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From molecular optimization to therapy combination: novel strategies to improve cancer DNA vaccines

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FOREWORD

Cancer is one of the leading causes of death worldwide. According to the International Agency for Research on Cancer, in 2018, there were 18 million cases of cancer with 10 million deaths; by 2040, the number of cancer cases is projected to be 30 million cases, with 16.5 million deaths (1).

The insufficient global survival benefits, the poor quality of life and the impossibility to cure all types of cancer using conventional therapies (chemotherapy, radiotherapy and surgery) left space to other therapies, such as immunotherapy, which tries to fight cancer by stimulating the patient's immune system against tumor cells (2-5). The excitement aroused by the potential of immunotherapy inspired the development of therapeutic cancer vaccines. In several clinical trials, the ability of vaccines to induce a specific and long-lasting immune response against cancer cells has been demonstrated (4). These exciting results led to the approval of the first vaccine against prostate cancer, Sipuleucel-T. However, the low benefit/cost ratio and the difficulty of the manufacturing process prevented this product from becoming a standard of care in cancer treatment (6). Further studies explored other more cost-effective and versatile vaccine platforms, such as DNA vaccines.

Cancer DNA vaccines are plasmids encoding tumor antigens, delivered to mount a specific immune response against tumor cells (7). Beside the low costs and the easiness associated to the manufacturing process, DNA vaccines showed a good safety profile and the ability to stimulate the innate and the adaptive arms of the immune system. Nevertheless, in the first clinical trials, they demonstrated a poor immunogenicity (8), especially due to the limited DNA uptake into the cells and the central and peripheral tolerance towards the encoded antigen(s) (9). The use of delivery techniques that increase the cellular uptake of DNA vaccines and the optimized design of the vector backbone, through the insertion of a strong promoter, polyadenylation (polyA) tail etc., already improved the efficacy of prophylactic vaccination in preclinical models (10). However, their use in therapeutic vaccination was far from the reality, due to the highly immunosuppressive tumor microenvironment.

Today, the better knowledge of the tumor immunology and the mechanisms that cancer uses to escape the immune system open new possibilities to enhance the effectiveness of DNA vaccines (4, 8). New strategies are needed to potentiate the immunogenicity of DNA vaccines and to overcome the immune suppressive tumor microenvironment and the central tolerance, which prevent the activation of a strong immune response (4).

My PhD thesis aims at overcoming the main limitations of cancer DNA vaccination, thus improving its efficacy. The strategies that will be used are the modification of the DNA vaccine from molecular codon optimization to rational antigen design, and its combination with strategies having a complementary mechanism of action. During this project, optimized DNA vaccines and specific combinations will be tested in three different tumor models (P815 mastocytoma, B16F1 melanoma and GL261 glioblastoma) to broadly validate our findings.

This work will expand the knowledge of therapeutic DNA vaccination against cancer, providing new therapeutic strategies for different tumor types and opens the possibility towards a more "personalized" approach to cancer. It also leads the way for a translation into the clinic, hoping that, in the future, DNA vaccines can become part of the standard of care for cancer patients.

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ABBREVIATIONS

ATC	Adoptive T cell
APCs	Antigen presenting cells
BBB	Blood brain barrier
CAR	Chimeric antigen receptor
CMV	Cytomegalovirus
CNS	Central nervous system
CO	Codon optimization
CTAs	Cancer/testis antigens
CTLs	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte Associated Protein 4
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
EP	Electroporation
FDA	Food and drug administration
GAAs	Glioma-associated antigens
GBM	Glioblastoma
GET	Gene electrotransfer
ICB	Immune checkpoint blockers
ICD	Immunogenic cell death
ID	Intradermal
IDO	Indoleamine-2,3-dioxygenase
ILs	Interleukins
IM	Intramuscular
IP	Intraperitoneal
IT	Intratumoral
MAGE	Melanoma-associated antigen
MDSCs	Myeloid-derived suppressor cells
MST	Median survival time
OAd	Oncolytic adenovirus
OVs	Oncolytic viruses
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein-ligand 1
PSA	Prostate-specific antigen
SC	Subcutaneous
TAs	Tumor antigens
TAAs	Tumor-associated antigens
TAMs	Tumor-associated macrophages
Th	T-helper lymphocytes
TILs	Tumor-infiltrated lymphocytes
TLRs	Toll-like receptors
TMB	Tumor mutation burden
TME	Tumor microenvironment
Tregs	Regulatory T cells
VSV-G	Vesicular stomatitis virus glycoprotein

CHAPTER I.
INTRODUCTION

Adapted from:

1. **Lopes A**.*, Lambricht L.*, Kos S., Sersa G., Préat V., Vandermeulen G. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opinion on Drug Delivery. 2016;13(2):295-310. doi: 10.1517/17425247.2016.1121990. (2016)*
2. **Lopes A**., Vandermeulen G., Préat V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. *Journal of Experimental and Clinical Cancer Research. 2019 Apr 5;38(1):146. doi: 10.1186/s13046-019-1154-7. (2019)*

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I. CANCER IMMUNOEDITING AND IMMUNOTHERAPY

Since decades, cancer therapy has been mainly focused on surgery, chemotherapy, radiotherapy, and endocrine therapy. However, these strategies frequently reach a refractory period leading to treatment failure and disease recurrence (1, 2). Furthermore, they are not adapted for all cancer types and the presence of metastasis limits the efficacy of certain treatments, such as surgical therapy (1). One new option is the immunotherapy, which enhances the patient's own immune system to attack the tumor. Indeed, once cancer is initiated, it can progress as a result of tumor cells escaping from the immune system (3). This concept is called "cancer immunoediting", which describes the evolution of cancer and the role of the immune system during the 3 phases of cancer development: (a) elimination, (b) equilibrium and (c) escape (4) (Figure 1).

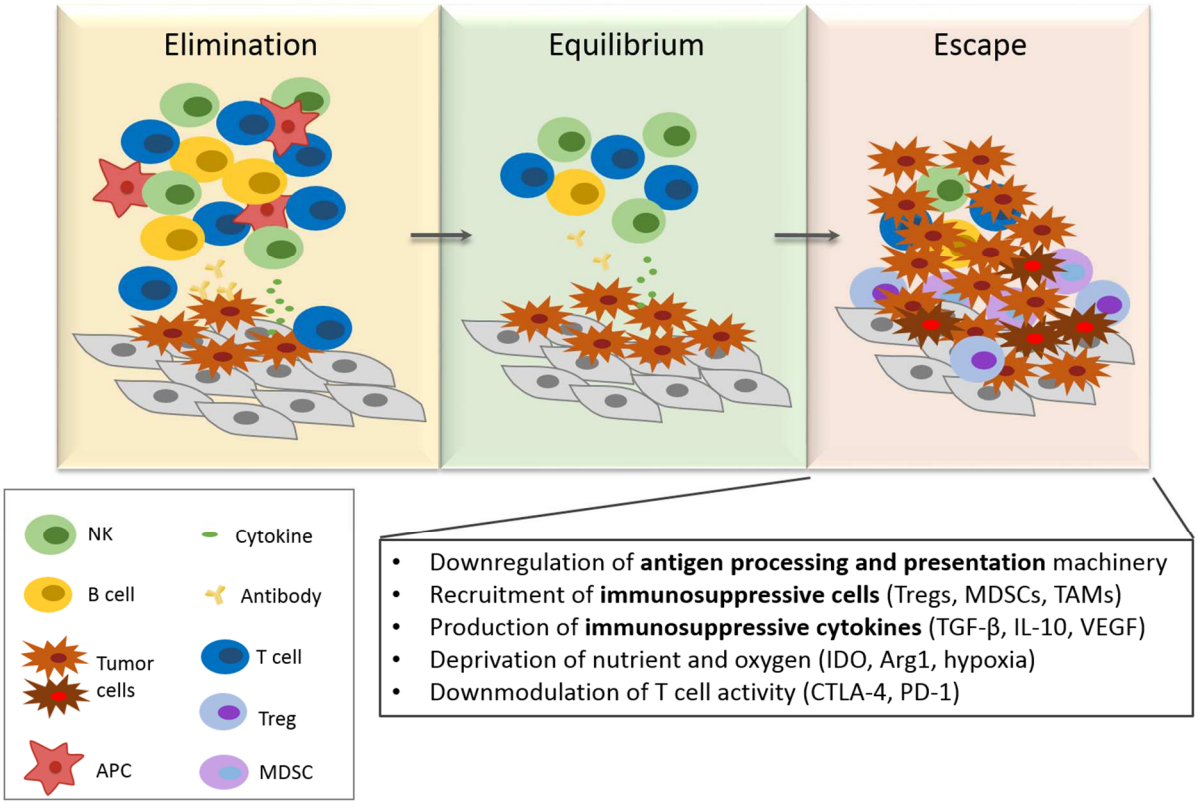

Figure 1: The immunoediting process. Tumor evolution is characterized by 3 different phases: Elimination, where the immune system recognizes and fights cancer cells; Equilibrium between the tumor and the immune cells, where tumor cells are in a dormant state; Escape of the cancer cells from the immune control, because of different modifications in the TME. Adapted from (5, 6).

During the *elimination,* both the innate and adaptive immune systems detect and destroy early tumors before they become clinically visible. This phase is characterized by an increased expression of tumor antigens (TAs), MHC class I, Fas and TRAIL receptor on tumor cells and perforin, granzymes, IFN-a/b/g, Interleukin (IL)-1, IL-12, TNFa in the tumor microenvironment (TME)

(7). In the *equilibrium* phase, the tumor is in a state of immune-mediated dormancy and there is a balance between anti-tumor (IL-12, IFNg) and tumor promoting cytokines (IL-10, TGFb). Tumor cell variants evolve that resist immune recognition (antigen loss or defects in antigen-presentation) and induce immunosuppression (7). The adaptive immunity is responsible for maintaining the occult tumor cells in equilibrium. In the *escape* phase, tumor cells that can circumvent immune recognition and destruction become invisible to the immune system and emerge as progressively growing, visible tumors (8). Different mechanisms induce tumor cell escape: downregulation of antigen processing and presentation machinery, recruitment of immunosuppressive cells (regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs)), production of immunosuppressive cytokines (TGFb, IL-10, VEGF), deprivation of nutrient and oxygen (Indoleamine-2, 3-dioxygenase (IDO), Arg1, hypoxia), down modulation of T cell activity (cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1)) (7).

The observations of the role of the immune system in tumoral processes inspired the development of cancer immunotherapies. They include: (i) *Strategies to enhance the T cell activity*: immune checkpoint blockers (ICB), which release "brakes" that keep T cells from killing cancer cells; adoptive T cell (ATC) therapy, which boosts the natural ability of T cells taken from patients' tumor to fight cancer; cytokines, such as IL-2, IL-12, which play important roles in the immune system's ability to respond to cancer; co-stimulatory receptor agonists, antibodies that target receptors on T cells, such as OX-40 or 4-1BB, to stimulate T cell activity (9). (ii) *Strategies that recognize and attack cancer cells*: targeted therapy using monoclonal antibodies, also known as therapeutic antibodies, which recognize and attach specific targets found on cancer cells, e.g., HER-2, CD19, CD20, BiTE antibodies etc. (10, 11); oncolytic viruses (OVs), which specifically infect and kill cancer cells. (iii) *Strategies to eliminate the immunosuppressive TME*: depletion of immunosuppressive cells, such as Tregs, MDSC, through antibodies against CCR4 and CSF1, which are expressed on the surface of Tregs and MDSC, respectively or through ICB (9); suppression of IL-10, TGFb, arginase, IDO etc., produced by immunosuppressive cells. (iv) *Strategies to enhance cancer cell recognition*: cancer vaccines, which boost the immune system's response against TAs, proteins overexpressed or uniquely expressed by cancer cells (12, 13); OVs, which release TAs following the killing of the cancer cells. Figure 2 shows the main different types of cancer immunotherapies and the targets they have in the context of antigen presentation and in the immunosuppressive TME.

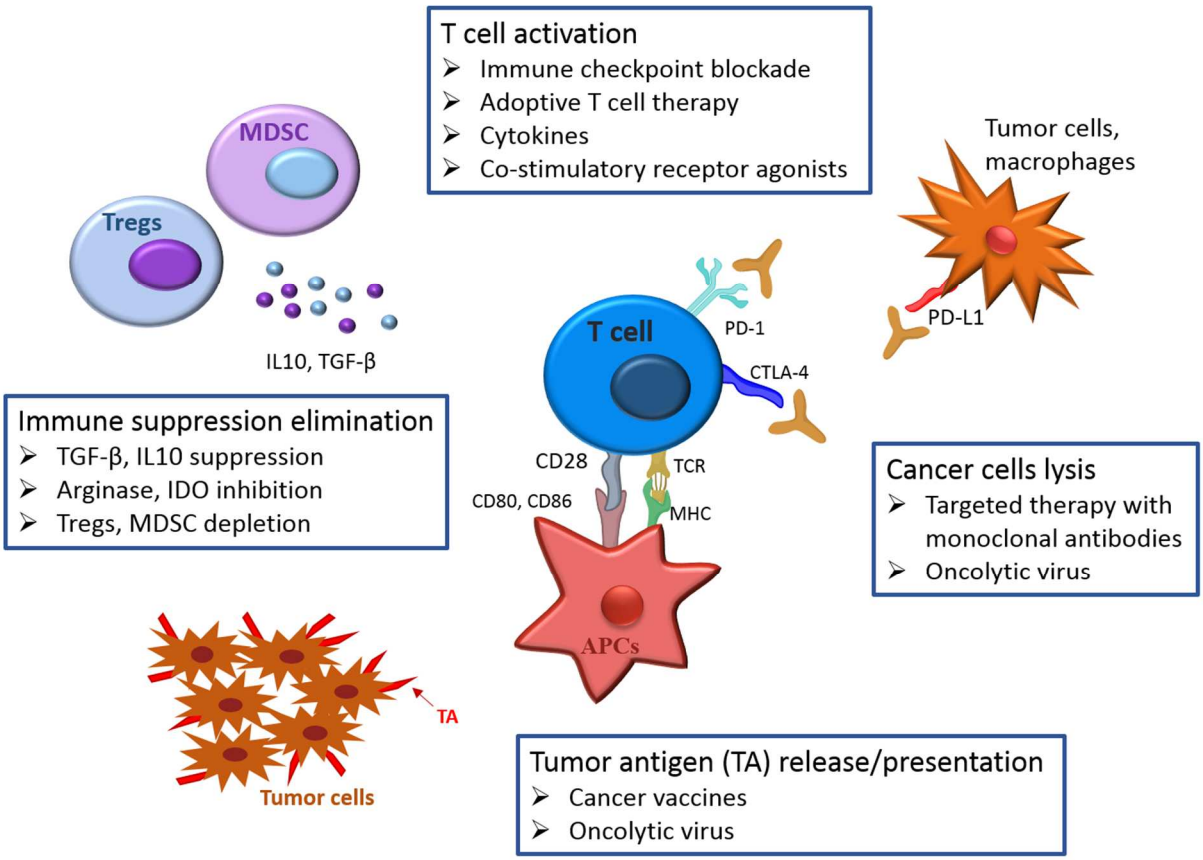

Figure 2: The main types of cancer immunotherapies and respective targets. Adapted from (14).

The different immunotherapies play a role in several steps of the "cancer-immunity cycle". This cycle describes the different stepwise events from the antigen release from dying cancer cells to tumor killing by T cells that lead to an antitumor immune response. The cycle and the impact of the different cancer immunotherapies (15) are illustrated in Figure 3.

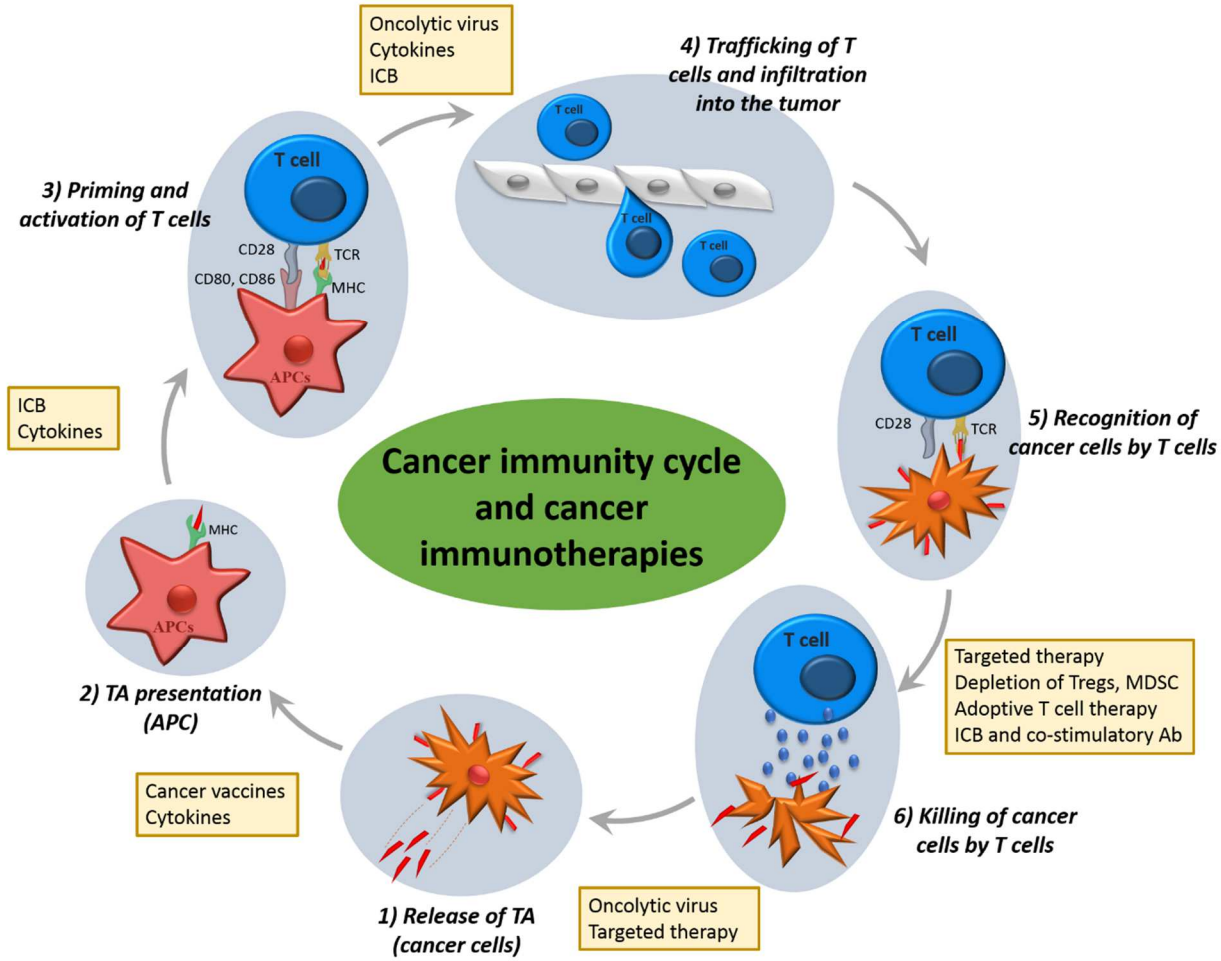

Figure 3: The cancer immunity cycle and the impact of cancer immunotherapy in the different steps. Adapted from (16).

II. THERAPEUTIC CANCER DNA VACCINES

Among the different strategies in immunotherapy, therapeutic cancer vaccines represent a specific, safe and well-tolerated therapy, able to induce a long-lasting immune response because of the establishment of the immunological memory (17). Cancer vaccines deliver TAs able to elicit an immune response to arrest cancer progression and prevent it from recurring (18, 19). They play a role in the first step of the cancer immunity cycle, as they strengthen patient's own immune response against TAs (20). Different types of cancer vaccines have been developed: cell-based vaccines, such as dendritic cell vaccines (e.g., Sipuleucel-T) (21) or whole tumor cell vaccines, protein/peptide vaccines (22, 23), viral/bacterial-based vaccines and gene-based vaccines (24, 25), including RNA and DNA vaccines (26) (Figure 4).

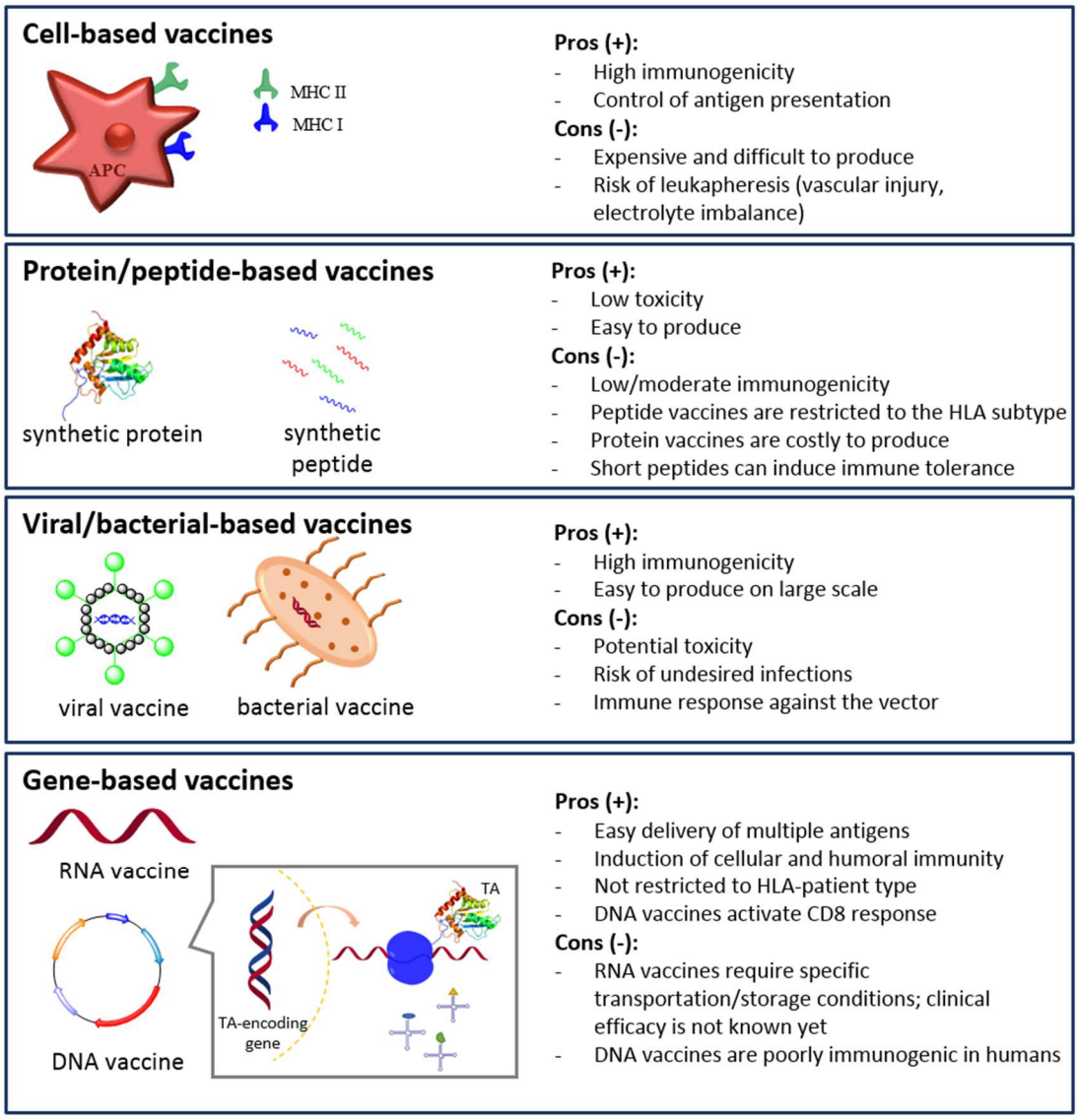

Figure 4: Pro and cons of the different types of cancer vaccines.

In particular, DNA vaccine is one of the most cost-effective cancer vaccine and offers different advantages compared to the others, as discussed in section II.5. DNA-mediated immunization began in the '90s, when a plasmid DNA encoding the influenza A nucleoprotein led to a protective and specific CTL response (27). In the same years, it was demonstrated the ability of mammalian cells to express gene encoded on plasmid DNA after transfection; it was also demonstrated that intramuscular (IM) injection of an antigenic DNA can lead to long-term gene expression and to a broad immune response against the encoded antigen (28, 29). Furthermore, the development of DNA vaccines was accelerated by the discovery of melanoma antigens encoded by normal genes, but activated only in melanoma cells or other tumor cells (30). The deeper understandings about the mechanism of action of DNA vaccines and their applicability in the field of cancer led to their rapid development to treat different tumor types in animals and humans.

II.1. STRUCTURE AND DESIGN OF CANCER DNA VACCINES

Cancer DNA vaccines consist in engineered DNA plasmids encoding one or several recombinant antigens, and sometimes adjuvants, that are delivered in vivo to solicit the immunity against TA-bearing tumor cells (31). To guarantee both optimal production in bacteria and strong expression in eukaryotic cells, the plasmid backbone requires: (i) an origin of replication (ORI), which allows their replication in bacteria; (ii) a selection sequence, to ensure stable inheritance of plasmids during bacterial growth (e.g., antibiotic resistance gene); (iii) a promoter, for optimal expression of the encoded genes in mammalian cells; (iv) a polyA sequence to stabilize the mRNA transcripts (32, 33). To be a vaccine, the plasmid should also encode the antigen gene(s) and, optionally, adjuvant gene(s) (Figure 5).

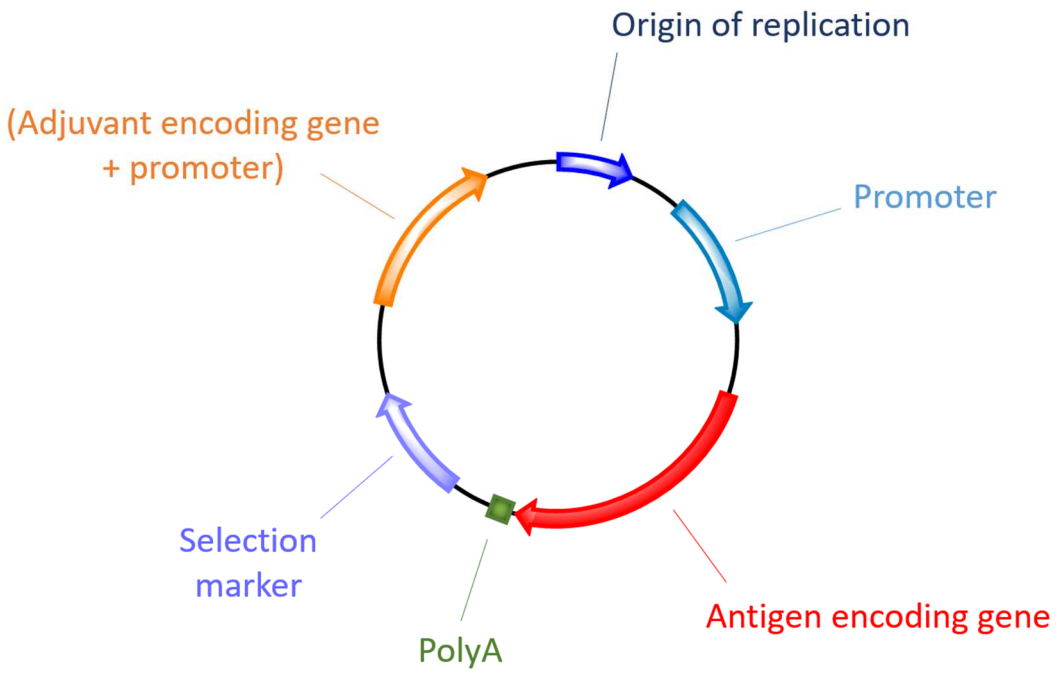

Figure 5: The DNA vaccine structure.

To ensure a good vaccine efficacy, plasmid design is a critical step. It should ensure a high antigen expression to promote its presentation by antigen-presenting cells (APCs) and the activation of the adaptive immunity. To increase antigen expression, many strategies have been adopted while designing the plasmid. For example, viral promoters, which are ubiquitously active at high level, have been widely employed to drive transgene expression. Among them, cytomegalovirus (CMV) promoter drives higher constitutive expression levels than alternative viral promoters (e.g., SV40) (34). Another strategy to increase the antigen expression is the insertion of a Kozak sequence upstream of the antigen gene, which has been demonstrated to facilitate the initiation of the translation process (35). Indeed, this sequence decreases the rate of mRNA scanning by the ribosome, thus improving the chance to recognize the start codon (35). Other critical features in plasmid design that increase antigen expression have been reviewed in (34, 36, 37).

Plasmid design can also play a role in increasing the innate immune system activation. It is already known that the dsDNA is recognized as a pathogen-associated molecular pattern (PAMP) by the cells and activates the innate immunity (38). Indeed, DNA contains immunostimulatory motifs, which are recognized by sensors inside the cells, such as CpG motifs, which stimulate Toll-like receptor 9 (TLR-9) (39, 40). Other motifs in the DNA structure have been demonstrated to activate different arms of the immune system (41). Hence, the plasmid could become more immunogenic, by inserting more immunostimulatory motifs. Several studies demonstrated that insertion of CpG

motifs in a plasmid could strengthen the induced immune response against different type of pathologies, e.g., hepatitis B, tuberculosis, anthrax etc (42-45). In other studies, CpG oligonucleotides were administered in combination with cancer vaccines leading to a slowdown in the tumor growth (reviewed in (46, 47)).

A promising strategy to increase both antigen expression and innate immune activation is the use of codon optimization (CO). This is a gene engineering approach that use synonymous codon changes to increase protein production, exploiting the genome degeneracy (48). It has been widely used to permit the expression of a protein in a heterologous system, i.e. the expression of a protein from a specific organism in another organism of a different species (49, 50).

Finally, a very important element of DNA vaccines is the choice of the right antigen, as discussed in section III. Figure 6 summarizes the discussed strategies in plasmid design and their effects in DNA vaccine immunogenicity.

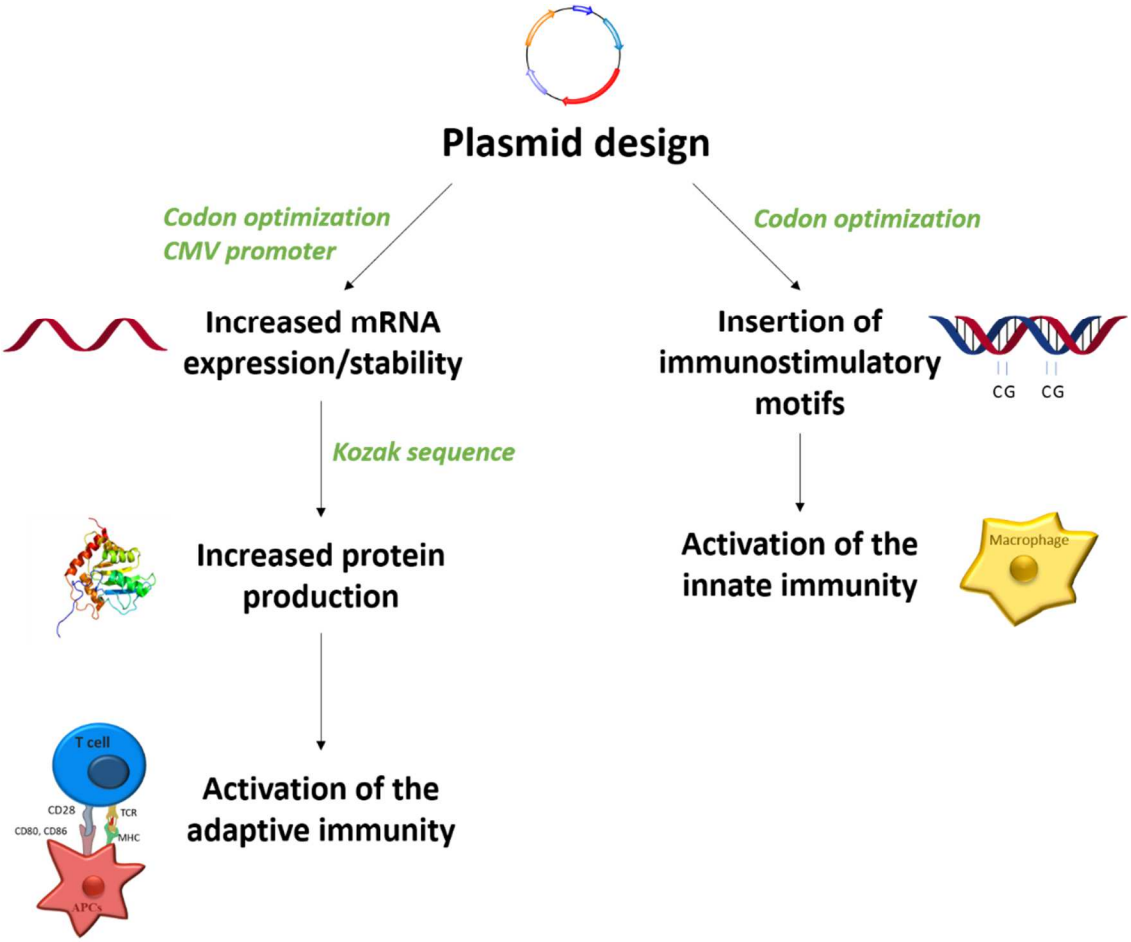

Figure 6: Strategies of DNA vaccine design and their effects in the immune activation.

II.2. ROUTES OF ADMINISTRATION AND DELIVERY METHODS FOR CANCER DNA VACCINES

DNA vaccines need to be delivered into the cells to be expressed by the host cellular machinery. Two key parameters should be considered to ensure an efficient cell transfection and antigen expression: the route of administration and the delivery method (51).

In preclinical and clinical studies, several routes of administration have been tested, including intramuscular (IM), intradermal (ID), intratumoral (IT), and subcutaneous (SC) (52). An analysis of the last 10-years clinical trials and 5-years preclinical studies showed that IM and ID administration are the most commonly used routes of administration for naked (non-formulated) DNA vaccines. Indeed, muscles are a protein factory as myocytes can produce extensive quantities of antigens for months (53). Delivery to muscle allows a long duration in gene expression and a delivery of a large amount of liquid; however, muscle injection can be painful. 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 APCs (54, 55). Hence, the site of administration has an impact on the immune activation (51), but also on the dose of vaccine injected, which typically varies from some μg (in mice) to mg (in humans) (56, 57).

The delivery method is the second important parameter for an efficient DNA transfection into the cells, which also influences the dose and the immune activation. Indeed, DNA plasmids must enter host cell nuclei to be transcribed into mRNA. Hence, a simple needle injection, which deposits the DNA in intercellular spaces, rather than within cells, does not to ensure an efficient DNA transfection and translation. The early failure of DNA vaccines to elicit strong responses in humans was largely due to the lack of an efficient delivery method (58). Improved delivery technologies, based on non-viral delivery, have been used to facilitate transport of DNA into the cells, resulting in much better immunogenicity in both clinical and preclinical studies. Despite the efficacy of viral vector delivery, the pre-existing immunity or the induction of an immune response against the viral vector limits its use for DNA delivery until now (59).

DNA non-viral delivery methods are usually classified in two categories: the chemical and the physical methods (60). Several interesting reviews have been published related to the different delivery strategies for DNA vaccination and their influence on the immune activation (52, 60-64). Briefly, chemical delivery approaches use biopharmaceuticals to increase DNA vaccine transfection efficiency (52), such as lipidic or polymeric nanoparticles (60). These systems will both protect nucleic acids and help them to cross the numerous barriers to reach the cell nucleus (65). Furthermore, nanoparticles can be functionalized to increase cellular uptake and to induce a sustained release of the encapsulated DNA vaccine (66). In some cases, the activation of the

immune system due to the delivery vector is described (67, 68). However, in clinical and preclinical studies, physical methods demonstrated a better transfection efficacy compared to the others and, thus, they are the most used delivery techniques. In particular, in oncological studies, the most used techniques include electroporation (EP) (69), DNA tatooing (70, 71) and gene gun (72), which are all able to overcome the extra- and intra-cellular barriers to a fast DNA transport into the nucleus. Indeed, as DNA is a negatively charged macromolecule, it will not easily pass through the membrane of cells. In such methods, DNA enters into the cells using electric pulses, mechanical or biolistic force (73, 74). Specifically, gene gun involves bombarding the skin with plasmid-coated gold particles by employing ballistic devices. Despite the high potency in relation the low dose of DNA required, this technique is not widely employed, due to its high cost and the poor induction of a Th1 immune response, which is the response required against cancer (60, 75, 76). DNA tattooing is a promising technique, as DNA does not need to be formulated, the cost of the device is low and the delivery into the skin induces the activation of the immune response. However, the transfection efficiency is not higher compared to simple IM DNA injection (61). Further studies need to be conducted to better elucidate its potential in the context of DNA delivery.

Until now, the delivery by EP of naked DNA vaccines via the IM or ID routes is the form of DNA-based anticancer vaccination currently preferred in clinical trials (64, 77). In different studies, EP demonstrated elevated transfection rates, a minimal extent of tissue injury that exerts immunostimulatory effects (upon the release of damage-associated molecular patterns (DAMPs)) and no significant toxicities (64, 69, 78).

II.3. ELECTROPORATION

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. 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. Some water molecules can enter the lipid bilayer, leading to membrane rearrangement to orient the polar heads of phospholipids toward water. Consequently, nanopores are formed in cell membrane (79). This event occurs spontaneously, but it is neither frequent nor thermodynamically stable (80). During the EP process, exposure of cell membrane to an electric field polarizes the membrane and thus reduces the energy required for water penetration in the bilayer. Therefore, the probability and stability of pore formation is improved (79). However, a direct visualization of pores after EP is still challenging and the term electropermeabilization or electrotransfer is sometime preferred to prevent any misuse by describing not fully elucidated molecular events (81, 82).

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 (53, 79, 83-86). Here, we will focus on the GET and its potential for DNA vaccination, with a special focus on DNA vaccination against cancer.

GET requires DNA 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 DNA, necessary for its transfer across the cell membrane (87, 88). Once into the cell, DNA translocates into the nucleus (89). The GET process is illustrated in Figure 7.

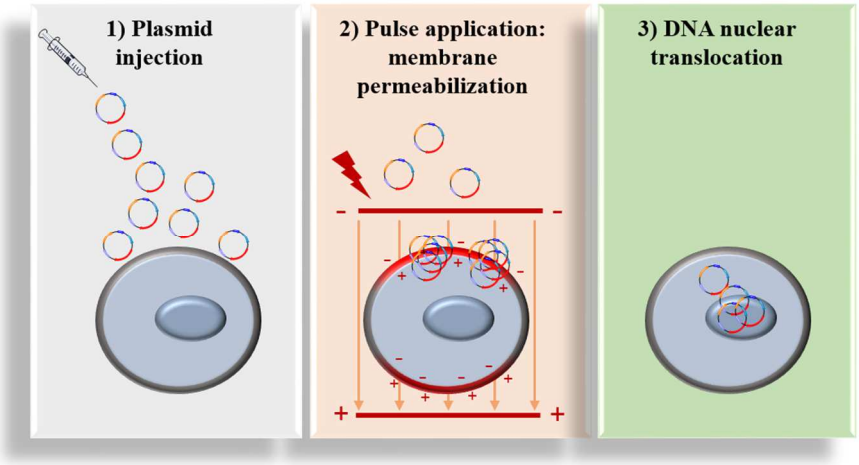

Figure 7: The EP process. Three different steps are involved: 1) Plasmid injection in the delivery site; 2) Electric pulse application, which permeabilizes cell membranes and allows the entry of the plasmid inside the cells; 3) The cellular membrane is resealed and DNA can translocate into the nucleus.

EP devices are pulse generators that deliver current through electrodes. Potential difference between electrodes generates an electric field around the cells that causes an induced transmembrane voltage (79). 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 (79, 81, 89).

EP not only increases the number of transfected cells and enhances the magnitude of gene expression compared to the injection of DNA alone, but also allows the co-transfection of several plasmids (90, 91). 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. It is effective for DNA delivery to several tissues, explaining its attractiveness for in vivo applications (91, 92). 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 (93, 94).

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 must be considered, 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 (95). EP requires the empirical optimization of many parameters to be able to target the right cells. Furthermore, specialized equipment is necessary, with the choice of an appropriate generator and electrodes (96-98). 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 (88).

To optimize the efficiency of EP, some technical parameters should be considered (Figure 8), in particular the electrode design and the pulse parameters.

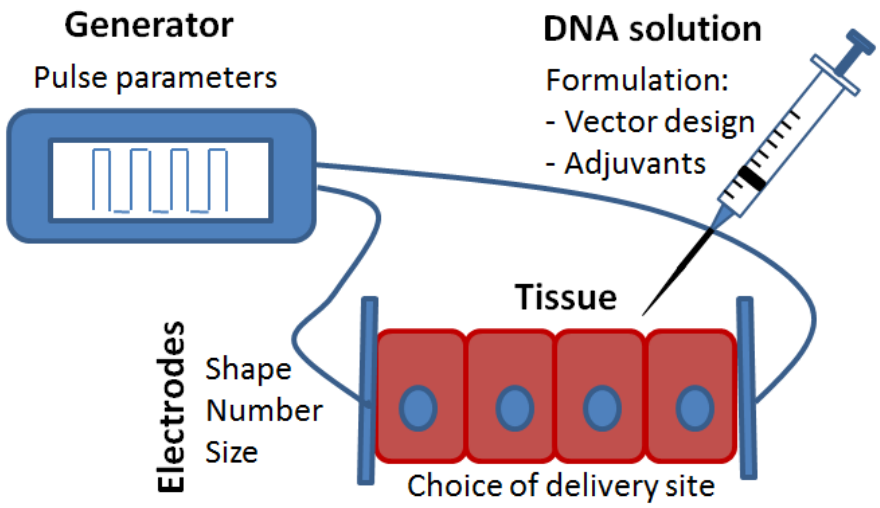

Figure 8: Schematic representation of EP procedure for GET and description of the protocol parameters that can be handled to optimize treatment outcome. DNA is injected prior electric field application via electrodes. The plasmid formulation, the choice of the delivery site, appropriate electrodes and pulse parameters have to be accurately adjusted to obtain optimal patient compliance and therapeutic efficacy (69).

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 (88, 99). The invasive electrodes consist of different needle configurations that are penetrated to the skin, muscle or

tumor (88, 91, 99-101) (Figure 9). 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.

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 (102). Further advancements have been made using non-penetrating electrodes such as caliper and plate electrodes. The depth of penetration of electric field is rather small (103), basically in the skinfold, which is in certain situations not convenient (103), and would not be optimal for use in humans. Furthermore, these electrodes require high voltages to enhance delivery and therefore can cause tissue damage (104). 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 DNA into the skin (104-106) and have promising expectations in cancer gene therapy (104). 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 (104). 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.

Figure 9: Different types of electrodes. A) Needle electrode; B) Plate electrode; C) Multi-electrode array. Adapted from (69).

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 (106, 107), low voltage (LV) pulses alone (108) or a combination of one or several HV and LV pulses (109-112). 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) (99).

The optimal pulse conditions reported in the different studies 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 tumors, because they require lower field strengths to reach the efficient gene delivery (113). 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 (114). 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.

Other important parameters are brilliantly reviewed in (53, 81, 89) and include the waveform, the molecular size, charge and formulation of the permeant, the site of administration etc.

Several clinical trials have been using GET against several infectious diseases and cancer. Up to now, 81 gene therapies have been recorded (clinicaltrials.gov), with the term "DNA electroporation". Most of them are currently in phase I and only few have been completed (Figure 10A, updated from (69)). Of relevance, two studies using DNA vaccines against cervical cancer have recently entered in phase III. The pathologies addressed are many forms of cancer and infectious diseases (Figure 10B, updated from (69)).

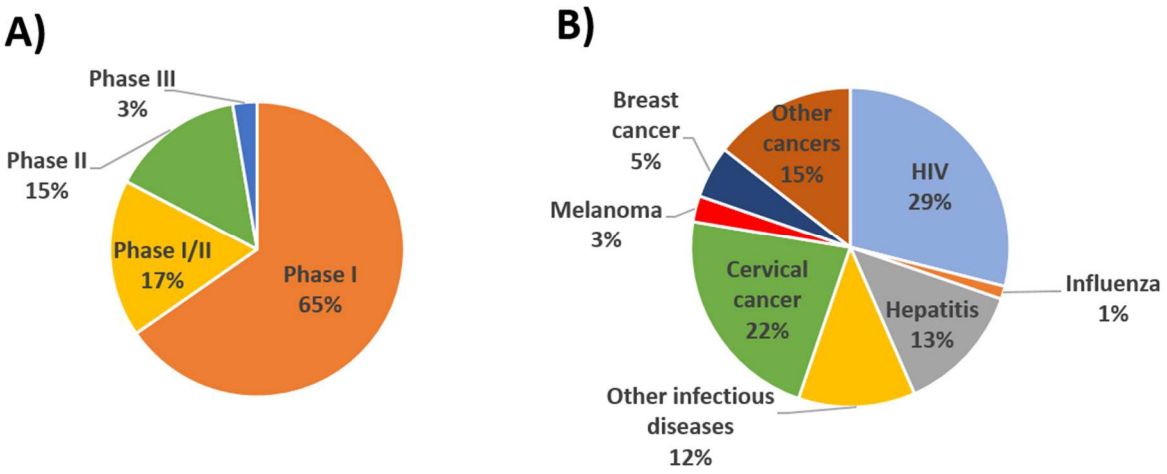

Figure 10: Clinical trials using EP for DNA vaccination. A) Phases of the studies. B) Types of pathologies addressed in clinical trials using EP delivery of DNA vaccines. Updated from (69).

In clinical studies, the adverse events associated to GET are mild and transient. EP can be easily performed after local anesthesia of the delivery area (115). However, in several clinical trials, the EP procedure did not request the use of a local anesthetic (55, 116, 117). 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 IT) with no systemic toxicity or clinically relevant side effects (Table 1).

Table 1: EP devices used in clinical trials. The name, the delivery route, the common side effects and their duration are described. IM = intramuscular, ID = intradermal, IT = intratumoral.

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	(55)
Easy Vax™	- ID: 80 needles, 600 µm depth	- Burning sensation - Tingling	- Few seconds Few minutes (< 5)	(118)
MedPulser	- IM: 4 needles, 1.5 cm depth - IT (no more information available)	- IM injection pain - Transient local reaction	- One minute - Few minutes	(119)
Trigrid	- IM: 4 needles - ID (no more information available)	- Mild-moderate local pain - Local reaction	- Few minutes - One day	(120-122)
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	(123)
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	(115)

Most of the current GET clinical trials against cancer use an IM injection of DNA followed by EP (Table 2). The cost-effectiveness and the safety profile of the DNA vaccines allow repeated low-dose administrations (in the order of mg) for a long-term protection (12). Most of the studies are still in progress and only few results are available. Here, some already completed studies involving DNA EP against cancer. Other studies will be discussed in Section V. In a phase I clinical trial, DNA coding for the melanoma tyrosinase, 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 tyrosinase-reactive CD8 T cell responses and 14% of those that received all the 5 doses had an increase in tyrosinase-reactive CD8+ IFNg+ T cells (121). It was also shown that a plasmid encoding HPV E6 and E7 viral oncoproteins which promote p53 and retinoblastoma degradation decreased the possibility of cervical cancer progression. DNA was IM electrotransfered, 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 IFNg response after a single immunization and it reaches 55% after the second vaccination. High levels of IFNg 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 (122). In a clinical study against prostate cancer, DNA 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 (123). In Table 2, we provide an overview of the clinical studies using the EP, starting from 2007.

Table 2: 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, when available. Combinations with other treatments are mentioned: chemotherapy (C), radiotherapy (R), chemoradiotherapy (CR), surgery (S), immunotherapy (I) and endocrine therapy (E). The clinicaltrials.gov identifier of the study and the principal investigator's name (when available) are provided. M = month, W = week, D = day. Keywords of the research "DNA vaccine", "cancer" and "electroporation", in clinicaltrials.gov; accessed the 1/03/2019.

Biological	Phase	Year	Adjuvant	Additional treatments	Delivery site	Dose	EP device	Additional information
------- DNA VACCINES AGAINST HPV-INDUCED CANCERS (17 clinical trials) -------								
VGX-3100 = plasmid DNA (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 3M	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 3M	CELLECTRA	- NCT01304524, Trimble, Parker, Valenzuela et al.
	I/II	2014	plasmid encoding hIL-12 (INO-9012)	R, CR	IM	6mg of VGX-3100 and 1mg adjuvant 1 dose	CELLECTRA	- NCT02172911, Gelder et al.
	I/IIa	2014	INO-9012	S, CR	IM	6mg of VGX-3100 and 1mg adjuvant 4 doses	CELLECTRA	- NCT02163057, Yang et al.
	II	2018	/	/	IM	4 doses in W 0, 4, 12, and 24	Not mentioned	- NCT03603808
	II	2018	/	/	IM	1 ml, on D0, W4 and 12, and potentially W40	CELLECTRA	- NCT03499795
	III	2017	/	/	IM	1 ml, D0, W4 and 12.	CELLECTRA	- NCT03185013

	III	2019	/	/	IM	1 ml, D0, W4 and 12.	CELLECTRA	- NCT03721978 (follow up of the NCT03185013)
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 1 dose	Trigrid	- NCT01634503, Kim et al. - Results published in (122)
	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 3M	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.
	II	2015	/	/	IM	1 mg, at D0, W4 and 12	Not mentioned	- NCT02596243
	/	2017	GX-I7 encoding IL7	/	IM	1 mg, 3 times	Not mentioned	- NCT03206138
pNGVL4a-CRT/E7 (detox) = pNGVL4a vector coding for calreticulin (CTR) and E7 (detox), a mutated form of E7 genes	I	2011	/	C	IM	0.5, 1, 2 or 4mg/dose 3 doses over 43D	Trigrid	- NCT01493154, Califano et al.
INO3112 = plasmid targeting HPV 16/18 (VGX-3100)	II	2018	INO-9012	I	IM	1, 3, 7, and 12W, then every 8W	Not mentioned	- NCT03439085
------- 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 3W	Trigrid	- NCT00471133, Wolchok et al. - Results published in (121)

SCIB1 = Plasmid DNA encoding a human antibody molecule engineered to express T cell epitopes derived from the TRP-2 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 6M	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 (4 clinical trials) -------								
Personalized polyepitope DNA vaccine	I	2015	/	/	IM	4 mg 3 doses over 57D	Trigrid	- NCT02348320, Gillanders et al.
Mammaglobin-A DNA Vaccine	I	2014	/	E	IM (2 sites)	2X3 doses over 84D	Not mentioned	- NCT02204098, Gillanders et al.
INO-1400 = Immunotherapy designed to target a gene known as the human telomerase reverse transcriptase (hTERT)	I	2014	INO-9012	/	IM	2 or 8mg of INO-1400 with or without 0.5mg or 2mg adjuvant 4 doses over 3M	Not mentioned	- NCT02327468, Vonderheide et al. - Also tested for lung and pancreatic cancers
Neoantigen DNA vaccine	I	2018	/	I	IM	D1, 29, 57, 85, 113, 141;	Trigrid	- NCT 03199040
------- DNA VACCINES AGAINST OTHER TYPE OF CANCER (11 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 3M	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, 4W apart	DermaVax	- NCT00859729, Yachnin et al. - Pathology: prostate cancer - Results published in (123)

Neoantigen DNA vaccine	I	2018	/	I	IM	4 mg, 6 treatments every 28+/-7 D	TriGrid	- NCT03532217, prostate cancer
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 24M
INVAC-1 = DNA vaccine encoding human telomerase reverse transcriptase (hTERT)	I	2014	/	/	ID	100, 400 or 800 µg 4W X 3 cycles	Not mentioned	- NCT02301754, Culine et al. - Pathology: solid tumors
INO-3106 = Vaccine against HPV6	I	2014	INO-9012	/	Not mentioned	3 mg or 6 mg INO-3106 alone or in combination with 1 mg adjuvant 4 doses over 9W	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 vector genomes/mL (low dose) or 2X10^11 vector genomes/mL (high dose) every 2W	Not mentioned	- NCT00753415 - Pathology: solid tumors
INO1400, encoding hTERT	I	2014	INO-9012	/	IM	2 mg, D0, W4, 8, and 12	Not mentioned	- NCT02960594, many solid tumors
INO-5401 = 3 separate DNA plasmids targeting Wilms tumor gene-1 (WT1) antigen, prostate-specific membrane antigen (PSMA) and human telomerase reverse transcriptase (hTERT) genes	I/II	2018	INO-9012	I, CR	IM	3 mg at D0 of each plasmid, every 3W for 4 doses, and then every 9W	CELLECTRA	- NCT03491683, glioblastoma
Neoantigen DNA vaccine	I	2018	/	/	IM	W1, 5, 9, 13, 17, and 21	Trigrid	- NCT03122106, pancreatic cancer
Neoantigen DNA vaccine	II	2018	/	I	IM	6 doses every 28D	Trigrid	- NCT03598816, renal cell carcinoma

II.4. MECHANISM OF ACTION OF CANCER DNA VACCINES AND IMMUNE ACTIVATION

Once injected into the body, the DNA vaccine can activate the different arms of the immune system. As previously mentioned, as a PAMP, DNA vaccines can activate the innate arm of the immune system. The dsDNA structure of DNA vaccines plays a "built-in" adjuvant effect (124). TLR-9, which is expressed in the endosomes of different immune cells, including APCs, recognizes the unmethylated CpG islands, typical of bacterial DNA, that are included in the backbone of the plasmid and/or in the antigen gene. This recognition activates a proinflammatory cascade, thus inducing the DNA vaccine immunogenicity. However, other mechanisms CpG-independent activate the innate immunity in response to DNA vaccines. Indeed, dsDNA is also recognized by cytosolic DNA sensors, such as stimulator of interferon genes (STING), TANK-binding kinase-1 (TBK1), cyclic GMP-AMP (cGAS), absent in melanoma 2 (AIM2), IFNg-inducible protein 16, polymerase III, etc. (125). Binding of intracellular nucleic acids to these sensors activates downstream signaling cascades, resulting in the production of type I IFNs and pro-inflammatory cytokines to induce a broad immune response directed against cancer cells. Type I IFN induced via the STING/TBK1 pathway was found to be crucial for both direct and indirect antigen presentation via DCs and muscle cells, respectively (124, 125). Hence, the innate immune activation further stimulates the adaptive one.

Upon delivery, the plasmid can transfect either APCs, e.g., dendritic cells (DCs), or other cells present at the delivery site (such as myocytes or keratinocytes) (63). According to the cells that are transfected with the plasmid, the antigen could be presented in different ways to the immune system (Figure 11):

1) *The antigen is expressed by APCs.* In this case, the TA will be loaded on major histocompatibility complex class I (MHC class I) molecules and will be presented to CD8 T cells in the draining lymph nodes (DLNs) to promote the proliferation of cytotoxic T lymphocytes (CTLs) (63, 126).

2) *The antigen is expressed by other cells (e.g., keratinocytes, myocytes).* In this case, different immune pathways could be activated. First, antigen can be loaded on MHC class I molecules at the cell surface. 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 shed from the cell, 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. At that moment, the exogenous antigen is loaded on MHC class II molecules, thus activating CD4 T cells and further inducing T helper (Th) cell proliferation (63, 126, 127). Th cells

play a critical role in supporting the activity of CTLs (Th1 cells) as well as B cells (Th2 cells) by producing specific cytokines. They also support the activity of memory T cells, leading to a long-lasting immunological memory (128).

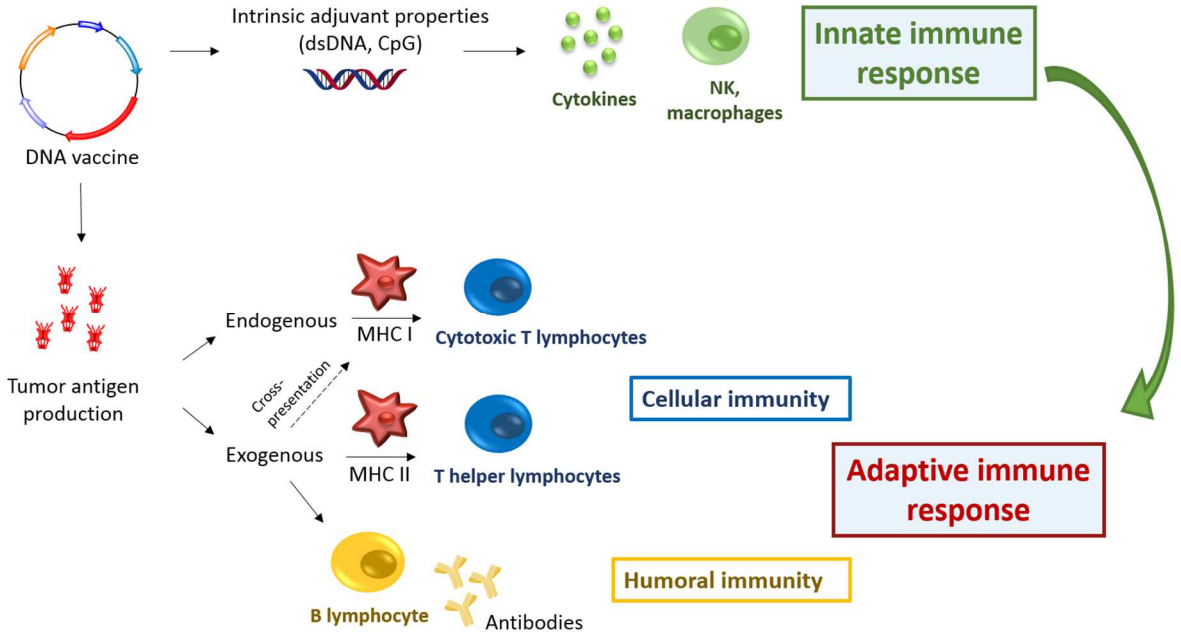

Figure 11: The different mechanisms of immune system activation induced by DNA vaccines. The dsDNA structure is recognized by sensors and receptors in the cytosol of the cells or in the endosomes and activates the innate immunity. The TA produced by DNA vaccine can be presented in different ways to T cells and B cells and, thus, activate the adaptive immunity, which is further activated by the innate arm of the immune system.

II.5. ADVANTAGES AND LIMITATIONS OF DNA VACCINES FOR CANCER VACCINATION

The use of genetic material as a vector for immunization has many advantages over traditional vaccines (62), under different point of view. (i) *Easy design and rapid production.* In silico programs permit to easily design and optimize DNA sequences that will be inserted in a plasmid vector. Production of DNA vaccines is fast, and they can be easily purified in large quantities. Interestingly, the same production platform can be used for different plasmids, reducing the costs of procedures (12). Furthermore, they can be formulated and injected as naked plasmids, i.e. formulated only in buffer and without a carrier, because of their long-term stability (129). ii) *High stability.* DNA vaccines have a good stability profile that permits a long-term storage and no cold chain is required for the transport (130). iii) *Versatility.* The antigen sequence inserted in a plasmid backbone could be easily adapted to another cancer type; more than one antigen/adjuvant could be encoded in the same plasmid vector; multiple plasmids can be administered at the same time; the same vaccine could be used for different cancer types (131, 132), because some tumors share the same TAs (e.g., melanoma and glioblastoma (GBM) have the same prenatal origin) (132). iv) *Safety.* DNA vaccines

present a quite good safety profile and the risk-benefit balance appears often favorable for both therapeutic and preventive vaccination approaches. Unlike virus attenuated vaccines, DNA vaccines do not carry the potential to return to virulence, particularly in immunocompromised patients (130, 133). Many clinical trials demonstrated the safety profile of DNA vaccines and showed that the side effects associated to DNA vaccine were similar to placebo (12, 134-137). The initial concerns regarding the integration of partial or complete plasmid sequences into the host genome by insertion mutagenesis, thereby risking inactivation of tumor suppressor genes or activation of oncogenes or causing chromosomal instability, have been disproven by further studies (138, 139). Indeed, integration occurs at slower rates than a spontaneous mutation (140). v) *Broad immune response and immunological memory.* As previously explained, DNA vaccines can induce both the innate and the adaptive immune responses, in addition to a long-lasting immunity (immunological memory). The ability of cancer DNA vaccines to broadly stimulate the immune response allows to overcome the limitations of traditional approaches (radiotherapy, chemotherapy and surgical resection) that have only limited roles against metastatic malignancies and have many side effects due to the non-specificity of the treatment that causes extensive damages to normal tissues (141). vi) *Production of the antigen in the natural conformation.* DNA vaccines allow the production of antigens that maintain their natural conformation and appropriate post-translational modifications (142).

However, the broad range of applications of DNA vaccines is somehow limited by the fact that plasmids can encode only proteins or peptide mimetics of carbohydrates (143) and they are not applicable for immunization against non-protein immunogens. In addition, even if the safety profiles are good so far, there might be a risk of producing some DNA-specific antibodies. However, some studies have shown a rare probability of anti-vector autoimmunity after DNA vaccination, even after multiple administrations (12, 139). Another potential problem would be the induction of tolerance if the danger signals induced by the plasmid is not strong enough during the priming phase.

Despite promising results in small animal models, the first clinical studies showed a limited efficacy of DNA vaccines in humans (12, 144). Hence, their main limitation remains the poor immunogenicity in the clinic, mainly due to the immunosuppressive tumor microenvironment (TME). However, this is a disadvantage shared by all the cancer vaccines. For this reason, until now, only one therapeutic cancer vaccine has been Food and Drug Administration (FDA)-approved for the human use (a cancer DC vaccine, Sipuleucel T) and most of the other cancer vaccines, including DNA vaccines, are still in clinical phase I or II (17, 145). A schematic overview of the advantages and limitations of DNA vaccines is given in Table 3.

Table 3: Advantages and limitations of cancer DNA vaccines.

Advantages	*Easy design and rapid production*
	High stability
	Versatility
	Safety and low toxicity
	Broad immune response and immunological memory
	Production of the antigen in the natural conformation
	Activation of the CD8 immune response
Limitations	*Only for protein/peptide antigens*
	Mild risk of anti-vector immunity
	Poor immunogenicity in humans

III. TUMOR ANTIGENS

Tumor antigens (TAs) are proteins overexpressed or uniquely expressed in the tumor tissue that can play a role in tumor initiation, progression and metastasis (141, 146). Since the characterization of the first TA, the melanoma-associated antigen (MAGE) in 1991 (147), a growing number of TAs has been identified. Coulie et al. classified human antigens in two categories (146) (Table 4):

- *Antigens of high tumoral specificity*. They are antigens that can elicit a strictly specific anti-tumor response. Three categories of TAs belong to this group: (i) Viral antigens, derived from cancers of viral origin (e.g., hepatocarcinoma, cervival cancer, nasopharyngeal carcinoma) (148, 149). (ii) Antigens that result from a mutation or genetic rearrangements. Many tumor-specific T cells recognize mutated antigens, derived from the change in one or more aminoacids, the alteration of the reading frame, or the transcription beyond the stop codon. In many papers, these TAs are described as "neoantigens" (150). They often derive from passenger mutations, often found on oncogenes, such as CDK4, BCR-ABL (146, 151, 152)(iii) Antigens encoded by cancer-germline genes. They are antigens normally not expressed in non-cancerous adult tissues, except in placenta and testis, which do not express the MHC class I and, thus, cannot present the antigen. Hence, these antigens are tumor-specific. The MAGE gene family is the main representative of this category. (146, 151)

- *Antigens of low tumoral specificity*. These are TAs subjected to a higher tolerance, as they are not uniquely expressed by the tumor. This category includes: (i) Differentiation antigens. They are expressed only in the cancer cells and in the non-tumoral tissue of origin, such as tyrosinase, Melan-A/MART1, GP100, TRP2 well documented in patients with melanoma, and involved in the melanin production; T cell responses to these antigens can lead to vitiligo (skin depigmentation), generally associated with a good prognosis (146, 151). Also the carcinoembryonic antigen (CEA) belongs to this category, as it is often expressed in colorectal cancer, but also by normal epithelial cells in the intestine. (151) (ii) Antigens derived from proteins that are overexpressed in tumors, such as HER2/neu, Wilm's tumor protein (WT1), mucin 1 (MUC1), overexpressed in epithelial tumors, leukemia and adenocarcinomas, respectively (146).

Table 4: Categories of tumor antigens

TA category	TA subcategory	Examples
Antigens of high tumoral specificity	Viral antigens	HPV E6-E7, EBV latent-membrane protein
	Mutational antigens (neoantigens)	Bcr-Abl, P53, Kras, Patient-specific mutations
	Antigens encoded by cancer-germline genes	MAGE family, GAGE, BAGE, NY-ESO1
Antigens of low tumoral specificity	Differentiation antigens	tyrosinase, Melan-A/MART1, GP100, TRP2
	Overexpressed antigens	MUC1, WT1, HER2/neu

When designing a cancer DNA vaccine, antigen(s) choice is crucial. TA should be chosen to straighten the DNA vaccine immunogenicity and to induce a broad immune response, overcoming the problem linked to the antigen loss, modification and tolerance.

Based on the antigen choice, we can distinguish 3 main types of DNA vaccines: chimeric, neoantigen and polyepitope DNA vaccines.

1) Chimeric DNA vaccines

Chimeric DNA vaccines are vaccines that encode xenogeneic antigens. They are proteins or peptides derived from different species, for which sequence is significantly homologous with the self-ortholog (153). The subtle differences between epitopes of the orthologue and the native protein elicit T and B cell responses against the xenoantigen (153, 154). Hence, xenogeneic antigens are recognized as "non-self-antigens", thus circumventing immune tolerance, while preserving an optimal homology to allow T cell recognition (154, 155). Different studies demonstrated the higher efficacy of xenogeneic antigens compared to the autologous antigen (155, 156). A complex DNA vaccine construct that delivers several xenogeneic epitopes dramatically increased the CTL anti-tumor activity (157). The efficacy of DNA xenovaccines was also tested in dogs (158), leading to the approval of the first xenogeneic DNA vaccine against human tyrosinase, Oncept, for the treatment of oral malignant melanoma in dogs (153).

It is also possible to design hybrid plasmids, which code for chimeric proteins that include both xenogeneic and homologous antigen domains (154). In this type of plasmids, the xenogeneic moiety can circumvent the immune tolerance and induce a more potent cellular response, while the homologous sequence can stimulate the activation of a broader immune response (153). Indeed, the chimeric protein produced by transfected cells can be taken-up by DCs, thus activating

the T cell immune response, but also can be recognized and internalized by B cells (153). Quaglino et al. found that the plasmid encoding the chimeric neu-Her-2 antigen was superior to both the fully autologous and to the fully xenogeneic vaccines in inducing a protective anti-tumor immune response against ErbB2$^+$ tumors (159). Starting from these results, other DNA vaccines were constructed by shuffling genes from mouse, rat, human and other species, improving the antigen immunogenicity and the vaccine efficacy (160-163). DNA xenovaccination has also been tested in clinic in melanoma patients, with encouraging results (164, 165) and one clinical study (NCT00096629) using human and murine PSA is ongoing (Table 5).

2) Neoantigen DNA vaccines

Most of anti-cancer DNA vaccines, both past and present, immunize using non-mutated TAs. However, these antigens are often present in normal or germline tissues, which can prevent a strong immune activation, because of immune tolerance (166). Several clinical trials using non-mutated TAs have failed to demonstrate beneficial effects compared with the standard of care treatment (167). In contrast, neoantigens are the result of tumor-specific DNA alteration that creates new epitopes. Due to their specific expression in cancer tissue and the potential reduction of side effects, they represent ideal targets against cancer and can be used in the design of cancer vaccines (168). Vaccination with neoantigens can turn "cold" (not highly immunogenic) tumors into "hot" (highly immunogenic) ones and mediate up-regulation of Programmed cell death protein ligand-1 (PD-L1) in the TME, thus extending the applicability of anti-PD-1/PD-L1 immunotherapy (169). Neoantigens are presented by APCs to CD4 and CD8 T cells to activate an immune response. They are highly tumor-specific and not affected by T cell tolerance (170). Their identification starts with exon sequencing from a tumor biopsy. Then, mutations are identified compared to an RNA data of a normal tissue. Prediction algorithms select those antigens that are recognized by MHC class I or II. Finally, in vitro and in vivo studies validate their ability to stimulate CD8 immune response, but especially a CD4 response (171, 172). However, not all peptides are immunogenic and identifying which mutations are targeted by the immune system is currently a subject of intense interest. Hence, prediction of immune response to neoantigens needs to be optimized. Assessing the immunogenicity of each neoepitope is not reasonably applicable on a large scale. Current computational approaches are being refined to improve neoantigen identification accuracy and are discussed in detail in (173). Integrated pipelines will need to be developed beginning with tumor genomic characterization, variant analysis, and accurate prediction of which mutations are likely to give rise to tumor-specific neoantigens (174). Other hurdles are associated to the use of personalized neoantigens for cancer immunotherapy, such as the manufacturing time. The median period for the discovery and the production of a personalized vaccine is around 3.5-4.5 months

(175, 176). In particular, the phase of selection of mutations to vaccine release ranges around 89-160 days (176, 177). This time has to be reduced to cure patients with metastatic disease. Another issue concerns the genetic heterogeneity of tumor (178). Thus, targeting a unique neoantigen would probably lead to selection of antigen non-expressing tumor cells. It has been demonstrated that the use of poly-epitope neoantigen RNA vaccine encoding up to 10 neoantigens was effective in 8/13 melanoma patients, that were totally tumor-free after one year (177). Compared to RNA and to peptide vaccines, DNA vaccines seem to elicit a more potent CD8 response against the encoded neoantigens, making them more attractive for cancer vaccination (179). Hence, once identified, the neoantigen can be cloned into a DNA vaccine. This personalization permits to design cancer vaccines tailored to each patient.

3) Polyepitope DNA vaccines

An advantage of DNA vaccines is the possibility to deliver several antigen genes in the same construct, at the same time and with the same delivery method. The presence of immunodominant and unconventional epitopes simultaneously delivered by a polyepitope DNA vaccine can induce a broad CTL response specific to multiple antigens (180). In this way, it is possible to overcome antigen mutation or deletion by tumor cells or the variation or absence of the appropriate T cell repertoire and MHC haplotype in the patients (180).

When designing a poly-epitope DNA vaccine, many parameters should be taken in consideration. Firstly, the competition for antigen recognition at the surface of the APC and the affinity of the selected epitopes for MHC molecules should be considered (181, 182). Palmowski et al. demonstrated that the use of an MHC class I polyepitope vaccine leads to preferential expansion of CTLs with a single immunodominant specificity (183, 184). In addition, the affinity of the selected epitopes for MHC molecules and transporters could influence the CTL immunodominance and the consequent immune response (181).

Secondly, although the CD8 T cell response has been considered to be the main protagonist in the anti-tumor immune response resulting from vaccination, the insertion of an epitope/antigen recognized by CD4 T cells into a DNA vaccine could activate a broader and stronger immune response. Several studies suggest the importance of the CD4 T cell population for cancer immunotherapy (185, 186). Recently, it has been demonstrated that CD4 T cells recognize a higher number of neoantigens than was previously known and can generate potent antitumor response (172, 187). Hence, a coordinated CD4 and CD8 response is necessary for the complete eradication of the tumor (187). T helper (Th)-peptides have been already used in combination with DNA vaccines to increase the activation of Th cells, thus further eliciting CTL immune response (188-

193). An example of Th epitope is the pan DR epitope (PADRE). This synthetic Th epitope, encoded in a DNA vaccine and administered with an antigen-encoding plasmid, increased the number of antigen-specific CD8 T cells, resulting in potent protective and therapeutic anti-tumor effects (194). Other studies demonstrated that a PADRE-encoding DNA generated CD4 Th1 cells that play an important role in maintaining long-term memory responses, helping the activity of CD8 T cells (195).

Many technics have been developed to find new epitopes. These studies lead to the identification of NY-ESO-1, MelanA/MART-1, SSX4, MELOE-1 and TRAG-3 in melanoma, EphA2 and MAGE-6 in renal cell carcinoma, CEA, MAGE-3 and telomerase in lung carcinoma, TRAG-3 in breast carcinoma, NY-ESO-1, p53 and SSX4 in ovarian cancer, among others (196). Some of these tumor antigens recognized by CD4 T cells belong to the same categories of those recognized by cytotoxic CD8 T cells (186).

Finally, it is important to identify the most immunogenic epitopes derived from TAs. New in silico techniques are being developed to improve the prediction of epitope immunogenicity to design a poly-epitope vaccine. They not only consider the binding affinity to the MHC and the different HLA subtypes, but also the conformation and interaction with the HLA, immunodominance *vs* tolerance etc. (197).

Many recent preclinical studies investigated the use of polyepitope DNA vaccines to reach a broad immune response. As a result, an increased IFNg production, higher Th and CTL response (197, 198), and a general decrease in the tumor growth rate and metastasis formation were observed in different types of cancer models (199, 200). Some preclinical studies focus on HPV model, using DNA vaccines encoding E6, E7 molecules (201), or E7 with a helper epitope (199). Another example is SCT-KDR2, encoding the mouse β2microglobulin + KDR2 (VEGFR2 antigen peptide) + MHC class I H-2D^{b}, in a B16 melanoma tumor model (200). A non-exhaustive list of the most recent preclinical trials (the last 5 years) using DNA vaccines against cancer can be found in Table 6. Many clinical trials are also testing the safety and efficacy of polyepitope DNA vaccine, such as: NCT02348320, NCT02157051 in breast cancer, NCT02172911 for cervical cancer, NCT01322802, NCT03029611 for ovarian cancer etc. In particular, in the clinical studies NCT02348320 and NCT03199040, a personalized polyepitope vaccine against breast cancer is being used, as well as in the NCT03122106 for pancreatic cancer and results will help us to establish the relevance of this vaccine strategy. This would address tumor heterogeneity and the loss of immunogenicity associated to low tumoral-specific antigens, which accounts for the failure of the current anticancer treatments (169). A complete list of the ongoing clinical trials can be found in Table 5.

Table 5: Clinical trials using DNA vaccines against cancer (years 2009-2019). A description of the vaccine, the study start year and the current trial phase are provided. Information regarding the use of adjuvant, the injection site, the doses and additional key parameters are presented, when available. Combinations with other treatments are described. M = month; W = week; D = day; EP = electroporation; IM = intramuscular; ID = intradermal; IV = intravenous. Keywords of the research "DNA vaccine" and "cancer", in clinicaltrials.gov; accessed the 1/03/2019.

Cancer type	Phase	Study start	DNA vaccine/ encoded antigen	Combination therapy	Treatment schedule	DNA delivery	References
Breast cancer	I	2015	Personalized polyepitope	/	- Vaccine: 4 mg, at D 1, 29 and 57	IM EP	NCT02348320
	I	2015	Mammaglobin-A antigen	Anastrozole, Letrozole, Tamoxifen, Exemestane, Goserelin, endocrine therapy	- Vaccine: 2 injections in the deltoid or lateralis muscles, at D 28, 56 and 84 - Endocrine therapy: to be determined by the treating physicians	IM EP	NCT02204098
	I	2015	pUMVC3-CD105/Yb-1/SOX2/CDH3/MDM2-polyepitope: mammalian expression vector pUMVC3 + CD105, Y-box binding protein-1, SRY-box 2, cadherin 3, murine double minute 2	rhuGM-CSF, adjuvant therapy	- Vaccine: every 28 D for 3 M, then an injection at 6 and 12 M - rhGM-CSF: ID every 28 D for 3 M	ID	NCT02157051
	I	2016	pUMVC3-IGFBP2-HER2-IGF1R: pUMVC3 vector + insulin-like growth factor binding protein-2 (IGFBP2), HER2 and ILGF-1 receptor precursor (IGF-1R)	GM-CSF (Sargramostim), adjuvant therapy	- Vaccine: D 1 and every 28 D, 3 times - GM-CSF: the same as the vaccine	ID	NCT02780401
	I	2018	Neoantigens	Durvalumab (anti-PD-L1 antibody), immune therapy	- Vaccine: 2 injections in 2 different sites, 3 M after the standard of care (D 1) and then D 29, 57, 85, 113 and 141 - Durvalumab: 1500 mg every 4 W, at D 85	EP	NCT03199040
Prostate cancer	II	2009	pTGV, encoding prostatic acid phosphatase (PAP)	rhGM-CSF, adjuvant therapy	- Vaccine: 100 µg, every 2 W for the first 12 W and then every 12 W, according to the immune response - rhGM-CSF: 200 µg every 3 M	ID	NCT00849121

	II	2011	pTGV-HP	rhGM-CSF, adjuvant therapy	- Vaccine: 100 µg, every 2 W for 6 times and then every 3 M for 2 years - rhGM-CSF: the same as the vaccine, but 208 µg of dose	ID	NCT01341652
	II	2013	pTGV-HP	Sipuleucel-T, autologous peripheral blood mononuclear cells with antigen presenting DCs that have been activated *ex vivo* with a recombinant fusion protein (PA2024, PAP linked to GM-CSF), immune therapy	- Vaccine: at W 6, 8, 10, 12, M 6 and 12 - Sipuleucel-T: W 0, 2 and 4	/	NCT01706458
	I	2015	pTGV-AR	GM-CSF, adjuvant therapy	- Vaccine: 100 µg, 6 doses every 2W and then 12, 24, 36 and 48 or 100 µg at W 0, 2, 12, 14, 24, 26, 36, 38, 48, 50 - GM-CSF: 200 µg co-injected with the vaccine	/	NCT02411786
	I/II	2015	pTGV-HP	Pembrolizumab (anti-PD-1 antibody), immune therapy; rhGM-CSF, adjuvant therapy	- Vaccine: 100 µg, at D 1, 15, 29, 43, 57 and 71 - Pembrilizumab: 2 mg/kg, IV, every 3 W, at D 1, 22, 43, 64 or D 85, 106, 127 and 148 - rhGM-CSF: 208 µg, ID, every 3 W	ID	NCT02499835
	II	2018	pTGV-HP	Nivolumab, GM-CSF	- Nivolumab: 240 mg IV every 2 W x 6 beginning D 1, then every 4 W x 9 beginning W 12) - rhGM-CSF: 208 µg, ID every 2 W x 4 beginning W 4, then every 4 W x 9 beginning W 12 - pTVG-HP: 100 µg, every 2 W x 6, beginning at D 1; then every 4 W x 9, beginning at W 12	ID	NCT03600350
	I	2018	Neoantigens	Nivolumab/Ipilimumab and Prostvac	- Priming with Prostvac (1 mg/kg, every 3 W, 2 doses) and the ICB (3 mg/kg, every 3 W, 6 doses) - Vaccine: 4 mg at 2 different sites, 6 times every 28 D	IM EP	NCT03532217

Cervical cancer	I/II	2015	VB10.16: composed by E6/E7 antigen of HPV16 + dimerization entity + APC binding protein	/	- Vaccine: 3 mg, W 0, 3, 6 or W 0, 4, 12	Lateral deltoid mucle	NCT02529930
	II	2015	GX-188E, encoding E6/E7 fusion protein of HPV 16 and 18, plus the immune-enhancer, Fms-like tyrosine kinase-3 ligand (FLT3L)	/	- Vaccine: 1 mg, W 0, 4 and 12	IM	NCT02596243
	I/II	2017	MEDI0457 = INO3112 = VGX-3100 (, encoding E6 and E7 proteins of HPV types 16 and 18) + INO-9012 (hIL-12)	Darvalumab (anti PD-L1 antibody), immune therapy	- Vaccine: / - Darvalumab: 1500 mg, IV, every 4 W	IM EP	NCT03162224
	/	2017	GX-188E	GX-I7 encoding IL7 receptor agonist, Imiquimod, adjuvant therapy	- Vaccine: 1 mg, 3 times - GX-I7: 3 mg, locally on the cervix, 4 times - Imiquimod: 12.5 mg, administered locally on the cervix, 8 times	IM	NCT03206138
	III	2017	VGX-3100	/	- Vaccine: 1 ml on D 0, W 4 and W 12	IM EP	NCT03185013
	I/II	2018	GX-188E	Pembrolizumab	- Vaccine: 1.0mg/0.5ml - Pembrolizumab: 100mg/4mL, IV	IM EP	NCT03444376
	II	2018	VGX-3100	/	- Vaccine: over 10 seconds for 4 doses in W 0, 4, 12, and 24	IM EP	NCT03603808
	II	2018	VGX-3100	Darvalumab	- Vaccine: W 1, 3, 7, 12 - Darvalumab: W 4, 8, 12	IM EP	NCT03439085
	III	2019	VGX-3100	/	- Vaccine: 1 ml on D 0, W 4 and W 12	IM EP	NCT03721978
Ovarian cancer	I	2012	pUMVC3-hIGFBP2 multiepitope: mammalian vector pUMVC3 + hIGFBP2	/	- Vaccine: monthly, for 3 M	ID	NCT01322802
	II	2017	pUMVC3-hIGFBP2 multiepitope	Carboplatin, Paclitaxel, chemotherapy	- Vaccine: 2 W after the chemotherapy and every 3 W - Chemotherapy: IV, 2 W before the vaccine	ID	NCT03029611

Pancreatic cancer	I	2018	Personalized neoantigen: pING vector + prioritized neoantigens + mesothelin epitopes	Chemotherapy	- Vaccine: W 1, 5, 9, 13, 17 and 21 - Chemotherapy: at W 1, 5, 9, 13, 17, 21, 25 and 77, after surgery and before vaccination	IM EP	NCT03122106
Glioblastoma	I/II	2018	INO-5401 (3 separate DNA plasmids targeting Wilms tumor gene-1 (WT1) antigen, prostate-specific membrane antigen (PSMA) and human telomerase reverse transcriptase (hTERT) genes)	Cemiplimab, radiation and chemotherapy; INO-9012	- Vaccine: 3 mg at D 0, every 3 W x 4 doses, and then every 9 W - INO-9012: 1 mg at the same time of the vaccine - Cemiplimab: IV, every 3 W at a dose of 350 mg per dose - Radiation therapy: 42 days after surgical intervention, and should start approximately 2 W after D 0 - TMZ: daily during radiation therapy, dose of 75 mg/m²	IM EP	NCT03491683
Melanoma	Early I	2018	IFx-Hu2.0 coding for Emm55 Streptococcal Antigen	/	- Vaccine: 100 µg in 200 µL per lesion	Intralesion	NCT03655756
Renal cell carcinoma	II	2019	Neoantigens	Darvalumab Tremelimumab	- Durvalumab: IV at a dose of 10 mg/kg over the course of 60 minutes, every 2 W, 8 doses - Tremelimumab: at a dose of 1 mg/kg over the course of 60 minutes, every 4 W, 4 cycles - Vaccine: D 1 of the first 28 D cycle of treatment with durvalumab and tremelimumab, every 2 W	IM EP	NCT03598816
Solid tumors	I	2014	hTERT	/	- Vaccine: 100, 400 and 800 µg as a single agent, every 4 W x 3 cycles	ID EP	NCT02301754
Anal neoplasm	II	2018	VGX-3100	/	- Vaccine: 1 ml on D 0, W 4 and W 12, and potentially W 40	IM EP	NCT03499795
Urothelial carcinoma	I/II	2018	INO-5401	INO-9012, Atezolizumab	- Vaccine: 9 mg, every 3 W x 4 doses then every 6 W x 6 additional doses, thereafter every 12 W - INO-9012: administered with the vaccine -Atezolizumab: IV infusion every 3 W	IM EP	NCT03502785

A good option to further optimize the efficacy of DNA cancer vaccination could be the combination of the three cited approaches, designing a poly-epitope chimeric vaccine containing specific neoantigens. In clinic, this could reduce the number of non-responding patients, by developing a stronger and more complete immune response.

IV. COMBINATION THERAPY FOR IMMUNOTHERAPY OF CANCER

In the analyzed preclinical (Table 6) and clinical (Table 5) studies, DNA vaccines can delay tumor growth and elicit a strong immune response, especially an antigen-specific CTL response, but rarely are able to totally reject the tumor. These modest gains were reached by optimizing DNA vaccines in several aspects, such as plasmid design and delivery, administration strategies etc., as previously described (51, 63, 202, 203). However, DNA vaccines alone are not able to overcome tumor immune escape caused by natural selection of tumor cell clones lacking immunogenic antigens or by immunosuppressive cells that are recruited in the TME (MDSCs, Tregs among others), that lead to the exhaustion of T effector cells (204). Cancer DNA vaccines can reach their optimum efficacy if combined with other strategies able to potentiate the antigen response but also to silence immunosuppression in the TME (205).

Table 6: Preclinical studies using DNA vaccines against cancer (years 2015-2018). A description of the vaccine and the year of the study are provided. Information regarding the use of adjuvant of combination therapies, the injection site, the doses and additional key parameters are presented, when available. Legend: M = month, W = week, D = day. Keywords in Pubmed: "cancer", "DNA vaccine", "cancer DNA vaccination", "plasmid"; studies from 2015 until 2018.

Cancer type	Animal	DNA vaccine	Combination therapies	Protocol	DNA vaccine delivery	Results	Year, ref.
Cervical cancer (TC-1 cells)	C57BL/6 mice	HPV plasmid encoding E6 and E7 antigens	pVAX1-ISG15 encoding an optimized mouse adjuvant ISG15	**Therapeutic vaccination** Tumor: 5 x 10^4 TC-1 tumor cells, SC Vaccine and adjuvant: 7 D after tumor implantation, followed by 3 boosts weekly	IM EP, in the tibialis anterior muscle	-Strong HPV E7-specific CD8 T cell immune response -Increase in IFNgsecretion -6/10 mice were tumor-free at D 42	2015 (201)
	C57BL/6 mice	pcDNA3.1-E7, encoding E7 antigen of HPV16	Monophosphoryl lipid A (MPL, TLR-4 agonist) and α-galactosylceramide (GalCer)	**Therapeutic vaccination** Tumor: 2 x 10^5 TC-1 tumor cells, SC Vaccine: 100 µg, 7 D after tumor implantation, followed by 2 boosts weekly Adjuvant: 25 µg of MPL and 1 µg of GalCer, SC with the vaccine	SC	-CTL-specific cytolytic activity -Higher INF-γ, IL-4 and IL-12 production -Decrease in tumor growth if both adjuvants were administered	2016 (206)
	C57BL/6 mice	HELP-E7SH, encoding E7 antigen of HPV16 and a helper epitope to stimulate CD4 T cell response	Abs against CD70, CTLA-4, PD-1; agonistic Ab to CD27	**Therapeutic vaccination** Tumor: 1 x 10^5 TC-1 tumor cells, at D 0 before the vaccination, SC Vaccine: 15 µl of a 2 mg/ml DNA solution, on D 0, 3 and 6 Abs: 100 µg, at D 0, 3 and 6 (CD70 also at D 9), IP	Tattoo (intraepidermal vaccination)	-Help epitopes increased E7-specific CD8 response in lymph nodes and spleen -CTLA-4 and PD-1 did not promote CTL priming, if not combined with CD27 or the vaccine -CD27 agonism + anti-PD-1 improved mice survival, albeit CD27 + anti-CTLA-4 further increased the CTL response	2016 (199)
	C57BL/6 mice	pVAX1-gDE7, encoding HPV-16 E7 protein fused to HSV-1 gD protein	pcDNA3-IL-2 encoding murine IL-2; anti-Gr1 Ab	**Therapeutic vaccination** Tumor: 7.5 x 10^4 TC-1 tumor cells, at D 0 before the vaccination, SC Vaccine and adjuvant: 50 µg alone or in combination with 50 µg of the adjuvant, at D 3, 2 doses, weekly	IM	-Vaccination with the 2 plasmids avoided MDSC accumulation -Combination of the vaccines and anti-Gr1 antibody increased mice survival, completely eradicating the tumor	2016 (207)

				Anti-Gr1: 200 µg, once/W for 6 IP injections, at D 7-10, 5 doses, weekly			
	C57BL/6 mice	pcDNA3.1-E7, encoding HPV-16 E7 antigen	melatonin	**Therapeutic vaccination** Tumor: 2 x 10^5 TC-1 cells at D0, before the vaccination, SC Vaccine: 90 µg, 3 times, at 7 D interval Melatonin: 50 or 100 mg/kg	SC	-Production of HPV16 E7-specific CTL -Increase of IFNg and TNFa in the TME -Tumor volume reduction	2018 (208)
	C57BL/6 mice	dbDNA, encoding HPV16 E6 and E7	/	**Therapeutic vaccination** Tumor: 5 x 10^4 TC-1 cells, SC, at D0 Vaccine: at D3, 25 µg/hind, boost after 7 W	IM in the anterior tibialis and IM EP in the quadriceps	-Delay in the tumor growth -High levels of IFNg-secreting Th cells -Production of IgG1 -IL-12 production and low IL-10	2018 (209)
Cervical cancer (HPV)	BALB/c mice	pNGVL4a-hCRTE6E7L2, expressing the HPV16 E6, E7 and L2 antigens	/	**Prophylactic vaccination** Vaccine: 3 injections, biweekly Tumor: 12 µl of HPV16 PsV, 19 D after the last vaccination	IM EP	-Production of Ab against E6, E7 and L2; -Protection of 3/5 of mice from the challenge, but without a significant difference compared to the control group	2017 (210)
Lobular carcinoma (TUBO cells)	BALB/c mice	pAmot, coding human p80 Amot (Angiomotin), antiangiogenic	/	**Therapeutic vaccination** Tumor: 10^5 TUBO cells, SC Vaccine: 50 µg, at D 7	IM EP in the quadriceps muscle	-Delay in tumor progression -Heterogeneous changes in the tumor region following antiangiogenetic treatment	2015 (211)
Murine breast cancer (D2F2 cells)	BALB/c mice	pVAX-E2A, encoding Her2/neu antigen	pVAX-CCL4, encoding CCL4, chemoattractant for immune effector cells	**Prophylactic vaccination** Vaccine: 2 x 100 µg, on D 1 and 15 Tumor: 2 x 10^5 Her2/neu$^+$ cells, on D 25, SC	IM	-With the combined therapy, 26% of mice remained tumor-free (CCL4 improved tumor protection) -CCL4 produced a Th1 anti-Her2/neu response	2016 (212)
Murine breast cancer (4T1 cells)	BALB/c mice	CpVR-FAP, encoding fibroblast associated protein (FAP)	Cyclophosphamide, chemotherapy agent	**Therapeutic vaccination** Tumor: 2 x 10^4 4T1 cells, at D 0, SC Vaccine: 100 µg, on D 2, 9 and 16 after tumor injection Cyclophosphamide: 50 mg/kg, on D 1, 8, 15, IP	IM in the tibialis anterior muscle	-Combination therapy increased median survival time of mice -Suppression of IL-10, VEGFα and CXCL12 mRNA expression	2016 (213)

	BALB/c mice	CpVR-FAP, encoding FAP	/	**Prophylactic and therapeutic vaccination** Tumor: 2 x 10^4 4T1 cells, SC Vaccine: 100 µg, on D 2, 9 and 16 after tumor injection or 3 times every 2 W before tumor injection	IM in the tibialis anterior muscle	-Specific CTL response against FAP -Increased IL-2 production -Delay of the tumor growth also in therapeutic setting -Decrease in FAP expression without impairing wound healing	2016 (214)
	BALB/c mice	pVAX1-mCr-1, encoding mouse Cripto-1 oncofetal protein	/	**Prophylactic vaccination** Vaccine: 40 µg Tumor: 2 x 10^5 4T1 mCr-1 cells, W12	ID EP	-Humoral response against Cr-1 -Protective immune response against cancer stem cells -Reduced lung metastasis	2018 (215)
Colon cancer (colon 26/β-gal cells)	BALB/c mice	pcDNA3/β-gal encoding β-galactosidase	pCAGGS/FasL encoding Fas ligand	**Prophylactic vaccination** Vaccine and adjuvant: 50 µg + 1 µM cardiotoxin to facilitate DNA uptake Tumor: 10^6 Colon 26/β-gal cells, 21 D after vaccine injection, SC	/	-The combined therapy decreased tumor growth rate -Production of Abs anti-β-gal	2015 (216)
Colon cancer (CT26/HER2 cells)	BALB/c mice	pVAX1-HER2, coding HER2 antigen	Gemcitabine, chemotherapy agent; anti-Gr1 antibody; anti-PD-L1 Ab	**Prophylactic and therapeutic vaccination** Tumor: 3-5 x 10^5 CT26/HER2 cells, SC Vaccine: 50 µg Anti-PD-L1 and Gr-1 Ab: 200 µg and 250 µg, respectively, IP Gemcitabine: 75 µg/g, 2 times/W, IP	IM EP	-In prophylactic vaccination, the combination of vaccine + anti-PD-L1 Ab failed to delay tumor growth -The addition of anti-Gr1 or gemcitabine delayed tumor growth	2017 (217)
Colon cancer (CT26 cells)	BALB/c mice	CpVR-MS and CpDV-IL-2-MS, encoding a fusion gene of human surviving S8 and human 33 MUC1, plus IL-2)	Ad-MS (Adenovirus)	**Therapeutic vaccination** Tumor: 10^6 CT26 cells, SC Vaccine: 100 µg, twice Ad-MS: 10^8 pfu, D1, 15 and 29	IM	-Specific immune response in splenocytes -Upregulation of CCL-19 and GM-CSF -Downregulation of PD-L1 and MMP-9	2018 (218)
Colorectal cancer (CT-	BALB/c mice	pcDNA-hNIS, expressing human sodium/io-	/	Vaccine: 100 µg, 3 times at 2 W intervals	ID	-Increase of IgG2a/IgG1 ratio -Increase of IFNg secreting cells and IFNg production -Th1 response	2018 (219)

26/NIS cells)		dide symporter (hNIS)		Tumor: 2W after the final hNIS DNA injection, 5×10^5 (left) or 1×10^5 (right) CT-26/NIS cells, SC		-Slower tumor growth	
Melanoma (B16F10-β-hCG cells)	C57BL/6 mice	CAVE = pSVK-VEGFR2-GFc-IL-12, Semliki Forest Virus expressing VEGFR2 and IL-12	CAVA = SFV replicon DNA vaccine targeting surviving and hCG antigens	**Prophylactic vaccination** Vaccine: 10 µg of CAVA/CAVE, 3 times at 10 D of interval Tumor: 7.5 x 10^4 B16F10-β-hCG cells, 7 D after the last immunization, SC	IM EP	-Combination of the 2 vaccines delayed tumor growth more efficiently than the single vaccine and increased mice survival -CAVE + CAVA decreased microvessel density	2015 (220)
Melanoma (B16F10 cells)	C57BL/6 mice	pSPD-gp100-CD40L, encoding gp100 inserted between mouse Surfactant Protein D (SPD) and CD40L	pIL-12, encoding IL-12p70; pcDNA3.1-GM-CSF encoding GM-CSF	**Therapeutic vaccination** Tumor: 5 x 10^4 B16F10 cells, ID Vaccine and adjuvants: 80 µg vaccine + 20 µg of each adjuvant plasmid, on D 3, 10 and 17	IM in hind quadriceps muscles	-Vaccine alone did not delay tumor growth, but the combination with the 2 adjuvants was very effective in increasing mice survival	2015 (221)
	C57BL/6 mice	pVAX1-MUCI, encoding mucin I glycoprotein	pVAX1-Flt3L, encoding Fms-like tyrosinase 3-ligand	**Therapeutic vaccination** Tumor: 1 x 10^6 B16F10 cells, SC Vaccine + adjuvant: 50 µg, priming when tumors were palpable, boosts after 7 and 14 D	IM EP	-Specific CTL and antibodies -Tumor growth suppression	2018 (222)
Melanoma (B16 cells)	C57BL/6 mice	p-mBAP31 and p-LAMP/mBAP31 = p43 and p43-Lysosomal Associated Membrane Protein (LAMP) vectors + mouse B-cell receptor-associated protein (mBAP)	/	**Therapeutic vaccination** Tumor: 5 x 10^4 B16 cells, at D 0, SC Vaccine: 50 µg, at D 3, 10, 17 and 24	SC	-No evidence of autoimmune disorders -High IFNg production, especially using LAMP vaccine -LAMP vaccine increased the CTL cytotoxicity -Suppression of tumor growth, especially using LAMP vaccine	2015 (223)
Melanoma	Horses	Minimalistic immunogenically defined gene expression (MIDGE)-Th1 vector + eqIL-12 and IL-1beta receptor	hgp100MIDGE-Th1; htyrMIDGE-Th1	**Therapeutic vaccination** Tumor: horses were already affected by melanoma Vaccine and adjuvants: 500 µg ID peritumorally and 500 µg IM into the semimembranosus muscle, 3 times	ID and IM	-Vaccine was safe and well-tolerated, except an increase in the body temperature on the day after injection and signs of acute inflammation -Tumor volume was reduced by 28.5%, but without significant differences if adjuvants were added	2015 (224)

		antagonist protein (ILRAP)-eqIL18					
Melanoma (B16F10-OVA cells)	C57BL/6 mice	pVAX2-OVA, encoding ovalbumin; pVAX2-gp100, encoding gp100	pVAX2-HIV-1 Gag	**Prophylactic and therapeutic vaccination** Vaccine: 1 µg (p-OVA) or 50 µg (p-gp100), at D 2, 9 and 16 (therapeutic) or 3 times every 2 W before the tumor challenge (prophylactic) Adjuvant: 1 µg, co-administered with the vaccine Tumor: 1 x 10^5 B16F10-OVA cells at D 0 (therapeutic) or 2 W after the last vaccine injection (prophylactic)	IM EP	-Delay of tumor growth and increase in mice survival -Codelivery of the adjuvant-encoding plasmid polarized the immune response towards a Th1-like phenotype	2016 (225)
Mastocytoma (P815 cells)	DBA/2 mice	Differently optimized pVAX2-P1A vaccines, encoding P815A	/	**Prophylactic and therapeutic vaccination** Vaccine: 50 µg, at D 2, 9 and 16 (therapeutic) or 3 times every 2 W before the tumor challenge (prophylactic) Tumor: 1 x 10^6 P815 cells at D 0 (therapeutic) or 2 W after the last vaccine injection (prophylactic)	IM EP	-Delay of tumor growth and increase in mice survival Activation of innate immunity related to the different CpG motif amount inside the P1A gene	2017 (203)
	DBA/2 mice	Optimized pVAX2-P1A vaccine, encoding P815A	Anti-CTLA-4, anti-PD-1	**Therapeutic vaccination** Tumor: 1 x 10^6 P815 cells at D 0 Vaccine: 50 µg, at D 2, 9 and 16 Anti-CTLA-4, anti-PD-1: 100 µg at D 3, 6 and 9	IM EP	-Survival reached 90% -Increase of specific T cell infiltration in the TME -Increase of IL-12 production -Decrease of metastasis formation	2018 (226)
Malignant tumor	HLA-A2.1/Kb transgenic mice	p-GST-YL66, against multiepitope YL66 (from COX2 and MAGE-4), linked with membrane permeable Tat-PTD and the universal Th epitope	/	Not available	/	-CTL-mediated tumor cell lysis in vitro and in vivo	2017 (198)

Colorectal cancer (MC32 cells)	C57BL/6 mice	Pc-DNA3-CEA, carcinoembryonic antigen (CEA)	Ab 4-1BB	**Prophylactic and therapeutic vaccination** Tumor: 1-5 x 10^5 MC32 cells, SC Vaccine: 50 µg, at 1 W of interval Ab anti-4-1BB: 50 µg, systemically, after vaccine injection	IM EP	-Antigen-specific CTL activity and tumor-protective immune response in prophylactic model -Ab 4-1BB increased CTL lytic activity -MC32 cells resisted to CEA DNA vaccination by loss of antigen presentation to CEA-specific CTL in therapeutic model	2015 (227)
Melanoma (B16), carcinoma (3LL)	C57BL/6 mice	SCT-KDR2, encoding the mouse β2microglobulin + KDR2 (VEGFR2 antigen peptide) + MHC class I H-$2D^b$, subcloned into pdDNA3.1	/	**Prophylactic vaccination** Vaccine: 50 µg, 3 times, at 1 W of interval Tumor: 10^5 B16 cells or 2 x 10^5 3LL cells, 10 D after the last vaccination, SC	ID	-CTL response to VEGFR2 -Inhibition of tumor-induced angiogenesis -Inhibition of tumor metastasis	2015 (200)
Sarcoma	HHDII-DR1 mice	SSX2-optimized vaccine	Ab anti-PD-1/L1	**Prophylactic and therapeutic vaccination** Vaccine: 100 µg, 6 times, every 2 W (prophylactic) or weekly the day after the tumor injection (therapeutic) Tumor: 2 x 10^4 SSX2-expressing sarcoma cells An anti-PD-1/L1: 100 µg, IP on the day following each vaccination	ID	-Optimized vaccine elicited inferior antitumor effect relative to the native vaccine -Increase of PD-L1 expression on tumor cells -CTL from immunized mice expressed more PD-1, increasing the antitumor efficacy of the combination with ICB	2015 (228)
Kidney cancer (RenCa cells)	BALB/c mice	pVAX1-G250-F2A-CTLA-4, containing the co-expression gene G250-CTLA-4, linked by Furin-2A (F2A)	/	**Therapeutic vaccination** Tumor: 10^5 RenCa cells, SC Vaccine: 50 µg, at D 7, 17 and 27	EP	-Humoral and cellular-specific immune response against CTLA-4 and G250 -Increase in INFγ and IL-4 (Th1/2 response) -Tumor growth rate decreased	2017 (229)

There is evidence that rationally combining therapeutic cancer DNA vaccines with other strategies may be synergic. In Figure 12, the main mechanisms of action postulated to mediate synergistic effects in combination with DNA vaccines are shown.

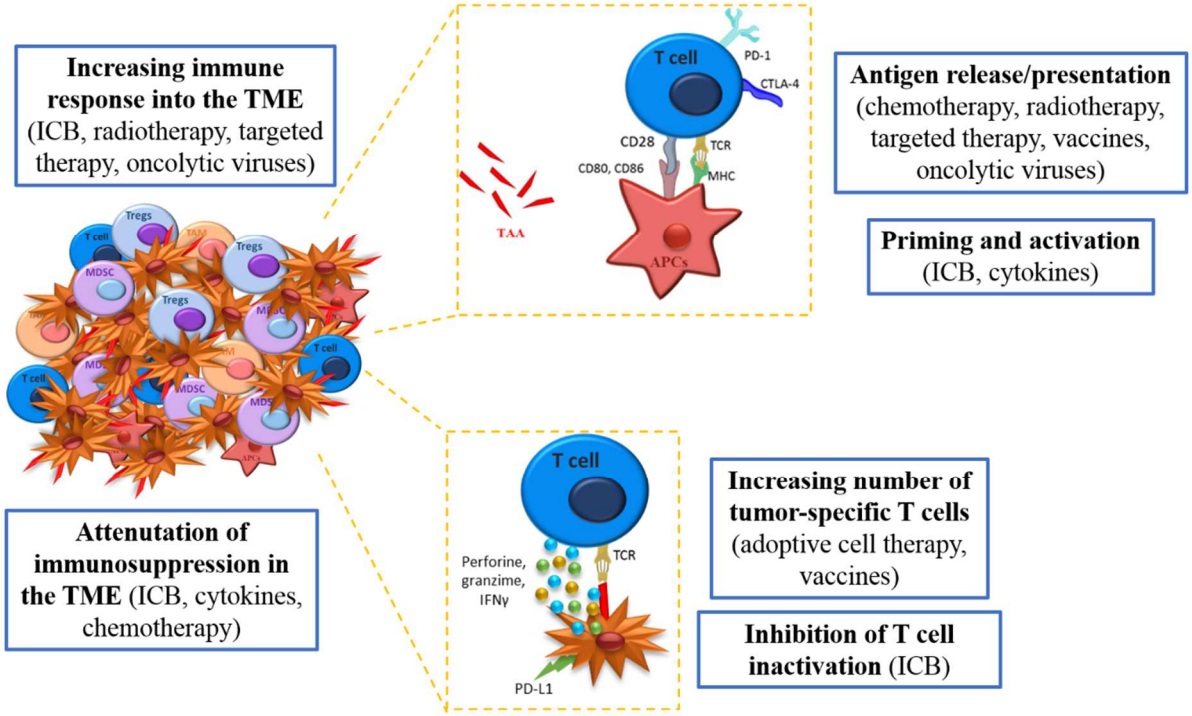

Figure 12: Mechanisms of action of therapies postulated to mediate synergistic effects in combination with DNA.

Therapies that can be rationally combined with DNA vaccination are:

1) Cytokines/adjuvants

Immunostimulatory cytokines can increase the effect of the vaccine on the effector T cells. They are generally encoded by the antigen-encoding vaccine or by another plasmid or injected as proteins in combination with the vaccine. The most used cytokines in recent studies include IL-2, IL-12 and GM-CSF. IL-2 is involved in differentiation of immature T cells into both Tregs and effector T cells. Its great efficacy against metastatic melanoma and metastatic renal cell carcinoma leads to its approval by the FDA (204, 230). IL-12 is another important cytokine involved in T cell activation and effector function and its combination with vaccine increases vaccine efficacy (221). A plasmid encoding IL-12 combined with a DNA vaccine against cervical cancer, promoted mice survival and decreased the number of MDSC in the TME (207). GM-CSF is used in many clinical trials (Table 5) for its activity on DC maturation, T cell activation and proliferation. However, this molecule can also attract MDSC and it is not clear how this cytokine plays a balance between immune activation and inhibition in vivo and current clinical studies are trying to answer to this

question (204). Other cytokines could be used in combination with DNA vaccines, e.g., INFg, IL-15, IL-7 etc. (202, 204).

Combination with other type of adjuvants could also be tested, such as TLR-activators. As already mentioned, CpG oligonucleotides are able to activate TLR-9, thus inducing memory B cell proliferation and DC activation (47).

2) Chemotherapy

In the last few years, it has been reported that anti-cancer chemotherapy can play a double role on tumor eradication. Many chemotherapeutic drugs, such as gemcitabine (231), paclitaxel (232), cyclophosphamide (233) and others, can target tumor cells inducing TA release and the release of DAMPs, thus activating the immune response (a process known as "immunogenic cell death" (ICD)) (234). Furthermore, the same drugs, applied in ultra-low (metronomic), frequently repeated and non-cytotoxic doses, can also target the TME to enhance T cell infiltration/activity in the TME, reduce the angiogenesis and remove immunosuppressive cells.

In a preclinical study, the combination of cyclophosphamide with DNA vaccines enhanced mice survival and decreased the expression of immunosuppressive cytokines, such as IL-10, VEGF etc. (213). Based on preclinical and clinical studies, the combination of chemotherapy and vaccine therapy may play a substantial role in future cancer treatments, especially when patients do not respond to ICB (235). Indeed, it has been demonstrated that treatment with chemotherapy restored sensitivity to checkpoint blockade through TLR-4 stimulation (235). Further clinical studies are necessary to better define the optimal agents and schedule of administration.

3) Targeted therapy

DNA vaccine could also be combined with targeted therapies that are able to mediate tumor cell antigen release and enhance T cell priming. Sunitinib, a multi-targeted receptor tyrosine kinase inhibitor, was found to decrease Tregs, MDSC and increase INFg-producing T cells in renal cell carcinoma patients (236). The combination of sunitinib with a viral vaccine encoding CEA decreased the tumor volume in a mouse model (237). Albeit not already tested with DNA vaccines, other tyrosine kinase inhibitors already approved by FDA, such as pazopanib, axitinib, cabozantinib, could improve patient response to vaccination.

4) Endocrine therapy

In hormonally-driven tumors such as prostate cancer and breast cancer, endocrine therapy is part of the standard of care and the effect of letrozole in decreasing the Tregs in the TME has been already demonstrated (238). Furthermore, androgen deprivation in prostate cancer induces thymic

regeneration and increases the number of effector T cells (204). In an ongoing clinical trial (NCT02204098, phase I), the effect of MamA vaccine administration in combination with Anastrozole, Letrozole, Tamoxifen, Exemestane, Goserelin is under investigation.

4) Radiotherapy

Preclinical data have demonstrated the additive effect of RT and vaccine, with enhanced destruction of tumor cells, release of TAs, increase of IFNg production and a global decrease of the tumor volume. T cells specific for other antigens not included in the vaccine were also generated (239, 240). In some cases, radiation treatment induced the "abscopal effect" in metastatic cancers. The abscopal effect describes a phenomenon of tumor regression that occurs distant from the site of treatment (241), probably due to the induction of ICD (242). This concept, described for the first time for the radiotherapy, could be applied to other therapies, which are administered in the tumor and that induce a systemic effect, such as OVs or IT EP (243, 244).

Since RT is a part of the standard of care, many trials using vaccines after radiation try to evaluate the mutual effects from the two therapies (Table 5).

5) Immune checkpoint blockade (ICB)

The signaling mechanism mediated by co-stimulatory/inhibitory molecules plays an important role in the T cell-mediated immunity. Many cells in the TME can express ligands for inhibitory receptors on the T cells, leading to their inactivation (245). Inhibitory receptors include CTLA-4, PD-1, TIM-3, LAG-3 etc. (245). ICB are antibodies directed against inhibitory receptors on T cells that allow the release of the "breaks" to enhance T cell activity, which ultimately results in increased antitumor immunity (246). In several studies, the in vivo blockade of CTLA-4 delayed tumor growth in animal models and resulted in tumor rejection in patients affected by melanoma (247, 248). This effect was mainly due to the inhibition of the TGFb and IL-10 secreting Tregs, but also to an increased T effector cell activation (249). Interestingly, this also resulted in immunity against secondary exposure to tumor cells, suggesting that the memory component of immune response can be evoked by anti CTLA-4 antibodies (250). Antagonist antibodies that target the PD-1 and its ligand PD-L1 have also achieved impressive and durable results in many solid tumors, leading to their FDA approval for different cancer types (204). Recently, a relationship between the ICB administration and the neoantigen burden has been demonstrated (251). Snyder *et al.* sequenced 64 patients with advanced melanoma and showed that somatic mutation burden was strongly associated with clinical response to anti-CTLA-4 (252). Similarly, Rizvi *et al.* demonstrated that mutation burden was a strong predictor of clinical response in non-small cell lung cancer (NSCLC) patients treated with anti-PD-1 therapy and that this therapy enhanced neoantigen-specific T cell

reactivity (253). The higher prevalence of somatic mutations in cancer cell genomes was a common feature among cancers with a higher probability of response to ICB. Thereafter, the link between mutation burden and clinical benefit following ICB immunotherapy was validated multiple times and in multiple tumor types (173). This is related to the concept that with increased tumor mutation burden (TMB), the probability of a cognate T cell clonally expanding against a specific tumor antigen will increase. In other words, tumors with high TMB often have more neoantigens that could be recognized by processes involved in antitumor immunity, making such cancers more likely to respond to ICB therapy (170, 254).

The number of clinical trials using ICB therapy is continuously increasing in the last years. Figure 13 shows the number of clinical trials using anti-PD-1 checkpoint blockade starting from 2013 until now. However, only a minority of patients responds to ICB therapy suggesting the need of a rational use of ICB based on biomarkers predictive of the immune response, to avoid the non-responsiveness to the therapy and undesired side effects (255, 256). To increase T cell activity in the TME and to broad the number of patients responding to ICB, combinations of ICB with different strategies were tested for a variety of malignancies in preclinical and clinical studies (245). Examples are: combination with radiation therapy (257), other antibodies (258), chemotherapy (259), cancer vaccines (260) etc. The number of trials using combination therapies is increasing, even if most of the trials are still in phase I or II (261). In particular, combination with DNA cancer vaccination seems to be promising to couple the benefits of ICB with the ability of vaccines to prime the antigen-specific CTL response (199, 226). Indeed, one of the reasons for ICB failure could be the absence of a pre-existing immunity (262). Hence, a potent cancer vaccine that could induce a T cell response against TAs might increase the number of responders to ICB (173).

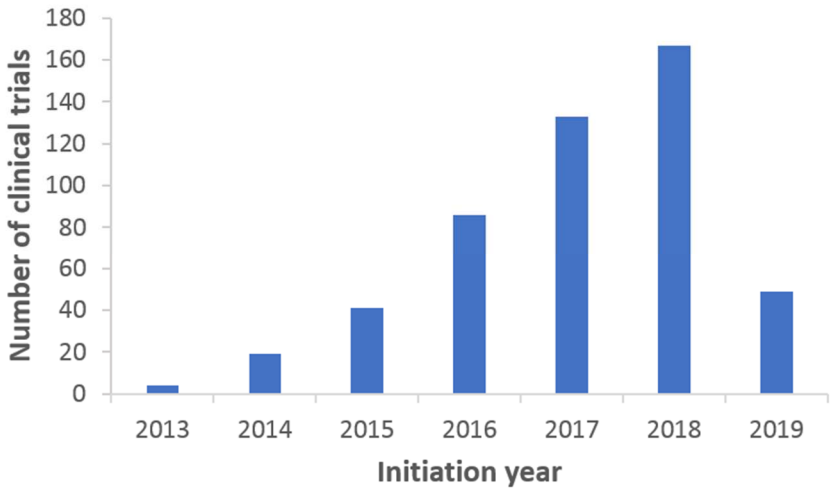

Figure 13: The number of clinical trials using anti-PD-1 ICB per year, starting from 2013 until March 2019. Keywords in clinicaltrials.gov: "anti-PD-1", "cancer", accessed 1/03/2019.

6) Oncolytic viruses (OVs)

OVs are native or genetically modified viruses selected or designed to specifically infect and kill cancer cells (263). Although OVs can enter both normal and cancer cells, the intrinsic abnormalities in the cancer cell response to stress, cell signaling and homeostasis, allow OV replication in the tumor cells. Indeed, compared to normal cells, cancer cells have an altered cell physiology, such as self-sufficiency in growth signals, insensitivity to growth inhibition signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis and tissue invasion and metastasis. OVs can exploit these characteristics for their selective replication in cancer cells (264). Some viruses are naturally selective for cancer cells (e.g., Vaccinia), while others need to be modified to specifically attack tumor cells (265). To obtain OVs (or more selective OVs), many viruses have been engineered, either by deleting viral genes required for virus replication in normal cells but dispensable in tumor cells or by using tissue/tumor specific promoters for critical viral genes (24, 263, 266).

OVs antitumor effect is due to two different mechanisms of action: (1) Direct lysis (i.e. oncolysis) of the infected cancer cells. (2) Immune response activation, by inducing an anti-viral immune response against the OV (24, 267), by generating proinflammatory signals due to cell damage (IFNg, TNFa, IL-12 etc.), but also by a possible release of TAs from the killed cancer cells (263, 268) (Figure 14).

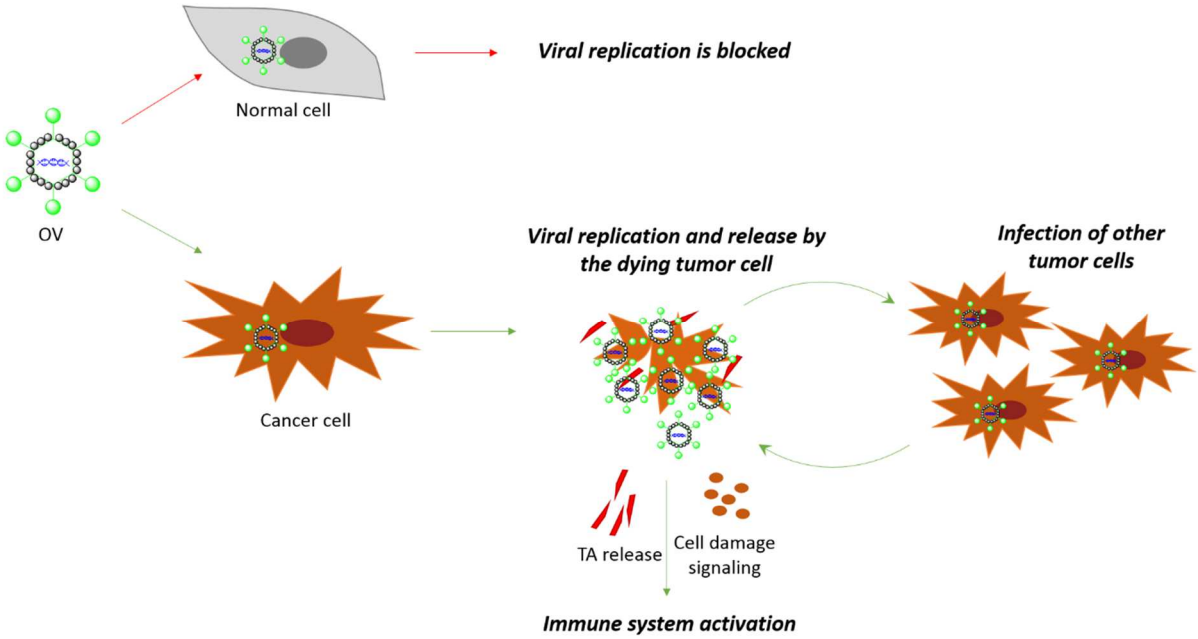

Figure 14: Mechanism of action of OVs in infecting normal vs cancer cells. The ability of OVs to selectively proliferate in cancer cells and to lysate them allow the TA release and the generation of cell damage signaling to activate a specific immune response against tumor cells.

However, the activation of the immune system could induce the clearance of the virus, thus reducing its therapeutic activity (263). The main limitation to OV therapy in the clinic is the poor/modest anti-tumor immune response (267). For this reason, many studies try to potentiate the OV immune response, by modifying the OV and/by combining OV with other therapies (268).

OV anti-tumor immune response can be increased by inducing the viral expression of pro-inflammatory cytokines and/or T cell co-stimulatory molecules (e.g., GM-CSF (269) or different ILs (270, 271)), by enhancing their lytic activity (e.g., TNFa insertion in the virus) (272), or by limiting the antiviral immune response against the virus, thus prolonging its half-life in the body (e.g., by using another virus serotype), etc. (263, 268, 273, 274). It is also possible to modify the genome of an OV to encode TAs (271, 275, 276) or to link antigen peptides on the viral surface (267, 277).

Another approach to increase OV immune response against cancer is its combination with other therapies. The good safety profiles of OVs and their ability to recruit T cells in the TME and to take advantage from the tolerogenic and immunosuppressive TME provides a rational strategy for combination treatment with other anti-tumor therapies (15, 271).

Many OVs have been clinically evaluated in combination with conventional anticancer therapies, such as chemotherapy and radiation therapy. It has been shown that the combination of an oncolytic adenovirus (OAd) and gemcitabine enhanced the anti-cancer apoptotic response, probably due to increased sensitivity of tumor cells to chemotherapy in the presence of OAd (15). In the case of the doxorubicin, the rationale under the combination with OAd is the positive effects of doxorubicin on virus replication.

OVs may act as cancer-selective radiosensitizers, enhancing the therapeutic activity of radiotherapy on tumors while sparing normal tissues. Indeed, viruses can inhibit cellular DNA repair pathways to protect themselves from unwanted interference by cell processes that are normally triggered by DNA damage. Exploiting these abilities to inhibit cellular DNA repair following damage by therapeutic irradiation may increase the anticancer potency of the combination (278, 279).

In several clinical studies, one of the most used approach is the association of OVs with ICB (e.g., NCT03206073, NCT02977156, NCT02798406). Indeed, OVs often induce IFN release in the local TME (280), which is known to upregulate PD-L1 expression on tumor cells; for this reason, the combination with anti-PD-1 or anti-PD-L1 would be especially interesting (15, 210, 263, 281). Furthermore, OV infection induces an anti-tumor immunity, thus recruiting the T cells in the TME; the further addition of the ICB ensures that those T cells remain active (275). In a phase Ib clinical trial, the combination between T-VEC and anti-PD-1 was tested against melanoma. The

combination therapy allowed a change in the TME, by increasing the T cell infiltration, IFNg signature and PD-L1 protein expression compared to the single therapies. An objective immunological response was reached by the 35-40% of the patients and a disease control rate in 76% of the patients (282).

Other strategies have already been tested in combination with OVs, such as ACT or heterologous prime-boost strategies, i.e., a series of multiple immunizations using different vectors (other viruses or different vaccine platforms) to deliver the same TA, thus potentiating the activated immune response (283).

One proposed reason for the decreased efficacy of ACT in solid tumors involves the inefficient trafficking of adoptively transferred cells to the tumor or their poor persistence in the TME. In this respect, OV can establish an inflammatory environment, which could facilitate the recruitment and expansion of the transferred cells. Furthermore, adoptively transferred cells can be used as OV carriers to combine the effects of OV therapy and ACT (284).

Until now, only few studies have analyzed the combination between OVs and cancer vaccines (285-287). Furthermore, at our knowledge, none of them showed the efficacy of DNA vaccines combined with OAd in a non-heterologous prime-boost vaccination. Further studies are needed to better characterize this interaction and the mutual role of the DNA vaccination and oncovirotherapy in the global anticancer effect.

7) Tumor resection

For decades, surgical therapy (with or without chemotherapy or radiotherapy) was the only treatment associated with improved long-term survival and the ability to provide palliation in patients affected by different types of resectable cancers (288-291). However, non resectable, recurrent or metastatic tumors often remained very difficult to treat (291, 292).

Nowadays, tumor resection still represents one of the most used technics to control the growth of resectable tumors and to increase patient survival. However, it is not used anymore as a single therapy approach. In the case of cancers such as GBM or colorectal cancer, complete or partial resection remains the first line treatment, when tumors are operable (293, 294). Nevertheless, the presence of residual cells, metastasis or recurrences arises the necessity of using other therapies to try to eradicate the pathology (293, 294). Beside the classically used chemotherapy or radiotherapy, there is an urgent need for additional therapeutic options, given the limitations of the current standard therapies (295, 296).

Since surgical removal of tumors induces an inflammatory response (297), tumor resection could be the ideal partner for cancer DNA vaccination. Indeed, the inflammatory responses produced by the surgery, locally in the TME, could recruit the adaptive T cell response generated by the vaccine and induce the regression of the residual cancer cells. In this way, the role of tumor resection could change from the simple tumor bulk removal to the adjuvant treatment in the immunotherapeutic context. If the combined effect of the two treatments could be demonstrated, this will leave a place in the standard of care treatment for cancer vaccination also in resectable tumors, to ensure the complete eradication of the residual tumor cells and a long-lasting protection against recurrences.

V. CLINICAL TRIALS USING THERAPEUTIC CANCER DNA VACCINATION

V.1. RESULTS OF COMPLETED CLINICAL TRIALS

Many already completed clinical trials tested the efficacy of DNA vaccines against different tumor types, such as breast, cervical, pancreatic and prostate cancers, multiple myeloma, and melanoma. These trials aimed at principally evaluating the safety and immunological response of DNA vaccines. A search for studies with "cancer" and "DNA vaccines" in clinicaltrials.gov (298) revealed 48 studies in the last 10 years with the following criteria: "completed", "suspended" and "terminated". Among the trials using DNA vaccines in a therapeutic approach, only a few of them have published results to date. Here, a non-exhaustive list of completed studies using naked DNA vaccines and containing results is described.

The NCT01304524 phase IIb clinical study tested the safety and efficacy of VGX-3100, a DNA vaccine targeting HPV 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia grade 2/3. Six milligrams of the vaccine were delivered by IM EP at 0, 4 and 12 weeks without any severe side effects, but only mild reactions in the injection site, fatigue, nausea and general malaise in some of the patients. The vaccine was generally well tolerated and showed great efficacy against the pathology in almost 50% of the treated patients, as shown in the histopathological and immunological analysis. Indeed, VGX-3100 elicited significantly increased frequencies of antigen-specific activated CD8 T cells and a higher humoral response compared to the placebo, making it the first therapeutic vaccine to elicit a complete adaptive immune response in patients with preinvasive cervical disease caused by HPV-16 and 18 (299). Two phase III clinical trials (NCT03185013 and NCT03721978) using VGX-3100 are ongoing, as shown in Table 2.

Recently, Kim et al. published the results of the clinical trial NCT01634503 concerning the safety and efficacy of GX-188E, another plasmid DNA encoding the E6 and E7 proteins of HPV serotypes 16 and 18. The vaccine was injected 3 times (weeks 0, 4 and 12) IM to alternating deltoid muscles, and three different doses were tested (1, 2 or 4 mg). Importantly, 8/9 of the patients exhibited an enhanced polyfunctional HPV-specific CD8 T cell response, and 7/9 of the patients displayed a complete regression of their lesions and viral clearance within 36 weeks of follow-up. The vaccine administration did not elicit serious vaccine-associated adverse events and was estimated to be safe and well tolerated (300).

Other published results show the properties of mammaglobin-A (Mam-A) DNA vaccination for patients with breast cancer. Mam-A is a tumor-specific secretory protein overexpressed in 80% of

human breast cancers. In a phase I clinical trial (NCT00807781), 4 mg of a pING-Mam-A DNA vaccine was administered at weeks 1, 4 and 8 IM to patients with metastatic breast cancer. The first results demonstrated the safety of the vaccine, with no significant side effects. The main observations about its efficacy were (i) an increase in the generation of specific Mam-A CD8 T cells and IFNg production; (ii) a decrease in the frequency of Tregs and lower levels of IL-10; and (iii) an improved progression-free survival compared to the control group. These encouraging results suggest that Mam-A DNA vaccination can induce antitumor immunity in breast cancer patients and increase survival time (301-303).

In another phase I/II study (NCT00859729), 50–1600 μg of a pVAXrcPSAv531 plasmid coding for the full-length PSA protein were ID injected and electroporated in patients with relapsed prostate cancer. The vaccine followed radiotherapy and endocrine therapy with an LH-RH analogue (leuprorelin). No systemic toxicity was observed, and discomfort from EP did not require the use of topical anesthetics. A general increase in T cell reactivity was observed in most patients, although IM immunization seemed to result in more potent antibody responses (116).

A personalized DNA vaccine was tested in patients with multiple myeloma in a phase I clinical trial. The DNA encoded a patient-specific single chain variable fragment linked to fragment C of the tetanus toxin. Six doses of 1 mg of the vaccine were injected IM after chemotherapy or autologous stem cell transplant. In total, 72% of the patients generated a cell-specific immune response, and the overall survival was 64% after a median follow-up of 85.6 months (304).

A phase II clinical trial (NCT01334060) evaluated the safety and efficacy of a pDOM-WT1-37 and pDOM-WT1-126 DNA fusion gene vaccine encoding the Wilms tumor antigen 1 for leukemia patients. The plasmid was injected using IM EP, with no severe side effects. However, combination strategies to expand T cell responses with immunomodulatory antibodies are in development (305).

Interestingly, Niethammer et al. reported a phase I clinical trial (NCT01486329) using an oral vaccine (VXM01) against the VEGF-Receptor 2 with Salmonella typhimurium as a carrier, in addition to chemotherapy with gemcitabine, in patients with stage IV and locally advanced pancreatic cancer. The doses consisted of a solution containing 106, 108, 109, and 1010 colony forming units of VXM01. Even if this formulation is not a naked DNA vaccine, VXM01 represents a novel strategy, which does not target a tumor cell-resident antigen but instead a tumor stroma-resident antigen overexpressed by the nonmalignant endothelial cells of the tumor neovasculature, giving the vaccine the potential to target many cancer types (306). The same vaccine is also being tested in GBM patients (NCT02718443).

Other 19 studies were found in PubMed using the following criteria: "cancer DNA vaccine", article type "clinical trial", starting from 2013 until now. Most of the studies focus on prophylactic immunization with HPV DNA vaccines. Two phase I studies show some results of therapeutic cancer DNA vaccination (NCT00250419 and NCT00647114). Both of them used the HER2/CEA DNA vaccine V930 and showed the instauration of both humoral and cellular immune responses with no detectable immune response against the vaccine itself. As CEA and HER2 are expressed by many solid tumors, patients with different types of cancer were recruited. The vaccination dose was on the order of a few milligrams every 14 days for 5 injections, and the plasmid was injected by IM EP. However, in this case, no evidence of an increase of a HER/2 or CEA-specific response was observed (307).

Overall, vaccination is used after conventional therapies. Completed, terminated and suspended clinical trials reported only minor discomfort after vaccination, no important side effects and, generally, an increased number of CD8 T cells specific for the antigen encoded by the DNA vaccine. Most of the trials used DNA vaccines encoding non-mutated TAs, and only a few tested personalized approaches (neoantigens).

V.2. ONGOING CLINICAL TRIALS

In searching all the cancer DNA vaccine interventional clinical studies in the last 10 years with the criteria "not yet recruiting", "recruiting", "enrolling by invitation" and "active non-recruiting", we found 56 studies. Among them, 27 studies used DNA vaccines as naked plasmids not encapsulated in cells or in virus-like nanoparticles. These studies are listed in Table 5. They are all in clinical phase I or I/II or II, and DNA vaccines are generally administered after the standard of care for each cancer type, including surgical ablation, radiotherapy and/or chemotherapy. The results for these trials are not yet available, except for the trial NCT00849121. This study used a DNA vaccine encoding prostatic acid phosphatase (PAP), with GM-CSF as an adjuvant, administered ID into patients with prostate cancer. Only one of the 17 patients experienced a vaccine-related adverse event of grade 2 or more, more than half had a great PAP-specific CTL response, and in 7/17 patients, the PSA doubling time increased during the treatment period. Twelve of the 17 patients (70%) were metastasis-free after one year of treatment (298).

Another study with the criteria "DNA electroporation" and "cancer" led to 3 more trials ("not yet recruiting", "recruiting", "enrolling by invitation" and "active non-recruiting") in the last 10 years: NCT03499795, NCT03491683, and NCT02301754. With the criteria "plasmid" and "tumor", we found 2 additional studies: NCT02531425 and NCT03502785. These are all listed in Table 5.

Of particular interest are the only 2 studies we found in phase III (NCT03721978 and NCT03185013) using VGX-3100 delivered by IM EP against cervical cancer.

Breast, prostate and cervical cancers are the most studied in the trials (Figure 15A). Most of the vaccines encode well-known non-mutated TAs (here indicated as tumor-associated antigens (TAAs), such as E6/7 HPV protein for cervical cancer, Mam-A or HER2 for breast cancer, PAP for prostate cancer, etc.). Only 20% of the clinical trials used personalized/neoantigen vaccines (e.g., NCT02348320 and NCT03122106), as shown in Figure 15B. This number has increased in recent years: 80% of the trials using neoantigens started in 2018–2019. Generally, more than one epitope is encoded by the DNA vaccines in both TAA and neoantigen vaccines (Figure 15B).

DNA vaccines are mostly associated with other therapies: immunotherapies (antibodies anti-CTLA-4, anti-PD-1, anti-PD-L1, and cell vaccines), immune adjuvants (GM-CSF, hIL-12, etc.) generally injected with the DNA vaccine or encoded in the vaccine itself, chemotherapy (carboplatin, paclitaxel, cyclophosphamide), and endocrine therapies (anastrozole, letrozole, tamoxifen, exemestane, and goserelin). In recent years, the number of studies using other therapies in combination with DNA vaccines has also increased (Figure 15C). DNA vaccines are usually injected IM or ID, in rare cases SC or in the lesion/tumor, and electroporated after the injection. The doses can vary from 100 μg to a few mg. The regimen of administration depends on the type of vaccine, but in all trials, vaccines are injected more than once, at 2–4 weeks of intervals, and the therapy lasts for a few months.

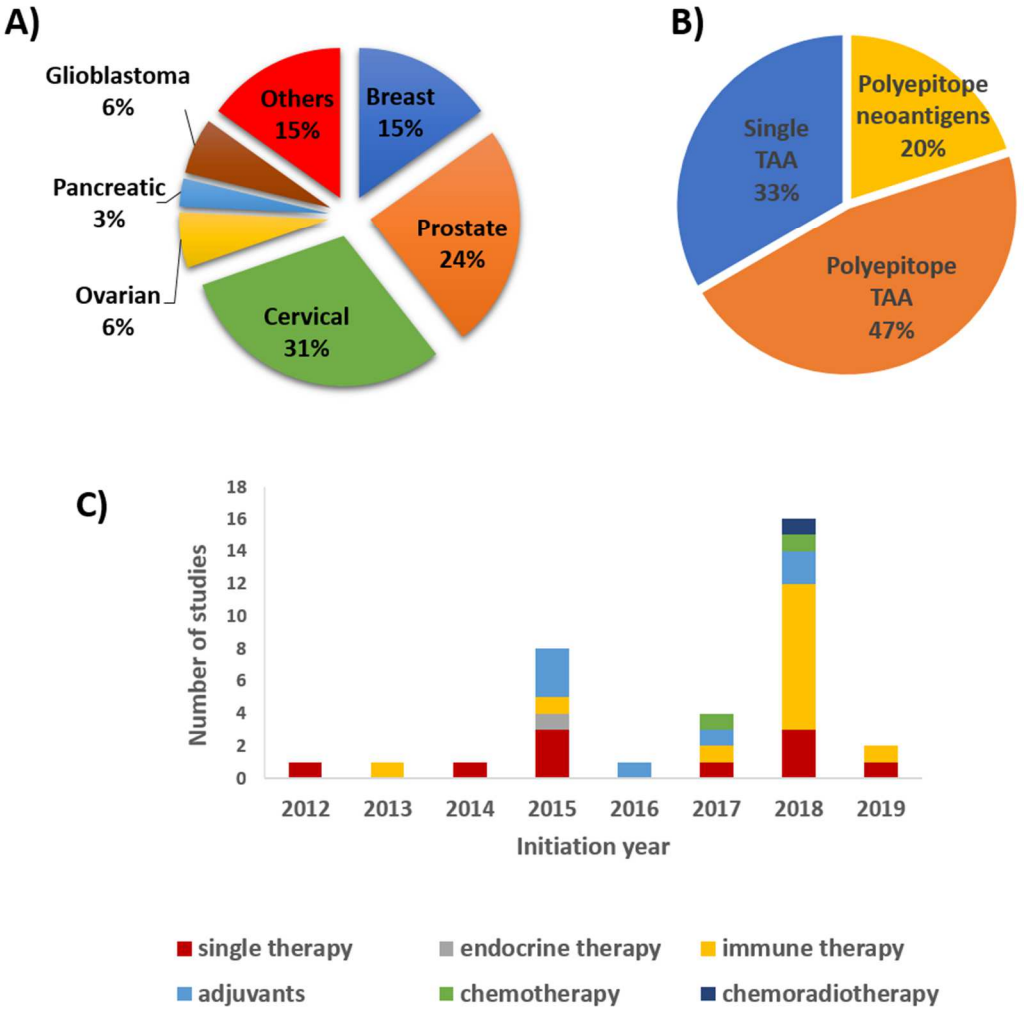

Figure 15: Ongoing clinical trials of the analyzed studies. A) Cancer types using cancer DNA vaccines in clinical trials. B) Type of antigens encoded in the DNA vaccine. C) Studies combining cancer DNA vaccines with other therapies (endocrine therapy, immunotherapy, chemotherapy, chemoradiotherapy or adjuvants) or using DNA vaccines as a single therapy.

VI. TUMOR PRECLINICAL MODELS AND RELATED ANTIGENS

DNA vaccination can be used for many different types of cancer. The real challenge is to design the right vaccine, according to the tumor specificities, and to combine it with the most appropriate therapeutic strategy for the single patient and the single cancer. Indeed, each tumor has its own characteristics, and therapeutic approaches should consider this heterogeneity.

From an immunological point of view, not all cancers have the same T cell infiltration, nor the same TAs expression and/or tumor mutation load (or tumor mutation burden, TMB) (308). The TMB is a measure of the number of mutations within a tumor genome, defined as the total number of mutations per coding area of the tumor genome (definition of the European Society for Medical Oncology, ESMO) (309). These effects affect the response to immunotherapy and the overall survival of patients (310, 311).

According to the T cell infiltration, tumors are generally classified as "hot" when the immune cell infiltration is high in the tumor bed and in the invasive margins, or "cold" or "immune desert" when the amount of tumor-infiltrated T lymphocytes (TILs) is low or absent. In between, there are hot tumors with exhausted or ineffective TILs, and "excluded" tumors, where immune cells are present only in the periphery of the tumor (312).

Cancers can also be classified as either high or low TMB tumors, based on the prevalence of somatic mutations in their genome, which is often related to the number of neoantigens (254). Although a tumor's genotype can shape its microenvironment, higher mutation rates are not necessarily related to high immune infiltration. Thus, a high TMB tumor doesn't always have an immunologically hot phenotype (254). Furthermore, a different TMB or TIL infiltration degree can be observed within the same cancer type (313).

Here, we will underline the most important characteristics of the tumor models used in this thesis, especially from an immunological point of view. A brief state of the art of the treatments available for the clinically relevant tumors will also be discussed.

VI.1. P815 MASTOCYTOMA PRECLINICAL MODEL

Mastocytomas are clinically rare tumors, often already present at birth or developed within the first months of life, but extremely sporadic in adults. They belong to the family of cutaneous mastocytosis (314).

From a preclinical point of view, the P815 mastocytoma represents a relevant model in the immunological studies, as it expresses the murine homologues of MAGE-type antigens, such as P815 AB, C, D and E, capable of inducing a CTL response (315). Like the MAGE-family antigens, P815 antigens are not expressed in most adult tissues, except for testis and placenta, which do not present the antigen in the context of MHC class I, and, therefore, they are highly tumor-specific (316).

The TAs expressed by P815 were among the first identified TAs and, for several years, P815 represented one of the best-studied mouse tumors in immunology and immunotherapy (317). In particular, the P1A gene encodes the antigen P815AB, in the form of a nonameric peptide containing two epitopes, P815A and P815B (315). This antigen has been used for different immunological preclinical studies using cancer vaccines, such as viral vaccines or DNA vaccines (318-320).

In Table 7, a non-exhaustive list of studies using DNA vaccination in the P815 model. Most of the studies use P815 cells transfected with a potent TA, to induce a strong immune activity; only few of them use P815 antigen. Generally, the vaccine is administered before the tumor injection (prophylactic vaccination) to significantly delay the tumor growth; following the vaccination, the presence in the tumor of antigen-specific CTLs is also shown. We can also remark that the most used techniques for the delivery of the DNA vaccine were the co-administration of transfecting agents (e.g., cardiotoxin and bupivacaine) or the GeneGun to increase the cell uptake of the plasmid. In particular, the bupivacaine is a local anesthetic, which induces a temporary damage to muscle fibers, thus recruiting inflammatory cells in the injection site. It forms a complex with the plasmid, which protects the DNA from nuclease degradation, thus increasing the gene expression and the immune activation (321). EP started to be used only recently.

Table 7: Preclinical studies using DNA vaccination in P815 model. Keywords in Pubmed: "P815" and "DNA vaccine" (accessed 08/03/2019). D = day, W = week.

Antigen	Vaccination schedule	Tumor injection schedule	Results	Year and reference
Large tumor Ag (T-Ag) of the papovavirus, SV40	Prophylactic: 1 or 10 µg, IP or SC	10^4 P815 cells, SC	TA-specific CTL	1996 (322)
P815A	Prophylactic: 100 µg, IM, 3 doses at 10 D interval	10^6 P815 cells, SC, 3 W after the last immunization	CTL response, prolonged survival	1997 (323)
Nucleoprotein of influenza virus	Prophylactic: 100 µg, IM, 2 doses at 3 W interval	10^4 P815-NPep cells, encoding the nucleoprotein of influenza virus, IP, 2 W after the last immunization	TA-specific CTL	1998 (324)
Human carcinoembryonic antigen (CEA)	Prophylactic: 50 µg for each paw, IM, 5 doses,	10^6 CEA^+ P815 cells, SC, 1 W after the last immunization	Humoral and cellular immune response, but only partial inhibition of the tumor growth	2000 (325)
Oncofetal alpha-fetoprotein (AFP)	Prophylactic: 50 µg IM or 1 µg ID (GeneGun)	10^4–10^6 P815-neo-AFP cells, SC	Mean survival was prolonged for 35 D	2002 (326)
SV40 large T-Ag	Prophylactic: 50 µg for each tibialis muscle (GeneGun)	10^3 $T\text{-}Ag^+$ P815 cells, SC	CTL stimulation by cross-priming	2002 (327)
P815	Prophylactic and therapeutic: 100 µg with bupivacaine HCl, IM, 3 doses over a 2-W interval	$2x10^5$ P815 cells, SC, 10 D after the last immunization; $5x10^4$ 4 D before the first immunization	Slowdown in the tumor growth and increased survival, especially using the prophylactic vaccination	2004 (328)
HIV-1 multigene, containing fused full-length sequences of rev, nef, tat, gag	Prophylactic and therapeutic: 1 µg, at W 2,4 and 5 (GeneGun)	10^6 P815-MultiHIV cells, SC, 2W after the last immunization; $0.2x10^6$ for the therapeutic vaccination	Delay in the tumor growth for the prophylactic vaccination	2007 (329)
P1A or $P1A_{35–43}$ epitope	Prophylactic: 50 µg for pP1A or equimolar amount for $pP1A_{35-43}$, IM (EP)	10^6 P815 cells, SC	TA-specific CTL, delay in the tumor growth	2014 (318)
Beta-galactosidase (b-gal)	Prophylactic: 10 or 50 µg, IM	10^6 $b\text{-}gal^+$ P815 cells, SC, 21 D after the vaccine injection	Delayed tumor growth	2015 (330)

VI.2. MELANOMA AND THE B16 PRECLINICAL MODEL

Cutaneous melanoma is the most potentially lethal and most aggressive skin tumor (331, 332). Its metastatic form is known for its resistance to traditional cancer treatments, including chemotherapy and radiotherapy, as well as its relative responsiveness to immunotherapy, compared with other cancer types (333). Currently, in clinic, systemic treatment of unresectable or metastatic melanoma mostly includes targeted therapy and immunotherapy (334-336). The 20% of patients treated with anti-CTLA-4 alone survived more than 3 years after the treatment (337). When anti-CTLA-4 is combined with anti-PD-1/PD-L1, almost half of the patients have an increased overall survival up to 3-5 years (331, 332). However, not all the patients respond to ICB and the immune-related grade 3 or 4 adverse events are very common, especially when the combination of two ICB is used (331, 338). Other treatments include OV therapy (e.g., T-VEC), ACT and chimeric antigen receptor (CAR)-T cell therapy, chemotherapy and the different combinations among these agents, as reviewed in (332, 336). Currently, new tested strategies include antibodies against other immune checkpoints (e.g., LAG-3, TIM-3), antibodies targeting T cell agonistic receptors (e.g., OX-40) (336), and therapeutic cancer vaccines (339). The results with DNA vaccines showed that the vaccination was well tolerated and, only in few patients, grade 1–2 toxicity was observed (339). From an immunological point of view, melanoma is thought to be one the most immunogenic (hot) tumor due to its exceptionally genomic instability (UV-driven), which leads to a high TMB and high number of potential neoantigens (333). Another reason to consider melanoma as a "hot" tumor concerns the immune infiltration. Indeed, in melanoma TME, a general high immune cell infiltration is observed, and this is often correlated with a good prognosis (340, 341). However, TILs found in the TME could be anergic (341).

The B16 murine melanoma is a rapidly growing and metastatic tumor of spontaneous origin (342). From an immunological point of view, this model is considered poorly immunogenic (342-345). Indeed, the TAs shed by B16 melanoma could induce an immune response in syngeneic mice, which only delays the tumor growth (342), because of the tumor immunosuppressive activity and the low presence of immuno-stimulatory signals (346, 347). B16 model presents TAs associated to the specific function of melanocytes, i.e., the melanocyte differentiation antigens (MDAs): gp100, tyrosinase, TRP1-2, MART-1/Melan-A (348, 349). They are all non-mutated proteins overexpressed in malignant melanocytes (350). Several neoantigens, recognized by tumor-specific CTL or by CD4 T cells, were also identified for a B16 melanoma cell line (351, 352). The B16F1 and B16F10 cell lines derive from the metastasis of the parental line B16F0. In particular, B16F1 derives by a one-time selective procedure, while B16-F10 was obtained by a ten-time selective procedure (353, 354). Compared to B16F1, the B16F10 has a more metastatic nature; however,

this aspect is not necessarily correlated with the aggressiveness of the two cell lines, which is quite similar.

Contrarily to the B16F10 model, the B16F1 has not been extensively used for DNA vaccination. The different studies in the B16F1 and B16F10 models, using naked DNA vaccines to encode melanoma antigens, are listed in Table 8.

Table 8: Preclinical studies using DNA vaccination in B16F1and B16F10 models. Keywords in Pubmed: "B16F1", "B16F10", "DNA vaccine" (accessed 15/03/2019). D = day, W = week.

Antigen	Vaccination schedule	Tumor injection schedule	Results	Year and reference
Murine heat shock protein65 (mHSP65)	Prophylactic: 2 µg, in 3 different portions of the abdominal skin, 4 times, every 2 W (GeneGun)	2 x 10^5 mHSP65$^+$ B16F1, SC, 2 W after the last immunization	TA-specific CTLs; decrease in the tumor growth	2008 (355)
MUC (Mucine) 1	Therapeutic: 100 µg, IM, 19 and 26 D after tumor injection	10^5 MUC1$^+$ B16F1, SC	Antitumor effect of the vaccine alone was weaker; the vaccine needed to be combined with an apoptosis-inducing adenine nucleotide translocator (ANT) short hairpin RNA to decrease the tumor growth	2011 (356)
HMGN1 (nucleosome binding protein)-gp100 fusion protein	Prophylactic: 4 µg, ID, once/W for 3 W (GeneGun)	2x10^4-5x10^5 B16F1, SC	HMGN1 has a great adjuvant effect and the vaccine induced full prophylactic protection	2014 (357)
Human IgG1 antibody molecule into which engineered epitopes from gp100 and TRP-2 melanoma TAs have been inserted	Therapeutic: 1 µg, ID, at D 3, 7, 10 and 14 (GeneGun)	2.5x10^4 or 1.5x10^5 B16F1 cells, SC, at D 0	Increased CD4 and CD8 infiltration, when the vaccine was combined with anti-PD-1; increase of the median survival only with the combination	2016 (358)
TRP-2 epitope	Prophylactic: 100 µg, IM, once/W for 3 W	2.5×10^3 B16F10 cells, SC	CTL induction and protective immunity, only when combined with a plasmid encoding TGFb	2005 (359)
MUC18	Prophylactic and therapeutic: 50 µg, IM, 6 doses, weekly	5x10^4 MUC18-expressing B16F10, SC	Humoral and CD8 T cell immune responses; not effective in a therapeutic model	2006 (360)
TRP-2, gp100	Therapeutic: 10 µg, IM EP, at D 6, 13, 20 and 27 after the B16F10 injection	2x10^5 B16F10 cells, SC	Only the combination with an angiostatic treatment showed an antitumor effect.	2009 (361)

Aquaporin-1 (AQP-1) fused to the ubiquitine	Prophylactic and therapeutic: 6 µg, GeneGun, 4 times, biweekly	10^5 cells, SC, 10 D after the last vaccination (prophylactic) or 5000 cells (therapeutic), SC, 1 D before the priming	CTL-mediated tumor growth suppression	2012 (362)
P42.3, a protein expressed during the mitotic phase in many types of cancer	Prophylactic: IM EP	10^5 B16F10 cells, SC, 7 D after the last immunization	Antigen-specific CTLs and anti-tumor immunity	2013 (363)
Cripto-1	Prophylactic: 40 µg, ID EP	$5x10^4$, SC or $2x10^5$, IV Cripto1-overexpressing B16F10 cells	Slowdown in the tumor growth, but not improved survival	2016 (364)
Macrophage Inflammatory Protein-3α (MIP-3α)-gp100 fusion protein	Therapeutic: 50 µg on D 3, 10 and 17	$5x10^4$ B16F10 cells, SC	Slowdown in the tumor growth, but not improved % of survival	2016 (365)
Ovalbumin (OVA) or gp100	Prophylactic and therapeutic: 1 µg (p-OVA) or 50 µg (p-gp100), at D 2, 9 and 16 (therapeutic) or 3 times every 2 W before the tumor challenge (prophylactic)	10^5 B16F10-OVA cells, SC	Slowdown in the tumor growth and improved survival in the prophylactic setting	2016(366)
Carcinoembryonic antigen (CEA)	Prophylactic: 50 µg, IM EP, every 2 W, 5 doses	$5x10^5$ CEA-expressing B16F10 cells, SC, 10 D after the last immunization	Humoral and cellular responses and tumor growth slowdown or tumor elimination	2017 (367)
TRP-2	Prophylactic: 40 or 80 µg, ID EP, biweekly	$2.5x10^5$ B16F10 cells, SC or $4x10^5$ IV	TRP-2-specific CTL response and decrease in lung metastasis	2017 (368)
OVA or gp100	Prophylactic: 40 µg, ID EP	10^6 B16F10-OVA cells, SC	Incomplete tumor protection when the gp100 antigen was used, but generation of memory T cells	2018 (369)
MUC1	Therapeutic: 50 µg, IM EP, priming when tumors were palpable, boosts after 7 and 14 D	10^6 $MUC1^+$ B16F10 cells, SC	Specific CTL and antibodies, tumor growth suppression	2018 (222)

Most of the time, the DNA vaccination in B16F1 model involves TAs that are overexpressed in many different cancers, such as alarmins, HSPs etc., which are expressed in case of danger and cellular stress, or antigens stably delivered into the injected cells (e.g., Mucine1). In the B16F10, the most studied antigens are gp100 and TRP2. Other antigens are also used, such as OVA, when B16F10 are modified to express this specific TA. Most of the studies perform the vaccination before the tumor challenge (prophylactic vaccination). Generally, the vaccine generates a specific CTL response but, when used alone, it is not strong enough in the therapeutic setting, when the tumor is already established.

VI.3. GLIOBLASTOMA AND THE GL261 PRECLINICAL MODEL

Glioblastoma is the most common and most aggressive primary malignant brain tumor in adults. It is an often-fatal brain malignancy and has a high recurrence rate even after surgical resection (370). The current standard treatment for GBM patients is maximal safe resection of the tumor (whenever possible) followed by radiotherapy with temozolomide (TMZ), but survival is poor, with a median survival of just 12-15 months (371-373). Among the treatments that have been FDA-approved for GMB, such as Gliadel wafers (carmustin), bevacizumab (antibody anti-VEGF) and tumor treatment fields (alternating electrical fields that damage the rapidly dividing tumor cells) (374), none significantly affects overall survival or is cost-effective (374).

Despite the initial believe of the brain as an immune privileged site, not able to mount an appropriate immune response, immunotherapy is emerging as a promising therapy for the treatment of GBM (370, 375, 376). However, immunotherapy has to overcome many limitations linked to the immunobiology of the brain and of the GBM. The first issue is the presence of the blood brain barrier (BBB) and the low amount of lymphatic vessels, which regulate the entry of the immune cells (377). Under physiological conditions, only few immune cells are present in the brain parenchyma; however, various pathological conditions disrupt the BBB, thus slightly increasing the permeability of the immune cell into a part of the brain (377). Second, GBM is an immunologically quiet ("cold") tumor, with low TMB and few TILs (378, 379). Furthermore, its high heterogeneity, the presence of glioma stem cells (GSCs) and the suppressive TME facilitate immune escape (377, 379, 380). Third, the immune stimulation in the intracranial space poses clinical safety risks including complications of cytokine release syndrome and autoimmune encephalitis (381).

For these reasons, until now, no FDA-approved immunotherapy for GBM has been approved. Many clinical trials using ICB, OVs, CAR-T cells and vaccines are ongoing (370, 375, 382). Some non-mutated TAs (e.g., IL-13Rα2, HER-2, gp100, survivin, WT1, TRP-2, MAGE-A1, MAGE-A3,

AIM2), or mutated TAs (e.g., EGFRvIII, p53, isocitrate dehydrogenase 1 ($IDH1^{R132H}$)) associated to GBM have been identified (383), and many I-III clinical trials using peptide and DC vaccines are exploring their safety and efficacy (383-387). To overcome GBM heterogeneity, multivalent peptide vaccines have been tested (383). Despite their ability to activate a specific immune response, cancer vaccines did not show significant survival benefits for the GBM patients (387-389).

From a preclinical point of view, GL261 is a syngeneic murine cell line that has been used in immune-competent mice to test the anti-tumor activity of GBM drugs (390, 391). This model shares characteristics similar to human GBM, for its invasive and angiogenic properties (392). GL261 cells have a fast growth rate with no contact inhibition in vitro and in vivo (393). GL261 tumors destroy the BBB and are partially immunogenic, as they express detectable levels of MHC I, but the expression of MHC II, B7-1, and B7-2 is limited or absent (392). This contributes to their escape from immune surveillance (391). While invasive and aggressive (untreated mice die within 4 weeks) (390), GL261 tumors are not known to be metastatic (391) neither in the SC nor in the intracranial models (393). However, survival time depends on the injected tumor cell number (393). This model has been used to test ACT therapy, IL-12-expressing DNA plasmid, vaccines, among other immunotherapies (391).

Regarding the GL261 TAs, this model carries both p53 and K-ras mutations and has elevated c-myc and p53 expression (393). Like melanoma, glioma cells also express tyrosinase, gp100, TRP-1, TRP-2 and p97 antigens (394), due to the common embryonic origin of the glial cells and the melanocytes from the neural ectoderm (395). Iizuka et al. identified a point-mutated form of GARC-1 as unique antigen for CTL in GL261 cells (396). One study has found a prolonged survival in GL261-bearing mice, by vaccination with the heat-shock protein 65 (HSP65) (397). In another study, the vaccination with a survivin peptide induced a specific CTL response and prolonged the survival in an intracerebral GL261 model (10^5 cells injected) (398). Only few studies using DNA vaccination against GL261 have been found in the literature and they are described in Table 9.

Table 9: DNA vaccination studies in a GL261 glioma model. Pubmed research: "DNA vaccine", "GL261" or "glioma". Accessed: 28/03/2019.

Antigen	Vaccination schedule	Tumor injection schedule	Results	Year and reference
human (h)TRP-2	Prophylactic: 50-100 µg, into both quadriceps (IM), 3 times	1.5×10^5 GL261 cells for SC, 10^5 for IV and 4×10^4 for intracranial injection	TRP-2-specific CTL response and increased survival	2003 (399)
SOX6	Prophylactic: 1.25 µg, ID, GeneGun, 4 times, weekly Therapeutic: D4, 11, 18 and 25	5 x 10^4 GL261 cells for intracranial injection	SOX6-specific CTL, CD4 activation and anti-tumor response	2008 (400)

As shown in the three Tables 7, 8 and 9, in many studies, DNA vaccination generates a tumor-specific immune response. However, this is not always translated in a prolonged survival or in a significant slowdown of the tumor growth. Especially in the therapeutic setting the results are different and unpredictable. This is mainly due to several variables between the studies: (i) A wide range of antigens have been explored, which can have a different immunogenicity and, thus, lead to a different outcome. (ii) The DNA vaccine is administered according to different protocols. Specifically, the administration schedule, the injection site and the doses vary in the different studies, even for the same tumor model. (iii) The number of injected tumor cells differs not only between the models, as the rate of the tumor growth is different, but also in the same preclinical model. For instance, in the B16F10 model, this number can vary from $5x10^3$ to 10^6 cells for the same administration site (SC). Furthermore, the tumor injection site can be different (predominantly SC, but also IP, IV, orthotopic etc.), and this can also influence the efficacy of the treatment. All these variables make difficult a comparison between the different studies and tumor models. A harmonization of these variables, at least in the same tumor type, could be benefic for the field of DNA vaccines, as it could help to choose the more suitable protocol, to reach the best outcome following DNA vaccination.

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CHAPTER II.
AIM OF THE THESIS

Conventional therapies against cancer typically fail to provide lasting antitumor benefits, owing to their inability to specifically eliminate all malignant cells. Immunotherapy is currently being evaluated as a means to direct the patient's immune system against residual cancer cells and eradicate the tumor. In particular, DNA vaccines represent an easy and safe strategy to activate a complete, specific and long-lasting immunity against several types of cancer. However, until now, their poor immunogenicity limited their application for human medicine and even for therapeutic vaccination in preclinical models. Many obstacles need to be overcome, such as the immunosuppressive TME and its heterogeneity, in term of cellular infiltration, antigen expression etc. The deeper knowledge of the tumor biology and immunity opens the way to new rational improvements in DNA vaccine field to try to completely eradicate cancer, giving a new hope to cancer patients.

This project is based on the hypothesis that a global optimization of the DNA vaccine efficacy could induce a potent, safe and specific immune response to eradicate an established tumor. Hence, the aim of my thesis is to improve the DNA vaccine immunogenicity against cancer, thus improving the survival in established cancer models. To this end, the strategies adopted mainly include the modification of the DNA vaccine from molecular codon optimization to rational antigen design and insertion in the vaccine, and the combination with strategies having a complementary mechanism of action.

During my thesis, DNA vaccines will be tested in different tumor models. The first model is the mastocytoma P815 to test the effects of codon optimization (chapter III) and ICB combination with the DNA vaccination (chapter IV). Then, the chapter V of my thesis explores for the first time the effects of a new poly-epitope DNA vaccine encoding TAAs and neoantigens against B16F1 melanoma and its combination with oncolytic viral therapy. Finally, in the chapter VI, the effects of DNA vaccination and tumor resection in an orthotopic GL261 glioblastoma model will be evaluated. For each combination, the mechanism of action will be explored, together with the contribution of the single therapy to the global immune response.

Specifically, the research questions and objectives of my thesis can be summarized as follows (Figure 1):

1. How can the DNA vaccine efficacy be improved? → Evaluation of the use of different strategies in antigen design and optimization: from a whole gene codon optimization to multi-epitope TAAs and neoantigen insertion, for a more "personalized" approach.

2. Is the DNA vaccine optimization strong enough to eradicate an established tumor? → Evaluation of the weaknesses of DNA vaccine as a single therapy and investigation of combination

approaches that help the antigen-specific immune cells generated by the vaccine to be more effective or to reach the TME.

3. How versatile are these approaches? In other words, are these findings applicable in different tumor models? → Validation of the optimized DNA vaccines for different cancer models, from a relevant preclinical model (P815 mastocytoma) to an immunologically hot tumor (melanoma) and, lastly, to a cold tumor (glioblastoma), using different therapy combinations.

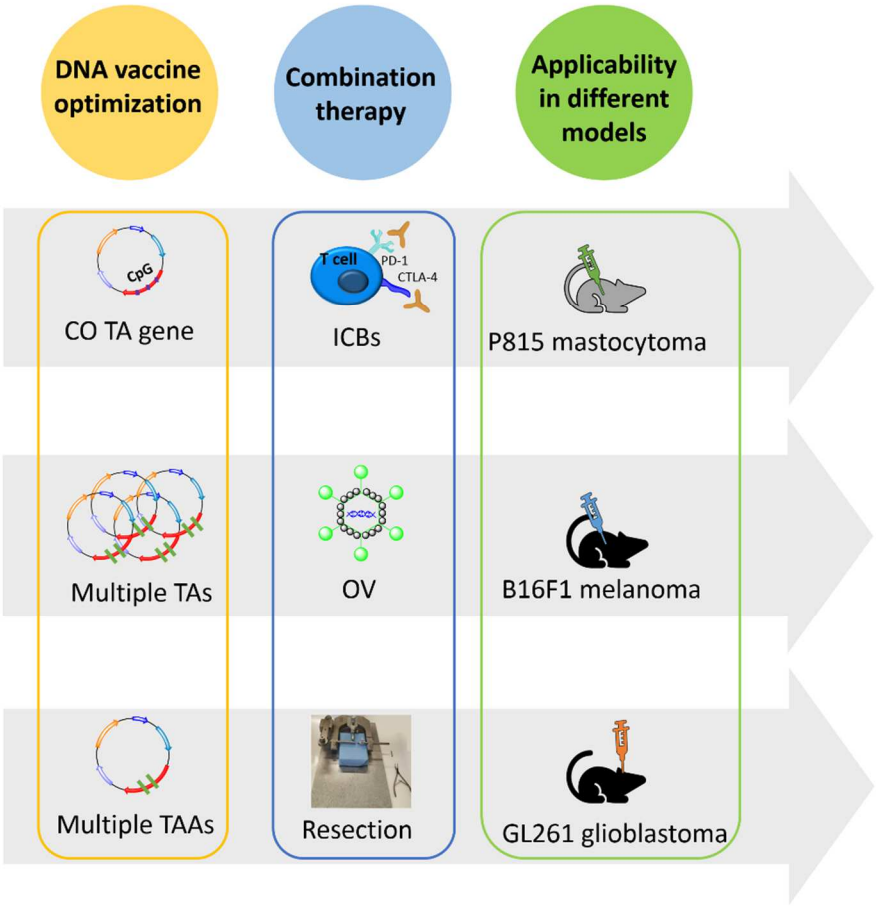

Figure 1: Specific aims of this PhD thesis: DNA vaccine optimization, combination therapy and applicability in different preclinical models. A specific DNA vaccine and combination therapy will be used for a precise cancer model.

This work will expand the knowledge of DNA vaccination for cancer therapy, providing new therapeutic strategies for different tumors and opens the possibility to a more "personalized" approach to cancer. It also leads the way for a translation into the clinic, with the hope that, in the future, DNA vaccines can become part of the standard of care for cancer patients.

CHAPTER III. CODON-OPTIMIZED P1A-ENCODING DNA VACCINE: TOWARDS A THERAPEUTIC VACCINATION AGAINST P815 MASTOCYTOMA

Adapted from:

Lopes A., Vanvarenberg K., Préat V., Vandermeulen G. Codon-optimized P1A-encoding DNA vaccine: towards a therapeutic vaccination against P815 Mastocytoma, *Molecular Therapy-Nucleic Acid (2017), 8: 404–415. doi: 10.1016/j.omtn.2017.07.011*

GENERAL INTRODUCTION

Previous studies in our lab on a P815 mastocytoma tumor model have shown that the efficacy of a prophylactic DNA vaccine encoding the entire P1A antigen gene was limited. Indeed, the vaccine failed to cure mice after a P815 challenge. However, the induction of an antigen-specific cytotoxic T lymphocytes immune response and the significant delay in the tumor growth were promising (1). The DNA vaccine needed to be optimized to have a higher efficacy. Codon optimization (CO) was already known as a technique to increase the protein production from an organism in a different host (2). CO gave also the possibility to modulate the number of immunostimulatory motifs, such as CpG motifs, inside a DNA sequence. We hypothesized that CO of the P1A antigen gene sequence could increase the antigen production and to activate the innate immune response, through the insertion of CpG motifs.

In this chapter, we explore the effects of CO and CpG modulation on the DNA vaccine efficacy, by analyzing the antigen production and the innate immunity following the vaccine delivery by electroporation. An impressive increase in the survival in a prophylactic setting (60% of mice survived to a lethal P815 challenge with the CO plasmid containing the highest number of CpG) was observed. The optimized vaccine was also able to slowdown the tumor growth and to increase the median survival time of mice vaccinated after the tumor injection (therapeutic vaccination). Furthermore, the therapeutic efficacy of the vaccine was higher even compared to the positive control (L1210.P1A.B7.1 cells, which have been stably transfected to express the P1A antigen). However, the low percentage of cured mice leaves the room for improvement.

ABSTRACT

DNA vaccine can be modified to increase protein production and modulate immune response. To enhance the efficiency of a P815 mastocytoma DNA vaccine, the P1A gene sequence was optimized by substituting specific codons with synonymous ones while modulating the number of CpG motifs. The P815A murine antigen production was increased with codon-optimized plasmids. The number of CpG motifs within the P1A gene sequence modulated the immunogenicity by inducing a local increase in the cytokines involved in innate immunity. After prophylactic immunization with the optimized vaccines, tumor growth was significantly delayed, and mice survival was improved. Consistently, a more pronounced intratumoral recruitment of CD8+ T cells and a memory response were observed. Therapeutic vaccination was able to delay tumor growth when the codon-optimized DNA vaccine containing the highest number of CpG motifs was used. Our data demonstrate the therapeutic potential of optimized P1A vaccine against P815 mastocytoma and show the dual role played by codon optimization on both protein production and innate immune activation.

KEYWORDS

Codon optimization, cancer DNA vaccines, P815 mastocytoma, P1A, prophylactic vaccination, therapeutic vaccination

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I. INTRODUCTION

Harnessing the immune system to fight cancer has become a priority in the last few years and is supported by an increasing knowledge of tumor-host interactions. Among the various immunotherapy strategies that are currently being developed, DNA vaccines have many advantages, such as low cost and high stability and versatility, which allows the modulation of the encoded antigen and its intrinsic immunogenicity. Interestingly, DNA vaccines can induce the activation of the innate immune response, but also the cellular and humoral arms of the adaptive immune system (3, 4). However, human applications of DNA vaccines have lagged, largely due to suboptimal immunogenicity compared to traditional vaccines (3, 5). Different approaches have been investigated in order to overcome this problem and enhance their efficacy (6-9).

Several elements appear critical for optimizing DNA vaccination. Antigen expression should be high enough to promote its presentation by antigen-presenting cells (APCs) and the activation of the adaptive immunity (10, 11). Codon optimization is an *in silico* technique originally based on the selection of codon triplets that have the highest tRNA frequency in the cytoplasm of the target species. Hence, codon optimization algorithms allow for an increase in the protein translation rate and mRNA stability, while maintaining the typical 3D structure of the protein (12). This technique is generally used to induce greater production of a foreign protein (e.g., when a viral or a bacterial protein must be expressed in mammalian cells) (13, 14). Codon optimization also allows for a modulation of the number of CpG motifs in the gene sequence. Indeed, it has been demonstrated that CpG motifs directly stimulate B cells and are recognized by Toll-like receptor 9 (TLR9) in dendritic cells (DCs), B cells, and macrophages, allowing the activation of the innate immune system (6). Hence, the addition of CpG motifs as "built-in" adjuvants into the plasmid sequence improves the immunogenicity of DNA vaccines (5, 15).

The DNA delivery method must also be carefully selected because it not only has an influence on the magnitude of the gene expression, which depends on the delivery efficacy, but it also contributes to the immunogenicity of the DNA vaccine (16). Electroporation (EP) is a non-viral delivery method that can improve *in vitro* and *in vivo* plasmid uptake and, thereby, increase the expression level of the transgene in many cell types and tissues (17). EP utilizes electric pulses at the site of immunization that transiently destabilize the cell membrane and promote the electrophoretic movement of negatively charged DNA into the cells (18, 19). In particular, intramuscular EP promotes long-lasting gene expression and the generation of a local and systemic immune response (20-22). This technique also reduces the amount of DNA required to activate the immune system (by up to 100 times) while increasing the potency of the immune response that

is generated compared to conventional DNA vaccinations (16). For these reasons EP is being used in many clinical trials for the delivery of DNA vaccines for different pathologies (18).

The P1A gene is a cancer-germline gene in mice that encodes the major tumor rejection antigen of mastocytoma P815, named P815A. This gene is activated in several tumors but is silent in normal cells, except placental trophoblasts and male germline cells. Since these cells do not bear the surface MHC class I molecules, they are not able to present the antigen (23). Hence, immunization against this antigen does not induce autoimmune side effects. P815A shares many characteristics with human MAGE-type tumor antigens (24), suggesting that the P815 mastocytoma tumor model is relevant for future applications in human medicine.

This study aims to generate a potent immune response against P815 by optimizing the P1A antigen gene sequence. We hypothesize that optimization of the codon sequence could improve the vaccine efficacy by enhancing antigen production and inducing a stronger activation of the innate immune system. These modifications would lead to a stronger protection of mice against P815 mastocytoma. Several P1A-expressing DNA vaccines were constructed that encoded exactly the same antigenic protein but differed in terms of nucleic acid sequence. First, the impact of these modifications on gene and protein expression and on plasmid immunogenicity was studied. Then, the vaccine efficacy in DBA/2 mice challenged with P815 after a prophylactic vaccination was evaluated, analyzing mice survival and $CD8^+$ tumor infiltration. Finally, the therapeutic efficiency of the optimized plasmid was investigated.

II. MATERIALS AND METHODS

II.1. PLASMID OPTIMIZATION AND PRODUCTION

Three CO P1A sequences with different numbers of CpG motifs were designed *in silico,* using the GeneOptimizer algorithm from GeneArt™ Gene Synthesis (Thermo Fisher Scientific), to enable codon optimization in mice (25). Then the genes were synthesized (Gene Art, Thermo Fisher Scientific, Massachusetts, USA) and subcloned into a pVAX2 vector containing the cytomegalovirus (CMV) promoter, as previously described (1). The plasmids were named pVAX2-P1A_CO0 (CO0), pVAX2-P1A_CO21 (CO21) and pVAX2-P1A_CO50 (CO50), where CO means that the P1A sequence has been codon optimized and 0, 21 and 50 refer to the number of CpG motifs in the P1A sequence. A fourth non-optimized plasmid, pVAX2-P1A_21 (21), containing the wild-type P1A sequence was also used in this study. Upstream of the gene sequence a Kozak sequence was inserted in order to improve the translation efficiency (26). For the analysis of *in vitro* expression, a 6X-His-tag motif was added downstream of the P1A gene sequence to facilitate protein quantification. To avoid any bias, no tag was included for the immunization experiments. All the sequences encoding the tumor rejection antigen P1A with the related modifications are shown in Figure 1. All plasmids were sequenced to ensure the correct nucleotide sequence (Beckman Coulter Genomics, Villepinte, FR) and the vaccines were amplified and purified using an EndoFree Plasmid Giga Kit (Qiagen, Venlo, NL), according to the manufacturer's protocol. Optical density at 260 nm was used to determine DNA concentration. Plasmids were diluted in Phosphate Buffer Saline (PBS) and stored at −20 °C before use.

II.2. CELL LINES

C2C12 murine myoblasts were kindly provided by Prof. Marc Francaux (Université Catholique de Louvain, Louvain-la-Neuve, BE). They were cultivated without reaching more than 70% confluence to avoid their differentiation into myotubes. P815B mastocytoma cells and L1210.P1A.B7.1 leukemia cells were obtained from Dr. Catherine Uyttenhove (Ludwig Institute for Cancer Research, Brussels, BE). L1210.P1A.B7.1 cells derived from a DBA/2 mouse were stably transfected to express the P1A antigen and the B7-1 costimulatory molecule (27). All the cells were cultured at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Specifically, L1210.P1A.B.7.1 cells were cultured adding 1.5 μg/ml of puromycin.

II.3. ANIMALS

DBA/2 female mice were obtained from Janvier (Le Genest-Saint-Isle, FR). Mice were between 5 and 6 weeks old at the beginning of the experiments. Water and food were provided *ad libitum*. All experimental protocols using mice were approved by the Ethical Committee for Animal Care and Use of the Medical Sector of the Université Catholique de Louvain (UCL/MD/2011/007 and UCL/MD/2016/001).

II.4. IN VITRO PLASMID TRANSFECTION

Ten microliters at the concentration of 1 μg/μl of tagged plasmids were electroporated into 10^6 C2C12 cells suspended in 100 μl of Pulsing Buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4), using the following protocol: 230 V, 4 ms, 1 pulse. Electroporation was performed in a 2 mm gap cuvette in a BTX™ Gemini X2 Electroporation System (Thermo Fisher Scientific, Waltham, MA). After pulsing, 25 μl of FBS was added and the cuvette was incubated for 5 minutes at 37 °C. Cells were suspended in media and seeded in a 96-well plate (10^6 cells/well).

II.5. EVALUATION OF ANTIGEN EXPRESSION

II.5.1. qPCR

Twenty-four hours after transfection, cells were lysed and total RNA was isolated using TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA). The quality and quantity of RNA were evaluated using a nanospectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA). One microgram of RNA was reverse transcribed using a first standard synthesis system (SuperScript™, Thermo Fisher Scientific, Waltham, MA) and oligo(dT) primers (Eurogentec, Liege, BE) according to the supplier's protocol. The resulting cDNA was used as template for 30 cycles of semi-quantitative polymerase chain reaction (PCR) in a T100™ thermocycler (Bio-Rad, Hercules, CA). Primers for β-actin (housekeeping gene) and the 4 different P1A genes were used to amplify respective cDNA by PCR. The PCR products were subjected to electrophoresis on a SYBR™ Safe (Thermo Fisher Scientific, Waltham, MA)-stained 1.5% agarose gel.

SYBR™ green real-time qPCR (GoTaq qPCR MasterMix kit, Promega, Fitchburg, WI) was conducted on a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) in order to detect the expression of P1A *in vitro* 24 hours after vaccination and *in vivo* 6 hours after the vaccine injection in the tibial muscle of DBA/2 mice. Analysis of the melting curves was performed to ensure the purity of PCR products. The results were analyzed with the StepOne Software V2.1. The P1A mRNA expression was calculated relative to the expression of corresponding β-actin, according to the delta-delta Ct method and all the results were normalized

to non-transfected cells. Primers for P1Awere designed using Primer Blast software based on the consensus of sequences from GenBank.

II.5.2. Western blotting

P815A protein production was evaluated in C2C12 cells 24 hours after plasmid transfection. Cells were lysed and proteins isolated using RIPA buffer (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocol. Proteins were quantified using a micro BCA test (Thermo Fisher Scientific, Waltham, MA). Ten micrograms of purified proteins were loaded in a Mini-PROTEAN TGX Precast Gel (Bio Rad, Hercules, CA) and separated at 300 V for 10 minutes. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (Bio Rad, Hercules, CA) and blocked with 5% milk in 0.05% Tween 20-PBS for 1 hour at room temperature and washed with a solution of 0.05% Tween 20-TBS. Mab against 6X-His-tag (Abcam, Cambridge, UK) diluted 1:1000 in 0.2% milk was added in order to detect P815A and incubated for 2 hours at room temperature with shaking. Mab anti-actin was used as the control (Abcam, Cambridge, UK). After washing with Tween 20-TBS the membrane was incubated with a solution of streptavidine-HRP (R&D systems, Minneapolis, MN, dilution 1:200) for 20 minutes at room temperature and then washed with 0.05% Tween 20-TBS. Membranes were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) and X-ray Film for Western Blot Detection (Thermo Fisher Scientific, Waltham, MA). Dot values were semi-quantitated with GelQuantNET software and the actin dots were used for normalization. Non-transfected C2C12 cell lysate was used as the negative control (Ctr).

II.6. IN VIVO DELIVERY OF DNA VACCINE

Mice were anesthetized with 140-150 µl of a solution of 10 mg/ml ketamine (Ketalar, Pfizer, New York, NY) and 1 mg/ml xylazine (Sigma, St. Louis, MO). The left paw was shaved using a rodent shaver (Aesculap Exacta shaver, AgnTho's, Stockholm, SE). DBA/2 mice were vaccinated with P1A encoding plasmids: 50 µg of plasmid diluted in 30 µl of PBS was injected in the left tibial cranial muscle and electroporated (200 V/cm, 8 pulses, 20 ms with 500 ms pause between pulses) as described in (1). Briefly, after the vaccine injection, a conductive gel was placed on the left paw to ensure electrical contact with the skin (Aquasonic 100, ultrasound transmission gel, Parker labs, Fairfield, NJ). The paw was then placed between 4 mm plate BTX caliper electrodes (VWR International, Leuven, Belgium). The pulses were delivered by a BTX™ Gemini Electroporation System (VWR International, Leuven, Belgium).

II.7. CYTOKINE EXPRESSION

The mice were sacrificed 6 hours after a single vaccine injection. The tibial cranial muscle was withdrawn and stored in RNA later solution (Thermo Fisher Scientific, Waltham, MA) at -20 °C before RNA extraction, according to the previous protocol (section II.5.1). The muscle was used to detect the local expression of inflammatory cytokines by qPCR. Primers for TNFα and IL-6 were designed using Primer Blast software based on the consensus of sequences from GenBank. The other primers can be found in (28, 29). A complete list of the primers used is shown in Table 1. All experiments were performed in triplicate.

Table 1: Primer sequences for PCR.

Oligo name	**Primer sequence (5′ → 3′)**
CO0-tag for	AGA-TGG-GGA-TGG-CAA-CAG-ATG
CO0-tag rev	GGC-CAC-ATC-CCT-CTC-ATA-CT
CO21-tag for	GGA-AGA-GAT-CCT-GCC-CTA-CC
CO21-tag rev	CTG-TTC-CTC-ATA-CAG-GGC-GT
CO50-tag for	GCG-ACG-GCA-ACA-GAT-GTA-AC
CO50-tag rev	GTA-CAG-GGC-GTC-GAT-GAA-CA
21-tag for	CCA-CGA-CCC-TAA-TTT-CCT-GGT
21-tag rev	GTG-GTG-ATG-GTG-ATG-ATG-AGG-T
β-actin for	TAC-AAT-GAG-CTG-CGT-GTG-GCC-C
β-actin rev	AGG-ATG-GCG-TGA-GGG-AGA-GCA-T
IL-6 for	CCG-GAG-AGG-AGA-CTT-CAC-AG
IL-6 rev	TCC-ACG-ATT-TCC-CAG-AGA-AC
TNFα for	CAT-CTT-CTC-AAA-ATT-CGA-GTG-ACA-A
TNFα rev	TGG-GAG-TAG-ACA-AGG-TAC-AAC-CC
IL-1β for	AAC-TGT-TCC-TGA-ACT-CAA-CTG-T
IL-1β rev	GAG-ATT-TGA-AGC-TGG-ATG-CTC-T
IL-12 for	GGA-AGC-ACG-GCA-GCA-GAA-TA
IL-12 rev	AAC-TTG-AGG-GAG-AAG-TAG-GAA-TGG

II.8. PROPHYLACTIC AND THERAPEUTIC VACCINATIONS

Each mouse received 3 vaccine administrations (one priming and two boosts). Vaccine injections were performed biweekly before the tumor challenge (n = 9-10) or weekly after the tumor challenge (n =18) for the prophylactic and therapeutic DNA immunization studies, respectively (Figure 4a and 6a, respectively). The therapeutic study is a combination of 2 separate experiments; the total number of mice is 18. As a positive control, mice were immunized with two intra-peritoneal injections of L1210.P1A.B.7.1 cells (10^6 living cells) in 100 µl PBS at two weeks or one week of interval for prophylactic (1, 30) and therapeutic vaccination, respectively. L1210.P1A.B7.1 positive control is a leukemia cell line derived from DBA/2 mice. These cells were stably transfected with the cosmid C1A.3.1 and the cDNA of B7, as described in (31). Non-immunized mice were used as a negative control.

II.9. TUMOR IMPLANTATION AND TUMOR GROWTH MEASUREMENT

Two weeks after the second boost (prophylactic) or 2 days before the priming (therapeutic), 10^6 P1A-expressing P815B cells diluted in 100 µl PBS were injected into the right flank of mice. An electronic digital caliper was used to measure the tumor length, width and height three times a week. Tumor volume was calculated as length × width × height (in mm^3). Mice were sacrificed when the tumor volume was larger than 1500 mm^3 or when they were in poor condition and expected to die shortly.

Immediately after sacrifice, tumors from mice that received a prophylactic vaccination were withdrawn and immediately fixed overnight in 4% PFA and then cryopreserved in 30% sucrose before Tissue-Tek OCT embedding (Sakura Finetek, Alphen aan den Rijn, NL). Ninety days after the first challenge, surviving mice were re-challenged on the left flank using the same protocol as the first challenge.

II.10. IMMUNOHISTOCHEMISTRY

To determine the tumor $CD8^+$ T lymphocyte infiltration, tumors embedded in OCT were sectioned at 10 µm using a cryostat (Leica Microsystems, Wetzlar, DE) and the sections were stained with antibodies directed against the murine CD3 and CD8. After permeabilization with 0.2% (*v*/*v*) Triton X-100 (Sigma, St.Louis, MO) in PBS and blocking in 10% (*w*/*v*) goat serum, 5% rat serum and 2% BSA in PBS for 1 hour at room temperature, the primary antibodies (rat CD8a-APC 1:250 (clone 53-6.7, BD biosciences) and CD3e-FITC 1:1000 (clone 145-2C11, BD biosciences)) were applied to the slides for 1 hour at room temperature. After being washed with PBS, the sections were mounted using Vectashield Mounting Medium (Vector laboratories, Burlingame, CA)

containing DAPI to visualize the cell nuclei. The slides were imaged using a structured illumination AxioImager microscope (Zeiss, Jena, DE, 10X objective).

II.11. STATISTICAL ANALYSIS

Statistical analyses were performed using the software GraphPad Prism 5 for Windows. Survival curves were compared using a Mantel–Cox (log-rank) test. Values of $p < 0.05$*, $p < 0.01$** and $p < 0.001$*** were indicative of statistically significant differences.

III. RESULTS AND DISCUSSION

III.1. CODON OPTIMIZATION ENHANCED *IN VITRO* EXPRESSION OF P815A ANTIGEN

To modulate the expression of the P815A antigen and optimize a DNA vaccine against P815 mastocytoma, four different P1A-encoding plasmids were constructed. In all the constructs, a strong viral promoter (CMV) and a Kozak sequence were inserted upstream of the antigen sequence. Four different P1A sequences were designed. The first one contained the non-optimized wild-type P1A sequence naturally carrying 21 CpG motifs (P1A_21). The three other sequences were codon-optimized (CO) and adapted to contain zero (P1A_CO0), 21 (P1A_CO21) or 50 (P1A_CO50) CpG motifs. To estimate the production of the P815A antigen, a 6X-His-tag was added downstream of the gene sequence in the plasmids used for P1A mRNA and P815A quantification. For all the other experiments, plasmids without 6X-His-tag were used. The alignment of the 4 gene sequences is shown in Figure 1.

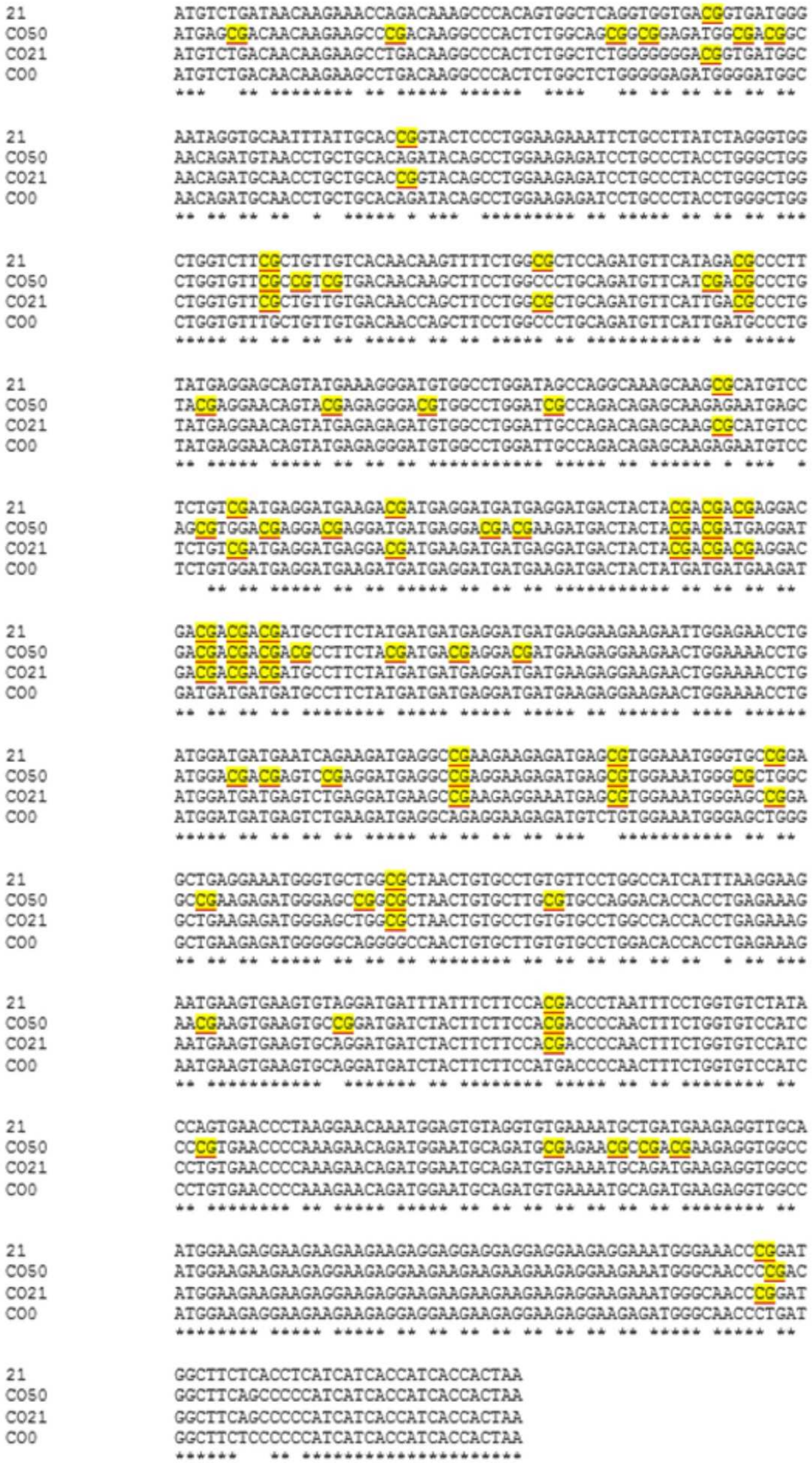


Figure 1: Alignment of the 4 different P1A gene sequences. The CpG motifs are highlighted. The stars indicate the presence of the same nucleotide in the 4 sequences. CO means codon optimization; 0, 21 and 50 refer to the number of unmethylated CpG motifs inside the P1A gene sequence.

After EP of the plasmids in C2C12 murine myoblasts, the mRNA and protein expression were evaluated *in vitro*, using RT-PCR/qPCR and western blot, respectively. Twenty-four hours after EP, the P1A mRNA levels were the same for all the plasmids, without any significant differences between the optimized and non-optimized genes (Figure 2a and 2b). As expected, the western blotting analysis revealed higher levels of P815A protein for the CO genes compared to the non-optimized (Figure 2c). No difference between the three CO plasmids was noticed. P1A mRNA level was also evaluated in the electroporated muscle of DBA/2 mice 6h after plasmid delivery. *In vivo* results confirmed what observed in vitro, showing no differences between the plasmids (Figure 2d). A similar result was obtained 48h after the plasmid electroporation (data not shown), supporting the hypothesis that the CpG motif amount did not influence the transgene mRNA expression in a short-term period.

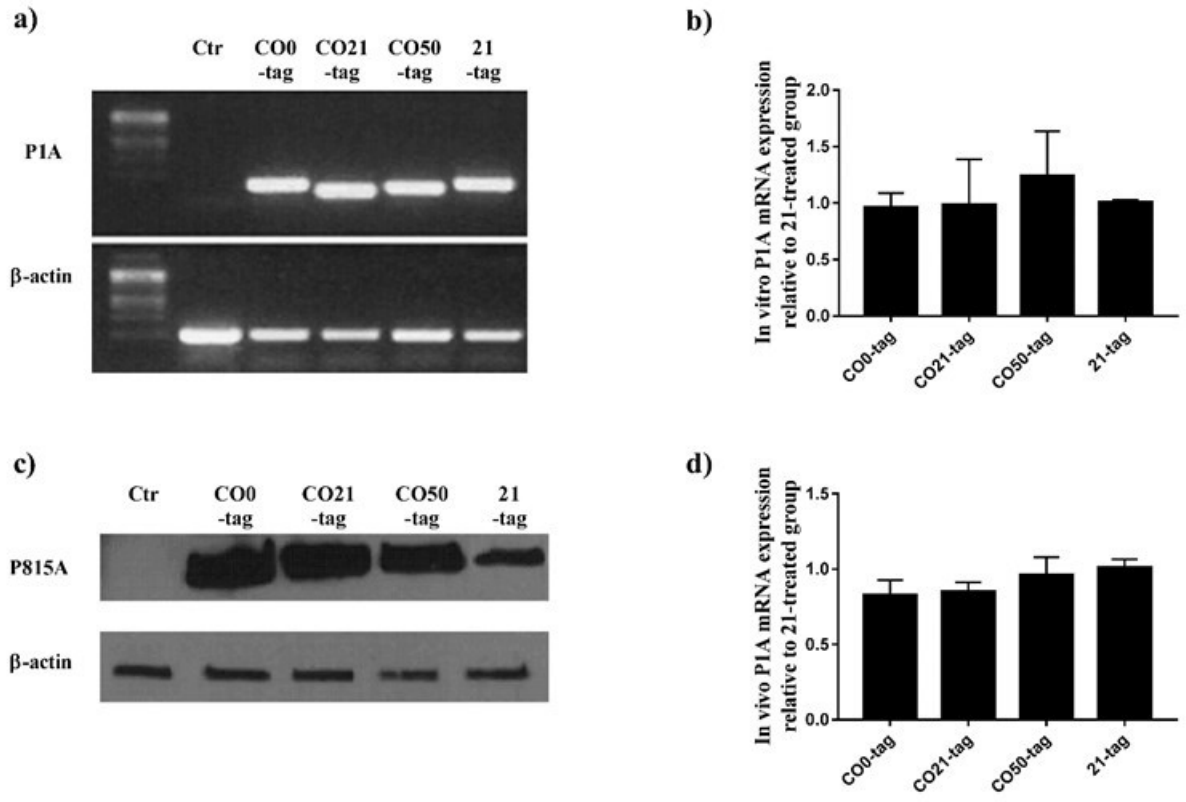


Figure 2: Evaluation of P1A and P815A antigen expression after 6X-His-tagged plasmid transfection by electroporation. (**a**) RT-PCR and (**b**) qPCR analysis of P1A mRNA expression 24 hours after vaccine delivery in C2C12 murine myoblasts. (**c**) Western blot analysis of P815A protein expression 24 hours after vaccine delivery in C2C12 murine myoblasts. (**d**) qPCR analysis of P1A mRNA expression 6 hours after vaccine injection in the tibial cranial muscle of DBA/2 mice. All the experiments were performed in triplicate (n = 3).

Codon optimization is often critical to enhance the production of viral or bacterial proteins in a foreign organism (13, 14). It is commonly accepted that this technique can increase the efficiency of the transgene expression, mostly acting on the codon usage (32). However, the different amount

of CpG motifs inside the transgene could also influence the protein production. Controversial data are reported in the literature. Some authors demonstrated that CpG-free plasmids induced a longer protein expression (33). Interestingly, Bauer *et al.* showed a clear loss of the transgene expression following the depletion of CpGs from the coding region (34). Also, the effect of CpG motifs on the protein expression can vary depending on the cell type (35). Here, our results confirmed that, at short term, the protein production was increased when optimizing a mouse gene sequence for expression in murine cells, independently from the intragenic CpG amount. This was due to improved protein translation, rather than gene transcription, as the mRNA levels were not influenced by the codon modifications. The increase in antigen translation can significantly improve the immune response activation to the DNA immunogens (36-38). Hence, the use of P1A CO vaccines can be an interesting strategy to produce a strong and specific immune response in the host.

III.2. CODON OPTIMIZATION INFLUENCED LOCAL CYTOKINE EXPRESSION *IN VIVO*

In addition to an increase in the protein translation rate and protein expression, codon optimization allows for an adjustment of the number of unmethylated CpG motifs within the gene sequence. The number of CpG motifs within the P1A gene sequence was modulated. To construct the CO0 vaccine, all the CpG motifs were removed from the P1A gene sequence, while the CO50 vaccine contains 50 CpG motifs within the P1A gene. The other two vaccines, 21 and CO21, contain the wild-type number of CpG motifs. To estimate the "built-in" adjuvant properties of the CpG motifs and to determine the activation of the innate immune response, qPCR amplification of IL-1β, IL-6, IL-12 and TNFα was performed. Their relative mRNA expression was assessed in the tibial cranial muscle of mice treated with the four vaccines (CO0, CO21, CO50 and 21), mice only electroporated (EP) or untreated mice. Six hours after the plasmid injection, the mRNA level of all the cytokines was significantly up-regulated at the vaccine injection site for all the treated groups compared to the untreated and EP control groups (Figure 3: all the treatments are significantly different ($p < 0.001$***) compared to untreated or EP groups, if not differently indicated).

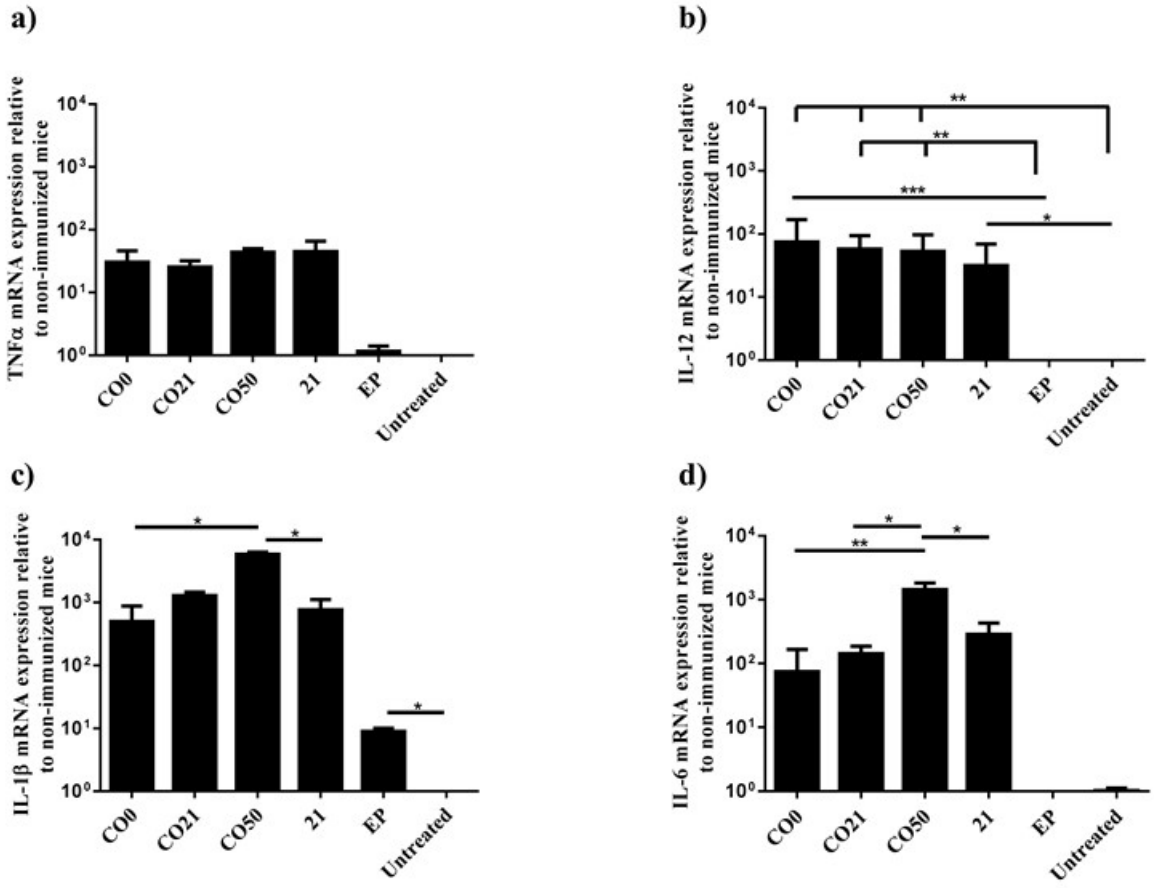


Figure 3: In vivo evaluation of local cytokine production 6 hours after a single vaccine injection and electroporation in the tibial cranial muscle. (**a**) TNFα, (**b**) IL-12, (**c**) IL-1β, (**d**) IL-6. All the treatments are significantly different ($p < 0.001$***) compared to untreated or electroporation (EP) groups, unless otherwise indicated. The results are related to the untreated mice and are expressed in a logarithmic scale (n = 3).

These results clearly indicate an activation of the innate immunity in response to the dsDNA structure itself, due to the different DNA sensing pathways (39, 40). Indeed, EP increases the permeability of the cell membranes thus allowing a greater DNA uptake in the cytoplasm. This process could allow the interaction of the plasmid DNA with cytosolic DNA sensors other than TLR-9, such as Zbp-1, HMGB, Dhx36, Ifi16 etc. (41) and the production of proinflammatory cytokines and chemokines (42). These features can explain the high cytokine expression in all the treated groups and the vaccine-independent cytokine production in the case of TNFα and IL-12 (Figure 3a, b). More interestingly, IL-1β and IL-6 levels increased according to the number of CpG motifs in the P1A gene. The pVAX2 backbone already contains 193 CpG motifs and is thus recognized as foreign dsDNA by TLR-9 in the endosomes, as plasmid molecules could enter the cells by electroendocytosis following the electric pulses application (43, 44). However, a dramatic induction of these cytokines was observed for the vaccine containing the highest CpG number, CO50 (Figure 3c, d). Indeed, these cytokines are directly correlated to the presence of CpG, as it

is less up-regulated in the presence of CpG-free plasmid.(41) Hence, even minor modifications in the number of CpG motifs might generate different innate immune responses. It has been demonstrated that the insertion of only few CpG motifs (16-20) in an antigen-encoding plasmid augmented the production of inflammatory cytokines and allowed the activation of the adaptive immune response (45, 46). Furthermore, these motifs could have a different effect on the immune activation, depending on their adjacent nucleotides (47-49). For instance, the motif RRCGYY seems to be more immunogenic than other types of CpG (50, 51). Seventeen RRCGYY motifs were present in the pVAX2 backbone while 6, 3 and 0 motifs were found in the CO50, CO21/21 and CO0, respectively. According to Coban et al., the addition of 3-5 strongly immunogenic CpG motifs was sufficient to increase the IL-6 and IFN-γ levels (47). In a gene sequence, immunoinhibitory motifs can also be present that prevent innate immune system activation (52). The transgene in the CO50 construct does not contain some of the immunoinhibitory motifs (e.g., TTAGGG) that were present in the P1A gene sequence of the other 3 plasmids.

The delivery method also influenced the cytokine production, as EP alone significantly increased the IL-1β level compared to the untreated group. Roos *et al.* (53) also demonstrated a dramatic up-regulation of several cytokines, among others IL-1β, and chemokines involved in defense responses, immune responses, inflammatory responses, chemotaxis and MHC class I receptor activity when DNA was delivered by EP (53). The up-regulation of these cytokines was correlated with the potentiation of the immune response induced by the delivered DNA (54, 55).

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG-ODN) have been widely used alone or as vaccine adjuvants that are co-delivered with the antigen-encoding vaccine in order to accelerate the induction, increase the maximum level and extend the duration of the induced immune response (56-58). Indeed, CpG-ODN can activate signaling by Toll-like receptor 9 (TLR9) on cells of the innate and adaptive immune system, leading to the production of several pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα and IL-12 (58-62). In particular, IL-6 and IL-1β play an important role in activation of the innate immune system. Indeed, IL-6 helps naïve $CD8^+$ T cells to proliferate and acquire lytic capability in the absence of stimulation by specific TCRs. It has also been demonstrated that IL-6, in synergy with IL-1, can augment IL-2 responsiveness of $CD8^+$ T cells and prime them for subsequent stimulation via the TCR (31, 63).

III.3. CODON OPTIMIZATION OF PLASMIDS AND MODULATION OF THEIR CPG MOTIF CONTENT SIGNIFICANTLY INFLUENCED P815 TUMOR GROWTH AND MOUSE SURVIVAL AFTER PROPHYLACTIC VACCINATION

To evaluate the impact of plasmid optimization on mice survival and tumor growth, mice were vaccinated and then challenged with P815 mastocytoma tumor cells (protocol shown in Figure 4a). The initial evolution of the tumor growth was similar for all the groups (Figure 4b). Three days after the challenge, all the mice had palpable tumors, demonstrating the aggressive nature of the tumors. Tumor volume reached a size of 50-100 mm^3 around days 5-7. Between days 10 and 20, the tumors of the untreated mice reached a plateau and then they started to re-grow faster than the others; at day 32 the 60% of untreated mice were already dead (Figure 4c). However, the tumors of all treated mice were found to be impalpable between days 10 and 20. The tumors that did not start to re-grow between days 20 and 35 were considered to be definitively rejected. Interestingly, 40-60% of mice treated with a CO vaccine completely rejected the tumor 10-15 days after the injection. The most significant tumor growth delay was observed in mice treated with the CO50 vaccine (Figure 4b). Furthermore, the mice treated with the CO sequences survived longer than the mice in the untreated group or the group treated with the non-optimized vaccine (21). Again, the greatest survival among the vaccine-treated mice was reached in mice treated with the CO50 vaccine (Figure 4c), as 60% of the mice survived the challenge. In the L1210.P1A.B7.1 treated group, only one mouse did not survive the challenge, confirming the efficiency of this treatment for prophylactic vaccination (64). As previously studied, immunization with L1210 cells expressing P1A and B7.1 efficiently protect mice against a P815 challenge (64), by induction of a very strong P1A-specific cytotoxic activity. These cells were used as positive control in (1, 64, 65).

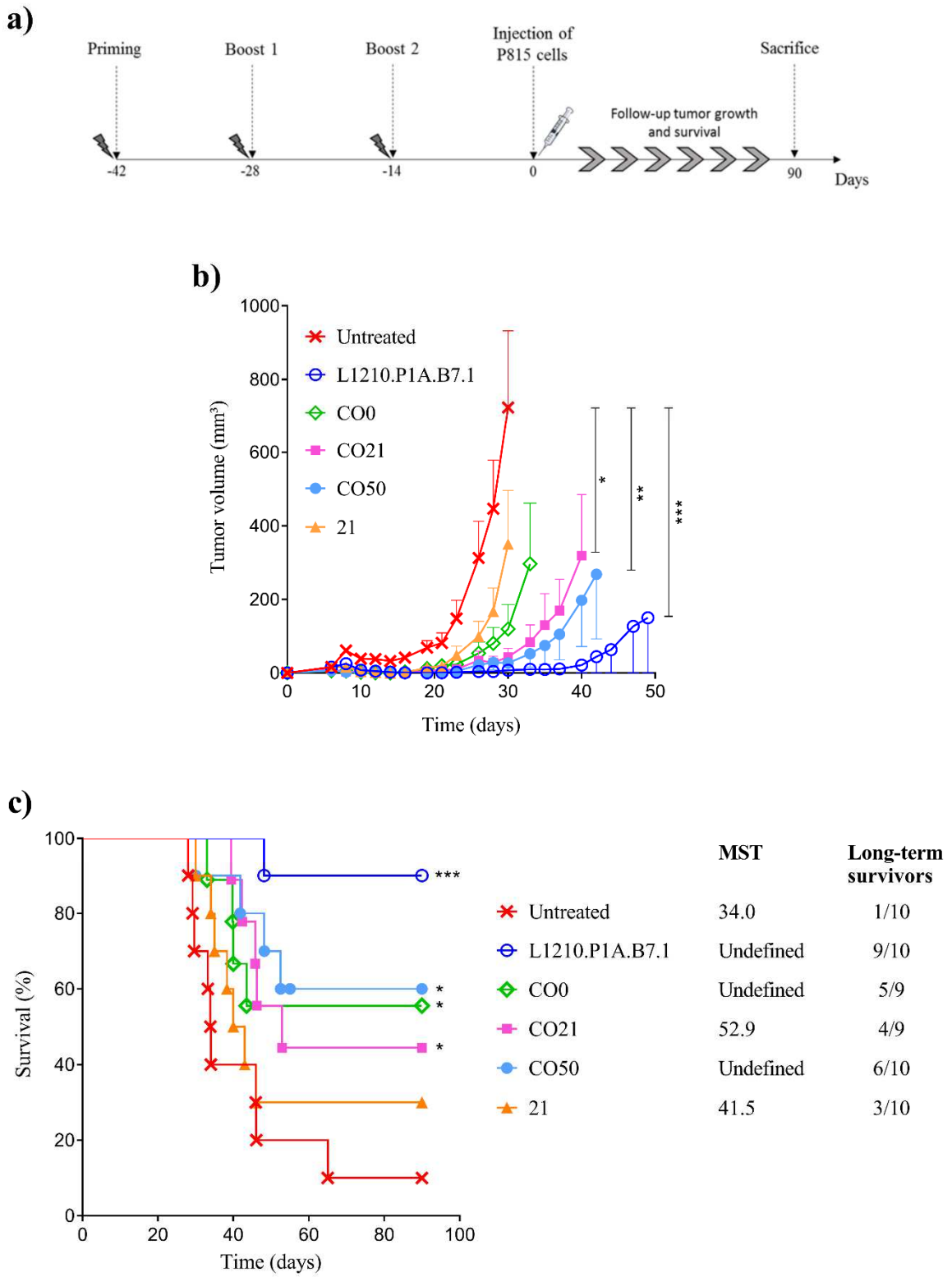


Figure 4: P815 challenge after immunization with intramuscular electroporation of 4 different pVax2-P1A vaccines: CO0, CO21 (n = 9), CO50, 21 or by intraperitoneal injection of L1210.P1A.B7.1 cells (n = 10). (**a**) Prophylactic vaccination protocol. (**b**) Evolution of tumor volume (mm^3) after P815 challenge as a function of time (days) (mean ± SD). Statistical analysis is referred to the untreated group: two-way Anova, column factor (***p value < 0.001, **p value < 0.01, *p value < 0.05) (**c**) Survival curve representing the percentage of mice alive (%) as a function of time (days). Statistical analysis: Log-Rank (Mantel–Cox) test (***p value < 0.001, *p value < 0.05). In the legend, the median survival time (MST, days) of P815 challenged DBA/2 mice after prophylactic vaccination and the number of long-term survivors are shown.

It also has been demonstrated that the administration of the wild-type P1A gene (the same as the 21 vaccine in our current study) could delay tumor growth and increase mouse survival, due to an augmented CTL activation (1). Here, better results were obtained using CO vaccines. Codon optimization induced an increased production of antigen and a modulation of the CpG motifs that further strengthened the *in vivo* effect of the vaccine and generated a stronger immunity.

To evaluate the presence of memory T cells, long-term survivors were re-challenged 4 weeks after the end of the experiment in the other flank with 10^6 P815 mastocytoma cells. All the mice rejected the tumor 8-10 days after the re-injection (data not shown). This result is representative of the generation of long-lasting immunity, a critical feature for successful vaccinations.

III.4. $CD8^+$ LYMPHOCYTE INFILTRATION IN TUMOR WAS HIGHER USING CODON-OPTIMIZED PLASMIDS WITH HIGHER CPG CONTENT

To evaluate the potency of the anti-tumor immune response in mice that received a prophylactic vaccination but did not survive to the P815 challenge, tumors were collected and analyzed by immunohistochemistry and the $CD8^+$ lymphocyte infiltration was evaluated. Figure 5 depicts a series of representative sections for all the groups of animals (n = 3 for all the groups, except L1210.P1A.B7.1, as only one mouse developed the tumor in this group). CD3 (green) and CD8 (red) staining were merged (yellow) in order to exclude NK cells from the $CD8^+$ T lymphocyte evaluation. In the groups treated with CO plasmids containing 21 or 50 CpG motifs more $CD8^+$ cells were observed than the untreated group or the group vaccinated with the non-optimized plasmid or with the CO0. Furthermore, in the CO21 and CO50-treated groups the formation of several $CD3^+/CD8^+$ aggregates all over the tumor was detected, especially in the tumor stroma and in the invasion front of the tumor. Again, the highest $CD3^+/CD8^+$ tumor infiltration was reached using the CO50 vaccine. Conversely, in the untreated and 21 groups the presence of $CD3^+/CD8^+$ cells was sporadic. An elevated presence of $CD8^+$ T lymphocytes in the tumor stroma of the CO21 and CO50 groups but not of the untreated, CO0 or 21 groups was confirmed by H/E staining with DAB directed against the anti-$CD8^+$ antibody (data not shown). The images from the only mouse treated with L1210.P1A.B7.1 cells that developed the tumor are also shown in Figure 5 and the result was similar to the CO50 group.

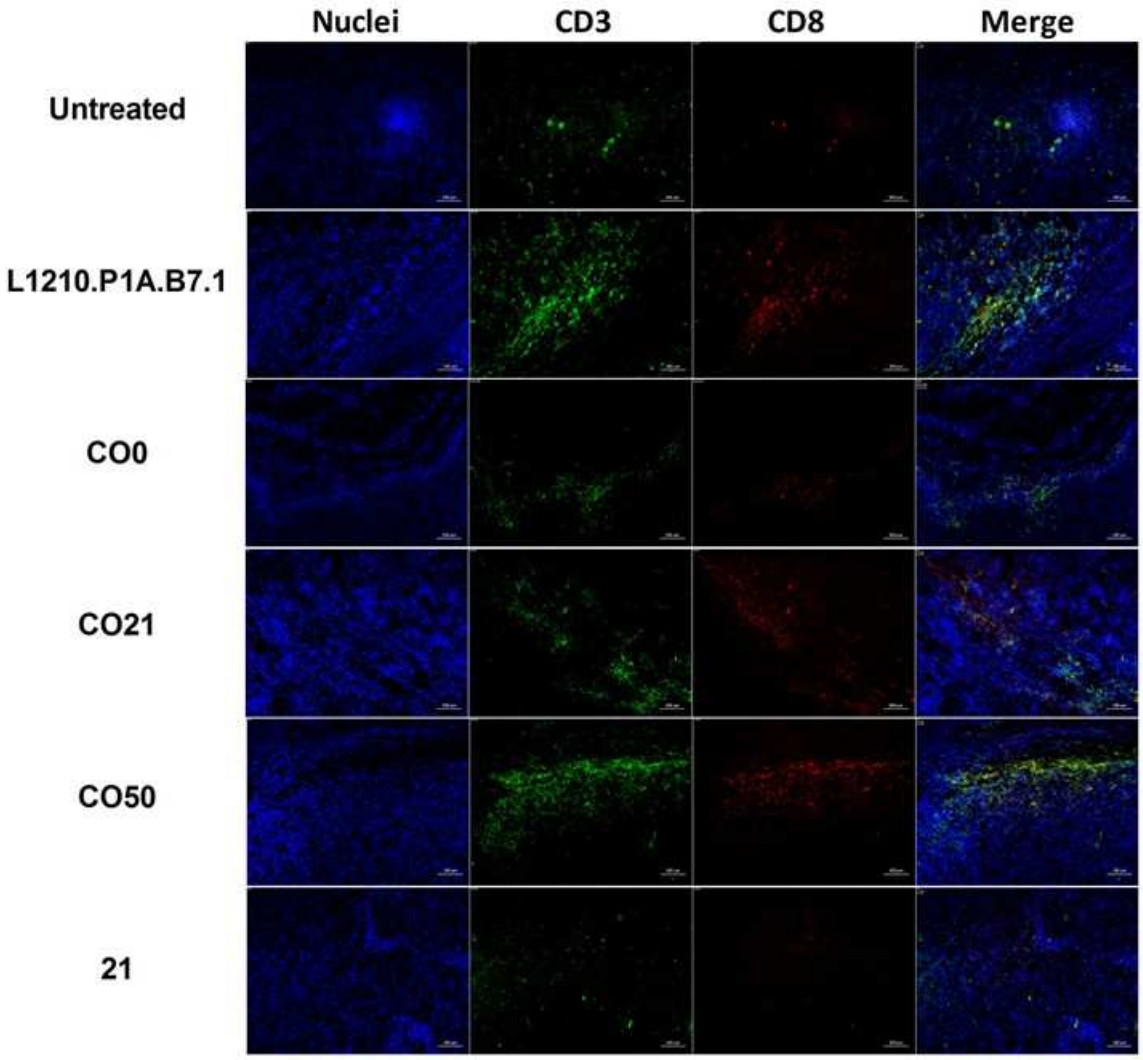


Figure 5: Representative images of immunostaining of CD3 and CD8 cells in tumors. Mice were injected and electroporated in the tibial muscle with the different P1A vaccines (CO0, CO21, CO50 and 21) or injected intraperitoneally with L1210.P1A.B7.1 cells (positive control) and compared to the untreated group. When the mice were sacrificed, tumors were harvested and fluorescent immunohistochemistry was performed for $CD3^+$ (FITC, green) and $CD8^+$ (APC, red) T cells. Sections were counterstained with DAPI. The final pictures represent merged images. The objective used was 10X and the scale bar = 100 μm. n = 3 for all the groups, except L1210.P1A.B7.1 (n = 1), as only one mouse developed the tumor.

Recently, Sobottka *et al.* (66) showed that a higher number of $CD8^+$ tumor-infiltrating lymphocytes, especially infiltrative-margin lymphocytes (i.e., lymphocytes resident in the peripheral areas of the invasive tumor cells representing the invasion front of the tumor) was associated with increased disease-free survival in human breast cancer (66). Hence, even in mice that eventually developed the tumor, the elevated lymphocyte infiltration obtained in mice treated with the CO vaccines containing higher number of CpG motifs could be responsible for the slower tumor growth and the prolonged survival time.

III.5. THERAPEUTIC VACCINATION USING THE CODON-OPTIMIZED P1A-ENCODING VACCINE WITH THE HIGHEST NUMBER OF CPG MOTIFS DECREASED TUMOR GROWTH RATE AND PROLONGED MOUSE SURVIVAL

To assess the therapeutic potential of P1A vaccines, the best plasmid from the prophylactic vaccination, pVAX2-P1A_CO50 was evaluated. P815 tumor cells were injected two days before the priming with the vaccine, followed by 2 boosts administered weekly (Figure 6a). Two days after the tumor cell injection, all the mice developed a tumor and no difference was seen until day 10, when the tumor growth rate decreased for the CO50-treated group, reaching a plateau between days 10 and 16 (Figure 6b). At day 16, the tumor volume in the CO50-treated mice was approximately half of the tumor volume in the untreated mice. Surprisingly, the L1210.P1A.B7.1 treatment was ineffective in delaying the tumor growth despite its efficacy in the prophylactic approach. Starting from day 16, all the tumors grew approximately at the same rate, leaving a gap between CO50-treated group and the others. Furthermore, treatment with the CO50 vaccine led to an increase in mouse survival (Figure 6c). However, only 6% of mice completely rejected the tumor, suggesting the necessity of a combined therapy to counteract the tumor-immunosuppressing microenvironment.

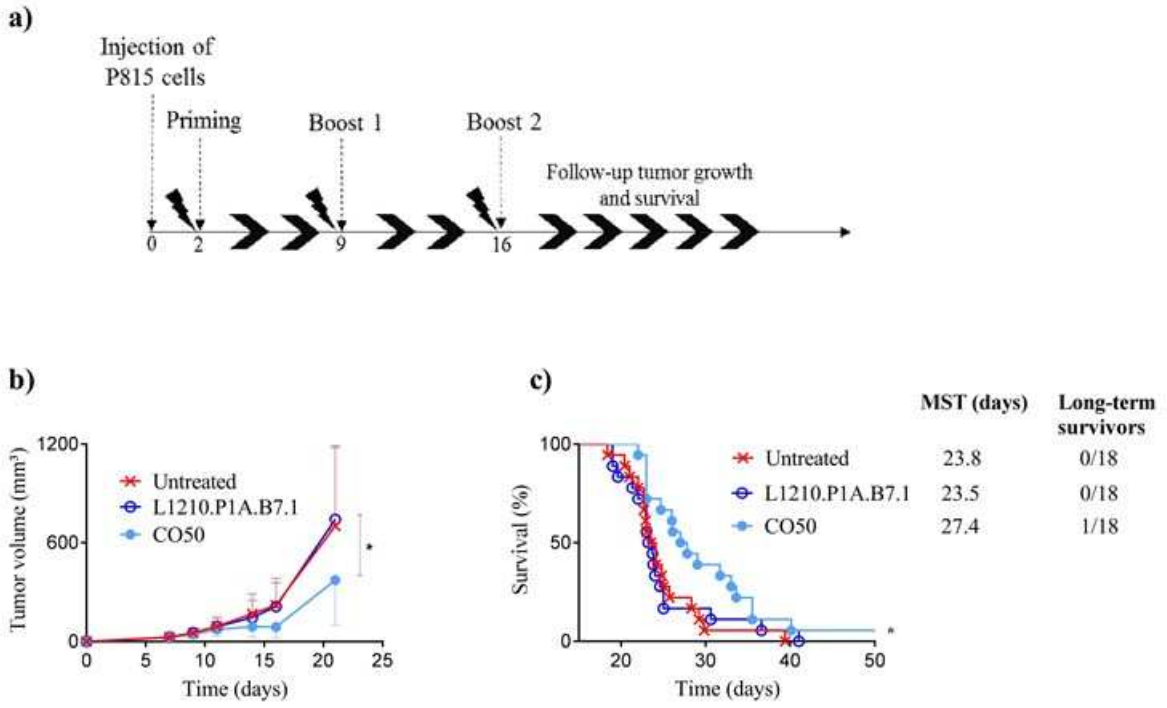


Figure 6: P815 injection followed by immunization with intramuscular electroporation of CO50 or intraperitoneal injection of L1210.P1A.B7.1 cells, compared to the untreated (n = 18). (**a**) Therapeutic vaccination protocol. (**b**) Evolution of tumor volume (mm^3) after P815 challenge as a function of time (days) (mean ± SD). Statistical analysis is compared to the untreated group: two-way Anova, column factor (*p value < 0.05). (**c**) Survival curve representing the percentage of mice alive (%) as a function of time (days). Statistical analysis: Log-Rank (Mantel–Cox) test (*p value < 0.05).

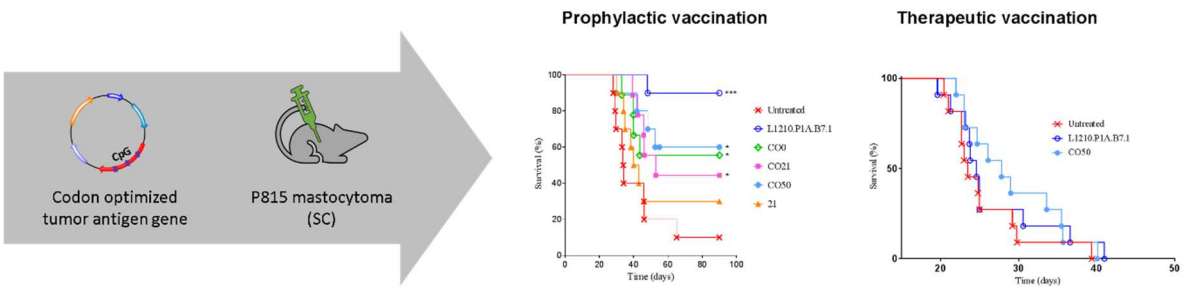
Although several DNA vaccines directed against P815 have been already tested in prophylactic cancer vaccination (1, 67, 68), only one previous study has obtained a promising result after a therapeutic vaccination. In 2004, Ni *et al.* tested P1A replicon DNA as a therapeutic vaccine and found a significant increase in mice survival (69). However, the dose of P815 cells injected was lower (5 x 10^4 cells) compared to the dose used in the current study (10^6 cells). Other studies have demonstrated the potential of P1A vaccines for a therapeutic approach if used in combination with adjuvants or other therapies that can slow tumor development (70, 71). Here, the administration by EP of an optimized P1A vaccine alone effectively increased the survival of the tumor-bearing DBA/2 mice after P815 tumor injection.

IV. CONCLUSIONS

Despite promising results in preclinical models, the low immunogenicity of DNA vaccines currently limits their development as cancer vaccines for human applications. This study aimed to assess whether the optimization of the antigen gene sequence could improve the immune response generated by a P1A DNA cancer vaccine against the P815 tumor, a model for vaccines directed against human MAGE-type tumor antigens (64).

Four P1A sequences were constructed and delivered *in vivo* by intramuscular EP. Three of them were codon optimized and contained various numbers of CpG motifs. All the CO sequences were able to significantly delay the tumor growth and increase the survival of the mice following a prophylactic vaccination (Figure 4). Among the different P1A constructs, the most successful vaccine was the one that contained the highest number of CpG motifs. Indeed, this vaccine had the combined effect of enhanced antigen production coupled with the induction of IL-1β and IL-6 cytokine expression at the site of administration as well as greater $CD8^+$ lymphocyte infiltration in the tumors of the vaccinated mice. The same vaccine was also able to delay the tumor growth and increase the survival of the mice under therapeutic vaccination conditions. For the therapeutic vaccination, the codon optimized vaccine containing 50 CpG motifs in the P1A sequence was more efficient than the L1210.P1A.B7.1 positive control (30), which was completely ineffective in that context (Figure 6). However, vaccine therapy still needs to address the immunosuppressive tumor microenvironment, which prevents the establishment of a strong immunity in an established tumor. Hence, a combination of DNA vaccination with other treatment modalities that simultaneously aim to reduce tumor growth or inhibit the immunosuppressive tumor environment may be the best strategy to eradicate cancer and improve the efficacy of DNA cancer vaccines.

Graphical conclusion



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CHAPTER IV.
COMBINATION OF IMMUNE CHECKPOINT BLOCKADE WITH DNA CANCER VACCINE INDUCES POTENT ANTITUMOR IMMUNITY AGAINST P815 MASTOCYTOMA

Adapted from:

Lopes A., Vanvarenberg K., Kos S., Lucas S., Colau D., Van den Eynde B., Préat V., Vandermeulen G. Combination of Immune Checkpoint Blockade with DNA cancer vaccine induces potent antitumor immunity against P815 Mastocytoma. *Scientific Report. 2018 24;8(1):15732. doi: 10.1038/s41598-018-33933-7.*

GENERAL INTRODUCTION

In the previous chapter, we showed that the optimization of a DNA vaccine allows to significantly increase the survival of mice vaccinated before the tumor challenge (prophylactic vaccination). Despite the impressive and uncommon tumor growth delay observed even in a therapeutic setting, the percentage of cured mice was low. We hypothesized that, in the presence of an established tumor, the DNA vaccine had to overcome the negative effects of the immunosuppressive tumor microenvironment (TME). The great success of immune checkpoint blockers (ICB) in the preclinical and clinical trials and their ability to "remove the brakes" of T cells (1), inspired the idea of combining them with our optimized vaccine.

In this chapter, the effects of the combination between the previously validated optimized P1A DNA vaccine and the ICB are explored. Interestingly, the combination allowed to reach 90% of survival, decreased the formation of liver metastasis and increased the infiltration of antigen-specific cytotoxic T cells (CTLs) in the TME. This result suggested that the main limitation of DNA vaccines used as a single therapy is that the specific CTLs are not able to reach or to be active in the TME, in the presence of an established tumor. This reflection inspired the idea that DNA vaccination, rationally combined with other therapies, can be used to increase the survival of mice, and maybe to cure them, even from other more aggressive and more clinically relevant tumor types.

ABSTRACT

DNA vaccination against cancer has become a promising strategy for inducing a specific and long-lasting antitumor immunity. However, DNA vaccines fail to generate potent immune responses when used as a single therapy. To enhance their activity into the tumor, a DNA vaccine against murine P815 mastocytoma was combined with antibodies directed against the immune checkpoints CTLA4 and PD1, which are involved in T cell activity. The combination of these two strategies delayed tumor growth and enhanced specific antitumor immune cell infiltration in comparison to the corresponding single therapies. The combination also promoted IFNg, IL12 and granzyme B production in the tumor microenvironment and decreased the formation of liver metastasis in a very early phase of tumor development, enabling 90% survival. These results underline the complementarity of DNA vaccination and immune checkpoint blockers in inducing a potent immune response, by exploiting the generation of antigen-specific T cells by the vaccine and the ability of immune checkpoint blockers to enhance T cell activity and infiltration in the tumor. These findings suggest how and why a rational combination therapy can overcome the limits of DNA vaccination but could also allow responses to immune checkpoint blockers in a larger proportion of subjects.

KEYWORDS

DNA vaccines, immune checkpoint blockade, P815 mastocytoma, tumor microenvironment, tumor-associated antigen, metastasis

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I. INTRODUCTION

Immunotherapy is an established approach to treat cancer based on the observation that the immune system can mount destructive responses against tumors. A major goal of immunotherapy is to develop a specific immune response against tumor-associated antigens (TAAs), which are derived from proteins that are specifically or preferentially expressed in tumor cells in comparison to non-transformed healthy cells (2). DNA vaccines represent a good strategy to prime T cell responses against TAAs (3). Furthermore, DNA vaccines can be used to deliver one or more antigens in their native conformation to develop a broad immune response (3).

The amplitude of the developed immune responses is determined not only by the antigen recognition of T cells but also by co-stimulation/co-inhibition at the immunological synapse. Indeed, activated T cells express inhibitory receptors on their surface, such as CTLA4 and PD1, aiming at preventing autoimmunity (4). These receptors are also responsible for the lack of effective antitumor immune responses in cancer patients, dampening T cell effector activity against tumor antigens. In particular, CTLA4 inhibits T cell activation during the priming phase of immunity (5). PD-1 transmits inhibitory signals into T cells after ligation with PD-1 ligands and promotes tolerance (6). Antibodies blocking these molecules can increase the effector activity of tumor-specific T cells (7). These antibodies, which are known as immune checkpoint blockers (ICBs), have already been approved by the FDA/EMA and used in the standard of care for different tumor types, such as melanoma and non-small-cell lung cancer (4, 8). However, ICBs have immune-related adverse effects, which commonly harm the gastrointestinal tract, endocrine glands, skin, and liver (8). Moreover, ICBs are effective only in a minority of patients. In most advanced cancers, the response rate with anti-PD-1/PD-L1 monotherapy is only ~20%(9), and the response rate with anti-CTLA4 is approximately 12% (4), indicating the need for improvement. This low efficacy may be due to a lack of pre-existing tumor-associated T cell immunity.

The murine mastocytoma P815 tumor model was used in the present study. This model is characterized by the expression of different TAAs, particularly P815A, encoded by the P1A gene as previously described (10). P815A shares many characteristics with human MAGE-type (melanoma antigen gene) tumor antigens (10), suggesting P815 mastocytoma as a good preclinical tumor model for future applications in human medicine. We have previously developed a codon-optimized vaccine encoding P815A (11). We demonstrated that the optimized vaccine increased antigen expression and activated innate immunity while retarding tumor growth in both preventive and therapeutic settings (11). However, therapeutic vaccination delayed tumor growth but only slightly increased the survival of mice.

In this study, we aimed to generate a more potent immune response by combining DNA vaccination with ICBs. We hypothesized that this combination can improve the therapeutic efficacy of the DNA vaccine and increase the number of mice responding to ICB by "releasing the brakes" of T cell activity and by activating a higher number of antigen-specific T cells. We also evaluated the effects of the two strategies in the tumor microenvironment (TME) in an early phase of tumor development and metastasis formation that, until now, has been poorly explored.

II. MATERIALS AND METHODS

II.1. PLASMID OPTIMIZATION AND PRODUCTION

The P1A DNA vaccine encoding the P1A antigen gene (here named plasmid P1A, pP1A) has been previously described (11). The plasmid was amplified and purified using an EndoFree Plasmid Giga Kit (Qiagen, Venlo, NL) according to the manufacturer's protocol. Optical density at 260 nm was used to determine DNA concentration. Plasmid was diluted in phosphate buffered saline (PBS, Thermo Fisher, Waltham, Massachusetts, USA) and stored at -20°C before use.

II.2. CELL LINES

P1A-expressing P815 murine cells were obtained from Dr. Catherine Uyttenhove (Ludwig Institute for Cancer Research) and cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, Massachusetts, USA) and 1% penicillin/streptomycin (Thermo Fisher, Waltham, Massachusetts, USA).

II.3. ANIMALS

DBA/2 female mice were obtained from Janvier (Le Genest-Saint-Isle, FR). Mice were between 5 and 6 weeks old at the beginning of the experiments. Water and food were provided ad libitum. All experimental protocols using mice were approved by the Ethical Committee for Animal Care and Use of the Medical Sector of the Université Catholique de Louvain (UCL/MD/2011/007 and UCL/MD/2016/001). All experiments were performed in accordance with relevant guidelines and regulations.

II.4. TUMOR IMPLANTATION AND TUMOR GROWTH MEASUREMENT.

At day 0, $1x10^6$ P815 cells diluted in 100 µl of PBS were injected subcutaneously into the right flank of each mouse. Tumors were measured with an electronic digital caliper three times per week. Tumor volumes were calculated as length × width × height (in mm^3). Mice were sacrificed when the tumor volume was greater than 1500 mm^3 or when they were in poor condition and expected to die shortly. Tumors were collected and used for further experiments.

II.5. THERAPEUTIC VACCINATIONS AND ICB ADMINISTRATION

Before each vaccine injection, mice were anesthetized with ±150 µl of a solution of 10 mg/ml ketamine (Ketalar, Pfizer, New York, USA) and 1 mg/ml xylazine (Sigma, St. Louis, MO, USA).

The left paw was shaved using a rodent shaver (Aesculap Exacta shaver, AgnTho's, Stockholm, SE). Mice in the pP1A (n = 10) and pP1A + anti-CTLA4/PD1 (n = 10) groups were injected with 50 μg of pP1A diluted in 30 μl of PBS in the left tibialis cranial muscle. The paw was then placed between 4 mm plate BTX caliper electrodes (VWR International, Leuven, BE) and electroporated (200 V/cm, 8 pulses, 20 ms with 500 ms pause between pulses). The pulses were delivered by a BTX™ Gemini Electroporation System (VWR International, Leuven, BE). The vaccine was administered 2, 9 and 16 days after tumor injection. Immune checkpoint blockade (ICB) antibodies directed against CTLA4 (clone 9D9) and PD1 (clone 29F.A12) were purchased from Bioconnect (Huissen, NL). Mice in the anti-CTLA4/PD1 and pP1A + anti-CTLA4/PD1 (n = 10) groups were injected intraperitoneally (IP) with 100 μg of each antibody in 100 μl of PBS 3, 6 and 9 days after tumor injection. Nonimmunized mice were used as a negative control (n = 10). The protocol is presented in Figure 1.

II.6. CD4 AND CD8 T CELL DETECTION BY FLOW CYTOMETRY

For the detection of CD4 and CD8 T cells in the tumor microenvironment, tumors were surgically removed, and tumor volume was measured 10 days after P815 cell injection. To prepare single cell suspensions, tumors were digested for 1 hour in 1 mg/ml collagenase type II (Sigma, Saint-Louis, Missouri, USA). Cells were collected, counted using an automatic cell counter (BioRad, California, USA) and washed with PBS containing 5 mM EDTA and 1% albumin. Cells were then incubated with an anti-CD16/CD32 antibody for 10 minutes on ice (clone cl93, Biolegend, San Diego, California, USA). Cells were washed and incubated for 60 minutes at 4°C with a cocktail of the following antibodies: anti-CD3-APC-Cy7 (clone 17A2, Biolegend, San Diego, California, USA), anti-CD4-FITC (clone GK1,5, Biolegend, San Diego, California, USA), anti-CD8-FITC (clone 53-6,7, Biolegend, San Diego, California, USA) and P1A tetramers-phycoerythrin (PE). For staining with anti-Ki67-BV421 (clone B56, BD Bioscience, Franklin Lakes, New Jersey, USA), antiFoxP3-APC (clone FJK-16s, Thermo Fisher, Waltham, Massachusetts, USA) and anti-IFNg-APC (clone XMG1.2, Biolegend, San Diego, California, USA), cells were previously incubated overnight at 4°C with a permeabilization/fixation solution (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Thermo Fisher, Waltham, Massachusetts, USA). Cells were then incubated with anti-CD16/CD32 antibody for 10 minutes on ice (Biolegend, San Diego, California, USA). Cells were washed and incubated for 60 minutes at 4°C with two different cocktails of antibodies diluted in the permeabilization/fixation solution: anti-Ki67, anti-IFNg (first cocktail) and anti-Ki67 and anti-FoxP3 (second cocktail). Samples were washed with PBS containing 5 mM EDTA and 1% albumin, and the cells were then suspended in PBS. For each staining panel, a negative control

(non-stained cells) was performed. For each antibody, we performed a control with cells stained with one antibody at the time. As the mice were sacrificed 24h after the last vaccine and ICB injection and tumors analyzed right after the sacrifice, no stimulation was needed to test the production of IFNg (12). Sample data were acquired with FACSverse (BD bioscience, Franklin Lakes, New Jersey, USA) and analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA). The number of cells was normalized by the tumor volume (mm^3).

II.7. DETERMINATION OF GRANZYME B AND IL12 EXPRESSION IN TUMORS USING QPCR ANALYSIS

To further determine the activity of immune cells present in the tumor microenvironment, tumors extracted at day 10 were analyzed by qPCR. Total RNA was isolated using TRIzol reagent (Thermo Fisher, Waltham, Massachusetts, USA) and phenol separation. The quality and quantity of RNA were evaluated using a nanospectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). Extracted RNA was considered pure if the 260/280 absorbance ratio of the sample was approximately 2 and the 260/230 absorbance ratio was 1.8-2.2. One microgram of RNA was reverse transcribed using a first-strand synthesis system (SuperScript™, Thermo Fisher Scientific) and oligo(dT) primers (Eurogentec, Liege, BE) according to the supplier's protocol. The resulting cDNA was used as template for 40 cycles of PCR amplification. SYBR™ green real-time qPCR (GoTaq qPCR MasterMix kit, Promega, Fitchburg, WI, USA) was conducted on a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) to detect IL12 and Granzyme B mRNA expression in the tumors. Analysis of the melting curves was performed to ensure purity of PCR products. The results were analyzed with StepOne Software V2.1. The mRNA expression of the cytokines was calculated relative to the corresponding expression of β-actin (housekeeping gene) according to the delta-delta Ct method. The results were normalized compared to the untreated control group. Primers for IL12 and Granzyme B were designed using Primer Blast software based on the consensus of sequences from GenBank. To determine PD-L1 mRNA expression, RNA from tumors at day 10 and from P815 cells was extracted and converted into cDNA as described previously. The resulting cDNA was used as a template for 30 cycles of semiquantitative PCR with a T100 thermocycler (Bio-Rad). Primers for β-actin (housekeeping gene) and PD-L1 were used to amplify respective cDNA by PCR. The PCR products were subjected to electrophoresis on a SYBR Safe (Thermo Fisher Scientific)-stained 1% agarose gel. A 1 bk DNA ladder (Sigma, Saint Louis, Missouri, USA) was used to check the length of the amplicon. A complete list of the primers used in this study is shown in Table 1.

Table 1. List of primers used for qPCR analysis.

Oligo name	**Primer sequence (5' → 3')**	**Amplicon length**
Granzyme B for	GAAGCCAGGAGATGTGTGCT	183 bp
Granzyme B rev	GCACGTTTGGTCTTTGGGTC	
IL-12 for	GGAAGCACGGCAGCAGAATA	180 bp
IL-12 rev	AACTTGAGGGAGAAGTAGGAATGG	
β-actin for	ACTCCTATGTGGGTGACGAG	206 bp
β-actin rev	CATCTTTTCACGGTTGGCCTTAG	
PD-L1 for	TAATCAGCTACGGTGGTGCG	273 bp
PD-L1 rev	AAACATCATTCGCTGTGGCG	

II.8. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 7 for Windows. Survival curves were compared using a Mantel–Cox (log-rank) test. p-Values less than 0.05 were considered statistically significant. Data with no common superscript letter (a, b, c) were considered significantly different ($p < 0.05$) according to ANOVA.

III. RESULTS AND DISCUSSION

III.1. THE COMBINATION OF pP1A VACCINE AND ICBs DELAYED TUMOR GROWTH AND INCREASED MOUSE SURVIVAL

To assess the therapeutic efficacy of the combination of pP1A with ICBs, tumor-bearing mice were treated with pP1A alone or in combination with anti-CTLA4 and anti-PD1. The protocol is shown in Figure 1a. Tumor growth was significantly slower for all the treatments compared to the untreated group and, importantly, was significantly slower in the group receiving pP1A in combination with ICBs than the group receiving ICBs alone or pP1A alone (Figure 1b). Indeed, tumors in the untreated group started to grow 8 days after tumor injection, and their growth was exponential. In the other groups, tumor volumes remained constant between day 8 and 13. Some of the tumors started to regrow after that period. In particular, pP1A alone delayed tumor growth, with the tumors starting to regrow at day 15 after a slight regression/plateau around day 10. The growth of tumors in individual mice is shown in Figure 1c. Compared to the untreated mice, all the other mice survived longer (Figure 1d). Survival was 20% or 60% when pP1A or ICB was used alone, respectively (Figure 1d). Using the combination therapy of pP1A with ICBs, 90% of mice survived (Figure 1d). However, the difference between ICBs and pP1A + ICBs was not statistically significant despite the higher survival trend observed in the group treated with the pP1A + ICB combination. The result obtained with pP1A alone confirmed our previous work on this tumor model, indicating the ability of a DNA vaccine to slowdown the tumor growth, but not to permit a complete and significant tumor rejection (11). The combination of pP1A with ICB further decreased tumor growth even if the doses of ICBs used in this study were lower than others that employed the same therapeutic combination (100 μg compared to 200-250 μg per dose, per antibody) (4, 13). This encouraging result suggests the possibility to reduce the dose of ICB when they are administered in combination with DNA vaccination, to decrease the toxicities associated to these antibodies in human patients (14).

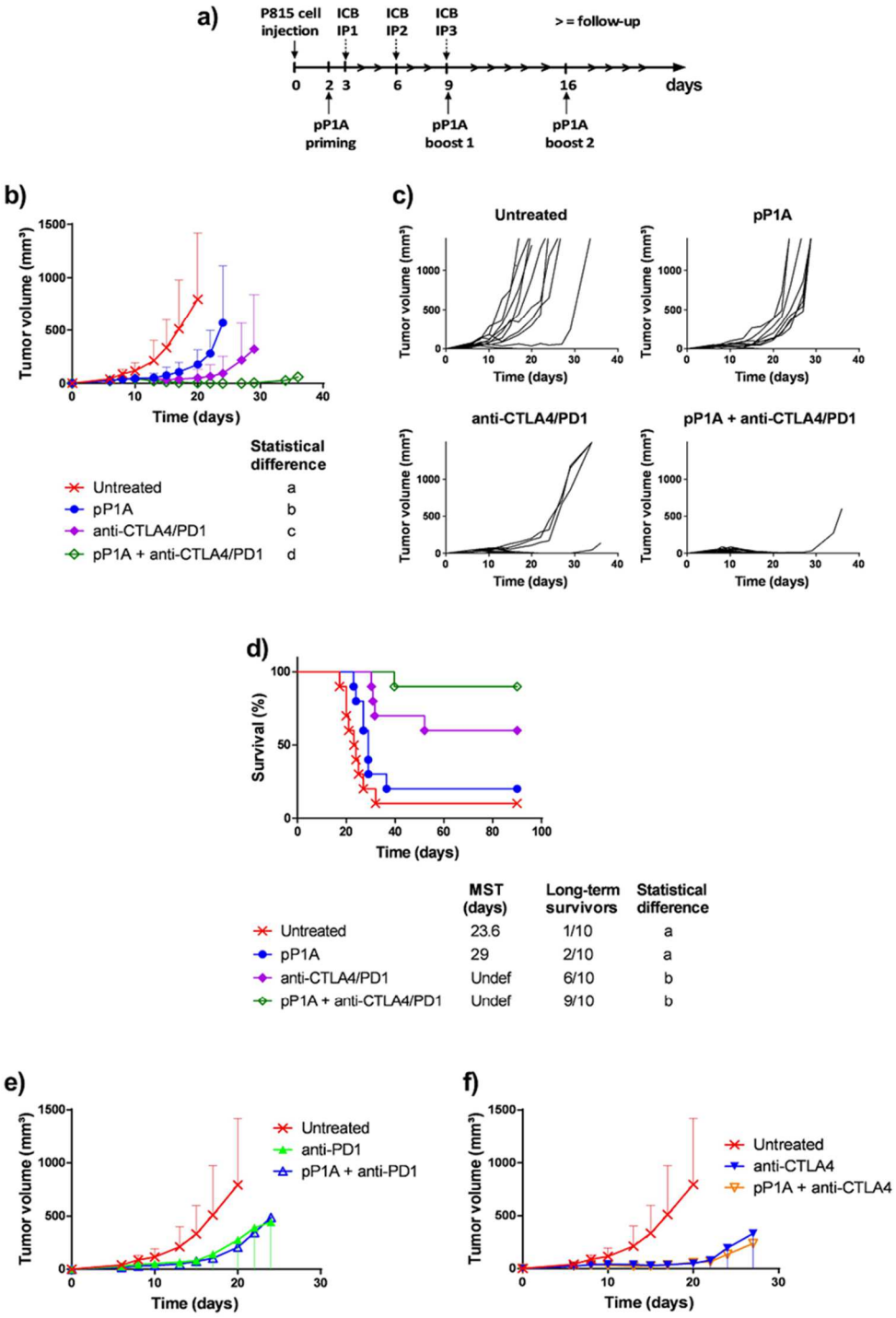


Figure 1: Therapeutic combination of pP1A and ICBs (anti-CTLA4/PD1).
(a) Therapeutic vaccination protocol. ICBs were administered intraperitoneally (IP) 3 times every 3 days starting at day 3. The pP1A vaccine was administered 3 times weekly starting at day 2 by intramuscular electroporation. **(b)** Evolution of tumor volume (mm^3) after P815 challenge as a function of time (days) (mean ± SD). All the groups were statistically compared to the others using two-way ANOVA, column factor ($p < 0.05$, n =10). **(c)** Tumor growth in individual mice and for every group of mice (n = 10). **(d)** Survival curve representing the percentage of living mice (%) as a function of time (days). Statistical analysis using log-rank (Mantel-Cox) test (significant difference when $p < 0.05$, n = 10). MST = median survival time. **(e, f)** Measurement of tumor volume (mm^3) after P815 challenge as a function of time for the anti-PD1 and pP1A + anti-PD1 groups (d) or for the anti-CTLA4 and pP1A + anti-CTLA4 groups (e) (days) (mean ± SD, n = 10). The absence of common superscript letters (a, b, c, d) indicates statistically different results.

The efficacy of anti-PD1 in our model may be due to the expression of PD-L1 mRNA in P815 cells and in the untreated tumors (Supplementary data 1). The rationale for the use of a second ICB is their additive effect on increasing mouse survival compared to the use of a single ICB (15, 16). Indeed, in the current experiment, the combination of pP1A with one ICB at a time was also tested by measuring the tumor growth in treated mice. No significant differences were observed between a single ICB used alone and one used in combination with the vaccine (Figure 1e, f). Das et al. (17) observed that the blockade of CTLA-4, PD-1, or the combination of the two leads to distinct genomic and functional signatures *in vivo* in purified human T cells and monocytes. Combination blockade induces non-overlapping changes in gene expression (17). This complementarity justifies the high interest in using more than one ICB in preclinical and clinical studies (7, 18-21). In particular, the anti-CTLA4 antibody can enrich and amplify the T cell repertoire (22), which is improved by priming with cancer vaccination, thereby inducing a proliferative signature to tumor-infiltrating T cells (23). In contrast, the anti-PD1 antibody reactivates disabled intratumoral T cells and modulates their cytolytic function (23). Furthermore, anti-PD1 therapy is more effective when tumor cells express PD-L1, as its expression is significantly associated with greater clinical response rates to anti-PD1 treatments (24). Hence, the combination of ICBs and cancer vaccination may transform poorly immunogenic tumors into 'inflamed' tumors, which are more sensitive to treatments and the host immune response (7, 23). The rational of this effect could be the complementary mechanism of DNA vaccination and ICB. Vaccination can help the phase of antigen processing and presentation that is dampened during tumor development, while ICB can sustain T cell activation and trafficking into the tumor. Hence, ICB could enhance the infiltration and activity of antigen-specific T cells generated by DNA vaccination. To verify this hypothesis, an analysis of T cell infiltration in an early phase of tumor development was performed.

III.2. TUMOR INFILTRATION BY CD4 T CELLS WAS HIGHER WHEN MICE WERE TREATED WITH A COMBINATION OF PP1A VACCINE AND ICBS

To assess the early phase cellular immune response in the tumor microenvironment, CD4 T cell infiltration was evaluated in tumors resected 10 days after P815 injection. Single cell suspensions were analyzed via flow cytometry to detect CD3+CD4+FoxP3- T cells (non-Treg CD4 T cells) and CD3+CD4+FoxP3+ cells (Tregs). In general, a higher amount of non-Treg CD4 T cells were detected in tumors of mice treated with ICBs and pP1A + ICBs (Figure 2a), and the ratio of Tregs to total CD4 T cells was not different between the groups (Figure 2b). This result means that the treatments did not reduce Treg infiltration in the early phase of tumor development. The reason of this result is not clear, as ICBs can reduce Treg infiltration in the TME according to the literature

(25). Our hypothesis is that the effects of ICBs on Treg depletion are visible in a later phase of tumor development. It would be interesting to follow the evolution of Treg infiltration and activity in the tumor microenvironment as a function of time. Nevertheless, there was a higher density of non-Treg CD4 T cells in the tumors of mice treated with ICBs. According to the literature, this effect may be principally due to the anti-CTLA4 antibody. The role of monoclonal antibodies that block inhibitory immune checkpoint molecules in enhancing T cell infiltration has been previously described (26, 27). Bengsch et al. found that disruption of the CTLA-4 interaction with CD80 using an anti-CTLA4 antibody induces CD4 T cell infiltration into tumors (28). However, although the vaccine alone did not have any effect on CD4 T cell infiltration, its combination with ICBs further increased the number of non-Treg CD4 T cells into the tumor. In some preclinical models, cancer vaccines enhanced effector T cell infiltration into tumors when combined with other therapies (29). Furthermore, the group receiving the vaccination alone promoted a higher non-Treg CD4 proliferation (Ki67+ cells) compared to the untreated group (Figure 2c, 2d and Supplementary data 2b). Ki67 is a nuclear protein associated to cell proliferation. It is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), making it an excellent marker for determining the growth fraction of a given cell population (30). This means that the vaccine can influence the proliferative capacity of infiltrated CD4 and increase the number of CD4-non Tregs inside the tumor, as observed in the combination group. Finally, CD3-positive cells were labeled with anti-IFNg antibodies to detect IFNg-secreting cells, and CD8 T cells were excluded from this analysis (Figure 2e, 2f). This experiment clearly demonstrated that there is higher IFNg production from both proliferating and non-proliferating (Ki67- cells, cells in G(0) phase) non-CD8 T cells (including CD4 T cells) when the combination therapy is used. Currently, the important role of CD4 T cells in tumor regression is being elucidated (31). In a clinical study of esophageal squamous cell carcinoma, increased CD4 T cell infiltration was significantly associated with longer overall survival (32). In the P815 model, Rahir et al., demonstrated that the infiltration of CD8-specific T cells requires CD4 T cells (33). For this reason, a growing number of cancer vaccines have been designed to contain at least one CD4 epitope (13, 34-36).

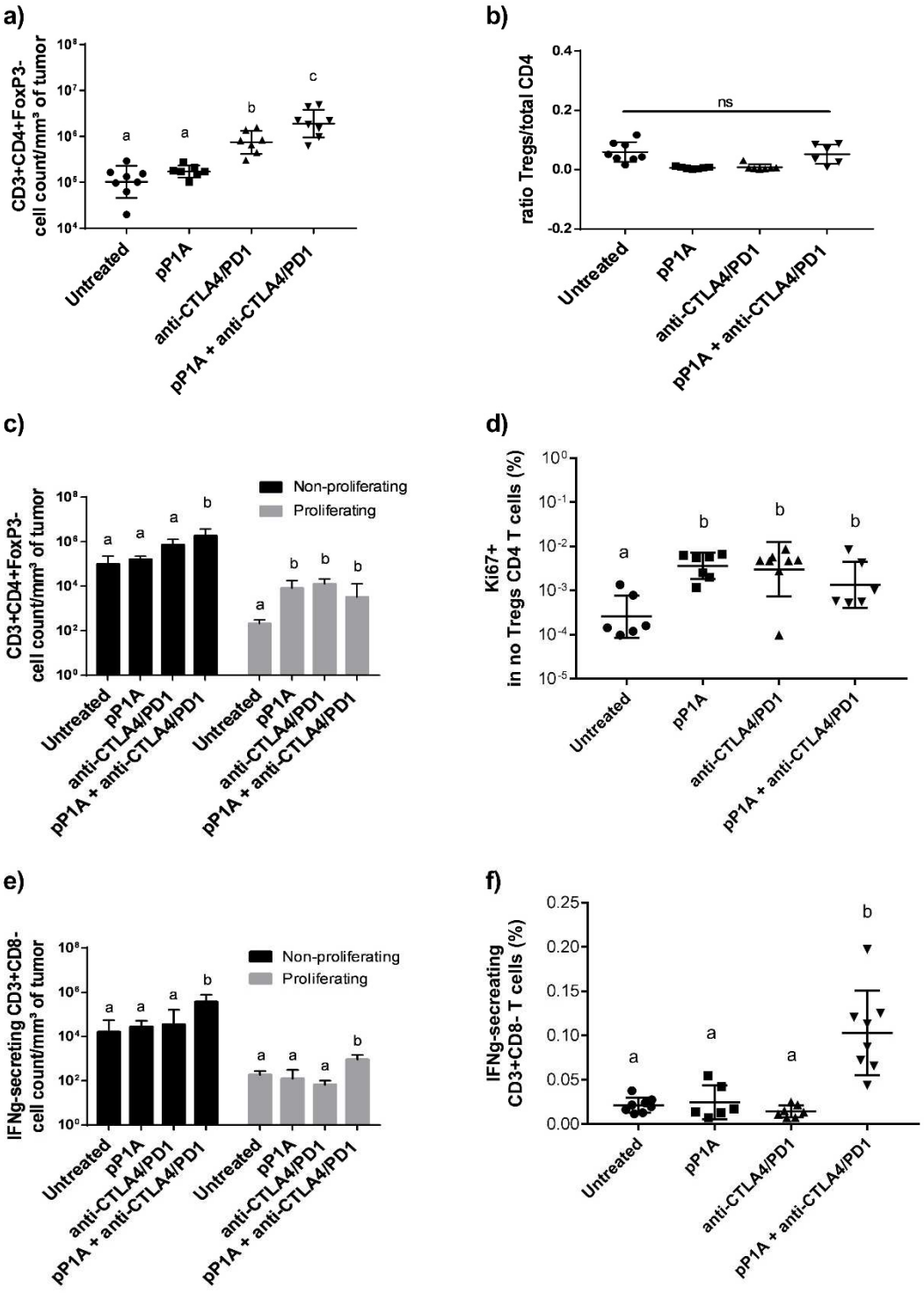


Figure 2: Evaluation of CD4 infiltration in tumors.
(a) Number of non-Treg CD4 T cells (CD3+CD4+FoxP3-) per mm³ of tumor. **(b)** Ratio of Treg cells (CD3+CD4+FoxP3+) compared to the total number of non-Treg CD4 T cells. **(c)** Number of proliferating and non-proliferating non-Treg CD4 T cells per mm³ of tumor. **(d)** Percentage of proliferating non-Treg CD4 T cells per mm³ of tumor. **(e)** Number of IFNg-secreting CD3+CD8- T cells per mm³ of tumor. **(f)** Percentage of IFNg-secreting CD3+CD8- T cells per mm³ of tumor. All the results are expressed as the mean ± SD (n = 6-8) and were considered statistically significant when $p < 0.05$ (indicated by the absence of common superscript letters) according to one-way ANOVA.

III.3. CD8 T CELL PROLIFERATION, ACTIVITY AND CYTOKINE PRODUCTION WERE HIGHER IN THE COMBINATION GROUP.

To evaluate the CD8 T cell activity and specific response induced by the vaccine alone or in combination with ICBs, mice were injected with P815 mastocytoma cells. Tumors were removed 10 days later and analyzed by flow cytometry. In general, a greater amount of CD8 T cells was detected in the group treated with ICBs and ICBs + pP1A, which was significantly different compared to the untreated or the pP1A groups (Figure 3a). Among the CD8 T cells, the number of IFNg-secreting CD8 T cells was significantly higher in the pP1A + anti-CTLA4/PD1 group than in all the other groups (Figure 3b). In this same group, there was also a significant increase in proliferating IFNg-secreting (Figure 3c, Supplementary data 2a) and P1A tetramer-specific CD8 T cells (Figure 3d, e, f and g), indicating that the analyzed CD8 T cells were not only antigen specific but also active against tumor cells because of their high secretion levels of IFNg (37). Furthermore, qPCR analysis of the extracted tumors showed significantly higher IL12 production and a shift in the granzyme B production in the combination group (Figure 3h, i). IFNg-producing T cells are critical for antitumor immunity (37, 38). The best characterized role of IFNg in CD8 T cell immunity is in enhancing MHC class I antigen presentation pathway, which facilitates cytotoxic T cells to recognize tumor cells. IFNg also enhances CD4 type 1 helper T (Th1) phenotype development (39), regulates MHC class I expression, has weak cytolytic activity and upregulates the expression of enzymes and cytokines such as Granzyme B and IL12 (37). In particular, the increased production of IL12 observed in the combination-treated mice followed the same trend as IFNg production. Indeed, IL12 initiates or increases IFNg secretion in a positive feedback loop (40). IL12 also polarizes CD4 T cells into Th1 and regulates the tumor vasculature, thereby playing an important role in tumor rejection (40, 41). IFNg production by antitumor-specific T cells also upregulates PD-L1 on tumor cells as a resistance mechanism to adaptive immunity, thereby promoting PD-L1/PD-1 blockade after vaccination(23).

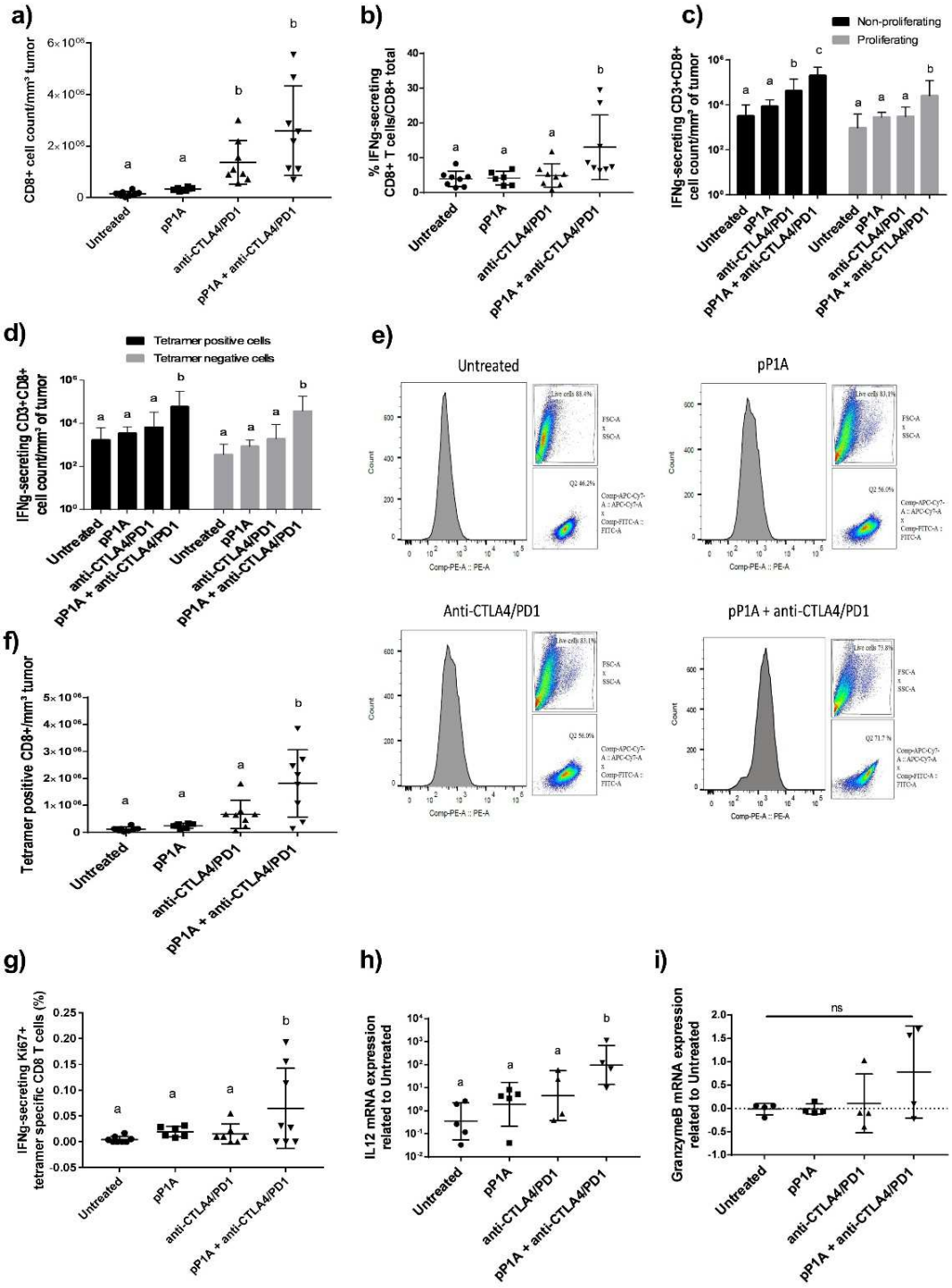


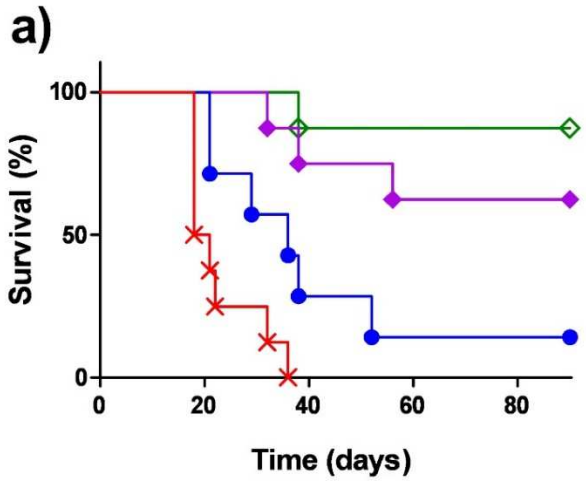
Figure 3: Evaluation of CD8 T cell infiltration, specificity and activity in tumors.
(a) Number of total CD8 T cells in the tumor (n = 6-8). **(b)** Percentage of IFNg-secreting CD8 T cells compared to the total number of CD3+CD8+ cells (n = 6-8). **(c)** Number of proliferating (Ki67+) and non-proliferating (Ki67-) IFNg-secreting CD8 T cells per mm³ of tumor (n = 6-8). **(d)** Number of antigen-specific (tetramer-positive) and antigen non-specific (tetramer-negative) IFNg-secreting CD8 T cells per mm³ of tumor (n = 6-8). **(e)** Representative images of flow cytometry analysis for tetramer-specific CD8 T cells in the single tumor cell suspension. Gating strategy: singlets → live cells → CD3+CD8+ cells → tetramer+ cells. **(f)** Antigen-specific CD8 T cells per mm³ of tumor. **(g)** Percentage of P1A antigen-specific IFNg-secreting and proliferating CD8 T cells per mm³ of tumor. **(h)** qPCR analysis of IL12 mRNA expression related to the untreated group (n = 4-5). **(i)** qPCR analysis of Granzyme B mRNA expression related to the untreated group (n = 4). All the results are expressed as the mean ± SD and were considered statistically significant when $p < 0.05$ (indicated by the lack of common superscript letters) according to one-way ANOVA.

The infiltration of antigen-specific CD8 T cells in the group treated with pP1A alone was not significantly different from that in the untreated group. These results suggest that the vaccine alone is not effective in inducing a sufficiently potent immune response in a therapeutic setting due to the immunosuppressive tumor microenvironment, confirming our previous findings (11). Most likely, the vaccine alone may not be able to ensure sustained CD8 T cell infiltration into the tumor, which could explain the rare presence of antigen-specific CD8 T cells in the tumor. On the other hand, treatment with ICBs alone did not show any significant difference compared to the untreated in priming CD8 T cells against the P815A antigen, but their role in enhancing intratumoral CD8 T cell infiltration has been already demonstrated (4, 21). This complementarity could explain the increased presence of antigen-specific CD8 T cells found in the combination group, as ICBs allow immune cell infiltration, while the vaccine induces their antigen specificity.

III.4. AFTER TUMOR RESECTION, MICE TREATED WITH THE COMBINATION THERAPY LIVED LONGER AND WITHOUT METASTASES

To evaluate the long-term effect of the treatments, tumors were surgically resected 10 days after tumor injection without sacrificing the mice. As shown in Figure 4a, 8 days after tumor resection, untreated mice started to die without any visible subcutaneous tumor. Within 3 weeks, all the untreated mice were dead. To understand the reason for their death, postmortem analyses were performed. Their liver was abnormally large and full of metastases (white spots shown in Figure 4b). The medium weight of these livers was 2.6 ± 0.8 g (n = 4), which was approximately 3 times higher than the weight of a healthy mouse liver. Hence, 10 days after tumor injection, untreated mice had already developed metastasis, and they died shortly later. Mice in the other groups lived longer. As expected, pP1A significantly prolonged mouse survival even when compared to the model without tumor resection (MST = 36 days instead of 29 days, as observed in Figure 1b and 4a), which may be due to a slowdown in metastasis formation induced by the vaccine. This effect has been observed in other studies using cancer vaccines (42, 43) and attests the capacity of these vaccines to act systemically. However, pP1A alone was not sufficient to completely avoid metastasis formation and death. The combination of the vaccine with ICBs significantly prolonged mouse survival compared to the pP1A group, seemingly due to the ICBs blocking metastasis development. A correlation between PD-L1 and PD-1 expression and metastasis has been described (44), which may explain the efficacy of anti-PD1/PD-L1 therapy against metastasis. Recently, the role of ICB in restoring the tumor-suppressive capacity of NK cells has also been demonstrated (45). These cells are involved in the control of metastasis, and their activity depends on the expression of IL12 and Granzyme B, among others (45, 46). Hence, the increased IL12

expression that was observed in this study could explain the metastasis regression and the higher survival in the ICB and ICB + pP1A groups.



	MST (days)	Long-term survivors	Statistical difference
Untreated	19.6	0/8	a
pP1A	36	1/7	b
anti-CTLA4/PD1	Undef	5/8	c
pP1A + anti-CTLA4/PD1	Undef	7/8	c

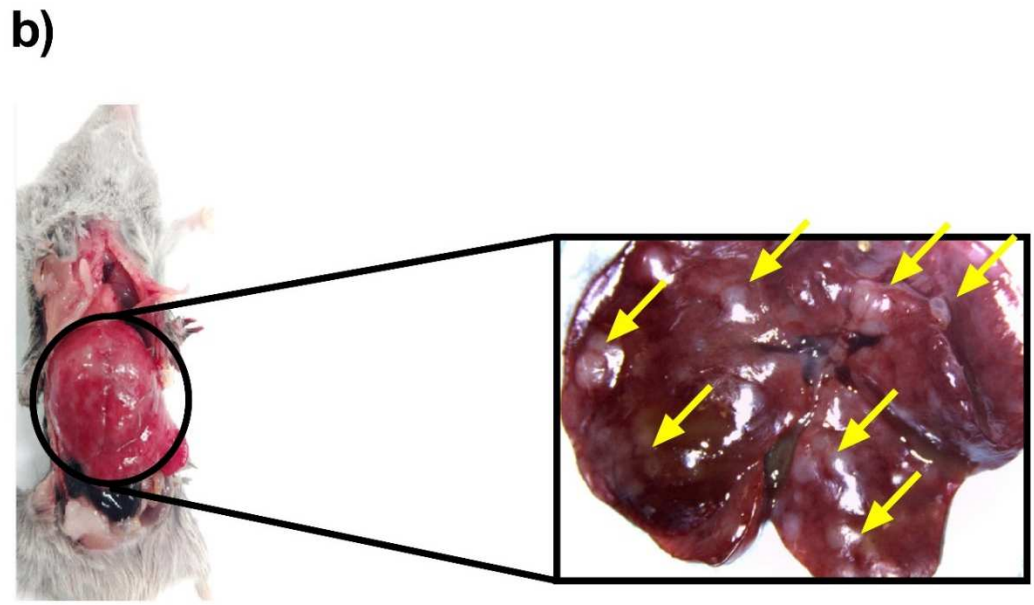


Figure 4: Mouse follow-up after tumor removal at day 10 post-P815 injection.
(a) Survival curve representing the percentage of living mice (%) as a function of time (days). MST = median survival time. Statistical analysis using log-rank (Mantel-Cox) test to compare each group to the others. Data with no common superscript letter (a, b, c) are significantly different ($p < 0.05$, $n = 8$). **(b)** Representative image of mouse postmortem analysis with magnification of liver metastases.

IV. CONCLUSIONS

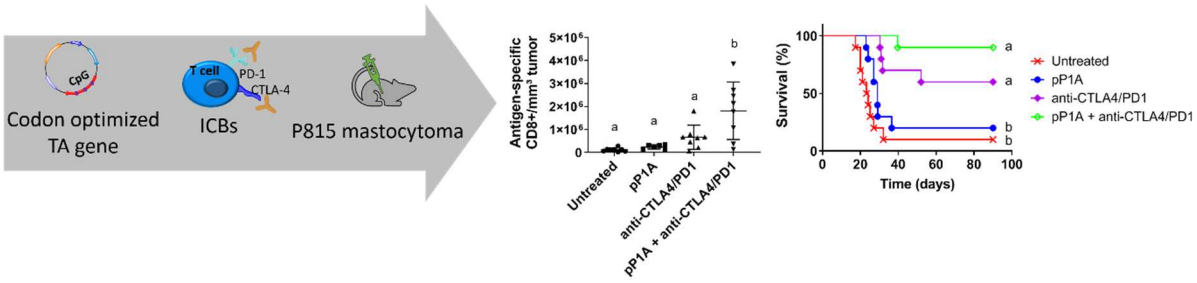
This study evaluates the main limitations of the use of DNA vaccines and ICB as single treatment against cancer. It also analyses the reasons of the successful combination of these two therapies, giving a future perspective on how to optimize anticancer immunotherapies. CTLA4 and PD1 antagonists have been widely used in preclinical and clinical trials due to their ability to enhance tumor-reactive T cell responses (47, 48). However, they are not able to specifically prime T cells and they are toxic in the majority of the patients(49).

In this study, the combination of ICBs and DNA vaccination permitted to decrease the doses of the antibodies compared to what is reported in the literature (50, 51) and generated a more potent immune response compared to the single therapies. Their effects in the tumor microenvironment were analyzed in a very early phase of tumor development, i.e., 10 days after the tumor injection, which was shown to be a critical stage for metastases formation. This synergy was due to the ability of DNA vaccination to prime T cells against a specific cancer antigen and of ICBs to increase T cell activity and infiltration in the tumor. Hence, ICBs created a favorable microenvironment for the action of the cancer DNA vaccine. Both the pP1A vaccine and ICB as single therapies showed an increase in CD4 T cells. ICB significantly increased also the number of IFNg-secreting CD8. When used in combination, however, pP1A and ICBs significantly increased IFNg and IL12 production but also CD4 and CD8 T cell infiltration 10 days after tumor implantation. This result explains the significant delay in tumor growth among mice treated with pP1A alone, ICB alone or pP1A + ICB, demonstrating an improved outcome in response to the combination therapy.

Compared to the untreated group, the group treated with ICBs and pP1A showed a protective effect when the tumors were removed, as they retarded the formation of liver metastases appearing in the early phase of tumor growth. These results indicated that immune activation starts to appear in the early phase of tumor development, especially when the combination therapy of the DNA vaccine and ICBs is used.

Overall, this study suggests and supports the idea of a rational combination of cancer DNA vaccines able to generate a tumor-specific and long-lasting immune response with therapies that increase immune cell infiltration, proliferation and activity in the tumor, such as ICB. In addition, it demonstrated that the beneficial effects of DNA vaccine in combination with ICBs appeared as soon as one week after its administration, allowing 7 out of 8 mice to survive after a tumor injection and avoiding metastases formation.

Graphical conclusion



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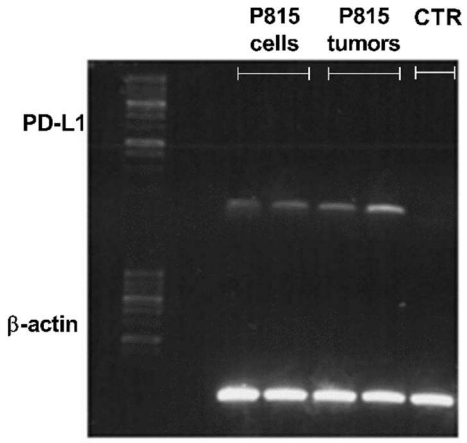
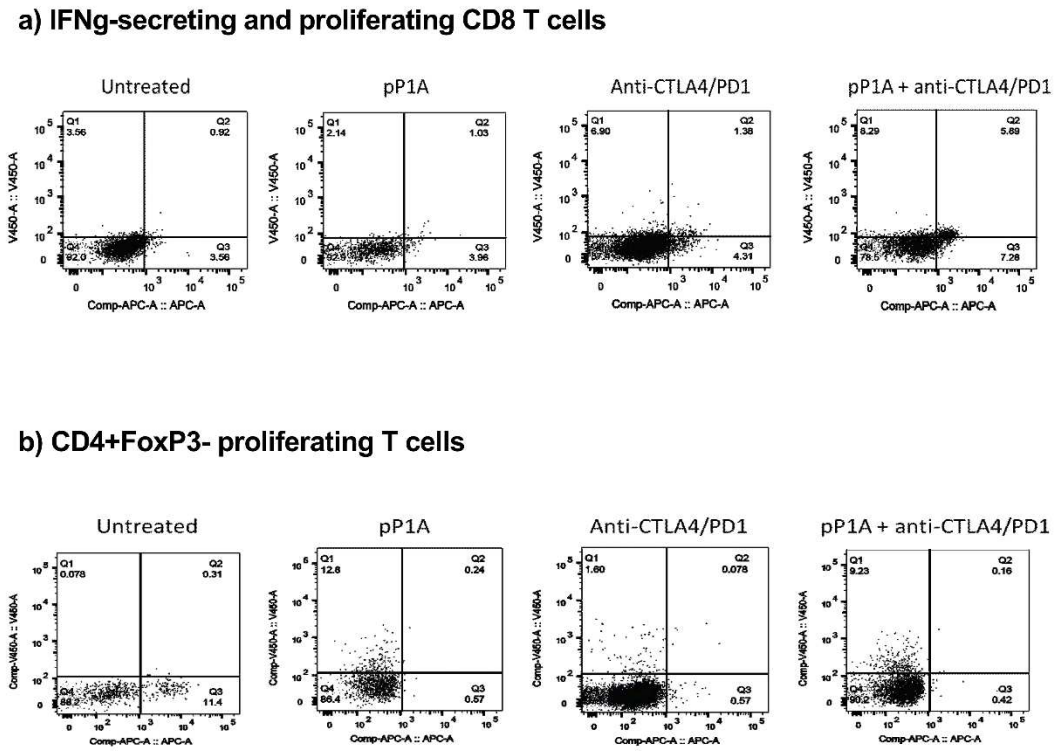
Supplementary data 1



Supplementary data 1: RT-PCR of PD-L1 mRNA expression in P815 cells and untreated tumors (n = 2). Control group is a sample without the PD-L1 primers (CTR).

Supplementary data 2



Supplementary data 2: FACS data of tumor-infiltrated T cells.
a) IFNg-secreting and proliferating CD8.
The gating strategy used was as follow: singlets → live cells → CD3+CD8+ cells → Ki67-IFNg cells
b) CD4+FoxP3- proliferating T cells.
The gating strategy used was as follow: singlets → live cells → CD3+CD4+ cells → FoxP3-Ki67

CHAPTER V. ONCOLYTIC ADENOVIRUS DRIVES SPECIFIC IMMUNE RESPONSE GENERATED BY A POLY-EPITOPE pDNA VACCINE ENCODING MELANOMA NEOANTIGENS INTO THE TUMOR SITE

Adapted from:

Lopes A., Feola S., Ligot S., Fusciello M., Vandermeulen G., Préat V., Cerullo V. Oncolytic Adenovirus drives specific immune response generated by a poly-epitope pDNA vaccine encoding melanoma neoantigens into the tumor site. *Journal of immunotherapy for cancer, 2019. doi : 10.1186/s40425-019-0644-7*

GENERAL INTRODUCTION

Until now, we have demonstrated the ability of optimized DNA vaccines to cure P815 mastocytoma-bearing mice, when combined with therapies that recruit/activate antigen-specific immune cells in the tumor. At this stage, we wanted to validate our findings in a more clinically relevant tumor model, known to be highly aggressive, poorly immunogenic and with a high tumor mutational burden, the B16 melanoma.

As previously discussed (Table 8 of the Introduction), the DNA vaccine efficacy in the B16 models was limited, especially in a therapeutic setting. Some studies already explored the possibility to administer more than one antigen to extend the immune activation also to CD4 T cells, considered crucial for tumor eradication (1, 2). Furthermore, the immunogenicity of neoantigens was starting to become a hot topic at the beginning of this study, but only few very recent studies are now exploring their applicability on DNA vaccination (3, 4).

We hypothesized that the delivery of more than one antigen, including non-mutated and well-known tumor-associated antigens (TAAs) and neoantigens, could increase the efficacy of the DNA vaccination. To this aim, we needed a new vaccine platform to be able to insert more than one epitope belonging to both TAAs and neoantigens. In our lab, we were exploring the possibility to insert in a pVAX2 vector the sequence of a viral protein, the Vesicular Stomatitis Virus Glycoprotein (VSV-G), that could (i) activate an innate immune response thanks to its viral nature, and (ii) to be modified for the insertion of different antigenic epitopes (5). At the beginning of this study, the possibility to modify in two different sites the VSV-G protein was validated. From the literature, we selected three B16 neoantigens (Kif18b, Cpsf3l and Pbk), known to be immunogenic, and two differentiation TAAs (gp100 and TRP2). However, when we screened the B16F10 cells to detect the presence of neoantigen mutations at different cell passages, we observed that some neoantigens mutated too rapidly. In particular, the mutation frequency for one of the analyzed neoantigens (the Cpsf3l) was higher in the B16F10 cells compared to the B16F1 (Table 0); hence, the design of a Cpsf3l neoantigen-encoding DNA vaccine for B16F10 would have been challenging.

Table 0: B16F1 and B16F10 mutation for the neoantigen gene Cpsf3l, at different cell passages.

Cpsf3l non-mutated epitope sequence	Cell passage number	Sequence in B16F1	Sequence in B16F10
EFKHIKAFDRTFADN PGPMVVFATPGM	8, 10 and 12	EFKHIKAFDRTFADNPGPMV **RPWQSAS**	EFKHIKAFDRTFADNPGPMV **RPWQSAS**
	16	The same as above	EFKHIKAFDRTFA**N**NPGPMV **RPWQSAS**

This would have been incompatible with the construction of a vaccine against specific antigens. The risk would have been a decreased efficacy after few tumor cell divisions. On the contrary, the B16F1 model was more stable in term of mutation frequency and it made possible the construction of a DNA vaccine directed against the predicted and detected neoantigens. For this reason, this study was performed in the B16F1 model.

Then, we constructed 4 different plasmids. Every plasmid was modified with one CD4 (gp100 or Cpsf3l or Kif18b) and one CD8 epitope (Pbk or TRP-2), keeping at least one TAA for every plasmid. Hence, the four generated plasmids were: pTRP2-gp100, pTRP2-Kif18b, pTRP2-Cpsf3l, pgp100-Pbk. The efficacy of the different plasmids has been tested in vivo. The use of only 2 TAAs (pTRP2-gp100) failed to show a significant decrease in the tumor growth rate (Figure 0A). When also the neoantigens were administered to B16F1-bearing mice (the four plasmids pTRP2-gp100, pTRP2-Kif18b, pTRP2-Cpsf3l and pgp100-Pbk administered in the "pDNA mix"), the median survival time (MST) was longer, even if this was not enough to significantly prolong their survival (Figure 0B).

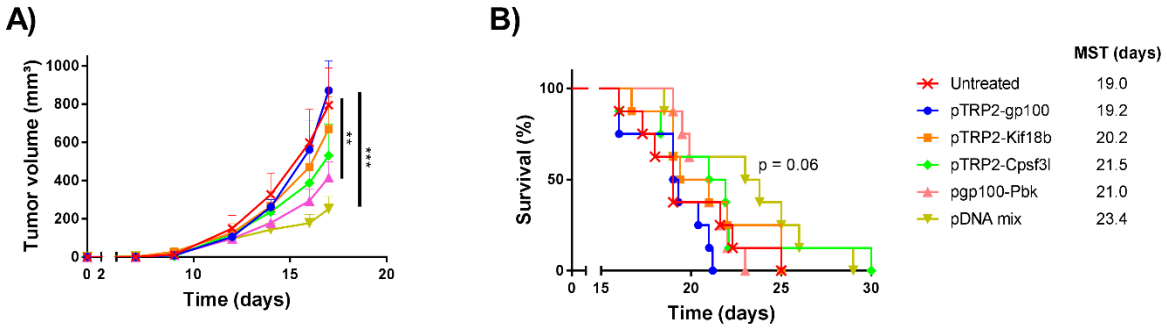


Figure 0: Tumor evolution and survival following DNA vaccination with the single plasmids encoding 2 TAs (pTRP2-gp100, pTRP2-Kif18b, pTRP2-Cpsf3l and pgp100-Pbk) or with the mix of the 4 plasmids (pDNA mix). A) Evolution of tumor volume (mm^3) after B16F1 challenge as a function of time (days) (mean ± SEM; n = 8). Statistical analysis is compared to the untreated group: two-way Anova, column factor (*p value < 0.05). B) Survival curves representing the percentage of alive mice (%) as a function of time (days); MST = median survival time. Statistical analysis: Log-Rank (Mantel–Cox) test.

Once the new vaccine was produced, we hypothesized that it needed to be combined with a treatment that could recruit the immune cells generated by the vaccine in the tumor site. One ideal candidate was an oncolytic virus, intratumorally injected, able at the same time to lysate tumor cells and to activate the immune system in the injection site.

In this chapter, we explore the effects of the combination of a new poly-epitope DNA vaccine encoding TAAs and neoantigens, and an oncolytic adenovirus in the generation of a potent immune response against B16F1 melanoma. The impressive delay in the tumor growth and the recruitment of antigen-specific CTLs obtained only when the two therapies where combined, further supported our previous findings.

The applicability of DNA vaccination in a poorly immunogenic tumor, such as B16 melanoma, represents a great hope for the future of cancer immunotherapy.

ABSTRACT

Background - DNA vaccines against cancer held great promises due to the generation of a specific and long-lasting immune response. However, when used as a single therapy, they are not able to drive the generated immune response into the tumor, because of the immunosuppressive microenvironment, thus limiting their use in humans. To enhance DNA vaccine efficacy, we combined a new poly-epitope DNA vaccine encoding melanoma tumor associated antigens and B16F1-specific neoantigens with an oncolytic virus administered intratumorally.

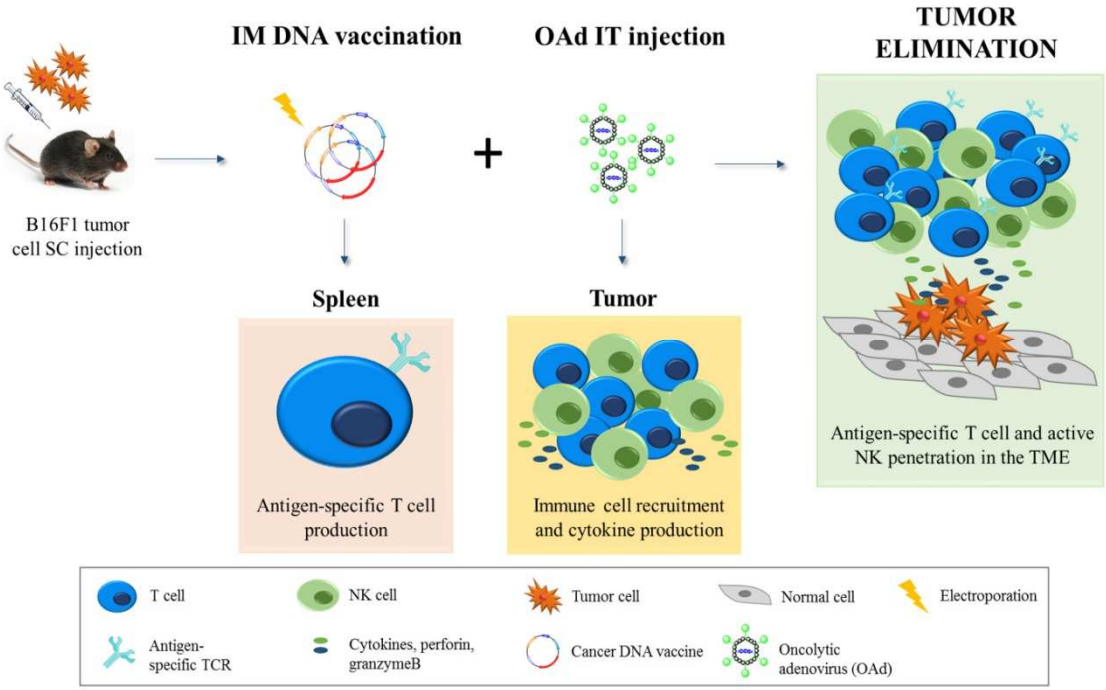
Methods - Genomic analysis were performed to find specific mutations in B16F1 melanoma cells. The antigen gene sequences were designed according to these mutations prior to the insertion in the plasmid vector. Mice were injected with B16F1 tumor cells (n = 7-9) and therapeutically vaccinated 2, 9 and 16 days after the tumor injection. The virus was administered intratumorally at day 10, 12 and 14. Immune cell infiltration analysis and cytokine production were performed by flow cytometry, PCR and ELISPOT in the tumor site and in the spleen of animals, 17 days after the tumor injection.

Results - The combination of DNA vaccine and oncolytic virus significantly increased the immune activity into the tumor. In particular, the local intratumoral viral therapy increased the NK infiltration, thus increasing the production of different cytokines, chemokines and enzymes involved in the adaptive immune system recruitment and cytotoxic activity. On the other side, the DNA vaccine generated antigen-specific T cells in the spleen, which migrated into the tumor when recalled by the local viral therapy. The complementarity between these strategies explains the dramatic tumor regression observed only in the combination group compared to all the other control groups.

Conclusion

This study explores the immunological mechanism of the combination between an oncolytic adenovirus and a DNA vaccine against melanoma. It demonstrates that the use of a rational combination therapy involving DNA vaccination could overcome its poor immunogenicity. In this way, it will be possible to exploit the great potential of DNA vaccination, thus allowing a larger use in the clinic.

Graphical abstract



KEYWORDS

Oncolytic adenovirus, cancer, DNA vaccine, melanoma neoantigens, tumor microenvironment

TABLE OF CONTENTS

I. INTRODUCTION

Tumor immunogenicity is not only patient specific but inherently specific to the individual tumor itself (6). Cancer vaccines allow the delivery of different tumor associated antigens (TAAs) and neoantigens that can be tailored to the individual tumor (7). This strategy would overcome the self-tolerance associated to TAAs (6, 8) and the issue of the cancer heterogeneity (9-12). Indeed, neoantigens represent ideal targets against cancer, due to their specific expression in cancer tissue and the potential lack of side effects, and can be used in the design of cancer vaccines. (8) In particular, cancer DNA vaccines are stable, cost-efficient, easy to manufacture, safe and allow the delivery of different antigens in the same plasmid (13). The use of CD4 epitopes, in addition to the CD8 epitopes, increases the DNA vaccine activity by activating the T helper (Th) response, as already demonstrated in preclinical essays (14-16). Indeed, immune recognition of mutation-derived epitopes seems to be mostly driven by CD4+ T cells (17, 18). However, DNA vaccines alone fail to drive a strong immune response in the tumor, probably due to the highly immunosuppressive tumor microenvironment (TME) (13, 19). For this reason, DNA cancer immunization usually needs the co-administration of other immunotherapeutic agents able to drive the generated immune response in the tumor border (6, 19). Among the different combinatorial approaches, the oncolytic viruses (OVs) represent the perfect immunological adjuvant to lead specific T cell response within the TME (20, 21).

OVs are biological agents that selectively infect and kill tumor cells without causing damages in healthy cells (22, 23). They can generate a strong immune response, including: (i) activation of a systemic pro-inflammatory state, (ii) attraction of cytotoxic immune cell populations to the sites of infection to eliminate virus-containing cells, and (iii) alarming neighboring uninfected cells of viral infection (24). In particular, Oncolytic Adenovirus (OAd) activates the innate immune system, through the activation of TLRs, NOD2 and other cytoplasmic sensors (25). However, OAd, which is not able to replicate in mice, *per se* is not able to fully eradicate highly aggressive cancers, but it can be modified to be more immunogenic (25). We previously studied and validated a modified OAd virus in a murine melanoma cell line, whose genome has been modified to introduce a series of CpG motifs (26). The TLR9 activation that resulted from the presence of CpG inside the virus allowed the activation of NK cells and cytokine production that were responsible of the tumor regression in nude mice (26).

We hypothesized that the combination in a no heterologous prime-boost manner of a poly-epitope and tumor-specific pDNA vaccine with a modified CpG-rich OAd could generate a full and specific immune response both systemically and in the TME. In this purpose, we designed new DNA vaccines encoding different B16F1-TAAs and B16F1-specific neoantigens inserted in a VSV-G gene sequence. VSV-G is a viral protein well known to improve the immune response (27). The efficacy of a pVAX2 DNA vaccine encoding one CD4 and one CD8 epitope in the VSV-G sequence has been validated in different tumor models, including B16F10 melanoma (5). Furthermore, the plasmid containing a single epitope demonstrated a selective activation of the CD4 response, when a CD4 epitope is encoded, or the CD8 response, when a CD8 epitope is encoded (Vandermeulen et al, in preparation). The advantage of delivering neoantigens would be to overcome the immune tolerance associated to the TAAs.

In this study, we aimed to improve the DNA vaccine efficacy by combining the poly-epitope DNA vaccines (here called pDNA) and an oncolytic adenovirus serotype 5-CpG (here called OAd) to drive in the TME the antigen-specific immune response generated by the DNA vaccine. To our knowledge, this is the first time that a DNA vaccine and an OAd are combined in a no heterologous prime-boost manner to induce the eradication of an already established tumor. We also explored the mechanisms and the contribution of each therapy to the observed tumor regression, which, until now, has been poorly explored, especially in the context of oncolytic virus treatment.

II. MATERIALS AND METHODS

II.1. CELL LINES

B16F1 cells, a melanoma cell line from C57BL/6 mice, were purchased from American Type Culture Collection (ATCC; Manassas, Virginia) and cultured in MEM complete medium, containing 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin (Life Technologies, California). The human lung carcinoma cell line A549 were purchased from ATCC and cultivate in DMEM supplemented with 10% FBS, 1% glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin.

II.2. GDNA EXTRACTION AND DETECTION OF B16F1 NEOANTIGEN MUTATIONS

B16F1 neoantigens were selected from immunogenic B16F10 mutations described in the literature, according to their low MHC I score, which indicates a high binding affinity, the response to RNA vaccination and the reactive T cell subtype (CD4 or CD8) (17). The presence of three selected neoantigens was verified in B16F1 cell line. Specific mutations were detected in the gDNA of the cells at passage number 7, 16, and 27 to verify the presence of the mutations at different time points (passage number 1 is when cells were purchased from ATCC). gDNA was extracted using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher, Massachusetts). Briefly, 20 µl of Proteinase K and 20 µl of RNAseA were added to 200 µl of cells (10^6 cells) and incubated for 2 minutes at room temperature (RT). Then, 200 µl of the Lysis/binding buffer were added to the cells and incubate at 55°C for 10 minutes, before adding 200 µl of pure ethanol. The lysate was purified using a spin column after 2 steps of washings with the washing buffers provided in the kit. gDNA was collected using 50 µl of elution buffer and stocked at -20°C, before performing the PCR amplification (see section II.8). Sanger sequencing was performed by Genewiz (United Kingdom) to verify the presence of specific mutations.

II.3. PDNA VACCINE DESIGN AND PRODUCTION

Four different plasmids encoding melanoma antigens TRP2, Pbk, Kif18b, Cpsf3l and human gp100 were designed (Supplementary data 1). Among the chosen antigens, 3 are recognized by MHC class II (Gp100, Kif18b and Cpsf3l), to stimulate CD4 T cell response, while the others belong to MHC class I epitopes (TRP2 and Pbk). The antigens TRP2 and gp100 are already known melanoma antigens (28, 29). Their presence has been verified in B16F1 cell line (Supplementary data 2). The other three are neoantigens described in the literature for the B16F10 melanoma cell line (17). They have been chosen among the B16F10 immunogenic mutations, based on their MHC

class I binding (low score for higher affinity) and their response after RNA and peptide vaccination in B16F10 tumor model (17). These neoantigens were specifically redesigned according to the mutations found in B16F1 cell line (Table 1). The mutations were verified at different B16F1 cell passages and compared with the gene expression in the spleen of C57Bl/6 mice as a non-mutated control. They have been cloned in a pVAX2 vector encoding the VSV-G viral protein. Two antigens for each plasmid, one CD4 and one CD8, were inserted inside the VSV-G sequence, as described in (5). Four plasmids were obtained: pTRP2-Gp100; pTRP2-Cpsf3l; pTRP2-Kif18b; pGp100-Pbk (Supplementary data 1). The mix of the four plasmids in a 1:1:1:1 proportion (1 µg for each plasmid) was called pDNA. In Table 1, the nucleotide and peptide sequences of each antigen are shown. The underlined amino acids indicate the presence of a mutation specific in B16F1 melanoma cell line.

As an additional control for the survival experiment, an irrelevant plasmid has been used, encoding two OVA epitopes in the VSV-G sequence (one CD4 and one CD8 epitopes). This plasmid has been called "pTOP-OVA CD4-OVA CD8".

Table 1*:* Antigen nucleotide and peptide sequence and MHC specificity (reactive T cell subtype (1)). List of the antigens used in the study. Mutated amino acids of the peptide sequence are in bold and underlined in the table.

Antigen name	Reactive T cell subtype (1)	Nucleotide sequence	Peptide sequence
TRP2	CD8	AGCGTGTACGACTTCTTCGTGTGGCTG	SVYDFFVWL
Pbk	CD8	GACAGCGGCAGCCCTTTTCCTGCTGCCGTGATC CTGAGAGATGCCCTGCACATGGCTAGAGGCCT GAAGTACCTGCACCAA	DSGSPFPAAVILR**D**ALHMARGL KYLHQ
Kif18b	CD4	CCTAGCAAGCCCAGCTTCCAAGAGTTCGTGGAC TGGGAGAACGTGTCCCCTGAGCTGAACTCTACC GACCAGCCTTTCCTG	PSKPSFQEFVDWE**N**VSPELNST DQPFL
Cpsf3l	CD4	GAGTTCAAGCACATCAAGGCCTTCGACAGAAC CTTCGCTGACAACCCCGGACCTATGGTTCGACC TTGGCAGTCTGCTAGC	EFKHIKAFDRTFADNPGPMV**RP WQSAS**
Gp100	CD4	TGGAACAGACAGCTGTACCCCGAGTGGACCGA GGCCCAGAGACTGGAT	WNRQLYPEWTEAQRL

II.4. VIRUS PRODUCTION

Ad5D24-CpG is an OAd bearing a CpG-enriched genome in the E3 gene (30). It was generated, propagated, and characterized using standard protocols, as previously described (31).

II.5. ANIMAL EXPERIMENTS AND ETHICAL PERMITS

All animal experiments were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland and the Belgian national regulations guidelines in accordance with EU Directive 2010/63/EU. The animal experiments were approved by the ethical committee for animal care of the faculty of medicine of the Université catholique de Louvain (UCL/MD/010/2019). Five weeks-old C57BL/6 mice were purchased from Envigo (Harlan, Netherlands) or by Janvier (France). Water and food were provided ad libitum.

II.5.1. Tumor Implantation and Tumor Growth Measurement

At day 0, $1x10^5$ B16F1 cells diluted in 100 µl of PBS were injected subcutaneously into the right flank of each mouse. Tumors were measured with an electronic digital caliper daily, starting from day 6 post tumor injection. Tumor volume was calculated as length × width × height (in mm^3). Mice were sacrificed when the tumor volume was greater than 1500 mm^3 or when they were in poor condition and expected to die shortly. Tumors and spleens were collected and used for further experiments. In another experiment, mice survival has been followed until the end.

II.5.2. pDNA vaccine injection and electroporation

Before each vaccine injection, mice were anesthetized with ±150 µl of a solution of 10 mg/ml ketamine (Ketalar, Pfizer, New York) and 1 mg/ml xylazine (Sigma, St. Louis, Missouri). The left paw was shaved using a rodent shaver (Aesculap Exacta shaver, AgnTho's, Sweden). Mice were injected with 1 µg of every plasmid (pDNA), or with 1 µg of pTOP-OVA CD4-OVA CD8 irrelevant plasmid, diluted in 30 µl of PBS in the left tibialis cranial muscle. The paw was then placed between 4 mm plate caliper electrodes (BEX Co., Ltd., Japan) and electroporated (200 V/cm, 8 pulses, 20 ms with 500 ms pause between pulses). The pulses were delivered by a CUY21EX electroporator (BEX Co., Ltd., Japan). The vaccine was administered 2, 9 and 16 days after tumor injection (Figure 1A).

II.5.3. Virus injection

Before each virus injection, mice were anesthetized in an isoflurane chamber. Then, they were injected intratumorally with 10^9 virus particles (VP) CpG-rich OAd, at day 10, 12 and 14 after

tumor injection. The protocol schedule of tumor, pDNA and virus injection is shown in Figure 1A.

II.6. ENZYME-LINKED IMMUNOSPOT (ELISPOT)

ELISpot was performed according to the manufacturer's instruction (Immunospot, The ELISPOT source, Germany). Briefly, 3 x 10^5 fresh splenocytes diluted in 100 µl CTL-Test medium (Immunospot, The ELISPOT source, Germany) were cultured overnight at 37°C in anti-IFNg coated 96 well plate. For stimulation, 10 ng/µl TRP2 peptide was added to the splenocytes and incubated for 3 days. As positive control for splenocyte activation, Cell Stimulation Cocktail (Invitrogen, California) were used; PBS was used as negative control. The development of the ELISpot plate followed the manufacturer's instruction (Immunospot, The ELISPOT source, Germany). Spots were counted by using an ELISPOT reader system (Immunospot).

II.7. FLOW CYTOMETRY ANALYSIS

NK, CD4 and CD8 T cell populations were analyzed by FACS. Tumors and spleen were surgically removed 17 days after B16F1 cell injection and FACS analysis of tumors and spleens T and NK population was performed. To prepare single cell suspensions, cells were passed through a 70 µm cell strainer (BD Falcon, New Jersey). Then, they were collected, counted using an automatic cell counter (Invitrogen, California) and washed with PBS, before adding the blocking solution with anti-CD16/CD32 antibody for 10 minutes on ice (clone 93, Biolegend, San Diego, California). Cells were washed and incubated for 60 minutes at 4°C with the following antibodies: CD49-APC, CD335-FITC, CD11b-PerCP-Cy5.5 (for NK detection), CD3-PerCPCy5.5, CD4-PeCy7, CD8-FITC (for CD4 and CD8 detection). For staining with anti-FoxP3-PE (clone FJK-16s, eBioscience, Thermo Fisher, Waltham, Massachusetts) or anti-IFNg-PE (clone XMG1.2, Biolegend, San Diego, California), cells were previously incubated overnight at 4°C with a permeabilization/fixation solution (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Thermo Fisher, Waltham, Massachusetts). Cells were then incubated with anti-CD16/CD32 antibody for 10 minutes on ice (Biolegend, San Diego, California), washed and incubated for 60 minutes at 4°C with anti-IFNg-PE or antiFoxP3-PE diluted in the permeabilization/fixation solution. Samples were washed with PBS fixed for 10 minutes with 4% formalin and, then, suspended in PBS. Sample data were acquired with FACS Fortessa or FACS Accuri (BD bioscience, Franklin Lakes, New Jersey) and analyzed with FlowJo software (FlowJo LLC, Ashland, Oregon). For the tumor analysis, the number of cells was normalized by the tumor volume (mm^3).

II.8. QPCR ANALYSIS

Tumors extracted at day 17 were analyzed by qPCR. Total RNA was isolated using TRIzol reagent (Thermo Fisher, Waltham, Massachusetts) and phenol separation, as previously described (19). The quality and quantity of RNA were evaluated using a nanospectrophotometer (NanoDrop 2000, Thermo Fisher, Waltham, Massachusetts). Extracted RNA was considered pure if the 260/280 absorbance ratio of the sample was approximately 2 and the 260/230 absorbance ratio was 1.8-2.2. One microgram of RNA was reverse transcribed using a first-strand synthesis system (SuperScriptTM, Thermo Fisher, Waltham, Massachusetts) and oligo(dT) primers (Eurogentec, Liege, BE) according to the supplier's protocol. The resulting cDNA was used as template for 40 cycles of PCR amplification. SYBR™ green real-time qPCR (GoTaq qPCR MasterMix kit, Promega, Fitchburg, Winsconsin) was conducted on a StepOne Plus Real-Time PCR System (Thermo Fisher, Waltham, Massachusetts) to detect different interleukins, chemokines, perforin and granzyme B. mRNA expression in the tumors and antigen gDNA and mRNA expression in the B16F1 cells. Analysis of the melting curves was performed to ensure purity of PCR products. The results were analyzed with StepOne Software V2.1. The mRNA expression of the cytokines was calculated relative to the corresponding expression of β-actin (reference gene) according to the delta-delta Ct method. The results were normalized compared to the mock control group. A complete list of the primers used in this study is shown in Table 2.

Table 2: List of the primers used in this study.

Primer name		Primer sequence (5' → 3')	Amplicon length (bp)
Granzyme B	For	GAAGCCAGGAGATGTGTGCT	183
	Rev	GCACGTTTGGTCTTTGGGTC	
Perforin	For	TCACACTGCCAGCGTAATGT	419
	Rev	AGGGCTGTAAGGACCGAGAT	
TNFa	For	CATCTTCTCAAAATTCGAGTGACAA	175
	Rev	TGGGAGTAGACAAGGTACAACCC	
IL2	For	TCACATTGACACTTGTGCTCCT	191
	Rev	CATCCTGGGGAGTTTCAGGTTC	
IL15	For	TTGGGCTGTGTCAGTGTAGG	182
	Rev	TGCAATTCCAGGAGAAAGCAGT	
IL12	For	GGAAGCACGGCAGCAGAATA	180
	Rev	AACTTGAGGGAGAAGTAGGAATGG	
IL1b	For	AACTGTTCCTGAACTCAACTGT	150
	Rev	GAGATTTGAAGCTGGATGCTCT	
IL10	For	GGTTGCCAAGCCTTATCGGA	115
	Rev	TCAGCTTCTCACCCAGGGAA	
CCL5	For	CTGCTGCTTTGCCTACCTCTC	149
	Rev	GAACCCACTTCTTCTCTGGGT	
b-actin	For	ACTCCTATGTGGGTGACGAG	206
	Rev	CATCTTTTCACGGTTGGCCTTAG	

II.9. ADDITIVITY VS SYNERGY ANALYSIS: THE SPECTOR'S FORMULA

The synergy vs additivity analysis has been performed by using the Spector's formula, as described in (32) and in (33). Briefly, for an additive effect, the combination index (CI) is in between +/- 2 times the standard error (SE): -2SE < CI < +2SE; while, for a synergic effect: CI > +2SE. The SE is defined as the derived as the square root of the total variance divided by the number of samples. The CI is defined by the following formula:

$$CI = \ln(\overline{X1}) + \ln(\overline{X2}) - \ln(\overline{X1+2}) - \ln(\overline{X0})$$

Where:

$\overline{X1}$ = mean of the effect of the control 1 (in our case, the mean of the tumor volumes for pDNA alone)

$\overline{X2}$ = mean of the effect of the control 2 (in our case, the mean of the tumor volumes for OAd alone)

$\overline{X1+2}$ = mean of the effect of the combination (in our case, the mean of the tumor volumes for the pDNA + OAd group)

$\overline{X0}$ = mean of the effect of the non-treated group (in our case the mean of the tumor volumes for the mock group).

II.10. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 7 for Windows. Survival curves were compared using a Mantel–Cox (log-rank) test. p-values less than 0.05 were considered statistically significant and indicated with different letters on the graphs (a, b, c, d). In particular, the presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups. The annotation "a,b" indicates no significant differences compared to a and b statistical groups.

III. RESULTS

III.1. CPG-ENRICHED ONCOLYTIC ADENOVIRUS AND POLY-EPITOPE PDNA VACCINE SYNERGY ENHANCED TUMOR REGRESSION IN MELANOMA-BEARING MICE

To improve the efficacy of DNA vaccination, a pDNA vaccine made of a mix of 4 plasmids encoding 2 melanoma TAAs and 3 neoantigens (Supplementary data 1) was injected and electroporated in the tibialis muscle of mice and combined with a CpG-enriched OAd virus (26), administered intratumorally (IT) (Figure 1A). Neoantigen mutations have been detected in the genomic DNA (gDNA) of the B16F1 cells at different passages to verify the stability of the mutations for the therapeutic vaccination. These neoantigens were specifically redesigned according to the mutations found in B16F1 cell line (Table 1).

Compared to the neoantigens described in the literature for the B16F10 cell line (17), the Cpsf3l sequence that we found in the B16F1 cells showed some differences, as described in Table 1.

To evaluate the therapeutic efficacy of the combination, the tumor growth was followed for each group (Figure 1B) and for every single mouse (Figure 1 C-F). All the mice developed a tumor 9 days after the B16F1 cell injection. In the pDNA+OAd group, tumor growth was significantly slower compared with the control groups mock, pDNA or OAd (Figure 1B). To determine if this effect was additive or synergic, we used the Spector formula (32). In particular, we calculated the reduction in the tumor volume at day 15 (when the tumor growth curves are significantly different) and we calculated the total standard error (SE) and the combination index (CI), as described in Spector's study (32). As our CI was > 2SE, we concluded that the effect was synergic (see materials and methods). When the vaccine was combined with the viral therapy, the tumors never reached 300 mm^3 in volume until the end of the experiment (day 17), as shown in Figure 1F, while in all the other groups, 46-100% of mice developed a bigger tumor (red lines in Figure 1 C-E).

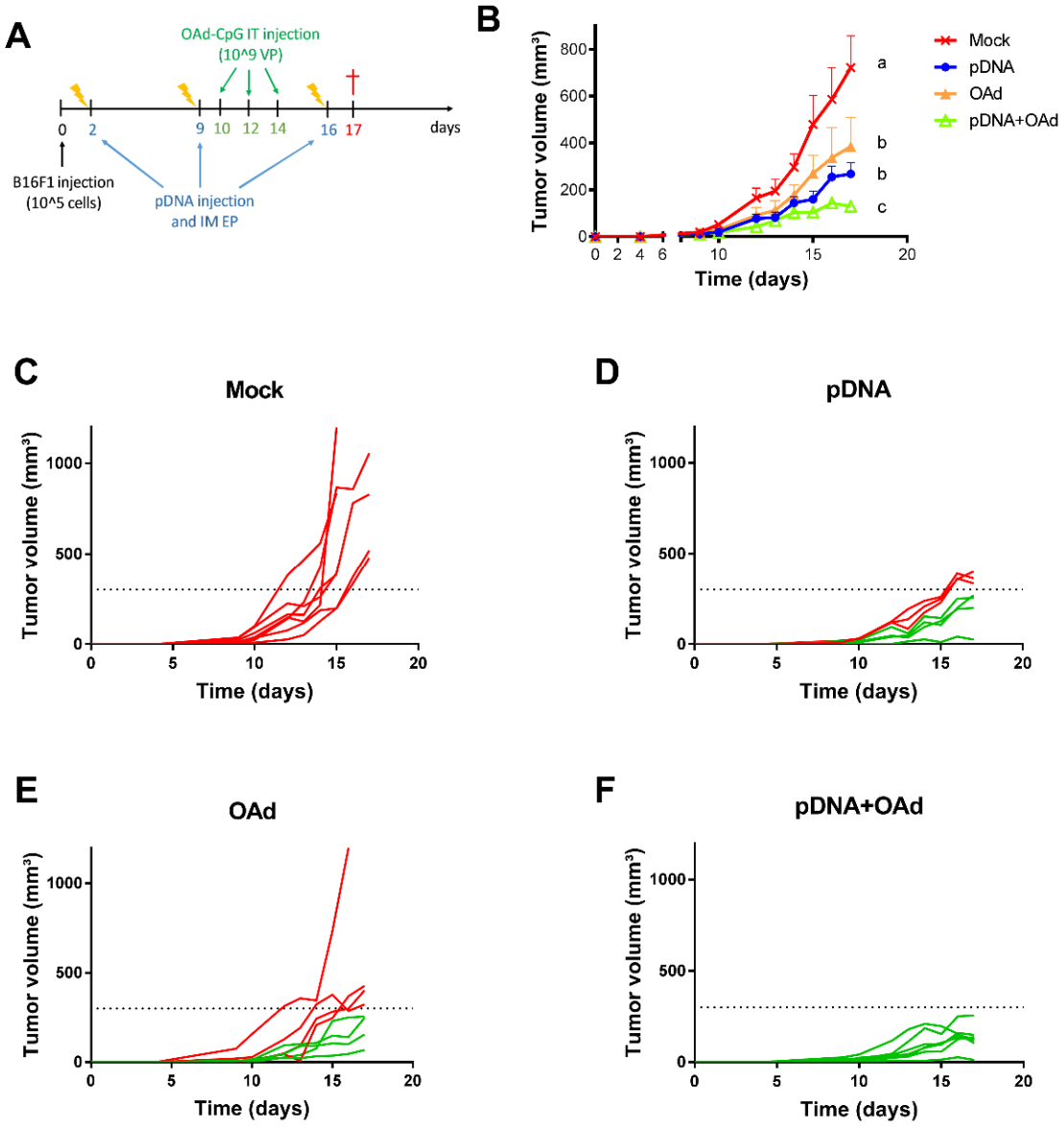


Figure 1: In vivo pDNA and OAd combination. A) Therapeutic DNA vaccination protocol in combination with oncolytic virus therapy. B16F1 cells were injected at day 0; pDNA vaccine was intramuscularly (IM) injected and electroporated 2, 9 and 16 days after tumor injection, while 10^9 OAd virus particles (VP) were IT injected 10, 12 and 14 days after tumor injection. Mice were sacrificed at day 17. B) Evolution of tumor volume (mm^3) after B16F1 challenge as a function of time (days) (mean ± SEM). All the groups were statistically compared to the others using two-way ANOVA, column factor ($p < 0.05$, $n = 8$). C-F) Tumor growth measurement for the single mouse and for each group of mice. In red, mice that developed a tumor > 300mm^3 in volume; in green, mice with a tumor volume < 300 mm^3. The a, b and c letters indicate statistical differences: the presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups.

III.2. pDNA AND OAd INCREASED THE IMMUNE CELL INFILTRATION IN THE TME; THEIR COMBINATION ALLOWED HIGHER ANTIGEN-SPECIFIC T CELL INFILTRATION AND NK ACTIVITY

To study the mechanism underpinning the synergy between the plasmid and the oncolytic virus therapy (pDNA+OAd group), infiltration of immune cells was assessed in the tumors of mice 17

days after the tumor injection (Figure 2). We first assessed the NK cells at the tumor, given their important role in innate rejection of tumor (34). Interestingly, we found that the total amount of NK cells was increased in all the treated groups and, significantly, in the groups treated with the virus (OAd and pDNA+OAd, Figure 2A). The number of NK cells that express the CD335 activating receptor (35) (active NK) was significantly higher only in the combination group compared to the mock (Figure 2B). This data is particularly interesting as it has been so far very poorly studied in the context of oncolytic virus treatment. The number of CD4 and CD8 adaptive immune cells also increased in the treated groups (Figure 2C and D), as well as the number of IFNg-secreting CD8 T cells (Figure 2E). As the pDNA vaccine encoded the TRP2 melanoma antigen, the number of TRP2-specific CD8 T cells was evaluated to test the ability of the vaccine to produce an antigen-specific immune response. As TRP2 is encoded in 3 of the four plasmids, it was chosen as the representative epitope to test the antigen-specific T cell infiltration. When mice were treated with the single therapies (pDNA or OAd), the number of TRP2-specific T cells was not increased compared to the mock group. Only when the two therapies were combined, the amount of TRP2-specific T cells was found to be significantly higher compared to all the other groups (Figure 2F). Different correlation analyses were performed to find the contribution of the different immune populations in the tumor growth. Almost no TRP2-specific T cells were found in bigger tumors (mock group), while a higher infiltration was found in smaller tumors (Figure 2G). Furthermore, a linear correlation ($R^2 > 0.90$) was observed between the number of CD8 T cells and active NK for all the treated groups (Figure 2H), but also between the TRP2-specific T cells and active NK cells for the combination group (Figure 2I).

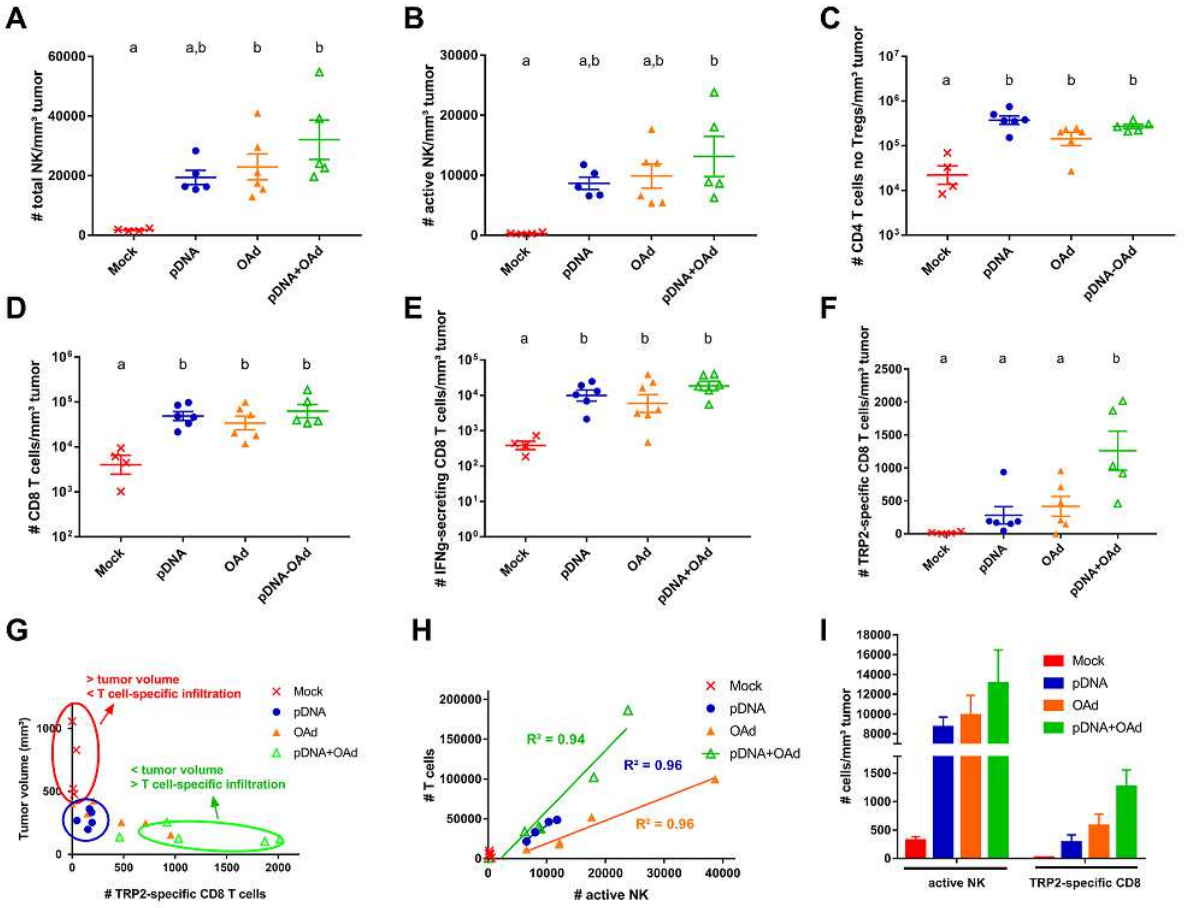


Figure 2: Immune cell infiltration in the TME. A) Number of total NK cells/mm³ tumor. B) Number of CD335+ (active) NK cells/mm³ tumor. C) Number of non-Treg CD4 T cells/mm³ tumor. D) Number of CD8 T cells/mm³ tumor. E) Number of IFNg-secreting CD8 T cells/mm³ tumor. F) Number of TRP2 antigen-specific CD8 T cells/mm³ tumor. G) Correlation between the tumor volume and the number of TRP2 antigen-specific CD8 T cells in the TME. H) Correlation between the number of active NK and CD8 T cells. R^2 was calculated by using a linear regression analysis. I) Correlation between active NK and TRP2-specific CD8 T cells. The results in A, B, C, D, E, F and I are expressed as mean ± SEM (n = 4-6). a and b letters on the graphs indicate significantly different results when the superscript letters are different. The annotation "a,b" indicates no significant differences compared to a and b statistical groups. The presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups.

III.3. CYTOKINE INVOLVED IN NK AND CD8 RECRUITMENT AND ACTIVITY ARE HIGHLY EXPRESSED IN THE TME OF PDNA+OAD-TREATED MICE

To evaluate the activity and the contribution of the NK and T cells in the TME, an evaluation of the cytokine and perforin/granzymeB expression was performed. A general increase in the cytokine expression was observed when mice were treated with both pDNA and OAd (Figure 3). In particular, a higher expression of proteins related to NK, Th1 and CTL activity was observed, such as Granzyme B (36) (Figure 3A), perforin (37) (Figure 3B), TNFa (38) (Figure 3C) and IL2 (39) (40) (Figure 3D). Many other interleukins were overexpressed, such as IL15, IL12 and IL1b (Figure

3E, F and G). Finally, an increased CCL5 expression was observed in the OAd-treated groups, OAd and pDNA+OAd (Figure 3H). Generally, all the cytokines, chemokines and enzymes were also overexpressed in the OAd group, but without observing significant differences compared to the mock group. Interestingly, IL10 was significantly overexpressed only in the OAd group (Figure 3I). This was the only cytokine expressed in higher amount in OAd group but not in the pDNA+OAd group.

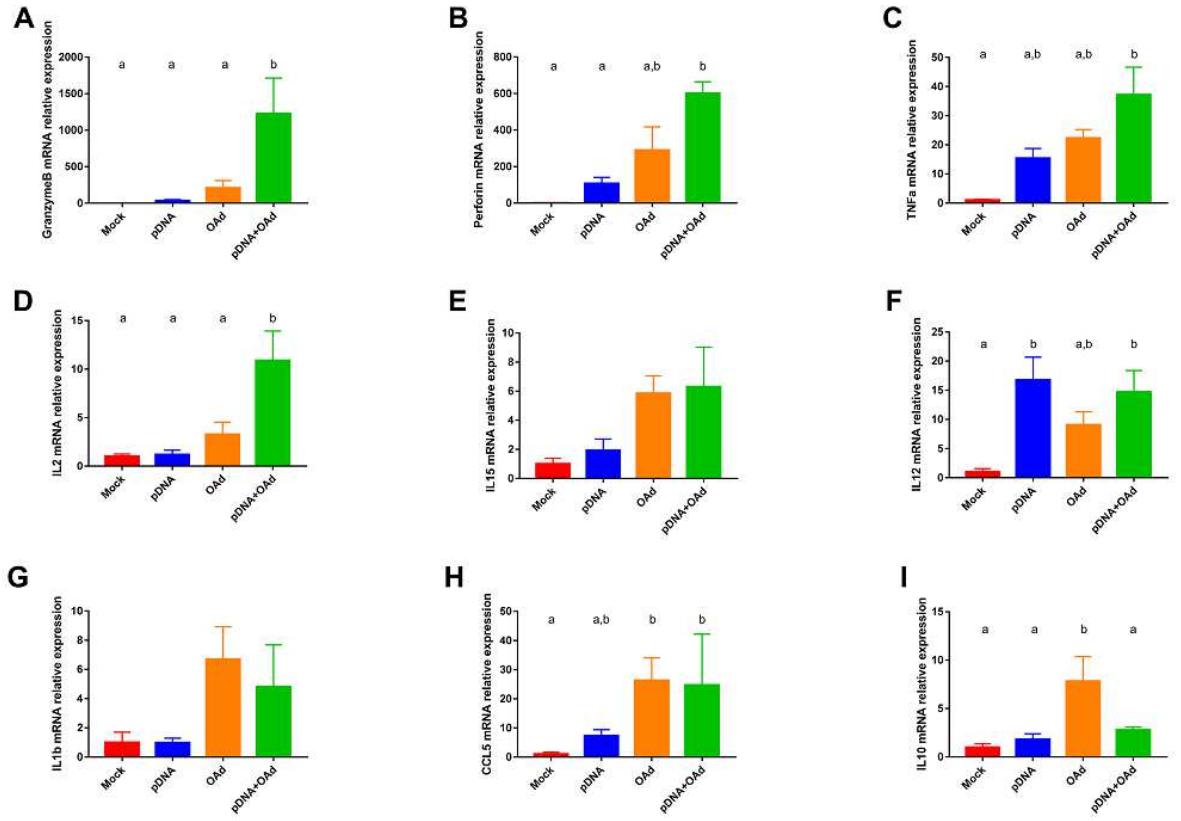


Figure 3: Cytokine expression in the TME: Granzyme B (A), Perforin (B), TNFa (C), IL2 (D), IL15 (E), IL12 (F), IL1b (G), CCL5 (H), IL10 (I). All the results are expressed as mean ± SEM (n = 4-6). a and b letters on the graphs indicate significantly different results when the superscript letters are different. The presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups. The annotation "a,b" indicates no significant differences compared to a and b statistical groups.

III.4. PDNA AND OAD INDUCED HIGHER IMMUNE CELL INFILTRATION IN THE SPLEEN AND THE COMBINATION INDUCED A GREATER ANTIGEN-SPECIFIC IMMUNE RESPONSE

Next, we wanted to evaluate the systemic immune activity and compare it with the response in the TME. To this end, splenocytes were collected 17 days after the tumor challenge and analyzed (Figure 4). All the treatments significantly increased not only NK infiltration but also the number of active NK in the spleen, compared to the mock group (Figure 4A and B). In addition to that,

the number of CD8 and non-Treg CD4 T cells was higher in the treated groups (Figure 4C and D). In particular, OAd and pDNA+OAd group showed the highest number of CD8 T cells (Figure 4C). ELISPOT analysis revealed high TRP2 specificity for pDNA and pDNA+OAd conditions, which was significantly different compared to the other groups (Figure 4E and F). pDNA+OAd group showed a significantly higher number of IFNg-secreting and TRP2-specific splenocytes compared to pDNA alone (Figure 4E and F).

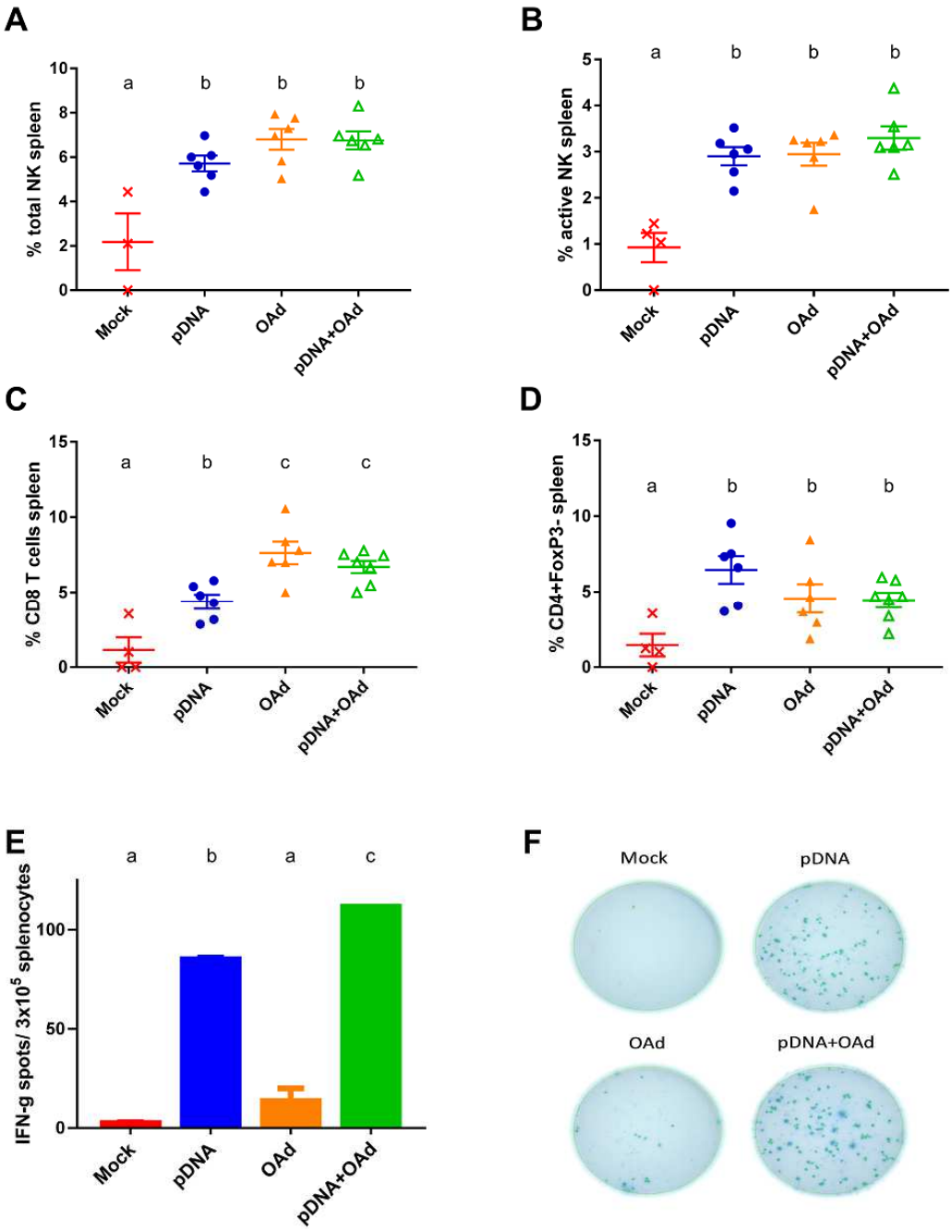


Figure 4: Immune cell analysis in the spleen. A) Percentage of total NK. B) Percentage of active NK. C) Percentage of CD8 T cells. D) Percentage of non-Treg CD4 T cells. E-F) ELISPOT analysis of the splenocytes stimulated with TRP2 peptide. All the results are expressed as mean ± SEM (n = 4-6). a, b and c letters on the graphs indicate significantly different results when the superscript letters are different. The presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups.

III.5. THE COMBINATION OF PDNA AND OAD INCREASED THE LONG-TERM SURVIVAL

Previously, we have addressed the short-term efficacy of the combination between pDNA and OAd, showing a significant decrease in the tumor growth and a higher CTL infiltration and activity when the two therapies are combined. To better understand the o, we performed a new experiment to follow-up the long-term survival. The vaccine and the virus have been administered following the same protocol used to evaluate the tumor growth, but this time, mice were followed-up until day 45 (Figure 5A). Our results showed that the median survival time (MST) was significantly longer in the group treated with pDNA and OAd. Furthermore, this combination cured almost 30% of mice (2/7 mice), compared to 0% in the other groups (Figure 5B).

To exclude an unspecific systemic effect of our vaccine, we evaluated the efficacy of the pDNA compared to an irrelevant plasmid. To this aim, a group of mice has been treated with a plasmid encoding for chicken ovalbumin-derived epitopes restricted for CD4 and CD8 in the VSV-G sequence (pTOP-OVA CD4-OVA CD8). This group did not show any differences compared to the untreated mock group, thus supporting the contribution of the adaptive immune effect induced by the pDNA encoding B16F1 TAAs and neoantigens.

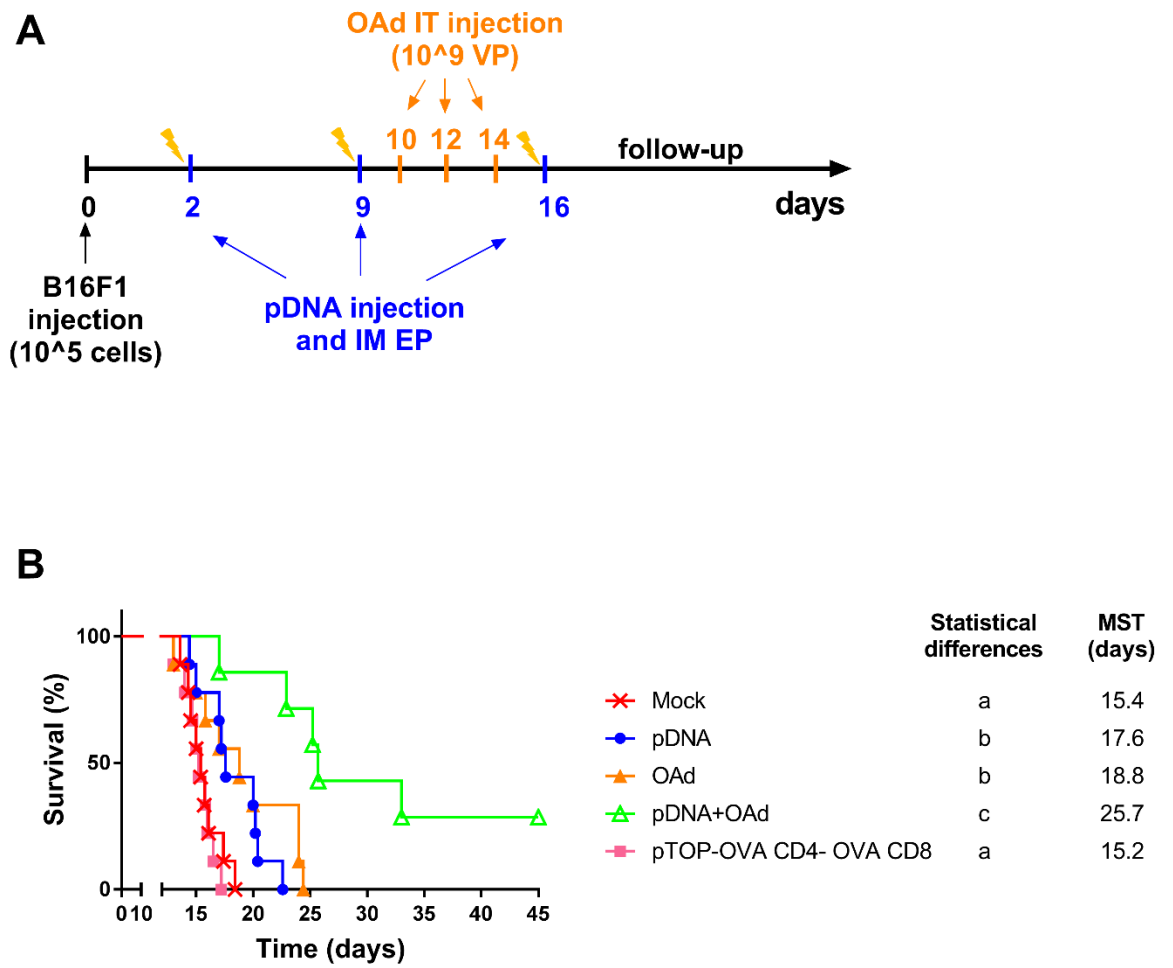


Figure 5: Evaluation of the long-term survival. A) Therapeutic DNA vaccination protocol in combination with oncolytic virus therapy. B16F1 cells were injected at day 0; pDNA vaccine was intramuscularly (IM) injected and electroporated 2, 9 and 16 days after tumor injection, while 10^9 OAd virus particles (VP) were IT injected 10, 12 and 14 days after tumor injection. B) Survival curves representing the percentage of alive mice (%) as a function of time (days); MST = median survival time. Statistical analysis: Log-Rank (Mantel–Cox) test (p value < 0.05; $n = 7$-9). The presence of two different letters in two groups indicate a statistical difference ($p < 0.05$) between them; the same letter in two different groups indicates the absence of a statistical difference between these two groups.

IV. DISCUSSION

In the field of cancer immunotherapy, DNA vaccines showed many promises but also failures due to their poor immunogenicity, especially in the clinic (41). A renovated interest was aroused by the use of "personalized" and poly-epitope DNA vaccines encoding different TAAs and neoantigens and the possibility to combine them with other strategies that can drive the generated immune response in the TME (19, 42). In the current study, a DNA vaccine (pDNA) against B16F1 melanoma has been tested. This pDNA was a mix of four different plasmids globally encoding three melanoma neoantigens, specifically designed in silico according to the B16F1 mutations (Kif18b, Cpsf3l and Pbk), and two TAAs (TRP2 and gp100). The neoantigens Kif18b and Pbk presented the same mutations described in the literature for B16F10 melanoma (17, 43), while the Cpsf3l neoantigen sequence that we found in B16F1 cells was different (Table 1). Seven amino acids were found to be mutated in the Cpsf3l gene when compared to the wild type sequence. These results confirm the high mutational burden of melanoma that many authors describe (17, 43-46). These neoantigens were selected based on the results obtained by other researchers. In particular, the mutated form of Kif18b (K739N) was found to be a dominant mutated antigen, and mice immunized with mutated Kif18b peptide could slow tumor growth and improve survival. (43) Also a mutated form of Cpsf3l (D314N) has been shown to induce a strong immune reaction preferentially against the mutated peptide. (43). Furthermore, the three selected neoantigens, including the mutated Pbk (V145D), induced an immune response after vaccination with RNA (47). In particular, Pbk has been described as a CD8 neoantigen, with a low MHC I score, which predicts the binding affinity to the MHC I complex (47).

The antitumor activity of the pDNA vaccine was drastically improved by combining it with an OAd virus whose genome was enriched with CpG motifs (Figure 1) (26).

We hypothesized that the OAd virus injected IT could activate the innate immune response locally and recruit in the TME the adaptive immune cells generated by the pDNA vaccine. Indeed, the IT viral therapy enhanced the recruitment of NK cells in the TME (Figure 2A) through the local production of several cytokines and chemokines, such as CCL5, IL15, IL1b, IL10, TNFa, among others (Figure 3) (38, 48, 49). It has been already demonstrated that the CpG-enriched OAd could stimulate the TLR9 response and that the anti-tumor immune response was related to the NK recruitment and activity (26). In particular, beside the higher number of NK cells in the TME, a significantly increased infiltration of CD335+ NK cells (active NK cells) was observed only in the combination group compared to the mock (Figure 2B). CD335 was originally identified as a receptor with the ability to mediate the killing of tumor-transformed cells. This receptor is also

involved in the control and elimination of several pathogens and has a role in immune homeostasis by regulating the expression of several immune cell types (50). The increased tumor infiltration of CD335+ NK cells can be correlated with a higher granzymeB, perforin and TNFa secretion in the combination group (Figure 3A, B and C), as their cytotoxic activity is mainly associated to CTL and NK cells (49, 51). Specifically, granzymeB plays a critical role in triggering apoptotic cell death, while perforin plays an important role in NK cell-mediated suppression of tumor initiation and metastasis (51). The significant increase in the number of active NK observed only in the combination group indicates the involvement of the pDNA in this response. Indeed, pDNA alone significantly increased IL12 expression compared to the mock (Figure 3F). Furthermore, contrarily to the OAd group, pDNA did not increase the IL10 levels (Figure 3I). These two ILs are important for the NK recruitment/activity. IL12 activates NK cells to destroy a variety of tumors in a perforin-dependent manner (49). IL10 plays a double role in the TME: from one side, it recruits NK cells, hence initiating immune cell infiltration. However, an excessive IL10 production can prevent the NK activity and transform the Th response in a Th2 response (52). The low levels of IL10 and the increased expression of IL12 in the pDNA-treated group confirm the ability of the DNA vaccines to shift the immune response towards a Th1 phenotype (38, 53, 54). IL12, as well as TNFa, not only activate NK cells but are also secreted in response to the innate immune activation to recruit the cells of the adaptive immunity (38, 54, 55). The high IL12 secretion following pDNA vaccination could be related to the Th1 switch induced by the vaccine itself, probably strengthen by the presence of a CD4 epitope in the pDNA.

Another important cytokine in the TME was IL2. This last is produced predominately by antigen-simulated CD4 T cells, CD8 cells, NK and activated DCs (39). It plays a critical role in the differentiation of CD4 T cells into a variety of subsets, recruits Tregs, and promotes CD8 T cell and NK cytotoxicity activity (55, 56). In preclinical and clinical studies, IL2 is administered in combination with cancer vaccines to dramatically enhance their anti-tumor activity (56, 57). It has been demonstrated that the Tregs recruitment induced by IL2 could be reduced by co-administering IL12 (58). This association further stimulates Th1, CTL and NK in a positive loop (57). In the current study, the pDNA vaccine itself permitted to increase the levels of IL12, which in turn could have enhanced the antitumor properties of IL2, contributing to the global antitumor efficacy. Indeed, the involvement of NK, CD8 and CD4 T cell influences the cancer immunity cycle in several aspects. In particular, vaccine-induced CD4 T cells promote an inflammatory tumor microenvironment, by producing IFNg, which improves CTL killing activity and sensitizes tumor cells for recognition and direct killing by cytotoxic Th1 effectors (12). This cycle will broad the antitumor T cell repertoire and restore the cancer immunity cyle (12).

Finally, the higher NK activity in the TME was directly correlated with the number of TRP-2 specific CD8 infiltrated in the TME (Figure 2I), which was inversely correlated with the tumor volume (Figure 2G). All these effects can explain why the NK cells are significantly more active in the TME and the immune response is stronger only when the two therapies are combined.

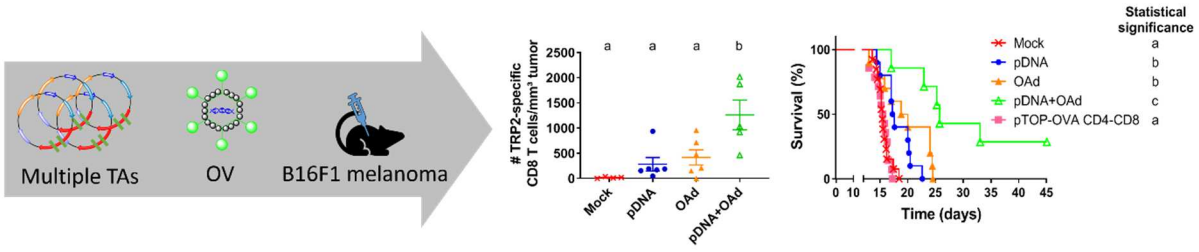
In the spleen, both pDNA and OAd had an effect in increasing NK, CD4 and CD8 cells (Figure 4A, B). In particular, antigen-stimulated splenocytes produced significantly higher amount of IFNg in the combination group, compared to all the other groups (Figure 4E, F). pDNA mainly contributed to this effect by generating a high amount of TRP2-specific T cells. This means that the vaccine can induce the production of antigen-specific T cells systemically, but it cannot drive the mounted immune response in the TME, probably due to the immunosuppressive TME. The concomitant IT administration of the virus can activate the innate immunity and recall the antigen-specific T cells generated by the vaccine into the tumor site. For this reason, we found an increased infiltration of TRP2-specific CD8 cells only in the TME of the combination group. This result confirms our previous study in a mastocytoma tumor model, where we observed that the DNA vaccine alone failed to significantly increase the survival, due to a poor immune T cell infiltration in the TME. Only when it was combined with immune checkpoint blockades the antitumor efficacy was significantly enhanced, due to the higher specific T cell infiltration and activity induced by the immunocheckpoint blockers (19).

Globally, these results explain the drastic decrease of the tumor growth rate and the significant improved survival observed when pDNA was combined with the OAd viral therapy, thus curing 30% of mice from a lethal melanoma cell injection.

V. CONCLUSIONS

In this study, we demonstrated that the potency of a poly-epitope cancer DNA vaccine against melanoma could be dramatically enhanced by the combination with an oncolytic virus administered in the tumor. The reason of the improved efficacy was the complementarity of the two therapies in activating the immune system. From one side, the local IT viral therapy was able to recruit NK innate immune cells in the TME. Therefore, different cytokines, chemokines and enzymes involved in the adaptive immune system recruitment and in the cytotoxic activity against the tumor were produced. On the other side, pDNA was able to produce antigen-specific T cells in the spleen, which reached the tumor when recalled by the local viral therapy. pDNA played also an important role in IL12 production in the TME, which created a positive loop in cytokine production and immune cell activity (57). The global effect was the dramatic slowdown in the tumor growth and a significant increase in the survival observed in the combination group compared to all the others. Our study demonstrates that a rational combination therapy involving DNA vaccination could overcome its poor immunogenicity in the TME, leading the way to a wider use of DNA vaccination in humans.

Graphical conclusion



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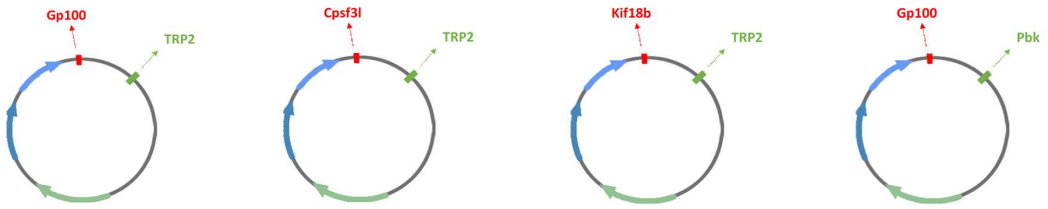
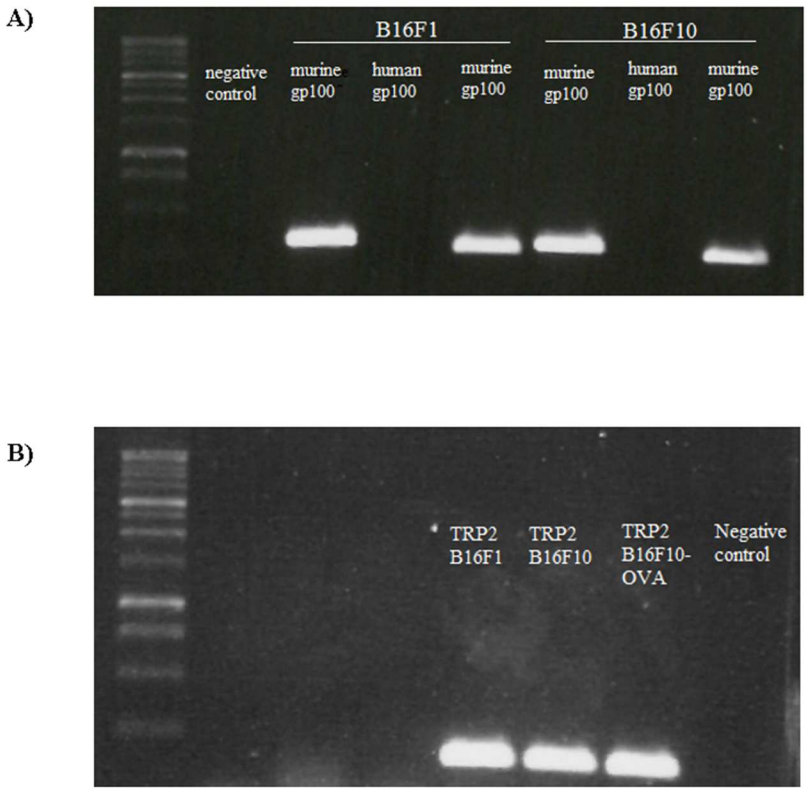
Supplementary data 1



Supplementary data 1: pDNA vaccine design. Four DNA vaccines encoding one CD4 epitope (in red) and one CD8 epitope (in green).

Supplementary data 2



Supplementary data 2: Expression of TRP2 and murine gp100 in B16F1 melanoma cell line.
A) Expression of murine and human gp100 in B16F1 and B16F10 (used as a positive control). In the second line, a negative control for the primers has been performed. Ladder of 1 Kb.
B) Expression of TRP2 in B16F1, B16F10 and B16F10-OVA cells (used as a positive control). In the last line, a negative control for the primers has been performed. Ladder of 1Kb.

CHAPTER VI.
THE ANTITUMOR ACTIVITY OF A DNA VACCINE ENCODING GLIOMA-ASSOCIATED ANTIGENS IS ENHANCED BY SURGICAL RESECTION IN A GL261 GLIOBLASTOMA ORTHOTOPIC MODEL

Adapted from:

Lopes A.*, Bastiancich C.*, Ligot S., Bausart M., Gallez B., Vandermeulen G., Préat V. The antitumor activity of a DNA vaccine encoding glioma-associated antigens is enhanced by surgical resection in a GL261 glioblastoma orthotopic model. *In preparation.*

* Equal contribution

GENERAL INTRODUCTION

In the previous chapters, we have demonstrated, in a mastocytoma model (chapter IV) and in a melanoma model (chapter V), the ability of DNA vaccination to mount an antigen-specific immune response that is driven and effective into the tumor microenvironment (TME) when a rational combined therapy is used.

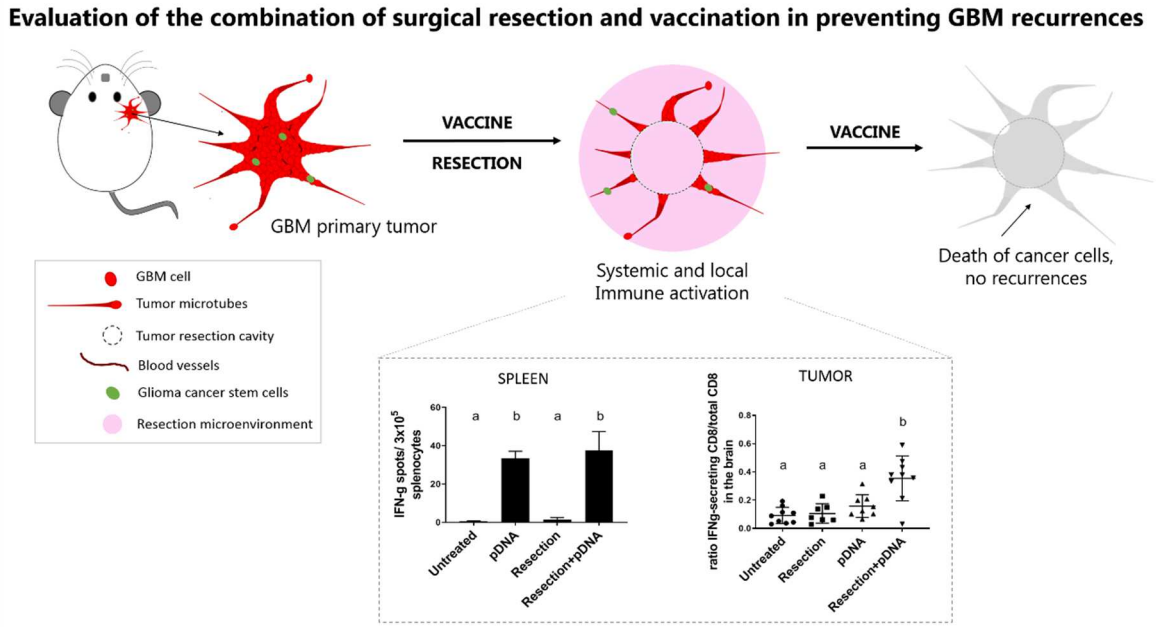
To demonstrate the broad applicability of our DNA vaccine platform, we tested a pTOP DNA vaccine encoding the epitopes of two glioma-associated antigens in a glioblastoma (GBM) tumor model. From an immunological point of view, this tumor is difficult to treat, because of the presence of the blood brain barrier and the lack of a classical lymphatic system (1). It is also considered "cold" for the low mutational burden and lymphocyte infiltration (2). Knowing that the main limitation of the DNA vaccines is their inability to drive activated and antigen-specific cytotoxic T lymphocytes (CTL) in the TME, we hypothesized that the combination with tumor surgical resection could improve the outcome. Indeed, the resection decreases the tumor mass, but also creates a local inflammation that could recruit the immune cells generated by the vaccine in the resection cavity, to eradicate the remaining tumor cells.

The vaccine was firstly tested subcutaneously and was very effective even without any combination therapy. However, when the tumor was injected orthotopically (in the brain), the vaccine needed to be combined with the surgical resection to increase the survival. An increased amount of antigen-specific CTLs was found in the brain, but also a decreased amount of immunosuppressive cells. These results could explain the 80% of survival observed with the combination therapy, compared to the 20-30% observed with the resection or the DNA vaccine alone.

ABSTRACT

This work evaluates the ability of a DNA vaccine encoding glioma-associated antigens (GAAs) to induce an antigen-specific immune response in a GL261 glioblastoma (GBM) model. A DNA vaccine encoding the GAAs TRP2 and gp100, expressed by GL261 cells, has been designed. A significant increase in the survival of vaccinated mice when the tumor was injected subcutaneously has been observed. However, when the tumor was inoculated into the brain (orthotopic model), only vaccinated mice that underwent surgical resection survived longer compared to the control groups (vaccine or resection). Immunological analysis shows a significant decrease of the infiltrated immunosuppressive cells 13 days after the priming dose of the vaccine and the presence of antigen-specific and immunologically active T cells in the brain. The combination between GBM resection and DNA vaccine immunotherapy can increase mice survival in a clinically-relevant preclinical model, opening the door to a new standard of care for GBM patients.

Graphical abstract



KEYWORDS

Glioblastoma, GL261, Immunotherapy, DNA vaccines, Surgical resection

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I. INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive brain tumor in adults. Its fast and unpredictable appearance, lack of effective treatments and poor prognosis (4-year survival rate <10%) make GBM a huge unsolved medical challenge; finding new and effective therapeutic strategies is an urgent global health need (3, 4).

Surgical debulking of the accessible tumor is the mainstay in the treatment of GBM and it is performed in all eligible patients (65% of the population affected by GBM) (5). The tumor resection allows to relieve the symptoms derived from the mass effect, removes the hypoxic and radio-chemoresistant tumor core, reduces the number of cells requiring treatment and provide material for molecular tumor characterization (6). Even though the surgical resection of GBM is generally considered as a safe procedure, the impact of the post-operative regenerative response in the brain on the formation of recurrences is often overlooked. Indeed, despite the important technical and imaging advances, GBM surgery inevitably causes an important brain injury leading to BBB disruption, edema, neuro-inflammation, angiogenesis, reactive gliosis and release of growth factors and cytokines (7). Several weeks after surgery, patients undergo radiotherapy and concomitant adjuvant oral chemotherapy with Temozolomide (TMZ) (8). However, the intrinsic properties of GBM - which include GBM cells high migratory and infiltrative patterns, the presence of tumor microtubes (TMs; ultra-long, thin, highly dynamic and infiltrative astrocytoma cells membrane protrusions) that cannot be surgically removed and the presence of glioma cancer stem cells (GSCs) chemoresistant to alkylating agents - promote the formation of recurrences that inevitably lead to patient's death (9, 10).

A strategy that has been recently reconsidered as a promising tool for GBM is immunotherapy. Indeed, the brain is no longer considered an "immune privileged" organ as it was shown that the Central Nervous System (CNS) possesses lymphatic vessels along the dural sinuses and meningeal arteries (11, 12). In mice, it has been shown that these vessels are functional and allow the drainage of immune cells and soluble constituents from the cerebrospinal fluid to the cervical lymph nodes, where CNS-derived antigens induce immune responses (12, 13). Moreover, in pathological conditions such as GBM, the blood-brain barrier is compromised allowing immune cells to infiltrate from the peripheral circulation (14). About 30% of the GBM tumor mass is composed of tumor associated macrophages (TAMs) - either tissue-resident microglia or bone marrow-derived macrophages derived from circulating monocytes recruited to the brain parenchyma (15, 16).

The microenvironmental landscape of brain tumors is very complex and pro- and anti-tumor immune responses rely on a delicate balance between TAMs, endothelial cells, pericytes, neurons, astrocytes, neutrophils and lymphocytes (14, 17). Immunotherapy strategies for GBM have mainly been focused on dendritic cells vaccines, peptide vaccines, CAR-T cell therapy, viral therapy as well as the use of check-point inhibitors to enhance T cells activation and boost the anti-tumor response (18-20). In most of the ongoing or completed clinical studies on newly diagnosed GBM patients (e.g., NCT02311920; NCT03018288; NCT02465268; NCT02546102; NCT00045968), the first immunotherapy treatment is planned after surgical resection and/or at the same time as radiotherapy and TMZ treatment. To our knowledge, only one study (phase I trials NCT02722512) envisages the first immunization as soon as possible after tumor resection (between 0-28 days and no more than 60 days post-operatively) and none has scheduled to begin immunotherapy prior to surgery.

Recent studies have shown that residual dormant glioma cells, TMs and GSCs in the resection margins are challenged by the healing post-surgical response which induces a vicious cycle of inflammation, angiogenesis and tumor regrowth, having a key role in the formation of local recurrences (7, 21-23). Indeed, the angiogenesis and inflammation involved in the regeneration and healing response to surgery possess both pro- and anti-tumorigenic functions (7).

Immunotherapy could target the tumor resection microenvironment, thus contributing to the removal of the residual tumor cells, by stimulating the patient's own immune system. In particular, cancer DNA vaccines hold great promise and showed several advantages, such as the induction of a long-lasting and specific anti-tumor immunity and a good safety profile (24). However, they are poorly immunogenic in the clinic due to the highly immunosuppressive TME (25). A renovated interest was aroused by the possibility to improve the DNA vaccine immunogenicity through a rational plasmid design and the combination with other therapies that can drive the generated immune response in the TME (26, 27).

The aim of this work is to evaluate the potential anti-tumor activity of a DNA vaccine encoding glioma-associated antigens (GAAs) against a GL261 GBM preclinical model. GL261 is a syngeneic model, which presents diffusive infiltrating pattern and specific tumor antigens, among others TRP2 and gp100 (28, 29). To this aim, a DNA vaccine encoding TRP2 and gp100 antigens has been designed. The gene sequences of the two antigens were inserted into the vesicular stomatitis virus glycoprotein protein (VSV-G) sequence encoded in a pVAX2 vector, as explained in (30). This vaccine has been initially developed within our group for the treatment of a subcutaneous B16F10 melanoma, where it showed a significant slowdown in the tumor growth (30).

In the current study, we test the therapeutic efficacy of the GAA DNA vaccine in a subcutaneous and in an orthotopic GL261 GBM model. To increase the DNA vaccine activity in the orthotopic model, the tumor has been surgically resected one day after the vaccination, with a 'biopsy punch' technique, recently validated in our group for U87 MG GBM and 9L gliosarcoma in Nude mice and Fischer rats (31, 32). This technique provides a reliable and clinically-relevant tool to test the efficacy of a wide range of treatments or drug delivery strategies. In this study, we adapted this technique to the GL261 GBM model in immunocompetent C57BL/6 mice.

The therapeutic efficacy of the vaccine in combination with the tumor resection has been evaluated, by analyzing the immunological activity in the brain and in the spleen two weeks after the vaccine priming. To our knowledge, this is the first study that combines the GBM resection with a DNA vaccine, evaluates the immunological response of the tumor resection microenvironment and explains its correlation with the observed mice survival.

II. MATERIALS AND METHODS

II.1. CELL LINES

GL261 murine glioblastoma cells were kindly provided by Professor Sophie Lucas (Université Catholique de Louvain). GL261 cells were cultured in Dulbecco's modified Eagle's Medium with 4.5 g/L glucose, 0.58 g/L L-glutamine and 0.11 g/L sodium pyruvate (Gibco, Life Technologies, USA). The media was supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies USA), 100 U/mL penicillin G sodium and 100 µg/mL streptomycin sulfate (Gibco, Life Technologies, USA). Cells were subcultured in 75 cm^2 culture flasks (Corning® T-75, Sigma-Aldrich, USA) and incubated at 37°C and 5% CO_2.

II.2. pDNA VACCINE DESIGN AND PRODUCTION

The pDNA vaccine used in this study encodes TRP2 and gp100 epitopes, which are already known glioblastoma antigens (33). The presence of the two antigens has been verified in GL261 cell line by PCR (Figure 1F). In Table 1, the reactive T cell subtype, the nucleotide and peptide sequences of these antigens are shown. The gene sequences of the antigen epitopes were inserted into the VSV-G sequence encoded in a pVAX2 vector, as reported by Vandermeulen et al (30). The plasmid was sequenced to ensure the correct nucleotide sequence (Genewiz, United Kingdom). Then, the plasmid was amplified and purified using an EndoFree Plasmid Giga Kit (QIAGEN, Germany), according to the manufacturer's protocol. Optical density at 260 nm was used to determine DNA concentration. Plasmids were diluted in PBS and stored at −20°C before use.

Table 1: Antigen nucleotide and protein sequence and predicted MHC specificity.

Antigen name	Reactive T cell subtype	Nucleotide sequence	Peptide sequence
$TRP2_{180-188}$	CD8	AGCGTGTACGACTTCTTCGTGTGGCTG	SVYDFFVWL
$gp100_{44-59}$	CD4	TGGAACAGACAGCTGTACCCCGAGTGGACC GAGGCCCAGAGACTGGAT	WNRQLYPEWTEAQRLD

II.3. ANIMAL EXPERIMENTS AND ETHICAL PERMITS

C57BL/6 female mice were obtained from Janvier (France). Mice were between 5 and 6 weeks old at the beginning of the experiments. Water and food were provided ad libitum; temperature and humidity were monitored daily in a conditioned room. All in vivo experiments were performed following the Belgian national regulations guidelines in accordance with EU Directive 2010/63/EU, and were approved by the ethical committee for animal care of the faculty of medicine of the Université catholique de Louvain (2014/UCL/MD/004 and UCL/MD/2016/001).

II.3.1. Subcutaneous tumor implantation and tumor measurement

At day 0, 2 x 10^6 GL261 cells diluted in 100 µl of PBS were injected subcutaneously into the right flank of C57Bl/6 mice. Tumors were measured with an electronic digital caliper 3 times per week, starting from day 6 post tumor injection. Tumor volume was calculated as length × width × height (in mm^3). Mice were sacrificed when the tumor volume was greater than 1500 mm^3 or when they were in poor conditions and expected to die shortly later.

II.3.2. pDNA vaccine injection and electroporation

The vaccine was administered 2, 9 and 16 days after SC tumor injection (Figure 1A) or 16, 23 and 29 days after orthotopic injection (Figure 2A). Before each vaccine injection, mice were anesthetized with ±150 µl of a solution of 10 mg/ml ketamine (Ketalar, Pfizer, New York, USA) and 1 mg/ml xylazine (Sigma, St. Louis, MO, USA). The left paw was shaved using a rodent shaver (Aesculap Exacta shaver, AgnTho's, Stockholm, SE). Mice were injected with 1 µg of the plasmid diluted in 30 µl of PBS in the left tibialis cranial muscle. The paw was then placed between 4 mm plate BTX caliper electrodes (VWR International, Leuven, BE) and electroporated (200 V/cm, 8 pulses, 20 ms with 500 ms pause between pulses). The pulses were delivered by a BTX™ Gemini Electroporation System (VWR International, Leuven, BE).

II.3.3. Orthotopic GL261 glioblastoma syngeneic model

Six-week-old female C57Bl/6NRj mice (Janvier, France) were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 13 mg/kg, respectively) and fixed in a stereotactic frame. A surgical high-speed drill (Vellman, Belgium) was used to perform a hole in the right frontal lobe and 5 x 10^4 GL261 cells were slowly injected using a Hamilton syringe fitted with a 32G needle as previously reported (31). To obtain cortical tumors, the injection coordinates were 0.5 mm posterior, 2.1 mm lateral from the bregma and 2.2 mm deep from the outer border of the cranium, respectively. The presence, volume and location of the tumors were determined by MRI, which was performed for all mice included in the study before the surgical resection of the tumor. Animals

presenting GL261 tumors were divided into four groups: untreated (n = 10); resection at day 16 (n = 9); vaccine at day 16, 23 and 29 (n = 12); resection at day 16 and vaccine at day 16, 23 and 29 (n = 9).

II.3.4. Magnetic Resonance Imaging

MRI was performed using a 11.7 T Bruker Biospec MRI system (Bruker, Germany) equipped with a 1 H quadrature transmit/receive surface cryoprobe after anesthetizing animals with isoflurane mixed with air (2.5% for induction, 1% for maintenance). Animal core temperature was maintained throughout the experiment by hot water circulation in the cradle and respiration was continuously monitored. Tumor volume was assessed using rapid acquisition with relaxation enhancement (RARE) sequence (repetition time = 2500 ms; effective echo time = 30 ms; RARE factor = 8; field of view = 2 x 2 cm; matrix 256 x 256; Slice thickness = 0.3 mm; twenty-five contiguous slices were acquired, $N_{average}$ = 4). Tumor volumes were calculated from a manually drawn region of interest.

II.3.5. Surgical resection of the tumor mass

At day 17 post-tumor inoculation, the tumor mass was surgically removed using the biopsy-punch resection technique adapted from Bianco, Bastiancich et al. (31). Briefly, animals were anaesthetized with ketamine/xylazine (100 mg/kg and 13 mg/kg, respectively) and immobilized in a stereotactic frame. An 8 mm incision was made in the midline along the previous surgical scar and a 2.1 mm diameter circular cranial window was created around the previous burr hole using fine tip tweezers (Dumont, Switzerland) to expose the brain. A 2 mm diameter biopsy punch (Kai Medical, Germany) was then inserted 3 mm deep and twisted for 15 s to cut the brain region surrounding the tumor. Once withdrawn, the tumor and brain tissues were aspired using a diaphgram vacuum pump (Vaccubrand GBMH+CO KG, Germany) connected to a Pasteur pipette and a 200 µl tip. Residual blood was removed from the surgical cavity using a haemostatic triangle (Fine Science Tools, Germany). The cranial window was then sealed with a 4 x 4 mm square piece of Neuro-Patch® (Aesculap, Germany) impregnated with a reconstituted fibrin hydrogel (25 mg/mL fibrin, 10 IU/mL thrombin, equal volumes; Baxter Innovations, Austria). Animals were then placed under a heating lamp (Infraphil, Philips, Belgium) for one hour to recover from surgery and avoid hypothermia.

All animals were then monitored daily and an MRI follow-up was performed 28 days after surgery. Eight-nine animals per group were sacrificed 29 days post-tumor inoculation for immunological analysis (FACS and PCR). The spleen and the brain of the animals were collected for further analysis. The remaining animals were sacrificed when they reached the end points (behaviour

changes e.g., lack of grooming and clinical signs of distress e.g.: paralysis, arched back, lack of movement plus 10% body weight loss and/or 20% body weight loss).

II.4. FLOW CYTOMETRY ANALYSIS

TAM, MDSC, CD4 and CD8 T cell populations were analyzed by FACS. Brains and spleens were surgically removed 29 days after GL261 orthotopical cell injection and FACS analysis of brains and spleens immune cell populations was performed. To prepare single cell suspensions, cells were passed through a 70 μm cell strainer (BD Falcon, New Jersey). Then, they were collected, counted using an automatic cell counter (Invitrogen, California) and washed with PBS, before adding the blocking solution with anti-CD16/CD32 antibody for 10 minutes on ice (clone 93, Biolegend, San Diego, California). Cells were washed and incubated for 60 minutes at 4°C with the following antibodies: anti-CD3-APC-Cy7 (Biolegend, San Diego, California), anti-CD4-PE (BD bioscience, United Kingdom), anti-CD8-BV421 (Biolegend, San Diego, California) for CD4 and CD8 T cell detection; with anti-CD11b-FITC (BD bioscience, United Kingdom), anti-F4/80-AF647 (BD bioscience, United Kingdom), anti-CD206-BV421 (Biolegend, San Diego, California) and anti-Gr1-PE (BD bioscience, United Kingdom) for TAMs and MDSCs; with anti-CD3-APC-Cy7, anti-CD8-FITC (Proimmune, United Kingdom) and Pentamers-TRP2-PE (Proimmune, United Kingdom) for the detection of TRP-2-specific CD8 T cells. For staining with antiFoxP3-AF488 (BD bioscience, United Kingdom) or anti-IFNg-APC (Biolegend, San Diego, California), cells were previously incubated overnight at 4°C with a permeabilization/fixation solution (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Thermo Fisher, Waltham, Massachusetts). Cells were then incubated with anti-CD16/CD32 antibody for 10 minutes on ice (Biolegend, San Diego, California), washed and incubated for 60 minutes at 4°C with anti-IFNg-APC or antiFoxP3-AF488 diluted in the permeabilization/fixation solution. Samples were washed with PBS fixed for 10 minutes with 4% formalin and, then, suspended in PBS. Sample data were acquired with FACSVerse (BD bioscience, Franklin Lakes, New Jersey) and analyzed with FlowJo software (FlowJo LLC, Ashland, Oregon).

II.5. ENZYME-LINKED IMMUNOSPOT (ELISPOT)

ELISpot was performed according to the manufacturer's instruction (Immunospot, The ELISPOT source, Germany). Briefly, 3 x 10^5 fresh splenocytes diluted in 100 μl CTL-Test medium (Immunospot, The ELISPOT source, Germany) were cultured overnight at 37°C in anti-IFNg-coated 96 well plate. For stimulation, 10 ng/μl of TRP2 peptide$_{180\text{-}188}$ (SVYDFFVWL) was added to the splenocytes and incubated for 2 days. As positive control for splenocyte activation, Cell Stimulation Cocktail (Invitrogen, California) was used; PBS and a P815 peptide (LPYLGWLVF)

were used as negative control. The development of the ELISpot plate followed the manufacturer's instruction (Immunospot, The ELISPOT source, Germany). Spots were counted by using an ELISPOT reader system (Immunospot).

II.6. qPCR ANALYSIS

Brains extracted at day 29 were analyzed by qPCR. Total RNA was isolated using TRIzol reagent (Thermo Fisher, Waltham, Massachusetts) and phenol separation, as previously described (34). The quality and quantity of RNA were evaluated using a nanospectrophotometer (NanoDrop 2000, Thermo Fisher, Waltham, Massachusetts). Extracted RNA was considered pure if the 260/280 absorbance ratio of the sample was approximately 2 and the 260/230 absorbance ratio was 1.8-2.2. One microgram of RNA was reverse transcribed using a first-strand synthesis system (SuperScriptTM, Thermo Fisher, Waltham, Massachusetts) and oligo(dT) primers (Eurogentec, Liege, BE) according to the supplier's protocol. The resulting cDNA was used as template for 40 cycles of PCR amplification. SYBR™ green real-time qPCR (GoTaq qPCR MasterMix kit, Promega, Fitchburg, Winsconsin) was conducted on a StepOne Plus Real-Time PCR System (Thermo Fisher, Waltham, Massachusetts). Analysis of the melting curves was performed to ensure purity of PCR products. The results were analyzed with StepOne Software V2.1. The mRNA expression of the cytokines was calculated relative to the corresponding expression of β-actin (reference gene) according to the delta-delta Ct method. The results were normalized compared to the untreated control group. The PCR products were subjected to electrophoresis on a SYBR Safe (Thermo Fisher Scientific) -stained 1.5% agarose gel to detect TRP2 and gp100 antigens in the GL261 cells. A complete list of the primers used in this study is shown in Table 2.

Table 2: List of the primers used in this study.

Primer name		Primer sequence (5' → 3')	Amplicon length (bp)
TRP2	For	CCAGGATGACCGTGAGCAA	171 bp
	Rev	TGGGCAGTCAGGGAATGGAT	
Murine-gp100	For	GGAGCTTCCTTCCCGTGCTT	321 bp
	Rev	GGCTCCCATTGATGATGGTGT	
Human-gp100	For	ATAGGTGCTTTGCTGGCTGT	263 bp
	Rev	ACCTGCCCATCTGGCAATAC	
b-actin	For	ACTCCTATGTGGGTGACGAG	206 bp
	Rev	CATCTTTTCACGGTTGGCCTTAG	

II.7. HEMATOXYLIN AND EOSIN STAINING

At the first signs of pain and discomfort, tumor-bearing mice were sacrificed, and the brain was removed and fixed in 10% formalin solution (Merck, Germany) for 24 h and then in PBS at 4 °C for at least two days. As explained in (35), brains were embedded in paraffin, sectioned in 10 μm sections using a MICROM 17M325 microtome (Thermo Fischer Scientific, USA) and collected on super-frost plus glass slide. For the histological analysis and evaluation of the cellular inflammatory response the samples were deparaffinized and stained with hematoxylin and eosin (H/E). Samples were processed using a Sakura DRS 601 automated slide stainer (Sakura Finetek Europe, The Netherlands).

II.8. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 7 for Windows. Survival curves were compared using a Mantel–Cox (log-rank) test. p-Values less than 0.05 were considered statistically significant and indicated with different superscript letters (a, b, c).

III. RESULTS

III.1. IN A SC MODEL, PDNA VACCINATION INCREASED THE SURVIVAL OF GL261 TUMOR-BEARING MICE

To validate the immunogenic potential of a pDNA vaccine encoding gp100 and TRP2 antigens in a GL261 GBM tumor model, mice were therapeutically vaccinated after subcutaneous (SC) injection of GL261 cells. The vaccination protocol is shown in Figure 1A. Tumor growth of vaccinated mice was significantly delayed compared to the untreated group (Figure 1B) and 6/7 mice were considered long-term survivors (Figure 1C). For the untreated group, the median survival time (MST) was 35.7 days (Figure 1C). Single curve tumor growth for untreated mice and vaccinated mice are shown in Figure 1D and 1E, respectively. The expression of TRP2 and gp100 in GL261 cells was demonstrated by PCR (Figure 1F).

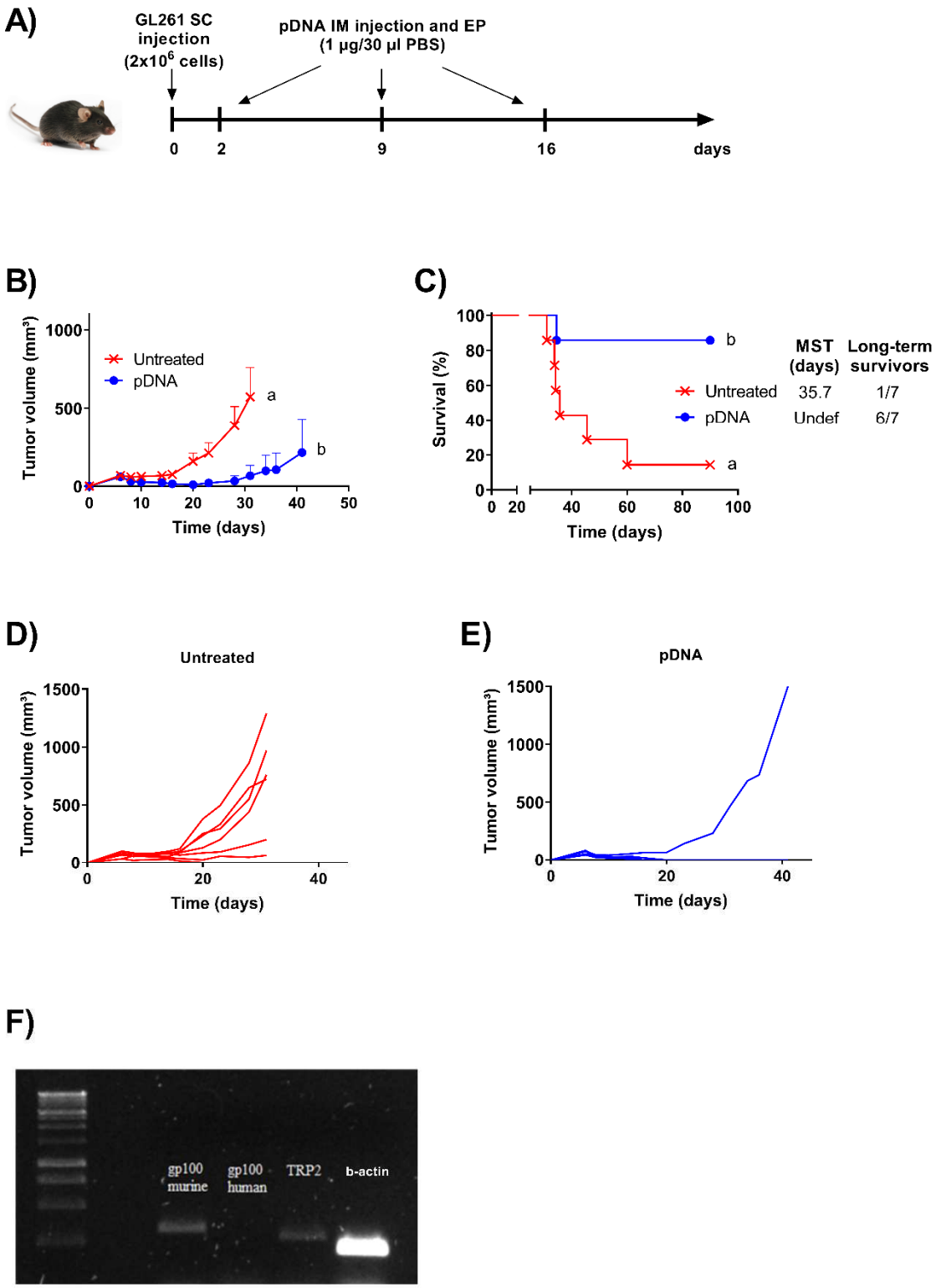


Figure 1: vaccination of mice SC injected with GL261 cells. A) Therapeutic vaccination protocol. B) Evolution of tumor volume (mm^3) after GL261 challenge as a function of time (days) (mean ± SD; n = 7). Statistical analysis is compared to the untreated group: two-way Anova, column factor (p value < 0.05). C) Survival curves representing the percentage of alive mice (%) as a function of time (days); MST = median survival time. Statistical analysis: Log-Rank (Mantel–Cox) test (p value < 0.05). D) and E) Single mouse tumor growth curves for the untreated and the vaccinated group, respectively. F) PCR in GL261 cells to verify the presence of murine gp100 and TRP2 antigens; b-actin has been used as housekeeping gene (positive control), while the human gp100 was a negative control. From a statistical point of view, a and b indicate significant differences between the groups.

III.2. IN A GL261 ORTHOTOPIC MODEL, TUMOR RESECTION AND PDNA VACCINATION SIGNIFICANTLY PROLONGED MICE SURVIVAL

To validate the efficacy of the pDNA vaccine in a murine orthotopic GBM model, C57Bl/6 mice were injected with 5 x 10^4 GL261 cells at the junction between the cortex and the striatum. Tumoral lesions of GL261-bearing mice were observed between the cortex and the striatum in all implanted animals at day 10 post-inoculation by magnetic resonance imaging (MRI). However, the hyperintense signal was less pronounced compared to the U-87 and 9L tumors (32, 36) and was often hard to visualize due to the limited contrast and brightness of the MRI T_2-weighted images with this tumor model. Mice were vaccinated at day 16, 23 and 29 and the tumor was resected 17 days after the GL261 inoculation (Figure 2A). In the control groups (untreated, resection or pDNA), most of the mice showed signs of discomfort and pain starting from day 27-30 after the tumor injection and their MST was less than 40 days (Figure 2B). MRI performed 27 days after tumor inoculation confirmed the presence of infiltrative and aggressive recurrences in control group mice (Figure 2C). Due to the infiltrative patterns of the GL261 tumors, we are unable to provide adequate volume estimation of the tumors at the designated time points but the presence of the tumor and of its infiltrative nature was confirmed post-mortem by hematoxylin and eosin staining (H/E) (Figure 2D). In the combination group (pDNA + Resection), reduced tumor lesions were observed by MRI eleven days after the vaccine priming and ten days after the resection (day 27 post-inoculation, Figure 2C) and 7/9 mice (80%) were considered as long-term survivors (Figure 2B).

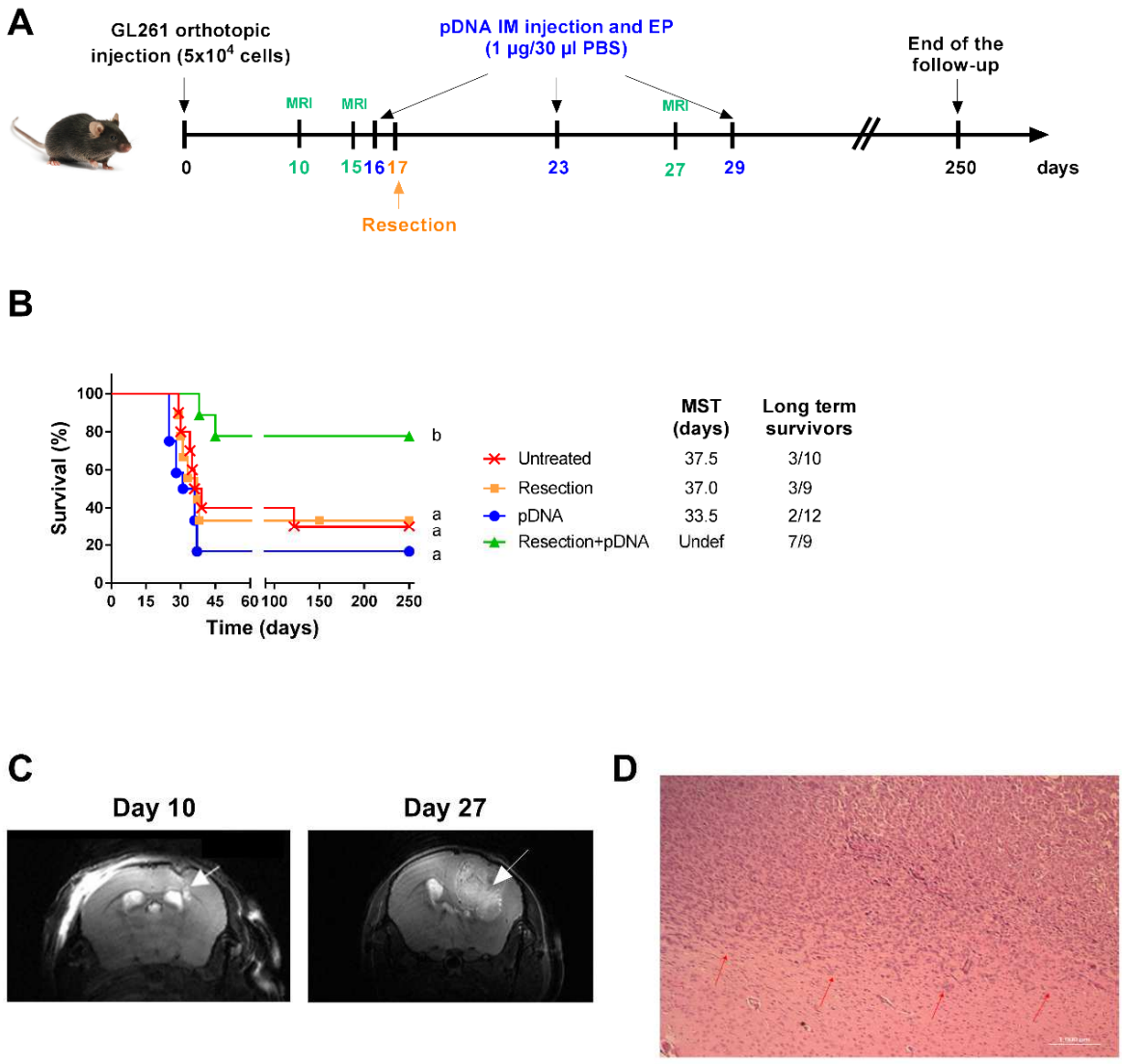


Figure 2: Vaccination and resection of mice orthotopically injected with GL261 cells. A) Injection, vaccination and resection protocol. B) Survival curves representing the percentage of alive mice (%) as a function of time (days); MST = median survival time. Statistical analysis: Log-Rank (Mantel–Cox) test (p value < 0.05). C) Representative axial T_2-weighted MRI image of an untreated mouse brain before (day 10 post-inoculation, left) and after tumor resection (day 27 post-tumor inoculation, right). The white arrows indicate the GL261 primary and recurrent tumor, respectively. D) Representative H/E image of the brain/tumor of an untreated mouse sacrificed due to signs of discomfort and pain. The red arrows indicate the infiltrative margins between the tumor (above) and the brain (below). EP = electroporation. From a statistical point of view, a and b indicate significant differences between the groups.

III.3. pDNA AND TUMOR RESECTION REDUCED THE NUMBER OF INFILTRATED IMMUNOSUPPRESSIVE CELLS IN THE BRAIN

To study the mechanism underpinning the synergy between the plasmid and the resection and their contribution in prolonging mice survival, the infiltration of different immune cells was assessed in the mice brains 30 days after tumor inoculation. The flow cytometry analysis revealed a significant decrease of the infiltrated immunosuppressive cells for all the groups compared to the untreated

group (Figure 3). This effect was seen for M2 macrophages (Figure 3A), myeloid-derived suppressor cells (MDSC) (Figure 3B) and T regulatory cells (Tregs) (Figure 3C). The greatest effect of the decrease in the tumor immunosuppression was observed when the vaccine was used alone or in combination with the resection. Furthermore, the ratio of Tregs compared to the total CD4 population was lower in all the groups compared to the untreated (Figure 3D). On the other hand, the ratio Tregs/CD8 population was significantly decreased only in the combination group compared to the untreated (Figure 3E).

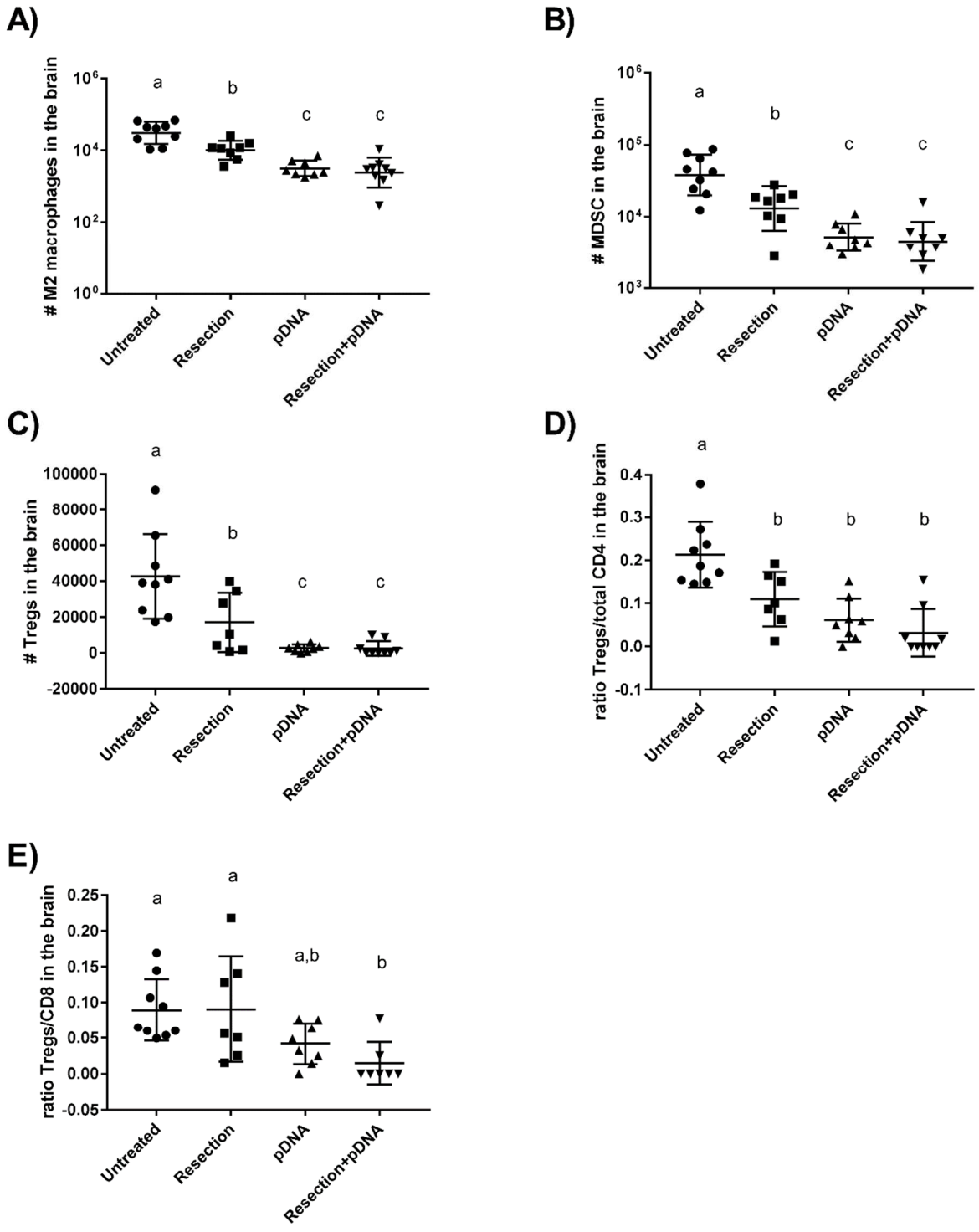


Figure 3: Evaluation of immunosuppressive cell infiltration in the brain, 29 days after GL261 inoculation. The number of M2 macrophages (A), MDSC (B) and Tregs (C) and the ratio Tregs/total CD4 (D) and Tregs/CD8 (E) are shown for all the groups and indicated as mean ± SD. n = 7-9 for each group. a, b and c letters on the graphs indicate significantly different results when the superscript letters are different.. The annotation "a,b" indicates no significant differences compared to a and b statistical groups.

III.4. pDNA AND TUMOR RESECTION REDUCED THE TOTAL NUMBER OF INFILTRATED CD4 AND CD8 T CELLS BUT NOT THEIR ACTIVITY

Further studies on the immune cell infiltration into the brain showed a decreased infiltration of CD8 T cells in the treated groups, especially in the combination group (Figure 4A). However, when we compared the number of total infiltrated CD8 with the number of IFNg-secreting CD8 (ratio), we can observe that the 40% of infiltrated CD8 produce IFNg, while in the other groups, only 10-15% of CD8 T cells produce IFNg (Figure 4B). Hence, the activity of CD8 in the combination group was significantly higher compared to all the other groups. The same trend was observed for the number of TRP2-specific CD8 (Figure 4C), compared to the number of total CD8 (Figure 4D), and for the CD4 T cells (Figure 4E). However, the slight increase in IFNg-secreting CD4 that can be observed in the combination group is not significantly different compared to the other groups (Figure 4F).

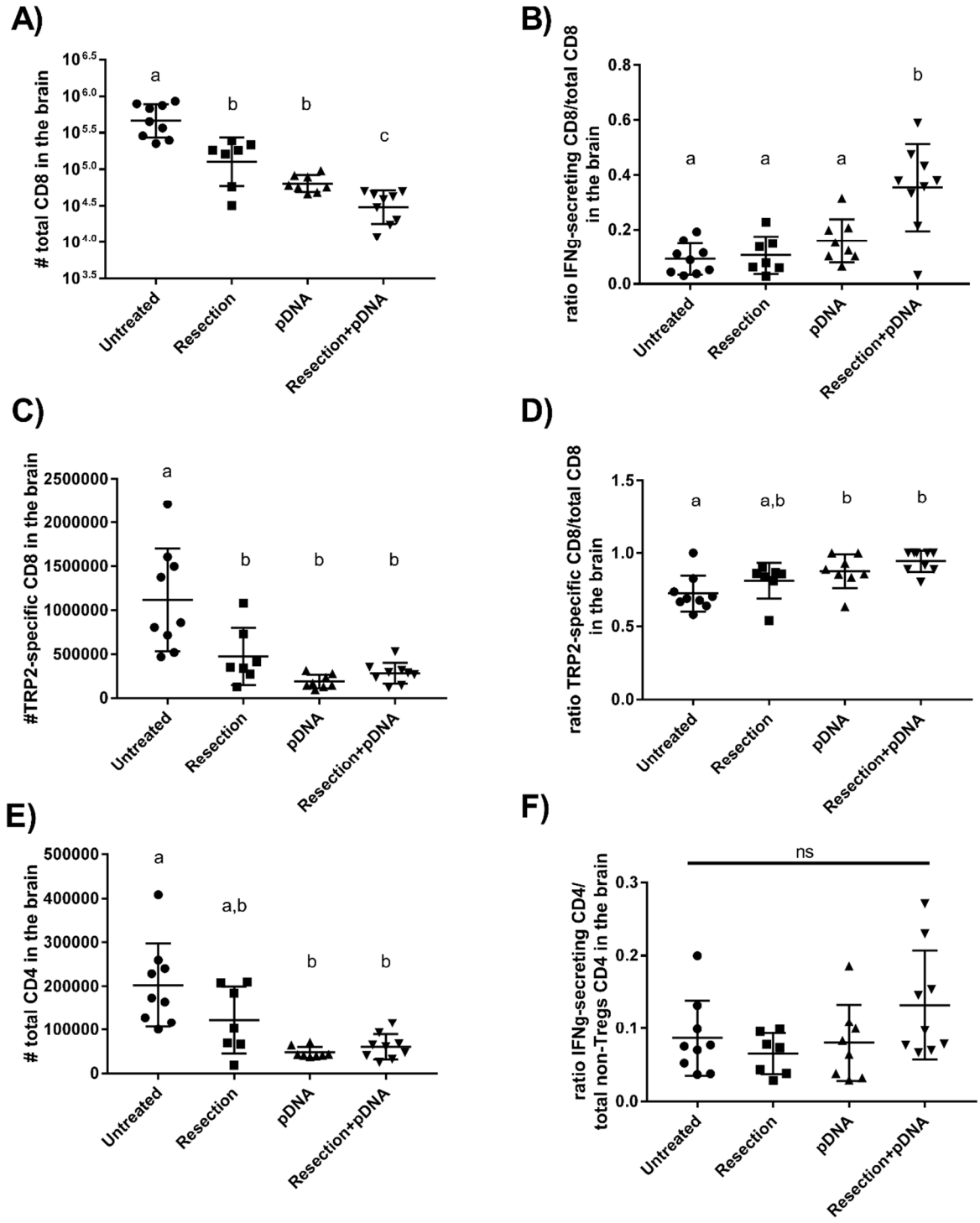


Figure 4: Evaluation of immune cell infiltration in the brain, 29 days after GL261 inoculation. The number of CD8 (A), TRP2-specific CD8 (C) and CD4 (E) and the ratio IFNg-secreting CD8/total CD8 (B), TRP2-specific CD8/total CD8 (D) and IFNg-secreting CD4/total no-Tregs CD4 are shown for all the groups and indicated as mean ± SD. n = 7-9 for each group. a, b and c letters on the graphs indicate significantly different results when the superscript letters are different. The annotation "a,b" indicates no significant differences compared to a and b statistical groups. ns = non-significant.

III.5. IN THE SPLEEN, PDNA INDUCED ANTIGEN-SPECIFIC IMMUNE RESPONSE, WHILE THE COMBINATION INCREASED THE NUMBER OF ANTI-TUMOR IMMUNE CELLS

Next, we evaluated the systemic immune activity and compare it with the response in the TME. To this end, splenocytes were collected 29 days after the tumor challenge and analyzed by flow cytometry and ELISpot (Figure 5). The combination group was the only treatment that significantly increased the number of M1 macrophages (Figure 5A), while decreasing the number of M2 macrophages (Figure 5B) compared to the untreated and resected groups. In addition, the number of MDSCs was lower in the groups treated with the vaccine (pDNA and pDNA+resection), as shown in Figure 5C. In the combination group, the CD8 infiltration was significantly higher compared to the untreated and resection groups (Figure 5D). The activation of TRP2-specific T cells by the vaccine in the pDNA and pDNA+resection groups was assessed by ELISpot for the IFNg analysis (Figure 5E and 5F). In the absence of vaccination, almost no spot has been detected (untreated and resection groups), whereas the pDNA and the pDNA+resection groups showed a significantly higher number of spots (Figure 5E and 5F).

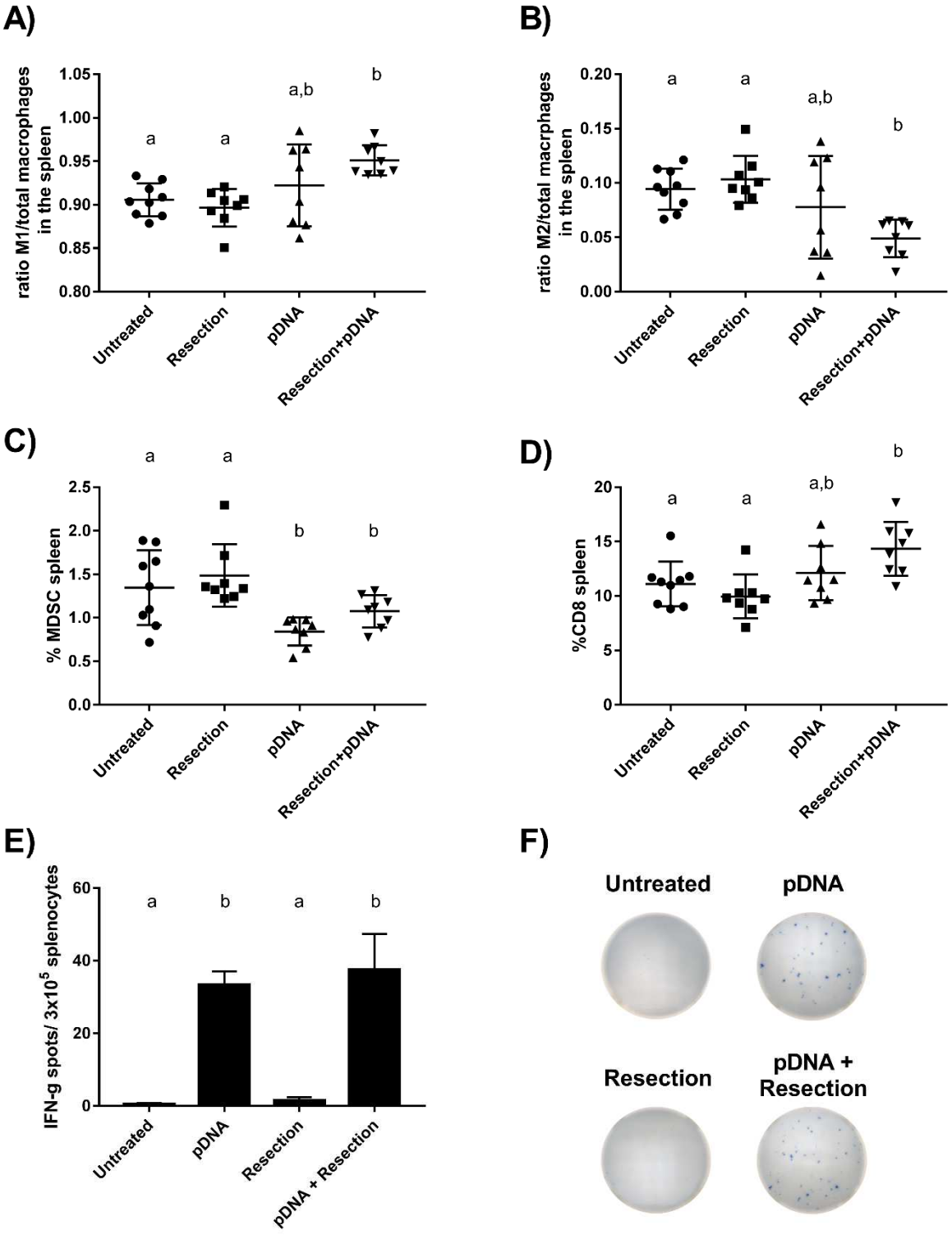


Figure 5: Evaluation of immune cell infiltration in the spleen, 29 days after GL261 inoculation. The ratio M1/total macrophages (A), M2/total macrophages (B) and the % of MDSC (C) and CD8 (D) are shown for all the groups and indicated as mean ± SD (n = 7-9 for each group). E-F) ELISPOT analysis for the IFNg production from splenocytes stimulated with TRP2 peptide (n = 7-9). a and b letters on the graphs indicate significantly different results when the superscript letters are different. The annotation "a,b" indicates no significant differences compared to a and b statistical groups.

IV. DISCUSSION

Immunotherapy is emerging as a new therapeutic option for different types of cancer, including GBM. In particular, the design of a DNA vaccine encoding GAAs, able to generate a specific and long-lasting immune response against GL261 GBM model, represents a promising treatment to target residual GBM cells after the surgical resection.

In the current study, a pDNA vaccine encoding GAAs has been tested in an orthotopic model of GBM, in combination with the surgical resection of the tumor, which is the first clinical step in GBM management. The encoded GAAs are TRP2 and gp100, which are antigens shared by melanoma, due to the common prenatal origin of the glial cells and the melanocytes from the neural ectoderm (37). This vaccine has been already successfully tested in B16F10 melanoma model, significantly slowing down the tumor growth and inducing both CD4 and CD8 immune response (30). The ability of the vaccine to activate a specific anti-tumor response was first validated in a GL261 SC model. A complete tumor regression was observed in 6/7 vaccine-treated mice (Figure 1C). Then, this vaccine was used to induce the immune response in a GL261 orthotopic model. In this case, neither pDNA alone nor the surgical resection alone increased mice survival. Only the combination of the two therapies allowed the long-term survival of 7/9 mice (Figure 2B).

We have previously demonstrated that, following tumor resection, fast and aggressive tumor recurrences develop in the 9L syngeneic model in immunocompetent rats but also in the xenograft U87 model in immunodeficient Nude mice (which lack of T cells but possess functional NK and B cells) (32, 36). Here, we report that recurrences were observed in >70% of our control animals (resected group) following tumor resection in the GL261 model, confirming the infiltrative and aggressive pattern of this preclinical GBM model. Interestingly, we observed that some untreated animals did not develop the tumor and, even though spontaneous regressions have rarely been observed in the GL261 model so far, several studies have shown regression of C6 GBM cortical tumors (32, 38). We hypothesize that the regression observed in 30% of our GL261 GBM-bearing untreated mice can be explained by the injection of GL261 cells in the cortex, in close proximity to the functional lymphatic vessels that have been observed in black-6 mice by Aspelund et al. and Louvaeau et al. (12, 13). The immune surveillance mediated by these lymphatic vessels may have had a role in the spontaneous regression. Another explanation could be the genetic background of our C57Bl/6JRj mice provided by Janvier (France) compared to previously reported studies. Indeed, it has been demonstrated that significant gene expression differences exist between sub-strains and these can have a strong impact on the immunological responses (39, 40).

The analysis of the immune infiltrating cells in the brain showed a strong decrease in the number of immunosuppressive cells (M2 macrophages, MDSCs and Tregs) (Figure 3), but also a global decrease of the CD8 and CD4 T cells (Figure 4) in all the treated groups (resection, pDNA and pDNA+resection). This effect could be explained by a possible tumor shrinkage in the treated groups, especially in the pDNA and pDNA+resection groups, which reduced the global tumor mass and, therefore, also the number of infiltrating immune cells. However, we could not calculate the immune cell "density" into the tumor (i.e., the ratio between the number of infiltrating cells/tumor volume), because the GL261 cells are highly infiltrative and do not form well-defined tumors (41), which makes difficult and approximate the measurement of the tumor volume. Indeed, histopathological analysis of the GL261 model demonstrates individual cell invasion several millimeters away from the tumor margins (42), as we also observed in Figure 2D.

For this reason, a series of comparative analysis have been performed. In particular, when we looked at the IFNg-producing T cells, we observed that the ratio IFNg-producing CD8/total CD8 is significantly higher only in the pDNA+resection group (Figure 4b), indicating the ability of the infiltrated CD8 T cells to produce IFNg. Furthermore, the vaccine-treated groups showed higher levels of antigen-specific T cells not only in the tumor but also in the spleen. This may indicate that the infiltrated CD8 T cells in the combination group, even if low in number, are not exhausted (43) and still able to recognize the antigen and produce IFNg. This aspect would revolutionize the immunotherapy of GBM, well known for having a severe T cell exhaustion signature, which damages the efficacy of immune-based platforms (44).

On the other hand, the ratio Tregs/CD4 and Tregs/CD8 T cells is lower in the treated groups. In particular, a significantly lower Tregs/CD8 T cell ratio, which indicates a poor Treg infiltration in the TME compared to the CD8 T cells, was observed only in the combination group, compared to the other groups. This suggests that the immunosuppressive activity was reduced when the vaccine was combined with the resection, thus permitting a higher CD8 T cell activation. Indeed, a low Tregs/CD8 ratio is often correlated with a good prognosis (45) and this aspect can explain the longer survival observed in the pDNA+resection group. Maes and colleagues demonstrated that the depletion of Tregs in a GL261 model was responsible for the tumor regression and for the decreased MDSC infiltration (46). In another study using the GL261 model, the decrease of the MDSC infiltration in the TME was associated with tumor growth inhibition (47). The correlation between the presence of Tregs and the high infiltration of MDSCs and TAMs M2 has also been demonstrated in different cancer types, including GBM (48, 49). Indeed, MDSCs recruited in the TME could become TAMs with an immunosuppressive phenotype (M2), which could promote glioma tumorigenesis. M2 TAMs are poor inducers of T cell response, are recruited within the

brain by GSCs and are implicated in brain tumor angiogenesis (14, 50-52). This immunosuppressive microenvironment is also responsible for the recruitment of Tregs (53). Other authors have already shown that treating mice with neutralizing antibodies against CD25 (present on Tregs' surface) eliminated the suppressive function of Tregs, thereby increasing CTL activity. Furthermore, when combined with dendritic cell vaccination, neutralization of CD25 resulted in 100% glioma cell rejection in mice (54). Hence, reprogramming of immunosuppressive T cell subsets in the context of GBM, and potentially other brain tumors, could activate or potentiate the anti-tumor immune responses (14).

Overall, in the brain, the infiltration of specific and IFNg-secreting CD8 T cells and a decreased immunosuppression were observed especially when the vaccine was combined with the surgical resection. Despite the immune activation in the brain induced by the DNA vaccine, when it is used as single therapy, we did not observe a positive effect on the survival. This is in accordance with what happens in clinical trials. In different clinical studies, cancer vaccines are able to induce an antigen-specific immune response, but do not have a good impact on the overall survival in patients (55, 56).

Several studies have demonstrated that surgery-related changes can have a strong impact on the TME. For example, surgery can induce excessive healing response mediated by TMs, GSCs and a phenotypic switch in reactive astrocytes promoting tumor proliferation and invasion (21, 23). Tumor debulking was also associated with an increased macrophage recruitment, both M1 and M2 (57). Hence, while surgery alone is not sufficient to significantly increase survival and could facilitate the proliferation and migration of non-resected tumor cells (58), it can contribute in the immunological changes that could further promote the vaccine activity. This could transform a potential side effect of the tumor resection in a useful tool to enhance vaccine therapy.

To our knowledge, this is the first study reporting the combination between GBM surgical resection and vaccine-mediated immunotherapy, with the first immunization being performed before the tumor debulking. We hypothesize that the vaccine administration prior to surgery might take advantage of the acute inflammatory response induced by the resection to activate a specific antitumor immune response (7), acting as double sword both on residual GBM cells and on the tumor resection microenvironment, thus avoiding the on-set of tumor recurrences. The employment of a concomitant therapy to induce a favorable microenvironment for the DNA vaccine activity has been already tested by our group in a B16F1 melanoma model. In this study, we combined the ability of an oncolytic virus, injected in the tumor, to locally activate the innate immune response, with the ability of the DNA vaccine to induce the adaptive (and specific) anti-tumor immunity. The antigen-specific immune cells generated by the DNA vaccine were driven in

the TME thanks to the cytokines expressed by the tumor in response to the viral infection. The result was a tremendous delay in the tumor growth observed in the combination group (59). Furthermore, very recently, a clinical study using anti-PD1 antibody in patients with resectable GBM, evaluated the efficacy of the immune checkpoint blockade therapy before the surgical resection. The result was the functional activation of tumor-infiltrating lymphocytes, producing an IFN response within the TME and the MST increased from 228.5 days to 417 days (60).

The analysis of the immune cells in the spleen confirmed the decreased immunosuppression and the generation of antigen-specific immune response that we observed in the brain. Indeed, the vaccine contributed to reduce the MDSC and M2 infiltration and to produce IFNg-secreting cells, following splenocyte stimulation with TRP2 peptide in both pDNA and pDNA+resection groups (Figure 5C and 5E-F). Furthermore, only in the combination group the levels of anti-tumor M1 macrophages and CD8 T cells were significantly higher compared to the other groups (Figure 5A-B and 5D).

Other than the difficulty to adequately measure the tumor volume, another limitation of this study concerns the timepoint of the sacrifice and immunological analysis. We chose to perform the immunological analysis 29 days after the tumor inoculation, i.e. 13 days after the first vaccination, to give the time to the vaccine to develop the adaptive immune response, but also because most of the control group mice did not survive longer (Figure 2B). However, an immunological follow-up in a later time point could probably reveal stronger differences between the groups and, in particular, between the pDNA and the combination group, which could better explain the significant differences in the survival in these 2 groups. On the other side, an analysis at an earlier time point could blind the immune response activation following the vaccination; furthermore, the inflammation induced by the resection could provide false positive/negative results to our analysis. Despite these limitations, this study looks at various aspects of the immune system activation, from the IFNg expression to the immunosuppression and the antigen-specificity, and is the first to explore the immune activity in an orthotopic GBM model following DNA vaccination and tumor resection.

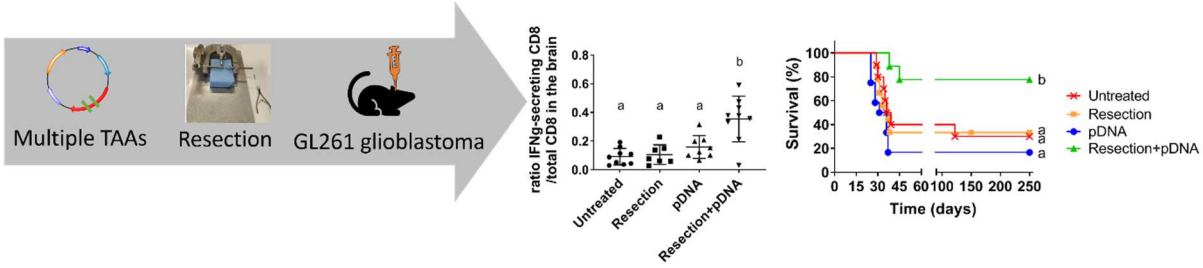
V. CONCLUSIONS

In this study, we investigated the combination of a DNA vaccine encoding GAAs and the tumor resection in a GL261 orthotopic model of GBM. Tumor resection, when applicable, represents a part of the standard of care for GBM patients and is the considered the first treatment option, before chemoradiotherapy. However, the insurgence of chemotherapeutic resistance and the presence of recurrences led the way to immunotherapy as a possible new curative strategy for GBM.

We demonstrated that the combination of DNA vaccination and tumor resection drastically increased mice survival. This was explained by the decreased number of immunosuppressive cells and the concomitant activity and antigen-specificity of T cells in the brain, when the two therapies were combined. The strength of this combination could overcome the limits of each single treatment: from one side the tumor resection reduces the number of tumor cells and induces a local inflammation that could strengthen the adaptive immunity activated by the vaccine. Moreover, the vaccine activates the host adaptive immune system against the residual tumor cells, thus avoiding the GBM recurrences.

A further step towards the clinic would consider the intra-patient and inter-patient GBM heterogeneity. Indeed, each tumor has its specificities and, even inside the same tumor, the cells could express different tumor antigens. One possibility would be to design a first vaccine containing well-known and non-mutated tumor antigens administered at the moment of the tumor diagnosis (several days prior to the surgery) to slowdown the tumor growth. After the resection, the tumor could be analyzed to find specific tumor neoantigens to design a polyepitope and personalized vaccine that could overcome the heterogeneity associated to GBM, one of the main causes of failure of the current treatments. Globally, this study opens the way to a new treatment option for GBM patients.

Graphical conclusion



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CHAPTER VII. DISCUSSION, LIMITATIONS AND FUTURE PERSPECTIVES

MAJOR FINDINGS OF THE PHD WORK

Cancer is one of the leading causes of death worldwide and conventional therapies typically fail to provide long-lasting benefits to the patients. Immunotherapy is emerging as a successful therapy against cancer and as a new hope for cancer patients. In particular, DNA vaccines showed to be safe and specific, and able to generate a broad immune response. However, they are poorly immunogenic, especially in the clinic.

My PhD work demonstrated the possibility to use DNA vaccination to significantly slowdown and even eradicate an established murine tumor in different cancer models. In particular, this project answered to the following three main research questions:

1. How can the DNA vaccine efficacy be improved?

2. Which are the limitations of DNA vaccines? And how to overcome them?

3. How versatile is DNA vaccination against cancer?

The answers to these questions and the major findings and contributions of my PhD thesis to the cancer DNA vaccine field can be summarized as follows (Table 1 and Figure 1):

1. The DNA vaccine itself can be improved by (i) codon optimization (CO), which increases the antigen production and the innate immune activity, through the introduction of in-built CpG immunostimulatory motifs in the antigen gene sequence. CO led to a drastic increase of the survival in a prophylactic approach compared to the non-optimized vaccine, but it only slowed-down the tumor growth in a therapeutic setting in the P815 model. (ii) rational selection of the TAs to insert in the DNA vaccine. This aspect improves the DNA vaccine efficacy in 3 different ways: (a) avoid the antigen loss or tolerance, by delivering different tumor antigens (TAs) (high- and low-specific TAs, mutated and non-mutated antigens) in one or more plasmids; (b) activate a broad immune response, by inserting both CD8 and CD4 epitopes; (c) allow a specific and "personalized" vaccination, by designing TAs according to the specific neoantigen mutations found in our cell lines. These strategies significantly slowed-down the tumor growth, but were not sufficient to cure mice bearing a fast-growing and poorly immunogenic tumor in the B16F1 subcutaneous (SC) model, or the GL261 tumor when inoculated orthotopically.

2. DNA vaccines alone are not able to completely eradicate an established tumor, because of the immunosuppressive tumor microenvironment (TME), which prevents the activity and/or the infiltration of T cells into the tumor bed. The use of combination therapies that either potentiate the T cell response, such as the immune checkpoint blockade (ICBs), or enhance their

migration into the TME, such as the oncolytic viruses (OVs) or the tumor resection, strongly improved the outcome.

3. The applicability of our findings has been demonstrated in three different tumor models (P815 mastocytoma and B16F1 melanoma in a SC model, and GL261 glioblastoma (GBM) in an orthotopic model), by testing different combinations. In all the models, the single therapies were less effective compared to the combination therapy. This effect was explained looking at the tumor immune cell infiltration and cytokine production. In particular, a stronger increase in the T cell activity and specificity in the TME was observed only when the DNA vaccine was combined with another therapy (ICB, OV or the resection).

Table 1: Research questions, approaches and main findings of my PhD thesis

Research questions	Approaches	Main findings
1. How can the DNA vaccine efficacy be improved?	Evaluation of CO, pTOP platform and TA choice	Optimized vaccines eradicate the tumor in a prophylactic model, but they only slowdown the tumor growth in a therapeutic model
2. Which are the limitations of DNA vaccines? Which are the treatments that can contribute to eradicate an established tumor?	Evaluation of the limits of DNA vaccines as single therapy and analysis of the combination approaches	DNA vaccines alone generate antigen-specific CTLs that do not reach or are not effective into the immunosuppressive TME; the combination therapy allows a stronger CTL migration/activity in the tumor, thus prolonging the survival or curing mice in a therapeutic setting
3. Versatility of this approach?	Validation of our findings in different tumor models	An increased survival and immune cell infiltration into the TME were observed in all the studied tumor models using optimized DNA vaccines in combination with complementary therapies

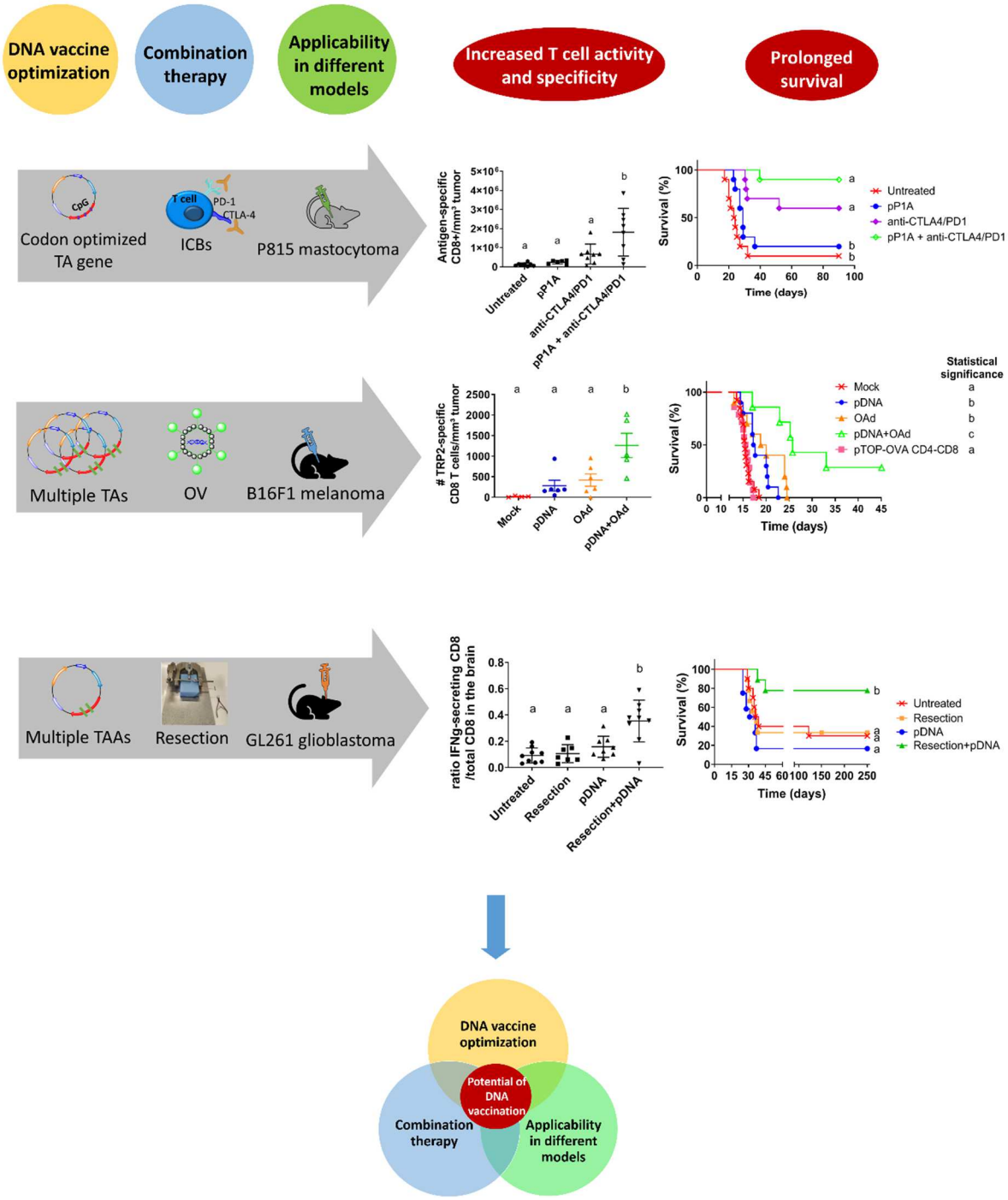

Figure 1: Potential of DNA vaccination: DNA vaccine optimization + combination therapy + applicability in different models.

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I. DISCUSSION

In the last 4-5 years, the use of DNA vaccination against cancer has strongly evolved. The first clinical studies (starting from 1998, NCT00019448) were principally evaluating the DNA vaccine dosage and safety. In these trials, cancer DNA vaccines encoded non-mutated TAs and were always used as a single therapy and administered after the conventional standard of care treatments. Only starting from 2015, DNA vaccines encoded neoantigens or more than one epitope, but, rarely, they were administered in combination with another therapy. In many cases, the strength of the immune response generated by the vaccine was still limited and only one DNA vaccine against HPV cervical cancer reached the phase III clinical trial, in 2017 (1). Until 2015, even in preclinical models, the number of studies exploring the efficacy of DNA vaccination in a therapeutic setting was limited and often not successful, due to antigen loss, tumor resistance, cancer cell heterogeneity etc. (2). Many studies tried to improve their immunogenicity principally based on a better delivery of the plasmid or by using different priming-boost strategies (2).

New concepts such as CO, neoantigen immunogenicity and the possibility to combine different therapies were the starting points of my PhD thesis. Hence, my thesis contributes to the evolution of DNA vaccines against cancer. In particular, it extends the knowledge of the effects of these new strategies in the field of DNA vaccination and evaluates the increased immunogenic activity of DNA vaccines and their mechanism of action.

In the following paragraphs, the scientific context of our findings, their meaning and implications for the future will be discussed.

I.1. INCREASE OF DNA VACCINE IMMUNOGENICITY

When this project started (in 2014), CO was greatly exploited by industries to increase the production of a protein from a specific organism in a different host (heterologous protein production) (3, 4). This technology significantly impacted the economic feasibility of microbial-based biotechnological processes (4). The great potential of CO for DNA vaccination was already known, but only for heterologous antigen production (e.g., to encode HPV viral antigens in mammalian cells) (5, 6).

To our knowledge, we were the first to exploit this technology for non-viral or non-bacterial proteins for cancer therapy and to use CO for the modulation of CpG motifs in an antigen gene to activate the innate immunity (Chapter III) (7). Indeed, oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG-ODNs) have been generally used as vaccine adjuvants

co-delivered with the antigen-encoding vaccine to accelerate the induction, increase the maximum level, and extend the duration of the induced immune response, but never as part of the vaccine itself (8, 9). The fact that the CpG modulation improved the DNA vaccine efficacy not only in a prophylactic but also in a therapeutic model, opens the possibility to introduce an innate immune stimulator to create a unique block with the antigen sequence and not as an extra component on the plasmid or on another plasmid.

To increase the DNA vaccine immunogenicity, another platform that we explored was the insertion of a viral protein gene, the Vesicular Stomatitis Virus Glycoprotein (VSV-G), into the pVAX2 vector. VSV-G is a viral fusion protein, essential for enveloped virus infection, which is recognized by the immune system to activate the innate immune response (10, 11). This gene was CO to contain more CpG motifs compared to its wild type sequence and inserted in the pVAX2 backbone. This system was patented and has been called "pTOP". To verify the activation of the innate immunity due to the presence of the VSV-G, we compared the empty (with no antigen inserted) pVAX2 with the empty pTOP vectors. The two plasmids were IM injected and delivered by EP in mice (pVAX2 and pTOP groups); mice that did not receive any plasmid have been used as control (untreated). The VSV-G mRNA expression has been verified to assess the transfection of the pTOP vaccine into the muscles of the pTOP group (Figure 2A). Then, the mRNA expression of some innate cytokines related to the anti-viral and to the anti-tumoral immunity has been evaluated in the injection site (muscles), 48 hours after the plasmid injection. A higher immune stimulation using the empty pTOP compared to the pVAX2 or to the untreated groups has been observed, even if this was not significantly different in each case, due to the low sample number (n = 3-4). In particular, the expression levels of IL6 and IL12 were increased (Figure 2B and C, respectively). IL12 is known to activate early NK cell-dependent IFNg production (in 3-5 days after the IL12 signal) and to enhance the CTL activity; it is also involved in the early inhibition of virus replication (12-14). IL6 plays an important role against viral infections, by inducing neutrophil-mediated viral clearance (15) and is involved in the switch from the innate to the adaptive immunity (16). IL6 has a double edge sword against cancer, being pro-tumoral when the inflammation becomes chronic, but helping the T cell recruitment and activity during the immune response activation (acute response) (17). Because of the higher IL6 levels following the pTOP transfection, CCL2 was overexpressed (Figure 2D). Indeed, this chemokine is secreted in response to the interaction between IL6 and its receptor and indicates the recruitment of T cells and monocytes in the tissue (16). This work is part of a more extended study aimed at characterizing the pTOP system for preclinical and clinical applications. Ongoing experiments are evaluating the cytokine production not only 48 hours (with an increased sample size) but also at 72 hours after

the plasmid injection and EP, to detect other time-dependent cytokines, such as IFNg, which would be expressed in response to the cytokines detected at 48 hours.

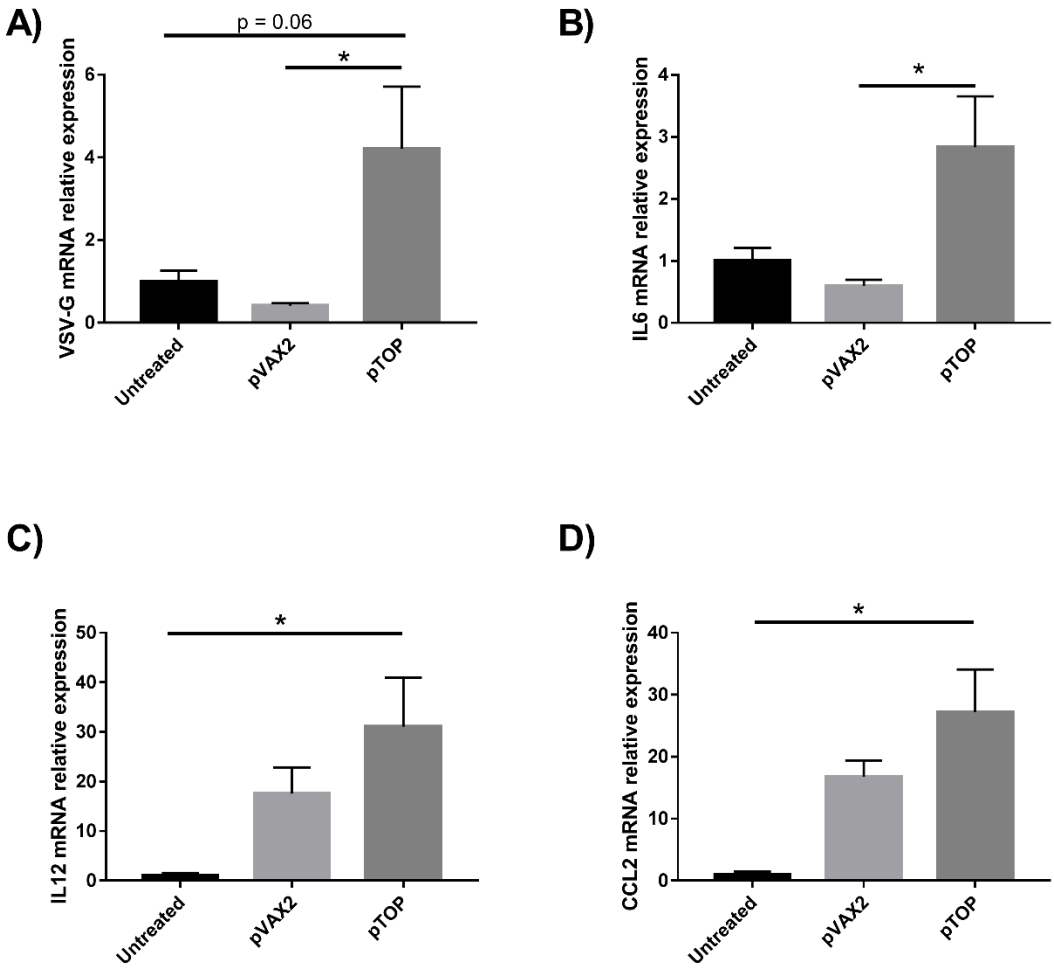

Figure 2: mRNA expression of VSV-G (A), IL6 (B), IL12 (C) and CCL2 (D) in the muscle of mice electroporated with pVAX2 vs pTOP. Mice that did not receive any plasmid (untreated) have been used as a control. The analysis has been performed 48 hours after the EP. The results are expressed as mean ± SEM; n = 3-4.

To exploit the potential of the pTOP platform for DNA vaccination, the VSV-G sequence was opened to insert TA epitopes. The possibility to modify in two different sites the VSV-G protein to insert two epitopes, one activating the CD4 and the other the CD8 T cell responses, has been previously demonstrated (18).

In this thesis, the pTOP system has been used as vaccine platform for the melanoma and GBM models. For the studies with GL261 GBM model, we inserted two non-mutated tumor-associated antigens (TAAs) shared by glioma and melanoma tumors: the TRP2, for the CD8 response, and the gp100, for the CD4. For the studies in the melanoma model, we used the two cited TAAs

(TRP2, gp100) and/or well-known B16 neoantigens (Cpsf3l, Kif18b and Pbk mutated genes) (19) into the pTOP vector. As explained in the "General introduction to the Chapter V", the presence of the neoantigen mutations in the B16F1 and B16F10 cells has been verified. The screening of the neoantigens in the gDNA for the two cell lines, showed a higher mutation rate of the B16F10 cells compared to the B16F1. To avoid a loss of efficacy of our vaccine after few cell divisions, we decided to work with the less mutagenic tumor model, the B16F1. We designed the neoantigen genes, according to the mutations found in these cells. The 5 TA epitopes (TRP-2, gp100, Cpsf3l, Kif18b and Pbk) were delivered in four different plasmids. Every plasmid was modified with one CD4 (gp100 or Cpsf3l or Kif18b) and one CD8 epitope (Pbk or TRP-2), keeping at least one TAA for every plasmid. Hence, the four generated plasmids were: pTRP2-gp100, pTRP2-Kif18b, pTRP2-Cpsf3l, pgp100-Pbk. Contrarily to the GBM model, in the B16F1 model, the use of only 2 TAAs (pTRP2-gp100) failed to show a significant decrease in the tumor growth rate. Only the combination of the 4 plasmids was able to delay the tumor growth (as explained in the "General introduction to the Chapter V").

In the field of cancer vaccination, the discovery of neoantigens arose the idea of the personalization in the cancer treatment to improve vaccine efficacy (20). Indeed, the past failure of cancer vaccines can, in part, be attributed to the selection of low tumor-specific TAs that elicit T cells of low avidity (21). In preclinical studies, peptide vaccination against two of the mutant melanoma antigens that we used, Kif18b (K739N) and Cpsf3l (D314N), significantly decreased the tumor growth and increased the survival (22). Until now, only few studies examined the DNA vaccination using neoantigens. In particular, in a TC-1 model, a DNA neoantigen vaccine only delayed tumor progression, without significantly impacting the survival (23). This result supports our findings in the B16F1 model. Nevertheless, the DNA vaccination using neoantigens could be a good option for cancer patients, for the potential high specificity and strength of the immune response associated to the neoantigens. Different clinical trials are ongoing to evaluate the efficacy of poly-neoepitope DNA vaccines (24). To our knowledge, only one study has results until now, which shows an overall survival of 64% after a median follow-up of 85.6 months (25). However, not all the tumor types are the same and the mutational load can be determinant to the response to neoantigen vaccination (20).

Despite further studies are needed to confirm or to disprove our results, it seems clear that optimized DNA vaccination is an excellent strategy to induce a CTL-specific immune response and to delay the tumor growth. However, when used alone, DNA vaccines cannot totally eradicate the tumor growth in a therapeutic setting. This consideration encouraged the use of other therapies in combination with DNA vaccines.

I.2. THE THERAPY COMBINATIONS

Generally, the interest of combining two therapies derives from the necessity to overcome the limitations of each single therapy. Since their discovery, ICB had an extraordinary success, as suggested by the exponentially increasing number of clinical studies in this field (Figure 13 – Introduction) and their increased use in clinic (26), but also a considerable toxicity (27). In our study, the combination of ICB with P1A-DNA vaccination in the mastocytoma P815 model led to the: (i) possibility to reduce the ICB doses to decrease the toxicity, compared to studies that use only ICB therapy; (ii) activation, migration and proliferation in the TME of the antigen-specific T cells generated by the DNA vaccine; (iii) increased survival and decreased tumor growth (28). Other researchers confirmed the ability of ICB to not only enhance the T cell activity but also to increase their migration in the TME (29-31). However, in contrast to other studies (31, 32), we did not observe any effect of ICB on Tregs depletion in our tumor model. Nevertheless, Tregs depletion is also considered to be context-dependent, i.e. dependent on the amount of CTLA4 expressed by Tregs and on the presence of other cells in the TME (32); probably, the early timepoint of our analysis (10 days after the tumor injection) did not allow us to see any variation in the Treg infiltration.

Despite the exciting results obtained using the combination between DNA vaccine and ICBs, not all the tumors nor all the patients positively respond to ICB therapy, as previously discussed. Furthermore, even if promising, ICBs are not cost-effectiveness treatments for many cancer types (33); the management of their related toxicities counts for the 6-18% of the total cost of treatment in human trials (34). Hence, the need to test new combination strategies. In addition, the use of new strategies would: (i) expand the knowledge of the current possibilities, (ii) enable to find new treatment combinations that would be effective in a broader cancer typology and (iii) give a stronger proof of concept to our findings concerning the potentials of DNA vaccination against cancer. Indeed, from our first combination study, between DNA vaccination and ICBs, we understood that the DNA vaccine limitation does not concern its ability to activate the immune response but could be mainly the inability of the primed T cells to reach the TME. Hence, we hypothesized that an agent that can recruit the activated T cells in the TME could be a valuable partner for the DNA vaccination. This idea inspired the combination of a DNA vaccine against melanoma with an Oncolytic Adenovirus (OAd). In the University of Helsinki, professor Cerullo's team already developed an OAd virus whose genome was enriched with CpG motifs, to increase its immunogenicity (35). We chose to work with the B16F1 melanoma tumor model, to test the

applicability of the DNA vaccination in a more clinically relevant tumor model, compared to the mastocytoma. Furthermore, the ability of B16F1 cells to form well-defined subcutaneous tumors allows an easy tumor growth monitoring and an accessible virus administration, to test the T cell recruitment in the TME after the intratumoral (IT) injection of the virus. To this aim, we used the four plasmids, previously constructed against B16F1 (section VII.1 and chapter V), to deliver a poly-epitope DNA vaccine encoding melanoma TAAs and neoantigens. The results confirmed our hypothesis: (i) The DNA vaccine alone can generate systemic antigen-specific T cells; (ii) antigen-specific T cells migrate into the TME when the vaccine is combined with OAd, which is administered in the tumor; (iii) these combined effects drastically decrease the tumor growth rate, thus prolonging the median survival time.

OVs have already been used in the immunotherapy of melanoma (e.g., T-VEC is an OV approved by FDA in 2015 for advanced melanoma), with great success, and are also known to activate the innate immunity if injected locally into the tumor (36, 37). Furthermore, OVs could be potentially used in several cancer types, as they exploit tumor-specific aberrations that predispose cancer cells to virus infection (38). However, despite the various combination of OVs with ICB, before us, nobody tried to combine them with the DNA vaccination (39). Their association with cancer DNA vaccines is benefic not only to enhance the immunogenicity of the vaccine but also to overcome the problem of the immune activation against the OV itself. Indeed, the use of the vaccine addresses this problem, by specifically expanding TA-specific T cells. Hence, our work expands the current knowledge in the field of both DNA vaccines and OVs and explores the mechanisms of interactions between these two therapies.

To further support our hypothesis, we worked with the GL261 GBM model, as a prototype of an immunologically "cold" tumor. We hypothesized that the resection made just after the vaccination could recruit the activated immune cells in the TME, because of the inflammation generated by the surgery (40). Concomitantly, the resection can contribute to the global therapeutic efficacy, by slowing down the tumor proliferation, due to the removal of the tumor bulk, and by disrupting the BBB to facilitate the T cell arrival. In this study, the most remarkable effect of the combination was a significant reduction of the immune suppressive cells, such as MDSCs, Tregs and M2 TAMs, but also an increased proportion of specific and active T cells in the TME. In agreement with our previous studies, the DNA vaccine induced the activation of a systemic immunity, without improving the survival; when the resection was used in combination to the vaccine, the survival was significantly higher (Chapter VI). Currently, tumor resection is part of the standard of care for operable patients; however, only few clinical trials are testing the DNA vaccination in GBM patients and none tries to first vaccinate and then operate the patients. We strongly believe that

vaccinating the patients at the first clinical manifestations of the tumor (when it is visible at MRI) and just before the resection could lead to a better outcome.

I.3. THE TRANSLATABILITY TO DIFFERENT PRECLINICAL MODELS

The long journey of this thesis allowed us to explore different preclinical models to strongly validate our findings. At the beginning of my thesis, a non-optimized DNA vaccine encoding the mastocytoma TAA P815 was being tested in our lab in a prophylactic setting. However, only 10% of mice survived to the challenge and the tumor growth was barely delayed, leaving room for improvement (41). The DNA vaccine improvements in an immunologically well-known preclinical model, such as mastocytoma, with defined TAAs that mimic the function of the MAGE-type tumor antigens, provided the proof of concept for my thesis. Indeed, the work with the mastocytoma underlined: (i) the possibility to improve the DNA vaccine efficacy, by optimizing the vaccine itself; (ii) the weaknesses of the DNA vaccination and the possibility to overcome these limitations by combining it with other therapies. The validation of this research hypothesis was performed in more clinically relevant tumors, the melanoma and the GBM models. Interestingly, we demonstrated that DNA vaccination could be effective in poorly immunogenic tumors, whenever the vaccination is combined with a rational strategy.

Until now, only few DNA vaccines have been tested against B16F1 model (reviewed in the Introduction) and none of them used neoantigens. The studies using only TAAs in B16F1 failed to decrease the tumor growth in a therapeutic setting, because of the high aggressiveness and poor immunogenicity of this tumor. Indeed, untreated mice die in ~ 2 weeks. For this reason, the vaccination has been performed only 2 days after the tumor injection, as we did for the mastocytoma model. Despite the "non-strictly clinical" therapeutic schedule (the vaccine was administered when the tumor was barely visible as a small black spot), the fact that our vaccine could slowdown the tumor growth is already a great success in this model and demonstrates that the optimization of the DNA vaccine could be benefic even against very aggressive tumors.

In the GBM model, when the GL261 were injected subcutaneously, the therapeutic protocol was the same as for the other two models. In this case, the slower tumor growth of GL261 cells (untreated mice die after 30 days) compared to the melanoma and mastocytoma, allowed the TAA-encoding DNA vaccine to cure 6/7 mice, without any combination therapy. To explain this effect, we hypothesized: (i) a relation between the slower mutation rate of the GBM model, compared to the melanoma, and a higher DNA vaccine efficacy; (ii) when the tumor growth is slow enough, the vaccine alone could have the time to mount an effective immune response, because the tumor is

still at his infancy and has not yet created a highly immunosuppressive microenvironment. On the contrary, when the tumor growth is too fast, the vaccine does not have the time to induce a strong immune response. In this case, the combination therapy is necessary to both slowdown the tumor growth and drive the immune cells in the immunosuppressive TME. However, when the GL261 tumor was implanted into the brain, the vaccine alone was not sufficient to cure the mice, despite the activation of the systemic immunity. This is because the brain is difficulty reachable by the T cells. Hence, the combination of DNA vaccination with tumor resection not only delayed the tumor growth and drove the T cells towards the tumor, but also facilitated the penetration of active immune cells in the TME. This combination also improved the outcome of the resection, which was ineffective when used as single therapy. In the orthotopic GBM model, the therapeutic protocol tried to imitate the situation in the clinic. Indeed, in this case, we first verified the presence of the tumor at the MRI (day 10 after tumor implantation) and, then, we vaccinated the mice (the vaccine priming was administered 16 days after the tumor inoculation). Even the combination approach was conceived thinking to the possible future clinical applicability, as the resection is still an important part of the standard of care for resectable GBM (42).

The main research questions and results that drove this PhD thesis are schematically summarized in Figure 3A and 3B.

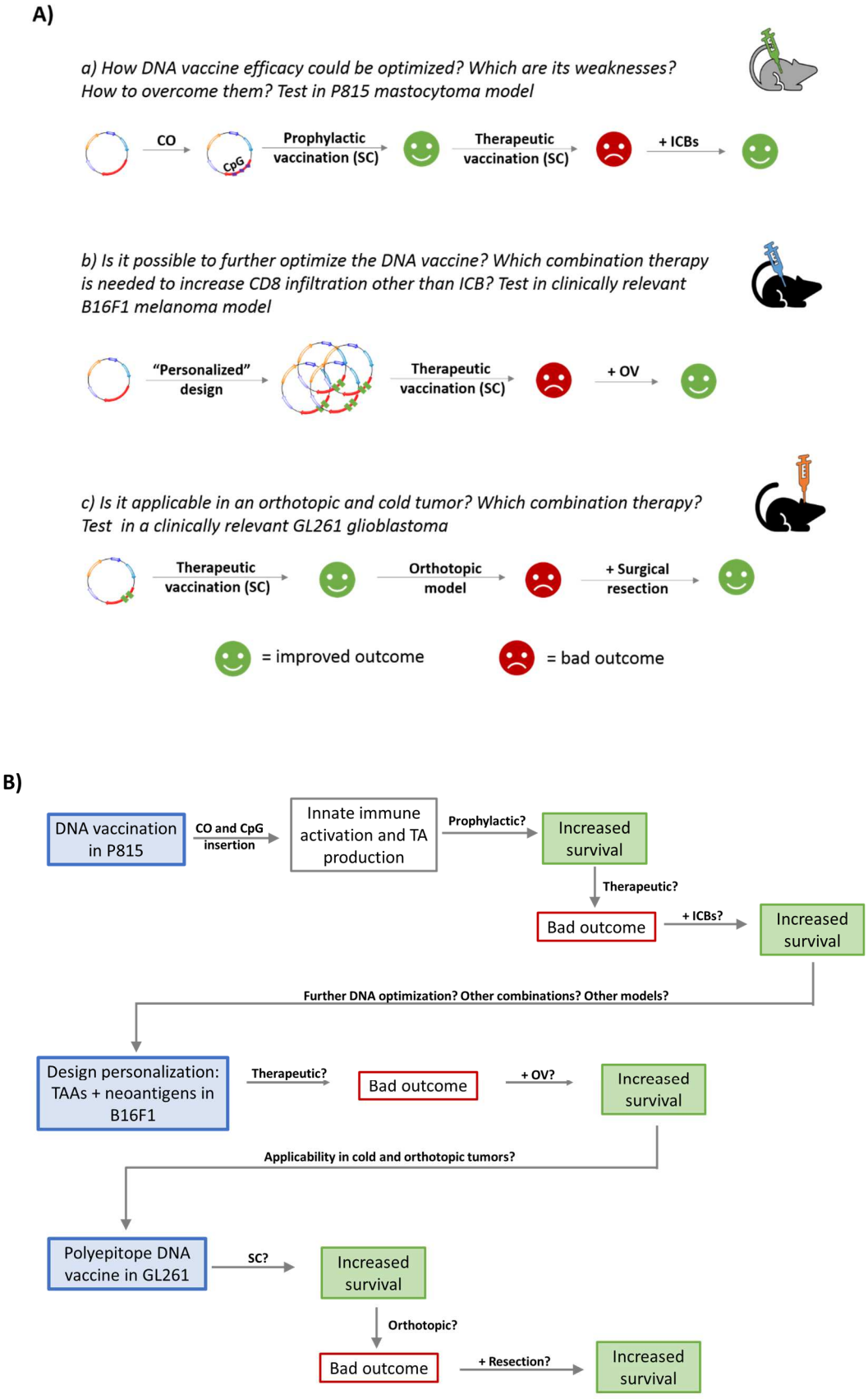

Figure 3: Research questions and answers of my PhD thesis. A) The three models used in the thesis and the main results. B) Questions that drove the PhD thesis and main outcomes. CO = codon optimization; OV = oncolytic virus; ICB = immune checkpoint blockade; SC = subcutaneous.

II. LIMITATIONS AND SHORT/MEDIUM-TERM PERSPECTIVES: WHICH ARE THE NEXT GAPS IN THE KNOWLEDGE?

My thesis demonstrated that the use of a codon-optimized, DNA vaccine encoding one or many TAs, in combination with therapies that modulate the immunosuppressive TME, seems to be a good therapeutic option against cancer. The applicability of our findings has been validated in different cancer models. However, many questions still need to be addressed and further studies are necessary to answer to these questions (Table 2).

Table 2: Limitations of this PhD thesis and ideas to overcome them

	Limitations	How to overcome the limitations	Ongoing/future experiments
Preclinical limitations	**DNA vaccines alone are not effective against fast-growing nor orthotopic tumors**	Possibility to further optimize DNA vaccines? a) Determine the TA to be encoded in the DNA vaccine and the criteria of the choice b) How many encoded TAs? In how many plasmids? Which is the antigen-insertion limit of the VSV-G system (pTOP)? c) Which is the effect of the non-coding regions TAs? d) Which would be the efficacy of DNA vaccines in another orthotopic and metastatic tumor model?	- Analysis of different criteria (mutation frequency, MHC affinity, tumor-specificity etc.) - Modification of VSV-G with as many epitopes as possible and delivery of one plasmid with all the epitopes vs many plasmids with one epitope - Design of plasmids encoding non-coding region TAs and test/analysis in mice - Test in CT26 model
	DNA vaccine administered too early	Design of more clinically relevant therapeutic schedule and combination a) Vary the administration schedule b) Toxicity management: possibility to decrease the doses of the administered therapies?	- Test of different treatment schedules to delay vaccine administration - Perform more analysis at different time points - Dose-descaling experiments

Clinical limitations	**Towards the clinic: what is missing?**	a) Toxicity and GLP condition studies b) Economical evaluation c) Design of the clinical protocol	- Administration of the GMP-produced DNA vaccine and evaluation of toxicity/DNA vaccine presence in several organs - Studies in GLP condition - Translational aspect (communication with clinicians, economists etc.)

In the following paragraphs, these limitations and question are examined, as well as the possible solutions and experiments to be conducted to answer to part of them.

II.1. HOW TO FURTHER OPTIMIZE THE DNA VACCINES?

In our therapeutic vaccination experiments, optimized DNA vaccines alone decreased the tumor growth rate and, in the case of GBM injected SC, to reject the tumors and cure a high proportion of mice. However, they failed to cure fast-growing tumors, as in the case of melanoma, or GBM when GL261 cells were inoculated orthotopically. This reflection arises a question: is it possible to further optimize the DNA vaccine efficacy to be more effective in a large spectra of tumor models and sites? To answer to this question, different critical aspects should be considered:

a) Which TA should be selected? Which criteria should be considered in the choice of the TA?

The choice of the TA to be selected into the DNA vaccine is not simple. When we look at non-mutated TAs and neoantigens, they all have advantages, but also drawbacks. Indeed, many relevant non-mutated antigens with low tumoral specificity have been identified for most tumors, but immune tolerance can limit their efficiency (43). Furthermore, in the case of differentiation antigens, such as gp100, Melan-A or TRP2, the presence of the antigens on non-tumor cells (e.g., melanocytes) could induce side effects (e.g., vitiligo) (44).

On the other hand, neoantigen identification is time consuming and expensive, and neoantigens do not reflect the tumor heterogeneity in the individual patient (e.g., in metastasis) (45, 46). Furthermore, neoantigen mutations could be passengers (47), with the risk to decrease the vaccine efficacy after a certain number of tumor cell replications. One way to address this specific point is to study the evolution of the mutations in vitro and in vivo, and not only in the main tumor site, but also in the metastases, at different time points. A time-line and multi-site study on neoantigen

mutations could help in creating databases able to predict the presence and the evolution of a specific neoantigen in a certain tumor and the influence of the immunotherapy on the mutation frequency. This can help to identify neoantigens distribution in the main tumor and metastasis and their mutation rate, but also to categorize neoantigens more suitable for therapeutic application.

The choice of the TA should also consider its affinity to the major histocompatibility complex (MHC) and their epitope specificity (CD4 vs CD8), predicted by different tools and databases (48, 49). However, the same epitope could generate a different immune response (e.g., CD4 or CD8) according to the type of vaccine platform. Recently, it has been demonstrated that the use of DNA vaccination can induce a CD8 immune response against neoantigens known to generate a CD4 response by using RNA or peptide vaccines (23). Hence, it is important to determine the epitope MHC affinity and CD4 or CD8 specificity for each antigen in the case of DNA vaccination to further investigate how the vaccine platform can modulate the immune response.

These reflections demonstrate that the TA choice should consider many conditions. Furthermore, current databases and tools that predict epitope binding and affinity lack the evaluation of other crucial factors that influence the antigen immunogenicity, e.g., the peptide processing by the proteasome, the stability of the MHC binding, the transport by the transporter protein associated with antigen processing (TAP) (50).

The main criteria to consider when selecting the TAs and the possible advantages (+) or disadvantages (-) of choosing non-mutated TAs or neoantigens, related to each criterion, are listed in Table 3:

Table 3: Main criteria to select TAs and the possible advantages (+) or disadvantages (-) of choosing non-mutated TAs or neoantigens.

Criteria to choose TAs	Non-mutated TAs	Neoantigens
Antigen identification	Easy identification (+)	Difficult identification (-)
Immunotolerance	TA-dependent (+/-), normally high (-)	None (+)
Mutation frequency	Low frequency (+)	High frequency (-)
Tumor specificity	TA-dependent (+/-)	Highly tumor-specific (+)
MHC affinity	Variable: to be determined according to the vaccine platform	

In resectable cancers, one possibility for an effective vaccination could be to administer one DNA vaccine before the tumor surgery/biopsy, encoding well-known TAs for the specific tumor. Once the tumor has been resected, the analysis of the specific mutations on the tumor biopsy would help the design of a more personalized DNA vaccine that will reflect the complexity of each tumor.

Sahin et al. already tried this approach, administering a NY-ESO-1- and/or tyrosinase RNA vaccine in melanoma patients, that were waiting the release of their personalized neoepitope vaccine (51). Their encouraging results suggest the possibility to broaden this approach to DNA vaccination.

b) How many antigens should be encoded in the same plasmid?

As already discussed, targeting multiple TAs, that can stimulate both CD4 and CD8 immune response, is a promising way towards an effective anti-tumor vaccination. However, it is important to consider that large plasmids (more than 5-6 kilobases) have a lower transfection/transcription efficacy when delivered by EP; hence, the number of antigens encoded in a single plasmid is limited (52, 53). For this reason, the use of different plasmids should be evaluated. In addition, it is still not known the number of antigens that are needed to have an effective anti-tumor immunity. In one study, it has been shown that a poly-specific and poly-functional DNA vaccine encoding 10 different neoantigens was a more efficient solution compared to a plasmid encoding only 2 neoantigens to prevent tumor growth in mice (54). However, further studies are needed to confirm these findings and to better determine the number of TA to be delivered, depending on the tumor type. The capacity of the VSV-G system to be modified with more than two epitopes is currently being explored in our lab. Hence, in the future, we might be able to answer at least to a part of these questions.

Once the number and the nature of the encoded TAs will be determined, a further question would be: is it worth encoding different non-mutated TAs and neoantigens in the same vaccine? In our hands, the administration of non-mutated TAs and neoantigens together proved a better efficacy compared to the non-mutated TAs alone and the use of many epitopes demonstrated to be the best choice in the B16F1 model (Chapter V). Indeed, non-mutated TAs and neoantigens have complementary advantages (Table 3) that could be exploited during vaccination. These findings need to be validated in other models or with other TAs.

c) Where does the new category of antigens derived from the non-coding regions stand?

The standard exome-based approaches miss the identification of TAs derived from non-coding regions (55). Most of them derive from non-mutated yet aberrantly expressed transcripts that could be shared by different tumors and patients (55). Others are generated by aberrant translation and degradation of introns (56). These TAs could potentially contribute to the development and further improvement of personalized cancer vaccines. An example of this TA category is the AH1 antigen

(SPSYVYHQF) in the CT26 colorectal cancer model, which derives from an endogenous retroviral gene product (57) and is almost not expressed in normal cells (58). Hence, the design of a DNA vaccine encoding non-coding region TAs and the evaluation of its in vivo anti-tumor efficacy would be a next area of research.

d) Which would be the efficacy of DNA vaccines in another orthotopic and metastatic tumor model?

The use of orthotopic models that can mimic the clinical situation is, probably, one the most affordable preclinical models. Indeed, orthotopic models can mimic the local TME and physio-pathological conditions of the real tumor, including the immune infiltrate and cytokine production (59, 60).

The CT26 model is a syngeneic colorectal model in Balb/c mice that can offer different advantages compared to the other models that we used until now. From an immunological point of view, the CT26 colorectal model is considered "warmer" than B16 melanoma and GL261 glioblastoma, according to its immune infiltration and the presence of co-stimulatory markers (61-63), even if, in human studies, the colorectal cancer model is situated between the melanoma and the glioblastoma in term of the mutational landscape (64). Some authors have already detected immunogenic neoantigen mutations in the CT26 model (19). Hence, we could exploit this model to design "personalized" vaccines, once the neoantigens mutations are detected. Contrarily to the GBM model, the CT26 is a metastatic model (65) that would allow us to study the potential differences in neoantigen expression in the metastasis vs the main tumor in vivo, as previously discussed. Furthermore, experiments in SC models that compared the tumor growth rate of CT26 and B16F10 showed that CT26 tumor growth was slightly slower compared to the B16F10 (66), which could be an advantage for DNA vaccination. Recently, our lab researchers successfully implanted the CT26 orthotopically, showing the feasibility of this process. For these reasons, we decided to follow-up our studies in this model. We have already cloned some DNA vaccines encoding CT26 non-mutated TAs and neoantigens. In the future, these vaccines will be tested in mice bearing an orthotopic CT26 tumor to determine the type of generated immune response for each antigen (CD4 or CD8), the follow-up of the mutations in vivo in the main tumor site and in the metastases and the immune infiltration at different time points. Furthermore, we will determine the maximum number of antigens that can be inserted in the pTOP system, without affecting its efficacy, and compare if it is better to deliver one plasmid encoding many TAs or many plasmids with few TAs (number of plasmid/encoded antigen ratio). We are convinced that this model could help us to answer to some of the limitations of this PhD thesis. In Figure 4, the reasons and advantages of

the tumor model choice and the short-term perspectives of our work in the CT26 model are illustrated.

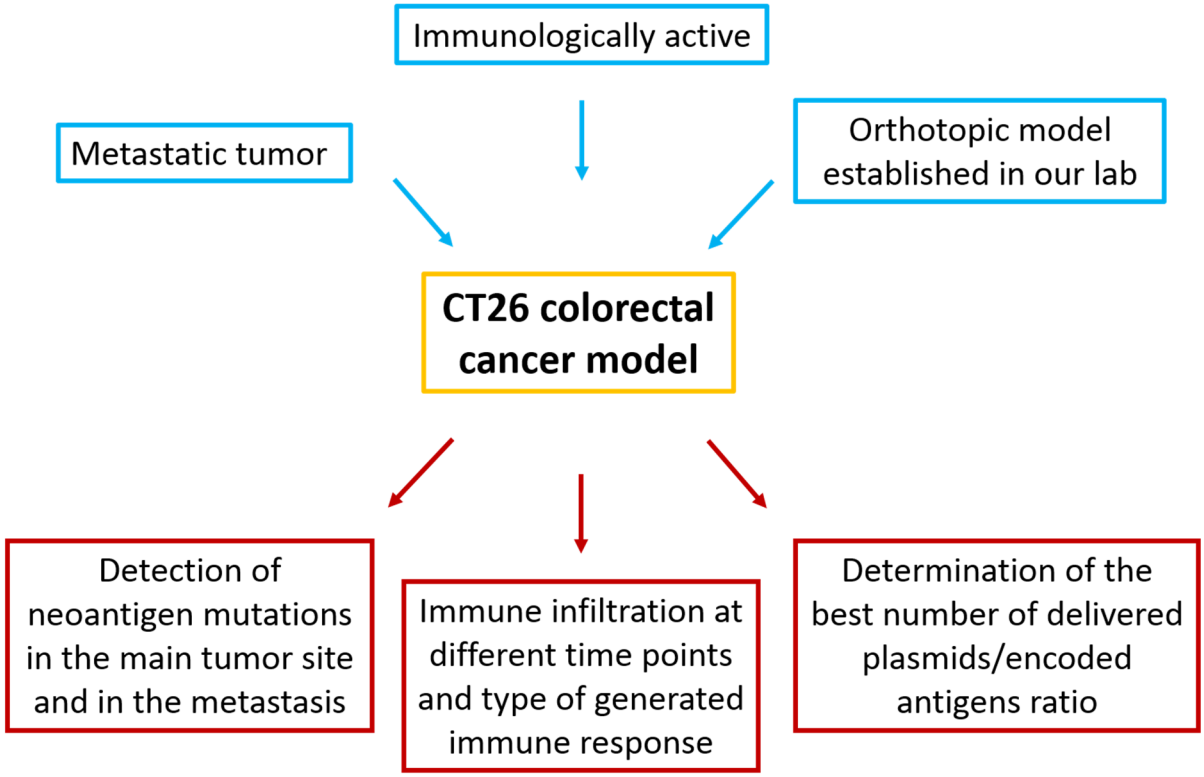

Figure 4: In blue, the reasons and advantages of the CT26 tumor model choice; in red the main research question that this model will answer.

II.2. VACCINE TREATMENT SCHEDULE: HOW TO OPTIMIZE THE COMBINATION STRATEGIES?

In our experiments in SC models, the DNA vaccine has been administered when the tumor was not visible or barely visible. This does not correspond to the real situation in the clinical trials, as the DNA vaccines are always administered as a last-line therapy, once all the others have failed. On the opposite side, a short-term evaluation of preventive vaccination in the clinic is challenging.

To facilitate the translation of the proof-of-concept in DNA vaccination from the murine model to the humans, our vaccines should be administered once the tumor is visible. Of course, this should consider the limits of the translatability from the preclinic to the clinic, as the faster tumor growth rate of murine cancers does not allow us to administer the vaccine too late. Hence, the use of a less fast-growing tumor would be a good starting model. Our experience with the GBM orthotopic model indicates that a later administration is possible, when the vaccine is rationally combined with the tumor resection. Indeed, the combined therapy allowed to slow-down the tumor growth and gave the time to the vaccine to activate the immunity. For these reasons, the

combination therapy and the tumor model should be carefully selected, and the vaccine should be immunogenic enough. The tumor type, its localization and growing rate determine the type of combinatorial therapy to be associated with the DNA vaccines.

These reflections arouse different questions: when is it better to administer the vaccine? Which type of combination therapy and schedule to choose? The answers should consider the time for the immune system to generate a specific immune response against the delivered antigen, the rate of the tumor growth, the need for multiple doses of administration and the interaction with the combined therapy. Furthermore, the doses should be consequently adapted to the combination regimen. In our experiments, the time of vaccine administration was the same for all the SC models, and the schedule of the combined therapy was carefully selected according to the literature or to the past experiences of our co-workers (e.g., in the case of OAd), leading to good results. However, if we want to administer the vaccine later, we need to have a stronger vaccine (as previously discussed) and we should understand when it is better to supply the other therapy. This implies the necessity to test different time schedules, perform more immunological analyses at different time points etc., to evaluate the influence of the schedule treatment on the global immune response.

When different therapies are used in combination, the toxicity might increase; therefore, the necessity of recognizing and managing adverse events will be critical for the treatment success. If the combination therapy is adequate, it would be possible to vary (decrease) the doses of the single therapy to decrease or better manage the related toxicities. Hence, different dose-descaling experiments should be performed.

From a clinical perspective, some studies in melanoma patients revealed that using ICB after the peptide/RNA vaccine treatment induced a sustained remission with no sign of disease recurrence during the observation period (51). In addition, it seems that checkpoint expression might change in a time-dependent way after the vaccine administration (39) and that the ideal timing for the combination should be evaluated for the specific therapy and patient. However, the clinical situation is complicated by the administration of therapies that could negatively influence the DNA vaccination. For instance, the use of dexamethasone in GBM patients, for the cerebral edema management, decreased the efficacy of concomitant immunotherapies, because of the corticosteroid-associated immune suppression (67).

II.3. TOWARDS THE CLINIC: WHAT IS MISSING?

In my PhD thesis, the efficacy of optimized DNA vaccines in combination with other therapies has been demonstrated in different murine models. Our exciting findings show the potential applicability of this tool in the clinic. However, before reaching the clinic, some aspects need to be

evaluated, starting from the toxicity studies in animals, going to an economical evaluation of the costs-benefits associated to the product, until the design of an adequate clinical protocol. In the following paragraphs, these aspects are discussed.

a) Toxicity studies

The translatability from the preclinic to the clinic could encounter some difficulties. One of the main barriers is the differences in the cancer oncoimmunology from animals to humans (68) and the assessment of the potential toxicity associated to the DNA vaccination. It is known that the rate of the tumor growth between mice and humans is different and it is impossible to predict the effect of our vaccines face to the human immune system. To try to overcome this problem, many genetically engineered mice, xenograph and humanized models have been developed. However, they all fail to recapitulate the chaotic way in which malignant transformation occurs during cancer development in human patients. Mouse models provide valuable insight into the mechanisms of action and provide important proof of concept for human studies, but there remains a need for larger animal models encompassing a fully competent immune system. Some researchers suggest the use of canine and porcine models, especially for skin cancers (68). However, housing, ethical regulation, and breeding difficulties limit the use of big animal models. Furthermore, even these models have limitations, and the idea of a universal model for oncoimmunology currently seems unrealistic (1). Hence, most of the critical points aroused in cancer DNA vaccination can ultimately be addressed only in clinical practice.

Before reaching the clinic, one of the main criteria that are required by the authorities is that the formulation is safe and well-tolerated. Generally, in clinical trials, DNA vaccines have already demonstrated a high safety profile (69). Furthermore, the single therapies (and the combination with ICB) that we tested in mice have been already tested in humans. However, we cannot forecast the possible side effects to the other combination therapies. In our models, mice did not present any side effect or any abnormal behavior, ascribable to the vaccine. Some studies are already being conducted in our lab to test the toxicity of our vaccines in different organs in mice at different time points and no relevant problems have been detected, until now. Before reaching the clinic, DNA vaccines should be produced in good manufacturing practice (GMP) conditions. Furthermore, toxicity studies should be repeated in good laboratory practices (GLP) conditions to ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of these non-clinical tests, from acute to chronic toxicity, especially to ensure a non-permanence of the vaccine in the gonads, and preclinical efficacy.

b) The economical aspect

Conducting a clinical trial is a very expensive procedure and an economical evaluation concerning the price of the drug development is fundamental at this stage. A great advantage of DNA vaccines is their high cost-effectiveness and the low costs associated to their manufacture. A veterinary study showed that, among the treatment options for canine oral melanoma, DNA vaccination was the therapy with the best cost-benefit ratio (70). However, the costs of a clinical trial include also other aspects. According to Sertkaya et al., the top three cost-drivers are related to the administrative staff costs, clinical procedure costs and site monitoring costs (71). The analysis of the costs depends also on the clinical phase, the therapeutic area, the number of enrolled patients etc. Hence, an economical evaluation and the research for funding is not a secondary aspect towards the clinic.

c) Design of the clinical protocol

As for the preclinical studies, a clinical trial should answer to specific research questions, following a precise study plan or protocol. For instance, the aim of the clinical phase I is to assess the safety and the dosage of the drug to proceed to the further studies. Once the objectives of the study are determined, it is necessary to decide: (i) The type of patients that will participate to the trial. This criterion includes the age, the sex, the pathology (in our case, the type of cancer) etc. In particular, the type of cancer is crucial to be determined and can influence many other criteria. The rarity of the pathology can influence the recruitment of the patients. Furthermore, the aggressiveness of the cancer determines the administration schedule and the concomitant therapies administered to the patient. (ii) The number of patients to be recruited. In a phase I this number is around 20-100 participants. However, it is becoming more and more difficult to recruit an adequate number of patients. This is due to many barriers, such as the low patient awareness about the existence of a clinical trial, public perception, complex protocols and procedures, competition with other clinical trials, etc. (72). Many strategies and advices from healthcare authorities are trying to overcome this problem (72). Furthermore, cancer vaccine personalization is already prohibitive for conducting large clinical trials, due to the specificity of each vaccine. (iii) The duration of the study. This depends from the type of cancer, the duration of the treatment, the time needed to have a response, the phase study. For a phase I, an answer is expected after some months. This aspect can have an impact on the design of the study. For instance, the application of DNA vaccination in a prophylactic model would require an undetermined time to have a feedback about the outcome; this makes difficult its applicability in clinical trials. (iv) The administration of the drug. We already know that the most effective way to deliver DNA vaccine is the electroporation. This technique

has been already validated in many studies on human patients and resulted to be safe and well-tolerated (73). However, the determination of the type of electrodes, the injection site and electrical parameters need to be adjusted to the clinical situation. (v) The dosage and administration schedule of the drug. As already discussed, the typical DNA vaccine dosage in clinical practice is around few hundred μg to some mg; the determination of an exact dose is required before starting a clinical trial. It could also be possible to envisage dose-escalation studies, if the number of enrolled patients is high enough. The administration schedule should consider the concomitant treatments and their influence on the efficacy of the tested vaccine, but also the stage of the disease. (vi) Determination of the type of data that should be collected and the type of data analysis. Before starting a clinical trial, it is necessary to determine the information that needs to be collected. We should also think in advance to the time point and the type of analysis, e.g., if blood collection or a tumor biopsy is necessary. This parameter should be carefully discussed with clinicians and patients, as it can influence the patient enrollment, the costs and the results. For the data analysis, the expertise of a statistician would be the best solution, as the data management from clinical trials is huge.

All these aspects underline the difficulty of the translation from the preclinical studies to the clinical trials. The translatability aspect should be considered in each step, as well as the need of collaborating with expert people, such as economists, clinicians, other researchers etc., to ensure the best quality to the clinical study.

III. PERSONAL OPINION ON THE LONG-TERM PERSPECTIVES IN CANCER DNA VACCINATION

Cancer is an evolving entity and we should look at it as a four-phase process: Elimination, Equilibrium, Escape and Evolution (4E model). Indeed, in the evolution phase, cancer acquires a different antigen landscape, inflammatory degree, antigen loss and mutations (74). Currently, DNA vaccines are not used as first line treatments in the clinical trials; hence, cancer is already in the evolution phase and there is a possibility that even optimized DNA vaccines could have a lower efficacy than expected. The way we currently look at vaccination and at its combination with other therapies should change, regarding two main aspects: (i) the personalized therapy due cancer heterogeneity: we need to find biomarkers to provide to the right patient the right therapy. However, the treatment personalization carries other problems, from the difficult to design large clinical trials to the economic impact for the industries and the society; (ii) the preventive vaccination, i.e., the possibility to vaccinate people either before the first clinical symptoms or at the first visible premalignant lesions.

III.1. THE VARIABILITY PROBLEM: THE NECESSITY OF BIOMARKERS; WHICH IS THE ECONOMIC IMPACT? HOW FAR FROM THERAPY PERSONALIZATION?

Cancer is a heterogeneous disease not only between patients, but also in the same patient. This happens because this disease mutates on its own as well as under selective treatment pressure and is able to evade the host immune system. This was the main reason of failures of the many late stage and costly clinical trials using cancer vaccines (75). Available immunotherapies, such as ICBs, have a variable response rate from one patient to another and are often associated with side effects. Furthermore, these drugs are often very expensive, and this raises concerns about a sustainable use of the public economic resources (76). A cost utility analysis is routinely performed, including for immunotherapeutic drugs, to consider the advantages in term of costs, overall survival and quality of life between two treatments and presents the outcome as an incremental cost per quality adjusted life year (QALY) (76, 77). Furthermore, the cost-effectiveness of a drug is not necessarily translated in the affordability and long-term sustainability of the drug (76). The economical aspect was the only reason of the withdrawal of the DC vaccine Provenge (Sipuleucel-T) in Europe (78). Hence, in an era of clearly limited resources and rising health care costs, the debate over the value of immunotherapeutics in oncology is not a secondary aspect and should be carefully considered. For these reasons, patients that could benefit from a certain therapy (responders) should be wisely selected. Therefore, it is important to identify biomarkers that could predict the patient response

to a specific therapy and to standardize the therapy according to the predicted biomarker. Furthermore, biomarkers may be useful for monitoring treatment response, predict risks, prognosis etc. and to understand the reason of the non-responsiveness of some patients to a certain therapy. In addition, there is a need to evaluate the potential cost-benefits of the combined therapy, e.g., vaccines can increase the number of ICB responders and vice versa (28, 79-81). However, the translation of biological data into predictive or prognostic biomarkers is complicated by the intricate interactions between tumors and the immune system and by host and tumor variability. Many studies are using bioinformatics tools and new genomic and proteomic technologies to predict specific tumor signatures, generating complex datasets that give rise to analytical challenges. Currently, we can rely on imperfect biomarkers, such as PD-L1 expression in the tumor, the immune cell infiltration, the expression of neoantigens or the TMB (Figure 5). This gap in knowledge leaves room for further studies that will help treatment selection and design the best combination therapy for each patient.

In response to these needs, many biobanks are growing to collect and analyze human samples to find more information about the relation biomarkers-response to immunotherapy (82). In this context, the data and material sharing between academies, industries and hospitals will become crucial. In addition, the design of clinical trials is evolving to consider small cohorts of patients with specific biomarkers (patient stratification) due to tumor heterogeneity and the personalized therapy (83). Recently, a phase I trial integrated highly individualized peptide vaccination with non-mutated TAs and neoantigens into standard care for patients with newly diagnosed GBM. Fifteen patients were treated with two vaccine, one targeting non-mutated TAs and the other targeting neoepitopes. Personalization was based on mutations and analyses of the transcriptomes and immunopeptidomes of the individual resected tumors. The vaccine was given after the surgery, 15 days after the beginning of the TMZ therapy. This approach was feasible in term of manufacturing time and treatment, and vaccines displayed favorable safety and strong immunogenicity (84). Hence, in the future, patients with GBM could be treated with a vaccine encoding well-known, non-mutated TAs before the resection and with a more personalized vaccine after the resection, to avoid recurrences.

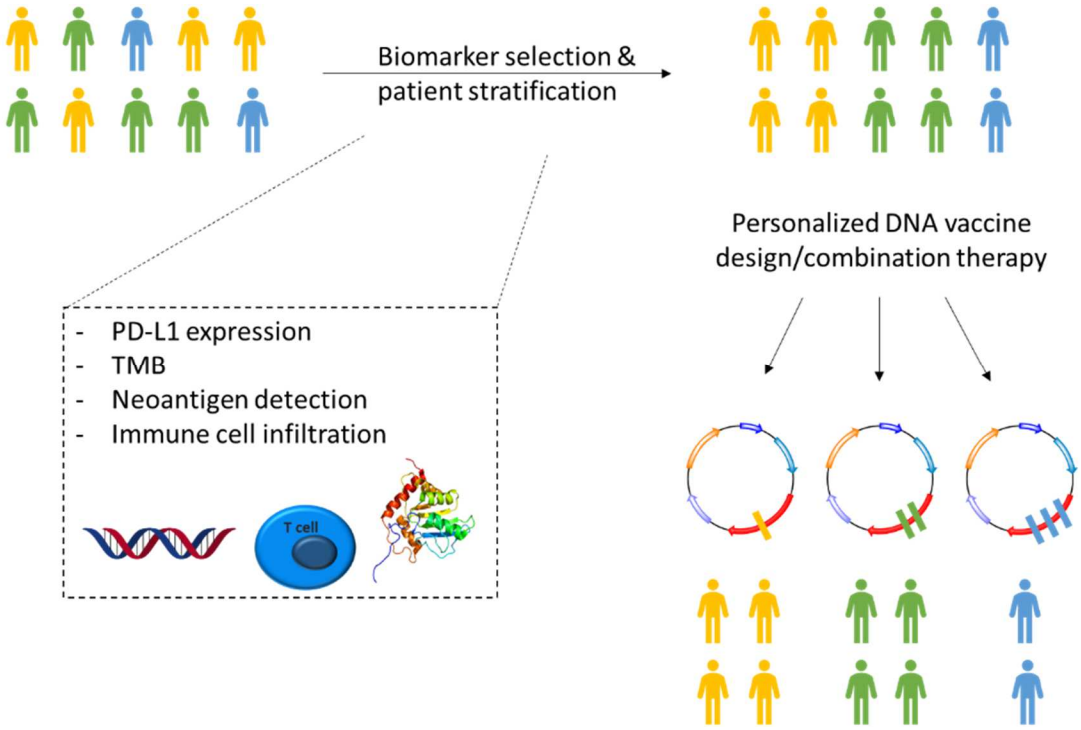

Figure 5: The therapy personalization: from patient stratification to the personalized DNA vaccine design. TMB = tumor mutational burden.

III.2. CANCER IMMUNE PREVENTION AND PROPHYLACTIC VACCINES: AN UTOPIC HOPE OR A FUTURE REALITY?

For many years, the definition of vaccination was a treatment that helps to prevent diseases. In the context of cancer, this concept was applied only in virus-derived cancers, e.g., HPV vaccine, which reduced the incidence and mortality of cervical cancer diseases in women (85). In these cases, the target of the vaccine is the virus; thus, these vaccines are effective due to their ability to prevent viral infections, thereby eliminating their oncogenic potential. Ideally, prophylactic vaccination should target antigens specific to cancer cells and essential for tumor survival in a selected high-risk population (83, 86).

The difficulty to create a prophylactic cancer vaccine for a true primary cancer prevention (in healthy individuals) is the inability to forecast the cell mutations that will evolve to cancer and the absence of early predictive biomarkers. Furthermore, the evaluation of the clinical efficacy in the short-term would be challenging, due to the absence of the tumor in a prophylactic approach.

A more feasible approach would be an early-phase vaccination (secondary prevention on pre-symptomatic disease), to start the therapy in a more effective time frame (87). A solution could be the discovery of biomarkers in pre-malignant lesions or on early cancers and a careful section of

the patient population (83). One recent clinical trial used a peptide vaccine (MUC1 peptide) in patients without cancer but with a history of colonic adenoma premalignant lesions. The production of antigen-specific antibodies and a long-lasting immunity were observed in half of the patients (88). The authors also showed the presence of MDSCs in some individuals even in a premalignant stage, which could be a possible biomarker for patient selection in clinical trials (88). A good strategy could be to target specific mutations involved in the oncogenesis and in the malignant transformation at the earliest genetic changes (e.g., early-phase Kras mutation was found in 90% of premalignant pancreatic intraepithelial neoplasm) (89). The use of genetic mice carrying this type of mutations and spontaneously developing tumors already provided a good proof-of-concept for prophylactic vaccination (87). However, a first requirement for preventive cancer vaccines will be a high safety profile and cheap costs. Under these perspectives, optimized cancer DNA vaccines might be employed as a first choice in the future of cancer immunotherapy.

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