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"Novel adenoviral vaccine encoding multiple tumor neo-antigens in combination with checkpoint blockade as a strategy for more effective cancer treatment"

Tutor

Prof. Alfredo Nicosia

Candidate

Maria De Lucia

Co-Tutors

Dr. Elisa Scarselli
Dr. Anna Morena D'Alise

COORDINATOR

Prof. Vittorio Enrico
Avvedimento

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ABSTRACT

Cancer is a genetic disease initiated by somatic mutations that activate oncogenic drivers or inactivate onco-suppressor brakes. This genetic diversity from non-cancer cells can further increase, considering that tumor development is accompanied by the accumulation of further mutations that strengthen the divergence from a normal cell, making tumor cells more recognizable as foreign by the immune system. It has been demonstrated that the mutational burden of tumors contributes to immune recognition of cancer and it may influence response to cancer immunotherapy, such as immune checkpoints blockade.

Neo-antigens are an important class of immunogenic tumor antigens and represent a promising target for vaccine therapy, having the potential to induce more robust and specific anti-cancer T cell responses than classical tumor-associated self-antigens. To exploit their immune potentiality for cancer immunotherapy, we developed a novel vaccine platform based on adenoviral vectors encoding multiple neo-antigens *in tandem*.

Starting from the mutanome of the murine colon carcinoma cell line CT26, we selected thirty-one neo-antigens, identified as the more confident neo-antigen candidates, according to the applied prediction pipeline.

Selected neo-antigens were inserted into a large adenovirus multi antigenic vaccine (GAd-CT26-31) and tested *in vivo* for immunogenicity and efficacy. Amongst the thirty-one neo-antigens, three were previously identified by others and tested in the context of an RNA-pentatope vaccine. Thus, a smaller GAd construct (GAd -CT26-5) was also generated for comparison with GAd-CT26-31. Immunogenicity testing in naïve mice resulted in eight immunogenic neo-antigens out of thirty-one analyzed. GAd-CT26-31 induced specific T cell immunity with improved potency and breadth than GAd-CT26-5. Both vaccines provided 100% tumor protection in prophylactic setting. In therapeutic setting, GAd-CT26-31 vaccine showed superior efficacy than smaller GAd-CT26-5 in combination with antibody-mediated blockade of the co-inhibitory receptor PD-1 (αPD-1), which was poorly effective to cure established tumors. A deep look at the immunological signature of complete responders to the combined therapy suggested that the more effective outcome of the vaccine correlated with increased potency and breadth of T cell responses induced by αPD-1.

ABBREVIATIONS

AAV	Adeno-Associated Virus
Ab	Antibody
Ad	Adeno
ADR	Adverse drug reaction
APC	Antigen presenting cell
B2M	beta-2 microglobulin
Bcl-2	B-cell lymphoma 2
BTLA	B and T lymphocyte attenuator
CD	Cluster of differentiation
CDK4	Cyclin-dependent kinase 4
CEA	Carcinoembryonic antigen
CIN	Cervical Intra-epithelial neoplasia
CT	Cancer/Testis
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
ELISpot	Enzyme-linked immunospot assay
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
FOXP3	Forkhead box P3
GAd	Great Apes-derived Adenovector
HA	Human influenza hemagglutinin
HDAC	Histone deacetylase
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen

ICB	Immune checkpoint blockade
ICS	Intracellular staining
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INK4	Inhibitors of CDK4
ITIM	Immunoreceptor tyrosine-based inhibition motif
LAG3	Lymphocyte-activation gene 3
MAGE-A1	Melanoma-associated antigen 1
MART-1	Melanoma-associated antigen recognized by T cells
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MPS	Massively parallel sequencing
NGS	Next generation sequencing
NK	Natural killer
NKG2D	Natural-killer group 2, member D
NK-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural killer T cells
ORF	Open reading frame
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1/2	Programmed death-ligand 1/2
PGE2	Prostaglandin E2
PSA	Prostate-Specific Antigen
SFC	Spot forming cells

SNV	Single nucleotide variant
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Teff	Effector T cell
Tex	Exhausted T cell
TGF	Transforming growth factor
Th	T helper cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumor-infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin-containing protein 3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VIN	Vulvar intraepithelial neoplasia
VISTA	V-domain Ig suppressor of T cell activation
Vp	Viral particle
VV	Vaccinia virus
WB	Western Blot

1. INTRODUCTION

1.1 Tumor immunology: from immuno-surveillance to tumor escape

Understanding how the immune system affects cancer development and progression has been one of the most challenging questions in immunology.In the midpoint of the twentieth century, the concept that the immune system can recognize and destroy nascent transformed cells was embodied for the first time in the 'cancer immunesurveillance' hypothesis of Burnet and Thomas.They both speculated that lymphocytes acted as sentinels in recognizing and eliminating continuously arising, nascent transformed cells. Because of the absence of strong experimental evidence supporting the concept (not enough was understood about mouse models of immunodeficiency), this hypothesis was abandoned shortly afterwards. The concept was resurrected nearly three decades later when new data clearly showed the existence of cancer immunosurveillance and also indicated it as a component of a more general process called 'cancer immunoediting'(Dunn et al., 2002). In cancer development, the immune system plays a dual role: on the one end it suppresses tumor growth by destroying cancer cells or by inhibiting their outgrowth, on the other end it also promotes tumor progression either by selecting for tumor cells that are more fit to survive in an immunocompetent host or by establishing conditions within the tumor microenvironment that facilitate tumor outgrowth (a phenomenon called 'immunoediting'). This conceptual framework integrates the immune system's dual host-protective and tumor-promoting roles.
During cancer immunoediting, the host immune system shapes tumor fate in three phases, "elimination", "equilibrium" and "escape", through the activation of innate and adaptive immune mechanisms (Figure 1).

The elimination phase is best described as an updated version of cancer immunosurveillance, in which the innate and adaptive immune systems work together to detect the presence of a developing tumor and destroy it before it becomes clinically apparent.
Initiation of the antitumor immune response occurs when cells of the innate immune system become alerted to the presence of a growing tumor, at least in part owing to the local tissue disruption that occurs because of the stromal remodeling processes (Gopal, 2015). The consequent production of pro-inflammatory molecules, together with chemokines that may be produced by the tumor cells themselves, leads to the recruitment of cells of the innate immune system (NKT, NK, γδ T cells, macrophages and dendritic cells). Infiltrating lymphocytes such as NKT, NK or γδ T cells recognize some structures on the transformed cells and are then stimulated to produce IFN-γ.

Tumor cells also express stress-induced molecules such as surface calreticulin, tumor antigens in context of MHC class I molecules, and/or NKG2D ligands recognized by $CD8^+$ effector cells and NK cells, respectively.

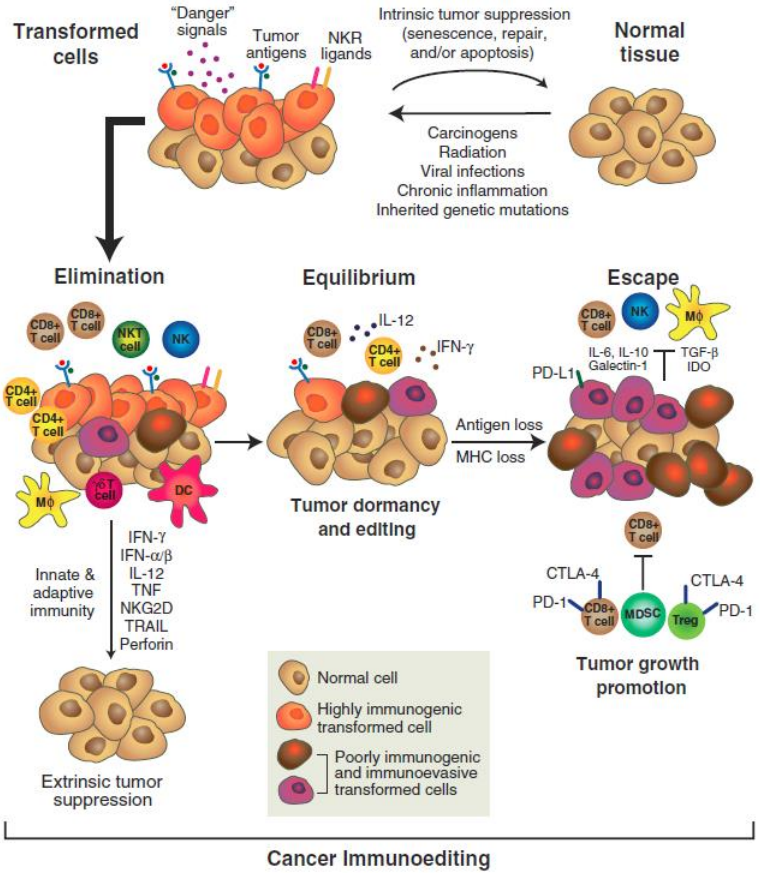

Figure 1. The three Es of cancer immunoediting (from Schreiber et al., 2011).
In its most complex form, cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immune response work in synergy to eliminate the rising transformed cells. The equilibrium phase provides evidence for a tumor-sculpting role of immunity. In this phase the immune system iteratively selects and/or promotes the generation of immune evasive tumor cell variants. When the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host the phase of escape is accomplished.

The initial production of IFN-γ initiates a cascade of innate immune reactions leading to some tumor cell death by both immunologic and non immunologic

mechanisms. The INF-γ initially produced may induce a limited amount of tumor death by means of antiproliferative and apoptotic mechanisms. However, it also induces the production of some chemokines from the tumor cells themselves as well as from surrounding normal host tissues. Some of these chemokines have potent angiostatic capacities and thus they block the formation of new blood vessels within the tumor, enhancing tumor cell death. Furthermore, the transactivation of tumor-infiltrating NK cells and macrophages by reciprocal production of IFN- γ and IL-12 contributes to tumor cell death by mechanisms involving TRAIL, perforin and reactive oxygen and nitrogen intermediates.

From dead tumor cells, a source of tumor antigens becomes available to the immature dendritic cells (DCs) recruited to the tumor site. These antigens might reflect one or more of the many mutated proteins that are typical of cancer (neo-antigens) or the products of non-mutated genes that are preferentially expressed by cancer cells (tumor associated antigens).
The activated, antigen-bearing mature DCs then migrate to the draining lymphnode, where they induce the activation of naïve tumor-specific Th1 $CD4^+$ T cells. Th1 cells facilitate the development of tumor-specific $CD8^+$ CTL induced via cross-presentation of antigenic tumor peptides on DC MHC class I molecules. Tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site, where the cytolytic T lymphocytes destroy the remaining antigen-bearing tumor cells whose immunogenicity have been enhanced by exposure to locally produced IFN- γ (Figure 2).

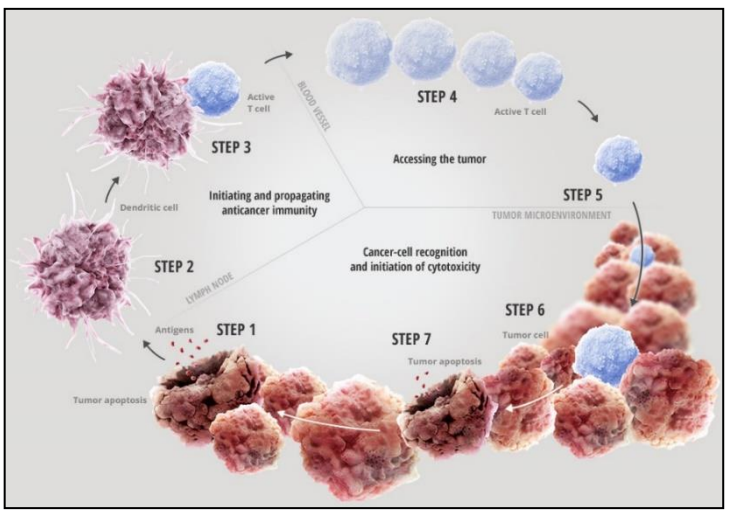

Figure 2. Cancer-immunity cycle.

The cancer-immunity cycle is a multistep process that involves (1) release of cancer cell antigens; (2) cancer antigen presentation; (3) priming and activation; (4) trafficking of T cells to the tumor; (5) infiltration of T cells into the tumor site; (6) recognition of cancer cells by T cells; (7) killing of cancer target cells ***(Chen and Mellman, 2013)***.

When the elimination phase is successful, it represents the complete editing process without progression to the subsequent phases.
If, however, rare tumor cell variants are not destroyed, they may survive the elimination phase and enter the equilibrium phase, in which the adaptive immune system prevents tumor cell outgrowth and also sculpts the immunogenicity of the tumor cells.
In this process, lymphocytes and IFN- γ exert potent selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed containing many genetically unstable and rapidly mutating tumor cells. During this period of Darwinian selection, many of the original escape variants of the tumor cells are destroyed, but new variants arise carrying different mutations that provide them with increased resistance to immune attack. So, in the equilibrium phase, occult tumor cells not destroyed in the elimination phase are held in a state of tumor dormancy as a consequence of adaptive immune system activity and undergo "editing". Recent work has demonstrated that T cells play a major role in shaping the immunogenicity of developing cancers and exert this effect by at least two mechanisms. First, T cells can shape tumor antigenicity/immunogenicity through an immunoselection process by destroying tumor cells that express strong tumor-specific mutant antigens, leaving behind tumor cells that either express weaker antigens or are incapable of expressing antigens (because of mutations in antigen processing or presentation)(Matsushita et al., 2012). Second, chronic T cell attack on a tumorhas been shown to silence expression of certain tumor-specific antigens through epigenetic mechanisms in a preclinical model (Dupage et al., 2012).

When tumor cell variants selected in the equilibrium phase can grow in an immunologically intact environment they enter into the escape phase. The immune system fails to restrict tumor outgrowth and tumor cells emerge causing clinically apparent disease.
Tumors escape immune attack by a variety of complementary mechanisms of immunosuppression, many of which operate in parallel:

- *Reduced immune recognition*: tumor cells can directly escape T-cell recognition by downregulating MHC class I, class I-like, or co-stimulatory molecules or by disabling other components of the antigen processing machinery.

- *Increased resistance or survival* (such as increased expression of STAT-3 or anti-apoptotic molecule Bcl2).
- *Development of an immunosuppressive tumor microenvironment.*
  - Among paracrine mediators, release of PGE2, arginase and IDO (all T-cell suppressors), and the release of VEGF (triggered in part by intratumoral hypoxia), exert multiple direct and indirect immunosuppressive activities. These mediators may function indirectly inhibiting T-cell diapedesis from the vasculature into the tumor bed or directly suppressing effector T-cell activation while enhancing the function of regulatory T cells (T_{reg}).
  - In addition, tumor cells may upregulate surface ligands, including PD-L1, PD-L2 and other ligands that engage receptors on the surfaces of activated T cells (PD-1), causing T-cell anergy or exhaustion.
- *Recruitment of a variety of leukocyte subsets infiltrating tumors able to suppress T-cell function.* In addition to Treg cells (the accumulation of which in tumors correlates with poor prognostic outcome), other suppressive lymphocyte subsets have been reported. They include IL-10 producing B cells and B regulatory cells, type II NKT cells, NK cells and γδ T cells. Myeloid lineage cells also promote immune suppression in tumors. Among these, myeloid-derived suppressor cells (MDSC) suppress T andNK cell activation, probably through several mechanisms including nitric oxide, reactive oxidative species, arginase, IL-10 andTGF-β; thereare also reports that MDSCs may specifically induce the expansion of Treg cells.

1.2 Vaccines in cancer therapy

1.2.1 Tumor-associated antigens

Cancer immunotherapy seeks to exploit the host's immune system to eliminate cancer cells. In this context, cancer antigens play a critical role, as they are responsible for triggering a cancer specific immune response.At the core of the design of immunotherapy strategies lies the fact that cancer patients can produce T lymphocytes that recognize tumor-specific antigens.
Ideally, cancer antigens should be highly immunogenic to induce strong immune responses and only be expressed by malignant cells for specific tumor killing. However, normal tissues may also express some. For this reason, human tumor antigens can be grouped in two main classes: 1) antigens of high tumoral specificity and 2) antigens of low tumoural specificity (Cooley et al 2014).

Three types of tumor antigens have the potential to elicit immune responses that are strictly tumor specific: 1) ***oncoviral*** antigens, 2) ***mutated*** antigens and 3) ***cancer-testis*** antigens. Instead, antigens of low tumor specificity include 4) ***differentiation*** antigens and 5) ***overexpressed/ accumulated*** antigens (Figure 3).

1) Approximately 12% of all human cancers worldwide are associated with oncogenic viruses. Human tumor viruses belong to a number of virus families, including the RNA virus families Retroviridae and Flaviviridae and the DNA virus families Hepadnaviridae, Herpesviridae and Papillomaviridae (McLaughlin-Drubin and Munger, 2008). They are associated to the onset of an important subset of human tumors, including cervical carcinoma, hepatocarcinoma, nasopharyngeal carcinoma and adult T cell leukemia (Parkin, 2006).
Even though human oncogenic viruses belong to different virus families and utilize diverse strategies to contribute to cancer development, they share many common features. One key feature is the tendency to establish long-term persistent infections. Consequently, they have evolved different strategies for evading the host immune response, which would otherwise clear the virus during these persistent infections.

Immunocompetent individuals normally mount a potent cytotoxic T-cell (CTL) response against infected cells expressing viral epitopes. However, oncogenic viruses generally avoid CTL surveillance by establishing latency in host cells. γ-herpesviruses express genes during the latency which can block TNF-induced pro-apoptotic signals generated by cell-mediated cytotoxic responses. Latent γ-herpesviruses remain under tight transcriptional regulation by NF-κB and histone deacetylase enzymes (HDACs). Disruption of viral latency in γ-herpesvirus lymphomas using the NF-κB and HDAC inhibitors results in lytic reactivation and cell death (Ramos and Lossos, 2011). Likewise, the chronic antigenic stimulation in HPV infections supports the establishment of an immunosuppressive microenvironment that triggers the transition from normal epithelium to cervical intra-epithelial neoplasia (CIN) and vulvar intraepithelial neoplasia (VIN) as well as their subsequent progression to invasive squamous cell carcinoma. In particular, IDO^+ cells, $FOXP3^+$ T cells, and $TGF\beta^+$ T cells increase across the disease spectrum in parallel with a sharp decline in IFN-γ^+ cells in invasive cancer (Kobayashi et al., 2008).

2) Mutated genes greatly contribute to the immunogenicity of human tumors. Gene mutations produce new antigenic peptides by changing one amino acid, by altering the phase of the reading frame or by extending the coding sequence beyond the normal stop codon. Due to their unique nature of being

expressed only on tumors and not on any other normal tissues, these tumor-specific TAAs can be recognized as non-self and not be subjected to central immune tolerance. These mutated proteins may play a critical role in the oncogenic process as products of mutated oncogenes or tumor suppressor genes and therefore survive immune selection in order to maintain tumor growth and proliferation. Mutated *CDK4* is an example of tumor antigens proved to be oncogenic. A point mutation in CDK4 results in the loss of binding of CDK4 to the inhibitor INK4A with a consequent disrupting of the cell cycle regulation (Plaen et al., 1995).
These type of mutations that confer a selective growth advantage, thus promoting cancer development, are commonly defined 'driver mutations'. Cancer cells also develop a large number of mutations that do not provide a growth advantage and are therefore called 'passenger mutations'. In most cancers, there are many more passenger mutations than driver mutations, with the latter ones more frequently giving rise to the so-called neo-antigens (discussed in 1.4.1).

3) Cancer/testis (CT) antigens are a category of tumor antigens with normal expression restricted to male germ cells in the testis but not in adult somatic tissues. In some cases, CT antigens are also expressed in ovary and in trophoblast. In malignancy, this gene regulation is disrupted, resulting in CT antigen expression in a proportion of tumors of various types.The first cancer antigen reported that could be recognized by T cells, MAGE-A1, belongs to this class (van der Bruggen et al., 1991).
Cancer-germline genes are an important source of tumor-specific antigens, with more than 60 cancer-germline genes having been identified. The mechanism that leads to the activation of these genes in tumor cells involves the demethylation of their promoter, which is methylated in all normal cells except in germline cells (De Smet et al., 1996).

4) Differentiation antigens are cell type specific and shared between tumors and the normal tissue of origin (Buonaguro et al., 2011). For example, both melanoma and normal melanocytes express GP100, Tyrosinase, and Melan-A/MART-1. Other differentiation antigens include PSA, Mammoglobin-A, and carcinoembryonic antigen (CEA) overexpressed in prostate cancer, breast carcinoma, and colon cancer respectively.

5) Some proteins shared by both normal and tumor cells may display overexpression only in cancer cells thus providing an opportunity for a specific T cell response. This is because a threshold level of antigen is required for recognition by T cells. If tumor cells present an amount of peptide–HLA complexes that is above the threshold of T cell activation and if

normal cells do not, a specific antitumor T cell response could occur. In this way, over-expression by malignant cells overrides tolerance and triggers T cell activation. An example of overexpressed tumor antigen is the oncogene and growth factor receptor ERBB2 (also known as HER2 and NEU) which is overexpressed in many epithelial tumors, including ovarian and breast carcinomas, owing to increased transcription and to gene amplification (Ross et al., 1998).

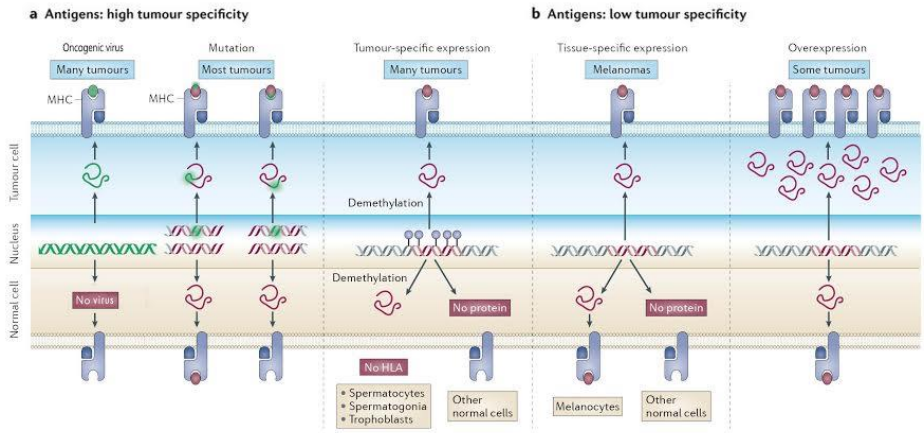

Figure 3. Classes of human tumor antigens recognized byT lymphocytes.
a. *Oncoviral antigens, mutated antigens and cancer-testis antigens show high tumor specificity. Point mutations can modify a peptide that already binds to the major histocompatibility complex or can enable a non-binding peptide to bind. Cancer-germline genes are selectively expressed in tumors and germline cells because of DNA demethylation. However, their antigens are not present on germline cells because of the lack of HLA molecules.* ***b.*** *Antigens of low tumor specificity include differentiation antigens and overexpressed/ accumulated antigens. In the figure, a melanocyte-specific gene is used as an example of tissue-specific gene expression. Both tumor cells and the normal tissue of origin (melanocytes) share the encoded protein. In the last panel, overexpression of particular proteins, such as ERBB2, beyond threshold levels can also trigger an antitumor immune response.*

1.2.2 Therapeutic cancer vaccines

Traditionally, vaccines have been used as a preventive measure against infectious diseases, triggering the immune system to produce neutralizing antibodies against specific pathogen antigens. More recently, vaccines have been applied as therapeutic strategies, aiming to induce immune system to activate cytotoxic T cells against infected cells and cancer. However, therapeutic vaccination against established diseases has proven much more

challenging than prophylactic vaccination against infectious diseases, because the vaccine intervention must overcome the hurdles posed by immune evasion by having to antagonize an immune system that has been restrained by tolerizing or polarizing mechanisms that sustain the disease in a misguided attempt at self-tolerance (Melief et al., 2015).

The idea of a therapeutic cancer vaccine originated with the discovery that patients can harbor $CD8^+$ and $CD4^+$ T cells specific for cancer antigens expressed in their tumors (Boon at al., 2006). Therefore, vaccination might reasonably be expected to amplify the frequency and strength of these pre-existing responses against tumor antigens or perhaps induce some *de novo* reactivities.

Based on their format/content, cancer vaccines may be classified into several major categories, which include cell based vaccines (tumor cell lysates, irradiated whole tumor cells, DCs), protein/peptide vaccines, and genetic vaccines (DNA, RNA and viral vectors) (Guo et al., 2013). Their development is based on the concept that the introduction of various tumor antigens into the host would facilitate immune mediated clearance of tumor cells. Ideally, therapeutic vaccination aims at expanding high-avidity $CD8^+$ T cells that can differentiate into CTLs able to kill cancer cells and to generate long-lived memory $CD8^+$T cells. This could be accomplished through either the priming of naïve T cells or the reprogramming of memory T cells that differentiate earlier in an environment not conducive to the generation of potent cytotoxic T cells. Indeed, cancer is a chronic disease and, as such, it is associated with skewed T cell memory, chronically activated and anergic $CD8^+$ T cells that express programmed cell death 1 (PD-1) (Freeman et al., 2006). In addition, vaccination should lead to the generation of long-lived memory $CD8^+$ T cells that will act to prevent relapse (Figure 4).

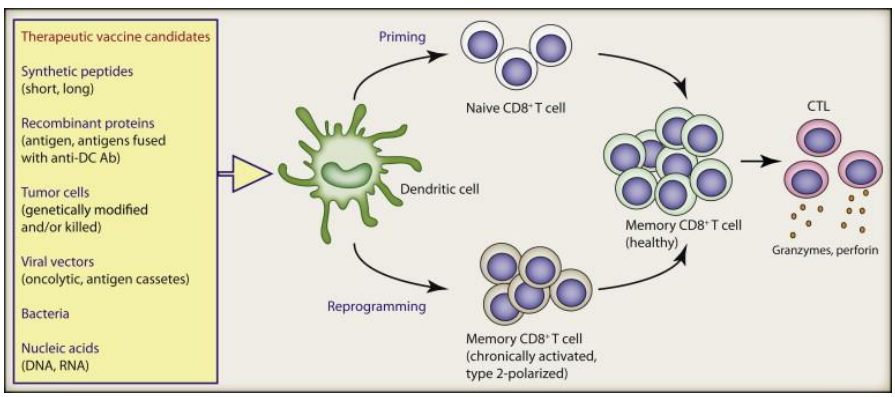

Figure 4. Therapeutic vaccines act to generate protective $CD8^+$ T cell immunity (from Palucka and Banchereau, 2013).

Therapeutic vaccines are expected to prime new T cells and induce a transition from chronically activated non-protective $CD8^+$ T cells to healthy $CD8^+$ T cells able to (1) generate CTLs that reject cancer and (2) provide long-lived memory CD8+ T, thereby preventing relapse.

1.2.3 Genetic cancer vaccines

Therapeutic vaccines have two objectives: priming antigen-specific T cells and reprogramming memory T cells. In this context, genetic vaccines represents a highly promising approach.
Genetic vaccination exploits the use of viral or bacterial vectors or nucleic acids to deliver one or more antigens *in vivo.* One major advantage of genetic vaccines is the easy delivery of multiple antigens in one vaccine and their ability to activate various arms of the immune system (Aurisicchio and Ciliberto, 2012).

- DNA vaccines consists of bacterial plasmid DNA into which specific sequences are incorporated under the control of an eukaryotic promoter. Genes in DNA vaccines can encode different antigens as well as various immunomodulatory molecules to manipulate the resulting immune response, after transduction into the target cells and subsequent *in vivo* expression by the host's gene expression machinery.

DNA vaccines were shown to be able to trigger both innate and adaptive immune response. The ability to stimulate the innate immune system arises from the bacterial origin of the backbone (Rice et al., 2008). The bacterial DNA appears to act as a pathogen-associated molecular pattern able to stimulate cells through Toll-like receptors (TLRs). Specifically, the hypomethilated CpG dinucleotides motif that is common in bacterial DNA, but rare in mammalian DNA, interacts with TLR9 expressed in immune cells, such as dendritic cells, B cells, and NK cells. Activation of TLR9 leads to a cascade of pro-inflammatory responses and results in the production of various cytokines. The local inflammation and increased production of cytokines from the innate immune responses can attract and activate additional immune cells, such as lymphocytes, and enhance subsequent antigen-specific immune responses.

DNA vaccines are delivered intradermically or more commonly by intramuscular injection, resulting in the transfection of keratinocytes or myocytes, respectively.
In the muscle, transfected myocytes express the vaccine-encoded antigens and act as a target for immune effector cells. In addition, they can also upregulate expression of MHC class I and co-stimulatory molecules, with

production of cytokines and chemokines. Consequent inflammation and production of cytokines attract professional APCs, like dendritic cells, to the transfection sites.
APCs have a dominant role in the induction of immunity of DNA vaccines by presenting vaccine-derived endogenous peptides on MHC I molecules. APCs can 'capture' these antigens by direct transfection or most commonly by cross presentation for example, owing to APC engulfment of apoptotic transfected cells. In addition, APCs mediate the display of peptides on MHC II molecules after secreted protein antigens from transfected cells are captured and processed within the endocytic pathway.
Antigen-loaded APCs travel to the draining lymph node via the afferent lymphatic vessel where they present peptide antigens to naïve T cells via MHC and the T cell receptor (TCR) in combination with co-stimulatory molecules, initiating an immune response and expansion of T cells. In response to peptide-bound MHC molecules and co-stimulatory secondary signals, activated CD4 T helper cells secrete cytokines during cell-to-cell interaction with B cells and bind to co-stimulatory molecules that are required for B cell activation (Figure 5).

The use of DNA plasmids in cancer immunotherapy offers several advantages. In addition to their safety, DNA vaccines allows for simple and flexible design, encoding wide range of antigens and immunomodulatory molecules. DNA vaccines are heat stable, easily stored and perfect for large scale production **(**Yang et al., 2014).
However, despite the promising features of DNA vaccines, they have been found to elicit immune responses less than other types of vaccines, including peptide vaccines, cellular vaccines, viral vector vaccines, and RNA vaccines. The relatively poor immunogenicity of DNA vaccines combines with other disadvantages: inefficient delivery of DNA into human cells, the need for DNA to cross both cell and nuclear membranes and be transcribed in order to allow for expression of the encoded antigen. Some of these considerations have driven a shift away from DNA vaccines and towards RNA vaccines.

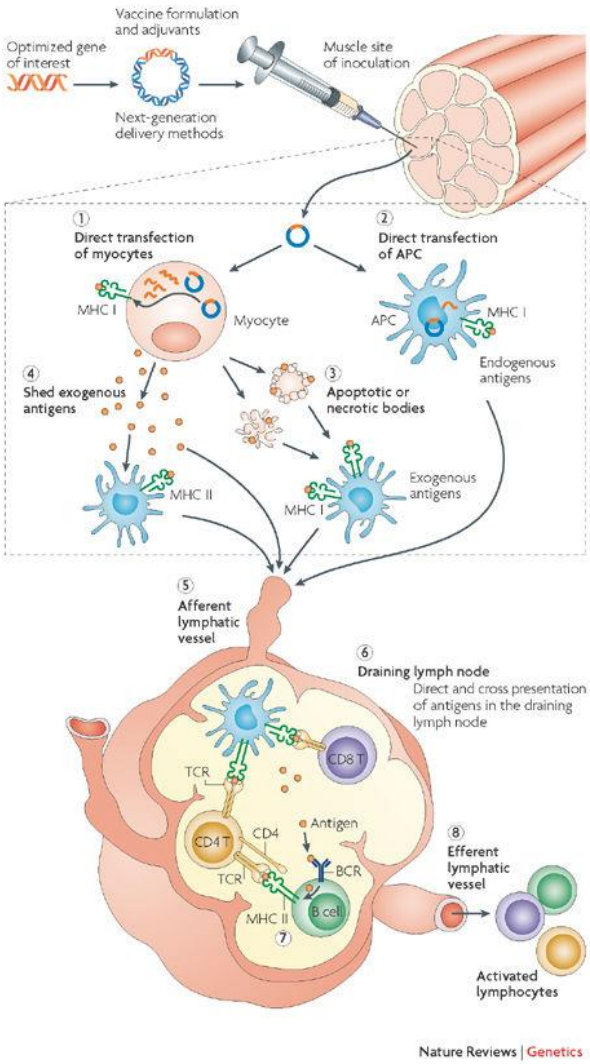

Figure 5. Induction of cellular and humoral immunity by DNA vaccines
The optimized gene sequence of interest is inserted into a plasmid backbone, purified, and then delivered to the inoculation site. Using the host cellular machinery, the plasmid enters the nucleus of transfected myocytes (1) and of resident antigen presenting cells (APCs) (2). Here, the plasmid components initiate gene transcription, which is followed by protein production in the cytoplasm and the consequent formation of foreign antigens, that can become the subject of immune surveillance in the context of both major histocompatibility complex class I (MHC I) and MHC II proteins. The presentation of vaccine-derived endogenous peptides on MHC I molecules by APCs can follow either direct APCs transfection by the plasmid vaccine (2) or cross-presentation of cell-associated exogenous antigens (3). In addition, APCs can capture secreted protein antigens that have been shed from transfected cell, process them within the endocytic pathway and finally display peptides on MHC II molecules (4). Antigen-loaded APCs travel to the draining lymph node via the

afferent lymphatic vessel (5) where they present peptide antigens to naïve T cells via MHC and the T cell receptor (TCR) in combination with co-stimulatory molecules, providing the necessary secondary signals to initiate an immune response and expansion of T cells (6). In turn activated CD4 T helper cells promote B cell activation (7). 'Armed' lymphocytes can finally leave the draining lymph node through the efferent lymphatic system (8).Kutzler and Weiner, 2008

- RNA vaccines consist in messenger RNA (mRNA) synthesized by *in vitro* transcription using a bacteriophage RNA polymerase and template DNA that encodes the antigen(s) of interest (McNamara et al., 2015). Once administered and internalized by host cells, the mRNA transcripts are translated directly in the cytoplasm and then, like DNA vaccines, the resulting antigens are presented to APC by major histocompatibility complex (MHC) class I and II proteins, with consequent induction of T cell-mediated immune responses. Alternatively, RNA vaccines can be constructed for the efficient production and secretion (or cell-surface expression) of extracellular antigens to stimulate B cell responses and antigen-specific antibody production. The effectiveness of RNA vaccines may also be related to the fact that RNA is known to be a potent stimulator of innate immunity (Ulmer et al., 2012).

Several techniques have been developed to improve the inherent instability of mRNA and translational efficiency and to optimize RNA vaccine delivery.

- One approach to induce a potent and targeted anti-tumor response is to use viruses to deliver tumor antigens to cells of the immune system**.**

Viral vectors are an attractive choice of antigen delivery system for cancer immunotherapy since they mimic a natural infection and provide potent danger signals, which are known to be important for the induction of an immune response (Harrop and Carroll, 2006).They enable intracellular antigen expression and induce a robust cytotoxic T lymphocyte (CTL) response, leading to the elimination of diseased cells.

Despite their efficacy, viral vectors present unavoidable problems that need to be addressed. Viral vector-based vaccines require assessment of efficacy and safety, including immunogenicity, genetic stability, ability to evade pre-existing immunity, replication deficiency or attenuation, and genotoxicity. For a high biological safety level, non- (or low-) pathogenic viruses are often selected (Ura et al., 2014).

Different viral vectors have been evaluated in cancer immunotherapy: adenoviruses, Adeno-Associated Virus (AAV), alphaviruses, flaviviruses, lentiviruses, measles virus, rhabdoviruses, retroviruses and Vaccinia Virus (VV) (Lundstrom, 2016).

The intrinsic properties of each virus have distinct advantages and disadvantages, which can determine their applicability in a particular therapeutic setting.

1.2.4 Adeno vector based cancer vaccines

Adenovirus vectors (Ads) are one of the most effective carriers for delivery of foreign antigens into the host cells. Ads have a large genome size and allow cloning of expression cassette for large antigens (ie: over 2000 amino acids). Furthermore, Ads do not integrate the viral genomic DNA into the hosts' genome, which reduces the risk of insertion mutagenesis (Zhang and Zou, 2016). Compared with other viral vectors, Ads are highly immunogenic and can induce robust adaptive immune responses, offering one of the most powerful technologies for cancer vaccine applications.

Adenoviruses are double-stranded DNA viruses with a genome of ~34–43 kb, with two inverted terminal repeats at both ends as origins for DNA replication. The genes that Ads express during the life cycle are generally clustered in early genes and late genes. The early genes include E1A, E1B, E2, E3 and E4, and they are mainly responsible for facilitating the replication of Ads by changing the expression levels of related host genes. The early genes can be further classified into two types: the immediate early genes (E1A) and the delayed early genes (E1B, E2, E3 and E4).
The E1A gene is transcribed first and, with the help of cellular factors, activates transcription of the other viral genes. Deletion of E1A renders the virus replication-defective. E1A stimulates viral DNA synthesis, dysregulates cell-cycle control, promotes apoptosis and plays a role in immunoevasion by inhibiting the activity of STAT1, which is needed for activation of interferon-responsive genes.
While E1A proteins promote apoptosis, E1B proteins have antiapoptotic functions. E1B polypeptides turn off host cell protein synthesis and help to stabilize, transport, and translate selectively viral RNA.
The E2 unit encodes DNA-binding proteins and a polymerase and is essentialfor viral replication.
E3 proteins allow the virus to escape immunosurveillance by different mechanisms: i) by reducing expression of major histocompatibility complex (MHC) class I determinants; ii) by direct binding to the groove of MHC class I molecules preventing binding and export to the cell surface of peptides; iii) by associating with TAP and thereby reducing efficient transport of peptides derived from proteolytic cleavage of *de novo* synthesized viral proteins from the cytoplasm tothe endoplasmic reticulum where they can associate with MHC class I molecules.

The E4 transcription unit encodes seven polypeptides, which affect viral transcription and a number ofhost cell functions including cell proliferation and apoptosis, in part by promoting degradation of p53. E4 is essential for nuclear export of viral RNA.
The late genes (L1-L5) are mainly responsible for the lysis of the host cells, assembly and release of the virions.
Ad virions mainly comprise two types of proteins: the capsid proteins and the core proteins. The core proteins mainly include proteins V, VII, X, and they mainly function as the DNA-associated proteins. The capsid proteins comprise Hexon, Penton, fiber, IIIa, VIII and IX.

Ads are isolated from different mammalian species, such as humans, dogs, sheep, bovines and non human primates. Among all, the human Ads and chimpanzee Ads are widely used in research or clinical studies. They have a broad tropism infecting a variety of dividing and non-dividing cells. They can be grown in human HEK293 cells and purified by CsCl gradient ultracentrifugation or chromatography, making them attractive for clinical use.

Adenoviruses are useful vectors for genetic vaccine delivery. To insert transgenes of interest viral E1 and E3 genes are commonly deleted and depending on space necessity, there is the possibility to delete also the E4 gene. By deletion of these genomic regions, the virus loses self-replication capacity, becoming replication-defective. This arrangement increases their predictability of their immunization properties and reduces unwanted side effects.
The most commonly used adenoviral vectors as genetic vaccines are derived from human adenovirus serotype 5. In particular, head-to-head comparisons with other genetic vaccine vectors (ie.: poxviruses, lentiviruses, alpha virus-based vectors and naked DNA) in animal models and the results obtained in human clinical trials, clearly showed that Ad5-based vectors currently represent one of the most potent delivery system for eliciting a $CD8^+$ T cell response against the encoded antigen(s). However, high titers of anti Ad5 neutralizing antibodies are commonly present in human population, impairing the immunogenicity of Ad5-based vaccines in humans.

To overcome this drawback, other human Adenovirus vectors based on rare serotypes such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49, and Ad50 have been proposed as potential alternatives to Ad5 because antibodies present in humans rarely neutralize them. However, they showed lower immunological potency than Ad5 in mice and non-human primates. Another approach is to use non-human Adenoviruses. Indeed, Colloca et al., (2012) generated a large collection of replication defective vectors based on Ad

isolated from chimpanzees. Functional screenings to assess growth capability in packaging cell lines as HEK293 and PER.C6, immunological potency in mice and non-human primates and sensitivity to neutralizing antibodies present in humans allowed the identification of novel vaccine carriers inducing potent cellular immunity, suitable for vaccine delivery in humans.

Among the large number of candidates screened for immunological potency by dose/response in mice, some ChAds were identified which showed immunological potency equivalent to Ad5 (ChAd3, ChAd63, ChAd83, PanAd1, PanAd2 and PanAd3). Importantly, the high level of immunogenicity of the top ranking ChAd3 and PanAd3 was confirmed in non-human primates, where they induced a level of T-cell response comparable to that of Ad5 even at low dose.

1.3 The blockade of immune checkpoints in cancer immunotherapy

1.3.1 Immune checkpoint receptors

The initiation and progression of immune responses are fine-tuned by a highly complex array of cytokines, chemokines, toll-like receptors and costimulatory molecules. Equally complex is the diversity of pathways and mechanisms employed by the immune system to regulate and/or terminate ongoing immune responses. When the regulatory mechanisms somehow fail, normal immune homeostasis is impaired leading to disastrous consequences to the host, as the onset of autoimmunity.

Co-stimulatory and co-inhibitory immune checkpoint receptors are critical modulators of the immune system, as they determine the functional outcome of T cell receptor (TCR) signalling.
Following recognition of cognate peptide–MHC complexes on APCs by the TCR, co-signalling receptors often colocalize with TCR molecules at the immunological synapse, where they synergize with TCR signalling to promote (co-stimulatory receptors) or inhibit (co-inhibitory receptors) T cell activation and function (Figure 6).

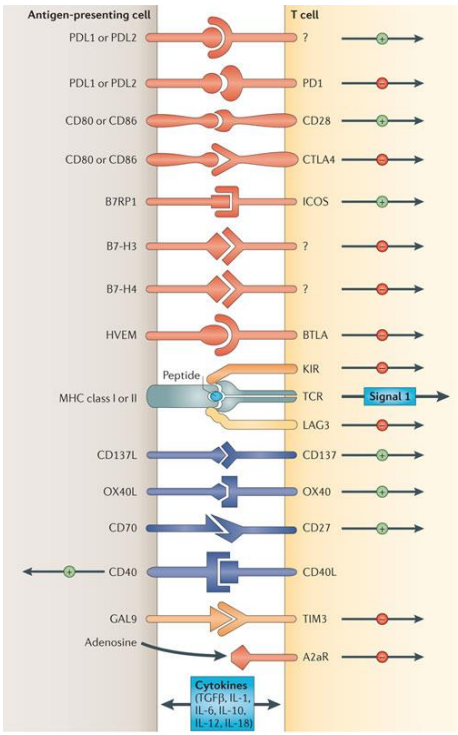

Figure 6. Multiple co-stimulatory and inhibitory interactions regulate T cell responses (from Pardoll, 2012).
Various ligand–receptor interactions between T cells and antigen-presenting cells (APCs) regulate the T cell response to antigen after the recognition of the cognate antigen through the TCR. These responses can occur at the initiation of T cell responses in lymph nodes or in peripheral tissues or tumors, where effector responses are regulated.

In this interactive environment, the repertoire of co-signalling receptors expressed on T cells is highly versatile and responsive to changes in overlapping spatiotemporal fashion. The costimulatory receptor CD28 and the inhibitory receptor CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) are a pivotal example. They bind the same ligands but they display distinct kinetics of expression, in order to modulate TCR signalling during the immune response (Intlekofer and Thompson, 2013).

The discovery of CD28 stimulatory receptor gave molecular confirmation to the theory according to which the TCR engagement is not itself sufficient to enact T cell clonal expansion and differentiation whereas a second signal is pivotal to drive lymphocyte clonal expansion.

CD28 is constitutively expressed on the surface of resting and activated T cells, while its ligands CD80 (or B7-1) and CD86 (or B7-2) belonging to the B7 ligands are expressed of the surface of APCs. The interaction between them provides a second signal to promote T cell activation, proliferation, survival and activation of effector functions.

The inhibitory receptor CTLA-4, expressed on the surface of activated T cells shares significant homology to CD28 and bounds the same B7 ligands, but their interaction counteracts CD28-mediated costimulatory signals and impairs the activation of T cells.
This opposed action is explained by a unique spatiotemporal regulation of CTLA-4.In resting T cells, CTLA-4 exhibits minimal expression and a peculiar pattern of intracellular localization and trafficking. It resides mostly within intracellular vesicles of the trans-Golgi network and endosomal compartments. Upon TCR engagement, CTLA-4 expression is induced, and intracellular vesicles containing CTLA-4 undergo relocalization to the immune synapse. At the cell surface, CTLA-4 competes with CD28 for access to B7 ligands. Compared with CD28, CTLA-4 has higher affinity and avidity for B7 ligands, which has been attributed to homodimer formation by CTLA-4 that allows for bivalent binding of B7 molecules, in contrast to the monovalent binding of B7 ligands by CD28.
The definitive role of CTLA-4 as a major negative regulator of T-cell activation was established with the description of CTLA-$4^{-/-}$ mice. They succumbed at three-to-four weeks of age from massive lymphoproliferation within the spleen and lymph nodes and end-organ infiltration by activated lymphocytes (Tivol et al., 1995).
Of note, CTLA-4 is constitutively expressed on $Foxp3^{+}$ T_{reg} cells that may use CTLA-4 to mediate suppression of T effector cells. The finding that conditional deletion of CTLA-4 in T_{regs}, but not in other cell types, results in impaired T_{reg} functions appears to support this notion (Wing et al., 2008).

CTLA-4 together with PD-1 (discussed in section 1.3.2) are the two negative checkpoint regulators that have been most actively studied. However, multiple additional inhibitory receptors have been discovered in the recent years. TIM-3 (T-cell immunoglobulin and mucin-containing protein 3), LAG-3 (lymphocyte-activated gene-3), TIGIT (T cell immunoreceptor with Ig and ITIM domains), BTLA (B and T lymphocyte attenuator) and VISTA (V-domain Ig suppressor of T cell activation) represent the main examples. Among them Lag-3, Tim-3 and TIGIT represent the next generation of co-inhibitory receptors to be translated to the clinic since they are highly expressed on dysfunctional or exhausted T cells in chronic diseases such as chronic viral infection and cancer.

A comparison concerning expression, signaling mofits and ligands between them is briefly summarized in Table 1.

	Lag-3	Tim-3	TIGIT
Expression			
CD4[a]	Tr1, nTreg, iTreg	Th1, Tr1, nTreg[b]	Tr1,Tfh, nTreg[c]
CD8[a]	dysfunctional T cells	Tc1, dysfunctional T cells	dysfunctional T cells
Natural killer cells	+	+	+
Dendritic cells	–	+	–
Monocytes/macrophages	–	+/–[d]	–
Signaling motifs	KIEELE	tyrosine[e]	ITT/ITIM
Ligands	MHC II, LSECtin	Galectin-9, Ceacam-1, HMGB-1, phosphatidyl serine	CD112, CD155

[a] Lag-3, Tim-3, and TIGIT are transiently upregulated on activated $CD4^+$ and $CD8^+$ T cells.
[b] In both mouse and human, Tim-3 is either not expressed or expressed on a very small fraction of $CD4^+Foxp3^+$ Treg cells in the normal circulation but is highly expressed on Treg cells at sites of tissue inflammation.
[c] In both mouse and human, TIGIT is expressed on about one-third of $CD4^+FoxP3^+$ Treg cells in the normal circulation and is highly upregulated on Treg cells at sites of tissue inflammation.
[d] In the mouse, Tim-3 is expressed on monocytes/macrophages only in inflammatory conditions. In humans, Tim-3 is expressed on peripheral blood monocytes and on macrophages.
[e] Tim-3 has five tyrosines in its cytoplasmic tail but no known signaling motif.

Table 1. Comparison of Lag-3, Tim-3 and TIGIT (from Anderson et al., 2015).

Several observations proved that these receptors have been shown to be important immune regulators in autoimmunity. Lag-3 plays a protective role in autoimmunity by dampening T helper (Th) cell responses directly through engagement of MHC-II. In addition, Lag-3 indirectly inhibits effector T cell responses via promotion of Treg-cell- and Tr1-cell-mediated suppression. In autoimmune diseases such as multiple sclerosis, Tim-3 is under-expressed on pathogenic Th1 cells. IFN-β therapy can increase Tim-3 on antigen-specific T cells directly or indirectly via promotion of IL-27 production from local antigen-presenting cells. Increased expression of Tim-3 is associated with reduction in disease relapses. TIGIT inhibits auto-pathogenic Th1/Th17 T cell responses through three different pathways: (1) TIGIT directly inhibits T cell activation and expansion; (2) TIGIT expressing effector and regulatory T cells engage CD155 on APCs thereby inducing tolerogenic APCs that secrete IL-10; (3) TIGIT promotes Treg-cell-mediated suppression through the induction of IL-10 and Fgl2, which potently and selectively suppress Th1 and Th17 cell responses.

1.3.2 PD-1/PD-L1 pathway

Immune checkpoints refer to a plethora of inhibitory pathways hardwired into the immune system that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage. Among the inhibitory immune mediators, the pathway consisting of the programed cell death 1 (PD-1) receptor (CD279) and its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) plays an important role in the induction and maintenance of peripheral tolerance and for the maintenance of the stability and the integrity of T cells. PD-1's immune-inhibitory function was elucidated by characterizing the autoimmune phenotype of PD-1–deficient mice, in which PD-1 deficiency leads to a loss of peripheral tolerance and the subsequent development of autoimmunity. PD-1–deficient mice develop different autoimmune diseases depending on their genetic background: C57BL/6-Pdcd1$^{-/-}$ mice develop lupus-like arthritis and glomerulonephritis with IgG3 and C3 deposits (Nishimura et al., 1999). BALB/c-Pdcd1$^{-/-}$ mice develop fetal dilated cardiomyopathy with a concomitant production of autoantibodies against cardiac troponin I (Nishimura et al., 2001).

The PD-1:PD-L1/L2 pathway also mediates potent inhibitory signals to hinder the proliferation and function of T effector cells. Similarly to CD80/CTLA-4, PD-L1/PD-1 are antagonists of CD80/CD28 co-stimulation. Engagement of PD-1 by its ligands PD-L1 or PD-L2 strongly counteracts TCR signal transduction and CD28 co-stimulation, transducing a signal that inhibits T cell proliferation, cytokine production, and cytolytic function.

Programmed death 1 (PD-1) receptor is a type I transmembrane protein preferentially expressed on activated CD4$^+$ and CD8$^+$ T cells, B cells, monocytes, natural killer (NK) cells, and dendritic cells (DCs). PD-1 consists of a single N-terminal IgV-like domain, an approximately 20 amino acid stalk separating the IgV domain from the plasma membrane, a transmembrane domain, and a cytoplasmic tail containing tyrosine-based signaling motifs: an immunoreceptor tyrosine-based inhibitory motif (ITIM) followed by an immunoreceptor tyrosine-based switch motif (ITSM). Both these motifs are implicated in PD-1 immunosuppressive effects, even if the ITSM appears to be the most important for mediating PD-1 suppression of lymphocyte activation (Chemnitz et al., 2004).
Upon binding to its ligands, PD-1 becomes phosphorylated on intracellular tyrosine residues within ITIM. Subsequently, protein phosphatases, such as SHP-2 are recruited to bind to the ITSM, become activated and inhibit proximal TCR signaling events (Sheppard et al., 2004) (Figure 7).

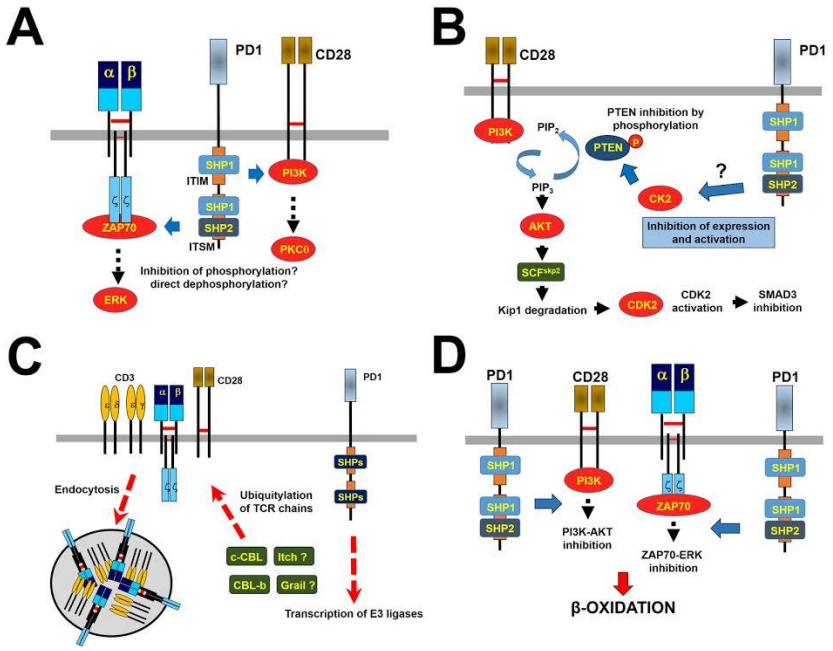

Figure 7. PD-1-dependent inhibitory mechanisms. (from Arasanz et al., 2017)
PD-1 mediates its immunosuppressive activity through direct and indirect inhibitory mechanisms over TCR signalling and T cell proliferation, by inhibiting membrane-proximal T cell signaling events. ***(A)*** *The direct inhibition of TCR signaling depends on the recruitment of SHP1 and SHP2 phosphatases to the tyrosine-based signaling motifs ITIM and ITSM. These phosphatases interfere with TCR signal transduction and CD28 co-stimulation by inhibiting ZAP70 and PI3K activities, respectively.****(B)****PD-L1 engaged PD-1 exerts an indirect inhibitory control over CD28 co-stimulation by reducing the expression levels and activities of CK2. As a result, active PTEN eliminates PIP3, shutting offAKT activation.Consequently,cell growth and survival is inhibited, because lymphocytes arrest at the G0-G1 phase.****(C)*** *PD-1 engagement regulates TCR surface expression, by promoting the expression of E3 ubiquitin ligases that ubiquitylate TCR chains. As a result, TCR is removed from the T cell surface, possibly by endocytosis.* ***(D)*** *Engaged PD-1 alters T cell metabolism by inhibition of ERK and PI3K-AKT activities.*

PD-1 is expressed on a large proportion of tumor-infiltrating lymphocytes (TILs) from many different tumor types. Among $CD4^+$ TILs, a generally high level of PD-1 expression is detectable on T_{reg} cells, which can represent a large proportion of intratumoral $CD4^+$ T cells. Increased PD-1 expression on $CD8^+$ TILs may either reflect an anergic or exhausted state, as has been suggested by decreased cytokine production by $PD\text{-}1^+$ compared with $PD\text{-}1^-$ TILs from melanomas (Ahmadzadeh et al., 2009).
The ligands of PD-1 (PD-L1 and PD-L2) are type I transmembrane glycoproteins, containing IgC and IgV domains. They share about 40% of amino acid identity. PD-Ls have distinct expression patterns: PD-L1 is

constitutively expressed on T and B cells, DCs, macrophages, mesenchymal stem cells and bone marrow-derived mast cells (Yamaki et al., 2002). In addition, PD-L1 is expressed on a wide variety of non-hematopoietic cells. In contrast, PD-L2 expression is restricted to activated DCs, macrophages, bone marrow derived mast cells, and more than 50% of peritoneal B1 cells.

Just as PD-1 is highly expressed on TILs from many cancers, the PD-1 ligands are commonly upregulated on the tumor cell surface from many different human tumors, correlating with adverse prognosis. On cells from solid tumors, the major PD-1 ligand that is expressed is PD-L1. Two general mechanisms for the regulation of PD-L1 by tumor cells have emerged: innate immune resistance and adaptive immune resistance. In the first case, constitutive oncogenic signalling pathways in the tumor cell drive PD-L1 expression (Parsa et al., 2007). On the contrary, in adaptive immune resistance, the tumor uses the natural physiology of PD-1 ligand induction that normally occurs to protect a tissue from infection-induced immune-mediated damage in order to protect itself from an antitumor immune response. Expression of PD-L1 as an adaptive response to endogenous antitumor immunity can occur because PD-L1 is induced on most tumor cells in response to interferons (IFNs), predominantly IFNγ (Taube et al., 2012).

PD-Ls mediate potent inhibitory signals after ligation with PD-1, causing a detrimental effect on antitumor immunity by allowing the tumor cells to escape immunosurveillance.
Indeed, although the PD-1: PD-L1/L2 pathway evolved to constrain such autoreactive T cells and maintain peripheral tolerance, it has been shown to have inimical effects on antiviral and antitumor immunity.
The hypothesis that engagement of PD-1:PD-L1 pathway might dampen immune responses for tumors was confirmed by the observation that overexpression of PD-L1 on a mouse mastocytoma cell line inhibits $CD8^+$ T cell cytolytic activity through PD-1 ligation, which intensifies tumor growth and invasiveness (Iwai et al., 2002).
When the PD-1/PD-L1 pathway is active in the tumor microenvironment, it promotes survival of cancer cells via antiapoptotic signals mediated via PD-L1 (Dong et al., 2002) and inhibits the activation of signaling pathways, which are critical for survival, expansion, and differentiation of T cells that recognize tumor antigens. The imbalanced activation of signaling events in T cells results in tumor tolerance by inhibiting T effector and memory cell generation and promoting the differentiation of T_{EX} and T_{reg} cells (Bardhan et al., 2016). These observations taken together with the general findings of increased PD-1 expression by TILs and the increased PD-1 ligand expression

by tumor cells provided an important rationale for the capacity of antibody blockade of this pathway to enhance intratumoral immune responses.

1.3.3 Antibody-mediated inhibition of co-inhibitory receptors

The working hypothesis of immunotherapy focuses on the premise that targeting specific molecules within the complex immunological mechanisms exploited by tumor cells to evade destruction can restore the antitumor immune response. A deep understanding of the complex interrelationships between the immune system and tumor cells led to the identification of several specific immunotherapeutic targets. Among them, immune checkpoint receptors emerged as a potential target for cancer treatment. Key targets of immune checkpoint inhibitory pathways include CTLA-4 and PD-1. These two immune-checkpoint receptors have been most actively studied in the context of clinical cancer immunotherapy. However, multiple additional immune checkpoints represent promising targets for therapeutic blockade.

In 1996, Allison and colleagues gave the first demonstration in mouse models of the ability of CTLA-4 antibodies to induce therapeutic antitumor immunity. *In vivo* administration of antibodies to CTLA-4 resulted in the rejection of tumors, including pre-established tumors. Furthermore, this rejection resulted in immunity to a secondary exposure to tumor cells (Leach et al.,1996). These preclinical findings encouraged the production and testing of two fully humanized CTLA-4 antibodies, ipilimumab and tremelimumab. The anti-CTLA-4 monoclonal antibodies (mAbs) ipilimumab, a fully human IgG1 (BristolMyers Squibb), and tremelimumab, a fully human IgG2 (Pfizer, MedImmune), were the first immune checkpoint blocking drugs to enter clinical testing in oncology, in 2000. In 2011, ipilimumab was approved in the US and Europe as first-line therapy for advanced unresectable melanoma, based on results from two phase III trials showing significant extensions in overall survival (OS) (Hodi et al., 2010; Robert et al., 2011). On the contrary, tremelimumab showed promise in early-phase melanoma trials, but it did not meet its designated endpoint when randomized against standard chemotherapy in a first-line phase III melanoma trial (Ribas et al., 2013).

Antibody blockade of CTLA-4/B7 interactions is thought to promote T_{eff} activation by interfering with negative signals transmitted by CTLA-4 engagement. Furthermore, these drugs have recently been postulated to have unique functions endowed by their specific isotypes, with evidence suggesting that ipilimumab may deplete T_{reg} cells over-expressing CTLA-4 (Selby et al., 2013).

Information garnered from trials of anti-CTLA-4 agents fast-forwarded the development of drugs blocking PD-1 or its major ligand, PD-L1. As predicted by murine models, these drugs have heightened tumor selectivity and reduced toxicity compared to anti-CTLA-4.A number of antibodies that disrupt the PD-1 axis have entered clinical trials (Figure 8).

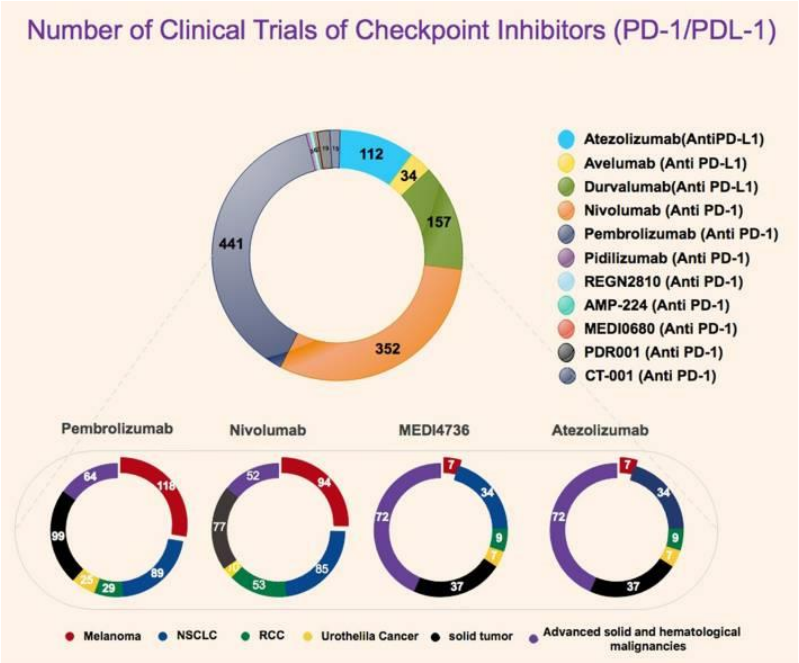

Figure 8. Statistics representative of number of clinical trials for PD-1 and PD-L1 inhibitor with highlight on currently for FDA approved PD-1/PDL-1 inhibitors (from Alsaab et al., 2017).

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PD-1 is more broadly expressed than CTLA-4. Its expression is also induced on activated non-T lymphocyte subsets, including B cells and natural killer (NK) cells. Therefore, although PD-1 blockade is typically viewed as enhancing the activity of effector T cells in tissues and in the tumor micro-environment, it also probably enhances NK cell activity in tumors and tissues and may also enhance antibody production either indirectly or through direct effects on PD-1^+ B cells. Furthermore, similarly to CTLA-4, PD-1 is highly expressed on T_{reg} cells. Because many tumors are highly infiltrated with T_{reg} cells that probably further suppress effector immune responses, blockade of the PD-1 pathway may also enhance antitumor immune responses by diminishing the number and/or suppressive activity of intratumoural T_{reg} cells (Pardoll et al., 2012).

In considering that many tumor cells express multiple inhibitory ligands, and TILs express multiple inhibitory receptors that regulate immune responses at different levels and by different mechanisms, it is rational to consider that concurrent or sequential combination of immunotherapies maybe more effective than monotherapy. These considerations led to the design of combinatorial strategies based on the dual or triple blockade of immune checkpoints in order to enhance antitumor immunity.
One such approach investigated co-targeting of PD-1 and CTLA-4. Preclinical models have shown that dual blockade, as compared with blockade of either pathway alone, synergistically improves antitumor responses. In clinics, combined immune checkpoint blockade (ICB) provided unprecedented efficacy gains innumerous cancer indications, with PD-1 inhibitor nivolumab plus CTLA-4 inhibitor ipilimumab in advanced melanoma as first-ever approved therapies for combined ICB.
However, combined ICB has considerable toxicity. Thus, gains in efficacy must be balanced against a higher frequency and severity of adverse drug reactions (ADR), therefore close monitoring and high experience in diagnosis and treatment of ADR is necessary (Hassel et al., 2017).

1.4 Neo-antigens as cancer immunotherapy targets

1.4.1 Tumor neo-antigens

All tumors arise because of somatically acquired changes in the DNA of cancer cells. However, among all the somatic abnormalities present in a cancer genome some of them are triggers of cancer development, while some others have no contribution in carcinogenesis. To embody this concept, the terms 'driver' and 'passenger' mutations have been coined.
A driver mutation is causally implicated in oncogenesis. It commonly occurs in genes that directly regulate the cell cycle or apoptosis. This class includes inactivating mutations in tumor suppressor genes and activating mutations in oncogenes that confer a selective advantage to the cells that carry them.On the contrary, passenger mutations are found within cancer genomes because somatic mutations without functional consequences often occur during cell division. They are neutral with respect to cell division or death, conferring no clonal growth advantage and therefore they do not contribute to cancer development (Stratton et al., 2009).
A significant subset of passenger mutations results in neo-antigens: mutated self-peptides expressed, processed and displayed by MHC proteins on the surface of the malignant cells, and subsequently recognized by autologous T cells as ‘non-self’ antigens. Because normal tissues do not possess these

somatic mutations, neo-antigen-specific T cells are not subject to central and peripheral tolerance, and lack the ability to induce normal tissue destruction. As a result, neo-antigens appear to represent ideal targets for T cell-based cancer immunotherapy.
The majority of relevant cancer somatic mutations are non-synonymous single nucleotide variants (SNVs) which change the amino acid translated by the respective codon. Other types of relevant mutations are insertions and deletions (indels), gene fusions and mutations in splice donor or acceptor sites of the open reading frame (ORF) of the resulting mRNA. Thereby, shifts may occur in the ORF and give rise to longer neo-antigen stretches harboring multiple immune recognition motifs (Vormehr et al., 2016).

A single altered amino acid may affect T-cell recognition in three ways (Figure 9):

- by creating an anchor amino acidby which the peptide acquires the ability to bind to an MHC molecule (Duan et al.2014);
- (II) by changing the T-cell receptor (TCR) binding properties resulting in a conformationally altered MHC-peptide complex, which is recognized by a different T cell clone not affected by central tolerance (Yadav et al. 2014)
- (III) by altering processing of the respective protein and its routing through MHC loading compartments, e.g. an altered proteasomal cleavage site preserving a ligand which normally would be degraded (Spierings et al., 2003, Pierce at al., 2001).

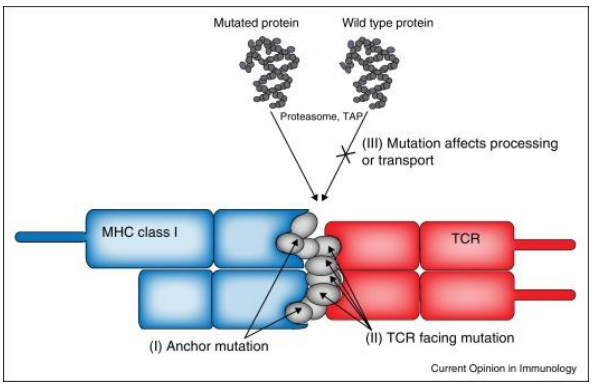


Figure 9. SNVs introduce neo-antigens through distinct mechanisms.
Mutations affecting anchor positions (I) or TCR facing residues (II) can create neo-antigens. Furthermore, novel epitopes can occur if a mutation alters the processing of a protein or the transport of a peptide into the ER (III).

1.4.2 Personalized cancer vaccines targeting the cancer mutanome

Neo-antigens represent potent targets for cancer immunotherapy vaccines, as they differentiate cancer from normal cells and can potentially be recognized as 'mutated self-antigens' by the mature T-cell repertoire, escaping central immune tolerance. Their systematic targeting by vaccine approaches, however, has been hampered by the fact that every patient's tumor possesses a unique set of mutations ('the mutanome') that must first be identified. Indeed, it is now appreciated that cancer is a patient-specific disease, where no two tumors are alike.

With the development of deep-sequencing technologies, it has become feasible to identify the mutations present within the protein-encoding part of the genome (the exome) of an individual tumor with relative ease. However, only a very small fraction of the non-synonymous mutations in expressed genes leads to the formation of a neo-antigen for which $CD4^+$ or $CD8^+$ T cell reactivity can be detected within tumor-infiltrating lymphocytes. The figure 10 depicts indeed the categories that indicate current estimates of the likelihood of neo-antigen formation in different tumor types, on the basis of their mutational load.

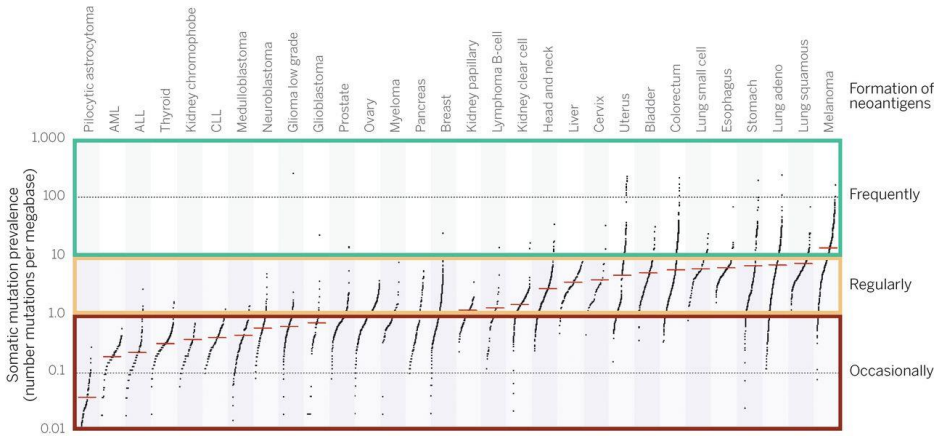


Figure 10. Estimate of the neo-antigen repertoire in human cancer (from Schumacher and Schreiber, 2015)

Data depict the number of somatic mutations in individual tumors. Every dot represents a sample while the red horizontal lines are the median numbers of mutations in the respective cancer types. Categories on the right indicate current estimates of the likelihood of neo-antigen formation in different tumor types.

Taken this into consideration, starting from the whole exome and transcriptome of tumor, computational algorithms are necessary for the prediction of likely neo-antigens to use for a personalized therapy in the clinical setting (Figure 11).

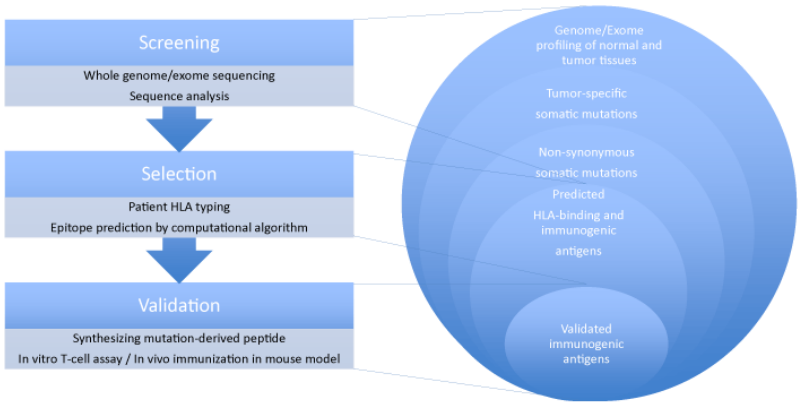


Figure 11. Cancer exome–based identification of neo-antigens.
The process of identifying cancer neo-antigens for targeted cancer immunotherapy consists of three steps: screening, selection, and validation of the candidate neo-antigens. First, the whole genome/exome sequence profile is comprehensively screened to identify tumor-specific somatic mutations (cancer neo-antigens) by massive parallel sequencing of tumor and normal tissues, respectively. When available, RNA sequencing data are used to focus on mutations in expressed genes. Second, computational algorithms are used for predicting the affinity of the mutation-derived peptides with the patient's own HLA and/or TCR. Alternative ways of epitope selection include "minigene" library screening and utilizing mass spectrometry analysis. Third, synthetic mutated peptides and wild-type peptides are used to validate the immunogenicity and specificity of the identified antigens by in vitro T-cell assay or in vivo immunization.

The identification of neo-antigens based on cancer exome data has been documented in a variety of experimental model systems and human malignancies. In 2012, two independent reports in mouse models provided a first preclinical proof that cancer exome–based approach can be used to identify neo-antigens that can be recognized by T cells.
Schreiber and colleagues demonstrated the feasibility of identifying spontaneously immunogenic tumor rejection antigens and their anti-tumor potency. Using massively parallel sequencing (MPS) and the MHC class I epitope prediction algorithm (IEDB algorithm), they characterized expressed mutations in highly immunogenic methylcholanthrene-induced sarcomas derived from immunodeficient $Rag2^{-/-}$ mice. Using class I prediction

algorithms, they identified mutant spectrin-β2 as a potential rejection antigen of the d42m1 sarcoma and validated this prediction. They also demonstrated that cancer immunoediting of d42m1 occurs via a T-cell-dependent immunoselection process that promotes outgrowth of pre-existing tumor cell clones lacking highly antigenic mutant spectrin-β2 and other potential strong antigens (Matsushita et al., 2012).

In the same year, Castle and colleagues presented a personalized immunotherapy approach to target the full spectrum of an individual tumor mutanome. They performed MPS and used the NetMHC algorithm to identify target neo-antigens for designing a cancer vaccine against B16F10 murine melanoma. They identified 962 non-synonymous somatic point mutations, 563 of which were actually expressed in tumor genes. They then selected 50 mutations for *in vivo* validation of immunogenicity and specificity, by administering either mutated or wild-type synthetic long peptides to the experimental mice. Approximately one third (16/50) showed the induction of a T-cell response, two of which were confirmed to have antitumor effects in both prophylactic and therapeutic settings, thereby qualifying mutated epitopes that include single amino acid substitutions as effective vaccines (Castle et al., 2012).

In 2015, the same group performed new vaccination studies employing the RNA vaccine technology. By using mRNA encoding mutated peptides identified in three different mouse tumor models (B16F10, CT26, 4T1 cancer cell lines)they revealed that a significant portion of mutations (21-45%) were immunogenic. Surprisingly, most neo-antigens were recognized by $CD4^+$ T cells (70–94%) and this subset controlled growth of advanced, highly aggressive mouse tumors. Building on these data they developed an *in silico* approach to extract the therapeutically effective vaccine candidates out of the dozens or hundreds of mutations which are typically identified by NGS (Kreiter et al., 2012).

Yadav et al. employed another approach for the identification of immunogenic neo-antigens in two tumor cell lines of MC-38 and TRAMP-C1 (Yadav et al.2014). They used mass spectrometry analysis combined with whole-exome/transcriptome sequencing. Of 1290 and 67 mutations expressed in MC-38 and TRAMP-C1, respectively, 170 and 6 were predicted to bind MHC-class I molecule by the NETMHC-3.4 algorithm. On the other hand, only 7 and 0, respectively, were shown to be present on the MHC-class I molecule by mass spectrometry. Two of the seven antigens were structurally predicted to be immunogenic, and both actually showed strong anti-tumor responses *in vitro*. Their study suggested that utilizing mass spectrometry, as another filter it is possible to reduce the burden of validation assays, which are extremely laborious, thereby simplifying the neo-antigen discovery process.

All the encouraging results obtained by several pre-clinical studies have promoted the use of personalized cancer vaccines in clinics, showing glimmers of success. Recently, two small clinical trials showed effective anti-tumor activity of vaccines tailored to match a patient's mutanome.

One group, led by Catherine Wu, evaluated a personalized peptide vaccine in a phase I study in patients with previously untreated high-risk melanoma after surgical resection (Ott et al., 2017). For each person, they formulated a vaccine that contained up to 20 protein fragments corresponding to the identified tumor mutations. Of six vaccinated patients, four had no recurrence at 25 months after vaccination, while two with recurrent disease were subsequently treated with anti-PD-1 therapy and experienced complete tumorregression, with expansion of the repertoire of neo-antigen-specific T cells. Indeed, they demonstrated that vaccination with neo-antigens both expands pre-existing neo-antigen-specific T-cell populations and induces a broader repertoire of new T-cell specificities in cancer patients, tipping the intratumoral balance in favor of enhanced tumor control.

The second group, led by Ugur Sahin, treated 13 melanoma patients with the RNA-based poly-neo-antigen approach (Sahin et al., 2017). Ten selected mutations per patient were engineered into two synthetic RNAs, each encoding five linker-connected 27mer peptides with the mutation in position 14 (pentatope RNAs). Eight patients who had no visible tumors at the time of vaccination remained tumor-free more than a year later. The remaining five participants' tumors had spread by the time they received the vaccine. Two of the five patients with metastatic disease experienced vaccine-related objective responses. One of these patients had a late relapse. A third patient developed a complete response to vaccination in combination with PD-1 blockade therapy.

These studies provide a strong rationale for further development of neo-antigens-targeted personalized cancer vaccines, alone and in combination with checkpoint blockade or other immunotherapies.

1.5 Combining neo-antigens-targeted cancer vaccines with checkpoint blockade

Both checkpoint blockade and neo-antigens-based cancer vaccines have shown promising results in the context of cancer immunotherapy but sometimes as monotherapy, they both revealed ineffective as anti-tumor treatment. Thus, a logical pursued approach has been the immunization of cancer patients with neo-antigens in combination with checkpoint blockade.

Several distinct supporting data have connected checkpoint blockade and cancer neo-antigens. Gubin and colleagues, who identified two biologically active neo-antigens in a mouse sarcoma, observed that used together, the two neo-antigens induced tumor rejection as efficiently as checkpoint blockade therapy (Gubin et al., 2014). These two observations highlighted the possibility that checkpoint blockade may operate even partially through amplification of the T-cell responses to neo-antigens. Duan and colleagues, who identified several biologically active neo-antigens of mouse sarcomas, tested the activity of a neo-antigen alone, CTLA-4 blockade alone, or both regimens together, and noted that the combination was significantly more effective than either agent alone (Duan et al., 2014).

Moreover, van Rooij and colleagues analyzed the tumor exome of a melanoma patient who had responded to CTLA-4 blockade and reported that T-cell response to a neo-antigen had increased significantly after CTLA-4 blockade (van Rooij et al., 2013).

Of note, two remarkable independent studies showed that the mutational landscape of a tumor determines sensitivity to checkpoints blockade. Starting from the malignant melanoma exomes of patients treated with CTLA-4 blockade, Snyder and colleagues elucidated a neo-antigens landscape that is specifically present in tumors with a strong response to CTLA-4 blockade. They validated this signature in a second set of patients with melanoma who were treated with anti–CTLA-4 antibodies, showing that predicted neo-antigens activated T cells from the patients treated with ipilimumab (Snyder el., 2014). Shortly afterwards, Rizvi and colleagues used whole-exome sequencing of non–small cell lung cancers treated with pembrolizumab to unravel the genomic determinants of response to this therapy. In two independent cohorts, higher non-synonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and progression-free survival (Rizvi et al., 2015).

Taken together, all these observations suggest that exploration of synergy between immunotherapy with neo-antigens and checkpoint blockade might be productive.

2. AIM OF THE STUDY

Among the several immunotherapeutic strategies for cancer treatment, therapeutic vaccination is emerging surely as a powerful approach to fight cancer. Therapeutic cancer vaccines are intended to boost the immune system's ability to recognize and destroy tumor antigens. To reduce off-target effects, neo-antigens represent an attractive target to direct the immune attack toward cancer cells, leaving safe the healthy ones. The improvements in omics science have surely increased the feasibility to easily identify this new promising class of tumor antigens that accumulates in cancer cell genome during tumor development, mainly as point mutations. Effective cancer treatment vaccines are intended to delay or stop cancer cell growth; to cause tumor shrinkage; to prevent cancer from recurrence. To be successful, a vaccine must stimulate specific immune responses against specific tumor targets and the immune responses must be powerful enough to overcome the barriers that cancer cells use to protect themselves from cytotoxic T cell-guided immune attack.

These premises have encouraged a promising research path in the lab where I carried on my PhD project, where the development of a new neo-antigens vaccine based on viral vectors has been pursued.
Starting from an *in silico* approach to extract the vaccine candidates out of thousands of mutations identified by NGS in a colon carcinoma cell line, Great Apes-derived adenovirus vectors, encoding these multiple neo-antigens *in tandem*, were developed.
Aim of my study has been the immunological validation of the selected neo-antigens and the assessment of the therapeutic efficacy of the developed neo-antigens encoded vaccine in the contest of arising or established cancer disease, in combination with PD-1 checkpoint blockade.

3. MATHERIALS AND METHODS

3.1 In silico analysis

3.1.1 Whole Exome and RNA sequencing

DNA and RNA library construction and next-generation sequencing of tumor sample were performed at Center for Translational Genomics and Bioinformatics (CTGB) - San Raffaele Hospital.

Genomic DNA was fragmented and used for Illumina library construction. Exonic regions were captured in solution using the Agilent mouse SureSelect All Exon kit 50Mb. Paired-end sequencing, resulting in 100bp from each end of fragments, was performed with the Hiseq2000 Genome Analyzer (Illumina) at target coverage of 120X.

RNA was fragmented and the sequencing library was prepared using Illumina TruSeq mRNA stranded kit. Sequencing was performed with the Hiseq2000 Genome Analyzer (Illumina) at target depth of 60 mln of paired-end reads.

3.1.2 Data processing

Whole exome sequencing NGS from BALB/c mouse tail was downloaded from SRA (experiment id: ERX391212) and used as control sample for comparison with tumor.

Quality control of sequenced reads was performed with FastQC 0.11.5 (Anders, 2010). Exome reads were trimmed by using trimmomatic-0.33 (Usadel, 2014) with the following parameters: HEADCROP:15; LEADING:5; TRAILING:5; SLIDINGWINDOW:4:15; MINLEN:75. RNAseq reads were trimmed by using as parameters: LEADING:5; TRAILING:5; SLIDINGWINDOW:4:15; MINLEN:50 HEADCROP:10;

Alignment of exome and RNAseq reads were performed by using HISAT2 2.0.4 (Salzberg, 2015) to mouse genome build mm10.

Reads that aligned to more than one locus with the same mapping score were filtered using Samtools 0.1.19 (Li, 2009).

Exome sequencing duplicated reads were marked using Picard's MarkDuplicates tool v1.14. Optimization of the alignment around indels and base recalibration was performed by using GATK software v3.4.46 (McKenna, 2010).

Somatic single nucleotide variants (SNV) were called by using mutect v1.1.17 (Cibulskis, 2013) and varscan2 v2.3.9 (Koboldt, 2012) with default parameters, by explicitly comparing the tumor sample vs the normal control sample.

Significant SNVs detected by at least one of the two variant callers were mapped onto the mm10 Refseq transcriptome by using Annovar (Wang, 2010) and retained only if induce a missense amino acidic change. For each missense SNV a 25 mer peptide was designed by selecting the mutated amino acid plus 12 wild type amido acids at both flanking regions.

MHC-I and MHC-II binding predictions were performed on 25-mer peptides by using the consensus method of IEDB 2.17 software (Moutaftsi, 2006). Within each 25-mer peptide, only the 9-mers that include the SNV and have a predicted IC50 ≤ 500nM were considered as likely to bind MHC-I. Similarly MHC-II binder epitopes were considered those that include the SNV and have a percentile rank score ≤ 1%.

Mutations were prioritized using filtering criteria within a funnel strategy. Initially were retained only SNVs with a positive MHC-I and MHC-II binding epitope prediction. Subsequently were selected the most confident SNVs that have a variant allele frequency in tumor ≥ 25%; at least 2 mutated reads in tumor and a ratio between the variant allele frequency in tumor and the variant allele frequency in control tissue ≥ 5. Only SNVs expressed with at least 1 mutated read in RNAseq were used to build a multi epitope construct.

3.2 Cell biological methods

3.2.1 Cell lines cultures

CT26 cells (ATCC) were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO, qualified performance, US origin) , 2mM L-glutamine (GIBCO), 1% (v/v) Penicilline/Streptomycin (GIBCO) at 37°C in 5% CO_2. FBS was heat inactivated (56°C, 30 min) before use.

HEK-293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; GIBCO) with 10% fetal bovine serum (FBS, GIBCO, qualified, Australia origin) at 37°C in 5% CO_2.

3.2.2 Infection and Western blot analysis

The expression of GAdN11-TPA 31ep was assessed by western blot of cell lysates from infected HEK293 (from human embryonic kidney carcinoma). Cells were cultured as described in 3.2.1. For infections, HEK293 cells were suspended in DMEM with 2% FBS and plated in 6-well plate (2.5 x 106 cells/well). Cells were infected at multiplicity of infection (MOI) of 150, thus 3,7 x 108 viral particle were used for each 6-well plate infection, in 1 ml of medium. Three hours after the infection, 2 ml of complete medium (10% FBS) were added to each well. 16h after the infection cells were harvested. Therefore, cells were scraped and centrifuged at 6000 rpm for 5 minutes at 4°C. After supernatant removal, pellets were washed once with PBS and centrifuged at 6000 rpm for 5 minutes at 4°C.Pellets were suspended in 100 ul of lysis buffer: Tris pH 7.5 (20 mM), NaCl (150 mM), EDTA pH 8 (1 mM), 1% TritonX-100 in the presence of protease inhibitors (Roche). Cell lysates were incubated on ice for 30 minutes, and then the soluble fraction was isolated by centrifugation at 14000 rpm for 20 minutes at 4°C.

The total protein concentration in cellular extracts was measured using Bio-Rad Protein Assay. 70γ of protein from each protein extract were denatured at 99°C for 5 minutes with NuPAGE® LDS Sample Buffer, in the presence of reducing agent (DTT).

Samples and protein marker were loaded on denaturing NuPAGE® 4-12% Bis-Tris Gradient gel. Proteins were transferred to a nitrocellulose membrane using i-Blot 2 Dry Blotting System (Invitrogen) according to manufacturer instructions. The membrane was blocked with 5% Milk in PBS 1X Tween 20 (0.1%) for 1 hour at room temperature with rocking. Then, it was incubated with primary antibody (rabbit α-HA, SIGMA H6908, or mouse α-GAPDH, SANTA CRUZ BIOTECHNOLOGY sc-32233) over-night, at 4°C with rocking. The proteins of interest were detected with HRP-conjugated goat α-mouse (SIGMA, A9044) or α-rabbit (SIGMA, A6154) and visualized with the Westar Super Signal (Cyanagen) substrate according to manufacturer's protocol. Western blot images were acquired using the ChemiDoc imaging system (Bio-Rad).

3.2.3 DNA and RNA extraction for NGS

CT26 tumors were collected from challenged BALB/c mice, freshly frozen, and then disrupted using TissueLyser (Qiagen). For RNA extraction, tumor tissues were homogenized in Buffer RLT (RNeasy Mini kit, Qiagen, cat

74104) with β-Mercaptoethanol. RNA was extracted as recommended by the instructions of manufacturer and solubilized in RNase-free water. DNA was extracted with DNAse blood and tissue kit (Qiagen, cat 69504) in accordance with the manufacturer's protocol and solubilized in Dnase-free water. RNA sequencing and whole exome sequencing were performed at San Raffaele Hospital, in Milan.

3.2.4 Validation of predicted tumor neo-antigen mutations

Two micrograms of tumor RNA, obtained as in section 3.2.3, were used for retrotranscription with Superscript first strand kit (Invitrogen). PCR was performed using the Phusion Hot start DNA Polymerase (Thermo scientific) with specific sets of primers. PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced at Bio-Fab Research srl.

The following primers were used:

Ag#4 Fw: 5'- accagaggagatgagtgtg -3'
Ag#4 Rv: 5'- ggcagccaggctatcatta -3'

Ag#5 Fw: 5'- gatgcagttggaagagcag -3'
Ag#5 Rv: 5'- tcgatgcttagcaccatgc -3'

Ag#10 Fw: 5'- cctatacagtggctgtcag -3'
Ag#10 Rv: 5'- gtaagaggagagatctccg -3'

Ag#11 Fw: 5'- tgctggaggagtaacagtg -3'
Ag#11 Rv: 5'- atgtgaaggtcacaccagc -3'

Ag#18 Fw: 5'- cagcatagacagcaccatc -3'
Ag#18 Rv: 5'- cacttcaggcttatcctgg -3'

Ag#23 Fw: 5'- acagtggtgtgctgagttc -3'
Ag#23 Rv: 5'- tactccagggcaaagcatg -3'

Ag#28 Fw: 5'- caacaagagctcacagtgg -3'
Ag#28 Rv: 5'- cactgatcttggaccttgg -3'

3.3 Animal experimental techniques

3.3.1 Animals

All experimental procedures were approved by the local animal ethics council and were performed in accordance with national and international laws and policies (EEC Council Directive 86/609; Italian Legislative Decree 26/14). The ethical committee of the Italian Ministry of Health approved this research. Animal handling procedures were performed under anesthesia, and all efforts were made to reduce animal numbers and minimize suffering. Six-week-old female BALB/c mice were purchased from Envigo. All day-to-day care of the mice was performed by trained mouse house staff at Plaisant, Castel Romano.

3.3.2 Tumor models

Tumor cells were cultured as described in section 2.2.1 and taken up in PBS. For subcutaneous (SC) tumor cells engraftment, mice were shaved at the right flank and anesthetized. Subsequently, the skin was lifted with forceps to inject CT26 tumor cells with a 1 ml syringe under the skin. Tumor growth was measured by caliper every 3-4 days using the formula $LxW^2/2$ (L as the largest and W the smallest diameter of the tumor).

For prophylactic studies and efficacy studies in early therapeutic setting, $2x10^5$ ($5x10^5$ to assess CT26 tumor cells sensitivity to CPIs) CT26 cells (injected volume 100 µl) were implanted sc at the right flank. For efficacy studies in established tumor setting, $2x10^6$ cells (injected volume 100 µl) were implanted. Before treatments start, animals were randomized (tumor size range 40-100 mm^3, tumor size average per group $\approx$ 70 mm^3). Animals were sacrificed as soon as signs of distress or a tumor volume above 2000 mm^3 occurred.

Tumor free mice from efficacy studies underwent to a second tumor cells engraftment on the left flank. After shaving and anesthetized, each mouse was sc implanted with $2x10^5$ cells (injected volume 100 µl).

3.3.3 Vaccinations

Viral vectors were administered via intramuscular injections in the quadriceps by delivering a volume of 50 µl per side (100 µl final volume). The injected dose for immunogenicity testing of GAd-CT26-31 vaccine was 5×10^8 or 5×10^7 viral particles (vp). All subsequent studies were performed by injecting 5×10^8 vp.

3.3.4 Antibodies administration

Antibodies were administered via intraperitoneal injection (IP).

For efficacy studies, 200 µg of α-mPD-L1 (BioXcell, clone 10F.9G2), α-mPD-1 (BioXcell, clone RMP114) or 100 µg of α-mCTLA-4 (BioXcell, clone 9H10) were used. Antibodies were diluted in PBS (injected volume 100 ul). For efficacy studies in early therapeutic setting, α-mPD-1 were injected at day0 (treatments start), day3, day6 or 7; in advanced therapeutic setting α-mPD-1 treatment was prolonged also at days 9,13,16.

For depletion studies, 200 µg of α-mCD8 (BioXcell, clone YTS169.4) or α-mCD4 (BioXcell, clone YTS191) were used. The injections were performed at: day-9, day-4, day-1 before vaccination (day0) and day6, day12.

3.4 Immunological assays

3.4.1 Ex vivo IFN-Ɣ ELISpot

MSIP S4510 plates (Millipore, Billerica, MA) were coated with 10 µg/ml of anti-mouse IFN-γ antibody (U-CyTech Utrecht, The Netherlands) and incubated overnight at 4 °C. After washing and blocking, mouse splenocytes were plated in duplicate at two different densities (1×10^6 and 5×10^5 cells per well) and stimulated overnight with single 25-mer peptides or peptide pool at a final concentration of 1µg/ml. The peptide diluent dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) and concanavalin A (Sigma-Aldrich, Milan, Italy) were used, respectively, as negative and positive controls. Plates were developed by subsequent incubations with biotinylated anti-mouse IFN-γ antibody (U-CyTech Utrecht, The Netherlands), conjugated streptavidin–alkaline phosphatase (BD Biosciences, San Jose, CA) and finally with 5-bromo-4-chloro-3-indoyl-phosphate/nitro blue tetrazolium 1-Step solution (Thermo Fisher Scientific, Rockford, IL). An automated ELISA –spot assay video analysis system automated plate reader was used to analyze plates.

ELISpot data were expressed as IFN-γ SFCs per million splenocytes. To have a positive ELISpot response these following conditions must occur: IFN-γ production present in Con-A stimulated wells, the number of spots seen in positive wells was three times the number detected in the mock control wells (dimethyl sulfoxide), at least 15 specific spots/million splenocytes.

3.4.2 Intracellular staining and FACS analysis

3×10^6 mice splenocytes were stimulated overnight at 37 °C in 5% CO_2 using single peptides or peptide pools as antigen at final concentration of 2 µg/ml for each peptide in presence of Golgi plug (BD Biosciences). Dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) was used as negative control, and phorbolmyristate acetate/ionomycin (Sigma-Aldrich, Milan, Italy) was used as positive controls. After overnight stimulation, mouse splenocytes were incubated with purified anti-mouse CD16/CD32 clone 2.4G2 (Fc block: BD Biosciences) and then stained in FACS buffer (phosphate-buffered saline, 1% fetal calf serum) with the following surface antibodies: allophycocyanin anti-mouse CD3e, clone 145-2C11; phycoerythrin anti-mouse CD4, clone L3T4; and PerCP anti-mouse CD8a, clone 53–6.7 (all from BD Biosciences). Intracellular staining was performed after treatment with Cytofix/Cytoperm and in the presence of PermWash (BD Biosciences) using fluorescein isothiocyanate anti-mouse IFN-γ, clone XMG1.2 (BD Biosciences). Stained cells were acquired on a FACS Canto flow cytometer and analyzed using DIVA software (BD Biosciences). At least 20,000 $CD8^+$, $CD3^+$ gated events were acquired for each sample.

3.5 Statistics

Statistical differences in medians between two groups were calculated with a nonparametric Mann–Whitney U test. All analyses were two-tailed and carried out using GraphPad Prism 6. n.s.: $P>0.05$, *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$, ****$P\leq0.0001$.

Two by two tables were used to compare two dichotomous variables (http://www.openepi.com/TwobyTwo/TwobyTwo.htm).

4. RESULTS

4.1 Generation of CT26 neo-antigens-encoded Adeno vaccine: pipeline for selection of CT26 neo-antigens

Neo-antigens represent ideal targets for T cell-based cancer immunotherapy. During oncogenesis, because of an uncontrolled tumor growth many mutations can arise and accumulate in cancer cells genome. Most of them are non-synonymous point mutations. If expressed, they cause amino acid substitutions in the relative encoded proteins.
The first step towards the generation of a neo-antigens-based cancer vaccine is the identification of cancer cells mutanome.
To select for high confidence neo-antigens to be encoded by the Adenoviral-based vaccine, CT26 cancer cell line was used. This murine colon carcinoma cell line is a well-known model of colon tumor engraftment, partially responsive to anti-PD1 therapy and characterized by high neo-antigens load.
CT26 tumors were harvested from engrafted syngeneic BALB/c mice, nucleic acids were isolated and underwent whole-exome sequencing and RNA sequencing. The alignment between the tumor exome and a normal control sample (exome of DNA extracted from BALB/c mouse tail) revealed tens of thousands somatic single nucleotides variants (SNVs). Of them, 2389 variants were retained because inducing an amino acid substitution in the encoded protein.
For each variant, a 25-mer peptide was designed by having the mutated amino acid in the center and 12 wild type amino acids at flanking regions.

A prioritization strategy followed to select the best neo-antigens candidates. Using filtering criteria within a funnel strategy, based on MHC class I and class II binding prediction, tumor allele frequency and RNA expression 31 neo-antigens were selected for vaccine development.

The first filter of binding prediction allowed the selection of 271 peptide variants. All 25-mer peptides bearing a 9-mer including the SNV and having a predicted IC50 $\leq$ 500nM were considered likely MHC-I binder, while all mutated 25-mers having a percentile rank score $\leq$1% were considered likely MHC-II binders.
To prioritize for mutations likely to be expressed in the majority of the tumor cells, variants that were present at a minimum of 25% allelic frequency were subsequently selected, resulting in 96 mutations.
Only SNVs found in expressed transcripts with at least one covering RNA-seq read were selected as candidate vaccine neo-antigens. The 31 selected antigens were inserted *in tandem* in a GAd vector to be tested *in vivo* as cancer vaccine (Figure 12).

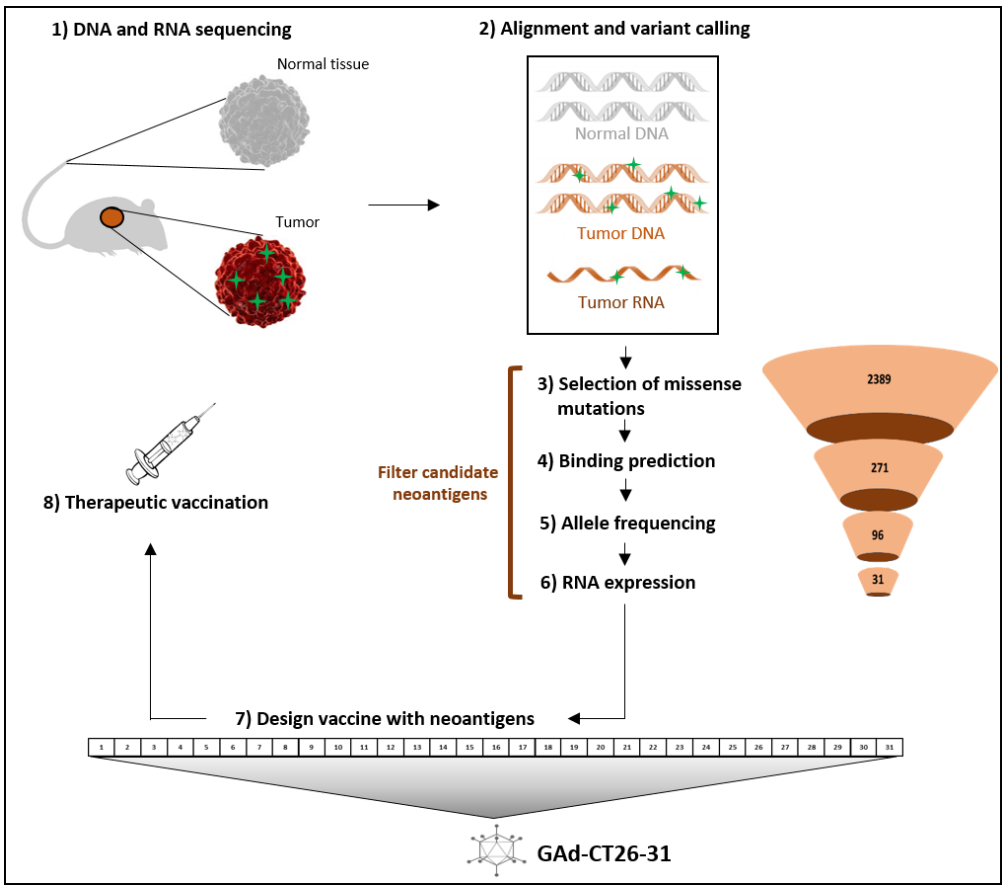


Figure 12. Identification and selection of the best neo-antigens candidates.

The flowchart resumes the approach used to generate a GAd-based vaccine encoding predicted neo-antigen candidates starting from the identification of CT26 mutanome.

Before testing the vaccine for immunogenicity and efficacy, GAd-CT26-31 was tested for expression of the insert after *in vitro* infection. HEK293 cells were chosen because they are easy to manipulate and are efficiently infected by adenoviral vectors. The expression of the insert was measured by using an antibody specific for a hemagglutinin tag (HA tag) present at the C-terminus of the neo-antigens expressing cassette. GAPDH expression levels were used as internal control for normalization. The adenoviral vector revealed good expression of the insert in infected cells (Figure 13).

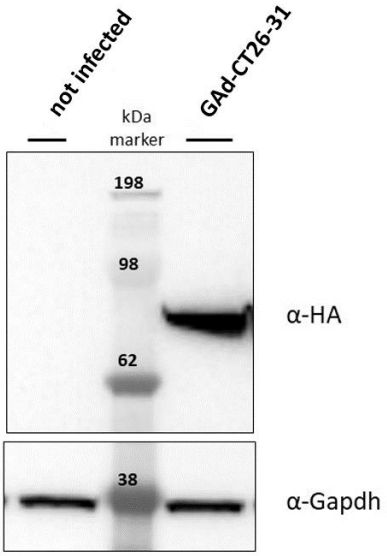


Figure 13. Western blot analysis to assess the expression of the 31 neo-antigens encoding cassette by infection in vitro.

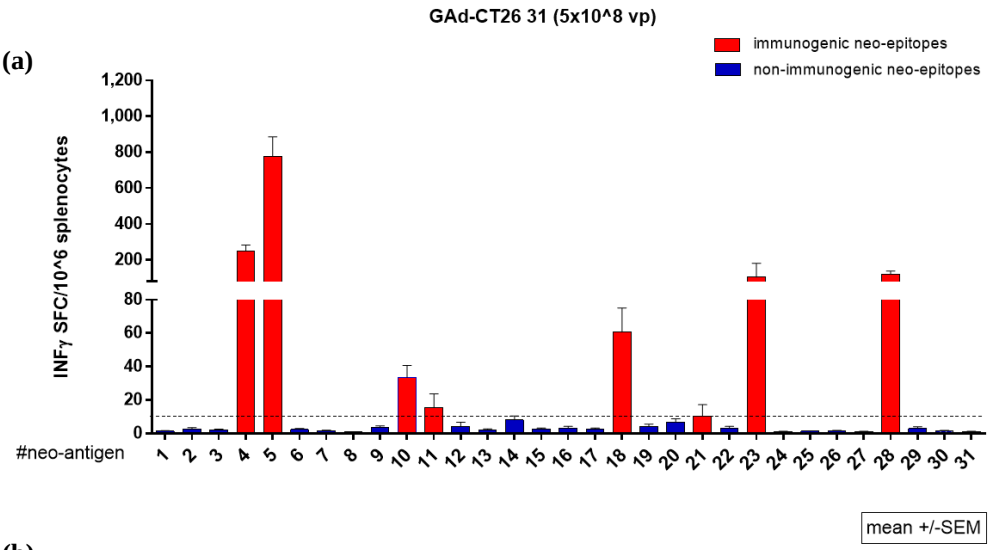
GAd-CT26-31 capability to express the insert encoding the 31 selected neo-antigens was evaluated by W.blot. Hek293 cells were infected with GAd-CT26-31 (MOI 150). The expected molecular weight for the HA tag (human influenza hemagglutinin) is 88,3kDa. Gapdh was used as a loading control. Not infected cells were included as negative control.

4.2 Immunogenicity of GAd-CT26-31 vaccine in naïve mice revealed eight immunogenic neo-antigens

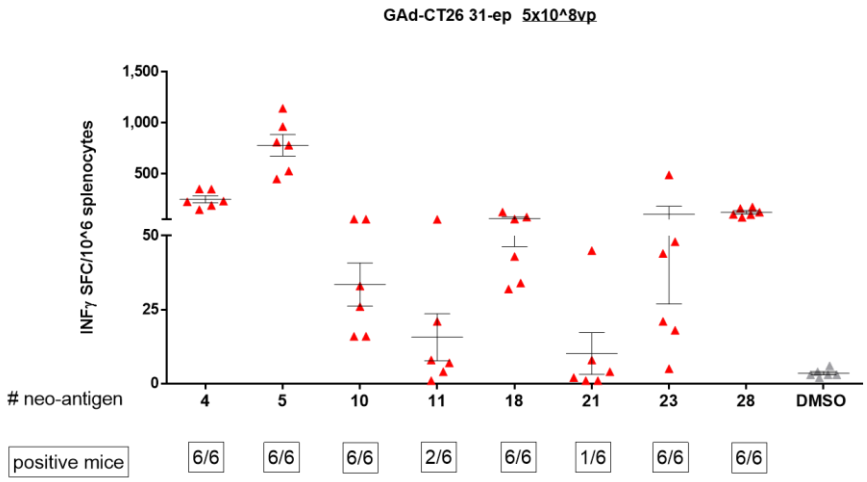
Immunogenicity of predicted neo-antigens represents a fundamental prerogative for a targeted vaccine approach as intended cancer vaccines. Therefore, the recognition by the host T cell repertoire of 'new' antigens encompassing mutated amino acids is the prerequisite for a likely therapeutic efficacy of the vaccine. To address this, we evaluated the potential capability of the thirty-one predicted neo-antigens encoded by GAd-CT26-31 to induce a specific immune response *in vivo*.
BALB/c mice were immunized at two different dosages, $5x10^8$ or $5x10^7$ viral particles (n=6 for each group). Three weeks post vaccination, splenocytes were harvested and analyzed for recognition of the encoded mutated peptides by IFN-γ ELISpot. Eight out of thirty-one predicted neo-antigens (26%) resulted to induce mutation-reactive IFN-γ -secreting T cells (Figure 14a,b).

To characterize the T-cell responses, T-cell subtype was determined by intracellular cytokine and surface marker staining. In a first step, the peptide pool was tested for recognition by the induced immune response via intracellular flow cytometric staining of INF-γ. This approach revealed that GAd-CT26-31 vaccine induced both $CD8^+$ and $CD4^+$ immune responses (Figure 14c). In a second step, the peptide pool was deconvoluted and each single peptide, resulted to be immunogenic by IFN-γ ELISpot was tested. $CD4^+$ T-cell reactivities against peptide #4, #18, #28 as well as $CD8^+$ T-cell reactivities against peptides #5 and #23 were revealed (Figure 14d). T-cell subtype reactivities against peptides #10, #11, #21 remain to be evaluated.

(a)



(b)



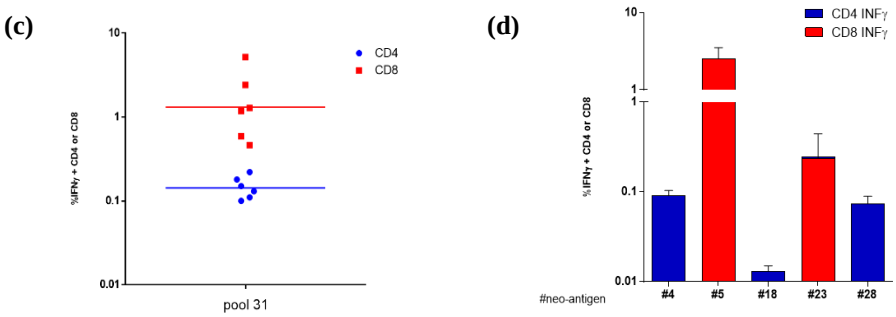


Figure 14. Immunogenicity of predicted neo-antigens.

(a) T-cell responses measured via IFN-γ ELISpot on splenocytes of mice vaccinated with 5x10^{8}vp of GAd-CT26-31. Immune responses were evaluated three weeks after immunization and expressed as number of T cells producing INF-γ per million of splenocytes. Neo-antigens are numbered in the graph according to their position in the vector. The dashed line shows the threshold of immunogenicity. Red or blue bars show immunogenic or non-immunogenic peptides, respectively. (b) T-cell responses evaluated for each immunogenic neo-antigens in individual mice. Number of positive mice (neo-antigen stimulated INF-γ SFC/10^6 splenocytes > 3x DMSO stimulated INF-γ SFC/10^6 splenocytes; INF-γ SFC/10^6 splenocytes > 15) is shown below.(c) Intracellular INF-γ staining and T-cell subtyping was performed after stimulation of splenocytes with the 31 peptide pool or (d) with each single peptide.

4.2.1 Comparison of GAd-CT26-31 versus GAd-CT26-5 vaccine in naïve: length does not affect immunogenicity

In a recent study, Kreiter and colleagues identified as immunogenic three out of the eight CT26 neo-antigens resulted to induce T-cell immune responses in our vaccine approach. They engineered a synthetic RNA pentatope encoding five predicted neo-antigens (according to their pipeline) connected by 10mer non-immunogenic glycine/serine linkers, that included neo-antigens #5, #18, and #28 (Kreiter et al., 2012). In order to assess whether increasing the number of neo-antigens in our adenoviral vector-based vaccine could affect the potency of immune responses, the neo-antigens predicted to be immunogenic in the above described pentatope construct were inserted into a GAd vector to develop a shorter vaccine, GAd-CT26-5 (Figure 15a). A comparison against GAd-CT26-31 was performed by testing both *in vivo.* BALB/c mice were immunized with GAd-CT26-31 or GAd-CT26-5 (n=6 for each group) at 5x10^8 viral particles. Three weeks post vaccination, an IFN-γ ELISpot assay was performed on splenocytes stimulated with each single shared mutated peptide. The immunological comparison revealed that the potency of T cell responses induced by the three shared immunogenic neo-

antigens was strictly comparable (Figure 15b), clearly showing that vaccine length does not affect immunogenicity.

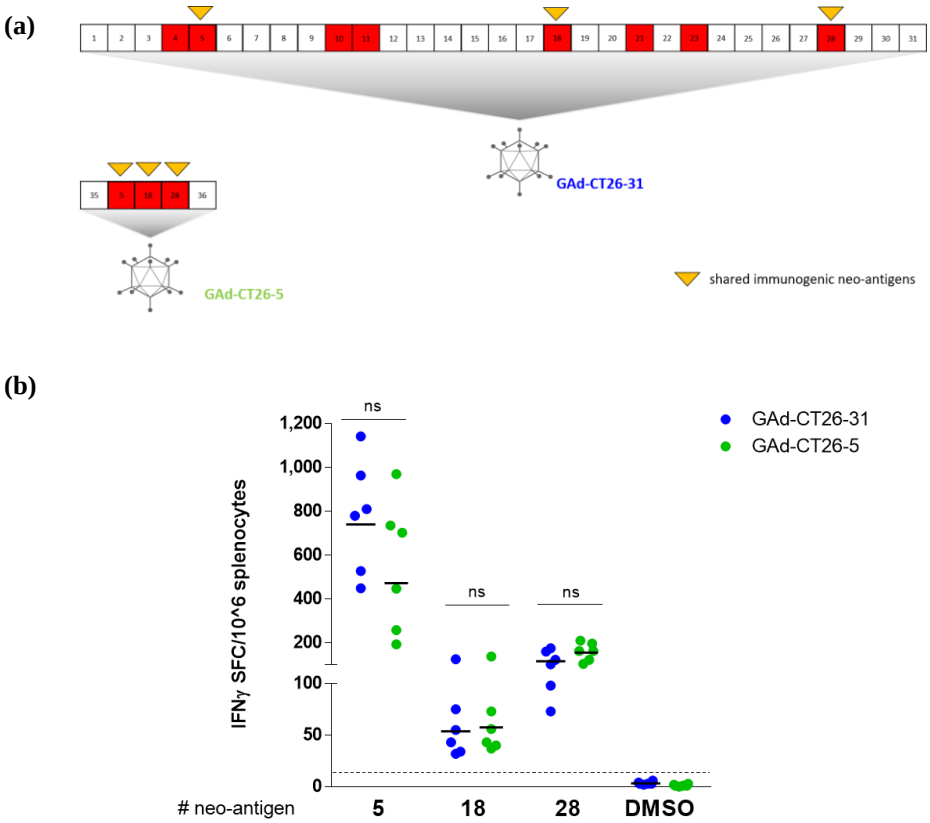


Figure 15. Immunogenicity testing of shared predicted neo-antigens in GAd-CT26-31 and GAd-CT26-5

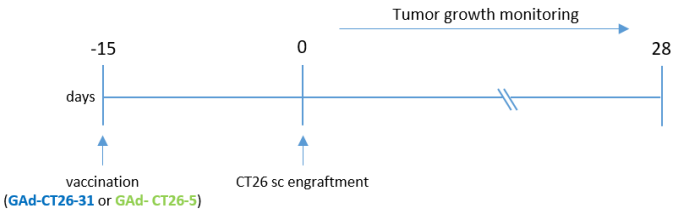
(a) Schematic representation of GAd-CT26-31 and GAd-CT26-5 vectors. Predicted neo-antigens resulted to be immunogenic are shown in red. Neo-antigens #5, #18, #28 are shared in the two vectors. (b) The immunological comparison of the three shared neo-antigens between GAd-CT26-31 (in blue) and GAd-CT26-5 (in green) was performed via IFN-γ ELISpot on splenocytes three weeks post immunization and expressed as number of T cells producing INF-γ per million of splenocytes.

4.3 GAd-CT26-31 vaccine resulted 100% effective in prophylactic setting on CT26 tumor

Immunogenicity testing showed that tumor mutations are frequently immunogenic and neo-antigens directed T cells are both $CD8^+$ and $CD4^+$. To

investigate whether these cancer mutations are good vaccine targets and, we first evaluated vaccine efficacy in a prophylactic setting of tumor engraftment. In this setting, the vaccine was administered before tumor cells inoculum (Figure16a).
BALB/c mice were immunized with GAd-CT26-31 vaccine at a dosage of 5x10^8vp. Two weeks later, all vaccinated mice were engrafted with $2x10^5$ CT26 cells by subcutaneous administration on the right flank. Tumor growth was then monitored over-time. In the same study, a comparison between GAd-CT26-31 and GAd-CT26-5 was performed to assess if a different number of immunogenic neo-antigens could affect anti-tumor immune-mediated protection. Results clearly showed a complete protection from tumor development in 100% GAd-CT26-31-treated mice, compared to mock vaccinated mice. No difference in terms of tumor protection was found between GAd-CT26-31 and GAd-CT26-5, since tumor growth was totally prevented also in GAd-CT26-5 vaccinated mice.

(a)



(b)

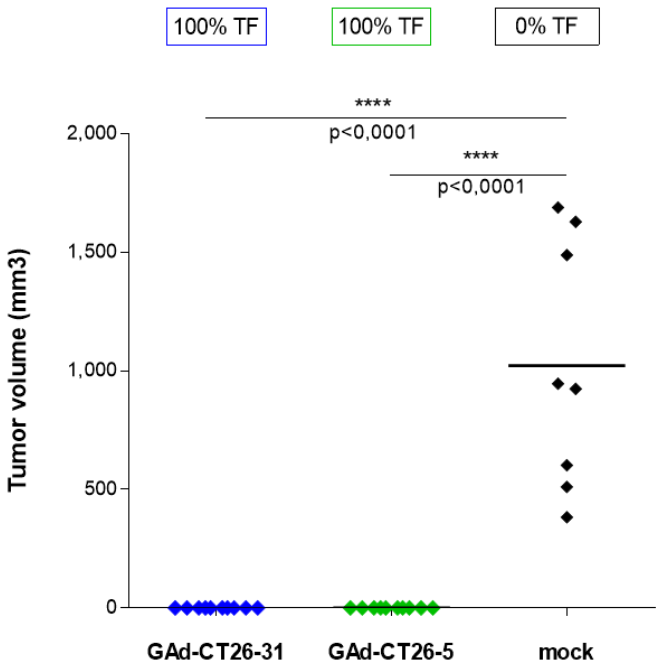


Figure 16. Efficacy of GAd-CT26-31 or GAd-CT26-5 vaccines in prophylactic setting.

(a) Schematic representation of the experimental plan adopted to evaluate GAd-CT26-31 efficacy in prophylactic setting, in comparison with GAd-CT26-5. Naïve BALB/c mice were immunized with 5x10^8vp of GAd-CT26-31 or GAd-CT26-5, two weeks after vaccination mice were challenged with CT26 tumor cells, by subcutaneous administration. Tumor growth was monitored over-time. (b) Tumor volume (mm^3) detected 28 days post tumor challenge is shown. In both groups, all mice resulted tumor free (TF) after single vaccination with CT26 neo-antigens-encoded vectors (each blue or green symbol represents a treated mouse with GAd-CT26-31 or GAd-CT26-5 respectively).

4.4 Combining GAd-CT26-31 vaccine with PD-1 checkpoint blockade improves anti-tumor efficacy in therapeutic setting

A therapeutic cancer vaccine can be considered effective if able to control tumor growth or better to induce a complete tumor regression in a contest of established disease. Therefore, to evaluate GAd-CT26-31 vaccine efficacy, it was tested in two different therapeutic settings: early and advanced tumor setting. In the first case, the vaccination was performed in an early stage of tumor growth, few days after CT26 tumor cells engraftment; whereas in the second case the vaccine was administered when the tumor was already visible, palpable and measurable in a defined range of tumor volume. In order to ensure the highest therapeutic efficacy possible, a combinatorial strategy was adopted. GAd-CT26-31 vaccine was combined with a treatment of checkpoint blockade targeting the inhibitory receptor PD-1.

Before choosing PD-1 blockade as combined treatment to the vaccine, the sensitivity of CT26 tumor cells to different checkpoints blockade was tested in an early therapeutic setting of efficacy. CT26 cells were sc injected three days before antibodies treatment targeting the inhibitory receptors CTLA-4 and PD-1 or the inhibitory receptor-ligand PD-L1. In each case, treatment was performed on days 0, 3, 6 (200 μg/mouse for PD-1 or PD-L1; 100 μg/mouse for CTLA-4). A different sensitivity of CT26 tumor cells emerged, in terms of complete responder mice. Given the high responsiveness to CTLA-4 blockade as monotherapy (Figure 17), the pathway PD-1/PD-L1 was chosen as target for blockade, in particular the inhibitory receptor PD-1 was selected since more relevant in current clinical studies.

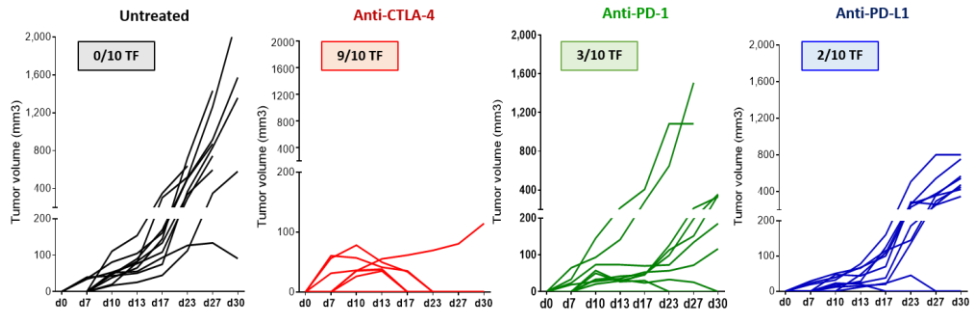


Figure 17. Sensitivity of tumor cells to CPIs.

BALB/c mice were challenged sc with CT26 cells three days before treatment start (day 0). Antibody-mediated checkpoints blockade was performed on days 0,3,6 (ip). Shown is tumor growth trend in individual mice over-time. The fraction of tumor free mice is reported in relative boxes.

The experimental protocol adopted in the efficacy studies for early therapeutic setting is shown in Figure 18a. BALB/c mice initially underwent to CT26 tumor cells engraftment by subcutaneous administration on the right flank ($2x10^5$ CT26 cells *per* mouse). Three days after tumor challenge mice were clustered into four groups for treatment start (day 0). Treatments included GAd-CT26-31 vaccine or PD-1 blockade as monotherapy and the combination of both (n=12 mice *per* group). An untreated group was also included. Vaccination was performed*via* intra-muscular route once, at day 0. PD-1 blockade by intra-peritoneal administration of αPD-1 mAb was performed three times (day 0, 3, 6). Tumor volume was monitored over-time to check tumor growth trend after treatments. Notably, the combination strategy revealed more effective than monotherapies. A pilot study, whose results are shown in Figure 18b,c,d, revealed a complete tumor shrinkage in 75% of mice treated with combined vaccine and αPD-1 versus 33% of tumor free mice obtained after treatment with αPD-1 alone. In this study a complete inefficacy of the vaccine was detected. However, a modest efficacy as monotherapy in this setting was observed in other studies (data not shown). Although, in this more advanced tumor setting, the combinatorial strategy did not lead to 100% of tumor free mice, a significant control over tumor volume was noted in not cured mice, still bearing a tumor mass, compared to vaccine or checkpoint blockade alone (Figure 18c). Furthermore, the combined treatment prolonged survival of treated mice,

100% of mice were still alive at the end of the study compared to other treatments (Figure 18d).

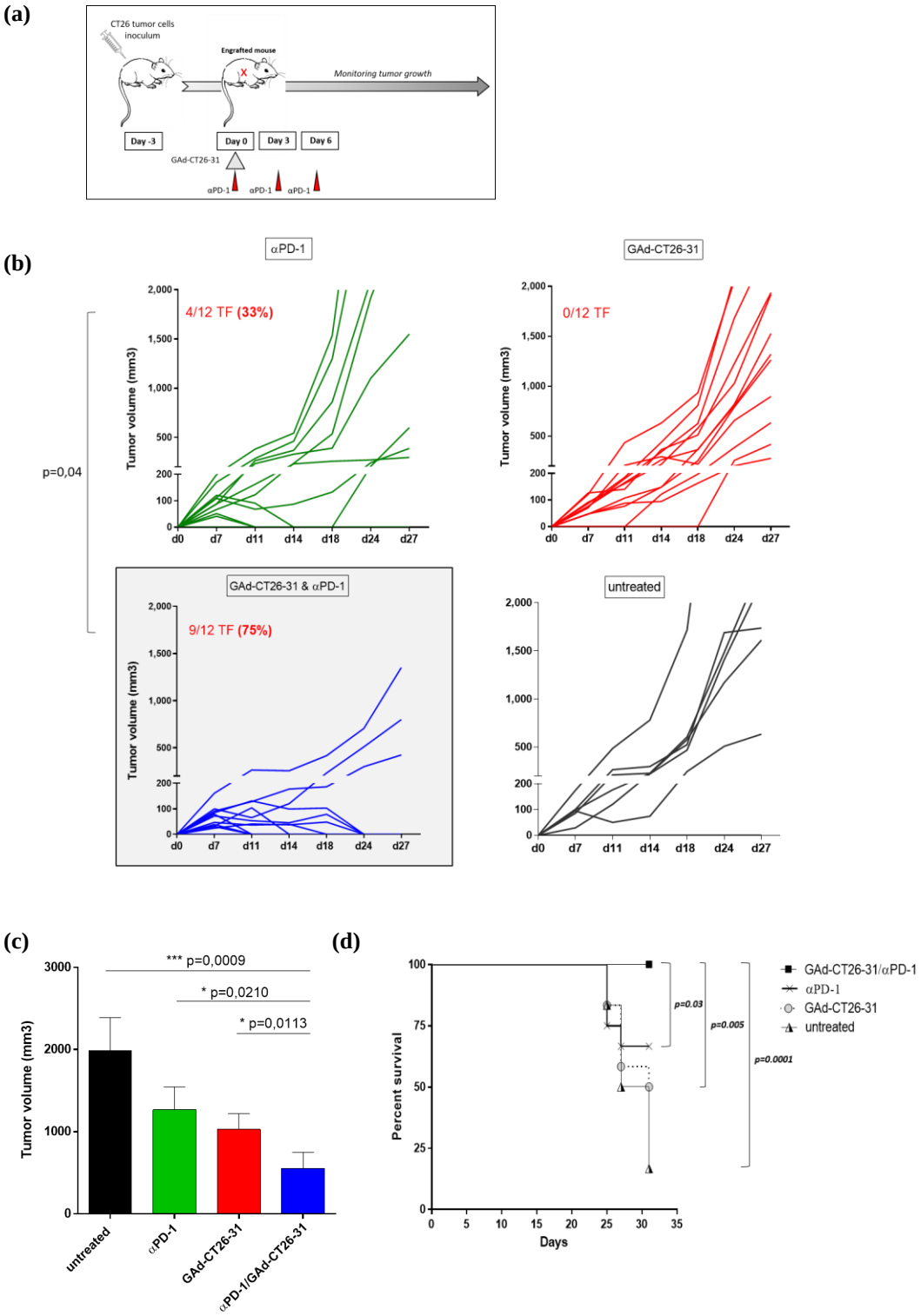


Figure 18. Efficacy of GAd-CT26-31 in combination with αPD-1 in early therapeutic setting.

(a) Schematic representation of treatments in early therapeutic setting. The therapeutic vaccination, in combination with PD-1 blockade, was performed three days after tumor cells engraftment (day 0). αPD-1 mAb administration was repeated at day 3 and day 6. The therapeutic effect of the two monotherapies was also evaluated, by monitoring tumor growth over-time. (b) Each line in each plot represents the trend of tumor growth for each treated mouse until day 27 from treatments start. In each plot the number of complete responders (tumor free mice; TF) is reported. Chi square is calculated on the number of tumor free mice (c) Tumor volume mean (+/- SEM) of remaining not responding mice is reported in the graph. (d) Survival of treated mice over-time until day 31 from treatments start.

In order to enforce the therapeutic efficacy of the combination GAd-CT26-31/αPD-1 a more advanced tumor condition was established *in vivo*.

BALB/c mice were engrafted with a higher dose of CT26 tumor cells ($2x10^6$ cells *per* mouse). About seven days after challenge, tumor masses were measured and all mice bearing a tumor volume ranging from 45-95 mm^3 were enrolled. Mice were randomized (tumor volume mean $\approx$ 70 mm^3) and treatments started at day 0. In the therapeutic setting two therapeutic combinations were tested and compared with αPD-1 blockade: GAd-CT26-31/αPD-1 and GAd-CT26-5/αPD-1. The latter combination was introduced to evaluate if a shorter vaccine, encoding fewer neo-antigens, could reveal less effective than a longer one. As performed in the early therapeutic setting, a single dose of vaccine was administered (day 0), whereas the PD-1 checkpoint blockade was prolonged over-time (day 0,3,6,9,13,16). A pilot study revealed that single agent therapy only had a very modest effect on tumor growth, while the combination of anti-PD1 and GAd-CT26-31 provided important tumor control, leading to tumor regression in 55% of mice. Remarkably, in presence of established tumors, the GAd-CT26-31 and GAd-CT26-5 demonstrated a different efficacy, with the longer GAd-CT26-31 vector showing superior efficacy than smaller GAd-CT26-5 (Figure 19).

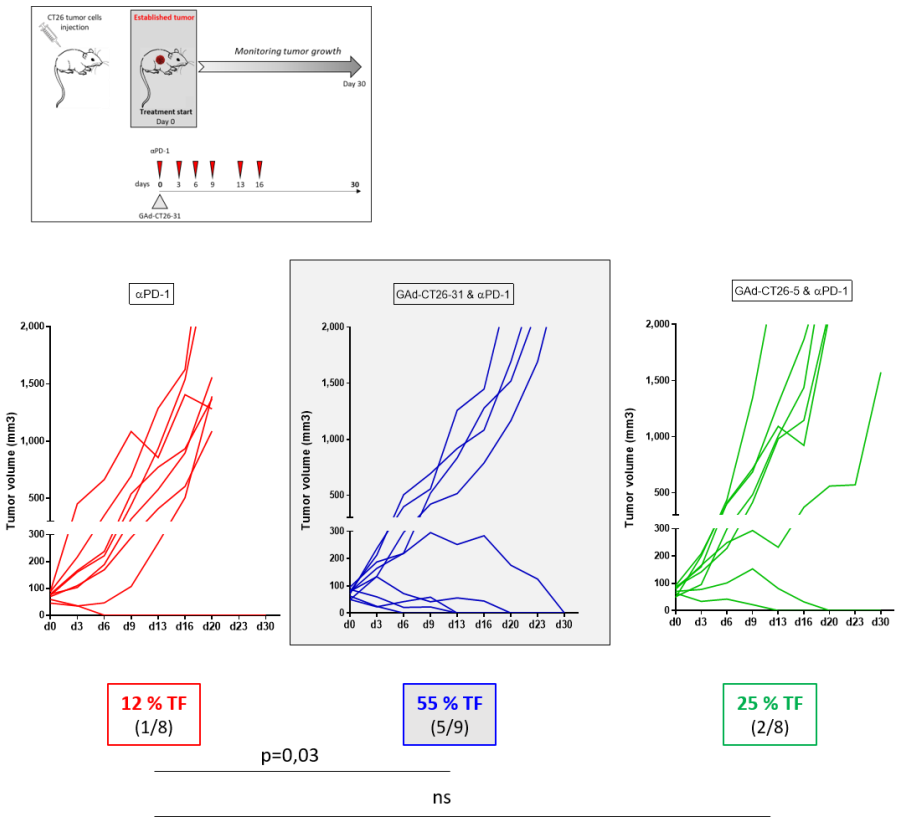


Figure 19. Efficacy of GAd-CT26-31 and GAd-CT26-5 vaccine in combination with αPD-1 in advanced therapeutic setting.

BALB/c mice were challenged sc with CT26 cells. One week later, mice with established tumors were randomized and treated with αPD-1 alone (red) or in combination with 5x10^8vp of GAd-CT26-31 (blue) or GAd-CT26-5 (green). Vaccine was administered at day 0 (im), while αPD-1 was given at day 0,3,6,9,13,16 (ip). Shown is tumor growth trend in individual mice over-time. The percentage of tumor free mice is highlighted below each group. Chi Square is calculated on the number of tumor free mice.

4.5 GAd-CT26-31 vaccine/ αPD-1 combination induces T cell-mediated protection against tumor re-challenge

The data shown demonstrated that vaccination combined with checkpoint blockade can induce efficient tumor control and survival benefit. However, long-lasting memory of tumor-specific T cells is necessary to permanently eradicate tumors.

Therefore, to check long-term immune responses and immune-mediated protection from the onset of a new tumor after a second challenge, all animals that had full tumor regression were again inoculated with CT26 tumor cells and tumor growth was monitored until day 155 from the first engraftment, 105 days after re-challenge. Complete responders mice from both therapeutic settings underwent second challenge. In figure 20 long term tumor growth monitoring is shown for complete responder animals from the advanced therapeutic setting. 100% of re-challenged mice remained completely tumor free along the monitoring time.

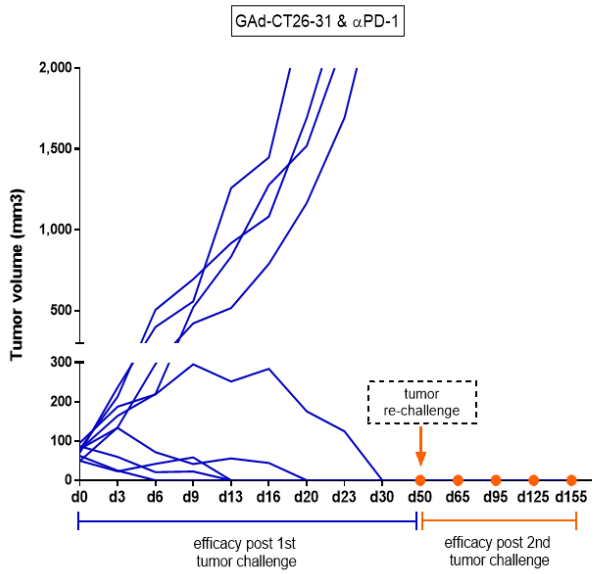


Figure 20. Long lasting efficacy of GAd-CT26-31/αPD-1 combination in complete responder mice from advanced therapeutic setting.

Fifty days after treatments start, five out nine BALB/c mice resulted to be completely cured by the combination of GAd-CT26-31 vaccine and αPD-1 were re-challenged (sc) with $2x10^5$ CT26 cells on the contralateral left flank. Tumor development was monitored over-time for 105 days long. Re-challenged tumor free mice (orange symbols) remained cured for all monitoring time.

4.6 Therapeutic efficacy of GAd-CT26-31/αPD-1 combination correlates with induction of neo-antigens T cell responses, higher and broader than αPD-1 monotherapy

Efficacy studies, both in early and therapeutic setting, showed that the combination of GAd-CT26-31 vaccine and αPD-1 is highly effective. However, a modest fraction of treated mice remained not cured by the adopted therapeutic strategy. Therefore, given the presence of two distinct groups of mice, either cured or not cured, we asked whether the efficacy of treatment could be associated with efficient induction of T cell responses just in responder animals but not in non-responder ones. To address this, splenocytes of responder and non-responder mice were isolated, stimulated with the pool of 31 peptides encoded by the vaccine and analyzed by *ex-vivo* IFN-γ ICS.
Results clearly showed that responders were able to mount an efficient tumor specific T cell response post vaccination (30-40 days after immunization), mostly with induction of CD8 IFN-γ secreting cells at much higher level than non-responder mice, that showed very weak, almost absent T cell responses (Figure 21).

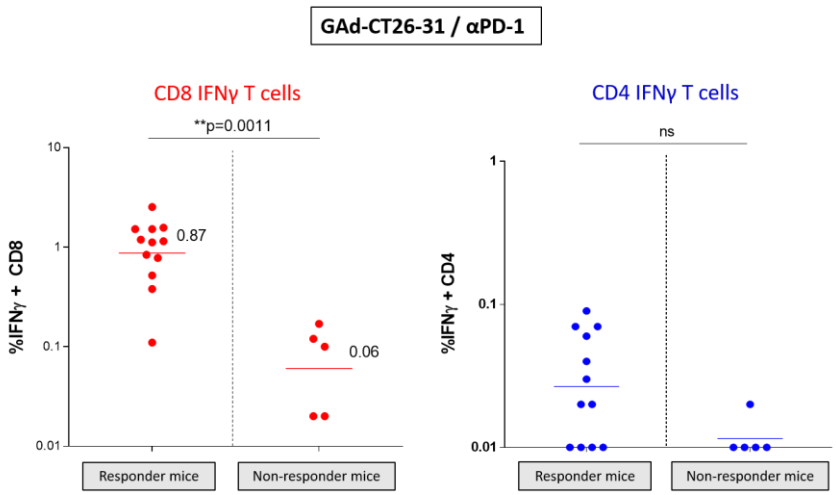


Figure 21. Immunological analysis in responder and non-responder mice receiving GAd-CT26-31 and αPD-1 in therapeutic setting.

T cell responses to mutated neo-antigens (pool of 31 peptides) were analyzed by INF-γ ICS in mice cured (responders) or not cured (non-responders) by combo treatment. $CD8^+$ and $CD4^+$ T cell responses were measured in both groups 30-40 days post treatment start. Geometric mean is shown in the two graphs.

Moreover, to understand the specific contribution of the vaccine over anti-PD-1 treatment, T cell responses to mutated neo-antigens were measured in responder mice, which received αPD-1 as monotherapy or αPD-1 combined to GAd-CT26-31 vaccine. Notably, whereas the treatment with αPD-1 only induced low T cells responses against a single mutated neo-antigen (neo-Ag#4), the vaccine increased and broadened the T cell responses induced by αPD-1, eliciting tumor-specific T cell immunity against each of the eight neo-antigens resulted to be immunogenic in naïve mice (neo-Ag#4, #5, #10, #11, #18, #21, #23, #28), suggesting that they all may contribute to enhance the therapeutic effect (Figure 22).

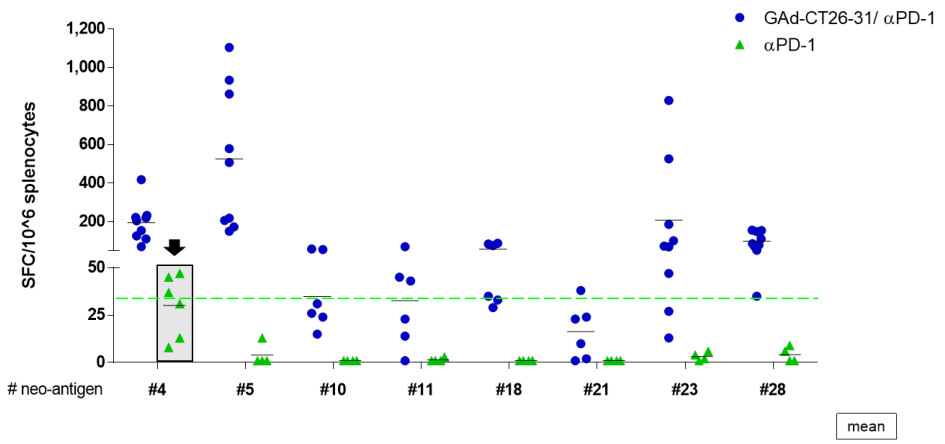


Figure 22. Specific T cell immune response in cured mice after treatment with GAd-CT26-31/ αPD-1 or αPD-1 in therapeutic setting.

4 weeks after treatment start (GAd-CT26-31/ αPD-1 in blue or αPD-1 in green) responder mice cured by each treatment (n=9/12 for combined strategies, n=4/12 for PD-1 blockade) in early therapeutic setting were analyzed for specific T cell immune responses against all immunogenic neo-antigens via IFN-γ ELISpot on splenocytes. For each sample the number of T cells producing INF-γ per million of splenocytes is reported. The black arrow highlights the unique specific T cell response induced by αPD-1. The green dashed line shows the threshold of positivity for αPD-1.

4.7 Therapeutic efficacy of GAd-CT26-31 in combination with αPD-1 depends on CD8⁺ T cell responses

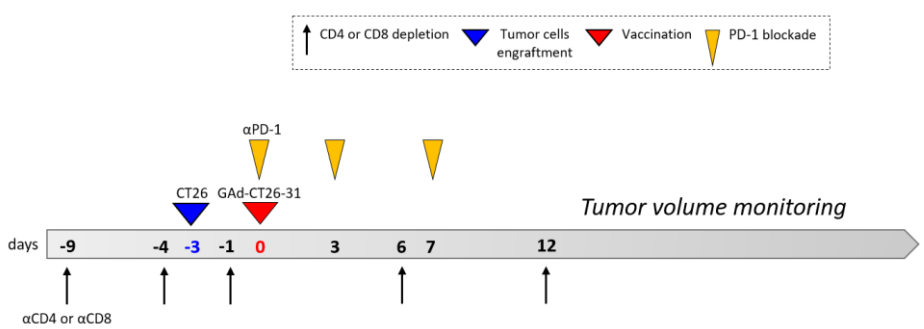
It was shown that predicted neo-antigens, resulted to be immunogenic, are recognized by $CD4^+$ or $CD8^+$ T-cells in a quite balanced manner (Figure 14d) and that therapeutic vaccination with GAd-CT26-31, directed against both

MHC class I and II mutations conferred tumor control in most of tumor-bearing mice, with 75% or 55% of complete tumor regression in early or advanced therapeutic setting, respectively (Figure 18b, 19). Moreover, therapeutic efficacy of GAd-CT26-31/αPD-1 combination correlated with induction of high-level neo-antigens T cell responses compared to non-responder mice (Figure 21). Thus, to investigate the contribution of $CD4^+$ and $CD8^+$ T cells to the therapeutic effect of GAd-CT26-31/αPD-1 in more details we performed studies of *in vivo* depletion *via* anti-CD4 (αCD4) or anti-CD8 (αCD8) antibodies. The early therapeutic setting was initially used for depletion studies: tumor cells were engrafted three days before vaccination and first injection of αPD-1 (repeated at day 3 and 7); anti-CD4 or anti-CD8 *in vivo* depletions were performed before (day -9, -4, -1) and after (day 6, 12) vaccination. From day 0 the monitoring of tumor volume started (Figure 23a).

It was also shown that the single-agent treatment with αPD-1 resulted less effective than the combination strategy with the vaccine. Moreover, the analysis of immune responses on fewer mice, completely cured by the PD-1 blockade, revealed induction of T cells against a single mutated neo-antigens (neo-Ag#4), previously resulted to be recognized by $CD4^+$ cells, compared to the broader reactivities, both $CD4^+$ and $CD8^+$, induced by GAd-CT26-31/αPD-1 in combination.
Therefore, to highlight a likely different T cell contribution to anti-tumor efficacy by the two different therapeutic approaches, we performed the same studies without vaccination as well.

Interestingly, results clearly showed a fundamental contribution of $CD8^+$ T cells for therapeutic efficacy in both approaches. After $CD8^+$ depletion, a significant reduction in the number of complete responders was shown (Figure 23b) and no control on tumor growth was appreciated in not cured mice (Figure 23c).

(a)



(b)

Treatment	Complete responder mice	Non-responder mice		
GAd-CT26-31/ αPD1	9	3	⇨ 75% TF	p=0,0001
GAd-CT26-31/ αPD1/ **αCD8**	0	12	⇨ 0% TF	ns
GAd-CT26-31/ αPD1/ **αCD4**	6	6	⇨ 50% TF	
αPD1	3	9	⇨ 25% TF	p=0,04
αPD1/ **αCD8**	0	11	⇨ 0% TF	ns
αPD1/ **αCD4**	3	9	⇨ 25% TF	

(c)

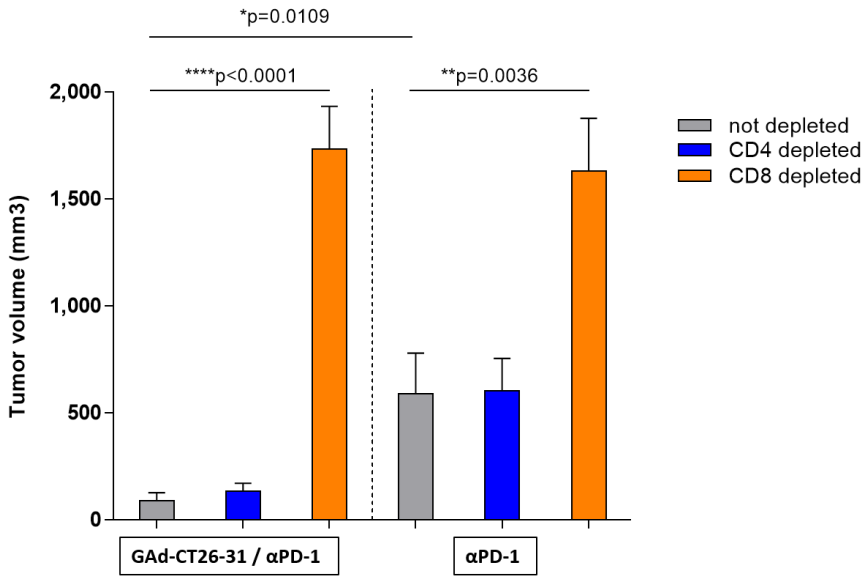


Figure 23. Therapeutic contribution of CD8⁺ and CD4⁺ T cells in GAd-CT26-31/αPD-1 or αPD-1 treatments.

(a) Schematic representation of the experimental setting adopted for in vivo depletions of CD8⁺ or CD4⁺ T cells. At day 0 the PD-1 blockade was performed as single-agent treatment (not shown in the schematic) or in combination with GAd-CT26-31 vaccine. αPD-1 was administered at day 0,3,7; αCD4 or αCD8 by ip injection at day -9, -4, -1 from treatment start. CT26 cells ($2x10^5$ cells/mouse) were engrafted at day-3. (b) The table summarizes results obtained post depletion in terms of complete responder mice or not cured, tumor-bearing mice. Chi Square is calculated on the number of tumor free mice. (c) Tumor volume mean of not cured mice is reported after GAd-CT26-31/αPD-1 treatment, with or without CD4 or CD8 depletion (on the left) or αPD-1 treatment, with or without CD4 or CD8 depletion (on the right). Grey bars for not depleted, blue bars for CD4⁺ depleted, orange bars for CD8⁺ depleted.

4.8 Dissecting the mechanisms underlying tumor escape: Can tumor mutations loss explain inefficacy of combined treatment?

Efficacy studies clearly showed that some tumors can escape the host adaptive immune response. To evade immune-mediated control several molecular mechanisms can be adopted. These mechanisms include: 1) alteration of the expression of classical and non-classical human leukocyte antigens (HLAs) and/or loss of tumor antigens, 2) loss of co-stimulatory molecules which are essential in inducing a powerful immune response, 3) the production of cytokines which are strongly immunosuppressive, and 4) induction of anergy or clonal deletion or suppressor cells.

To address the point we started to investigate some of the above mentioned mechanisms of immune evasion in some non-responding tumors, remained not cured in efficacy studies. Surely, the loss of tumor antigens may represent one of the crucial determinants underlying the lack of efficacy of a vaccine developed to encode for tumor-specific mutated proteins.

Our analysis has so far involved a restricted number of escapers. However, a huger cohort of non-responder tumors is planned to dissect.
After tumor collection and tissue digestion RNA was extracted from tumor homogenates and retro-transcribed. Predicted mutations were validated by PCR on cDNA. All mutations analyzed so far have been confirmed (Table 2), allowing to exclude the loss of tumor mutations as mechanism of immune-evasion of tumors.

	Neo-antigens						
#mouse	**#4**	**#5**	**#10**	**#11**	**#18**	**#23**	**#28**
#815	+	+	+	+	+	+	+
#816	+	+	+	+	+	+	+
#822	+	+	+	+	+	+	+

Table 2. Summary table of PCR validation of predicted mutations in tumors of escaper mice.

The symbol '+' indicates the positivity to the presence of the predicted mutations in the first three analyzed escaper tumors that did not respond to GAd-CT26-31/αPD-1 treatment in early therapeutic setting of efficacy.

5. DISCUSSION

There are compelling pieces of evidence that a primary function of the immune system is to confer protection against cancer. Alexandre Corthay briefly traced eight main evidences emerged over decades of studies to address this point in order to support the involvement of immune system in the host defense against cancer. First, primary immunodeficiency in mice and humans is associated with increased cancer risk. Second, organ transplant recipients, who are treated with immunosuppressive drugs, are more prone to cancer development. Third, acquired immunodeficiency due to infection by human immunodeficiency virus (HIV-1) leads to elevated risk of cancer. Fourth, the quantity and quality of the immune cell infiltrate found in human primary tumors represent an independent prognostic factor for patient survival. Fifth, cancer cells harbor mutations in protein-coding genes that are specifically recognized by the adaptive immune system. Sixth, cancer cells selectively accumulate mutations to evade immune destruction ("immunoediting"). Seventh, lymphocytes bearing the NKG2D receptor can recognize and eliminate stressed premalignant cells. Eighth, a promising strategy to treat cancer consists in potentiating the naturally occurring immune response of the patient, through blockade of the immune checkpoint molecules as CTLA-4, PD-1, or PD-L1 (Corthay, 2014).

In this scenario, the nature of the antigens that allow the immune system to recognize cancer cells represents a crucial point in order to direct immune attack specifically toward cancer. Tumor cells deviate from normal body cells in two immunologically important ways. First, tumor cells carry tens to hundreds of protein-changing mutations that are either responsible for cellular transformation or that have accumulated as mere passengers. Second, because of genetic and epigenetic alterations, tumor cells express a series of self-proteins that are normally not present or present at lower levels. These changes lead to the presentation of an altered repertoire of MHC-associated peptides.

As compared with non-mutated self-antigens, neo-antigens have been postulated to be of particular relevance to tumor control, as the quality of the T cell pool that is available for these antigens is not affected by central T cell tolerance.

With the development of deep-sequencing technologies, the identification of mutations within the exome of an individual tumor and the prediction of potential neo-antigens has become easier, allowing *ad personam* analysis more feasible. This need has become more and more important considering that a large fraction of mutations in human tumors is not shared between patients at meaningful frequencies and may therefore be considered patient-specific.

The pipelines used to identify neo-antigens can vary substantially. Anyway, a robust pipeline allowing the filtering of cancer exome data is essential, in particular for tumors with high mutational load.

The strategy adopted in this project to predict the best neo-antigen candidates relied on the "funnel strategy". Starting from 2389 missense mutations, the filtering was based on three main prerequisites: prediction of MHC-I or -II binding, allele frequency and RNA expression. They allowed restricting the selection to high confident thirty-one neo-antigens, meant as 25-mer peptides bearing the mutated amino acid in the center. However, neo-antigens immunogenicity represents a basic requirement to exploit predicted neo-antigens as good cancer vaccine targets. T-cells have to recognize MHC-bound mutated peptides to be activated and elicit their effector functions.
Several studies demonstrated that some peptides are more immunogenic than others and therefore more likely to be T-cell epitopes. Calis et al set out to determine which properties cause such differences in immunogenicity. To this end, they collected and analyzed a large set of data describing the immunogenicity of peptides presented on various MHC-I molecules. Two main conclusions derived from their analysis: first, they showed that positions P4–6 of a presented peptide are more important for immunogenicity; second, some amino acids, especially those with large and aromatic side chains, are associated with immunogenicity. This information was combined into a simple model that was used to demonstrate that immunogenicity is, to a certain extent, predictable (Calis et al, 2013). A recent study by Yadav et al strengthened this observation (Yadav et al.2014).
Combining whole-exome sequencing and transcriptome sequencing analysis with mass spectrometry they identified six neo-antigens in MC-38 tumors found to be presented on MHC-I. Immunogenicity of mutated peptides was further predicted *in silico* and validated using *in vivo* studies. Before *in vivo* testing, they applieda structural prediction algorithm to predict MHC-I peptide immunogenicity. Mutant peptides were modelled into peptide–MHC-I structures using existing crystal structures from the Protein Data Bank as starting models and optimizing the conformation of the bound mutant peptide using the program FlexPepDock (London et al., 2011). By this way, they analyzed the potential for the mutant residue in each neo-antigen to interact with the TCR. Two out of six predicted MHC-I binders were predicted as immunogenic peptides, also confirmed *in vivo*, by peptide vaccination. Indirectly, this PhD project demonstrated that a substantial further experimental effort is required to predict immunogenicity, highlighting a crucial influence of the vaccine strategy adopted into the determination of predicted neo-antigens immunogenicity.

The vaccine approach adopted in the present study was based on high capacity of novel adenovirus vectors derived from primates to encode long strings of cancer neo-antigens and induce cancer specific T cells. After approaching CT26 mutanome for cancer neo-antigens prediction, we developed a Great Apes Adenovirus-based vaccine encoding predicted neo-antigens *in tandem.* Immunogenicity testing, performed *in vivo,* showed that a good fraction of predicted neo-antigens resulted to elicit immune responses, both $CD8^+$ and $CD4^+$. Encouraged by this result and subsequent efficacy studies, we tried to consolidate the platform, by developing another vaccine encoding already known predicted neo-antigens, demonstrated to be effective in cancer therapy. Seven neo-antigens identified by Yadav and colleagues, of which three immunogenic by peptide vaccination, were inserted into the viral vector and tested for immunogenicity *in vivo.* Surprisingly, seven out of seven neo-antigens revealed to be immunogenic. This result clearly showed that this novel vaccine platform enhances the breadth of responses against neo-antigens that resulted to be not immunogenic by peptide immunizations.

Another relevant observation that support the platform of adenoviral-based vaccine emerged from the comparison between GAd-CT26-31 and GAd-CT26-5, encoding 31 or 5 neo-antigens respectively. Increasing the number of multiple neo-antigens encoded by the viral vaccine does not affect neo-antigens immunogenicity (Figure 15), showing a perfectly comparable magnitude of elicited immune responses. This result leads to considerate the possibility to increase the number of neo-antigens to insert into the vaccine without affecting immunogenicity of predicted neo-antigens that can actually elicit an immune response. Increasing the number of neo-antigens that can be inserted into the adenoviral vaccine becomes relevant for tumors with high mutational load and gives the possibility to include neo-antigens that could fit less with parameters of prediction algorithms, and perhaps eventually excluded from the vaccine, but that can reveal indeed effective response *in vivo*.

Indeed, this study showed that the presence of a higher number of neo-antigens encoded by the vaccine might have advantages in the therapeutic setting. A first effort to determine if $CD8^+$ and $CD4^+$ T cells induced against neo-antigens could provide protective anti-tumor immunity resulted in 100% efficacy also in the group of GAd-CT26-5 treated mice. The presence in both constructs of a likely shared immune-dominant neo-antigen could explain why no difference was observed between constructs. Furthermore, the absence of tumor cells at the time of immunization (tumor cells injection was performed two weeks post vaccination) might suggest the absence of an established suppressive microenvironment opposing the eliciting of immune responses.

The different load of neo-antigens in the vaccine became relevant in a more aggressive tumor state. In advanced therapeutic setting, the treatment of established tumors with the two combinations (αPD1/ GAd-CT26-5 or αPD1/ GAd-CT26-31) showed a significant difference in terms of cured mice compared to control (αPD1-treated mice). Remarkably, the GAd-CT26-31 and GAd-CT26-5 demonstrated a different efficacy, with the longer GAd-CT26-31 vector showing superior efficacy than shorter GAd-CT26-5, underlying the contribution of a greater number of neo-antigens in priming a greater number of specific T cells with effector functions.

However, the presence of tumor-specific antigens and of a repertoire of specific T cells is only one of a number of essential conditions for a successful immune attack on cancer cells, as well described by the cancer-immunity cycle introduced by Chen and Mellman (Figure 2). The perturbation of just one of the seven steps that characterize this cycle can prevent efficacy of immunotherapy. In such cases, in which the cancer-immunity cycle is disrupted at one of the cycle step, the number of neo-antigens produced is unlikely to still be of much relevance. Because of this interdependence of different phases of the cancer-immunity cycle, the combined use of immune therapies that report on these different phases appears warranted. An experimental evidence in support was observed in this study (Figure 18). In early therapeutic setting, the single treatment with GAd-CT26-31 revealed no efficacy on tumor shrinkage at all, as well as the PD-1 blockade as monotherapy resulted in just 33% of tumor free mice compared with the 75% of efficacy from the combination of both. A closer look at the enhanced efficacy derived from combined treatment allowed us to correlate the therapeutic efficacy of GAd-CT26-31/αPD-1 combination with the induction of higher and broader neo-antigens specific T cell responses than αPD-1 monotherapy (Figure 22). A huge effort to identify which specific T cell reactivity could emerged from PD-1 blockade to justify its partial efficacy brought us to investigate a high number of neo-antigens, beyond the 31 inserted in GAd-CT26-31 emerged by other not reported pipelines. However just one reactivity emerged, against the neo-antigen #4.

Despite the great efficacy emerged from GAd-CT26-31/αPD-1 treatment, some tumors did not benefit from the treatment, escaping from the host adaptive immune response.

In considering resistance mechanisms to immune-based therapies, an interesting review recently have reported some of the resistance mechanisms relevant to immunotherapeutic failure, categorizing them as primary, adaptive, and acquired resistance mechanisms (Sharma et al., 2017). The

table 3 briefly highlights the main mechanisms of primary and adaptive resistance to immunotherapy.

	Mechanism	Examples
tumor cell intrinsic	absence of antigenic proteins	low mutational burden lack of viral antigens lack of cancer-testis antigens overlapping surface proteins
	absence of antigen presentation	deletion in TAP deletion in B2M silenced HLA
	genetic T cell exclusion	MAPK oncogenic signaling stabilized b-catenin mesenchymal transcriptome oncogenic PD-L1 expression
	insensibility to T cells	mutations in interferon gamma pathway signaling
tumor cell extrinsic	absence of T cells	lack of T cells with tumor antigen-specific TCRs
	inhibitory immune checkpoints	VISTA, LAG-3, TIM-3
	immunosuppressive cells	TAMs, Tregs

Table 3. Mechanisms of primary and adaptive resistance to immunotherapy.

The most straightforward reason why a tumor would not respond to immune therapy is lack of recognition by T cells because of absence of tumor antigens (Gubin et al., 2014). Alternatively, cancer cells may have tumor antigens but develop mechanisms to avoid presenting them on the surface restricted by MHC, due to alterations in the antigen-presenting machinery, beta-2-microglobulin (B2M), or MHC itself.

The comparison of peripheral immune responses in tumor free mice and tumor bearing mice that did not respond to treatment led us firstly to evaluate a likely absence of the antigenic epitopes in not cured tumors. Taking into account the modest number of samples so far analyzed, predicted mutations were detected, giving the cue to investigate other likely mechanisms of immune evasion. Some preliminary studies aiming to analyze the repertoire of tumor infiltrating lymphocytes (TILs) in some non-responding tumors gave us some indications of the presence of neo-antigens specific INFγ-T cells *in situ.* Moreover, some efficacy studies on the same tumor setting with other combined therapies (not discussed in this thesis) showed that these tumors can 100% respond to the treatment. All these observations would exclude tumor cell intrinsic mechanisms as likely mechanisms of tumor evasion, giving support to tumor cell extrinsic mechanisms as focus of further investigations. Tumor-cell-extrinsic mechanisms that lead to primary and/or adaptive resistance involve components other than tumor cells within the

tumor microenvironment, including T_{regs}, myeloid derived suppressor cells (MDSCs), M2 macrophages, and other inhibitory immune checkpoints, which may all contribute to inhibition of anti-tumor immune responses.

6. CONCLUSIONS

Clinical response to PD-L1 and PD-1 pathway blockade have been seen across a wide range of solid and hematologic cancers, suggesting that many tumors have pre-existing T cell-mediated immunity that is restrained by the PD-L1/PD-1-induced suppression of T cells.
However, as effective as immunotherapy can be, only a minority of people exhibit dramatic responses, with the frequency of rapid tumor shrinkage from single-agent anti-PD-L1/PD-1 antibodies ranging from 10–40%, depending on the individual's indications (Zou et al., 2016). Therefore, a broader view of cancer immunity is required.
To unravel the genomic determinants of response to PD-1 blockade, Rizvi and colleagues used whole-exome sequencing of non–small cell lung cancers treated with pembrolizumab, an antibody targeting programmed cell death-1 (PD-1). In two independent cohorts, higher non-synonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and progression-free survival (Rizvi et al., 2015). A high neo-antigen burden might in turn be associated with the presence of tumor neo-antigen-specific T cells. Thus, expansion of such specific T cells could potentially increase the rate of clinical responses to immune checkpoint inhibitors. In this context, therapeutic cancer vaccines targeting neo-antigens identified using next-generation sequencing and prediction algorithms might represent a promising tool.

Purpose of my thesis has been indeed the development of a novel tumor neo-antigens encoded vaccine based on adenoviral vector to test in preclinics for immunogenicity and efficacy, in combination with PD-1 blockade, as a strategy for a more effective cancer treatment. Encouraging results emerged.

GAd-CT26-31 vaccine was generated by selecting thirty-one predicted neo-antigens from CT26 mutanome, eight of them resulted to be immunogenic *via* immunogenicity studies, performed *in vivo*. A comparison with a smaller vaccine GAd-CT26-5 sharing three immunogenic neo-antigens showed that the vaccine length does not affect immunogenicity. Indeed, comparable high potency of T cell responses between the two constructs emerged for shared neo-antigens.

Even if the two vaccines resulted to be 100% effective in prophylactic setting on CT26 tumor, in a context of established tumors larger GAd-CT26-31 vector was superior than GAd-CT26-5 in combination with αPD-1. Remarkably, in cured tumors larger GAd-CT26-31 vaccine increased and broadened the neo-antigens-specific T cells immune response rescued by PD-

1 blockade, suggesting that a higher efficacy correlates with increased breadth of T cell response induced by vaccination.

This is of relevance especially in presence of poorly immunogenic tumors, where the effect of checkpoint blockade is limited by the lack of antigenicity of tumor cells and the selective augmentation of antitumor T-cell responses with vaccines will likely increase the clinical activity of cancer immunotherapies.

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