cells[246]. pGag's ability to increase DNA vaccine potency was further proven as it increased protection against tumour challenge. The prophylactic immunisation of mice with an anti-OVA DNA vaccine slowed down the growth of B16F10-OVA tumours and improved mouse survival when the antigenic plasmid was combined with pGag (Figure 14). Moreover, the efficacy of this strategy was confirmed in a less immunogenic TAA model by combining pGag with a vaccine coding for the gp100 melanoma antigen. The results indicated that mice developed a more potent immune response to the B16F10-OVA tumour only when pGag was co-delivered with pGP100 (Figure 15). In this case, the delivery of the antigenic plasmid alone did not offer tumour protection, highlighting again the importance of the adjuvant plasmid in inducing an effective immune response. Finally, therapeutic immunization of the mice was tested with the anti-OVA DNA vaccine model. Once again, tumour growth delay was observed only with the combination of antigenic and adjuvant plasmids (Figure 16). These results demonstrated the ability of pGag to regulate adaptive immunity, as the addition of this plasmid promotes a Th1 immune response and, in a consistent way, improves the protective immunity against tumours.

Finally, the mechanisms lying behind the immune modulation induced by pGag were further studied. First of all, the improved vaccine efficacy was specific to pGag co-delivery and not due to an increase of DNA amount used for

immunizations. Indeed, using 1 µg or 10 µg of pOVA DNA vaccine led to similar protection efficacy following B16F10-OVA challenge (Figure 19).

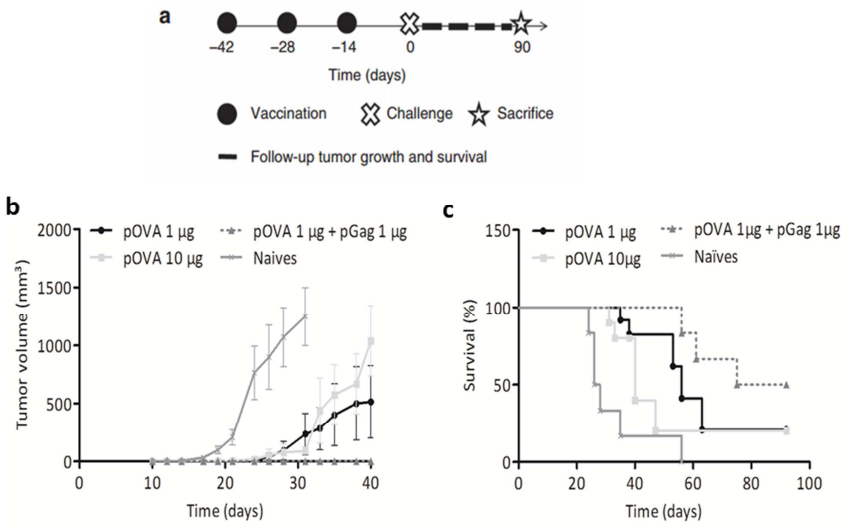

Figure 19 - The effect of pOVA dose during prophylactic anti-OVA immunization on the antitumour activity.

(**a**) C57BL/6 mice were immunized in a regimen of one prime and 2 boosts at a 2-week interval with the antigenic OVA plasmid combined or not with pGag. Two weeks after the last vaccination, they were challenged with B16F10-OVA cells. (**b**) Tumour growth and (**c**) survival follow up. No significant differences were observed between 1 µg pOVA and 10 µg pOVA DNA vaccine treatments (n=6) (ANOVA, Bonferroni post-test in (**b**) and Comparison of survival curves, Mantel-Cox test in (**c**)).

As pGag adjuvant effect could be driven by the formation of VLPs *in vivo*, the requirement of particles formation was analysed. Immunization of mice with a plasmid encoding a mutated form of the HIV-1 Gag protein that prevents VLP formation[243] led to similar results than immunization with the plasmid coding for the native protein (Figure 17, Figure 18). This suggests that the adjuvant effect is probably mediated by immune recognition of the DNA or protein sequence of HIV-1 Gag rather than by VLPs. For instance the HIV-1 Gag plasmid may contain CpG motifs that can be recognized by the TLR9 and activate innate immunity[167]. About HIV-1 Gag protein, some of its domains were shown to act as PAMPs[218-220]

and induce innate immunity. In addition, the protein may contains MHC-II restricted epitopes that can induce a strong T-helper response[247]. The anti-tumour effect is mediated by the antigen-specific response, as delivery of pGag alone did not offer protection against tumour challenge after prophylactic immunization (Figure 18c). Interestingly, pGag seemed to have an anti-tumour effect by itself in therapeutic vaccination setting (Figure 17c). This supports the important role played by pGag on immune system mediators. Future work may aim at better characterizing the mechanisms involved.

Taken together, these results support an adjuvant role for pGag when co-delivered by EP with a tumour antigen encoding plasmid. This strategy seemed to stimulate efficiently innate immunity, taking advantage of both the ability of DNA EP to stimulate innate immunity and of the immunomodulatory effects induced by pGag. This approach led to an efficient priming of an adaptive immune response and allowed for the augmentation of cancer DNA vaccine efficacy.

The use of pGag as a genetic adjuvant is of particular interest from a translational point of view. Indeed, genetic adjuvants hold a higher potential for enhancing DNA vaccine potency than traditional ones. First, they possess intrinsic adjuvant properties due to their recognition by cytosolic DNA sensors. In addition, both antigens and adjuvants often need to be delivered at the same time to the patient to obtain coordinated priming of the immune response. As the antigenic plasmid of DNA vaccines is not the physical antigen but its coding sequence, it first needs to be expressed in sufficient amounts by the host cells. Because many conventional adjuvants work immediately after injection, their effect might be lower at the time the antigen is present[84]. In contrast, the use of plasmid encoding immunomodulatory proteins permits the coordinated delivery of antigens and adjuvants, tailoring the immune response to the demands of each particular disease[106]. Until now, most of the genetic adjuvants used in trials encode proteins

that activate innate immune responses directly, such as cytokines, chemokines, signalling or co-stimulatory molecules. Here, the strategy is different and innovative as pGag most likely induces innate immune responses indirectly, through PAMP recognition by PRRs. Last but not least, HIV-1 Gag has already been involved in clinical trials in the context of HIV vaccine development, where it was shown to be safe[236]. Therefore, the transition from preclinical to clinical evaluation of this strategy would be easier.

In conclusion, this study supports the adjuvant effect of plasmid EP *in vivo*. It demonstrates that immunization with pGag favours Th1 immunity against the DNA vaccine-encoded antigen and consistently delays tumour burden. This work highlights the powerful adjuvant potential of pGag for enhancing DNA vaccine potency and opens interesting tracks for the design of new genetic adjuvants that could be used in a clinical setting in an attempt to overcome the current limitations of DNA vaccines in humans.

Chapter 3 – Cancer DNA vaccine coding for a modified VSV-G is a potent inducer of antigen-specific T-cell responses

Patent Pending: Vandermeulen G., Lambricht L., Préat V. Modified VSV-G and vaccines thereof. Patent Application: EP16188736.9, filed Sep. 14, 2016

Authors : Laure Lambricht[1], Ans De Beuckelaer[2], Kevin Vanvarenberg[1], Bernard Ucakar[1], Johan Grooten[2], Véronique Préat[1], Gaëlle Vandermeulen[1]

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Abstract

DNA vaccines are ideal candidates for inducing potent antigen-specific T-cell responses, but the low immunogenicity of DNA vaccines has hampered their application in cancer immunotherapy thus far. In this study, we hypothesized that a plasmid coding for a modified vesicular stomatitis virus glycoprotein (VSV-G) with a T-epitope insertion (pTOP) could be an effective strategy for cancer DNA vaccine development. The ability of pTOP-based DNA vaccines to induce potent and specific anti-vaccine T cells and protect against tumours was assessed with the ovalbumin antigen model, with insertion of either major histocompatibility complex (MHC) class I- or MHC class II-restricted epitopes in the VSV-G sequence (pTOP-OVA_MHCI or pTOP-OVA_MHCII). Administration of pTOP-OVA_MHCI and pTOP-OVA_MHCII induced the proliferation of specific CD8+ and CD4+ T cells, respectively, demonstrating that correct processing and presentation of the inserted epitopes occurred. Prophylactic immunization of mice with pTOP-OVA_MHCI led to a significant reduction in B16F10-OVA tumour growth as well as an increase in survival rates. The vaccine efficacy and cytotoxic T-cell response were reinforced by co-administering pTOP-OVA_MHCI and pTOP-OVA_MHCII. A weak protective anti-tumour therapeutic effect was observed in mice immunized with the combination of pTOP-OVA_MHCI and pTOP-OVA_MHCII, whereas immunization with a plasmid encoding OVA had no effect. These results highlight the promising therapeutic potential of these plasmids encoding a modified VSV-G containing specific T epitopes. The choice of appropriate epitope and epitopes combination is probably a key to alleviate the potency of this strategy.

1. Introduction

Since the discovery of the checkpoint inhibitors, cancer immunotherapy is emerging as one of the most substantial drug development stories of the decade. However, these drugs only elicit long-lasting remission in a minority of patients, which is likely due to the lack of T cells at the margins of tumours[248]. Researchers have postulated that if an effective T-cell response is raised first, then it might yield better therapeutic results[249]. Therefore, developing vaccines capable of inducing potent T-cell responses would represent a great advancement in cancer immunotherapy.

Historically, cancer DNA vaccines strategies have been focused on eliciting CD8+ cytotoxic T-cell responses because CTLs can directly kill tumour cells and because they respond to peptides presented in the context of MHC class I[9]. These peptides are frequently derived from intracellular proteins, which is the case for many tumour antigens. However, the central role of $CD4^+$ T helper cells in cancer immunity and immunotherapy has also been investigated over the years[250,251]. DNA vaccines are ideal candidates for stimulating the *de novo* activity of T lymphocytes [138]. In addition to the low cost, easy production and stability of manufactured DNA vaccines, the use of DNA vaccines in cancer vaccine development is particularly attractive as they induce a broad immune response [252]. However, the first results of the DNA vaccination clinical trials conducted in the nineties were disappointing and dampened interest in the further development of DNA vaccines[253].

Since the first clinical trials, many efforts have been made to improve the immunogenicity of DNA vaccines[97]. Recent advances and studies regarding delivery technologies have highlighted the benefit of using electroporation (EP) to deliver DNA vaccines[105,200]. Recently, we demonstrated that a plasmid encoding a

viral protein could enhance cancer DNA vaccine potency, as the efficacy of the cancer DNA vaccine was improved by the co-administration of a plasmid encoding HIV-1 Gag viral capsid protein[254]. The VSV-G protein is also an attractive candidate for boosting DNA vaccine efficiency. Indeed, administration of a plasmid encoding VSV-G enhanced the potency of a cervical cancer DNA vaccine [224]. The VSV-G protein possesses permissive sites in which several amino acids can be inserted without modifying protein function[221]. Therefore, we hypothesized that a plasmid encoding a modified VSV-G with inserted T epitopes could serve as an efficient anti-cancer DNA vaccine. The modified VSV-G plasmid may offer high specificity through the induction of an anti-epitope T-cell response and may elicit enhanced immunogenicity due to the encoded viral protein.

This study aimed to demonstrate that modified VSV-G plasmids have the potential to induce potent T cells that specifically recognize and destroy antigen-expressing cells for the development of novel cancer DNA vaccines. The ovalbumin model antigen was selected to precisely characterize the immune response and B16F10-OVA melanoma cells that constitutively express ovalbumin were used to evaluate the anti-cancer potential of the vaccine candidates. First, we assessed the ability of these modified VSV-G encoding plasmids to activate specific and potent T-cell responses. Second, we studied the prophylactic efficacy of the vaccines in a melanoma model. Next, we investigated the cytotoxic T-cell response induced by the vaccines, with a particular focus on epitope combination. Finally, we analysed the therapeutic potential of the DNA vaccines.

2. Materials and Methods

Plasmids

pTOP refers to the plasmids encoding VSV-G in which the foreign epitopes were inserted. Codon-optimized gene sequences of ovalbumin (pOVA), VSV-G (pVSVG), and VSV-G with an OVA MHC class I-restricted epitope inserted at amino acid 191 position and surrounded by restriction sites SpeI and EcoRI (pTOP-OVA_MHCI) were designed using GeneOptimizer and obtained by standard gene synthesis from GeneArt® (Thermo Fisher Scientific, Waltham, MA, US). These sequences were subcloned in the pVAX2 vector using cohesive-end cloning. pTOP-OVA_MHCII was obtained by opening pTOP-OVA_MHCI with SpeI and EcoRI restriction enzymes (Promega, Fitchburg, WI, US) successively (to avoid a start activity induced by co-digestion). Then, two complementary and overlapping phosphorylated oligonucleotides that encoded the MHC class II-restricted epitope (IDT-DNA, Leuven, Belgium) were incorporated in the digested vector using cohesive-end cloning. The different sequences can be found in the Appendix 1. The plasmids were prepared using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, Netherlands) and diluted in PBS (1×) (Life Technologies, Carlsbad, CA, US). The quality of the purified plasmid was assessed by the ratio of optical densities (260/280 nm) and by 1% agarose gel electrophoresis. Plasmids were sequenced by Sanger DNA sequencing (Beckman Coulter Genomics, UK) and stored at -20°C.

Cell culture

B16F10-OVA, a melanoma cell line from C57BL/6 mice that expresses ovalbumin, was cultured in MEM supplemented with GlutaMAX with 10 % FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin (Life Technologies, Carlsbad, CA, US).

Animals

Six- to eight-week-old C57BL/6 female mice were obtained from Janvier Labs (Le Genest Saint Isle, FR) and housed in a minimal-disease facility with ad libitum access to food and water. For tumour implantation and electroporation, the mice were anaesthetized with a 150 µL intraperitoneal injection of 10 mg/mL ketamine and 1 mg/mL xylazine. The ethical committee for Animal Care and Use of the Medical Sector of the Université Catholique de Louvain approved our experimental protocols (UCL/MD/2011/007 and UCL/MD/2016/001).

Tumour challenge

A total of 1×10^5 B16F10-OVA cells diluted in 100 µL PBS were injected subcutaneously into the right flanks of C57BL/6 mice. The tumour cells were inoculated before the plasmid treatment for the therapeutic experiments and two weeks after complete immunization for the prophylactic studies. Tumour size was measured three times a week with an electronic digital calliper. Tumour volume was calculated as the length × width × height (in mm^3). The mice were sacrificed when the tumour volume reached 1500 mm^3 or when the mice were in poor health and expected to die soon.

Immunizations

Intramuscular electroporation. After the mouse hair was removed using a rodent shaver (AgnTho's, Lidingö, Sweden), 30 µL of a PBS solution containing either 1 µg of pOVA, 1 µg of pVSV-G, 1 µg of pTOP-OVA_MHCI, 1 µg of pTOP-OVA_MHCII, or 1 µg of pTOP OVA_MHCI and 1 µg of pTOP-OVA_MHCII was injected into the tibial cranial muscle. The leg was placed between 4-mm-spaced plate electrodes, and 8 square-wave electric pulses (200 V/cm, 20 ms, 2 Hz) were delivered. For prophylactic immunizations, two boosts were similarly applied two and four

weeks after priming. For therapeutic immunizations, the treatment started two days after the tumour cell injection, and two boosts were delivered every week. *Ear pinna electroporation*. A 30-µL DNA solution containing 1 µg of pOVA, pTOP-OVA_MHCI or pTOP-OVA_MHCII was injected into the pinna of both ears (2 injections per mouse). The 2-mm-spaced plate electrodes were applied around the ear to deliver 10 square-wave electric pulses (500 V/cm, 20 ms, 1 Hz). For all electroporation protocols, electric pulses were generated by a Gemini System generator and delivered with BTX Caliper Electrodes (BTX; both from VWR International, Leuven, Belgium). A conductive gel was used to ensure electrical contact with the skin (Aquasonic 100; Parker Laboratories, Inc., Fairfield, NJ, USA).

OT-I and OT-II proliferation

A single-cell suspension was prepared from the spleens and lymph nodes of transgenic OT-I and OT-II mice (Ghent University, Ghent, Belgium). T cells were isolated using a CD8a+ and CD4+ T-cell isolation kit for mouse and an AutoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, the isolated T cells were labelled with CFSE (Thermo Fisher Scientific, Waltham, MA, US) by incubating 50×10^6 cells/ml PBS with 5 µM CFSE for 7 minutes at 37°C. The reaction was blocked by adding ice-cold PBS+10 % FBS (Life Technologies, Carlsbad, CA, US). A total of 2×10^6 OT-I or OT-II cells were injected into the tail vein of C57BL/6 recipient mice. The mice were treated 2 days later with plasmid injection and electroporation to the pinna. Mice were sacrificed 4 days later to collect the draining lymph nodes for single-cell suspension preparation. Flow cytometric measurement was performed after staining the cells with Aqua Live/Dead (Invitrogen, Carlsbad, CA, US), CD19 APC-Cy7, CD8 PerCP (for OT-I proliferation), CD4 eFluor 450 and Valpha2 TCR PE (for OT-II proliferation) and Fc block (all BD Biosciences, San Diego, CA, US). Dextramer SIINFEKL H-2kb PE (Immudex, Copenhagen, Denmark) was added for OT-I proliferation

measurement. The read out was performed using a BD FACSVerse (BD Biosciences, San Diego, CA, US).

In vivo killing assay

Mice were prophylactically immunized by intramuscular DNA electroporation. Three weeks after the last vaccine administration, splenocytes (2.5×10^6 cells/ml PBS) from naive mice were pulsed with 1 µg/ml PBS of SIINFEKL or an irrelevant peptide for one hour at 37°C. These pulsed splenocytes were stained with 5 µM (high) or 0.5 µM (low) CFSE. The two labelled cell populations were mixed at a 1:1 ratio, and a total of 1×10^7 cells were adoptively transferred into the immunized mice by intravenous injection. Two days after the transfer, the host mouse spleens were isolated and analysed by flow cytometry after staining with Aqua Live/Dead (Invitrogen, Carlsbad, CA, US), Fc Block and α-F4/80 (BD Biosciences, San Diego, CA, USA). The percentage of antigen-specific killing was determined using the following formula[241]: $100 - 100^* ((\%\ CFSE^{high}\ cells/\%\ CFSE^{low}\ cells)^{immunized\ mice}/(\%\ CFSE^{high}\ cells/\%\ CFSE^{low}\ cells)^{non\text{-}immunized\ mice})$.

Statistical analysis

The results are presented as the means±SEM. The differences in means between groups were analysed for significance using appropriate tests that are described in the figure legends (GraphPad Prism Software).

3. Results

pTOP-based DNA vaccines induced the proliferation of epitope-specific T cells.

To efficiently prime an immune response, the inserted epitopes must be correctly presented to their specific T lymphocytes. The pTOP-OVA_MHCI vector containing an OVA-MHC class I-restricted epitope (SIINFEKL) and the pTOP-OVA_MHCII vector containing an OVA-MHC class II-restricted epitope (ISQAVHAAHAEINEAGR) were tested for their ability to induce the proliferation of their corresponding lymphocytes isolated from OT-I and OT-II transgenic mice, respectively (Figure 20a). Mice were immunized by ear pinna electroporation so that the draining lymph nodes were close to the delivery site, and T-cell proliferation could be accurately assessed [71]. The positive control pOVA containing both class I and class II epitopes induced CD8+ and CD4+ T-cell proliferation (Figure 20b,c). pTOP-OVA_MHCI efficiently activated the CD8+ T-cell response with strong proliferation of OT-I cells (58.8±8.6 %), whereas proliferation induced by pOVA only reached 39.6±12.8 %. Immunization with pTOP-OVA_MHCII did not induce a CD8+ T-cell response (Figure 20b) but elicited the division of 24.8±1.9 % of the CD4+ T cells. The percentage of dividing cells reached 31.2±4.7 % following pOVA immunization, and no OT-II cell proliferation was observed in pTOP-OVA_MHCI-immunized mice (Figure 20c).

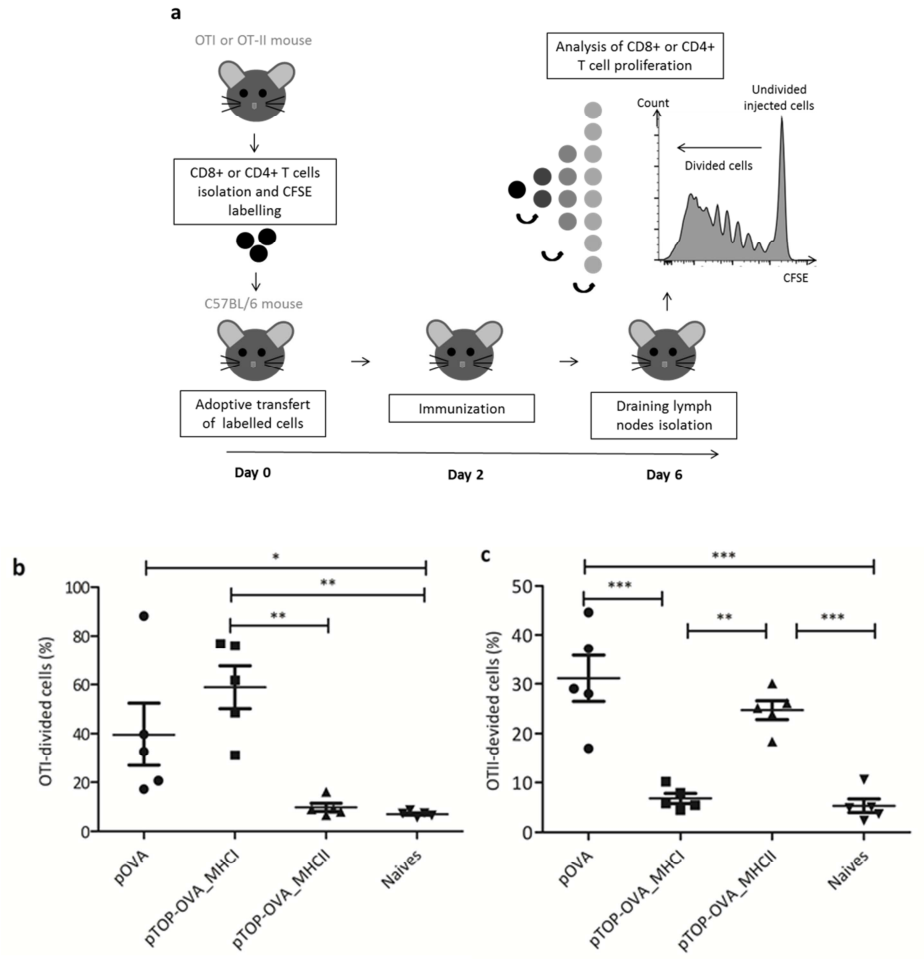


Figure 20 - Evaluation of the ability of pTOP constructs to specifically activate T lymphocytes.

(a) Schematic representation of OT-I/OT-II proliferation experiment designed to assess the ability of the vaccines to activate CD8+/ CD4+ T cells with a TCR designed to recognize SIINFEKL/ISQAVHAAHAEINEAGR. **(b)** Quantification of the dividing OT-I cells based on flow cytometry histogram analysis. Percentages were compared by Tukey's multiple comparison test (n=5), and significant differences are indicated with asterisks (*P<0.05, **P<0.01). **(c)** Quantification of the dividing OT-II cells based on flow cytometry histogram analysis. Percentages were compared by Tukey's multiple comparison test (n=5), and significant differences are indicated with asterisks (**P<0.01, ***P<0.001).

Prophylactic intramuscular immunization with pTOP-OVA_MHCI or pTOP-OVA_MHCI combined with pTOP-OVA_MHCII or pOVA delayed tumour growth.

The efficacy of the anti-ovalbumin pTOP-based vaccines was assessed in a B16F10-OVA melanoma model. Mice were prophylactically immunized by intramuscular electroporation (Figure 21a) with pOVA, pVSVG, pTOP-OVA_MHCI, pTOP-OVA_MHCII, or pTOP-OVA_MHCI combined with pTOP-OVA_MHCII. Tumours grew at a similar speed in mice immunized with pVSVG and pTOP-OVA_MHCII compared to naive mice, and there were no significant survival differences among these groups (Table 4). For tumour growth delay analysis, a mean tumour size of 300 mm^3 was chosen as a threshold value representing established growth. The mean size of the naive mouse tumours reached 300 mm^3 on the 17^{th} day, as did the tumours in the pVSVG-immunized mice. This threshold was reached one day later in the pTOP-OVA_MHCII-immunized mice. At the same time point, the tumour sizes were smaller in the pTOP-OVA_MHCI treated mice, and the mean size reached 300 mm^3 on the 22^{nd} day. A lengthy delay in tumour growth was observed in mice immunized with both MHC class I and MHC class II pTOP constructs and mice treated with pOVA that reached the threshold value at day 27 and 30, respectively (Figure 21b). For pTOP-OVA_MHCI, pTOP-OVA_MHCI combined with pTOP-OVA_MHCII- and pOVA-treated mice, the survival was significantly improved compared to untreated mice (Figure 21c). Long-term survival was observed in 50 % of pOVA-immunized mice and 40 % of mice immunized with pTOP-OVA_MHCI combined with pTOP-OVA_MHCII (Table 4).

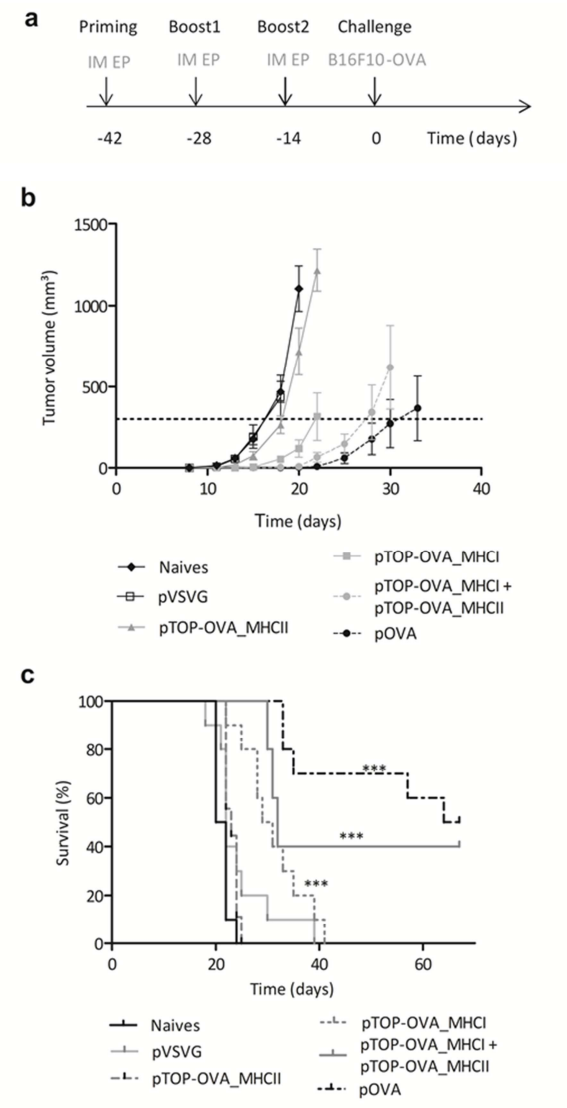


Figure 21 – Evaluation of the prophylactic potential of anti-ovalbumin pTOP-based DNA vaccines.

C57BL/6 mice were immunized by prophylactic IM EP with either pVSVG, pTOP-OVA_MHCII, pTOP-OVA_MHCI, pOVA or the combination of both pTOP-OVA_MHCI and pTOP-OVA_MHCII. Two weeks after the last vaccine administration, mice were challenged with an injection of B16F10-OVA cells. **(a)** Schematic timeline of the experiment. **(b)** Tumour growth follow up until the first mouse sacrifice. The error bars represent the mean±SEM (n=10) (ANOVA, Bonferroni post-test). **(c)** Survival rates after challenge. The asterisks indicate significant differences compared to naive mice (***P<0.001) (Comparison of survival curves, Mantel-Cox test).

Co-delivery of pTOP-OVA_MHCI and pTOP-OVA_MHCII reinforced the cytotoxic response to the vaccine antigen.

A CD8+ CTL can kill cells with surface expression of its specific epitope in the context of MHC class I. The cytotoxic responses triggered by the vaccines containing the OVA-MHC class I-restricted epitope were assessed by a killing assay (Figure 22a). Mice were immunized with pOVA, pTOP-OVA_MHCI or pTOP-OVA_MHCI and pTOP-OVA_MHCII, and their ability to recognize and eliminate SIINFEKL-pulsed target cells was assessed. The CTL response was drastically improved when both pTOP-OVA_MHCI and pTOP-OVA_MHCII were administered compared to immunization with pOVA or pTOP-OVA_MHCI alone (Figure 22b). Co-delivery of pTOP-OVA_MHCI and pTOP-OVA_MHCII induced 64.5±3.8 % target cell killing. Target cell killing decreased to 14.5±6.2 % or 23.3±11.9 % following pTOP-OVA_MHCI or pOVA immunization, respectively.

Immunization with both pTOP-OVA_MHCI and pTOP-OVA_MHCII induced therapeutic protection against B16F10-OVA cells.

The therapeutic potential of the pTOP-based vaccines was analysed. The same treatments were applied as those used in the prophylactic experiment but the treatment started two days after the injection of tumor cells and was repeated one and two weeks later (Figure 23a). The tumours grew quickly in all the groups. At day 15, the mean tumour size had already reached 300 mm^3 in all the mice, apart from those which were immunized with both pTOP-OVA_MHCI and pTOP-OVA_MHCII (Figure 23b). Regarding the survival curves, only mice immunized with both pTOP constructs lived significantly longer compared to the naive mice (Figure 23c). Surprisingly, the positive control pOVA was unable to protect mice from the tumour challenge.

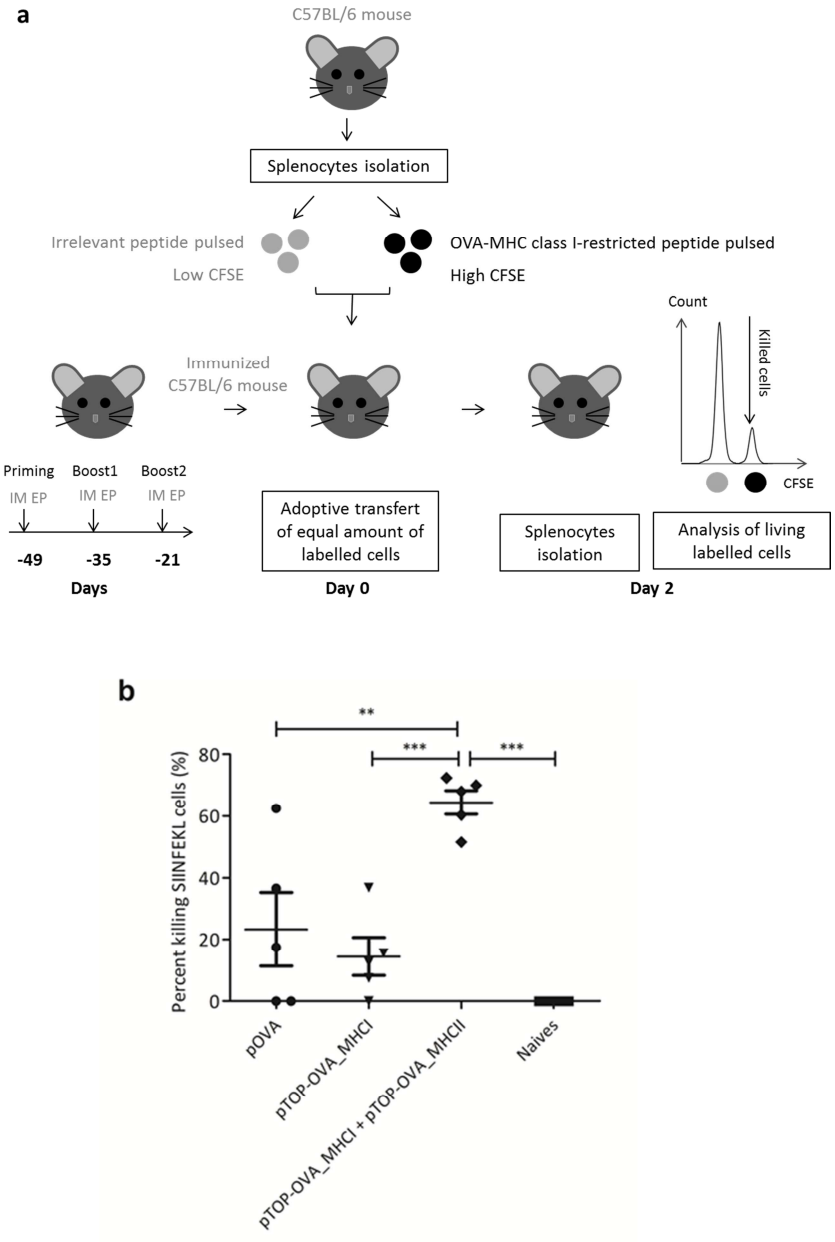


Figure 22 – Evaluation of the CTL response induced by OVA-MHC class I-based vaccines. (**a**) Schematic representation of the CTL assay evaluating the ability of the vaccines to induce effective cytotoxic T-cell responses and subsequent specific killing of cells expressing the target antigen. (**b**) Quantification of target OVA cell killing based on flow cytometry measurement. Percentages were compared by Tukey's multiple comparison test (n=5), and significant differences are indicated with asterisks (**P<0.01, ***P<0.001).

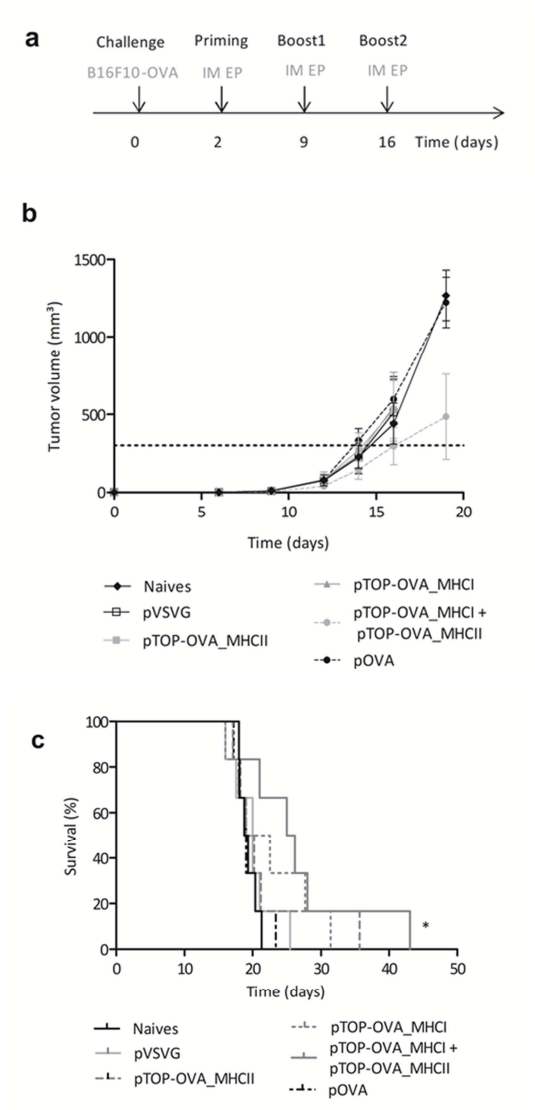


Figure 23 - Evaluation of the therapeutic potential of anti-ovalbumin pTOP-based DNA vaccines.

(a) C57BL/6 mice were injected with B16F10-OVA cells on day 0. Next, the therapeutic vaccines were administered at days 2, 9 and 16 by IM EP of either pVSVG, pTOP-OVA_MHCII, pTOP-OVA_MHCI, pOVA or the combination of both pTOP-OVA_MHCI and pTOP-OVA_MHCII. (b) Tumour growth follow up until the first mouse sacrifice. The errors bars represent the mean±SEM (n=6) (ANOVA, Bonferroni post-test). (c) Survival rate monitoring. The asterisks indicate significant differences compared to naive mice (n=6) (*P<0.05) (Comparison of the survival curves, Mantel-Cox test).

Table 4– Comparison between prophylactic and therapeutic pTOP-based DNA vaccines.

The median survival indicates the day when the survival rate reached 50 % and long-term survival indicates the percentage of mice still alive and tumour free at the end of the experiment.

	Median Survival (days)		**Long term Survival (%)**	
Group	Prophylactic n = 10	Therapeutic n = 6	Prophylactic n = 10	Therapeutic n = 6
Naives	21	19	0	0
pVSVG	22	20	0	0
pTOP-OVA_MHCII	23	20	0	0
pTOP-OVA_MHCI	30	21	0	0
pTOP-OVA_MHCI + pTOP-OVA_MHCII	32	26	40	0
pOVA	64	19	50	0

4. Discussion

Currently, there is a need to develop efficient cancer vaccines capable of inducing potent and specific anti-tumour T-cell responses. We hypothesized that pTOP, a plasmid encoding a modified VSV-G protein with defined T epitopes inserted, could be an efficient strategy for this purpose. We showed that pTOP-based DNA vaccines induced high-avidity, anti-epitope T cells and protected mice from tumour cells expressing the vaccine antigen. This protection appeared to be mainly driven by the action of CD8+ CTLs, and it was reinforced by co-activating CD4+ Th cells. An efficient therapeutic vaccine was obtained by combining class I and class II epitopes in pTOP, demonstrating the potential of this strategy for future applications.

We first demonstrated that pTOP-based DNA vaccines can activate high avidity CD8+ and CD4+ T-cell responses by inserting class I and class II epitopes, respectively. The inserted epitopes appeared to be correctly processed and presented on MHC molecules. MHC class I and class II molecules have a similar function: they present intracellular peptides at the cell surface to CD8+ T cells and exogenous peptides at the cell surface to CD4+ T cells, respectively[57]. MHC class I- and MHC class II-restricted epitopes are obtained via different proteolytic mechanisms, and the two pathways can be linked by mechanisms such as cross-presentation and autophagy[255]. Epitope insertion in pTOP could activate each pathway specifically, as demonstrated by the specific proliferation of OT-I and OT-II cells. Moreover, pTOP-based vaccines strongly activated T cells, and the proliferation percentages were close to those induced by the positive control pOVA.

The anti-tumour efficacy of pTOP-based DNA vaccines was assessed in a prophylactic immunization experiment. Mice were challenged with B16F10 cells

that stably express the OVA protein. pTOP-OVA_MHCI significantly delayed tumour growth and improved mice survival, whereas pTOP-OVA_MHCII had no effect on tumour burden. This result supports the observed major anti-cancer role of the CD8+ CTLs[9]. The co-administration of pTOP-OVA_MHCI and pTOP-OVA_MHCII tended to improve the long-term survival of the mice. This result emphasized the importance of $CD4^+$ T-helper cells that were already known to be necessary for optimal and sustained effector $CD8^+$ T cell responses, as well as the induction and maintenance of $CD8^+$ T-cell memory [256].

The importance of CD4+ Th activation was strengthened by analysing the anti-vaccine CTL response. Mice immunized against the class I epitope of OVA were able to specifically kill SIINFEKL-pulsed cells that mimic targeted tumour cells. The killing percentages were the highest in mice immunized with both pTOP-OVA_MHCI and pTOP-OVA_MHCII. The increased efficacy compared to mice immunized with pTOP-OVA_MHCI alone again supported the ability of CD4+ Th cells to strengthen the CD8+ CTL response to the vaccine antigen. In addition, mice immunized with pOVA exhibited a weaker ability to kill the target cells compared to mice immunized with pTOP-OVA_MHCI and pTOP-OVA_MHCII, suggesting an advantage of inserting the epitopes in the VSV-G protein to enhance vaccine potency.

The hypothesis that pTOP-based vaccines could contribute to the enhancement of DNA vaccine immunogenicity was supported by the analysis of the therapeutic potential of pTOP-based vaccines. In this experiment, pOVA was not able to protect mice against tumour burden, and the only efficient treatment was the combination of pTOP-OVA_MHCI and pTOP-OVA_MHCII. This result also suggested that pTOP has an immunoregulatory function and the ability to enhance DNA vaccine efficacy. Some studies have suggested mechanisms by which VSV-G could modulate the immune response. VSV-G was shown to induce

an immunogenic form of cellular fusion and necrosis, which could induce immune cell recruitment at the vaccine delivery site[223,224,257]. It was also shown to enhance cross-presentation of antigens[224,226,227], a phenomenon that may be enhanced by antigen concentration and transfer in VSV-G-induced nanovesicles[258]. Lately, VSV-G has been suggested to induce autophagy[225] and thus may be involved in modulation of the antigen processing pathways[259].

Altogether, these results indicate the ability of the plasmids encoding the VSV-G protein with insertion of defined T epitopes to induce potent and specific anti-epitope T-cell responses and elicit antigen-bearing tumour cell recognition and destruction. As shown in the therapeutic experiment, this strategy may help circumvent the low immunogenicity observed with DNA vaccines encoding the full-length antigenic protein. This result was confirmed in other therapeutic immunization experiments (unpublished data). However, the benefit of using this strategy in a prophylactic approach has not been clearly evidenced so far.

This work also highlighted the importance of using appropriate epitopes and epitope combinations. For cancer vaccine design, the main challenge resides in determining which tumour antigens recognized by T lymphocytes should be used[9]. The efficacy of pTOP-based vaccines could probably be further enhanced by playing on the choice of the inserted epitope. This can be easily done, as pTOP vectors are easy to modify and combine and could be used in a wide range of applications. Current work aims at optimizing epitopes choice and evaluating the therapeutic potential of the vaccine candidates.

Chapter 4 – General discussion

1. Achievements

The thesis relies on genetic tumour vaccination based on pDNA delivery. This method was chosen because DNA plasmids appear as a safe and effective method to immunize against cancer, and present low production costs and high stability compared to the alternative strategies[260]. Electroporation was used to deliver the DNA vaccines. This approach uses electrical fields to increase the DNA delivery efficiency and offers at the same time an adjuvant activity[105]. The high number of clinical trials based on pDNA EP highlights the potential of this strategy[200]. Cancer DNA vaccines can induce a broad immune response but their immunogenicity in patients has been quite low so far[253]. Therefore, the use of adjuvants to enhance DNA vaccine efficacy is under deep investigation.

The aim of the thesis was to assess whether plasmids encoding viral structural proteins could enhance cancer DNA vaccine potency. The idea behind this strategy was to benefit from a potential adjuvant effect of the encoded viral gene that would improve cellular immunity to the vaccine antigen. To assess whether this strategy would be beneficial, two approaches were pursued: (i) the co-delivery of a plasmid coding for the HIV-1 gag protein, pGag, with a plasmid encoding the antigen expressed by tumour cells and (ii) the delivery of pTOP, a plasmid that encode a modified VSV-G in which a specific T epitope is inserted.

We hypothesize that the *in vivo* electroporation of pGag could enhance cancer DNA vaccine immunogenicity. First, we performed preliminary studies to demonstrate that cell transfection with pGag led to the formation of HIV-1 Gag VLPs, and that electroporation of pDNA induced the production of type I IFNs *in*

vivo. Then, the effects of pGag on the immune response against the OVA model antigen and a tumour associated antigen were evaluated in B16F10-OVA melanoma. We showed that the administration of pGag with the anti-OVA DNA vaccine induced a clear shift of the anti-OVA immune response towards a Th1 polarisation, which is favourable for cancer cell elimination. Consistently, pGag co-delivery with either an anti-OVA or an anti-gp100 DNA vaccine increased protection against B16F10-OVA tumour challenge following the prophylactic immunisation of mice. Therapeutic immunization was tested with the anti-OVA DNA vaccine model and a tumour growth delay was observed only with the combination of pOVA and pGag. Finally, we showed that the immunomodulatory effects triggered by pGag *in vivo* were not mediated by the formation of VLPs. In conclusion, these results suggest the ability of pGag to regulate adaptive immunity, as the administration of this plasmid promotes a Th1 immune response to the vaccine antigen and improves the protective immunity against tumours.

The second strategy consisted in the use of a plasmid encoding a modified VSV-G protein in which a T-cell epitope is inserted. This strategy may have a dual therapeutic benefit to induce potent and specific T-cells responses by (i) enhancing the vaccine immunogenicity via the viral protein encoding gene and (ii) delivering specific epitopes recognized by T cells. The ability of pTOP-based DNA vaccines to induce potent and specific anti-vaccine T cells and protect against tumours was assessed with the ovalbumin antigen model, with insertion of either MHC class I- or MHC class II-restricted epitopes in the VSV-G sequence. Administration of pTOP-OVA_MHCI and pTOP-OVA_MHCII led to the proliferation of specific CD8+ and CD4+ T cells, respectively, demonstrating that correct processing and presentation of the inserted epitopes occurred. Prophylactic immunization of mice with pTOP-OVA_MHCI led to a significant reduction in B16F10-OVA tumour growth as well as an increase in survival rates. The vaccine

efficacy and CTL response were reinforced by co-administering pTOP-OVA_MHCI and pTOP-OVA_MHCII. Finally, a protective anti-tumour therapeutic effect was observed in mice immunized with the combination of pTOP-OVA_MHCI and pTOP-OVA_MHCII. These results indicate the ability of the plasmids encoding the VSV-G protein with insertion of defined T epitopes to induce potent and specific anti-epitope T-cell responses and elicit antigen-bearing tumour cell recognition and destruction. These plasmids are therefore promising for the design of epitope-based cancer DNA vaccines, combining ease of production, versatility and the ability to induce potent and specific anti-tumour T-cell responses.

These studies demonstrate the potential of plasmid encoding viral structural proteins to enhance cancer DNA vaccine potency. The first strategy presents the HIV-1 Gag plasmid as a genetic adjuvant for cancer DNA vaccines and highlights its ability to promote a Th1 response against the vaccine antigen. The second strategy combines high specificity and immunogenicity to induce potent and specific anti-epitope T-cell responses, presenting this modified VSV-G as a promising platform for delivering tumor epitopes and generating cellular immune response against cancer. However, this work is only a first step in this innovative field. Several parameters still need to be studied in order to make the best out of this strategy. This last chapter discusses some limitations of the current work and gives some clues to go further.

2. Limitations and reflections

Genetic adjuvant role

The basic postulate of this work is that plasmid encoding viral proteins could have an adjuvant effect to enhance the efficacy of cancer DNA vaccines. The studies mainly focused on the analysis of the anti-tumour immune response induced by the vaccines. Very little is known about the possible adjuvant mechanisms of these plasmids. We hypothesized that these plasmids could modulate immune responses induction either by stimulating innate immunity, by providing helper responses or by mechanisms associated with the structural properties of the viral protein. A brief overview about what is known and what can be studied is given for each possibility.

1- Stimulation of innate immunity via PRR sensing of the viral gene DNA sequence or the expressed protein.

It is believed that the viral structural protein encoding plasmid delivery can induce innate immune response. Indeed, the host may sense the gene sequence of the viral protein or it may sense the invading viral protein via its PRRs. Some receptors could sense specific DNA motifs, such as the TLR-9 or specific cytosolic sensors[166]. Other receptors can sense the expressed viral proteins. It has been shown for instance that the HIV-1 capsid can be detected via cyclophilin A and TRIM5 receptors[218,219]. It was also found that the MA and CA domains of Gag protein serve as PAMPs for TLR2[220]. The VSV-G protein was shown to activate a specific antiviral TLR4-dependent pathway[222] and has also been suggested lately to induce autophagy[225].

To study whether such sensing takes place, different parameters can be studied. In order to determine whether the gene or the protein is responsible for PRR

activation, a stop codon can be introduced at the beginning of the viral structural protein gene. Thereby, the DNA sequence will be conserved whereas the protein will not be expressed. Then, it is possible to assess whether some PRRs are over-expressed following the delivery of the plasmid[129]. Moreover, it is known that the main consequences of PRR engagement are the production of IFN and inflammatory cytokines as well as the induction of autophagy[37]. The ability of the plasmids to induce or reinforce such events could also be assessed. In addition, it could be interesting to analyze whether the enhanced DNA vaccine immunogenicity is still observed in transgenic mice lacking some key receptors involved in the PRR pathways, such as mice lacking the type I IFN receptor ($Ifnar^{-/-}$) or the TLR-9 ($TLR9^{-/-}$). However this may be trickier as it would certainly impair DNA vaccine immunogenicity[174].

2- Induction of strong helper responses thanks to viral T-cell epitopes.

$CD4^+$ T-helper are key players in the anti-tumour immunity. Particularly, Th1 cells are necessary for optimal and sustained effector $CD8^+$ T cell responses, as well as the induction and maintenance of $CD8^+$ T-cell memory[231,256]. Th1 cytokines stimulate APCs and also help foster the development of CTLs that are responsible for the cell-mediated immune response against viruses and tumor cells[250]. Of course, both viral structural proteins used in this work were shown to possess MHC class II epitopes (https://www.hiv.lanl.gov/)[261].

It would be interesting to study the induction of viral structural protein-specific Th1 responses upon vaccination. The identification of strong helper epitopes may be interesting for the development of potent tumour vaccination strategies. It could also be worthwhile to analyze in more details if the delivery of the plasmid encoding the viral structural protein is able to enhance tumour-specific CTLs activity and memory.

3- *Enhanced DCs maturation and T-cell priming related to the function of the protein.*

Finally, an adjuvant effect can be triggered by the function of the viral structural protein. These proteins play a major role in viral infectivity, allowing the virus to penetrate the host cells and to bud from them. In the case of the HIV Gag protein, cell transfection with pGag is known to lead to VLPs production by the host cells. These HIV-1 Gag VLPs were shown to improve DC maturation and T-cell priming[216]. Another study demonstrated that HIV-1 VLPs can propagate innate immune signaling by packaging cGAMP and delivering it to uninfected target cells[217]. Regarding VSV-G, the fusogenic envelope glycoprotein was shown to induce an immunogenic form of cellular fusion and necrosis, which could induce immune cell recruitment at the vaccine delivery site[223,224,257]. Besides this function, it was also shown that human cell transfection with a plasmid encoding VSV-G induced the release of fusogenic vesicles named gesicles, that are able to incorporate proteins from producer cells and deliver them to other recipient cells[258]. This is probably linked to the ability of VSV-G to enhance cross-presentation of antigens[224,226,227].

There is a huge panel of potential effects that could be linked to the viral protein function. In this context, the best approach to study whether the adjuvant effect is related to their function consists in introducing mutation in the viral gene to deprive the protein of its structural function. For pGag, this was done by using pGag*, a mutated form of HIV Gag that is unable to form VLPs[243]. We showed that VLP formation was not responsible for the enhanced immunogenicity of the DNA vaccine. The same approach could be pursued with VSV-G, by using mutations that abolish the fusogenic properties of the protein and its capacity to form gesicles[262,263].

Potential downsides of this adjuvant strategy

Even if the use of pDNA encoding viral structural protein may enhance cancer DNA vaccine potency, it may also have some downsides. A first question is related to safety concerns and potential side effects associated with these plasmids. Regarding the electroporation of the pDNA, it seems that this approach is safe and convenient[200]. It should be pointed out that the use of antibiotic resistance-free plasmids in clinical applications is usually required by the Regulatory Agencies and that alternative selection markers have been developed to take over[68]. The expressed viral protein may also exert a certain toxicity related to its function. The safety of HIV-1 Gag encoding plasmid has already been demonstrated in the context of HIV vaccine development[234-236]. Regarding the pTOP strategy, additional safety studies should be performed.

In addition to these toxicity concerns, a major risk of such adjuvant strategy is the induction of immunity against the viral protein. Even if this may be helpful (for instance if the Th1 immune response is induced), it may also lead to deleterious effects. The major risk is the occurrence of antigenic competition where the anti-viral responses dominate over the vaccine-specific responses[264]. Vaccination often results in high-magnitude responses to a small number of immunodominant epitopes, with only weak responses induced against subdominant epitopes[265]. Several variables can contribute to it, among which the ability of the epitope to bind appropriate MHC molecules, its processing and presentation efficiency, the co-stimulation quality and intrinsic features of the T cells[266]. The phenomenon of immunodominance is complex and difficult to predict. Simultaneous delivery of the different epitopes, the absence of previous antigenic encounters and the abundance of the various epitopes may help in favouring a broad immune response against multiple epitopes[266]. This highlights the drastic importance of the experimental settings in shaping immune response and vaccine efficacy.

Influence of the experimental settings

The experimental protocol used for immunization may have an impact on immune responses induction. This section describes some parameters used in this work that could influence the vaccine efficacy.

Vaccine delivery

As explained in the introduction, the pDNA design, the dose, the delivery site, the prime-boost strategy, the type of electrode and the pulses parameters may have an impact on the type and magnitude of the immune response induction[71]. Hence, these parameters remained unchanged throughout the work to allow objective comparisons. They were chosen based on the work previously done in the lab by Vandermeulen et al. It is often more difficult to obtain efficient therapeutic vaccines than prophylactic ones because the vaccine intervention must combat an immune system that has been restrained by mechanisms that sustain the disease[267]. Interestingly, plasmid encoding viral structural proteins appeared particularly tailored for enhancing DNA vaccine efficacy in therapeutic approaches. Indeed, pOVA automatically failed to hold back tumours, whereas the combination of the tumour antigens and the viral protein encoding plasmids could delay tumour growth. Maybe this can be explained by the ability of these plasmids to activate innate immune mediators (for instance the activation of NK cells or the secretion of cytokines) that can exert an anti-tumour effect[14,37]. Another difference between prophylactic and therapeutic vaccines is the type of T cell memory that they are expected to induce. Effector memory T cells (T_{EM}) would be needed for therapeutic vaccines, whereas the prophylactic approach would require central memory T cells (T_{CM}). Indeed, T_{EM} ensure a direct protective memory as they migrate to inflamed peripheral tissue and display immediate effector function. In the case of T_{CM}, a reactive memory is induced that involves

the proliferation of these memory cells and their differentiation to effector cells[268]. The generation of T_{CM} and T_{EM} greatly depends on the antigenic signal strength and exposure[269,270].

The viral structural protein encoding plasmid

The choice of the viral structural gene as well as the design of the plasmid may influence vaccine efficacy. In this work, we worked with a viral core gene as well as an envelope gene. These were also chosen based on the work previously done in the lab[209]. However, other viral structural genes could have been considered. First, other viral strains may be envisaged. For safety and regulatory reasons, the use of pDNA already involved in clinical trials is an asset. The use of viral protein genes from non-human viruses may also be advantageous as it insures that there is no pre-existing immunity against the encoded protein. This could maybe lower the risk of immune competition[266,271]. In the particular case of virus induced cancers, the use of the same strain genes may be more appropriate. Secondly, the use of other viral components may be evaluated, such as non-structural protein genes of the viral genome. However, these proteins may trigger deleterious effects in the host cells and may be associated with higher toxicity[272]. Using non-structural protein also takes away the option to display the antigen into self-assembling structures that may provide improved immunological properties[162,273]. Related to this point, the choice of the plasmid construct used to deliver the tumour antigen and the viral protein genes is also important. They can be delivered either on separate pDNA, on a polycistronic vector, or as a chimeric protein construct, by fusing the two genes or by inserting tumour epitopes sequence in the viral protein gene. The delivery form would probably impact immune responses induction. It may impact APCs activation as well as the processing and presentation of the expressed antigens. It could also be interesting to assess the effect of combining several viral structural protein encoding

plasmids. We evaluated the combination of pGag and pTOP-OVA_MHCI in a prophylactic experiment, but the combination of both plasmids suppressed the protective effect of the vaccine candidate. We also showed in a prophylactic experiment that combining pOVA and pVSV-G lead to similar efficacy than the delivery of pOVA alone (unpublished data). These strategies should also be tested in therapeutic approaches. So far, we demonstrated the benefit of pGag co-delivery in both prophylactic and therapeutic approaches, whereas the effect of pTOP-based vaccines was only suggested in a therapeutic approach. In conclusion, it is difficult to predict which viral protein genes would have a positive impact on the immune response to cancer DNA vaccines and the best way to ascertain it consists in experimentation.

The antigen and cancer model

The ovalbumin (OVA) model antigen was selected to evaluate the anti-cancer potential of the vaccine candidates. This antigen is stably expressed in the B16F10-OVA cell line of mice melanoma. This model is interesting as it has been extensively studied in various cancer immunotherapeutic strategies and several means can be used to characterize the OVA-specific immune response. However, OVA is not a real tumour antigen as it is a xenogenic protein that is derived from another species than the patient. By utilizing xenogenic antigens, it is possible to break immune tolerance to self-antigens and to induce a much stronger immune response[61]. Therefore, the ability of the viral structural protein encoding plasmids to enhance cancer DNA vaccine potency should be evaluated with tumour antigens too.

Differentiation antigens able to induce spontaneous T-cell responses have been documented in patient with melanoma. The main antigenic peptides that are recognized by such CTLs are derived from tyrosinase, melan-A and GP100. As

these antigens are expressed by the B16F10-OVA cells, they can be chosen as vaccine target. The ability of pGag to enhance the efficacy of an anti-GP100 DNA vaccine was stressed out in the third chapter. Regarding the pTOP strategy, some oligonucleotides coding for MHC class I restricted peptides were inserted in the VSV-G sequence, including melan-A_{26-35}, $GP100_{209-217}$ and $tyrosinase_{368-376}$. These vaccines failed to protect mice against tumour challenge and to induce tumour antigen-specific cytotoxic responses following prophylactic immunizations, even when changing the pDNA dose and the delivery site (unpublished results). These observations highlight the importance of identifying the ideal target epitopes and epitopes combination. Maybe that the delivery of peptides derived from antigens of low tumour specificity within pTOP is not efficient, but that the insertion of epitopes of high tumour specificity would be. In this vein, the insertion in pTOP of neo-epitopes identified in the B16F10-OVA cells could be worthwhile to test[274]. In addition, as more and more studies highlight the important role of MHC class II epitopes in anti-cancer immunity[266,275,276], efficient vaccines might be obtained by inserting universal or tumour-specific helper epitopes in other sites of the VSV-G sequence to enhance the cancer DNA vaccine efficiency. This strategy is currently under investigation (Vandermeulen et al.). Immunizing against several tumour-specific epitopes would probably also lower the risk of immune escape[277].

Another limitation of the B16F10-OVA model, consisting in subcutaneous injection of melanoma cells, is that it poorly reflects the real tumour development. The effects of pTOP and pGag should also be assessed in more representative models, such as spontaneous or carcinogen-induced tumours. Their efficacy on other cancer types may also be evaluated. The development of representative models is critical for pre-clinical studies, even if animal models are of course unlikely to validate vaccine strategies for humans[228,278].

3. Perspectives for immunotherapy

The development of efficient therapeutic cancer vaccines against cancer is a key challenge in cancer immunotherapy. These vaccines are believed to serve to enlarge the pool of tumour-specific T cells, to reactivate existing tumour T cells that may be in an anergic state and stimulate new naive antitumour T cells[9,17]. Even if the clinical benefits of therapeutic vaccines have been established for some viral and nonviral cancers, the proportion of patients benefiting from treatment with cancer vaccines leaves much to be desired[256].

To improve therapeutic efficacy, the vaccine design must be optimized. There are currently signs of success in the clinic for all vaccine strategies developed in the introduction (section 3.4.), but this does not mean that these platforms have reached their final developmental stage[17]. For instance, a major concern about cancer DNA vaccines is the choice of adjuvants that appropriately and efficiently stimulate CD4+ and CD8+ T effector and memory T-cell responses. This work presents an innovative strategy to address this issue. Therapeutic vaccines must achieve sufficient antigen concentration in DCs and must activate them with adjuvants[256].

The choice of appropriate tumour antigens is another key element of the vaccine design. Recent advances in genome sequencing combined with *in silico* epitope prediction algorithms have allowed the identification of patient specific subset of mutated antigen that can be source of neo-epitopes presented on MHC class I or II molecules and elicit T-cell responses[274,276,279]. This raises the hope among the scientific community that these epitopes will pave the way towards personalized cancer vaccines.

Besides their insufficient design, most vaccine trials have been a testimony of the immune suppressive and immune escape mechanisms that operate in cancer[9,256].

There is therefore a need to find proper co-treatments during vaccination to deal with the immunoregulatory mechanisms that promote immune resistance in cancer[17,248]. For instance, combination with chemotherapeutic agents that are known to deplete immunosuppressive Tregs or MDSCs without affecting effector and memory T cells[280], or the addition of checkpoint inhibitors[281] can enhance therapeutic vaccine efficacy. However, the choice of appropriate co-treatment is complex, as the regulatory balance in the Cancer-Immunity Cycle between stimulatory and inhibitory factors is delicate. Inappropriate equilibrium can induce severe immune-related adverse events[32,50]. Therefore, distinct therapeutic strategies may be required for restoring immune-mediated cancer elimination, depending on the mechanisms of immune evasion exploited by cancer. The choice of proper co-treatments could be based on fact-findings by molecular and cellular profiling in the patients' cancerous tissues[16,17] .

4. Conclusion and perspectives

In conclusion, this thesis demonstrates the potential of plasmid encoding viral structural proteins to enhance cancer DNA vaccine potency (Figure 24). However, this work is only a first step in this innovative field. To go further, the adjuvant mechanisms induced by these plasmids should be characterized in more details. The potential of this approach might also be assessed in various experimental settings. Finally, in a booming immunotherapeutic field, it could be interesting to understand the immune escape mechanisms exploited by cancer and, eventually, to combine this strategy with other treatments.

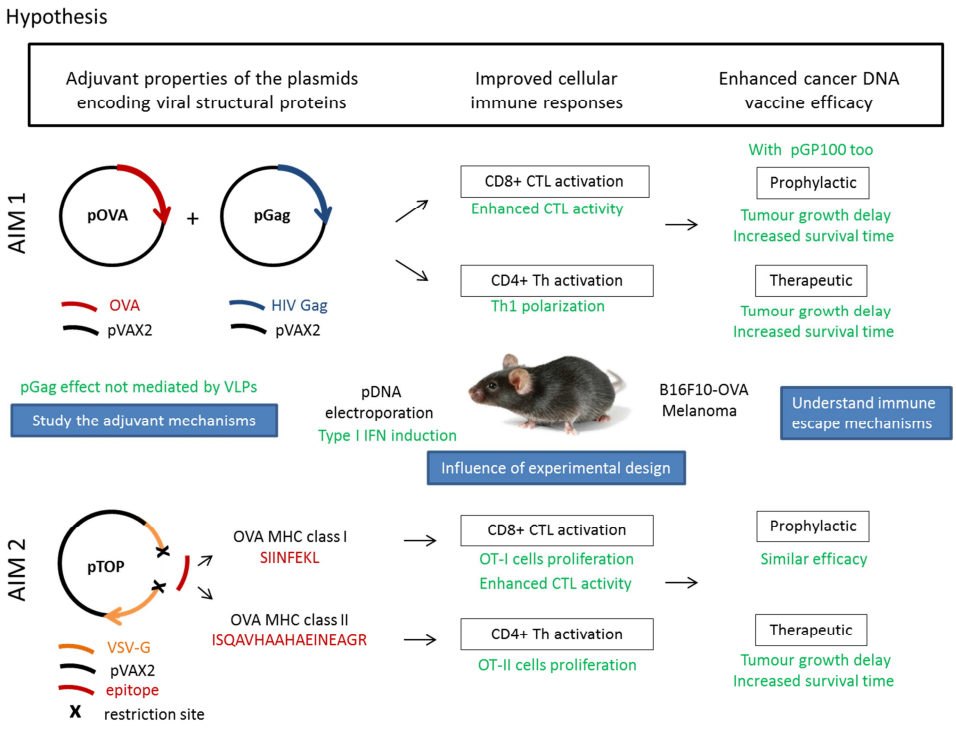
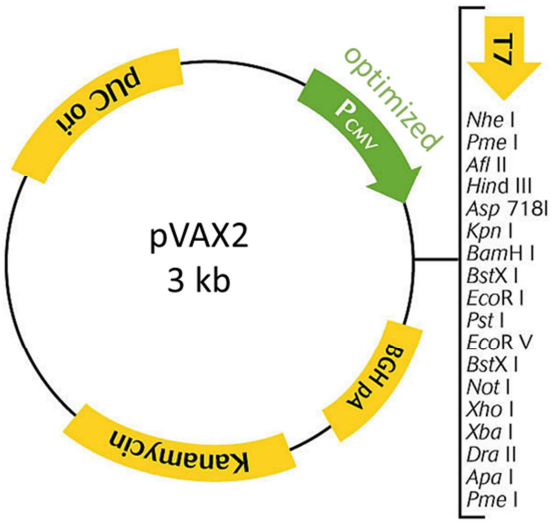


Figure 24 – Concluding picture: aims (black), main achievements (green) and perspectives (blue).

Appendix

Additional information about the plasmid constructs.



pVAX2: This vector possesses an origin of replication, an optimized CMV β promotor, a ploy-A tail and a Kanamycin resistance gene. Figure modified from https://www.thermofisher.com/order/catalog/product/V26020.

Codon-optimized sequences inserted in pVAX2 between BamHI and NotI.

1) Ovalbumin (pOVA)

ATGGGATCTATCGGCGCTGCCAGCATGGAATTCTGCTTCGACGTGTTCAAAGAACTGAA
GGTGCACCACGCCAACGAGAACATCTTCTACTGCCCTATCGCCATCATGAGCGCCCTGGC
CATGGTGTACCTGGGCGCCAAGGACAGCACCAGAACCCAGATCAACAAGGTGGTGCGA
TTCGACAAGCTGCCCGGCTTCGGCGACTCTATCGAGGCCCAGTGTGGCACCAGCGTGAA
CGTGCACAGCAGCCTGAGAGACATCCTGAACCAGATCACCAAGCCCAACGACGTGTACA
GCTTCAGCCTGGCCAGCAGACTGTACGCCGAGGAAAGATACCCCATCCTGCCCGAGTAC
CTGCAGTGCGTGAAAGAGCTGTACAGAGGCGGCCTGGAACCCATCAACTTCCAGACAGC

CGCCGACCAGGCCAGAGAGCTGATCAACAGCTGGGTGGAAAGCCAGACCAACGGCATC
ATCAGAAACGTGCTGCAGCCCAGCAGCGTGGACTCCCAGACAGCTATGGTGCTCGTGAA
CGCCATCGTGTTCAAGGGCCTGTGGGAAAAGACCTTCAAGGACGAGGACACCCAGGCC
ATGCCCTTCAGAGTGACCGAGCAGGAATCCAAGCCCGTGCAGATGATGTACCAGATCGG
CCTGTTCAGAGTGGCCTCCATGGCCTCCGAGAAGATGAAGATCCTGGAACTGCCTTTCGC
CAGCGGCACCATGAGCATGCTGGTGCTGCTGCCTGATGAGGTGTCCGGACTGGAACAG
CTGGAATCCATCATCAACTTTGAGAAGCTGACCGAGTGGACCAGCAGCAACGTGATGGA
AGAACGGAAGATCAAAGTGTACCTGCCCCGGATGAAGATGGAAGAGAAGTACAACCTG
ACCAGCGTGCTGATGGCTATGGGCATCACCGATGTGTTCAGCAGCAGCGCCAACCTGAG
CGGCATCAGCTCTGCCGAGAGCCTGAAGATCAGCCAGGCCGTGCACGCTGCCCACGCCG
AGATCAATGAGGCCGGCAGAGAAGTCGTGGGATCTGCCGAAGCTGGCGTGGACGCCGC
TTCCGTGTCCGAGGAATTCAGAGCCGACCACCCCTTTCTGTTCTGCATCAAGCACATTGC
CACCAACGCCGTGCTGTTCTTTGGCAGATGCGTGTCCCCCTGA

2) HIV-1 Gag (pGag)

ATGGGAGCTAGAGCCTCTGTGCTGTCTGGCGGCAAGCTGGACAGATGGGAGAAGATCA
GACTGAGGCCAGGCGGCAAGAAGAAGTACAAGCTGAAGCACATCGTGTGGGCCAGCA
GAGAGCTGGAAAGATTCGCCGTGAACCCCGGCCTGCTGGAAACCAGCGAGGGCTGCAG
ACAGATCCTGGGCCAGCTGCAGCCTTCTCTGCAGACCGGCAGCGAGGAAAGAAGATCCC
TGTACAACACCGTGGCCACCCTGTACTGCGTGCACCAGAGAATCGAGATCAAGGACACC
AAAGAGGCCCTGGACAAGATCGAGGAAGAACAGAACAAGTCTAAGAAGAAGGCCCAG
CAGGCTGCCGCCGACACAGGCCATTCTTCTCAGGTGTCCCAGAACTACCCCATCGTGCAG
AACATCCAGGGCCAGATGGTGCATCAGGCCATCAGCCCCAGAACCCTGAACGCCTGGGT
CAAGGTGGTGGAAGAGAAGGCCTTTAGCCCCGAAGTGATCCCCATGTTCAGCGCCCTGT
CTGAGGGCGCCACACCCCAGGACCTGAACACCATGCTGAACACAGTGGGCGGCCACCA
GGCCGCCATGCAGATGCTGAAAGAGACAATCAACGAAGAGGCCGCCGAGTGGGACAG
AGTGCACCCTGTGCATGCTGGCCCTATCGCCCCTGGCCAGATGAGAGAGCCTAGAGGCT
CTGATATCGCCGGCACCACCAGCACCCTGCAGGAACAGATCGGCTGGATGACCCACAAC
CCCCCCATCCCTGTGGGCGAGATCTACAAGAGATGGATCATCCTGGGACTGAACAAGAT
CGTGCGGATGTACAGCCCTACCAGCATCCTGGACATCAGGCAGGGCCCCAAAGAGCCCT
TCAGAGACTACGTGGACAGATTCTACAAGACCCTGAGAGCCGAGCAGGCCAGCCAGGA
AGTGAAGAACTGGATGACAGAGACACTGCTGGTGCAGAACGCCAACCCCGACTGCAAG
ACCATCCTGAAGGCTCTGGGCCCTGGCGCCACCCTGGAAGAGATGATGACAGCCTGTCA
GGGCGTGGGCGGACCTGGCCATAAGGCTAGAGTGCTGGCCGAGGCCATGAGCCAAGTG
ACCAACCCTGCCACCATCATGATCCAGAAGGGCAACTTCCGGAACCAGAGAAAGACCGT

GAAGTGCTTCAACTGCGGCAAAGAGGGCCATATCGCCAAGAACTGCAGAGCCCCCAGA
AAGAAAGGCTGCTGGAAGTGTGGAAAAGAGGGGCACCAGATGAAGGACTGCACCGAG
AGACAGGCCAACTTCCTGGGCAAGATCTGGCCTAGCCACAAGGGCAGACCCGGCAACTT
TCTGCAGAGCAGACCCGAGCCTACCGCCCCTCCTGAGGAAAGCTTCAGATTCGGCGAGG
AAACCACCACCCCCAGCCAGAAGCAGGAACCCATCGACAAAGAGCTGTACCCTCTGGCC
AGCCTGAGAAGCCTGTTCGGCAGCGACCCTAGCAGCCAG

3) Human GP100 (pGP100)

5’ATGGACCTGGTGCTGAAGAGATGCCTGCTGCACCTGGCCGTGATCGGCGCTCTGCTGG
CTGTGGGAGCTACCAAGGTGCCCAGAAACCAGGACTGGCTGGGCGTGTCCAGACAGCT
GAGAACAAAGGCCTGGAACAGGCAGCTGTACCCCGAGTGGACAGAGGCCCAGAGACTG
GACTGTTGGAGAGGCGGACAGGTGTCCCTGAAGGTGTCCAACGACGGCCCTACCCTGAT
CGGAGCCAACGCCAGCTTCTCTATCGCCCTGAACTTCCCCGGCAGCCAGAAGGTGCTGCC
TGACGGACAAGTGATCTGGGTCAACAACACCATCATCAACGGCTCCCAAGTGTGGGGCG
GACAGCCAGTGTATCCTCAGGAAACCGACGACGCCTGCATCTTCCCTGATGGCGGCCCTT
GTCCTAGCGGCAGCTGGTCCCAGAAAAGATCCTTCGTGTACGTGTGGAAAACCTGGGGA
CAGTACTGGCAGGTGCTGGGCGGACCTGTGTCTGGCCTGTCTATCGGCACAGGCAGAGC
CATGCTGGGCACCCACACCATGGAAGTGACCGTGTACCACAGAAGAGGCAGCAGATCCT
ACGTGCCCCTGGCCCACTCTAGCAGCGCCTTCACCATCATGGACCAGGTGCCCTTCAGCG
TGTCCGTGTCCCAGCTGAGAGCACTGGACGGCGGCAACAAGCACTTCCTGAGAAACCAG
CCCCTGACATTCGCCCTGCAGCTGCACGACCCTAGCGGCTATCTGGCCGAGGCCGACCT
GAGCTACACATGGGACTTCGGCGACAGCAGCGGCACCCTGATCTCTAGAGCCCTGGTCG
TGACCCACACCTACCTGGAACCTGGCCCTGTGACAGCCCAGGTGGTGCTGCAGGCTGCC
ATCCCTCTGACAAGCTGTGGCAGCAGCCCTGTGCCTGGCACAACAGACGGCCACAGACC
TACAGCCGAGGCCCCTAACACAACCGCTGGACAGGTGCCAACCACCGAGGTCGTGGGC
ACAACACCAGGCCAGGCTCCTACAGCTGAGCCAAGCGGCACAACCAGCGTGCAGGTGC
CCACAACCGAAGTGATCAGCACCGCCCCTGTGCAGATGCCTACCGCCGAGAGCACAGGC
ATGACCCCTGAAAAGGTGCCCGTGTCTGAAGTGATGGGCACCACCCTGGCCGAGATGAG
CACACCTGAGGCCACCGGCATGACACCAGCCGAGGTGTCAATCGTGGTGCTGAGCGGA
ACAACAGCCGCCCAAGTGACCACCACCGAGTGGGTGGAAACCACCGCCAGAGAGCTGC
CCATCCCTGAGCCTGAGGGCCCTGACGCCAGCAGCATCATGAGCACCGAGAGCATCACA
GGCAGCCTGGGCCCTCTGCTGGATGGCACAGCTACCCTGAGACTCGTGAAGCGCCAGGT
GCCACTGGACTGCGTGCTGTACAGATACGGCAGCTTCTCCGTGACCCTGGACATCGTGC
AGGGCATCGAGTCCGCCGAGATTCTGCAGGCAGTGCCTAGCGGAGAGGGCGACGCTTT
TGAGCTGACCGTGTCTTGTCAGGGCGGCCTGCCTAAAGAGGCCTGCATGGAAATCAGCA

GCCCCGGCTGTCAGCCCCCTGCTCAGAGACTGTGTCAGCCCGTGCTGCCTAGCCCTGCCT
GTCAGCTGGTGCTGCATCAGATCCTGAAGGGCGGCTCCGGCACCTACTGCCTGAATGTG
TCTCTGGCCGACACCAACAGTCTGGCCGTGGTGTCTACCCAGCTGATCATGCCCGGCCAG
GAAGCTGGACTGGGACAGGTGCCACTGATCGTGGGCATCCTGCTGGTGCTGATGGCTGT
GGTGCTGGCCTCCCTGATCTACAGGCGGAGACTGATGAAGCAGGACTTCTCTGTGCCCC
AGCTGCCTCACAGCAGCAGCCACTGGCTGAGACTGCCCAGAATCTTCTGCTCCTGCCCCA
TCGGCGAGAACAGCCCACTGCTGTCTGGCCAGCAAGTCTGA

4) Vesicular stomatitis Indiana virus glycoprotein G (pVSVG)

ATGAAGTGCCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAACTGCAAGTTTACCATC
GTGTTCCCCCACAACCAGAAGGGCAACTGGAAGAACGTGCCCAGCAACTACCACTACTG
CCCCAGCAGCAGCGACCTGAACTGGCACAACGACCTGATCGGCACCGCCATCCAAGTGA
AGATGCCCAAGAGCCACAAGGCCATCCAGGCCGATGGCTGGATGTGCCACGCCAGCAA
ATGGGTCACCACCTGTGACTTCAGGTGGTACGGCCCCAAGTACATCACCCAGAGCATCA
GATCCTTCACCCCCAGCGTGGAACAGTGCAAAGAGAGCATCGAGCAGACCAAGCAGGG
CACCTGGCTGAACCCCGGATTCCCACCTCAGAGCTGTGGCTACGCCACCGTGACAGATG
CCGAGGCCGTGATCGTGCAAGTGACCCCTCACCACGTGCTGGTGGACGAGTACACAGGC
GAGTGGGTGGACAGCCAGTTCATCAACGGCAAGTGCTCCAACTACATCTGCCCCACCGT
GCACAACAGCACCACCTGGCACAGCGACTACAAAGTGAAAGGCCTGTGCGACAGCAAC
CTGATCAGCATGGACATCACATTCTTCAGCGAGGACGGCGAGCTGAGCAGCCTGGGCAA
AGAGGGCACAGGCTTCAGAAGCAACTACTTCGCCTACGAGACAGGCGGCAAGGCCTGC
AAGATGCAGTATTGCAAGCACTGGGGCGTGCGGCTGCCTAGCGGAGTGTGGTTCGAGA
TGGCCGACAAGGACCTGTTCGCCGCTGCCAGATTCCCCGAGTGTCCTGAGGGCAGCAGC
ATCTCTGCCCCTAGCCAGACAAGCGTGGACGTGTCCCTGATCCAGGACGTGGAAAGAAT
CCTGGACTACAGCCTGTGTCAGGAAACCTGGTCCAAGATCAGAGCCGGCCTGCCCATCA
GCCCTGTGGACCTGTCTTACCTGGCCCCCAAGAACCCAGGCACAGGCCCTGCCTTCACCA
TCATCAATGGCACCCTGAAGTACTTTGAGACACGGTACATCCGGGTGGACATTGCCGCCC
CTATCCTGAGCAGAATGGTGGGAATGATCAGCGGCACCACCACCGAGCGCGAGCTGTG
GGATGATTGGGCCCCTTACGAGGATGTGGAAATCGGCCCCAACGGCGTGCTGAGAACC
AGCAGCGGCTACAAGTTCCCCCTGTACATGATCGGCCACGGCATGCTGGACTCCGACCT
GCACCTGTCTAGCAAGGCCCAGGTGTTCGAGCACCCCCACATCCAGGATGCCGCCAGCC
AGCTGCCTGACGACGAGTCTCTGTTCTTCGGCGACACCGGCCTGAGCAAGAACCCCATC
GAGCTGGTGGAAGGCTGGTTCAGCAGCTGGAAGTCCTCTATCGCCAGCTTCTTCTTCATC
ATCGGGCTGATTATCGGCCTGTTCCTGGTGCTGAGAGTGGGCATCCACCTGTGCATCAA

GCTGAAGCACACCAAGAAGAGGCAGATCTACACCGACATCGAGATGAACCGGCTGGGC
AAATGA

5) VSV-G with insertion of SIINFEKL surrounded by restriction sites (pTOP-OVA_MHCI)

ATGAAGTGCCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAACTGCAAGTTTACCATC
GTGTTCCCCCACAACCAGAAGGGCAACTGGAAGAACGTGCCCAGCAACTACCACTACTG
CCCCAGCAGCAGCGACCTGAACTGGCACAACGACCTGATCGGCACCGCCATCCAAGTGA
AGATGCCCAAGAGCCACAAGGCCATCCAGGCCGATGGCTGGATGTGCCACGCCAGCAA
ATGGGTCACCACCTGTGACTTCAGGTGGTACGGCCCCAAGTACATCACCCAGAGCATCA
GATCCTTCACCCCCAGCGTGGAACAGTGCAAAGAGAGCATCGAGCAGACCAAGCAGGG
CACCTGGCTGAACCCCGGATTCCCACCTCAGAGCTGTGGCTACGCCACCGTGACAGATG
CCGAGGCCGTGATCGTGCAAGTGACCCCTCACCACGTGCTGGTGGACGAGTACACAGGC
GAGTGGGTGGACAGCCAGTTCATCAACGGCAAGTGCTCCAACTACATCTGCCCCACCGT
GCACAACAGCACCACCTGGCACAGCGACTACAAAGTGAAAACTAGT**AGCATCATCAACT**
TCGAGAAGCTGGAATTCGGCCTGTGCGACAGCAACCTGATCAGCATGGACATCACATTC
TTCAGCGAGGACGGCGAGCTGAGCAGCCTGGGCAAAGAGGGCACAGGCTTCAGAAGC
AACTACTTCGCCTACGAGACAGGCGGCAAGGCCTGCAAGATGCAGTATTGCAAGCACTG
GGGCGTGCGGCTGCCTAGCGGAGTGTGGTTCGAGATGGCCGACAAGGACCTGTTCGCC
GCTGCCAGATTCCCCGAGTGTCCTGAGGGCAGCAGCATCTCTGCCCCTAGCCAGACAAG
CGTGGACGTGTCCCTGATCCAGGACGTGGAAAGAATCCTGGACTACAGCCTGTGTCAGG
AAACCTGGTCCAAGATCAGAGCCGGCCTGCCCATCAGCCCTGTGGACCTGTCTTACCTGG
CCCCCAAGAACCCAGGCACAGGCCCTGCCTTCACCATCATCAATGGCACCCTGAAGTACT
TTGAGACACGGTACATCCGGGTGGACATTGCCGCCCCTATCCTGAGCAGAATGGTGGGA
ATGATCAGCGGCACCACCACCGAGCGCGAGCTGTGGGATGATTGGGCCCCTTACGAGG
ATGTGGAAATCGGCCCCAACGGCGTGCTGAGAACCAGCAGCGGCTACAAGTTCCCCCTG
TACATGATCGGCCACGGCATGCTGGACTCCGACCTGCACCTGTCTAGCAAGGCCCAGGT
GTTCGAGCACCCCCACATCCAGGATGCCGCCAGCCAGCTGCCTGACGACGAGTCTCTGTT
CTTCGGCGACACCGGCCTGAGCAAGAACCCCATCGAGCTGGTGGAAGGCTGGTTCAGCA
GCTGGAAGTCCTCTATCGCCAGCTTCTTCTTCATCATCGGGCTGATTATCGGCCTGTTCCT
GGTGCTGAGAGTGGGCATCCACCTGTGCATCAAGCTGAAGCACACCAAGAAGAGGCAG
ATCTACACCGACATCGAGATGAACCGGCTGGGCAAATGA

Oligonucleotides to obtain pTOP-OVA_MHCII

CTAGT**ATCAGCCAGGCCGTGCACGCTGCCCACGCCGAGATCAATGAGGCCGGCAGA**G

A**TAGTCGGTCCGGCACGTGCGACGGGTGCGGCTCTAGTTACTCCGGCCGTCT**CTTAA

References

1 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. *CA Cancer J Clin* **66**, 7-30, (2016).

2 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70, (2000).

3 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, (2011).

4 Couzin-Frankel, J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* **342**, 1432-1433, (2013).

5 McCarthy, E. F. The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas. *The Iowa Orthopaedic Journal* **26**, 154-158, (2006).

6 Ehrlich, P. Über den jetzigen Stand der Chemotherapie. *Berichte der deutschen chemischen Gesellschaft* **42**, 17-47, (1909).

7 Van Pel, A. & Boon, T. Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. *Proc Natl Acad Sci U S A* **79**, 4718-4722, (1982).

8 Boon, T. & Kellermann, O. Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. *Proc Natl Acad Sci U S A* **74**, 272-275, (1977).

9 Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer* **14**, 135-146, (2014).

10 Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P. & Van Pel, A. Tumor antigens recognized by T lymphocytes. *Annu Rev Immunol* **12**, 337-365, (1994).

11 Shankaran, V. *et al.* IFN[gamma] and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107-1111, (2001).

12 Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998, (2002).

13 Mittal, D., Gubin, M. M., Schreiber, R. D. & Smyth, M. J. New insights into cancer immunoediting and its three component phases — elimination, equilibrium and escape. *Current opinion in immunology* **27**, 16-25, (2014).

14 Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **331**, 1565-1570, (2011).

15 Chen, D. S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* **39**, 1-10, (2013).
16 Beatty, G. L. & Gladney, W. L. Immune escape mechanisms as a guide for cancer immunotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **21**, 687-692, (2015).
17 van der Burg, S. H., Arens, R., Ossendorp, F., van Hall, T. & Melief, C. J. M. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer* **16**, 219-233, (2016).
18 Cichorek, M., Wachulska, M., Stasiewicz, A. & Tymińska, A. Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii I Alergologii* **30**, 30-41, (2013).
19 Scott, A. M., Allison, J. P. & Wolchok, J. D. Monoclonal antibodies in cancer therapy. *Cancer Immunity* **12**, 14, (2012).
20 Kaufman, H. L., Kohlhapp, F. J. & Zloza, A. Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev Drug Discov* **14**, 642-662, (2015).
21 Lee, S. & Margolin, K. Cytokines in Cancer Immunotherapy. *Cancers* **3**, 3856-3893, (2011).
22 Wu, R. *et al.* Adoptive T-cell Therapy Using Autologous Tumor-infiltrating Lymphocytes for Metastatic Melanoma: Current Status and Future Outlook. *Cancer Journal (Sudbury, Mass.)* **18**, 160-175, (2012).
23 Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* **12**, 252-264, (2012).
24 Wolchok, J. D. & Saenger, Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist* **13 Suppl 4**, 2-9, (2008).
25 Wolchok, J. D. PD-1 Blockers. *Cell* **162**, 937, (2015).
26 Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *New England Journal of Medicine* **373**, 23-34, (2015).
27 Johnson, D. B., Puzanov, I. & Kelley, M. C. Talimogene laherparepvec (T-VEC) for the treatment of advanced melanoma. *Immunotherapy* **7**, 611-619, (2015).
28 Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L. & Kroemer, G. Immunogenic cell death in cancer and infectious disease. *Nat Rev Immunol* **17**, 97-111, (2017).
29 Kroemer, G., Galluzzi, L., Kepp, O. & Zitvogel, L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* **31**, 51-72, (2013).
30 Woller, N., Gürlevik, E., Ureche, C.-I., Schumacher, A. & Kühnel, F. Oncolytic Viruses as Anticancer Vaccines. *Frontiers in Oncology* **4**, 188, (2014).

31 Calvet, C. Y., Famin, D., Andre, F. M. & Mir, L. M. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. *Oncoimmunology* **3**, e28131, (2014).

32 Kamensek, U., Kos, S. & Sersa, G. Adjuvant Immunotherapy as a Tool to Boost Effectiveness of Electrochemotherapy. *Handbook of Electroporation*, 1-16, (2016).

33 Yeh, S. *et al.* Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology* **116**, 981-989 e981, (2009).

34 Blanchard, T., Srivastava, P. K. & Duan, F. Vaccines against advanced melanoma. *Clinics in Dermatology* **31**, 179-190, (2013).

35 Maio, M. Melanoma as a model tumour for immuno-oncology. *Annals of Oncology* **23**, viii10-viii14, (2012).

36 Medzhitov, R. & Janeway, C. A. Decoding the Patterns of Self and Nonself by the Innate Immune System. *Science* **296**, 298, (2002).

37 Braciale, T. J. & Hahn, Y. S. Immunity to viruses. *Immunological reviews* **255**, 10.1111/imr.12109, (2013).

38 Clem, A. S. Fundamentals of Vaccine Immunology. *Journal of Global Infectious Diseases* **3**, 73-78, (2011).

39 Moser, M. & Leo, O. Key concepts in immunology. *Vaccine* **28 Suppl 3**, C2-13, (2010).

40 Vigneron, N. Human Tumor Antigens and Cancer Immunotherapy. *BioMed Research International* **2015**, 17, (2015).

41 Buonaguro, L., Petrizzo, A., Tornesello, M. L. & Buonaguro, F. M. Translating Tumor Antigens into Cancer Vaccines. *Clinical and Vaccine Immunology : CVI* **18**, 23-34, (2011).

42 van der Bruggen, P. *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643-1647, (1991).

43 Wolfel, T., Hauer, M., Schneider, J. & Serrano, M. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* **269**, 1281, (1995).

44 Coulie, P. G. *et al.* A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proceedings of the National Academy of Sciences* **92**, 7976-7980, (1995).

45 Lu, Y. C. & Robbins, P. F. Cancer immunotherapy targeting neoantigens. *Semin Immunol* **28**, 22-27, (2016).

46 Editors, N. B. The problem with neoantigen prediction. *Nat Biotech* **35**, 97-97, (2017).

47 Bobisse, S., Foukas, P. G., Coukos, G. & Harari, A. Neoantigen-based cancer immunotherapy. *Annals of Translational Medicine* **4**, 262, (2016).

48 Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74, (2015).

49 Quaglino, P. *et al.* Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study. *Ann Oncol* **21**, 409-414, (2010).

50 Gyorki, D. E., Callahan, M., Wolchok, J. D. & Ariyan, C. E. The delicate balance of melanoma immunotherapy. *Clin Trans Immunol* **2**, e5, (2013).

51 Vigneron, N., Ma, W., Michaux, A. & Van den Eynde, B. J. in *Antigen Processing: Methods and Protocols* (ed Peter van Endert) 187-207 (Humana Press, 2013).

52 Viatte, S., Alves, P. M. & Romero, P. Reverse immunology approach for the identification of CD8 T-cell-defined antigens: Advantages and hurdles. *Immunol Cell Biol* **84**, 318-330, (2006).

53 Backert, L. & Kohlbacher, O. Immunoinformatics and epitope prediction in the age of genomic medicine. *Genome Medicine* **7**, 119, (2015).

54 Weide, B. *et al.* Results of the First Phase I/II Clinical Vaccination Trial With Direct Injection of mRNA. *Journal of Immunotherapy* **31**, 180-188, (2008).

55 Srivatsan, S. *et al.* Allogeneic tumor cell vaccines: The promise and limitations in clinical trials. *Human Vaccines & Immunotherapeutics* **10**, 52-63, (2014).

56 Grullich, C., McGoldrick, S., Zeiser, R. & Finke, J. The death receptor pathway is not involved in alloreactive T-cell induced mitochondrial membrane permeability. *Leuk Lymphoma* **46**, 1207-1216, (2005).

57 Neefjes, J., Jongsma, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* **11**, 823-836, (2011).

58 Palucka, K. & Banchereau, J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* **12**, 265-277, (2012).

59 Campbell, D. J. & Butcher, E. C. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med* **195**, 135-141, (2002).

60 Keenan, B. P. & Jaffee, E. M. Whole Cell Vaccines — Past Progress and Future Strategies. *Seminars in Oncology* **39**, 276-286, (2012).

61 Srinivasan, R. & Wolchok, J. D. Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines. *Journal of Translational Medicine* **2**, 12-12, (2004).

62 Melief, C. J. M. & van der Burg, S. H. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* **8**, 351-360, (2008).

63 Khazaie, K., Bonertz, A. & Beckhove, P. Current developments with peptide-based human tumor vaccines. *Curr Opin Oncol* **21**, 524-530, (2009).

64 Collins, S. A. *et al.* Viral vectors in cancer immunotherapy: which vector for which strategy? *Curr Gene Ther* **8**, 66-78, (2008).

65 Weide, B., Garbe, C., Rammensee, H. G. & Pascolo, S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunol Lett* **115**, 33-42, (2008).

66 Senovilla, L. *et al.* Trial watch: DNA vaccines for cancer therapy. *Oncoimmunology* **2**, e23803, (2013).

67 Sahin, U., Kariko, K. & Tureci, O. mRNA-based therapeutics [mdash] developing a new class of drugs. *Nat Rev Drug Discov* **13**, 759-780, (2014).

68 Vandermeulen, G., Marie, C., Scherman, D. & Préat, V. New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials. *Molecular Therapy* **19**, 1942-1949, (2011).

69 Glenting, J. & Wessels, S. Ensuring safety of DNA vaccines. *Microb Cell Fact* **4**, 26, (2005).

70 Gurunathan, S., Klinman, D. M. & Seder, R. A. DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol* **18**, 927-974, (2000).

71 Vandermeulen, G. *et al.* The site of administration influences both the type and the magnitude of the immune response induced by DNA vaccine electroporation. *Vaccine* **33**, 3179-3185, (2015).

72 Calvet, C. Y., André, F. M. & Mir, L. M. Dual therapeutic benefit of electroporation-mediated DNA vaccination in vivo: Enhanced gene transfer and adjuvant activity. *Oncoimmunology* **3**, e28540, (2014).

73 Mann, J. F. *et al.* Enhanced immunogenicity of an HIV-1 DNA vaccine delivered with electroporation via combined intramuscular and intradermal routes. *J Virol* **88**, 6959-6969, (2014).

74 Enama, M. E. *et al.* Phase I Randomized Clinical Trial of VRC DNA and rAd5 HIV-1 Vaccine Delivery by Intramuscular (IM), Subcutaneous (SC) and Intradermal (ID) Administration (VRC 011). *PLoS ONE* **9**, e91366, (2014).

75 Kutzler, M. A. & Weiner, D. B. DNA vaccines: ready for prime time? *Nat Rev Genet* **9**, 776-788, (2008).

76 Donnelly, J. J., Wahren, B. & Liu, M. A. DNA vaccines: progress and challenges. *J Immunol* **175**, 633-639, (2005).

77 Grunwald, T. & Ulbert, S. Improvement of DNA vaccination by adjuvants and sophisticated delivery devices: vaccine-platforms for the battle against infectious diseases. *Clinical and Experimental Vaccine Research* **4**, 1-10, (2015).

78 Faurez, F., Dory, D., Le Moigne, V., Gravier, R. & Jestin, A. Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection. *Vaccine* **28**, 3888-3895, (2010).

79 Ledwith, B. J. *et al.* Plasmid DNA vaccines: assay for integration into host genomic DNA. *Dev Biol (Basel)* **104**, 33-43, (2000).
80 Roos, A. K. *et al.* Skin electroporation: effects on transgene expression, DNA persistence and local tissue environment. *Plos One* **4**, e7226, (2009).
81 Cattamanchi, A. *et al.* Phase I study of a herpes simplex virus type 2 (HSV-2) DNA vaccine administered to healthy, HSV-2-seronegative adults by a needle-free injection system. *Clin Vaccine Immunol* **15**, 1638-1643, (2008).
82 Mehendale, S. *et al.* Safety and immunogenicity of DNA and MVA HIV-1 subtype C vaccine prime-boost regimens: a phase I randomised Trial in HIV-uninfected Indian volunteers. *Plos One* **8**, e55831, (2013).
83 Vardas, E. *et al.* Indicators of therapeutic effect in FIT-06, a Phase II trial of a DNA vaccine, GTU((R))-Multi-HIVB, in untreated HIV-1 infected subjects. *Vaccine* **30**, 4046-4054, (2012).
84 Grunwald, T. & Ulbert, S. Improvement of DNA vaccination by adjuvants and sophisticated delivery devices: vaccine-platforms for the battle against infectious diseases. *Clin Exp Vaccine Res* **4**, 1-10, (2015).
85 Amanna, I. J. & Slifka, M. K. Current Trends in West Nile Virus Vaccine Development. *Expert review of vaccines* **13**, 589-608, (2014).
86 Ferraro, B. *et al.* Clinical Applications of DNA Vaccines: Current Progress. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* **53**, 296-302, (2011).
87 MacGregor, R. R. *et al.* First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* **178**, 92-100, (1998).
88 Cui, Z. DNA vaccine. *Adv Genet* **54**, 257-289, (2005).
89 Mestas, J. & Hughes, C. C. W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology* **172**, 2731-2738, (2004).
90 Bauer, S. *et al.* Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proceedings of the National Academy of Sciences* **98**, 9237-9242, (2001).
91 Dale, C. J. *et al.* Prime-boost strategies in DNA vaccines. *Methods Mol Med* **127**, 171-197, (2006).
92 Saade, F. & Petrovsky, N. Technologies for enhanced efficacy of DNA vaccines. *Expert Rev Vaccines* **11**, 189-209, (2012).
93 Welch, M., Villalobos, A., Gustafsson, C. & Minshull, J. You're one in a googol: optimizing genes for protein expression. *Journal of the Royal Society Interface* **6**, S467-S476, (2009).
94 Angov, E. Codon usage: Nature's roadmap to expression and folding of proteins. *Biotechnology Journal* **6**, 650-659, (2011).
95 Kotsopoulou, E., Kim, V. N., Kingsman, A. J., Kingsman, S. M. & Mitrophanous, K. A. A Rev-Independent Human Immunodeficiency Virus

Type 1 (HIV-1)-Based Vector That Exploits a Codon-Optimized HIV-1 gag-pol Gene. *Journal of Virology* **74**, 4839-4852, (2000).

96 Li, L., Saade, F. & Petrovsky, N. The future of human DNA vaccines. *Journal of biotechnology* **162**, 171-182, (2012).

97 Saade, F. & Petrovsky, N. Technologies for enhanced efficacy of DNA vaccines. *Expert Review of Vaccines* **11**, 189-209, (2012).

98 Papadakis, E. D., Nicklin, S. A., Baker, A. H. & White, S. J. Promoters and control elements: designing expression cassettes for gene therapy. *Curr Gene Ther* **4**, 89-113, (2004).

99 Vandermeulen, G. *et al.* Skin-specific promoters for genetic immunisation by DNA electroporation. *Vaccine* **27**, 4272-4277, (2009).

100 Danda, R. *et al.* Targeted expression of suicide gene by tissue-specific promoter and microRNA regulation for cancer gene therapy. *PLoS One* **8**, e83398, (2013).

101 Kos, S. *et al.* Improved Specificity of Gene Electrotransfer to Skin Using pDNA Under the Control of Collagen Tissue-Specific Promoter. *J Membr Biol*, (2015).

102 Bode, C., Zhao, G., Steinhagen, F., Kinjo, T. & Klinman, D. M. CpG DNA as a vaccine adjuvant. *Expert review of vaccines* **10**, 499-511, (2011).

103 Gil, A. *et al.* DNA vaccine prime followed by boost with live attenuated virus significantly improves antigen-specific T cell responses against human cytomegalovirus. *Hum Vacc Immunother* **9**, 2120-2132, (2013).

104 Li, L. & Petrovsky, N. Molecular mechanisms for enhanced DNA vaccine immunogenicity. *Expert review of vaccines* **15**, 313-329, (2016).

105 Calvet, C. Y., Andre, F. M. & Mir, L. M. Dual therapeutic benefit of electroporation-mediated DNA vaccination in vivo: Enhanced gene transfer and adjuvant activity. *Oncoimmunology* **3**, e28540, (2014).

106 Flingai, S. *et al.* Synthetic DNA vaccines: improved vaccine potency by electroporation and co-delivered genetic adjuvants. *Front Immunol* **4**, 354, (2013).

107 Lee, S. H., Danishmalik, S. N. & Sin, J. I. DNA vaccines, electroporation and their applications in cancer treatment. *Hum Vaccin Immunother* **11**, 1889-1900, (2015).

108 Kaestner, L., Scholz, A. & Lipp, P. Conceptual and technical aspects of transfection and gene delivery. *Bioorg Med Chem Lett* **25**, 1171-1176, (2015).

109 Mali, B., Jarm, T., Snoj, M., Sersa, G. & Miklavcic, D. Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. *Eur J Surg Oncol* **39**, 4-16, (2013).

110 Yarmush, M. L., Golberg, A., Sersa, G., Kotnik, T. & Miklavcic, D. Electroporation-based technologies for medicine: principles, applications, and challenges. *Annu Rev Biomed Eng* **16**, 295-320, (2014).

111 Denet, A. R., Vanbever, R. & Preat, V. Skin electroporation for transdermal and topical delivery. *Adv Drug Deliv Rev* **56**, 659-674, (2004).

112 Bennett, W. F., Sapay, N. & Tieleman, D. P. Atomistic simulations of pore formation and closure in lipid bilayers. *Biophys J* **106**, 210-219, (2014).

113 Rols, M. P. Electropermeabilization, a physical method for the delivery of therapeutic molecules into cells. *Biochim Biophys Acta* **1758**, 423-428, (2006).

114 Teissie, J., Golzio, M. & Rols, M. P. Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. *Biochim Biophys Acta* **1724**, 270-280, (2005).

115 Gothelf, A. & Gehl, J. What you always needed to know about electroporation based DNA vaccines. *Hum Vacc Immunother* **8**, 1694-1702, (2012).

116 Marszalek, P., Liu, D. S. & Tsong, T. Y. Schwan equation and transmembrane potential induced by alternating electric field. *Biophys J* **58**, 1053-1058, (1990).

117 Kotnik, T., Pucihar, G. & Miklavcic, D. Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport. *J Membr Biol* **236**, 3-13, (2010).

118 Kotnik, T. & Pucihar, G. in *Advanced Electroporation Techniques in Biology and Medicine* (eds A. G. Pakhomov, D. Miklavcic, & M. S. Markov) (2010).

119 Kotnik, T. *et al.* Electroporation-based applications in biotechnology. *Trends Biotechnol* **33**, 480-488, (2015).

120 Miklavcic, D. *et al.* The importance of electric field distribution for effective in vivo electroporation of tissues. *Biophys J* **74**, 2152-2158, (1998).

121 Hirao, L. A. *et al.* Intradermal/subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques. *Vaccine* **26**, 440-448, (2008).

122 Wells, D. J. Gene therapy progress and prospects: Electroporation and other physical methods. *Gene Ther* **11**, 1363-1369, (2004).

123 Bolhassani, A., Khavari, A. & Orafa, Z. in *Nanotechnology in Drug Delivery* (ed Ali Demir Sezer) (2014).

124 Gehl, J. Gene electrotransfer in clinical trials. *Methods Mol Biol* **1121**, 241-246, (2014).

125 Heller, L. C. & Heller, R. In vivo electroporation for gene therapy. *Hum Gene Ther* **17**, 890-897, (2006).

126 Kobiyama, K. *et al.* Innate Immune Signaling by, and Genetic Adjuvants for DNA Vaccination. *Vaccines* **1**, 278, (2013).

127 Babiuk, S. *et al.* Increased gene expression and inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. *J Biotechnol* **110**, 1-10, (2004).

128 Chiarella, P. *et al.* Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration. *Expert Opin Biol Ther* **8**, 1645-1657, (2008).

129 Znidar, K., Bosnjak, M., Cemazar, M. & Heller, L. C. Cytosolic DNA Sensor Upregulation Accompanies DNA Electrotransfer in B16. F10 Melanoma Cells. *Molecular Therapy—Nucleic Acids* **5**, e322, (2016).

130 Weaver, J. C. Electroporation theory. Concepts and mechanisms. *Methods Mol Biol* **55**, 3-28, (1995).

131 Andreason, G. Electroporation as a technique for the transfer of macromolecules into mammalian cell lines. *Journal of Tissue Culture Methods* **15**, 56-62, (1993).

132 Staal, L. & Gilbert, R. in *Clinical Aspects of Electroporation* (eds Stephen T. Kee, Julie Gehl, & Edward W. Lee) Ch. 5, 45-65 (Springer New York, 2011).

133 Heller, R. & Heller, L. C. Gene electrotransfer clinical trials. *Adv Genet* **89**, 235-262, (2015).

134 El-Kamary, S. S. *et al.* Safety and Tolerability of the Easy Vax™ Clinical Epidermal Electroporation System in Healthy Adults. *Molecular Therapy* **20**, 214-220, (2012).

135 Eriksson, F., Tötterman, T., Maltais, A.-K., Pisa, P. & Yachnin, J. DNA vaccine coding for the rhesus prostate specific antigen delivered by intradermal electroporation in patients with relapsed prostate cancer. *Vaccine* **31**, 3843-3848, (2013).

136 Wallace, M. *et al.* Tolerability of Two Sequential Electroporation Treatments Using MedPulser DNA Delivery System (DDS) in Healthy Adults. *Molecular Therapy: the Journal of the American Society of Gene Therapy* **17**, 922-928, (2009).

137 Diehl, M. C. *et al.* Tolerability of intramuscular and intradermal delivery by CELLECTRA(®) adaptive constant current electroporation device in healthy volunteers. *Hum Vacc Immunother* **9**, 2246-2252, (2013).

138 Yang, B., Jeang, J., Yang, A., Wu, T. C. & Hung, C. F. DNA vaccine for cancer immunotherapy. *Hum Vaccin Immunother* **10**, 3153-3164, (2014).

139 Yuan, J. *et al.* Immunologic responses to xenogeneic tyrosinase DNA vaccine administered by electroporation in patients with malignant melanoma. *Journal for Immunotherapy of Cancer* **1**, 20-20, (2013).

140 Kim, T. J. *et al.* Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nature Communications* **5**, 5317, (2014).

141 Bagarazzi, M. L. *et al.* Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. *Sci Transl Med* **4**, 155ra138, (2012).

142 Trimble, C. L. *et al.* Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet*, (2015).

143 Gothelf, A. & Gehl, J. Gene electrotransfer to skin; review of existing literature and clinical perspectives. *Curr Gene Ther* **10**, 287-299, (2010).

144 Gothelf, A., Mahmood, F., Dagnaes-Hansen, F. & Gehl, J. Efficacy of transgene expression in porcine skin as a function of electrode choice. *Bioelectrochemistry* **82**, 95-102, (2011).

145 Maruyama, H. *et al.* Skin-targeted gene transfer using in vivo electroporation. *Gene Ther* **8**, 1808-1812, (2001).

146 Daugimont, L. *et al.* Hollow Microneedle Arrays for Intradermal Drug Delivery and DNA Electroporation. *J Membrane Biol* **236**, 117-125, (2010).

147 Sersa, G. *et al.* Electrochemotherapy in treatment of tumours. *Ejso-Eur J Surg Onc* **34**, 232-240, (2008).

148 Heller, R., Cruz, Y., Heller, L. C., Gilbert, R. A. & Jaroszeski, M. J. Electrically mediated delivery of plasmid DNA to the skin, using a multielectrode array. *Hum Gene Ther* **21**, 357-362, (2010).

149 Guo, S. Q. *et al.* Electro-gene transfer to skin using a noninvasive multielectrode array. *J Control Release* **151**, 256-262, (2011).

150 Drabick, J. J., Glasspool-Malone, J., Somiari, S., King, A. & Malone, R. W. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. *Molecular Therapy* **3**, 249-255, (2001).

151 Donate, A., Coppola, D., Cruz, Y. & Heller, R. Evaluation of a Novel Non-Penetrating Electrode for Use in DNA Vaccination. *PLoS One* **6**, (2011).

152 Pavselj, N. & Preat, V. DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse. *J Control Release* **106**, 407-415, (2005).

153 Roos, A. K., Eriksson, F., Walters, D. C., Pisa, P. & King, A. D. Optimization of Skin Electroporation in Mice to Increase Tolerability of DNA Vaccine Delivery to Patients. *Molecular Therapy* **17**, 1637-1642, (2009).

154 Andre, F. M. *et al.* Efficiency of High- and Low-Voltage Pulse Combinations for Gene Electrotransfer in Muscle, Liver, Tumor, and Skin. *Human Gene Therapy* **19**, 1261-1271, (2008).

155 Rabussay, D. *et al.* Enhancement of therapeutic drug and DNA delivery into cells by electroporation. *J Phys D Appl Phys* **36**, 348-363, (2003).

156 Rabussay, D. *et al.* Toward the development of electroporation for delivery of DNA vaccines to humans. *Molecular Therapy* **9**, S209-S209, (2004).

157 Groselj, A. *et al.* Coupling treatment planning with navigation system: a new technological approach in treatment of head and neck tumors by electrochemotherapy. *Biomed Eng Online* **14 Suppl 3**, S2, (2015).

158 Vasan, S. *et al.* In Vivo Electroporation Enhances the Immunogenicity of an HIV-1 DNA Vaccine Candidate in Healthy Volunteers. *Plos One* **6**, e19252, (2011).

159 Spanggaard, I. *et al.* Gene electrotransfer of plasmid antiangiogenic metargidin peptide (AMEP) in disseminated melanoma: safety and efficacy results of a phase I first-in-man study. *Hum Gene Ther Clin Dev* **24**, 99-107, (2013).

160 Awate, S., Babiuk, L. A. & Mutwiri, G. Mechanisms of Action of Adjuvants. *Frontiers in Immunology* **4**, 114, (2013).

161 Clark, R. & Kupper, T. Old meets new: the interaction between innate and adaptive immunity. *J Invest Dermatol* **125**, 629-637, (2005).

162 Bachmann, M. F. & Jennings, G. T. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* **10**, 787-796, (2010).

163 Silva, J. M., Videira, M., Gaspar, R., Preat, V. & Florindo, H. F. Immune system targeting by biodegradable nanoparticles for cancer vaccines. *J Control Release* **168**, 179-199, (2013).

164 Silva, J. M. *et al.* In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model. *J Control Release* **198**, 91-103, (2015).

165 Pashine, A., Valiante, N. M. & Ulmer, J. B. Targeting the innate immune response with improved vaccine adjuvants. *Nat Med* **11**, S63-68, (2005).

166 Desmet, C. J. & Ishii, K. J. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. *Nat Rev Immunol* **12**, 479-491, (2012).

167 Ohlschlager, P., Spies, E., Alvarez, G., Quetting, M. & Groettrup, M. The combination of TLR-9 adjuvantation and electroporation-mediated delivery enhances in vivo antitumor responses after vaccination with HPV-16 E7 encoding DNA. *Int J Cancer* **128**, 473-481, (2011).

168 Babiuk, S. *et al.* TLR9(−/−) and TLR9(+/+) mice display similar immune responses to a DNA vaccine. *Immunology* **113**, 114-120, (2004).

169 Babiuk, S. *et al.* TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. *Immunology* **113**, 114-120, (2004).

170 Spies, B. *et al.* Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J Immunol* **171**, 5908-5912, (2003).
171 Ishii, K. J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451**, 725-729, (2008).
172 Ishikawa, H., Ma, Z. & Barber, G. N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788-792, (2009).
173 Tudor, D. *et al.* Type I IFN modulates the immune response induced by DNA vaccination to pseudorabies virus glycoprotein C. *Virology* **286**, 197-205, (2001).
174 Zitvogel, L., Galluzzi, L., Kepp, O., Smyth, M. J. & Kroemer, G. Type I interferons in anticancer immunity. *Nat Rev Immunol* **15**, 405-414, (2015).
175 Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nature reviews. Immunology* **14**, 36-49, (2014).
176 Petrovsky, N. & Aguilar, J. C. Vaccine adjuvants: Current state and future trends. *Immunol Cell Biol* **82**, 488-496, (2004).
177 Pasquale, A. D., Preiss, S., Silva, F. T. & Garcon, N. Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines (Basel)* **3**, 320-343, (2015).
178 Marichal, T. *et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med* **17**, 996-1002, (2011).
179 McKee, A. S. *et al.* Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells. *Proc Natl Acad Sci U S A* **110**, E1122-1131, (2013).
180 Marrack, P., McKee, A. S. & Munks, M. W. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* **9**, 287-293, (2009).
181 Mbow, M. L., De Gregorio, E. & Ulmer, J. B. Alum's adjuvant action: grease is the word. *Nat Med* **17**, 415-416, (2011).
182 Wang, S. *et al.* Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum phosphate. *Vaccine* **18**, 1227-1235, (2000).
183 Ulmer, J. B. *et al.* Enhancement of DNA vaccine potency using conventional aluminum adjuvants. *Vaccine* **18**, 18-28, (1999).
184 Quirk, E. K. *et al.* Safety Profile of the Merck Human Immunodeficiency Virus-1 Clade B gag DNA Plasmid Vaccine With and Without Adjuvants. *Open Forum Infect Dis* **1**, (2014).
185 Cristillo, A. D. *et al.* Induction of mucosal and systemic antibody and T-cell responses following prime–boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J Gen Virol* **92**, 128-140, (2011).

186 Petrovsky, N. & Cooper, P. D. Advax™, a novel microcrystalline polysaccharide particle engineered from delta inulin, provides robust adjuvant potency together with tolerability and safety. *Vaccine*.

187 Ara, Y. *et al.* Zymosan enhances the immune response to DNA vaccine for human immunodeficiency virus type-1 through the activation of complement system. *Immunology* **103**, 98-105, (2001).

188 Khatri, K., Goyal, A. K., Gupta, P. N., Mishra, N. & Vyas, S. P. Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. *International Journal of Pharmaceutics* **354**, 235-241, (2008).

189 Xia, Y. *et al.* Chitosan-based mucosal adjuvants: Sunrise on the ocean. *Vaccine*.

190 Underhill, D. M. Macrophage recognition of zymosan particles. *J Endotoxin Res* **9**, 176-180, (2003).

191 Jimenez, G. S. *et al.* Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. *Hum Vaccin* **3**, 157-164, (2007).

192 Vilalta, A. *et al.* Analysis of biomarkers after intramuscular injection of Vaxfectin®-formulated hCMV gB plasmid DNA. *Vaccine* **27**, 7409-7417, (2009).

193 Smith, L. R. *et al.* Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. *Vaccine* **28**, 2565-2572, (2010).

194 Raviprakash, K. *et al.* A dengue DNA vaccine formulated with Vaxfectin(®) is well tolerated, and elicits strong neutralizing antibody responses to all four dengue serotypes in New Zealand white rabbits. *Human Vaccines & Immunotherapeutics* **8**, 1764-1768, (2012).

195 Scheerlinck, J. Y. Genetic adjuvants for DNA vaccines. *Vaccine* **19**, 2647-2656, (2001).

196 Okuda, K., Wada, Y. & Shimada, M. Recent Developments in Preclinical DNA Vaccination. *Vaccines* **2**, 89, (2014).

197 Kobiyama, K. *et al.* Innate Immune Signaling by, and Genetic Adjuvants for DNA Vaccination. *Vaccines (Basel)* **1**, 278-292, (2013).

198 Sokol, C. L. & Luster, A. D. The Chemokine System in Innate Immunity. *Cold Spring Harbor perspectives in biology* **7**, a016303, (2015).

199 Song, M. Y., Park, S. H., Nam, H. J., Choi, D. H. & Sung, Y. C. Enhancement of vaccine-induced primary and memory CD8(+) T-cell responses by soluble PD-1. *J Immunother* **34**, 297-306, (2011).

200 Lambricht, L. *et al.* Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opin Drug Deliv* **13**, 295-310, (2016).

201 Weiss, J. M., Subleski, J. J., Wigginton, J. M. & Wiltrout, R. H. Immunotherapy of cancer by IL-12-based cytokine combinations. *Expert Opin Biol Ther* **7**, 1705-1721, (2007).

202 Dinarello, C. A. Proinflammatory cytokines. *Chest* **118**, 503-508, (2000).
203 Larocca, C. & Schlom, J. Viral vector-based therapeutic cancer vaccines. *Cancer J* **17**, 359-371, (2011).
204 Jensen, S. & Thomsen, A. R. Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion. *J Virol* **86**, 2900-2910, (2012).
205 Perot, B. P., Ingersoll, M. A. & Albert, M. L. The impact of macroautophagy on CD8(+) T-cell-mediated antiviral immunity. *Immunol Rev* **255**, 40-56, (2013).
206 Draper, S. J. & Heeney, J. L. Viruses as vaccine vectors for infectious diseases and cancer. *Nat Rev Microbiol* **8**, 62-73, (2010).
207 Zhao, C., Ao, Z. & Yao, X. Current advances in virus-like particles as a vaccination approach against HIV infection. *Vaccines* **4**, 2, (2016).
208 Naskalska, A. & Pyrć, K. Virus like particles as immunogens and universal nanocarriers. *Polish Journal of Microbiology* **64**, 3-13, (2015).
209 Vandermeulen, G. *et al.* Highly potent delivery method of gp160 envelope vaccine combining lentivirus-like particles and DNA electrotransfer. *J Control Release* **159**, 376-383, (2012).
210 Ludwig, C. & Wagner, R. Virus-like particles-universal molecular toolboxes. *Curr Opin Biotechnol* **18**, 537-545, (2007).
211 Pitoiset, F., Vazquez, T. & Bellier, B. Enveloped virus-like particle platforms: vaccines of the future? *Expert Review of Vaccines* **14**, 913-915, (2015).
212 Marsac, D., Loirat, D., Petit, C., Schwartz, O. & Michel, M.-L. Enhanced presentation of major histocompatibility complex class I-restricted human immunodeficiency virus type 1 (HIV-1) Gag-specific epitopes after DNA immunization with vectors coding for vesicular stomatitis virus glycoprotein-pseudotyped HIV-1 Gag particles. *Journal of virology* **76**, 7544-7553, (2002).
213 Bellier, B. *et al.* DNA vaccines encoding retrovirus-based virus-like particles induce efficient immune responses without adjuvant. *Vaccine* **24**, 2643-2655, (2006).
214 Desjardins, D. *et al.* Recombinant retrovirus-like particle forming DNA vaccines in prime-boost immunization and their use for hepatitis C virus vaccine development. *J Gene Med* **11**, 313-325, (2009).
215 Huret, C. *et al.* Recombinant retrovirus-derived virus-like particle-based vaccines induce hepatitis C virus-specific cellular and neutralizing immune responses in mice. *Vaccine* **31**, 1540-1547, (2013).
216 Chang, M. O., Suzuki, T., Suzuki, H. & Takaku, H. HIV-1 Gag-virus-like particles induce natural killer cell immune responses via activation and maturation of dendritic cells. *J Innate Immun* **4**, 187-200, (2012).

217 Gentili, M. *et al.* Transmission of innate immune signaling by packaging of cGAMP in viral particles. *Science* **349**, 1232-1236, (2015).

218 Manel, N. *et al.* A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. *Nature* **467**, 214-217, (2010).

219 Pertel, T. *et al.* TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* **472**, 361-365, (2011).

220 Henrick, B. M., Yao, X. D., Rosenthal, K. L. & team, I. s. HIV-1 Structural Proteins Serve as PAMPs for TLR2 Heterodimers Significantly Increasing Infection and Innate Immune Activation. *Front Immunol* **6**, 426, (2015).

221 Schlehuber, L. D. & Rose, J. K. Prediction and identification of a permissive epitope insertion site in the vesicular stomatitis virus glycoprotein. *J Virol* **78**, 5079-5087, (2004).

222 Georgel, P. *et al.* Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway. *Virology* **362**, 304-313, (2007).

223 Errington, F. *et al.* Fusogenic membrane glycoprotein-mediated tumour cell fusion activates human dendritic cells for enhanced IL-12 production and T-cell priming. *Gene Ther* **13**, 138-149, (2005).

224 Mao, C. P. *et al.* Combined administration with DNA encoding vesicular stomatitis virus G protein enhances DNA vaccine potency. *J Virol* **84**, 2331-2339, (2010).

225 García-Valtanen, P. *et al.* Autophagy-inducing peptides from mammalian VSV and fish VHSV rhabdoviral G glycoproteins (G) as models for the development of new therapeutic molecules. *Autophagy* **10**, 1666-1680, (2014).

226 Linardakis, E. *et al.* Enhancing the efficacy of a weak allogeneic melanoma vaccine by viral fusogenic membrane glycoprotein-mediated tumor cell-tumor cell fusion. *Cancer Res* **62**, 5495-5504, (2002).

227 Bateman, A. R. *et al.* Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells. *Cancer Res* **62**, 6566-6578, (2002).

228 Dranoff, G. Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nat Rev Immunol* **12**, 61-66, (2012).

229 Braumuller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, 361-365, (2013).

230 Rosenberg, S. A. *et al.* Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* **86**, 1159-1166, (1994).

231 Castellino, F. & Germain, R. N. Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annu Rev Immunol* **24**, 519-540, (2006).

232 Babiuk, S. *et al.* Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine* **20**, 3399-3408, (2002).

233 Li, L., Saade, F. & Petrovsky, N. The future of human DNA vaccines. *J Biotechnol* **162**, 171-182, (2012).
234 Kelleher, A. D. *et al.* A randomized, placebo-controlled phase I trial of DNA prime, recombinant fowlpox virus boost prophylactic vaccine for HIV-1. *AIDS* **20**, 294-297, (2006).
235 Jin, X. *et al.* Multiple factors affect immunogenicity of DNA plasmid HIV vaccines in human clinical trials. *Vaccine* **33**, 2347-2353, (2015).
236 Kalams, S. A. *et al.* Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults. *PLoS One* **7**, e29231, (2012).
237 Gottlinger, H. HIV-1 Gag: a molecular machine driving viral particle assembly and release. *Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, LA-UR*, 02-2877, (2001).
238 Melchjorsen, J. Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity - clues for treatments and vaccines. *Viruses* **5**, 470-527, (2013).
239 Vandermeulen, G., Uyttenhove, C., De Plaen, E., Van den Eynde, B. J. & Preat, V. Intramuscular electroporation of a P1A-encoding plasmid vaccine delays P815 mastocytoma growth. *Bioelectrochemistry* **100**, 112-118, (2014).
240 Garinot, M. *et al.* PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. *J Control Release* **120**, 195-204, (2007).
241 De Geest, B. G. *et al.* Surface-engineered polyelectrolyte multilayer capsules: synthetic vaccines mimicking microbial structure and function. *Angew Chem Int Ed Engl* **51**, 3862-3866, (2012).
242 Rizza, P., Moretti, F., Capone, I. & Belardelli, F. Role of type I interferon in inducing a protective immune response: perspectives for clinical applications. *Cytokine Growth Factor Rev* **26**, 195-201, (2015).
243 O'Carroll, I. P. *et al.* Functional redundancy in HIV-1 viral particle assembly. *J Virol* **86**, 12991-12996, (2012).
244 Bachmann, M. F. & Jennings, G. T. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* **10**, 787-796, (2010).
245 Seya, T. *et al.* Adjuvant for vaccine immunotherapy of cancer - focusing on TLR2 and TLR3 agonists for safely enhancing antitumor immunity. *Cancer Sci*, (2015).
246 Kidd, P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* **8**, 223-246, (2003).
247 Paul, S. & Piontkivska, H. Frequent associations between CTL and T-Helper epitopes in HIV-1 genomes and implications for multi-epitope vaccine designs. *BMC Microbiol* **10**, 212, (2010).

248 Ledford, H. Cocktails for cancer with a measure of immunotherapy. *Nature* **532**, 162-164, (2016).
249 Xue, W. *et al.* SCIB2, an antibody DNA vaccine encoding NY-ESO-1 epitopes, induces potent antitumor immunity which is further enhanced by checkpoint blockade. *Oncoimmunology* **5**, e1169353, (2016).
250 Knutson, K. L. & Disis, M. L. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. *Cancer Immunol Immunother* **54**, 721-728, (2005).
251 Ivanova, E. A. & Orekhov, A. N. T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview. *BioMed Research International* **2015**, 327470, (2015).
252 Liu, M. DNA vaccines: a review. *Journal of internal medicine* **253**, 402-410, (2003).
253 Coban, C. *et al.* Novel strategies to improve DNA vaccine immunogenicity. *Curr Gene Ther* **11**, 479-484, (2011).
254 Lambricht, L. *et al.* Coadministration of a Plasmid Encoding HIV-1 Gag Enhances the Efficacy of Cancer DNA Vaccines. *Mol Ther*, (2016).
255 Vyas, J. M., Van der Veen, A. G. & Ploegh, H. L. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* **8**, 607-618, (2008).
256 Melief, C. J., van Hall, T., Arens, R., Ossendorp, F. & van der Burg, S. H. Therapeutic cancer vaccines. *J Clin Invest* **125**, 3401-3412, (2015).
257 Bateman, A. *et al.* Fusogenic membrane glycoproteins as a novel class of genes for the local and immune-mediated control of tumor growth. *Cancer Res* **60**, 1492-1497, (2000).
258 Mangeot, P. E. *et al.* Protein transfer into human cells by VSV-G-induced nanovesicles. *Mol Ther* **19**, 1656-1666, (2011).
259 Gannage, M. & Munz, C. MHC presentation via autophagy and how viruses escape from it. *Semin Immunopathol* **32**, 373-381, (2010).
260 Prather, K. J., Sagar, S., Murphy, J. & Chartrain, M. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. *Enzyme and microbial technology* **33**, 865-883, (2003).
261 Burkhart, C. *et al.* Characterization of T-helper epitopes of the glycoprotein of vesicular stomatitis virus. *Journal of virology* **68**, 1573-1580, (1994).
262 Fredericksen, B. L. & Whitt, M. A. Vesicular stomatitis virus glycoprotein mutations that affect membrane fusion activity and abolish virus infectivity. *J Virol* **69**, 1435-1443, (1995).
263 Sun, X., Belouzard, S. & Whittaker, G. R. Molecular architecture of the bipartite fusion loops of vesicular stomatitis virus glycoprotein G, a class III

viral fusion protein. *Journal of Biological Chemistry* **283**, 6418-6427, (2008).

264 Perez, S. A. *et al.* A new era in anticancer peptide vaccines. *Cancer* **116**, 2071-2080, (2010).

265 Walsh, S. R. *et al.* Vaccination With Heterologous HIV-1 Envelope Sequences and Heterologous Adenovirus Vectors Increases T-Cell Responses to Conserved Regions: HVTN 083. *The Journal of Infectious Diseases* **213**, 541-550, (2016).

266 Sette, A. & Fikes, J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Current Opinion in Immunology* **15**, 461-470, (2003).

267 Melief, C. J. M., van Hall, T., Arens, R., Ossendorp, F. & van der Burg, S. H. Therapeutic cancer vaccines. *The Journal of Clinical Investigation* **125**, 3401-3412, (2015).

268 Farber, D. L., Yudanin, N. A. & Restifo, N. P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* **14**, 24-35, (2014).

269 Sallusto, F., Geginat, J. & Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* **22**, 745-763, (2004).

270 Lanzavecchia, A. & Sallusto, F. Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol* **17**, 326-332, (2005).

271 Saxena, M., Van, T. T. H., Baird, F. J., Coloe, P. J. & Smooker, P. M. Pre-existing immunity against vaccine vectors – friend or foe? *Microbiology* **159**, 1-11, (2013).

272 Emerman, M. & Malim, M. H. HIV-1 Regulatory/Accessory Genes: Keys to Unraveling Viral and Host Cell Biology. *Science* **280**, 1880-1884, (1998).

273 Wen, Y. & Collier, J. H. Supramolecular peptide vaccines: tuning adaptive immunity. *Current opinion in immunology* **35**, 73-79, (2015).

274 Castle, J. C. *et al.* Exploiting the mutanome for tumor vaccination. *Cancer Res* **72**, 1081-1091, (2012).

275 Kreiter, S. *et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696, (2015).

276 Vormehr, M. *et al.* Mutanome directed cancer immunotherapy. *Current Opinion in Immunology* **39**, 14-22, (2016).

277 Palucka, K. & Banchereau, J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* **39**, 38-48, (2013).

278 Davis, M. M. Immunology Taught by Humans. *Science translational medicine* **4**, 117fs112-117fs112, (2012).

279 Gubin, M. M. *et al.* Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* **515**, 577-581, (2014).

280 Alizadeh, D. & Larmonier, N. Chemotherapeutic targeting of cancer-induced immunosuppressive cells. *Cancer Res* **74**, 2663-2668, (2014).

281 Fu, J. *et al.* Preclinical evidence that PD1 blockade cooperates with cancer vaccine TEGVAX to elicit regression of established tumors. *Cancer Res* **74**, 4042-4052, (2014).

Curriculum Vitae

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1. Education

PhD – Biomedical and pharmaceutical Sciences
UCL, 2013-2017

Master's Degree – Chemistry and Bioindustries, Option in biomolecular and cellular engineering
UCL, 2011-2013

Bachelor's Degree – Biosciences Engineering, Option in chemistry
UCL, 2008-2011

2. Experience

Research Fellow – F.R.S-FNRS
PhD thesis in Biomedical and pharmaceutical sciences
Vaccination, Immunotherapy, Molecular biology, Drug Delivery, Biomaterials
2013-2017

Quality assistant – Chimay Fromages
Internship and student job as a quality assistant in a cheese factory
Microbiological testing, BRC audit preparation, quality management
July 2010 , 2011 and 2012

3. Certifications

Science & Medicine for laboratory animals, UCL, 2013

Nutrition and Nutritherapy, CERDEN, 2016-2018

4. Prices and distinction

Research Fellow (renewal), FNRS Grants, 2013-2017

Lauréate “Ma thèse en 180 secondes”, UCL finale, 2015

Selection for the Erasmus UE mobility program, Wageningen University, 2012

5. Publications

Lambricht L, Vanvarenberg K, De Beuckelaer A, Van Hoecke L, Grooten J, Ucakar B, Lipnik P, Sanders NN, Lienenklaus S, Préat V, Vandermeulen G. C*oadministration of a Plasmid Encoding HIV-1 Gag Enhances the Efficacy ofCancer DNA Vaccines.* Mol Ther. 2016 Sep;24(9):1686-96. doi: 10.1038/mt.2016.122. Epub 2016 Jun 20

Lambricht L, Lopes A, Kos S, Sersa G, Préat V, Vandermeulen G. *Clinical potential of electroporation for gene therapy and DNA vaccine delivery.*Expert Opin Drug Deliv. 2016;13(2):295-310. doi: 10.1517/17425247.2016.1121990. Epub 2015 Dec 19.

Lambricht L, Peres C, Florindo H, Preat V, Vandermeulen G, *Polymer-Based Nanoparticles as Modern Vaccine Delivery Systems,* Micro- and Nanotechnology in Vaccine Development. Elsevier. 2017; Chapter 10: 185-98

Vandermeulen G, Vanvarenberg K, De Beuckelaer A, De Koker S, **Lambricht L**, Uyttenhove C, Reschner A, Vanderplasschen A, Grooten J, Préat V. *The site of administration influences both the type and the magnitude of the immune response induced by DNA vaccine electroporation.* Vaccine. 2015 Jun 22;33(28):3179-85. doi: 10.1016/j.vaccine.2015.05.005. Epub 2015 May 14.

Lambricht L, De Berdt P, Vanacker J, Leprince J, Diogenes A, Goldansaz H, Bouzin C, Préat V, Dupont-Gillain C, des Rieux A. *The type and composition of alginate and hyaluronic-based hydrogels influence the viability of stem cells of the apical papilla.* Dent Mater. 2014 Dec;30(12):e349-61. doi: 10.1016/j.dental.2014.08.369. Epub 2014 Aug 31.

Publication in preparation:
Lambricht L, De Beuckelaer Ans, Vanvarenberg K, Ucakar B, Grooten J, Preat V, Vandermeulen G. *Cancer DNA vaccine coding for a modified VSV-G is a potent inducer of antigen-specific T-cell responses.* (2017)

Patent deposit:
Vandermeulen G., **Lambricht L.**, Préat V. *Modified VSV-G and vaccines thereof*. Patent Application: EP16188736.9, filed Sep. 14, 2016, Patent Pending

6. Conference contributions

Co-administration of a plasmid encoding HIV-1 Gag enhances the efficacy of cancer DNA vaccines. Meeting of the Belgian-Dutch Biopharmaceutical Society. Oral presentation. Nov. 2015, Leuven

Co-delivery of a Gag encoding plasmid enhances the Th1 immune response generated by DNA vaccine. ESGCT Congress. Poster presentation. Sep. 2015, Helsinki

Construire de faux virus pour vaincre le cancer. Ma thèse en 180 secondes. Oral presentations. Feb. and May 2015, Louvain-La-Neuve and Namur

Nanoparticular delivery systems of cancer vaccines. 3rd NanoFar autumn school. Poster presentation. Oct. 2014, Brussels

Nanoparticular delivery systems of cancer vaccines. 3rd Nanodrug International Scientific Meeting. Oral presentation. Jul. 2014, Portugal

Hydrogel properties influence dental stem cell viability in vivo and in vitro. Meeting of the Belgian-Dutch Biopharmaceutical Society. Poster presentation. Dec. 2013, Ghent