

UNIVERSITÀ DEGLI STUDI DI TORINO



Doctoral School in Life and Health Sciences

Ph.D. program in

Complex Systems for Quantitative Biomedicine

***ADENOVIRAL-BASED VACCINE PROMOTES NEOANTIGEN-SPECIFIC
CD8+ T CELL STEMNESS AND TUMOR REJECTION***

Internal tutor:

Prof. Enzo Medico

Candidate:

Nadia Brasu

External tutor:

Dr. Luigia Pace

XXXVI Cycle

Academic Years: 2020-2024

INDEX

ABSTRACT	4
INTRODUCTION	5
1) Immune system	5
2) The innate immune system	5
3) The adaptive immune system	5
4) B lymphocytes	6
5) T lymphocytes	7
6) CD8+ T cells	9
7) Subsets of CD8 T cells in both mouse and human	10
8) CD8+ T cell exhaustion	15
9) Tumor antigens	17
10) Cancer immunoediting	20
11) Cancer immunotherapies	23
11.1) Immune checkpoint therapy	24
11.2) Remodeling the TME through immunotherapy	25
11.3) Adoptive cell transfer therapy (ACT)	25
11.4) Cancer vaccines	26
AIMS	32
RESULTS	33
<i>αPD-1 and GAd treatment expands neoantigen-specific CD8 T cells in MC38 model.</i>	33
<i>GAd therapeutic vaccination increases the frequencies of neoepitope-specific memory precursors and effectors</i>	37
<i>scRNA-seq analysis of neoAg-specific CD8+ T cells demonstrate stem-like and effector cell accumulation after αPD-1 +GAd treatment</i>	41
<i>CD8+ T_{STEM} precursors are induced by the combination of GAd vaccine with PD-1 blockade</i>	45
<i>αPD-1 treatment plus Gad vaccine induces a broader multifunctional CD8+ T clonotypes</i>	50
<i>Strong and broad T cell responses was elicited by neoantigen-based vaccine Nous-209 in patients with dMMR tumors</i>	55
<i>CD8+ T cell responses to peptide pools in MSI CRC treated with Nous209 vaccine</i>	58
<i>Cytokines and transcription factor profiles of CD8+ T cells with Nous209 vaccine</i>	64
<i>Nous-209 vaccine leads to an expansion and diversification of neoAg-specific TCR clonotypes in dMMR tumor patients</i>	68
<i>Analysis of F24 neoantigen-specific CD8+ T cells in partial responders treated with NOUS209</i>	71
<i>Single cells RNA sequencing analysis of neoepitope-specific CD8+ T cells from PR Pt1</i>	74
<i>Nous-209 vaccination expand F24-specific TCR clonotypes in PR_Pt 1</i>	77

<i>Immune phenotype characterization of activated CD8+ T cells in melanoma patients treated with NOUS- PEV vaccination</i>	78
<i>Cytokines and transcriptional factor profile of FSP-specific CD8+ T cells in melanoma patients treated with NOUS-PEV vaccine.</i>	84
DISCUSSION	86
MATERIALS AND METHODS	90
REFERENCE	100
EXPERIMENTAL ROLES AND TECHNICAL CONTRIBUTIONS	121

ABSTRACT

Upon chronic antigen exposure, CD8⁺ T cells become exhausted, acquiring a dysfunctional state correlated with the inability to control infection or tumor progression. In contrast, stem-like CD8⁺ T progenitors maintain the ability to promote and sustain effective immunity. Adenovirus (Ad)-vectored vaccines encoding tumor neoantigens have been shown to eradicate large tumors when combined with anti-programmed cell death protein 1 (α PD-1) in murine models; however, the mechanisms and translational potential have not yet been elucidated. Here, we show that gorilla Ad vaccine targeting tumor neoepitopes from colon rectal cancer (CRC) enhances responses to α PD-1 therapy by improving immunogenicity and antitumor efficacy. Single-cell RNA sequencing (scRNA-seq) demonstrated that the combination of Ad vaccine and α PD-1 increased the number of murine polyfunctional neoantigen-specific CD8⁺ T cells over α PD-1 monotherapy, with an accumulation of Tcf1⁺ stem-like progenitors in draining lymph nodes and effector CD8⁺ T cells in tumors. Combined T cell receptor (TCR) sequencing analysis highlighted a broader spectrum of neoantigen-specific CD8⁺ T cells upon vaccination compared to α PD-1 monotherapy. The translational relevance of these data is supported by results obtained in the first 12 patients with metastatic deficient mismatch repair (dMMR) tumors were treated with a combination of immune check-point blockade antibody (Pembrolizumab) and a heterologous prime boost protocol of Modified Vaccinia Ankara (MVA) and Ad vaccine both encoding shared neoantigens across three different tumor types (gastric, colorectal and gastroesophageal junction cancers). Expansion and diversification of TCRs were observed in post-treatment biopsies of patients with clinical response, as well as an increase in tumor-infiltrating T cells with an effector memory signature. As proof of concept, we validated these findings by single-cell RNA sequencing of a partial responder, the analysis revealed an increase of transitory memory neoantigen-specific CD8⁺ T cells following Ad vaccination. In particular, TCR sequences of neoantigen-specific clones detected in peripheral blood mononuclear cells (PBMCs) after α PD-1 treatment were expanded following Ad vaccination. Additionally, the analysis identified the same TCR clones both in peripheral blood and tumor biopsies. Taken together all these findings indicate a new vaccination protocol to overcome resistance to PD-1 blockade by promoting immunogenicity and broadening the spectrum and magnitude of neoantigen-specific T cells infiltrating tumors.

INTRODUCTION

1) Immune system

Our body is constantly exposed to external pathogens, such as bacteria, viruses, and fungi, to cancer cells that are recognized and eradicated by a complex network of organs, tissue, and cells called the immune system. Serving as the body's primary defense mechanism, the immune system comprises two components: innate immunity and adaptive immunity (1).

2) The innate immune system

The innate immune system, also known as non-specific immunity, provides a rapid host response and defense once the pathogen invades the organism. The rapid response is due to various factors, first by different components which include physical barriers such as anatomical structure like the skin and mucous membranes, as well as physiological barriers, and second through receptors that are encoded in the germline genome that give rise to a pre-existing immune mechanism (1). The evolution of the pre-existing immune mechanism has led to swift detection and prompt initiation in the elimination of a broad spectrum of pathogens (2). In addition to the above-mentioned factors, an amplitude of cell types plays a pivotal role in this defense system, like phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, natural killer (NK), and innate lymphoid cells (3). Once infection has started, these immune cells are quickly recruited through a complex cascade of chemokines and cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin 1 (IL-1) and interleukin 6 (IL-6) (2). Last but not least, another key component of the innate immune response is the complement system, which comprises over 30 plasma proteins that, in the absence of inflammation, are found in an inactive state. When inflammation appears, a proteolytic cascade is triggered. The different steps of the cascade begin the recognition of pathogenic surfaces that, first generate the molecule anaphylatoxins, second lead to the opsonization of the pathogenic surface through various complement opsonins like C3b and finally lyse the pathogenic surface by assembling membrane-penetrating pores known as the membrane attack complex (MAC) (3).

3) The adaptive immune system

Adaptive immunity, also referred to as acquired or specific immunity, has evolved to provide a broader and more precisely targeted repertoire of recognition for both self- and non-self-antigen. The key features of adaptive immune systems comprise the activation of specific immunological effector pathways in response to pathogens, the generation of immunological

memory, and the regulation of host immune homeostasis. These processes are orchestrated by the interplay between the antigen-presenting cells (APC) and the major players of the adaptive immune system: the Bone marrow-derived lymphocytes, also known as B cells and Thymocyte-derived lymphocytes, called T cells. The specificity of the system is achieved during the development through a complex process of somatic recombination, where a variety of gene segments undergo rearrangement to create unique and highly specific antigen receptors in B and T cells (1). This rearrangement mechanism results in the generation of an extensive repertoire of receptor specificities capable of recognizing various components of potential pathogens, ranging from viruses to cancer cells. Alongside specificity, another important characteristic is immunological memory. Upon encountering an antigen, whether by T or B cells, if a sustained response occurs, the immunological memory will be established. This memory feature is vital because upon re-exposure to the same antigen, the immune system mounts a rapid and efficient response (4).

4) B lymphocytes

B lymphocytes, together with their antibodies, represent the essential components of humoral immunity. They originate from hematopoietic precursor stem cells (HSC) in the liver during the fetal period and bone marrow in adulthood (5). The development of HSC to mature B cells involves distinct stages of maturation and selection processes influenced by gene rearrangement of the immunoglobulin segments like the heavy chain (H-chain) together with the light chain (L-chain), leading to three developmental stages, pro-B cells, pre-B cells forming the antigen pre-B cell receptor complex, and immature B cells that express a complete IgM molecule, an important component that leads the egression of immature B cells from the bone marrow into circulation (6, 7). When reaching the spleen as transitional B cells, they receive signals determining their differentiation into the marginal zone (MZ) or follicular B cells. MZ B cells give rise to short-lived plasma cells post-antigen encounter in secondary lymphoid organs like lymph nodes, spleen, or gut-associated lymphoid tissue, while follicular B cells generate long-lived plasma cells residing in specialized niches within bone marrow germinal centers for long-term immune protection. Activated B cells release antigen-specific B cell receptors as soluble antibodies upon encountering their antigen and can also secrete cytokines, contributing to immune responses (7, 8).

5) T lymphocytes

T lymphocytes, pivotal components of the adaptive immune system, are the principal representatives of cellular immunity. They originate from common lymphoid progenitors in the bone marrow and subsequently migrate to the thymus, where they lose the potential to become B cells and natural killer cells (9). In the thymus, the double-negative lymphoid progenitors undergo a complex process of differentiation and maturation, which shapes these cells into functional T lymphocytes, ready to be released into the peripheral tissues. The thymus environment plays a crucial role in T cell development and commitment. In particular, thymic epithelial cells produce growth factors, such as the cytokine interleukin 7 (IL-7) and Notch ligands like Delta-like 4 (DLL4), which are essential components that initiate Notch signaling in T cell progenitors (10). Notch signaling induces the expression of three key transcription factors: the T-cell factor 1 (TCF-1), GATA binding protein 3 (GATA3), and B-cell lymphoma/leukemia 11b (Bcl11b) which are necessary for the initial expression of T-lineage specific genes, such as those encoding components of the CD3 complex, as well as Rag1, required for T cell receptor rearrangement (11, 12). The T-cell receptor (TCR) is a protein complex found on the surface of T cells, responsible for the recognition of foreign or altered self-antigens bound to major histocompatibility complex (MHC) in mice or human leukocyte antigens (HLA) in human, both presented by APC (13). The TCR is constituted by two different transmembrane glycoprotein chains, termed α and β chains, which are disulfide-linked. Each TCR chain is composed of two domains: a variable region (V), responsible for antigen recognition and uniquely characterizing all TCRs, and a constant region (C) Ig-like domains, followed by a transmembrane domain and a short cytoplasmic tail. The TCR also contains complementarity-determining regions (CDRs) that directly interact with the peptide-MHC (pMHC) complex (14). There are two types of MHCs: the MHC class I and the MHC class II. MHC class I presents short peptides (called neoepitopes) of 8-11 amino acids, derived primarily from intracellular degradation of proteins in the cytosol or altered self-peptides. This process alerts the immune system to activate against intracellular infections and targets infected cells for destruction through the action of cytotoxic CD8⁺ T cells. In contrast, MHC class II presents longer peptides (15-24 aa) that originate from proteolysis of extracellular antigens in endosomal compartments, leading to an intracellular cooperation between hematopoietic cells. This facilitates an immune response that helps combat extracellular infections through the action of helper and regulatory CD4⁺ T cells (14),(15). Binding between TCR and the MHC-peptide complex is fundamental for the selection and maturation of T cells. Double negative T cells (CD3-CD4-

CD8-) become double positive T cells expressing $\alpha\beta$ TCR (CD3+CD4+CD8+). These latter interact with a high density of self-peptides-MHC-I and MHC-II complexes expressed by medullary thymic epithelial cells and dendritic cells (16). T cells with TCRs that bind too strongly to these self-peptide-MHC complexes receive strong activation signals, which trigger their programmed cell death (apoptosis), leading to a negative selection process (17). In contrast, an intermediate level of binding between MHC-peptide complexes and TCRs initiates effective T cell maturation, also known as positive selection. Thymocytes that express TCRs that bind to self-peptides-MHC complex I become CD8+, whereas those that express TCRs that bind to self-peptides MHC class II ligands become CD4+ T cells. Once T cells have completed their maturation into single-positive CD4+ and CD8+ T cells, they migrate into the bloodstream to reach peripheral lymphoid sites, such as lymph nodes and spleen. These cells are known as naïve T cells. The migration of naïve T cells into the bloodstream is caused by the combination of chemokine receptors and different cell adhesion molecules, such as selectins, integrins, and members of the immunoglobulin families, as well as chemokines. Once naïve T cells are attached to the venule endothelium wall, the chemokine C-C motif ligand 21 (CCL21) induces a switch in the integrin lymphocyte function-associated antigen 1 (LFA-1) to a configuration that has affinity for the intercellular adhesion molecule 1 (ICAM-1) expressed in the venule endothelium. These steps initiate adhesion, diapedesis, and migration of the T cells into the T-cell zone of secondary lymphoid organs (18). Here, antigen-bearing dendritic cells activate naïve T cells through binding between the pMHC complex-TCR and binding between the co-stimulatory B7 molecules and CD28 on the T cell. Primed T cells proliferate and give rise to clonal expansion. This proliferation is driven by cytokines such as interleukin 2 (IL-2), which are produced by T cells themselves in the environment, as well as IL-7, and interleukin-15 (IL-15). Finally, proliferating T cells differentiate from naïve to cytotoxic T cells capable of directly killing pathogen-infected cells, whereas priming of naïve CD4 T cells leads to the development of a diverse array of effector cell types depending on the nature of the signals they receive during priming. They can mature into CD4+ T helper 1 (Th1) or T helper 2 (Th2) cells that are involved in the regulation of both humoral and cellular immunity, and also into effector T cells, which will differ according to the environment of the cytokines and the nature of the antigen they encounter (19). Naïve CD4+ can also become regulatory T cells (Treg) that have the ability to control and regulate cell homeostasis and prevent immunopathology. A subset of the proliferating T cells differentiates into memory T cells, which can provide long-term immunity to the same antigen (20).

6) CD8⁺ T cells

Together with CD4⁺ T cells, cytotoxic T cells or CD8⁺ T cells are the major players in the adaptive immune system against intracellular pathogens and tumors. Once APCs have exposed both a foreign antigen through the MHC-I and B7 family molecules, recognized respectively by the TCR/CD3 complex and the CD28 co-receptor (21), naïve CD8⁺ T cells become active effector cytotoxic T cells capable of killing cells carrying pathogenic antigens. One of the most important characteristics of the previously described process is the TCR antigen recognition binding. It is characterized by three different parameters: TCR affinity, avidity, and functional avidity (22). TCR affinity is defined as the strength of the interaction between a single TCR and the pMHC ligand, whereas TCR avidity measures the strength of multiple TCR-pMHC binding and also takes into account the effect of other molecules, such as TCR co-receptors (23). TCR functional avidity represents the fitness and activity of T cells in response to different concentrations of peptide epitopes. Despite TCR affinity is correlated with the ability of T cells to detect poor quantities of antigens, TCR functional avidity predicts the capacity of TCR-T cells to induce a tumor-specific reaction when the number of pMHC is low (24). Each of the aforementioned parameters is a key component of T-cell fate determination steps, such as activation, proliferation, differentiation, and effector function. T cell activation begins when, after encountering the antigen, naïve T cells undergo rapid and robust proliferation, and these newly generated cells possess effector functions such as cytokine production and cytolysis (17), however, they are short-lived cells with a percentage between 90-95% of the population that is eliminated through apoptosis following the peak of proliferation and expansion phase (25). After the expansion phase, naïve T cells become effector cells that are capable of killing through cytotoxic granules, such as secretory lysosomes. These lysosomes can be constituted by the pore-forming protein perforin plus a family of serine protease molecules referred to as granzymes. The function of perforin is to open the pores in the cell membrane of the target cells that should be eliminated and to allow the entry of granzymes, such as Granzyme B (GzmB) into the cytosol, which starts the apoptosis cascade either directly through the activation of the Caspase 3 or indirectly via interaction with BH3-interacting domain death agonist (BID) (26), (27). In addition, effector cytotoxic T cells can release cytokines, such as TNF- α and type I interferons (IFN- γ), that contribute to host defense. In particular, TNF- α is able to inhibit viral replication directly and induces increased expression of MHC class I molecules and other proteins involved in the peptide loading of these newly synthesized MHC class I molecules in infected cells, whereas

IFN- γ activates macrophages, recruiting them to sites of infection, where they serve both as effector cells and as APCs (28). Another important factor that dictates the fate commitment of effector T cells, leading to phenotypic and functional changes, is the transcriptional network. To promote effector development, the transcriptional factors associated are the T-box transcription factor TBX21 (T-bet) (29), Inhibitor of DNA binding 2 (ID2), B lymphocyte-induced maturation protein 1 (Blimp-1) and interferon regulatory factor (IRF-4)(30). Although most naïve T cells become effector cells, the remaining 5-10% that are able to survive the contraction phase give rise to long-lived memory populations. These populations are characterized by the transcriptional factors B cell lymphoma 6 (BCL-6), Eomesodermin (Eomes), Inhibitor of DNA binding 3 (ID3), T cell factor 7 (TCF-7) and Forkhead box O1 (Foxo1) (31).

7) Subsets of CD8 T cells in both mouse and human

During a disease, from acute infection to cancer, the diverse steps of differentiation of CD8⁺ T cells can be summarized into four main populations: from naïve to effector, memory, and exhausted cells, each of which is characterized by the acquisition and the loss of specific surface molecules that can be used to define different immune phenotypes (32). First, naïve T cells, in particular precursors, are characterized by the expression of homing receptors such as L-selectin (CD62L) and chemokine receptors like CCR7. These two receptors are necessary for cell extravasation and homing to T cell areas of secondary lymphoid organs. In humans, naïve cells are also characterized by a basal expression of CD45RA, co-stimulatory molecules such as CD27 and CD28 (33). Sufficient antigenic and costimulatory signals prime naïve T cells, which undergo clonal expansion and differentiation into effector cells. These effector cells migrate to the sites of infection and mediate pathogen clearance by killing infected cells. During this step, they underwent a dramatic shift in the expression of surface proteins that regulate cellular trafficking, in fact they lose the expression of CD62L and CCR7 (34). In contrast, the initial activation of CD8⁺ T cells is marked by an up-regulation of surface markers, including CD44 and CD69, signifying their transition from a naïve state to an activated state (31). The activation state of T cells is followed by a significant upregulation of various molecules, like the killer cell lectin-like receptor G1 (KLRG1) and the IL-2 receptor subunit-alpha (CD25), which facilitate their effector functions (31). As reported in the previous paragraph, after the killing of infected cells, most effector cells undergo the contraction phase, where the majority of these cells die, and only 5%-20% of these cells survive. The remaining effector cells give rise to the memory maintenance phase,

where there is the formation of a pool of long-lived memory cells. Memory cells are decisive for two major reasons: first, they provide immediate protection in peripheral tissue, and second, they mount recall responses to antigens in secondary lymphoid organs. The principal characteristics of memory T cells are the preservation of high proliferative capacity, a multipotent state where memory T cells can be memory cells but also quickly re-activated effector function upon reinfection, and long-term survival and self-renewal in the absence of antigen, thanks to IL-7 and IL-15. Memory cells can be divided into memory precursor cells, defined by a higher expression of CD127, CD27, CD44, reduced expression of CD25, and no expression of KLRG1. On the contrary, short-lived effector cells are identified by CD44+, CD62L-, CD27-, KLRG1+, and CD127- expression (35). Furthermore, immunological memory should also be divided into protective memory and reactive memory. The first one is mediated by effector memory T cells (T_{EM}) that travel to infected peripheral tissues and display rapid effector functions, while the second is mediated by central memory T cells (T_{CM}) that migrate to the T area of secondary lymphoid organs, have limited effector function, but when there is antigenic stimulation, they rapidly proliferate and differentiate into effectors (25),(36). In mice, T_{CM} are defined by the expression of both CD44 and CD62L molecules, whereas T_{EM} cells lose the expression of CD62L. In contrast, in humans, T_{CM} cells express CD45RO and constitutively express CCR7 and CD62L receptors. They exhibit heightened sensitivity to antigenic stimulation, reduced dependence on co-stimulation, and increased level of CD40L, enhancing their ability to provide stimulatory feedback to dendritic cells (DC) and B cells. Furthermore, upon TCR activation, T_{CM} cells primarily produce IL-2, they differentiate efficiently into effector cells capable of producing significant amounts of IFN- γ or interleukin-4 (IL-4). On the other hand, T_{EM} lose the expression of CCR7 and CD45RA, exhibit variability in the expression of CD62L and CD45RO expression, and possess distinct sets of chemokine receptors and adhesion molecules essential for their migration to inflamed tissues (37). One of the principal characteristics of T_{EM} is its rapid effector response and are notable for their high levels of Perforin expression, a key protein involved in cytotoxic activity (38-40). Additionally, a subset of CD8+ T_{EM} cells that express CD45RA (T_{EMRA}) are characterized by their high cytotoxic potential due to the fact that they carry the largest amounts of perforin among memory T cell populations. This unique profile of T_{EMRA} cells underscores their role in the development of rapid and powerful immune responses, particularly in situations requiring immediate effector functions against pathogens or infected cells (41). The development and different functions of T_{CM} and T_{EM} cells are due to the expression of different transcription factors. In fact, T-bet, Blimp1,

ID2, and signal transducer and activator of transcription 4 (STAT4) expression is associated with T_{EM} cells, while Eomes, TCF1, BCL-6, ID3, and signal transducer and activator of transcription 3 (STAT3) expression is associated with T_{CM} cells (42), (43). Another important subset for immunological memory is the one defined as stem cell memory (T_{STEM}), a small subset of long-lived CD8⁺ T cells that has the ability to self-renew and differentiate into effector cells after antigen re-exposure (44). In particular, they can differentiate into central memory, effector memory, and effector T cells (45). In mice, T_{STEM} cells are characterized by the expression of CD62L and are negative for CD44, similar to naïve T cells, but they also co-expressed other markers such as stem cell antigen 1 (Sca-1) and high levels of antiapoptotic molecule B cell lymphoma 2 (Bcl2), the β chain of the receptor IL-2, IL-15 receptor (IL-2R β) and the chemokine (C-X-C motif) receptor CXCR3 (46, 47). On the contrary, human T_{STEM} are defined as CD45RO⁻, CCR7⁺, CD45RA⁺, CD62L⁺, CD27⁺, CD28⁺ and IL7R⁺, these markers expressed also in naïve T cells. However, they also co-express CD95, IL-2R β , CXCR3 and LFA-1 (48, 49). As reported above, CD8⁺ T cells can differentiate into several subtypes of cells with distinct characteristics, from T_{NAIVE} to T_{MEMORY}, T_{STEM} and T_{EX}. However, the determination of the differentiation fate of CD8⁺ T cells is due to extrinsic and intrinsic factors, including that following:

- nature and antigen dose (50)
- TCR specific and affinity (51),(52)
- environmental stimuli (53),(54)
- mechanism of tolerance (51)
- immunosuppressive effects of the microenvironment (55),(56).

Today, although different immuno-phenotypes of CD8⁺ T cells have been identified, the mechanisms leading from one phenotype to another are not well understood. For this reason, several models that describe the lineage commitment of CD8⁺ T cells have been proposed (57). The major determinant in the different models is time, and according to it, the models could be divided in asymmetric cell decision model and a postponed or lagged temporal model of differentiation, where the development of effector and memory T cell subsets may not occur simultaneously but rather follow a delayed timeline (58),(59). In the asymmetric model, following initial cell division, the daughter cell positioned proximally to the immunological synapse, between the T cell and the dendritic cell, is designated to become an effector precursor, whereas the daughter cell situated distally will contribute to the formation of the memory T cell pool (60). In contrast, the timeline referred models are characterized by changes in gene expression caused by both transcriptional and epigenetic changes (58, 59,

61). The progressive differentiation model hypothesizes that CD8⁺ T cells advance through a series of stable developmental stages, implying that memory CD8⁺ T cells originate directly from naïve T cells, which sequentially give rise to T_{STEM}, T_{CM}, and T_{EM} CD8⁺ T cells, ending in effector cells. As this progression unfolds, the various subsets gradually lose their plasticity and reprogramming capacity (49, 62). In contrast, the model of transient and reversible differentiation emphasizes specific changes that occur during the differentiation process (58). Notably, certain genes expressed in naïve CD8⁺ T cells are temporarily silenced in effector memory subsets but are re-expressed in the later stages by memory subsets (58). Consequently, this model hypothesizes that both memory and effector precursor subsets exhibit transitory and/or reversible gene expression patterns. The differentiation of CD8⁺ T cells is typically accompanied by T cell clonal expansion (63). Recent advances in quantitative single-cell analysis have revealed the presence of intermediate subsets of cycling CD8⁺ T lymphocytes during acute infections in murine models (64). According to this framework, these intermediate subsets exhibit gene expression patterns of both memory and effector cells, suggesting that they likely represent a stage of bipotent effector/memory precursors (65). These bipotent intermediates may correspond to a subset that can either commit to effector functions or enter a state of long-term memory quiescence. Finally, the progressive exhaustion model is characterized by distinct stages of exhaustion that are regulated and maintained by transcription factor regulators. Specifically, TCF-1⁺ CD8⁺ T precursors (T_{PEX}) can differentiate into transient CD101-Tim3⁺ intermediate exhausted cell subsets (T_{TRANSITORY}), which subsequently evolve into CD101⁺Tim3⁺ terminally exhausted (T_{EX}) CD8⁺ T cells during the later stages of differentiation (66),(67). This observation suggests that these transient subsets retain both proliferative and effector capacities.

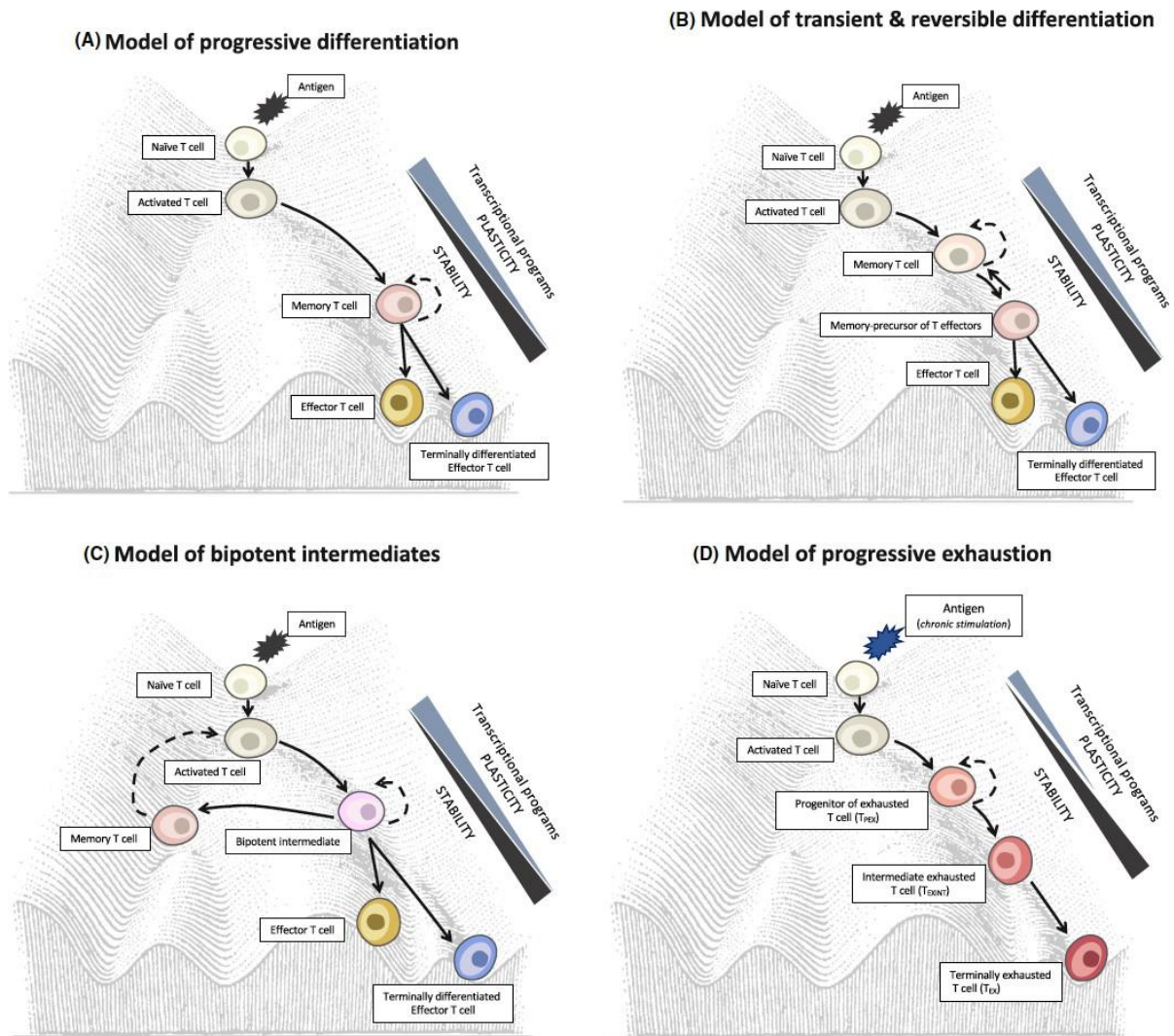


Fig. 1.1. Different models of T cells differentiation represented on a Waddington's epigenetic landscape.

(A) Model of progressive CD8⁺ T cell differentiation. **(B)** Model of transient and reversible differentiation. **(C)** Model of bi-potent intermediates. **(D)** Model of progressive exhaustion. Figure adapted from Pace L and Montacchiesi G, immunological review 2021.

TCF-1, encoded by the TCF7 gene, is a transcription factor important for T cell development and differentiation (68). It is highly expressed in naïve T cells and memory precursors, promote their formation by inducing the expression of the transcriptional factor Eomes (56, 69). Indeed, TCF-1 is downregulated in effector T cells during acute infection but persists during chronic viral infection (68). Furthermore, it contributes to stemness, together with its homologue lymphoid enhancer-binding factor-1 (LEF1), an effector transcription factor, fundamental for early T cell development (70). TCF-1 is highly expressed in T_{PEX} cells, identifying them distinctly from T_{EX} cells (71). Furthermore, the co-expression of TCF-1

with the inhibitory receptor PD-1, is defined as the bridge between T cell memory and exhaustion, as these TCF1+PD1+ T_{PEX} display some characteristics in common with central memory T cells, including the regeneration capacity and the production of differentiated effector T cells, but also features of the exhausted phenotypes such as the expression of some inhibitory receptor (72). Blockade of PD-1 via immunotherapy leads to a re-expansion of T_{PEX} that are able not only to resolve chronic infections, but also to control the tumor growth (73). The Thymocyte Selection-Associated High Mobility Group Box, also known as TOX, is another crucial player in the process of CD8⁺ T cell exhaustion (74). It is a transcription factor that plays a critical role in the development and function of CD8⁺ T cells, particularly in the context of chronic infections and cancer. Indeed, TOX is a central regulator of CD8⁺ T cell exhaustion, in particular, it is required for the development and the maintenance of terminal T_{EX} cells in chronic infections and cancer (75). TOX expression is correlated with impaired proliferation, cytokine production and degranulation (76). In addition, T_{EM} can express TOX, whereas naïve and early differentiated memory CD8⁺ T cells don't express TOX, but only TCF-1 (74). Another important receptor that plays a significant role in immune cell signaling and differentiation is the C-X3-C motif chemokine receptor 1, named CX3CR1 (77). This receptor is involved in migration, adhesion and retention of leukocytes at sites of inflammation and its expression has been correlated with distinct differentiation states, from naïve and terminally differentiated subsets (78). In fact CX3CR1⁻, combined with high expression of TCF-1 highlights an high proliferative potential (79), whereas CX3CR1⁺, co-expressed with CD62L, discriminates memory T cell populations with direct effector function (78). Furthermore, the presence of CX3CR1⁺ CD8⁺ T cells suggest a transition from naïve to effector phenotype, in fact within the transitory subset CD101-Tim3⁺ CD8⁺ T cells that proliferate and contribute to viral control, the CX3CR1 transcript was found upregulated, suggesting that CX3CR1⁺ T cells may retain some capacity for proliferation and differentiation into memory T cells, linking them to both effector and memory responses (66).

8) CD8⁺ T cell exhaustion

Persistent antigen stimulation and failure of the immune response lead to an altered state of T cells known as exhaustion. This phenomenon is commonly observed in chronic infections, viral infections and in cases of tumor progression (80). The first and most crucial aspect of exhaustion appears to be the persistent and ongoing exposure to the antigen. Indeed, the severity of exhaustion is directly correlated with the level of antigen stimulation (81), as

confirmed in HIV-1 studies (82) and LCMV studies (83). When CD8⁺ T cells are primed during infections, but are withdrawn from continuous antigen stimulation at early time point (1-week post-infection), they have the ability to mature into fully functional CD8⁺ memory T cells. By contrast, if these T cells are subject to a persistent antigen exposure for 2-4 weeks, an exhaustion state is set in, and these cells do not regain typical memory differentiation upon cessation of antigen exposure (84, 85). Furthermore, other phenomena, such as IL-2 production impairment, followed by a reduction in TNF- α production, are one of the first effector activities to be extinguished, and they can determine the exhausted state (81). This altered cytokine profile triggers a diminished capacity to proliferate and perform effector functions typically associated with robust, protective effector and memory T-cell populations (86). Accordingly, the intensity and persistence of chronic antigen stimulation and infection appear to be pivotal determinants that drive T cell exhaustion and are directly correlated with the degree observed during chronic infection (87, 88). Another hallmark of exhaustion is the sustained upregulation and co-expression of multiple inhibitory receptors, such as PD-1. The inhibitory signaling pathway mediated by PD-1 and its ligands is important for the exhaustion because, during a normal immune response, PD-1 expression is upregulated for T cell activation, and once the antigen is depleted, the expression of PD-1 decreases (89). However, during chronic infection, when there is continued epitope persistence, the expression of PD-1 augments drastically, causing a change in the transcriptional status of the *pdc1* gene promoter that remains demethylated and poised, even in the absence of antigen stimulation, leading to an exhausted state (90). Furthermore, exhausted PD-1⁺ T cells co-express other inhibitory receptors as lymphocyte activation gene 3 protein (LAG3), 2B4 (also known as CD244), CD160, T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM3; also known as HAVCR2) and the cytotoxic T-lymphocyte antigen 4 (CTLA-4) (90, 91). Beyond antigen persistence and co-expression of inhibitory receptors, exhausted T cells display metabolic abnormalities, characterized by impaired mitochondrial function, increased glycolysis, and altered lipid metabolism (92). Notably, these cells fail to transition into a quiescent state and acquire antigen-independent homeostatic responsiveness, which is a characteristic of functional memory T cells. Another class of molecules that govern the process of T cell exhaustion is the soluble factors that constitute a distinct group of signaling molecules. This category encompasses immunosuppressive cytokines like interleukin-10 (IL-10) and transforming growth factor-beta (TGF- β), along with inflammatory cytokines such as IFNs and IL-6 (93, 94). Commonly, the number of inhibitory receptors co-expressed by exhausted T cells is correlated the more pronounced the degree of exhaustion.

This altered expression profile is accompanied by dysregulated expression and utilization of critical transcription factors, including T-bet, Eomes, and the nuclear factor of activated T-cells (NFAT). In particular, during chronic infection, T-bet and Eomes were reciprocally expressed in exhausted CD8⁺ T cells in a different manner, leading to the distinction of two subpopulations of exhausted T cells: a small pool of T exhausted progenitor cells (T_{PEX}) and a large population of terminally exhausted T cells (T_{EX}) (95). The first has superior proliferative potential and survival capacity compared to the second, and T_{PEX} cells exhibit higher effector functions like cytokine production and cytolytic activity compared to T_{EX}, in addition they are more capable of responding to immunotherapy or reinvigoration strategies (96). T_{EX} are defined as the end stage of T cell exhaustion, resulting from prolonged antigen exposure and overstimulation, in addition they are derived from T_{PEX} and cannot revert to a functional state, representing a point of no return in the exhaustion pathway (96). The immune phenotype of CD8⁺ T_{PEX} cells is characterized by high expression of T-bet, TCF-1, SLAMF6, CXCR5, middle PD-1, and low expression of Eomes and Tim3 expression, whereas the T_{EX} has limited proliferative capacity and higher expression of inhibitory receptors when compared to their progenitors, so they express high PD1, EOMES, CD39, CD244 and Tim3 (97-99). Significantly, blockade of the PD-1 pathway appears to selectively rejuvenate the T-bet⁺ subset, with minimal effect on the EOMES⁺ cells (100).

9) Tumor antigens

As a consequence of malignant transformation, the repertoire of peptides displayed on the cell surface by MHC molecules undergoes significant alterations (101). These changes in the peptide landscape led to the generation of different classes of tumor antigens that vary on the basis of their genetic alteration, localization and expression levels (101). Tumor antigens can be classified into two main groups: tumor-associated antigens (TAA) and tumor-specific antigens (TSA). The former are derived from tumor cells that frequently express aberrant or overexpressed proteins due to the activation of oncogenic signaling pathways and epigenetic changes (102). These antigens are present not only in the tumor but also in normal tissue, and they can be shared between different types of tumor and tissue. TSA are generated from the genetic instability inherent to tumor cells, which leads to the accumulation of DNA alterations, including mutations, insertions, and deletions. These genetic changes result in the formation of entirely novel stretches of amino acid sequences. (103).

The two different categories of antigens could be further distinguished depending on their features, such as binding affinity and stability.

The TAA can be further classified into the following subcategories (Fig 1.2):

- Overexpressed tumor antigens are self-antigens expressed in an aberrant manner in tumors, compared to normal tissue (104). Examples of these tumor antigens are the human epidermal growth factor receptor 2 (HER-2), p53 and survivin (105). In particular, therapies that were developed are monoclonal antibodies, such as Trastuzumab, which can bind to the HER-2 receptor, inhibiting tumor growth and enhancing immune-mediated destruction of HER-2 positive breast and gastric cancer (106). Another type of therapies used against HER-2 positive cancer cells are the tyrosine kinase inhibitors like Lapatinib or Tucatinib (107).
- Tissue differentiation antigens (TDA) are proteins that are expressed in a tissue-specific manner, reflecting the differentiation stage of cells within that tissue. They are typically involved in normal physiological functions of tissue and can be overexpressed in malignant cells (108). These antigens are not exclusive to cancer, but are also found in normal cells of the corresponding tissue type, albeit at lower levels. Examples of TDA are: the mammoglobin A, that is expressed in mammary gland but overexpressed in breast cancer cells, or the melanoma antigen recognized by T cells 1 (MART1) expressed in melanocytes but overexpressed in melanoma cells (109). For MART1 TDA, novel therapies are under development, such as peptides vaccines that contains the MART1 derived epitopes (110) or adoptive cell transfer therapies of TILs or engineered TCR T cells (111).
- Cancer-germline antigens (CGA) are antigens not expressed in normal tissue but only in germline and trophoblast cells, however, they are aberrantly expressed in a variety of human cancers. An example is the cancer-testis antigen 1 (CTAG1, also known as NY-ESO) (112, 113). Currently, there are various therapies against NY-ESO, from TCR gene therapy in which a study of 17 patients reported that 9 of them showed clinical responses without serious side effects (114, 115) to peptide vaccines and combination therapies (115). In particular, the co-expression of co-stimulatory receptors such as NY-ESO1 and PD1-41BB in chimeric T cells enhances T cell proliferation, functionality, and antitumor activity by converting inhibitory signals from PD1-PDL1 axis (116). In addition, the combination of NY-ESO1 therapy with immune checkpoint inhibitors has shown some positive responses in patients (116, 117).

Although TAA can be recognized by the immune system, especially by T cells, which can build a strong antitumor response directly kill TAA-producing tumor cells (118), on the other hand, their expression in normal tissues poses the risk of self-immunity. This risk is due to the fact that some TAAs are also expressed in healthy tissues, thereby causing the immune system to mistakenly target these normal cells. The activation of T cells against TAAs can cause collateral damage to normal tissues and manifest itself as autoimmune reactions (119). In contrast to TAA, tumor-specific antigens are foreign to the body because they are expressed only by cancer cells (120). They arise from genetic alterations as non-synonymous point mutations that can induce an amino acid codon switch that results in the generation of a new sequence-altered protein named neoantigens (nAgs) (121). The resulting epitope that is recognized by T cells is termed neoepitope, and this recognition triggers an immune response, particularly by T cells, that target and kill cancer cells displaying these neoantigens (122). Neoantigens can be divided into two main groups:

- Shared neoantigens which are derived from mutations found across different patients with the same tumor type and often, they are driver mutations used as targets for “off-the shelf” vaccines (123).
- Personalized neoantigens that are unique to an individual and generally arise from somatic mutations, they are the main target for personalized immunotherapies (124). Currently, immunotherapies that have been developed using neoantigens are cancer vaccines, such as neoantigens cancer vaccine encoding different frame shift peptides in a non-human primate viral platform used to treat colon cancer vaccines (125) or adoptive transfer T cell therapy that involves modified T cells from patients to enhance their ability to recognize and attack cancer cell presenting neoantigens, such as neoantigens reactive T cells (NRT) against melanoma (126) or non-small cell lung cancer (NSCLC) (127). Furthermore, immune checkpoint blockade inhibitors can modulate and expand neoantigens specific CD8⁺ T cells leading to a better immune response (128). All these therapies were developed because neoantigens have some advantages, first, they are unique to tumor cells and their absence in normal tissue, drastically reduces the risk of provoking autoimmunity and minimizes damage to healthy cells and reduces side effect compared to traditional therapies (129),(130). Second, they elicit a strong immune response, making them effective targets to immunotherapies, and finally they can be used to the creation of personalized medicine, potentially leading to better outcomes (131). Although neoantigens have different advantages, they also show some disadvantages, because the process of identifying and validating them can be complex and time consuming, requiring genomic and bioinformatic techniques, the variability of the therapies because their effectiveness

can vary between patients depending on the tumor's mutation landscape and the immune system's status, and the potential for immune evasion, because the tumor can develop a mechanism to evade immune detection, including the down- regulation of neoantigen presentation or alteration of the tumor microenvironment (127). However, neoantigens features make them interesting, safety and relevant for cancer immunotherapy, with a low potential for damaging normal tissue (132).

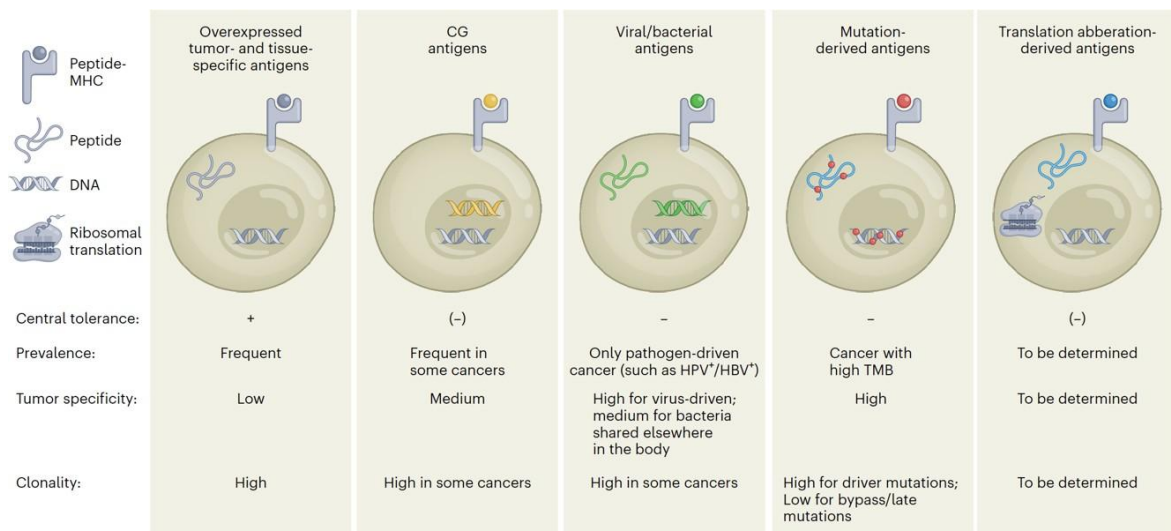


Fig. 1.2. Various types of tumor antigens recognized by T cells.

Tumor and tissue-specific antigens are often overexpressed in tumor cells but are also found in healthy tissues, leading to low tumor specificity and issues with central tolerance. In contrast, cancer/testis (CG) antigens are typically only present in the germline and reappear in tumors, offering moderate tumor specificity while still being influenced by central tolerance. Antigens derived from viral and bacterial infections linked to oncogenesis are highly tumor-specific, as they are not expressed in healthy tissues and bypass central tolerance. Neoantigens, which result from mutations such as single-nucleotide variations, insertions/deletions, or gene fusions, are unique to cancer cells, providing them with high tumor specificity and no central tolerance. Additionally, aberrant neoantigens arise from dysfunctional transcription and translation processes in cancer cells and are not encoded in the genome. This newer category of TSAs is still being studied for its prevalence and specificity in tumors. Figure adapted from Peri A. et al., Nature cancer 2023.

10) Cancer immunoediting

Cancer is a complex disease characterized by uncontrolled cell growth and proliferation that can ultimately lead to organ dysfunction and death. Causes of cancer are multifaceted and can be divided into two categories: exogenous and endogenous risk factors. Exogenous risk factors are external factors that increase the likelihood of developing cancer, including an unbalanced diet, smoking, and chronic inflammation (133). Endogenous risk factors, on the other hand, are factors within the body that contribute to the development of cancer, including

genetic mutations, hormonal imbalances, immune system dysregulation, and hereditary predispositions (134, 135). These endogenous factors can interact each other and with external environmental factors, such as exposure to carcinogens, to initiate and promote the growth of cancer cells. Another important feature in cancer is the interaction between the tumor microenvironment (TME) and the immune system, indeed solid tumors are infiltrated by different types of immune cells, the infiltrating leukocytes, such as dendritic cells, macrophages, mast cells, and the tumor infiltrate lymphocytes (TILs) such as T and B lymphocytes and Treg cells. They are defined as lymphocytes that surround the tumor and can control cancer progression. The immune system has a dual role, on the one hand, it can lead to tumor progression. An example of immune system players that can have these role are the tumor-associated macrophages (TAMs), which mainly promote tumor growth and may be crucial for angiogenesis, invasion, and metastasis (136), for this reason a high TAM content is generally correlated with poor prognosis (137). On the other hand, the presence of a large number of lymphocytes, especially T cells, has been reported to be an indicator of good prognosis in many cancers such as, melanoma, breast cancer, ovarian cancer, non-Hodgkin's lymphoma, head and neck cancer, non-small-cell lung cancer (NSCLC), esophageal cancer and urothelial carcinoma (138). The importance of tumor-infiltrating TILs in the tumor microenvironment is not solely determined by their quantity, but also by their spatial distribution within the tumor, in fact, they can be localized in: the tumor center (CT), the invasive margin (IM) or in the adjacent tertiary lymphoid structures (TLS). TILs found at the IM, the interface between the tumor and the surrounding healthy tissue, are associated with a better prognosis, as they indicate an active immune response at the tumor-host interface and a positive correlation with overall survival and disease-free survival when compared with TIL infiltrating the center of the tumor. However, the immune system can paradoxically constrain and promote tumor development and progression. This process is known as cancer immunoediting and, in its most complex form, proceeds through three phases termed, elimination, equilibrium, and escape (Fig. 1.3) (139, 140).

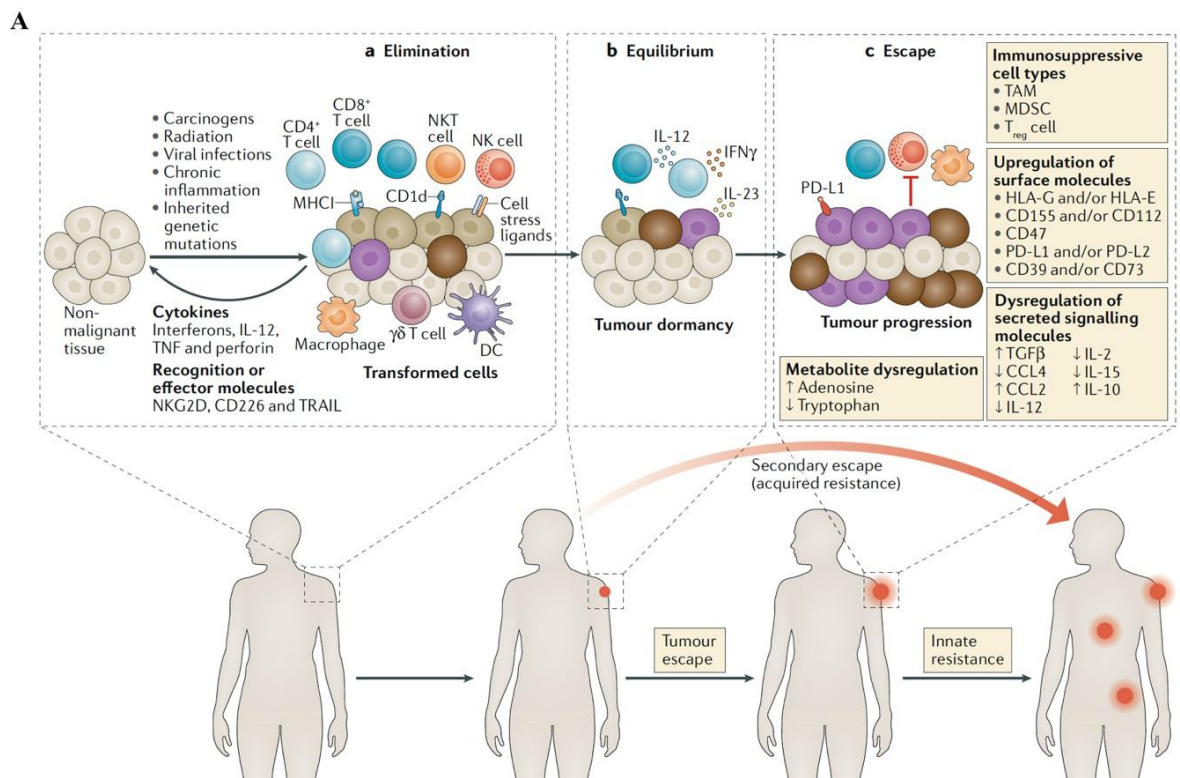


Fig. 1.3. Phases of cancer immunoediting

Cancer immunoediting is divided into three phases, elimination, equilibrium and escape. In the elimination phase (a), the innate and adaptive immune system work together for recognize tumors transformed cells that escape that have evaded the body's intrinsic tumor suppression mechanisms, thereby preventing the development of clinically detectable tumors. If the destruction of tumor cells is successful, this elimination represents the culmination of the immunoediting process. In cases where tumor cells survive this elimination phase, they may progress into the equilibrium phase (b), characterized by a halt in net growth and a modification of cellular immunogenicity mediated by the adaptive immune system. Subsequently, tumors that have undergone such editing may transition into the escape phase (c), where their proliferation is no longer constrained, primarily due to the activation of immunosuppressive and/or immunoevasive pathways. Tumors that have escaped these immune controls become clinically detectable as visible tumors. Key immune components involved in these processes include dendritic cells (DC), myeloid-derived suppressor cells (MDSC), MHC class I (MHCI), natural killer cells (NK cells), natural killer T cells (NKT cells), programmed cell death ligand 1 (PD-L1), tumor-associated macrophages (TAM), and regulatory T cells (Treg cells). Figure adapted from O'Donnell, W. L. Teng M and Smyth M, Nature review clinical oncology, 2019.

Initially, in the elimination phase, the immune system recognizes and kills tumor cells that have evaded the cell-intrinsic mechanism of tumor suppression. However, some subclones of tumor cells escape immune surveillance and enter an equilibrium phase where they co-exist with the immune system without being eliminated. In this phase, tumor cells are under constant pressure from the adaptive immune system, which, coupled with genetic instability,

leads to the acquisition of changes that enable them to escape immune recognition and destruction by altering antigen expression, upregulating alternative receptors such as PD-L1 and CTLA-4, secreting cytokines like IFN- γ and recruiting another type of TILs, the Tregs, that have the ability to inhibit the T cell function owing to the secretion of IL-10 or TGF- β (141-144). An additional determinant related to TILs capable of eliciting tumors is the presence of PD-L1 at both primary and metastatic tumor sites (145). PD-L1, which is absent in normal tissues, can be induced by inflammatory cytokines, notably IFN- γ , leading to its up-regulation in various cell types, including tumor cells. This up-regulation of PD-L1 in response to IFN- γ , released by TILs, serves as an adaptive immune evasion mechanism within the tumor microenvironment. This mechanism inhibits the activity of local effector T cells, reduces their proliferation, and decreases their cytokine production. The presence of this adaptive immune evasion mechanism, even in advanced stages of cancer, implies the existence of immunosurveillance (146). Consequently, contemporary immunotherapeutic strategies targeting checkpoint inhibitors, such as PD-L1, play a pivotal role in improving the longevity and efficacy of TILs (147), (148). In conclusion, in the escape phase, tumor cells successfully continue to grow successfully, leading to disease progression (149). The cancer immunoediting process is present not only during the natural growth of tumors, but recent studies highlight its recurrence in patients treated, in part or entirely, with immunotherapies (140). This suggests that the phenomenon may persist even after such treatments have been administered and could be related to a lack of immune response to some developing tumors (150). In particular, a loss of immune response can take place in patients with systemic immune suppression or in those who have a low mutational load, and consequently, the immune system cannot recognize them as foreign antigens. For these reasons, it is important to understand the mechanisms and pathways that cause tumors to be resistant or develop resistance to cancer immunotherapies (107).

11) Cancer immunotherapies

Immunotherapy endows the immune system with reactivities that are not present naturally, training the patient's immune system to eliminate cancer cells by targeting them. They are a pivotal treatment for eradicating a wide spectrum of immune disorders, from immunodeficiency to cancer. In the last century, immunotherapy has given rise to a new oncology revolution, becoming one of the most prominent approaches in the fight against cancer. One of the main advantages of this therapy is the durable antitumor response it produces and the minor adverse effect it can cause compared to traditional therapeutic

strategies such as surgery, radiation therapy (RT), and chemotherapy (151),(152). However, even immunotherapy has some restrictions. The first limitation in its efficacy is due to the low quality of response and the unavoidable effects caused by the systemic administration of therapy (153). Another limitation of the treatment is the defective presence of the antigen by the immune system, which results in the ineffectiveness of the treatment (154). Finally, the major obstacle is represented by immunosuppressive TME (155). Within the TME, tumor cells secrete immunosuppressive chemokines and cytokines like VEGF, TGF- β and other factors that recruit immune-suppressive cells like Treg and MDSCs. In this environment, these cells express immune checkpoint molecules such as PD-1 and CTLA-4 that play a pivotal role in immune evasion, leading to the inefficacy of immunotherapy (156). Although immunotherapy has some side effects, the research field studying these therapies is continually growing. In fact, current immunotherapies can be divided according to their intervention into active and passive therapies. The former refers to immune checkpoint therapy (ICB), therapies that model de novo TME such as cytokines or chemokines administration and cancer vaccines, while the latter includes antibody-targeted therapy, adoptive cell therapy (ACT) and oncolytic virotherapy (157, 158).

11.1) Immune checkpoint therapy

The regulation of immune system hyperactivation is governed by conserved negative regulators known as checkpoint molecules. Checkpoint molecules, like PD-1, CTLA-4, LAG3, and Tim3, ensure that T cell responses maintain tolerance to self-immunological homeostasis and regulate the duration of the immune response, decreasing tissue damage. (159). The PD-1 pathway, which includes both the PD-1 receptor and its ligand PD-L1, is a crucial signaling pathway to reinvigorate the functionality of cytotoxic T lymphocytes. The PD-1 receptor down-regulates the immune response, and its loss causes a dysfunction in the peripheral tolerance (160). Furthermore, like CTLA-4, it plays a central role in maintaining T cell homeostasis (161, 162). Although the mechanism driven by the PD-1 axis controls immune response, the pathway is also exploited by cancer cells. These cells induce the up-regulation of PD-1 expression, leading to T cell exhaustion, resulting in an inefficiency of the immune system in fighting the tumor. This, in turn, creates a tumor microenvironment that favors the growth and proliferation of malignant cells. To avoid these problems, a solution is represented by the inhibition of this pathway using monoclonal antibodies (163, 164) or the secreted extracellular domain of PD-1 (165). Indeed, studies that use the aforementioned therapy highlight a reversion of the PD-1 axis with enhanced cytotoxicity

toward tumor cells, so monoclonal antibodies that block PD-1 were designed and developed (166). The use of anti PD-1 therapy has different advantages such as, ameliorating the overall patient survival, indeed meta-analyses have shown that α PD1 therapy provides a significant survival advantage, for 0.76 to 0.81, over conventional therapies for metastatic or advanced cancers (167). The blockade of PD-1 leads to a durable response and it has a broad applicability, in fact, several studies demonstrate a response in advanced melanoma, renal cell carcinoma and NSCLC (168). Patient responses to α PD-1 are often prolonged compared to conventional therapies (169). Furthermore, check point blockade immunotherapy has lower toxicity compared to traditional therapies and it can be used in combination with chemotherapy, radiation, target therapies to enhance antitumor activity (170, 171). In 2014, the Food and Drug Administration (FDA) approved the first humanized PD-1 target therapies, referred to as Pembrolizumab (172) and Nivolumab (173), against unresectable melanoma. To date, anti-PD-L1 therapy shows a decrease in tumor growth in patients with melanoma, renal cell carcinoma, non-small cell lung cancer, and bladder cancer (174-176).

11.2) Remodeling the TME through immunotherapy

The tumor microenvironment is composed of different transformed cells, such as TAMs, tumor-associated fibroblasts (TAFs), MDSCs, and Tregs, as well as cytokines that promote tumor growth by inducing the suppression of antitumor immune activation (140). For these reasons, treatments that can regulate the activity of transformed cells and the secretion of cytokines and chemokines have been created, with the major ones being targeted nanomedicines and nanovaccines (177). The first includes nanoparticles capable of targeting specific components of the TME, such as transformed cells or immunosuppressive cytokines, whereas the latter remodel the TME targeting specific immune cells, such as TAMs, which cause an immunosuppressive environment (178).

11.3) Adoptive cell transfer therapy (ACT)

Adoptive cell transfer therapy is a personalized treatment that enables the manipulation and infusion of antitumor lymphocytes into cancer-bearing hosts. The most representative ACT approaches are the injection of tumor-infiltrating lymphocytes (TILs) and the administration of chimeric antigen receptor T cells (CAR-T). The first type of therapy is the most effective, especially in metastatic melanomas, because it induces their complete remission (179). The development of the therapy starts with biopsy analysis and its division into a single cell suspension. The individual cells are grown with IL-2, and the lymphocytes that are grown

and able to destroy the tumor cells are expanded and tested for reactivity against the tumor. Those with the best reactivity are rapidly expanded and then a number greater than 10^{11} is administered to the patient. In addition, for a more effective clinical response, it is important that the patient undergoes a preparative lymphodepletion regimen (180, 181). Instead, CAR-T administration involves genetically modified T cells with the introduction of anti-tumor receptors inside healthy T cells. The engineered cells are capable of expressing specific antigens, different intracellular domains, non-MHC restriction receptors, and co-stimulatory receptors such as CD28 (182) or CD137 (183) to fully activate T cells, and last functional transgenic protein like suicide genes or IL genes. All these feature lead CAR-T cells to recognize and attack malignant cells, resulting in a good therapeutic response, especially in hematological malignancies like acute lymphoblastic leukemia (ALL), diffuse large B cell lymphoma and myeloma (184). However, their efficacy in solid tumors is still low because there is a lack of antigens that target solid tumors (185). There are several key limitations of ACT therapy like T-cell dysfunction because T cells utilized in ACT can become dysfunctional due to terminal differentiation, exhaustion, senescence, and activation-induced cell death. This can undermine the potency of ACT (186). Tumors can develop resistance mechanisms like antigen escape, where they downregulate or lose the target antigen recognized by the engineered T cells (184). For some ACT approaches like CAR-NKT cell therapy, the starting cell population (NKT cells) is poor of numbers, making it difficult to obtain enough cells for treatment. Prolonged in vitro expansion to generate sufficient numbers can also impair T-cell function (187). Finally, the manufacturing capabilities and cost associated with the production of the therapy are very high and represent a significant barrier to broad implementation (188).

11.4) Cancer vaccines

Cancer vaccines are one of the most promising immunotherapies involved in the fight against cancer. Their principal function is to induce and amplify an antigen-specific immune response through active immunization against tumor cells (189). Cancer vaccines can be classified into prophylactic vaccines and therapeutic vaccines; the first are used to prevent the development of cancer targeting oncogenic viruses, like cervical cancer caused by human papillomavirus (190),(191), and hepatocellular cancer caused by hepatitis B (192). The second ones are designed to support the immune system in the killing of cancer cells. Another categorization of cancer vaccines can be performed based on their mode of action, including cell-based, peptide-based, protein-based or genetic (DNA, RNA and viral bacterial) based

vaccines (193),(194). The advantages of cancer vaccines comprise the potential to induce a robust immune response, the long-term prevention and treatment of cancer, minimal side effects compared to traditional therapies like chemotherapy, and the ability to specifically target cancer cells while sparing healthy cells (195). However, disadvantages of cancer vaccines encompass the difficulty in developing effective vaccines for all types of cancer, the variability of immune responses among individuals, and the potential for limited efficacy in advanced-stage cancers. Among the different kinds of cancer vaccines, a new therapeutic approach that is showing encouraging results is the neoantigen-specific cancer vaccine. The study and the discovery of neoantigens have led to a change in the development of new therapies for two reasons: first, they establish a complex immune tolerance inducing a selective immune response against only cancer cells that express the specific neoantigen. Second, they appear to be more suitable compared to TAA because they are present only in tumor cells and not in normal cells, leading to a reduction in autoimmune-related toxicities (119). The design of the personalized vaccine begins with a small tumor biopsy and healthy tissue from the patient. Both pieces are subjected to whole-exome sequencing and RNA sequencing, and bioinformatic tools and prediction are used to identify gene variants that encode tumor specific peptides, the neoantigens. Then, neoantigens are screened using prediction algorithms that estimate peptide-MHC (or HLA) binding, such as NetMHC or IEDB (196). This parameter is crucial because the stability between the peptide and the MHC is considered to be a better predictor of immunogenicity compared to the MHC affinity alone (197). Then Multiple predicted neoantigens can also be subjected to further validation through mass spectrometry (198). Once the predicted neoantigens are chosen, they are first coupled to an adjuvant to prime and activate T cell responses, and finally, encoded into viral platforms as adenoviruses, poxviruses, which are suitable for encoding a large number of antigens and inducing effective antitumor immunity (199).

As previously reported, there are different formulations for the creation of neoantigen vaccines such as:

- DNA vaccines involve the transfection of a DNA sequence that encodes neoepitopes of interest. However, the use of CG-rich DNA, which was intended to serve as an immune adjuvant by stimulating Toll-like receptors, resulted in suboptimal immunogenicity and limited clinical efficacy (200).
- RNA vaccines use RNA sequences encoding tumor-specific neoantigens to stimulate an immune response against cancer cells. The key features of these vaccines are the possibility to encode multiple epitopes, a good profile with a potential adjuvant effect, and they can be

rapidly manufactured and administered to patients, as they do not require tumor tissue for preparation like peptide-based vaccine (201, 202).

- Peptide-based vaccines are a type of vaccine that consists of short sequences of amino acids (peptides) engineered to mimic specific neoepitopes. At the beginning of the development of these vaccines, there was a problem of high immune escape induced by a single peptide approach; consequently, to avoid these problems, multi-peptides approach were developed. Another advantage that they possess is that they can be combined with any adjuvant (203) (204).

As reported in Table 1, various phase 1 and 2 clinical trials that use target neoantigens for therapeutic vaccination have yielded promising outcomes (205). For example, in a phase I clinical trial involving 13 melanoma patients vaccinated with mRNA vaccines encoding five non-synonymous single nucleotide variants (nsSNV), T cell responses against multiple neoantigens have been observed with a reduction of the metastatic events across the participants (206). Among these, two patients exhibited objective responses, one had mixed response, another had stable lymph node metastases that were removed using ICB therapy, and one achieved a complete response when treated in combination with α PD-1 therapy. The remaining eight patients-maintained tumor-free status for a duration of 12-23 months (207).

Year of publication	Investigator /sponsor	Clinicaltrials.gov identifier, phase	Indication	Platform /treatment	Key results
2014	NIH	NCT01174121 , 1	Metastatic cholangiocarcinoma	Adoptive transfer of neoantigen-specific (ERBB2IP E805G) CD4 ⁺ T cells isolated from tumor	Decrease in target lesions with stabilization of disease, reinjection led to tumor regression, single patient report
2015	Washington University	NCT00683670 , 1	Stage III or IV melanoma	Intravenous application of neoepitope peptide-loaded DC vaccine	CD8 ⁺ T cell responses and broadened antigenic breadth as well as clonal diversity
2016	NIH	NCT01174121 , 2	Metastatic colorectal cancer	Adoptive transfer of neoantigen-specific (KRAS G12D) CD8 ⁺ T cells isolated from tumor	Regression of multiple lung metastases upon infusion of four different T cell clonotypes
2017	BioNTech	NCT02035956 , 1	Stage III or IV melanoma	Intranodal application of naked mRNA vaccine encoding for multiple neoepitopes	CD8 ⁺ and especially CD4 ⁺ T cell responses against multiple neoantigens, significant reduction of cumulative rate of metastatic events after vaccination
2017	Dana-Farber Cancer Institute	NCT01970358 , 1	Stage III or IV melanoma	Subcutaneous application of peptide vaccine consisting of pooled mutated epitopes	Polyfunctional CD8 ⁺ and especially CD4 ⁺ T cell responses with durable memory response, recognition of autologous tumor, combination with anti-PD-1 therapy beneficial for clinical outcome
2019	Immatics	NCT02149225 , 1	Glioblastoma	Intradermal application of peptide vaccine consisting of shared and mutated epitopes	CD8 ⁺ and CD4 ⁺ T cell responses against multiple shared and mutated epitopes
2019	Dana-Farber Cancer Institute	NCT02287428 , 1/1b	Glioblastoma	Subcutaneous application of peptide vaccine consisting of pooled mutated epitopes	Polyfunctional CD8 ⁺ and CD4 ⁺ T cell responses with enriched memory phenotype and augmented T cell infiltration to the tumor
2020	Dana-Farber Cancer Institute /Neon Therapeutics / BioNTech US	NCT02897765 , 1	Advanced melanoma, NSCLC, bladder cancer	Subcutaneous application of peptide vaccine consisting of pooled mutated epitopes combined with PD-1 blockade	Durable CD8 ⁺ and especially CD4 ⁺ T cell responses with cytotoxic potential, observation of epitope spreading upon vaccination
2020	NIH/Moderna	NCT03480152 , 1	Metastatic gastrointestinal cancer	Intramuscular application of LNP-formulated mRNA vaccine encoding for multiple neoepitopes	CD8 ⁺ and CD4 ⁺ T cell responses against multiple mutated epitopes, small patient group (n=4), no objective clinical response
2021	NCT/ University of Heidelberg	NCT02454634 , 1	Newly diagnosed glioma	Subcutaneous application of a single IDH1 (R132H) peptide vaccine	Vaccine-induced CD4 ⁺ T cell responses across multiple MHC alleles in over 90% of the patients

Table 1. Currently clinical trials that use neoantigens cancer vaccines as targets

Figure adapted from Peri A. et al, Nature cancer 2023.

In clinical trials, the viral platforms most used are:

- Poxviruses such as modified vaccinia Ankara (MVA) or New York vaccinia virus (NYVAC)
- Alphavirus
- Rare human adenoviruses (Ad) serotypes
- Human Adenoviruses 5
- Chimpanzee adenoviruses (ChAd)

Initially in the generation of neoantigen vaccines, adenoviruses were considered the best candidate vaccine platform, because they infect different types of cells, like quiescent cells and APC, their genome is safer compared to the other viral platform and they drive a robust and selective T- and B-response (208, 209).

However, the major challenge associated to Ad viruses is the pre-existing adaptive immunity in humans, in fact the presence of circulating antibodies anti-HAdV-5 alters the ability of HAdV-5 vectors to transfer the gene of interest to target tissue (Fig1.4) (210, 211).

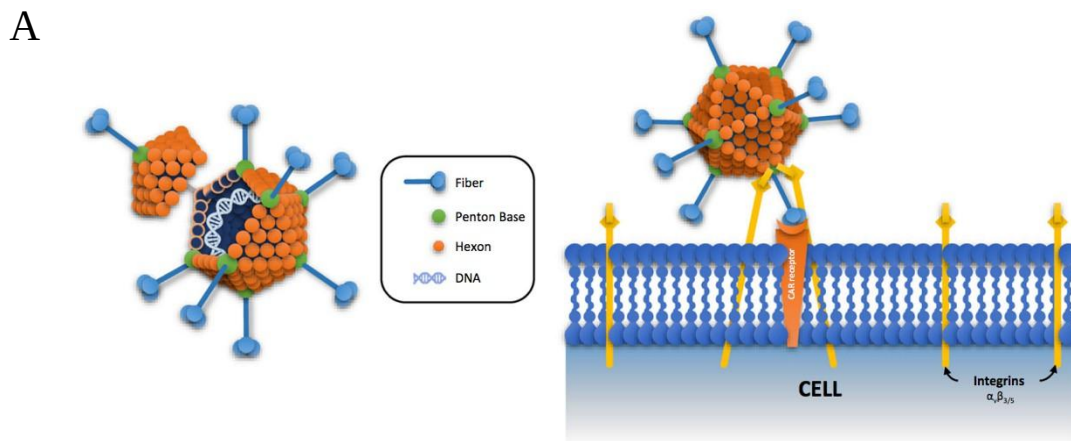


Fig. 1.4. Structural organization of Adenovirus

Adenoviruses are a non-enveloped, double-stranded DNA virus constitute by structural protein, hexon, penton base and fiber. The last two components mediate the interaction with Cocksackie and the adenovirus receptor, also known as CAR. Figure adapted from Mendorça et al., Nature partner journals, 2021.

To overcome the presence of pre-existing immunity against HAd, nonhuman primate-derived adenoviruses (NHPAdV) are candidates for research use (212). Although, they are closely related to HAdVs both in terms of genome structure and sequence homology, they differ markedly from the latter in the main neutralizing determinants that lead to a minor seroprevalence and lower neutralizing titers in human individuals worldwide (213). The principal vectors isolated from NHPAdV are Chimpanzees (ChAd), Bonobo (PanAd), Gorillas (GAd) and Macaques (SAd), but only two GAd vectors join the clinical stage (NCT04041310) (214). Another class of viral vectors that have characteristics similar to GAd vectors are the poxviruses, such as MVA (215). They can be used in combination creating a heterologous prime/boost vaccination protocol, which is necessary to overcome the issue of anti-adenovirus pre-existing immunity in humans (216). Nowadays, there are several clinical trials using GAd vaccine against various types of tumors (217),(218). Although various types of colorectal cancer are treated with GAd vaccine, often in combination with immune checkpoint therapy as pembrolizumab or nivolumab (217), the immune responses they

induce remains poorly studied. For this reason, the goal of our work aims to study the adaptive immune system responses of neoantigen-specific CD8⁺ T cells in both murine and human tumor-bearing models, treated with the combination of immunotherapies such as adenoviral vaccines and immune checkpoint blockade.

Overall, the principal advantage of administering cancer vaccines is the development of personalized medicine for each patient that leads to specific immune response and low side effect, whereas the limitations are that some antigens may induce autoimmune toxicities related to immune activation in non-target tissues, and the mechanism of action induced by these vaccines is currently poorly understood.

AIMS

The combination of immunotherapy, in particular of neoantigen-based adenovector vaccine and monoclonal antibodies against PD-1 acts synergistically to eradicate large established tumors (18). However, the mechanism underlying the antitumor effect and the synergy between the immunotherapies have not been elucidated. Understanding the mechanism and the T cell responses triggered by the immunotherapies, can lead to an improvement in the development of these one and in the creation of a new off shelf and personalized antitumor vaccines.

The majors' purposes of this project are:

- 1) To characterize the immune phenotype of neoantigen-specific CD8⁺ T cells responses in colon cancer following the combination of nonhuman adenoviral Great Apes adenoviral (GAd) vaccine and immunotherapies.
- 2) To identify the gene regulatory networks underlying the clonal heterogeneity of neoantigen specific T cells by single cell multi-omics approach in treated tumor bearing mice.
- 3) To study the vaccine immunogenicity, immune responses and the gene regulatory networks by single cell approach in MSI colorectal cancer and in melanoma patients treated with a heterologous prime/boost protocol characterized by the injection of Pembrolizumab (α PD-1), GAd and Modified Vaccinia Ankara (MVA) neoantigen-vaccine.

RESULTS

α PD-1 and GAd treatment expands neoantigen-specific CD8 T cells in MC38 model.

To develop an adenovirus (Ad) vaccine that targets tumor-specific neoantigens, we selected seven predicted immunogenic neoepitopes, derived from tumor-specific point mutations of the murine MC38 colorectal adenocarcinoma cell line. These peptides were chosen based on their high binding affinity for the class I major histocompatibility complex (MHC-I) compared to the corresponding wild-type peptides and were identified using mass spectrometry (MS) (198). To evaluate the stability and lifespan of neoepitope-MHC complexes, we used flow cytometry to measure the surface expression of MHC-I molecules in RMA-S cells after loading with the neoantigen peptides (Fig.2.1A)(219). The results showed that the Reps1, Cpne1, and Adpgk peptides exhibited higher and longer stability compared to the other neoepitopes (Fig.2.1B).

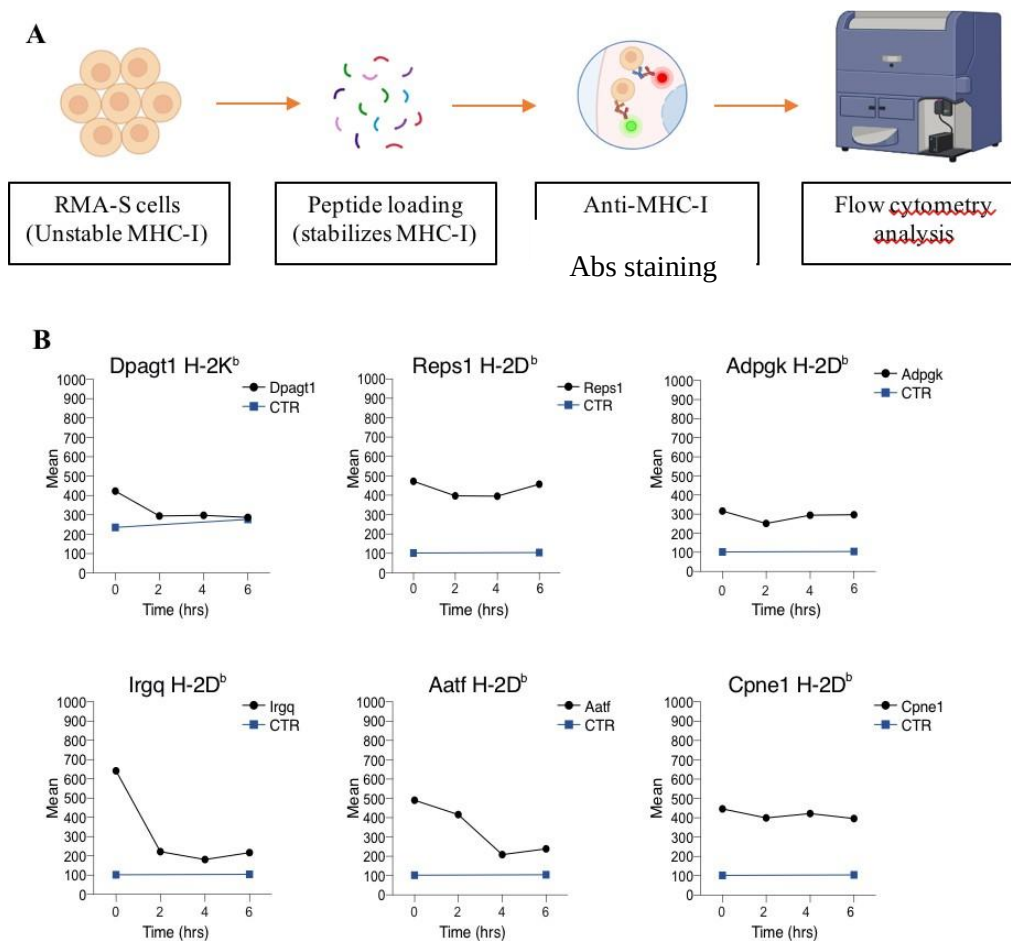


Fig. 2.1. Kinetics of MHC-I (major histocompatibility complex I)-peptide affinity and stability were evaluated over time.

(A) Experimental design of the RMA-S plus peptide stability assay **(B)** Dpagt1, Repts1, Adpgk, Irgq, Aatf and Cpne1 were analyzed. Mean of two independent experiments is shown.

To create a single synthetic antigenic molecule, the seven neoepitopes were joined head-to-tail and cloned into a GAd vector, as previously described (125). The efficacy of the GAd vaccine and α PD-1 treatment was evaluated in mice with established subcutaneous MC38 tumors (Fig. 2.2A). Mice were subcutaneously injected with the adenocarcinoma MC38 cell line and, after 10 days, immunized with the GAd vaccine and treated with an antibody blocking PD-1 (α PD-1) once the tumor was established. Consistent with our previous findings (125), we observed enhanced antitumor control in mice receiving combined GAd and α PD-1 treatment compared to α PD-1 monotherapy, increasing overall survival (Fig. 2.2B).

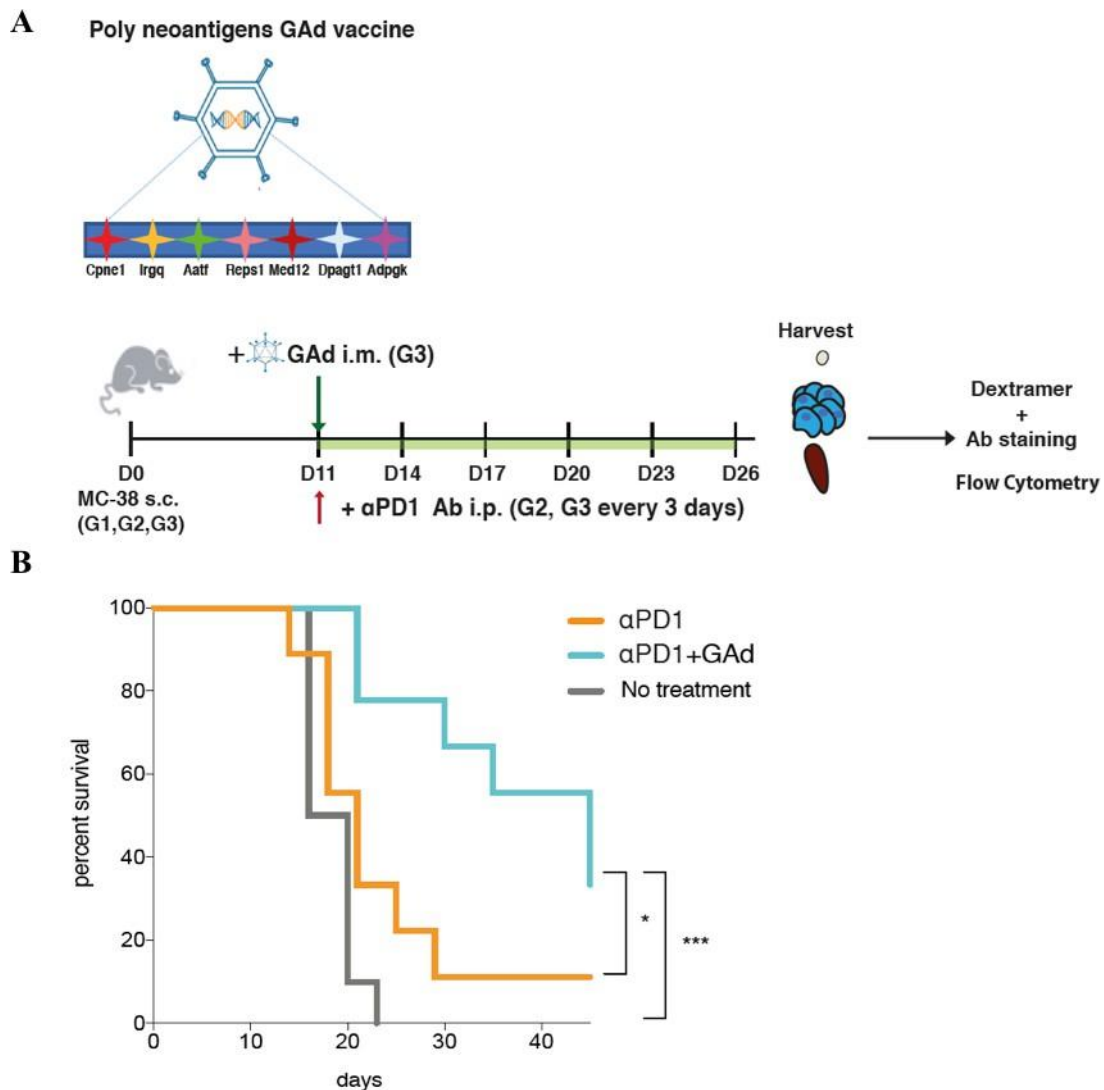


Fig. 2.2. Experimental design and overall survival of treated tumor-bearing mice.

(A) C57BL/6J female mice (G1 to G3) were subcutaneously (s.c.) injected with MC38 adenocarcinoma colon cancer cell line. Eleven days later, G2 and G3 were intraperitoneally (i.p.) injected, every 3 days, with the monoclonal antibody (Ab) α PD-1. Eleven days after tumor implantation (tumor size between 70 and 100 mm³), G3 were also intramuscularly (i.m.) injected with poly-neoantigen Gad vaccine. Twenty-six days after tumor implantation, Db-Adpgk⁺ and Db-Reps1+CD8⁺ T cells were analyzed, in the draining lymph node, tumor, and spleen, by flow cytometry. **(B)** Overall survival of tumor-bearing mice was measured over time in G1, G2, and G3.

Previous studies in the same MC38 tumor model identified the Adpgk neoepitope as the most effective in conferring protection after peptide combined with adjuvant immunization and exhibiting the most prominent expansion in the tumor (198). At day 26, mice were sacrificed and draining lymph nodes, tumor, and spleens were collected. The neoantigen-specific CD8⁺ T cell responses in these organs was detected using the H-2Db-Adpgk peptide–MHC-I dextramer after treatment with α PD-1 compared to the combination of GAd and α PD-1 (Fig. 2.3A, B, D, and F). In addition to analyzing CD8⁺ T cells specific for Adpgk, we also examined CD8⁺ T cells specific for Reps1, a neoepitope less effective in tumor rejection. As expected, in untreated control tumor-bearing mice, the frequency and number of both Db-Adpgk⁺ and Db-Reps1⁺ CD8⁺ T cells were very low (Fig. 2.3, A to C) (125). However, the frequency of Db-Adpgk⁺CD8⁺ T cells increased significantly in the draining lymph node, tumor, and spleen of tumor-bearing mice treated with GAd + α PD-1 compared to control tumor-bearing animals (Fig. 2.3,B,D and F,). In tumor-bearing mice treated with GAd immunization combined with α PD-1 antibody, we observed a significant increase in Db Adpgk⁺CD8⁺ T cells in the tumor and spleen, with approximately 100 and 10 times more cells, respectively, compared to mice receiving α PD- 1 treatment alone. In contrast, we did not observe substantial differences in CD8⁺ T cell responses with GAd + α PD-1 treatment against Reps1 in draining lymph nodes and tumors (Fig. 2.3, C and E). However, both frequency and number of Db-Reps1+CD8⁺ T cells were enhanced in the spleen (Fig. 2.3G). These findings indicate that GAd vaccination promoted neoepitope immunogenicity, leading to the expansion and accumulation of CD8⁺ T cells reactive to Adpgk and Reps1 neoepitopes in the spleen of tumor-bearing mice. Additionally, Adpgk-reactive CD8⁺ T cells were also expanded in the draining lymph nodes and tumors of these mice. These results suggest that two neoepitopes, Adpgk and Reps1, both identified by mass spectrometry on tumor cells and with similar features in terms of stability, predicted binding affinities, and in vivo immunogenicity, exhibit different capabilities to infiltrate the tumor bed.

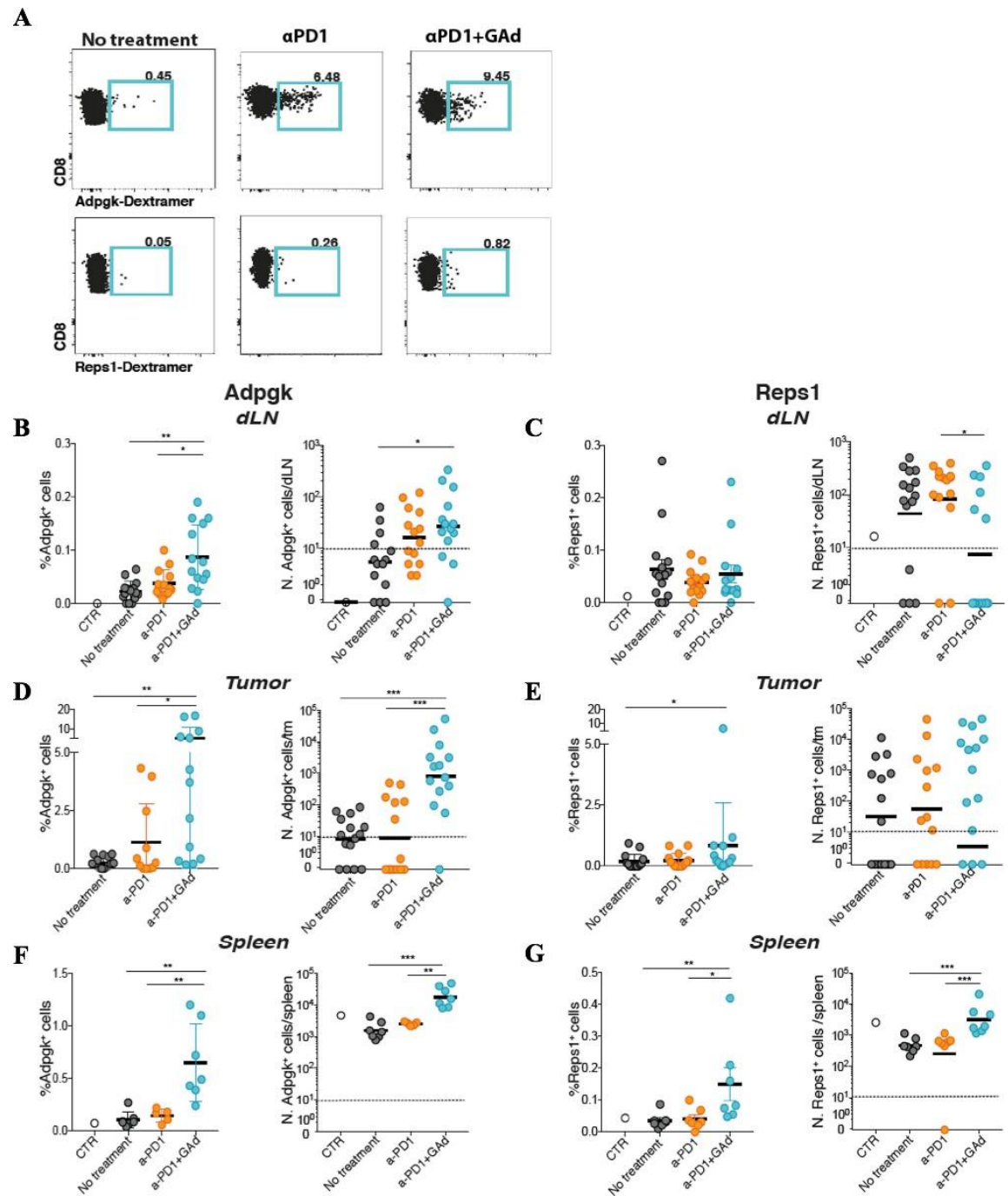


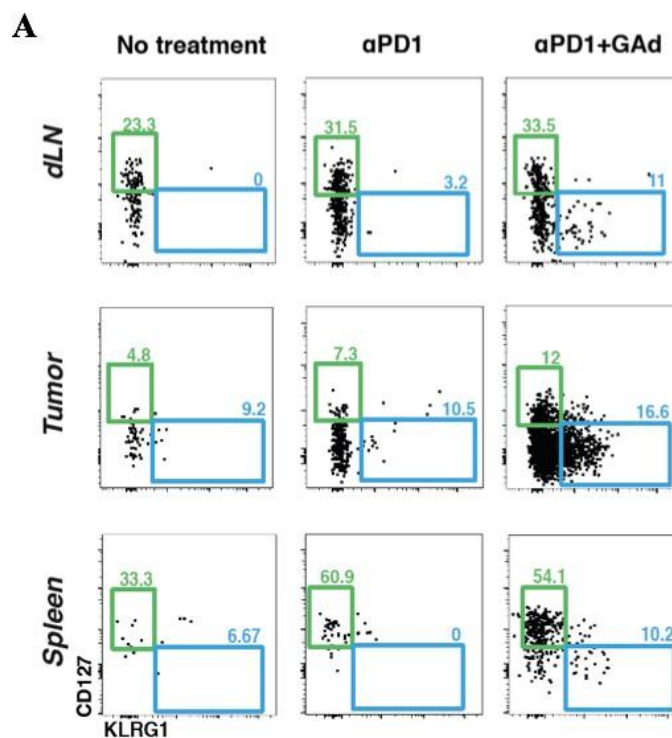
Fig. 2.3. GAd vaccination combined with α PD-1 treatment reduces tumor growth by increasing the number of neopeptide-reactive CD8⁺ T cells in mice.

(A) Representative dot plots of Db-Adpgk+CD8⁺ T cells and Db-Reps1+CD8⁺ T cells in the tumors; numbers represent percentages. (B, D and F) Percentages and numbers (N.) of Db-Adpgk+CD8⁺ T cells were measured in draining lymph node (dLN) (B), tumor (tm) (D), and spleen (F). (C, E and G) Percentages and numbers of Db-Reps1+CD8⁺ T cells were measured in draining lymph node (C), tumor (E), and spleen (G). Data are shown

as mean with SEM (**B to G, left**) and geometric mean (**B to G, right**). *P < 0.05, **P < 0.01, and ***P < 0.001 (Mann-Whitney test). Graphs are representative of six experiments with seven mice per group.

GAd therapeutic vaccination increases the frequencies of neoepitope-specific memory precursors and effectors

We further characterized the phenotype of Adpgk-specific CD8⁺ T cells induced by GAd vaccination in tumor-bearing mice by multiparametric flow cytometry, analyzing the frequency and number of CD127⁺ KLRG1[−] memory precursor, CD127⁺ CD62L⁺ KLRG1[−] central memory, CD127[−] KLRG1⁺ short-lived effector, or tissue-resident memory (T_{RM}) CD103⁺ CD69⁺ CD8⁺ T cells, as well as exhausted CD8⁺ T cells based on the expression of PD-1 and CD38 of Db- Adpgk⁺ CD8⁺ T cells as reported in Fig. 2.4 and Fig. 2.5 (51, 64, 90, 220). Although, αPD- 1 treatment alone resulted in a higher proportion of CD127⁺ KLRG1[−] memory precursors in the draining lymph node compared to the combined treatment with GAd (Fig.2.4D), we observed a significant increase in the number of CD127⁺ KLRG1[−] memory precursors in tumor-bearing mice treated with GAd in the draining lymph nodes, tumors, and spleens (Fig. 2.4 A and B). However, we found a higher proportion, a 10-fold difference of effector CD127[−] KLRG1⁺ Db-Adpgk-specific CD8⁺ T cells in draining lymph nodes, tumors, and spleens of mice injected with Gad in tumors and spleens as compared to mice receiving αPD- 1 alone (Fig. 2.4 A,C and E).



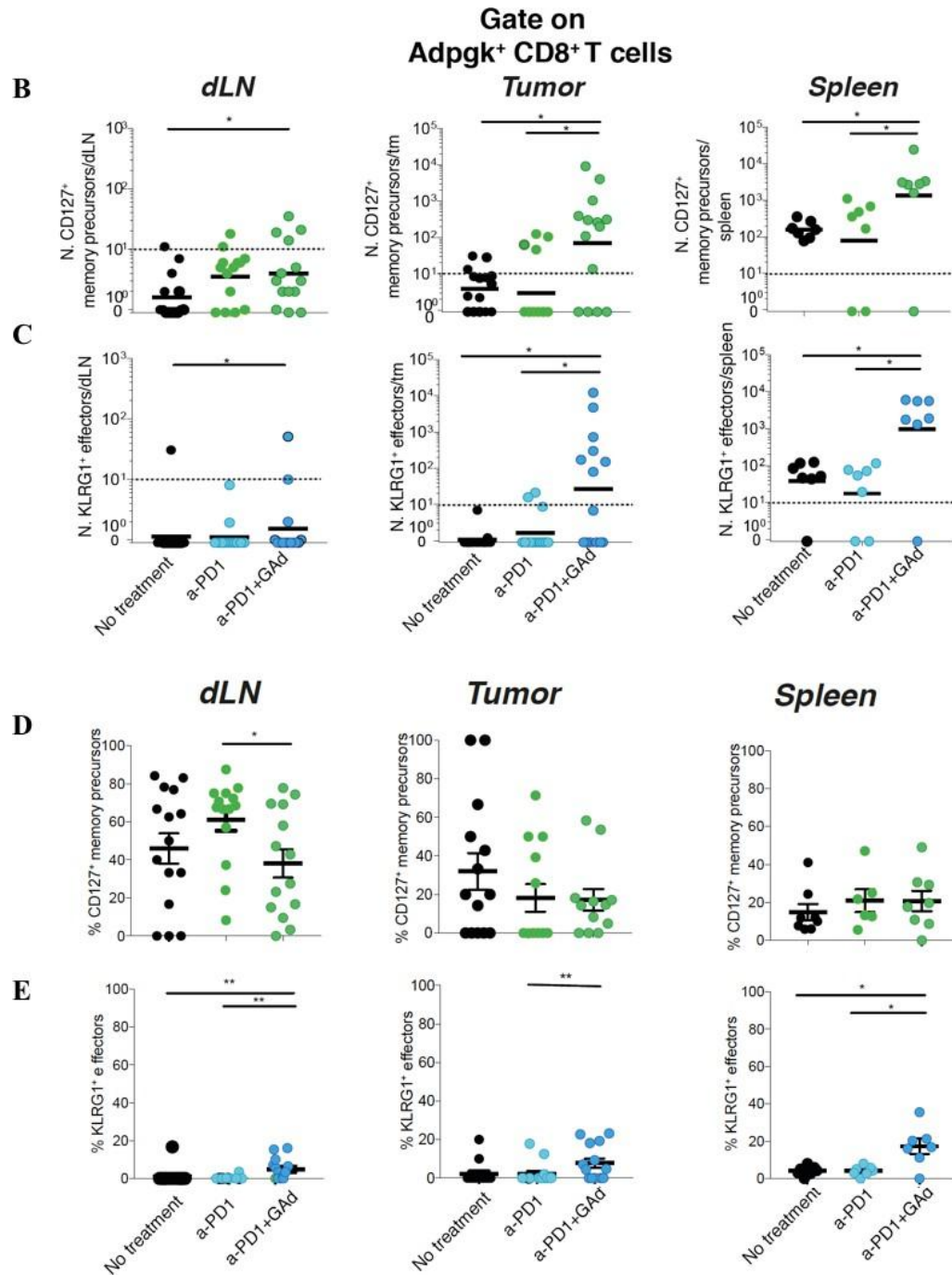


Fig. 2.4. GAd combined with α PD-1 treatment induces neopeptide-reactive memory precursor CD8⁺ T cells in mice.

(A) Representative dot plots of CD127 and KLRG1 markers (CD127⁺KLRG1⁻ memory precursors and CD127⁻KLRG1⁺ effectors); numbers represent percentages. (B to E) Numbers (B,C) and percentages (D,E) of Db-Adpgk⁺CD8⁺ T memory precursors and effectors were measured in draining lymph node (left), tumor (middle), and spleen (right).

We next investigated whether the memory precursor and effector subsets induced by GAd vaccination against neopeptides could also express the markers PD-1 and CD38, which are commonly associated with T cell exhaustion (Fig. 2.5 and Fig 2.6). Increased frequency and number of PD-1 and CD38 double-positive cells were found among the memory precursors within tumors, whereas low frequency and numbers were detected in draining lymph nodes (Fig. 2.5 A and B). Mice treated with Gad vaccine showed a reduction in the frequency of CD127 + CD8 + T cells co-expressing PD-1 and CD38 as compared to α PD-1 alone treated mice (Fig. 2.5B).

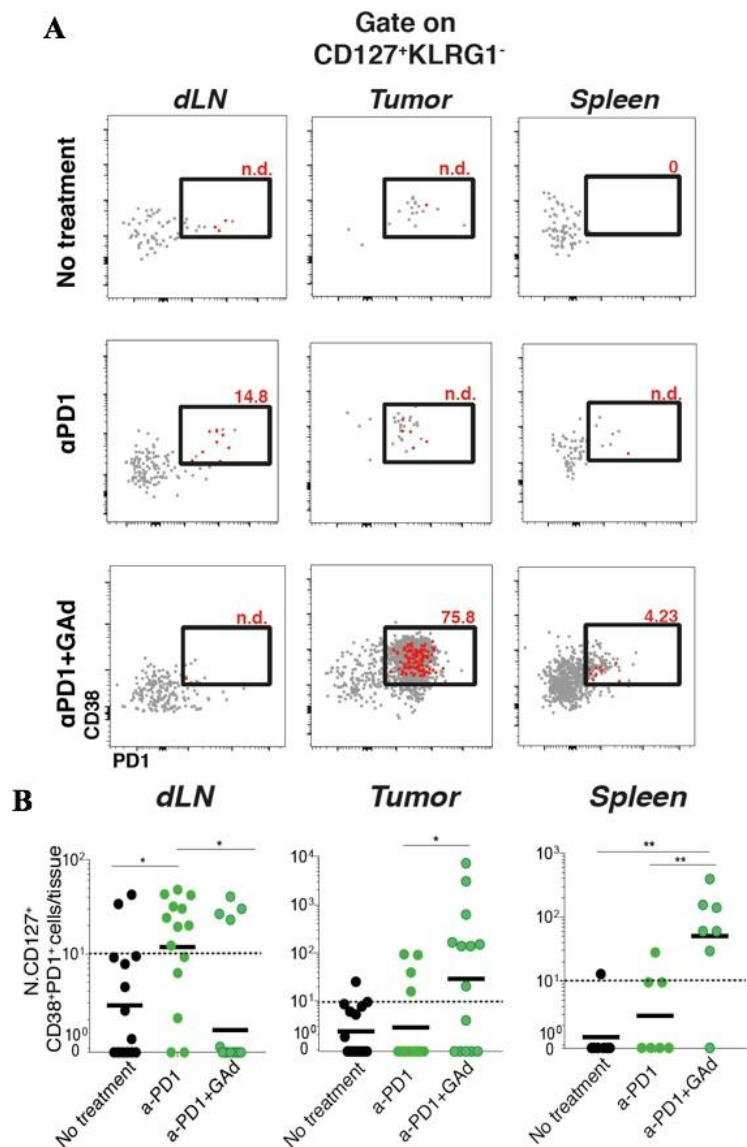


Fig. 2.5. Flow cytometry immune phenotypic analysis of memory precursors and effector cells.

(A) Representative dot plots of CD38 and PD-1 markers gated on CD127⁺KLRG1⁻ Db-Adpgk⁺CD8⁺ T cells.

(B) Numbers of CD38⁺PD-1⁺ cells gated on CD127⁺ Db-Adpgk⁺CD8⁺ T cells were measured in draining lymph node (upper), tumor (middle), and spleen (lower).

In GAd-treated mice, we found that approximately 58% of Db-Adpgk⁺ CD127⁻ KLRG1⁺ effector cells and 70% of CD127⁻ KLRG1⁻ CD8⁺ T cells in tumors expressed both PD-1 and CD38 (Fig. 2.6A). These observations indicate that, although a fraction of cells acquired exhaustion markers after GAd vaccination (Fig. 2.6, B and C), the number of exhausted T (T_{EX}) cells increased primarily among CD127⁻ KLRG1⁻ CD8⁺ T cells. In addition, the frequency of Db-Adpgk⁺ CD103⁺ CD69⁺ T_{RM} CD8⁺ T cells augmented in the tumors of α PD-1 + GAd-treated mice (Fig. 2.6 D and E).

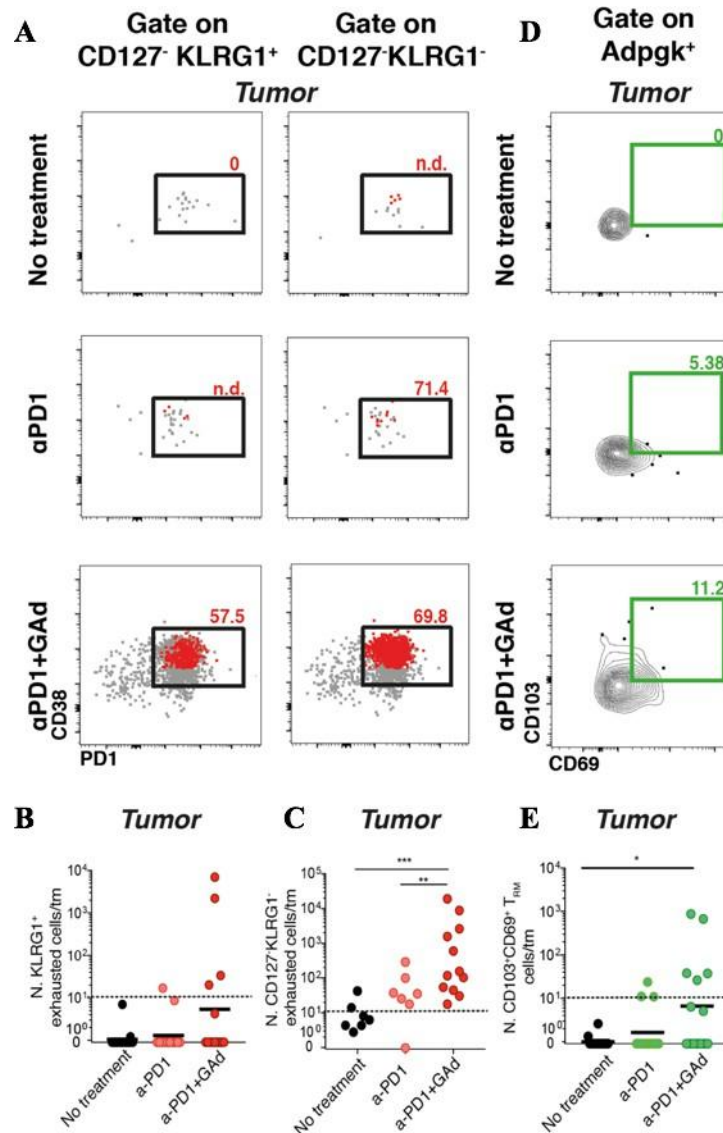


Fig. 2.6. Immune phenotypic characterization of exhausted and tissue resident memory cells.

(A) Representative dot plots of exhausted CD38+PD-1+ cells analyzed on gated CD127–KLRG1+ (left) and on gated CD127–KLRG1– (right) Db-Adpgk+ CD8+ T cells in tumor; numbers represent percentages. In red, memory precursors gated on Db-Adpgk+ CD8+ T cells were displayed; in gray, the total number of Db-Adpgk+ CD8+ T cells was displayed. (B) Numbers of exhausted Db-Adpgk+ CD8+ T cells on gated CD127–KLRG1+ were measured in tumor. (C) Numbers of exhausted Db-Adpgk+ CD8+ T cells on gated CD127–KLRG1– were measured in tumor. (D) Representative dot plots of CD103 and CD69 markers (CD103+CD69+ TRM cells) in tumor; numbers represent percentages out of the total CD8+ T cells isolated from the tumor. (E) Numbers of Db-Adpgk+ CD8+ TRM cells were measured in tumors.

Together these findings suggest that GAd immunization combined with α PD-1 enhances immunogenicity and induces distinct paths of differentiation and expansion of neoantigen-specific CD8+ T cells in tumor-bearing mice. This response is characterized by a higher number of memory progenitors in both draining lymph nodes and spleens, as well as effector T cells in the tumors.

scRNA-seq analysis of neoAg-specific CD8+ T cells demonstrate stem-like and effector cell accumulation after α PD-1 +GAd treatment

To further elucidate the heterogeneity and gene expression programs of neoantigen-specific CD8+ T cell subsets induced by Gad immunization and α PD-1 treatment, we conducted single-cell RNA sequencing (scRNA-seq) analysis on CD8+ T cells. Mice were subcutaneously injected with MC38 colon-rectal tumor cell line and they were divided in 3 groups, one group was no treated, the second group was treated with only α PD1, and the third group was injected with the combination of GAd neoantigen-vaccine plus α PD1. At day 27 mice were sacrificed and fluorescence-activated cell sorting (FACS) was performed from lymph nodes of control tumor-free mice and Db -Adpgk+ CD8+ T lymphocytes harvested from draining lymph nodes and tumors. (Fig. 2.7A).

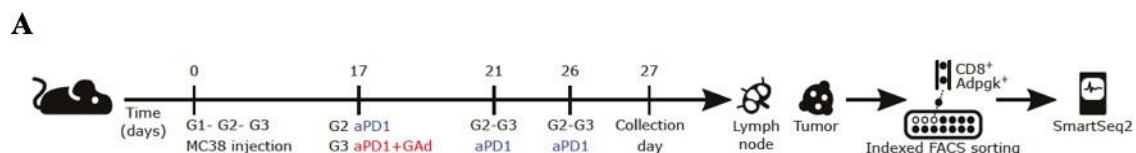


Fig. 2.7. Experimental design of neoantigen-specific single cell RNA sequencing.

(A) Three groups of mice (G1 to G3) were treated as shown; CD8+ T cells isolated from untreated mice were used as the control (CTRL).

Smart-Seq2 technology (221) was used to analyze index sorted cells from control and Db - Adpgk+ CD8+ T cells of individual mice. After sequencing and quality control filtering, we obtained 57 naïve T cells from two control tumor-free mice and 1132 Db -Adpgk+ CD8+ T lymphocytes from nontreated (157 cells), α PD-1-treated (226 cells), and α PD-1 + GAd-treated (749 cells) tumor-bearing mice (Fig. 2.8); for each condition, we processed nine, five, and eight biological replicates, respectively. A principal component analysis (PCA) of single-cell transcriptome data is visualized as a set of Uniform Manifold Approximation and Projection (UMAP) plots in Fig. 2.8 (A and B). As reported in Fig. 2.8A, we found a different distribution of Db-Adpgk+ CD8+ T cells isolated from the lymph nodes and tumors, underlining distinct gene expression profiles and to study and identify the distinct cell subsets, we next clustered the PCA results. The analysis of Db-Adpgk+ CD8+ T cells highlighted seven clusters (Fig.2.8 B and C), which were categorized as naïve (cluster 2), early activated (cluster 6), activated (cluster 1), T_{STEM} precursor (cluster 5), memory (cluster 7), effector (cluster 3), and T_{EX} (cluster 4) on the basis of distinct transcription profiles (Fig. 2.9 A to C).

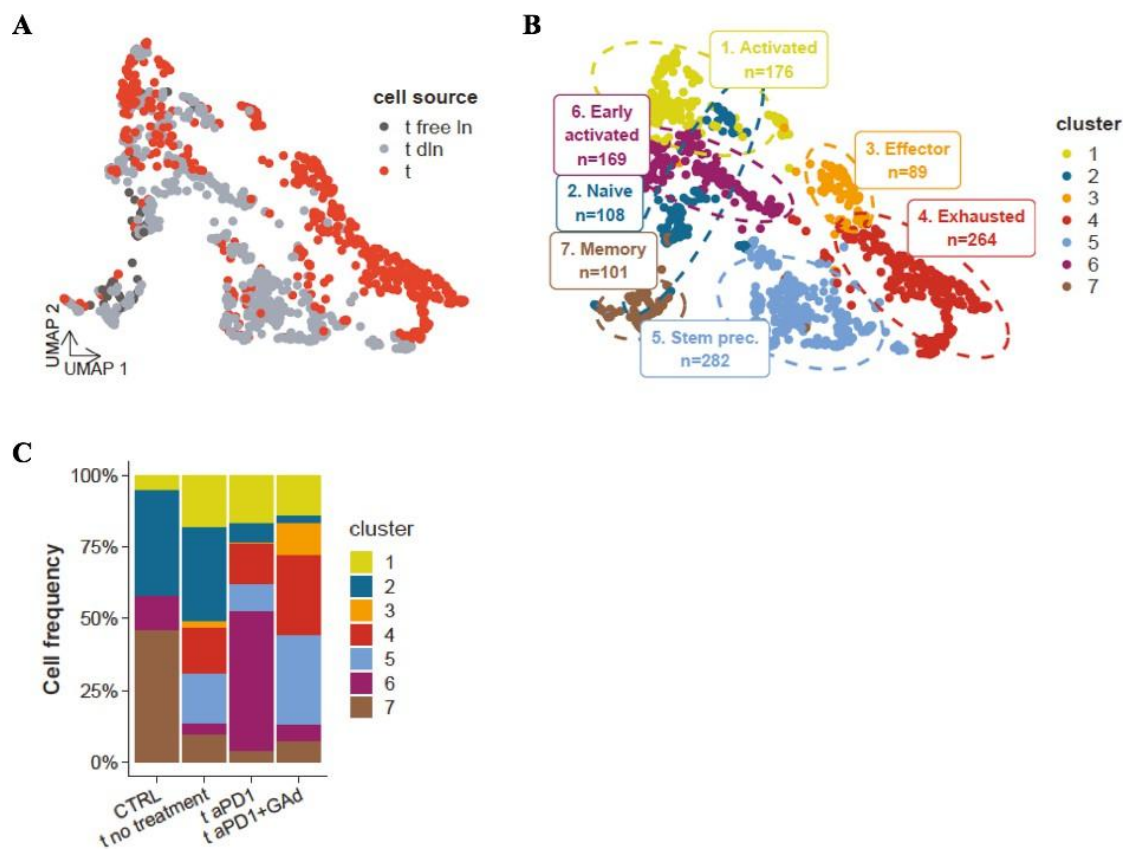


Fig. 2.8. Smart-Seq2 analysis of Adpgk+CD8+ T cells in dLN and tumors from free and tumor-bearing treated mice.

(A) UMAP visualization of CD8+ T cells (n = 1189) color-coded by tissue of origin (t, tumor; ln, lymph node, dln, draining lymph node). **(B)** UMAP visualization of clustering results with phenotype annotations. **(C)** Distribution of Db-Adpgk+ CD8+ T cell frequencies in each experimental condition, color-coded by cluster.

.

Neoantigen-specific CD8 T cells in the lymph node had a memory signature whereas effectors and exhaustion signature belonging to tumors; sorted control CD8⁺ T cells from tumor-free mice were found in the naïve and memory cluster (Fig. 2.8, A to C). The expressed genes that characterized naïve cells were *Tcf7* (Tcf1) and *Sell* (CD62L) (Fig. 2.9A). Early activated cells exhibited low expression of *Tcf7* transcriptional factor and *IL7r*, whereas effector and T_{EX} cell clusters displayed high expression of effector genes such as *Ifng*, *Gzmb*, *Prf1*, and *Cxcr6* (Fig. 2.9, A and C). Notably, the exhausted subset showed consistently higher expression of checkpoint genes *Pdcd1*, *Lag3*, and *Tigit* (Fig. 2.9A). In contrast, the T_{STEM} precursor population was characterized by the expression of *Tcf7*, *Cxcr3*, *Il7r*, and *Slamf6*, with *Eomes* being uniquely expressed in this cluster. Memory cells were distinguished by the expression of *Sell* and *Il7r* genes, and the transcriptional factor *Id3* was also transcribed in this subset. To investigate the distribution of cells among different clusters and their relationship with specific treatments, we analyzed the cell distribution among various clusters. In control tumor-free mice, the majority of total CD8⁺ T cells were localized in the memory and naïve clusters (46% and 37%, respectively), with lower frequencies in the early activated and activated clusters (12% and 5%, respectively) (Fig. 2.9C). In contrast, Db-Adpgk⁺ CD8⁺ T cells derived from nontreated tumor-bearing mice were predominantly found in the naïve cluster (32%) and, to a lesser extent, in the activated, T_{STEM} precursor, T_{EX}, and memory clusters (18, 17, 16, and 10%, respectively). In tumor-bearing mice treated with α PD-1, the majority of Db-Adpgk⁺ CD8⁺ T cells were found in the early activated cluster (49%), with smaller frequencies in the activated and T_{EX} clusters (17 and 14%, respectively). α PD-1-treated mice also exhibited a significantly lower frequency of the T_{STEM} precursor, naïve, memory, and effector clusters (10, 7, 3, and 0.4%, respectively). Db-Adpgk⁺ CD8⁺ T cells from tumor-bearing mice treated with α PD-1 + GAd combination was predominantly distributed in the T_{STEM} precursor and T_{EX} clusters (31 and 28%, respectively) and, in smaller proportions, in the activated, effector, memory, early activated, and naïve clusters (14, 11, 7, 6, and 3%, respectively). The distribution of Db-Adpgk⁺ CD8⁺ T cells across different clusters was not uniform between tumors and lymph nodes (Fig 2.9, D and E). In lymph nodes of α PD-1 + GAd-treated mice, Db-Adpgk⁺ CD8⁺ T cells were predominantly enriched in the T_{STEM} precursor cluster, accounting for 69% of the cells in cluster 5. Conversely, tumor-infiltrating Db-Adpgk⁺ CD8⁺ T cells from α PD-1 + GAd-treated mice were enriched in the T_{EX} (44%) and effector (19%) clusters. In contrast, Db-Adpgk⁺ CD8⁺ T cells from lymph nodes of α PD-1-treated mice were significantly augmented in the early activated cluster, comprising 64% of the cells. Furthermore, Db-

Adpgk⁺ CD8⁺ T cells from lymph nodes of untreated tumor-bearing mice were predominantly enriched in the naïve cluster, accounting for 55% of the cells.

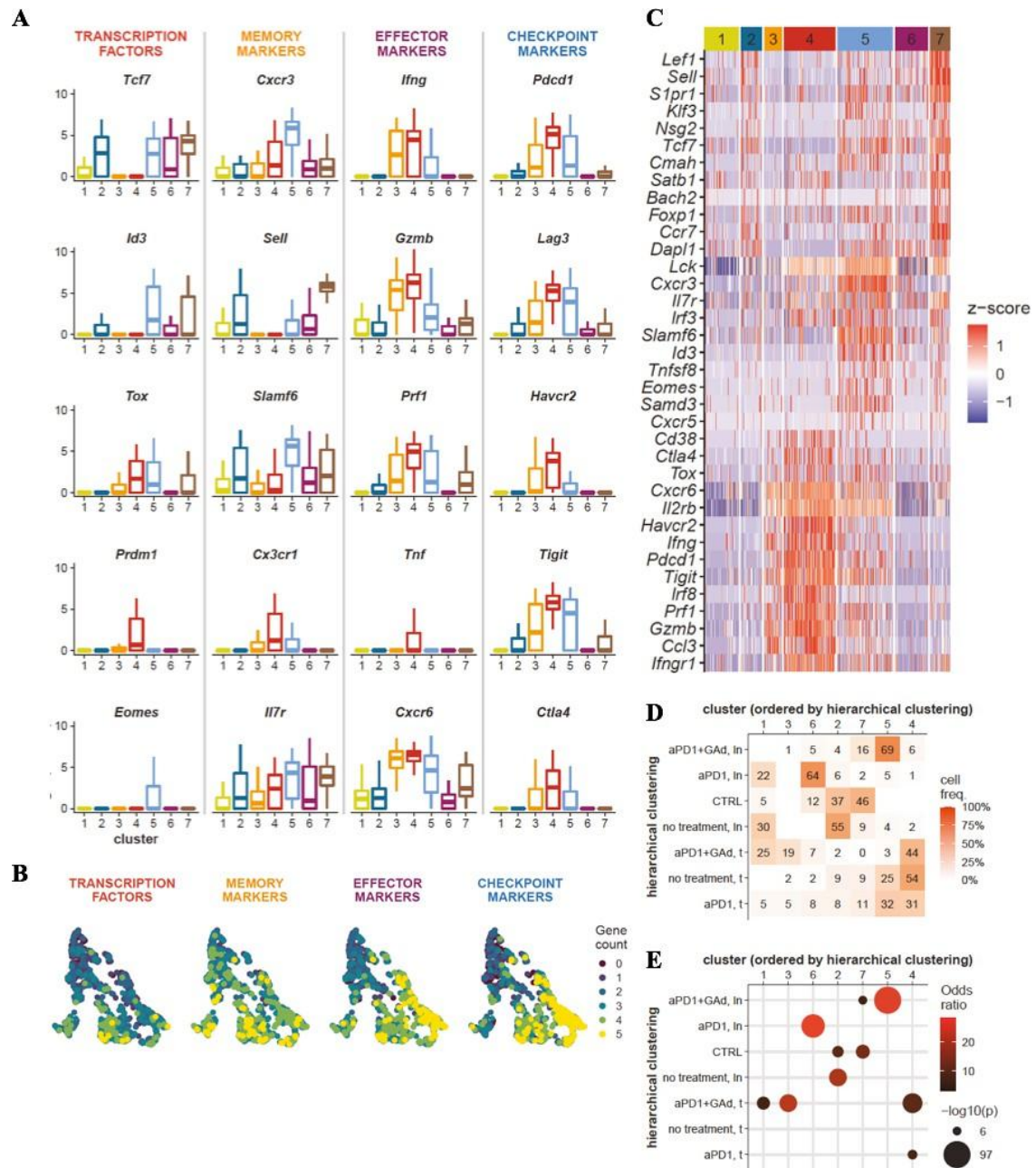


Fig. 2.9. scRNA-seq of CD8⁺ T cells identifies distinct subpopulations induced by α PD-1 + GAd vaccine in mice.

(A) Measurement of the expression of genes encoding transcription factors, memory, effector, and checkpoint markers is shown in boxplots. Kruskal-Wallis test, $P \leq 5.7-16$. (B) Number of expressed marker genes from (A) projected onto UMAP. (C) Heatmap of selected top differentially expressed (DE) genes, TSTEM progenitor, and TEX CD8⁺ T cell signatures (29), color-coded by cluster. (D) Heatmap of cluster-wise cell frequencies for each treatment and cell type (In, lymph node; t, tumor) and (E) results of Fisher's exact test of

those frequencies. The x- and y-axis orders were defined through hierarchical clustering. Frequencies were calculated from each combination of cell type and treatment (shown on rows) across all clusters; thus, the sum of the values of each row of the heatmap is 1 (=100%).

Overall, these results suggest that the combination treatment of α PD-1 and GAd promotes the differentiation of Db-Adpgk⁺ CD8⁺ T cells into T_{STEM} precursors in the lymph node and effector subsets in the tumor.

CD8⁺ T_{STEM} precursors are induced by the combination of GAd vaccine with PD-1 blockade

To determine the distinct immune phenotypes of each cluster, we performed differential expression analysis by comparing the gene expression profiles of each subset against all others. This approach enabled the identification of statistically significant genes (adjusted $P \leq 0.05$) in each cluster, with 7313, 2664, 2167, 6723, 4256, 5907, and 2449 genes exhibiting significant differences, respectively (Fig 2.10A).

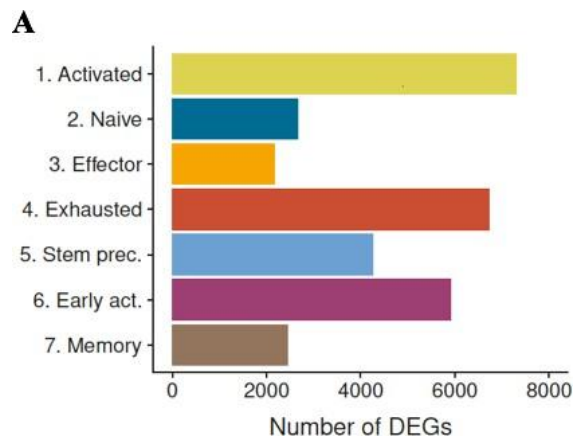


Fig. 2.10. scRNA-seq analysis of Adpgk specific CD8⁺ T cells from treated mice.

(A) Differentially expressed genes for each cluster.

Next, to investigate the gene expression programs and the stages of differentiation corresponding to each cluster, we employed a gene set enrichment analysis (GSEA), by pairwise comparison of each cluster against all other clusters, using published immunological signatures from the Molecular Signatures Database (MSigDB) (222). The analysis revealed differential enrichments in the gene expression profiles, with distinct signatures emerging for each cluster (Fig. 2.11A). In the naïve population (cluster 2), we observed positive enrichment (73),(223) for the TCF7⁺ T_{STEM} precursor signatures, with 26% mean signature

expression (Fig. 2.11B). In contrast, the naïve population exhibited a negative enrichment of memory, exhaustion, and effector signature (94, 224). In the effector population (cluster 3), we observed positive enrichment for the effector versus memory signature (94) and negative enrichment for the exhaustion signature. T_{EX} cells (cluster 4) exhibited a strong positive enrichment for exhaustion signatures (223), with a mean of 47% of genes expressed, and significant enrichment for T_{RM} gene expression profiles, with 44% of genes expressed (Fig. 2.11 A and B). T_{STEM} precursors (cluster 5) showed a positive enrichment for both memory and effector signatures, indicating a potential for both memory and effector differentiation. (224). This cluster also showed enrichment for T_{STEM} precursor (223) and T exhausted circulating cell signatures, with a mean of 38% and 30% of genes expressed, respectively (Fig. 4, A and B). In contrast, the early activated subset (cluster 6) presented negative enrichments for the exhaustion (225), memory, and effector signatures (94, 224) suggesting a lack of exhaustion markers and a potential for early activation. The memory population (cluster 7) displayed positive enrichments in TCF7⁺ cell tumor infiltration (223), T_{STEM} precursor, and circulating memory T cell signatures (226), but negative enrichment in the effector signature (94). These results confirmed that clusters 5 and 7 were enriched in Db-Adpgk⁺ CD8⁺ T_{STEM} precursor cells, with circulating and memory gene signatures, whereas cluster 4 identified the exhausted Db-Adpgk⁺ CD8⁺ T cell subset.

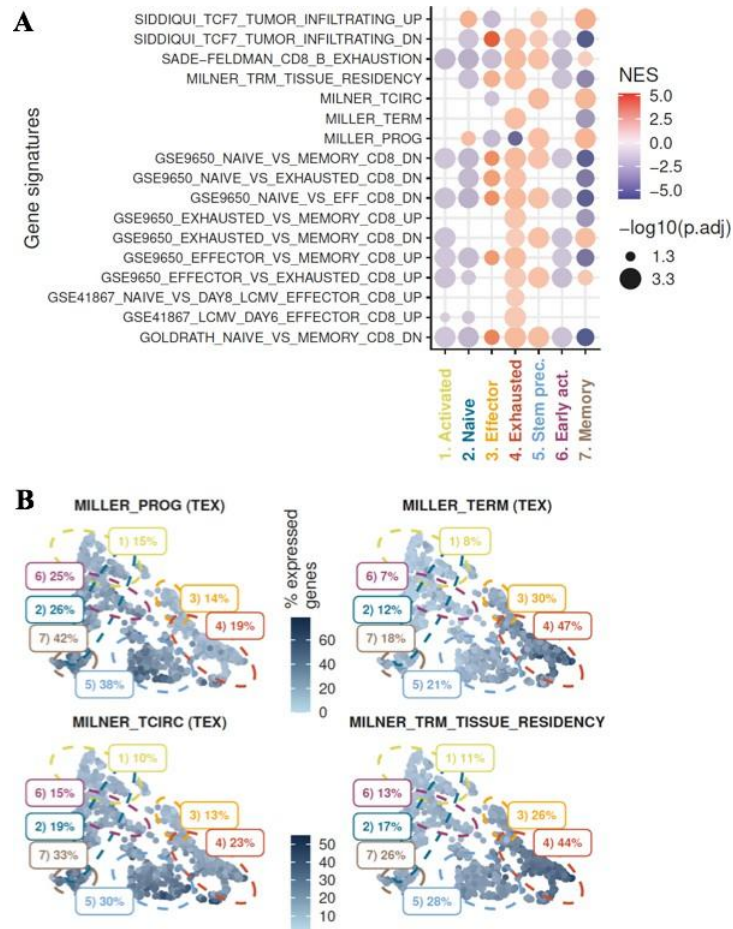


Fig. 2.11. Phenotype landscapes and trajectories of differentiation of Db-Adpgk+ CD8+ T cells from mice.

(A) GSEA of DE genes found in clusters (adjusted $P \leq 0.05$). NES, normalized enrichment score. **(B)** Percentage of signature expression of TSTEM progenitors (MILLER_PROG.) and TEX CD8+ T cells (MILLER_TERM), core circulating (MILNER_TCIRC), and core tissue-resident memory T cell (MILNER_TRM_TISSUE_RESIDENCY) signatures in each cell.

The scRNA-seq profiles revealed different immune phenotypes between lymph nodes and tumors, suggesting distinct stages of lineage commitment. Based on the insights gained from the gene set enrichment analysis, we further investigate whether CD8+ T cells reactive against Adpgk could follow distinct pathways of lineage differentiation after the combination of α PD-1 + GAd treatment. To determine the transcriptional trajectories corresponding to fate commitment of Db-Adpgk+ CD8+ T cells, we performed a supervised trajectory analysis (225), (227). This analysis revealed three distinct differentiation paths originating from the naïve cell cluster (Fig. 2.12, A and B). These trajectories likely represent the potential lineage commitments of Db-Adpgk+ CD8+ T cells in response to the combination treatment. By tracing these transcriptional trajectories, we gained insights into the dynamic transitions and

fate decisions of the neoantigen-specific CD8⁺ T cell population under the influence of α PD-1 and GAd. The supervised trajectory analysis revealed three distinct lineage trajectories originating from the naïve cell cluster. The first lineage trajectory, comprising 360 cells, was characterized by a progression toward T cell activation. The second lineage trajectory, consisting of 548 cells, exhibited a more complex progression through the early activated, effector, and T_{EX} cell populations.

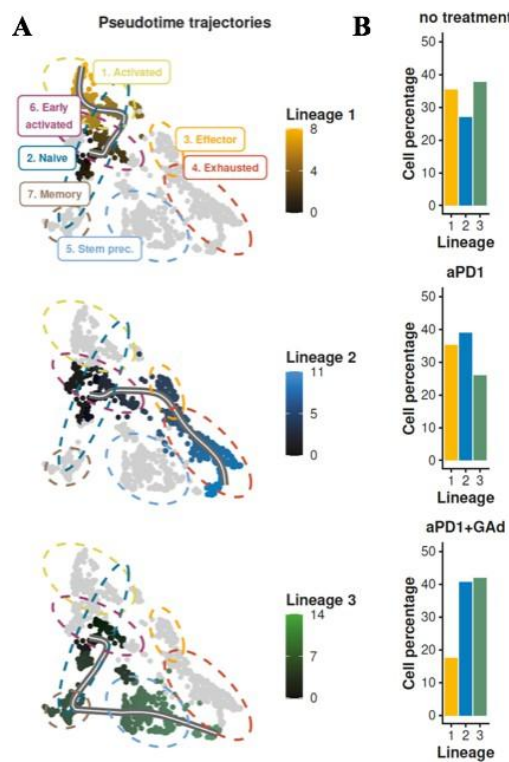


Fig. 2.12. Characterization of distinct lineage commitment via supervised trajectory analysis.

(A) Trajectory inference results shown as pseudotime projected onto UMAP. (B) Bar plots of cell percentages cross experimental treatments for each lineage.

Notably, we observed a significant increase in the expression of effector and checkpoint markers along this trajectory (Fig. 2.13A). The third lineage trajectory, comprising 561 cells, progressed through the memory, T_{STEM} precursor, and T_{EX} subsets, suggesting a potential for long-term memory and T cell exhaustion. In this trajectory, a transient higher expression of genes encoding some memory markers as well as *Cxcr3*, *Sell*, *Slamf6*, and *Il7r*, and transcription factors, such as *Tcf7* and *Id3*, was observed. The trajectory from T stem precursor to the exhausted stage displayed a progressive augmentation of checkpoint and effector marker expression. The distribution of Db-Adpgk⁺ CD8⁺ T cells from nontreated

tumor-bearing mice across the three lineage trajectories was found to be roughly equal, with 38% of cells belonging to lineage 3, 35% to lineage 1, and 27% to lineage 2 (Fig. 2.12B).

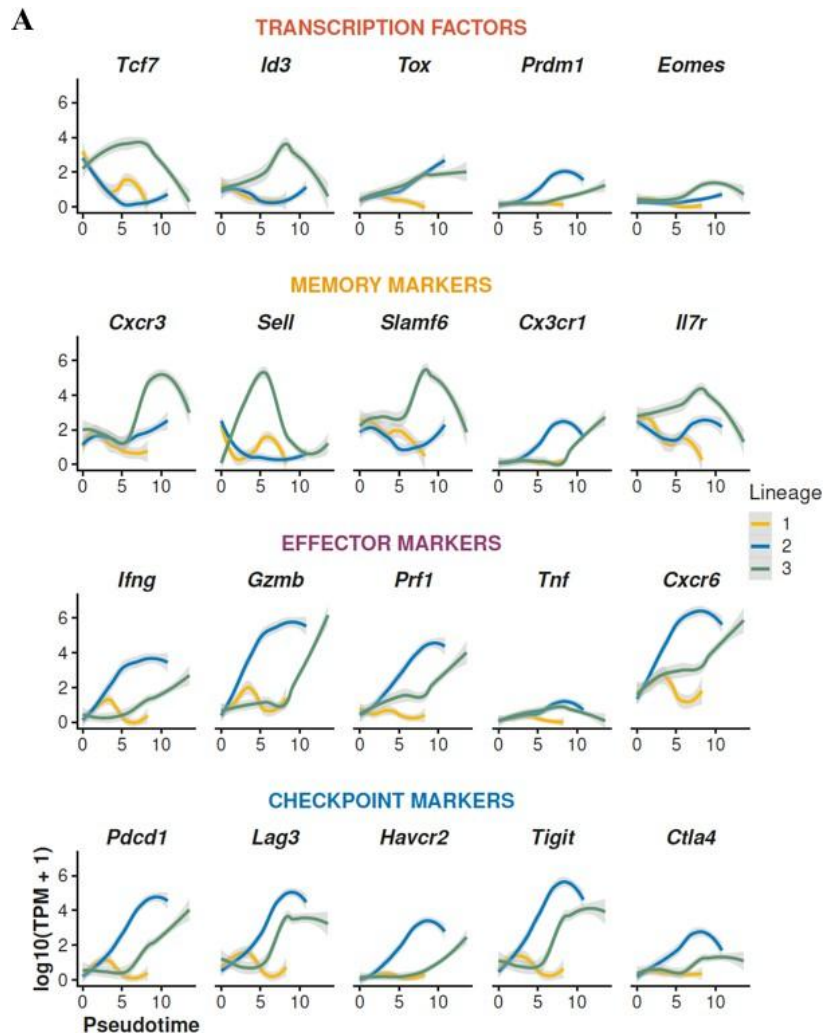


Fig. 2.13. Study of the phenotype landscape and trajectories of differentiation of Db-Adpgk⁺CD8⁺ T cells from mice.

(A) Locally weighted scatterplot smoothing of expression of transcription factors, memory, effector, and checkpoint markers along pseudotime trajectories.

In the case of PD-1-treated mice, the Db-Adpgk⁺ CD8⁺ T cells were distributed in similar proportions among the three lineages, with 39% belonging to lineage 2, 35% to lineage 1, and 26% to lineage 3. Conversely, the Db-Adpgk⁺ CD8⁺ T cells from mice treated with the combination of PD-1 + GAd exhibited a distinct distribution, with the highest proportions observed in lineages 3 and 2 (42% and 41%, respectively), and a smaller fraction in lineage

1 (17%). This distinct lineage distribution was further validated by the analysis of surface protein expression obtained during the FACS-indexed sorting of single cells (Fig 2.14).

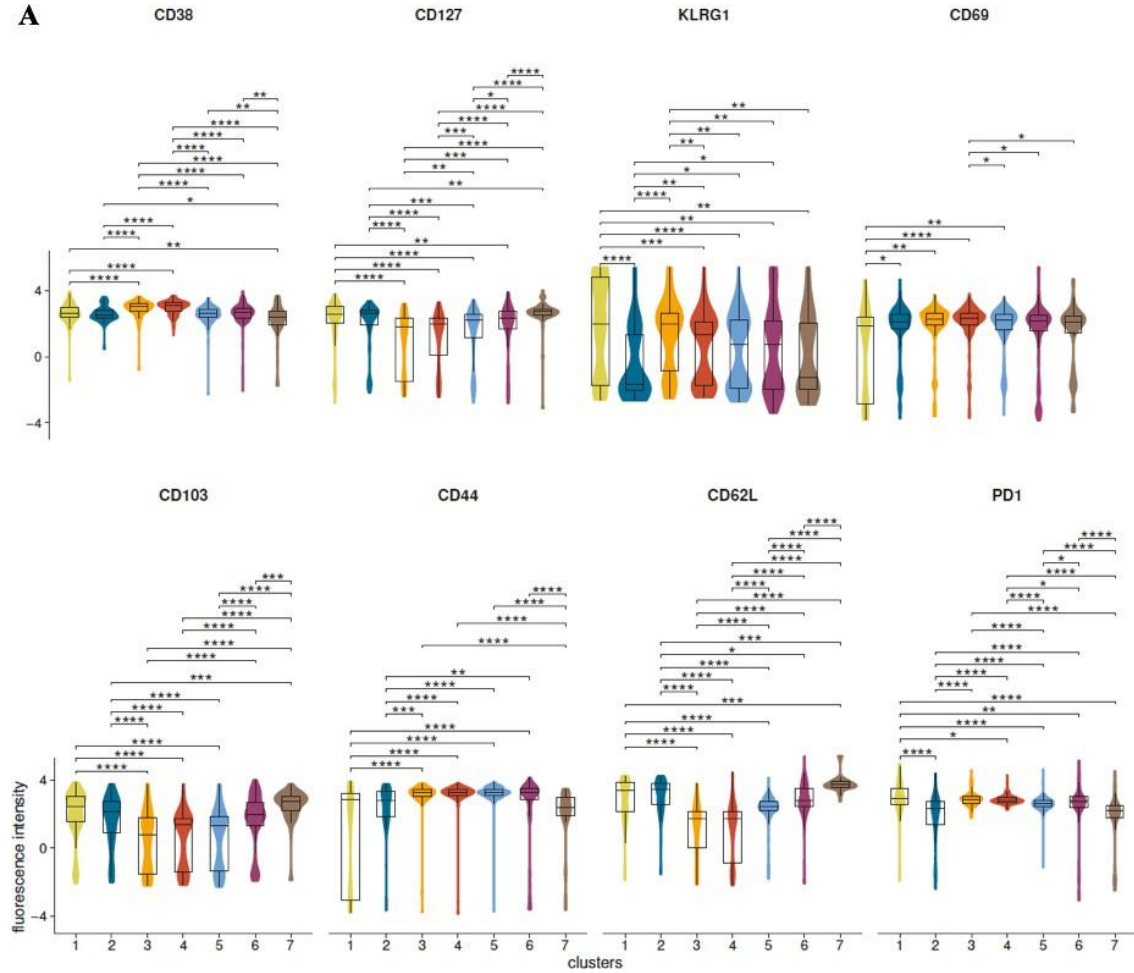


Fig. 2.14. Analysis of the fluorescence intensity projected onto cluster data.

(A) The geometric Mean Fluorescence Intensity (gMFI) values obtained from FACS sorting were projected onto cluster data, shown as $\log_{10}(x)$ and $-\log_{10}(x)$ of raw positive and negative values, respectively.

These findings suggest that the α PD-1 + GAd combined treatment drives Db-Adpgk+ CD8+ T cell fate commitment towards two main lineages of differentiation: one progressing through the memory, T_{STEM} precursor, and T_{EX} cell states, and the other through the effector and exhausted phenotypes.

α PD-1 treatment plus Gad vaccine induces a broader multifunctional CD8+ T clonotypes

To investigate whether the clonal expansion of CD8+ T cells reactive against Adpgk was associated with the increased percentage and number of T_{STEM} precursors and effectors observed in the PD-1- and PD-1 + GAd-treated mice, we leveraged the single-cell Smart-

Seq2 sequencing data to analyze the full-length T cell receptor (TCR) chain sequences at the single cell level. This analysis resulted in the identification of 330 unique and 34 expanded TCR clonotypes (Fig. 2.15 A and B). In naïve, memory and early activate subsets unique TCR were identified, whereas effector, T_{EX}, and T_{STEM} showed the presence of expanded clonotypes (clone size > 30 cells) with a percentage of 69,74 and 56 respectively (Fig. 2.15 A and B).

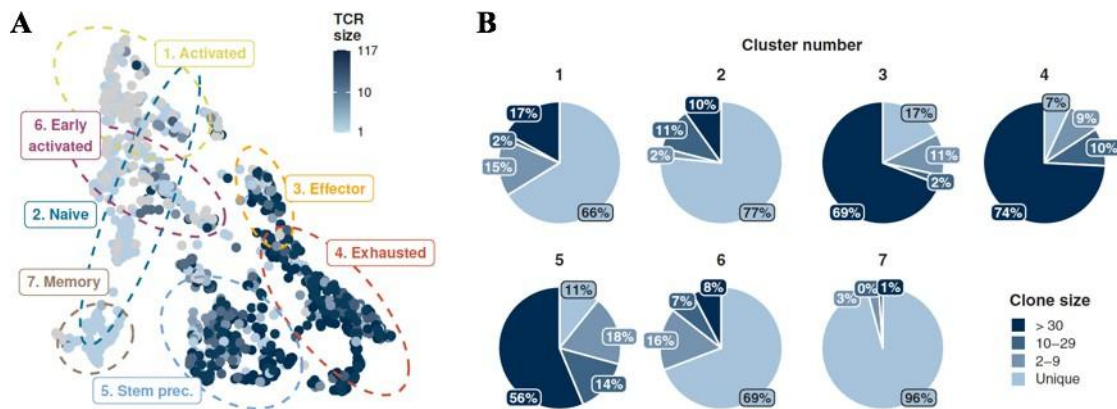


Fig. 2.15 Study of the TCR clonotypes in lymph nodes and tumors of mice, respectively.

(A) T cell receptor (TCR) clonotype size of each Db-Adpgk+ CD8+ T cell projected onto UMAP. **(B)** Pie charts of TCR clonotype size for each cluster.

The analysis of clonotype size across the naïve, early activated, effector, and exhausted subsets (Fig. 2.15B) revealed a significant increase in the proportion of expanded clonotypes throughout the progressive differentiation described in lineage 2 (Fig. 2.12A). Additionally, the results highlighted that the different treatments generated distinct TCR patterns (Fig. 2.16A), suggesting that the combination of PD-1 and GAd may have a unique impact on the TCR repertoire. Unique TCR sequence, precisely 51 singletons were displayed in cells from lymph nodes of control tumor-free mice, whereas in lymph nodes and tumors of non-treated mice a distinct TCR distribution was present. The former includes Db-Adpgk+CD8+ T cells that had a unique TCR, 48 singletons and 5 clones, while the latter comprised neoantigen-specific CD8+ T cells that displayed a higher number of expanded clones ~2 to 29 cells (16 singletons and 11 clones). In mice treated with α PD-1, the TCR clonotype distribution in both tumor and lymph nodes was characterized by a mix of expanded clones and singletons. Specifically, the tumor and lymph nodes contained 7 and 9 expanded clones, respectively, whereas 14 and 57 singletons were observed in these tissues, respectively. In contrast, the

highest number of expanded Db-Adpgk+ CD8+ T clones was observed in mice treated with the α PD-1 + GAd vaccine, with 14 clones and 73 singletons present in lymph nodes, and 15 clones and 71 singletons in tumors. To gain a deeper understanding of how the cells from each TCR clone were distributed between lymph nodes and tumors, we analyzed their frequency distribution in both compartments (Fig. 2.16, B and C). An 86% represented by six to seven clones of Db-Adpgk+CD8+ T cells were found in both tumor and lymph nodes in mice immunized with α PD-1, whereas only one clone was found exclusively among Db -Adpgk+ CD8+ T cells from lymph nodes (Fig. 2.16B). In mice treated with α PD-1 + GAd, a significant proportion of CD8 T cell clones (65%) were found to be present in both the tumor and lymph node microenvironments, with 11 out of 17 clones exhibiting this dual distribution. In contrast, four clones (23%) were exclusively localized to the tumor microenvironment, while two clones (12%) were restricted to the lymph node microenvironment (Fig. 2.16C).

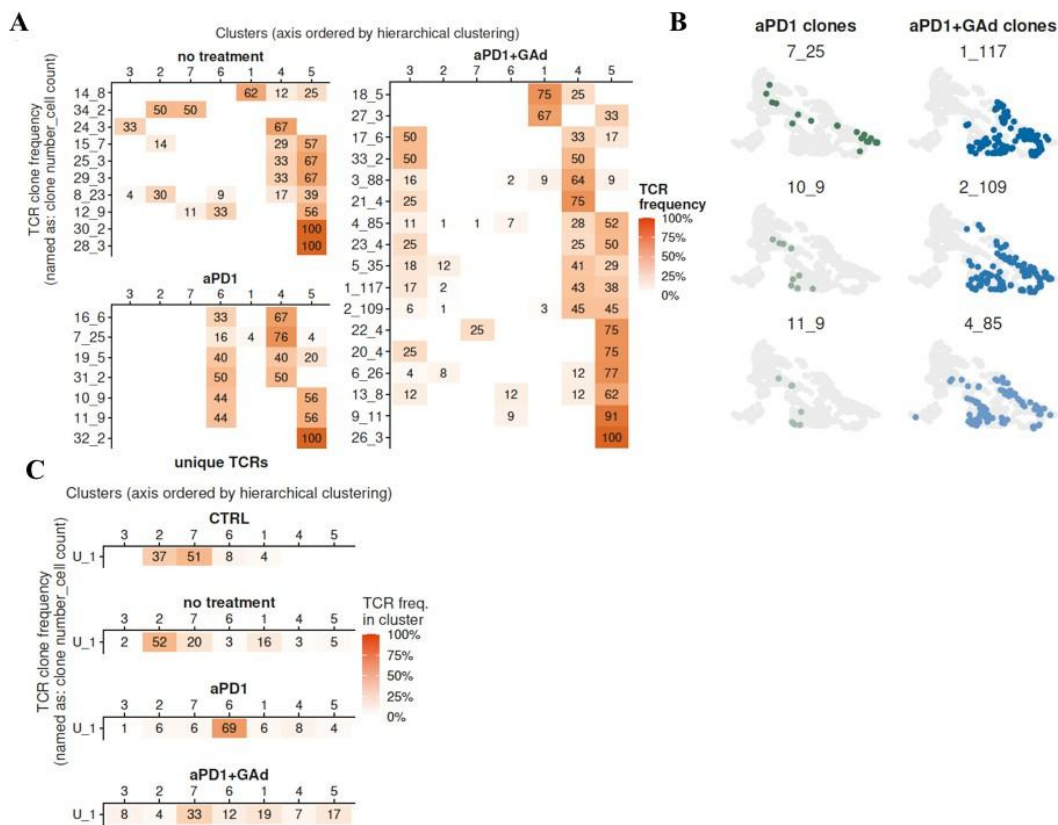


Fig. 2.16. Exploration of expanded TCR clonotypes in memory progenitors stem-like and exhausted stages in lymph nodes and tumors of mice, respectively.

(A) Distribution of TCR clonotype size in the different experimental groups, color-coded by TCR. The TCR label indicates the numeric ID of each clonotype and the number of cells belonging to the clonotypes before and after the underscore, respectively. “U” indicates unique TCRs. Numbers of clones and unique TCRs are annotated on the plot. **(B and C)** Scatterplot of cell frequencies for each TCR clonotype in lymph node and tumors isolated from **(B)** α PD-1–treated and **(C)** α PD-1 + GAd–treated mice, respectively.

These findings suggest that cells from the same TCR clone can be present in both lymph nodes and tumors but at different stages of differentiation. For instance, Fig. 2.17B illustrates examples of TCR clones derived from α PD-1 or α PD-1 + GAd treatment that are found in both tissues. Notably, TCR clones isolated from α PD-1–treated mice were predominantly enriched in the early activated, TEX, and TSTEM precursor populations, whereas clones from α PD-1 + GAd–treated mice were primarily found in TSTEM precursor, effector, and TEX cell subsets (Fig. 2.17A). Furthermore, we observed that unique TCRs were predominantly found in the memory, early activated, and naïve populations, with mean frequencies of 28%, 22%, and 19%, respectively (Fig 2.17C).

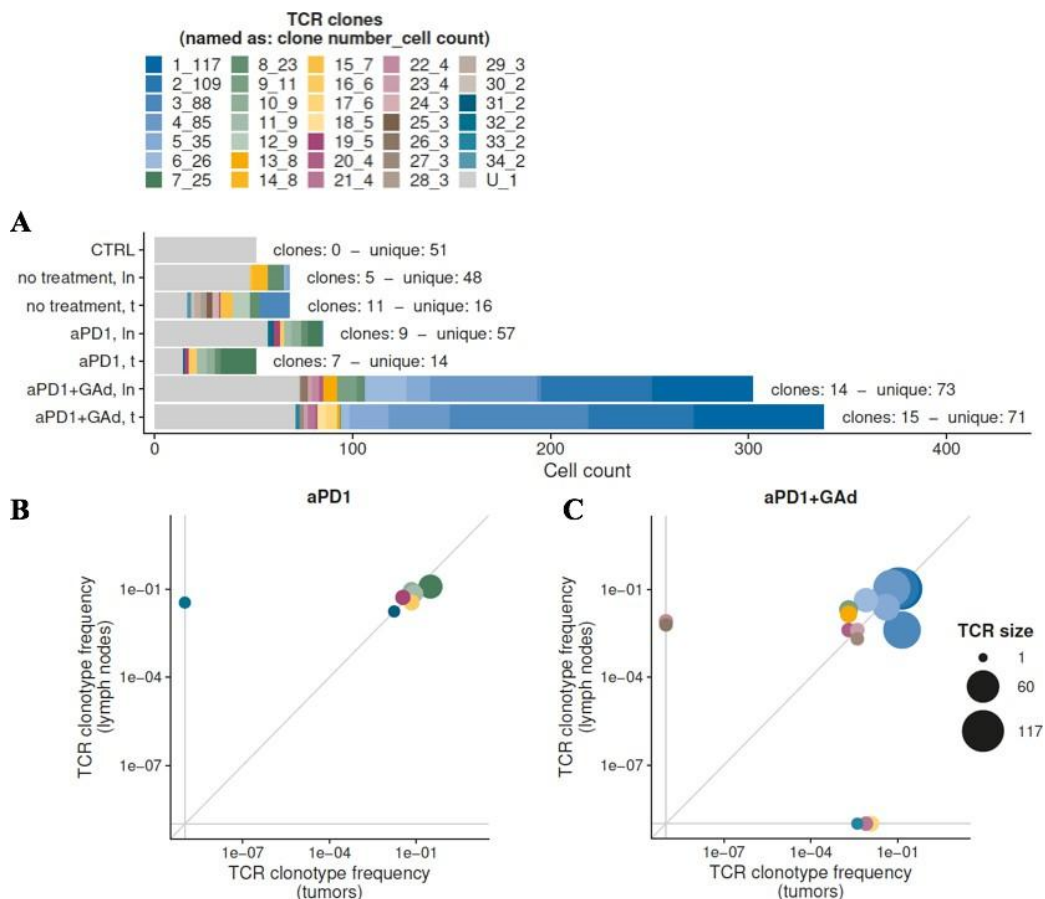


Fig. 2.17. Analysis of TCR clone frequency of Adpgk+CD8+ T cells.

(A) TCR-wise frequencies from cell counts of clonotypes across clusters. Axis order was defined through hierarchical clustering. (B) Examples of TCR distributions projected onto UMAP for α PD-1– and α PD-1 + GAd–treated mice, respectively. (C) Frequencies of unique clonotypes across clusters.

Notably, we identified 10 distinct TCR clones in nontreated tumor-bearing mice, with only one clonotype exhibiting a frequency of more than 10 cells distributed between the T_{STEM} precursor and naïve populations, with mean frequencies of 39% and 30%, respectively. In mice treated with α PD-1, only one TCR clone had more than 10 cells, indicating a limited TCR repertoire. The majority of TCR clones associated with α PD-1 treatment were found in the T_{EX}, T_{STEM} precursor, and early activated populations. In stark contrast, mice treated with α PD-1 + GAd exhibited a remarkable expansion of TCR clones, with a total of 17 distinct TCR clones identified. In particular, seven of these clones had more than 10 cells, and two clones had more than 100 cells, indicating a significant increase in TCR repertoire diversity and expansion. This expansion was particularly pronounced in the T_{STEM} precursor population (cluster 5), where the number of TCR clones increased from 8 in nontreated tumor-bearing mice to 14 in α PD-1 + GAd–treated mice. Furthermore, the effector population (cluster 3) of α PD-1 + GAd–treated mice exhibited a broader TCR repertoire, with 12 clones identified, compared to the limited number of TCR clones observed in nontreated controls. These findings are consistent with the possible lineages of differentiation (Fig. 2.12A) and suggest that the combined treatment leads to a more diverse and expanded TCR repertoire in T_{STEM} cells (cluster 5) (Fig. 2.18). Lineage 2, which traverses from the naïve to the T_{EX} cluster, and lineage 3, which spans from the naïve to the T_{STEM} precursor cluster, both exhibited a statistically significant increase in TCR clone frequency. These findings collectively indicate that the combined treatment with α PD-1 + GAd is the most potent inducer of TCR clonotype expansion and diversification in both lymph nodes and tumors.

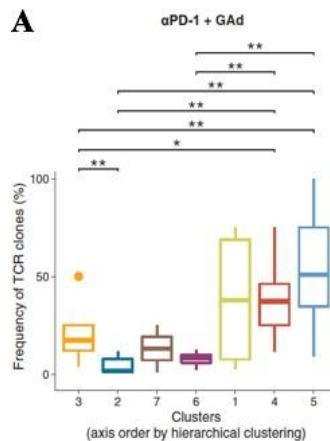


Fig. 2.18. Evaluation of TCR frequencies in mice treated with the combination of immunotherapies.

(A) Distribution of TCR cell frequencies from α PD-1 + GAd-treated mice among clusters (Wilcoxon signed-rank test).

Strong and broad T cell responses was elicited by neoantigen-based vaccine Nous-209 in patients with dMMR tumors

To explore the translational application of immunotherapies combination, immune-phenotype from patients PBMC's treated with Nous209 neoantigen cancer vaccine plus pembrolizumab from Phase I clinical trial were characterized by *ex vivo* IFN- γ ELISpot of PBMCs. Nous-209 vaccine is a vaccination protocol based on a heterologous prime-boost vaccination, the prime was done via a first injection of GAd vector followed by three boost immunizations with Modified Vaccinia Ankara (MVA) vector, both encode 209 frame-shift peptides (FSPs) that are tumor- specific neoantigens previously detected and shared across patients with deficient mismatch repair system, so microsatellite instability from different tumors, as colorectal cancer, gastric cancer and endometrial cancer. The vaccination was combined with ICB treatment, an anti- PD1 monoclonal antibody (pembrolizumab) every 3 weeks (Fig. 2.19A).

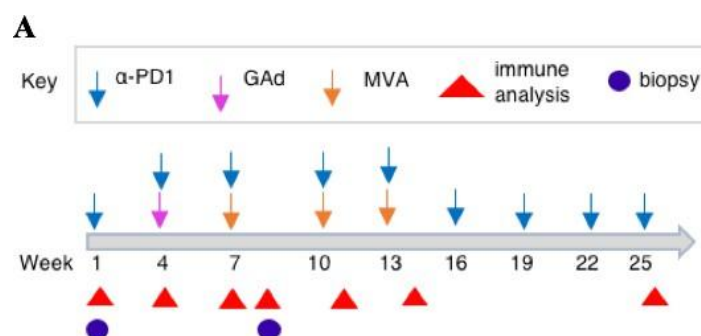


Fig. 2.19. Clinical protocol of Nous209 vaccine.

(A) Clinical event timeline for 12 vaccinated patients (Pt) from baseline to the latest time point; treatments include α PD-1 combined with GAd and MVA vaccine prime boost administrations.

Patients were categorized on the basis of the so-called Response Evaluation Criteria in Solid Tumors v1.1(RECIST). The RECIST criteria are a set of guidelines used to define the response of solid tumors to cancer treatment, the points under investigation are the dimension of target lesions, major or equal to 10 mm with CT or major of 20 mm with MRI, and the number of the lesions, maximum 5 target lesion and 2 lesions per organ. Using RECIST, patients in clinical trials can be categorized as complete responders (CR), when all target lesions disappear and pathological lymph-nodes have a reduction of short axis minor to 10 mm, partial responder (PR) there is a decrease of 30% in the sum of diameter of the target lesion, compared to baseline, progressive disease (PD), an increase of 20% in the sum of diameters of target lesions taking as reference the smallest sum on study and stable disease (SD), neither PR nor PD criteria highlighted (228, 229). This study consists of two sequential cohorts: a dose escalation cohort and a dose expansion cohort, both evaluating two doses of GAd-209-FSP and MVA-209-FSP). Notably, we observed durable partial responses in three patients enrolled in the dose 1 cohort, including two patients with colorectal cancer and one patient with gastroesophageal gastric cancer. In the dose 2 cohort, which included six patients with colorectal cancer and three patients with gastric cancer, we found four partial responses, two patients with stable disease, and three patients with progressive disease (Fig. 2.20A). The partial responder patients were considered as responder whereas patients that show progressive or stable disease were considered as no responder (230). The median follow-up period for patients in the dose 1 cohort was 20.5 months (range, 18.1 to 22.5 months), while for those in the dose 2 cohort, it was 9.4 months (range, 1.7 to 15.2 months) as of the cutoff date. Notably, no dose-limiting toxicities (DLTs) were detected during the study.

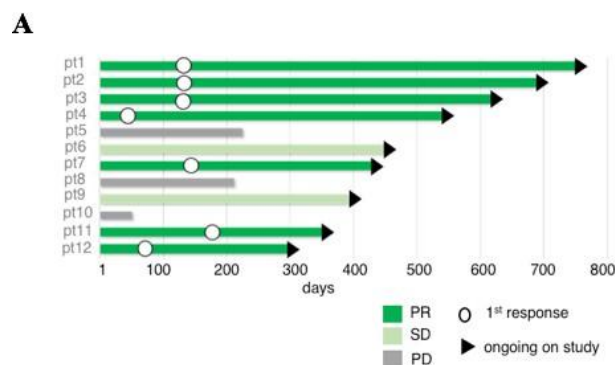


Fig. 2.20. Classification of the patients on the basis of RECIST Criteria.

(A) Clinical responses after treatment as assessed by tumor imaging per RECIST v1.1: response duration shown as a swimmer plot for tumor response over time. White circles indicate time of first response. The arrowheads on the right indicate continuing study treatment.

The secondary endpoint of the study was vaccine immunogenicity, which was evaluated using an ex vivo enzyme-linked immunosorbent spot (ELISpot) assay against 16 peptide pools covering the entire sequence of the 209 FSPs encoded by the vaccine. To assess the impact of Nous-209 vaccination on the activation of specific T cells against the 209 FSPs, we collected peripheral blood mononuclear cells (PBMCs) at various time points before and after vaccination (Fig.2.19A). The immunogenicity of the vaccine was investigated through ex vivo interferon- γ (IFN- γ) ELISpot assays, which showed that 67% of patients in the dose 1 cohort and 100% of patients with evaluable samples in the dose 2 cohort exhibited vaccine-induced responses (Fig. 2.21A). The peak responses after vaccination reached a mean of approximately 1500 spot-forming cells (SFCs) per million PBMCs, whereas the baseline pre-vaccination responses measured after the first dose of pembrolizumab reached a mean of approximately 200 SFCs per million PBMCs (Fig. 2.21B).

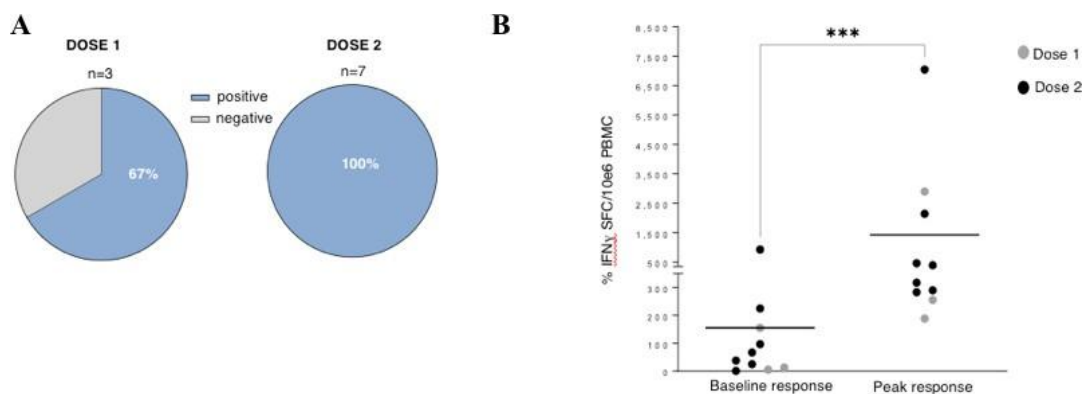


Fig. 2.21. Investigation of vaccine immunogenicity elicited by Nous209 vaccination protocol.

(A and B) Immune responses measured in patients (n = 10) after Nous-209 vaccine by ex vivo IFN- γ . Immunogenicity was assessed by ex vivo IFN- γ ELISpot on peripheral blood mononuclear cells (PBMCs) stimulated with 16 pools of overlapping peptides covering the entire vaccine sequence. **(A)** Frequency of patients showing a positive response after vaccination and number of SFCs/million PBMCs corresponding to the sum of the responses to the single pools. Dot plot in **(B)** represents peak responses for each individual subject, compared with baseline prevaccination responses after pembrolizumab. Lines represent the mean of immune response.

The T cell responses were directed against multiple peptide pools. The breadth and kinetics of the response are depicted for two patients (Pt3 and Pt6), one from each dose group (Fig. 2.22 A and B). Furthermore, the induction of high levels of IFN- γ + FSP-specific CD8+ T cells was measured in the two patients using intracellular staining followed by flow cytometry analysis (Fig. 2.22C). These findings collectively demonstrate the durable neoepitope-specific T cell responses in patients with cancer following vaccination with Nous-209 combined with PD-1 blockade.

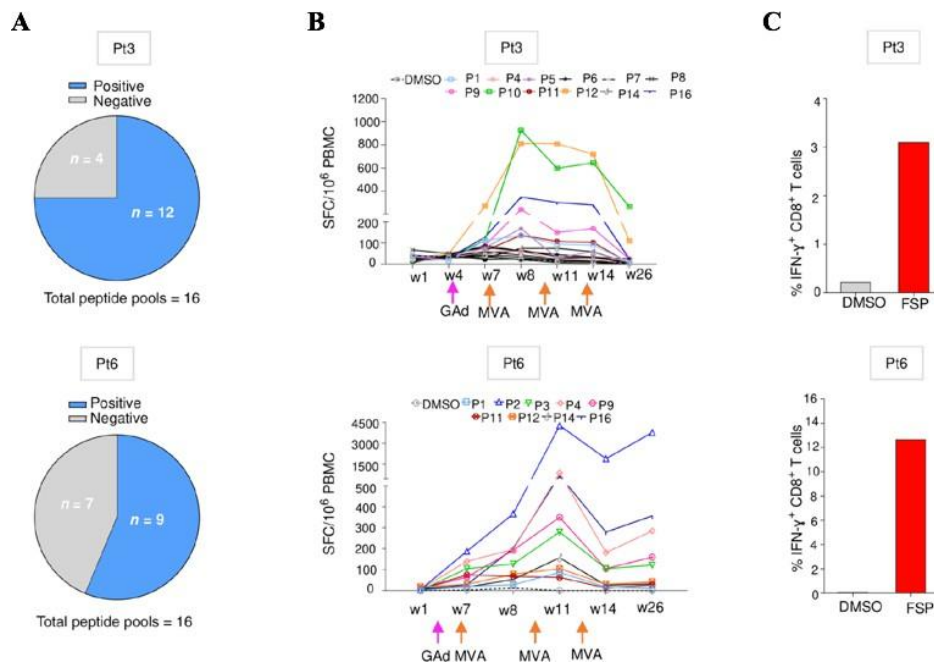


Fig 2.22 Characterization of immune response against different peptides pools at each clinical time point in partial responder Pt3 and stable disease patient Pt6

(A to C) Breadth of immune responses: number of FSP-positive pools (A), kinetic T cell responses measured by ex vivo IFN- γ ELISpot (B), and IFN- γ + FSP-specific CD8+ T cells measured by intracellular staining (ICS) and flow cytometry after vaccination (C) are reported for patients 3 (dose 1) and 6 (dose 2).

CD8+ T cell responses to peptide pools in MSI CRC treated with Nous209 vaccine

To further study and quantify CD8+ T cells responses against different peptides pools and the response elicited by Nous209 vaccination protocol in patients, we measured the frequency of activated CD8+ T cells expressing CD25 and CD69 in PBMCs from four patients with distinct disease outcomes, three with partial responses defined as Pt1, Pt3 and Pt12, one with stable disease named Pt6, and finally one with progressive disease, the Pt16 subject, using

flow cytometry. Frozen PBMCs were thawed and stimulated with specific individual peptide pools by ELISPOT, in order to select the peptide pool inducing the best immune responses in terms of IFN- γ production. Afterwards, Pool 2,3,4,5,7,9,10,12,16 and single peptides, F24 and V3_FSP29 (previously identified through RNA-seq) were used to stimulate PBMCs and analyzed by flow cytometry. The PBMCs were collected at different time points: baseline, following immune checkpoint blockade treatment with α PD1 (post-pembrolizumab), after priming with the GAd vaccine (post-GAd), and after boosting with the MVA treatment (post-MVA). Following 48 hours of stimulation at 37°C, the quantification was performed with background subtraction, at multiple time points (Fig. 2.19 and Fig.2.23).

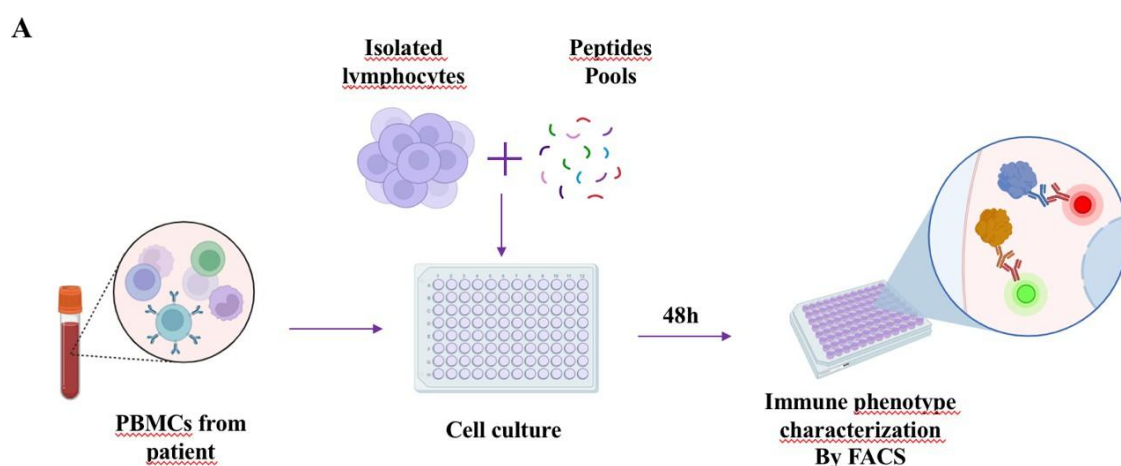


Fig. 2.23. Experimental design of stimulated PBMCs from patients.

(A) PBMCs from patients were isolated through lymphocyte density gradient centrifugation method, counted and plated with the respective peptide pools. Cells were incubated for 48h at 37°C, then they were collected and stained with labeled antibodies to characterize the immune phenotype.

First, we quantified expansion and functional status of the antigen-specific CD8⁺ T cell population in response to the different treatment components at different time points (Fig 2.19). Unfortunately, due to the limited availability of PBMC samples from some patients, our ability to characterize and analyze CD8⁺ T cell activation was constrained for certain time points. Despite these limitations, we were able to evaluate the activation of CD8⁺ T cells in response to patient-specific peptide pools at key time points. We observed that the activation of neoepitope-specific CD69⁺ CD25⁺ CD8⁺ T cells augmented after the first MVA boost, in responder patients with partial response disease outcome (PR_Pt1, PR_Pt3 and PR_Pt12; Fig 2.24, upper panel). In the subject with stable disease (SD_Pt6), an increase

in the percentage of CD25+CD69+CD8+ T cells was identified following MVA vaccination with all peptide pool stimulation. In contrast, patients with progressive disease (PD_Pt16) did not exhibit any significant CD8+ T cell activation at any of the analyzed time points (Fig 2.24, lower panel). These results suggest that the GAd vaccine and MVA boost can elicit CD8+ T cell responses in subjects with favorable clinical outcomes (PR), as evidenced by the upregulation of activation markers CD25 and CD69, whereas the lack of CD8+ T cell activation in PD patients (Pt16) across all time points may indicate an inability to mount an effective Ag-specific CD8+ T cell response, which could contribute to their poor clinical outcomes.

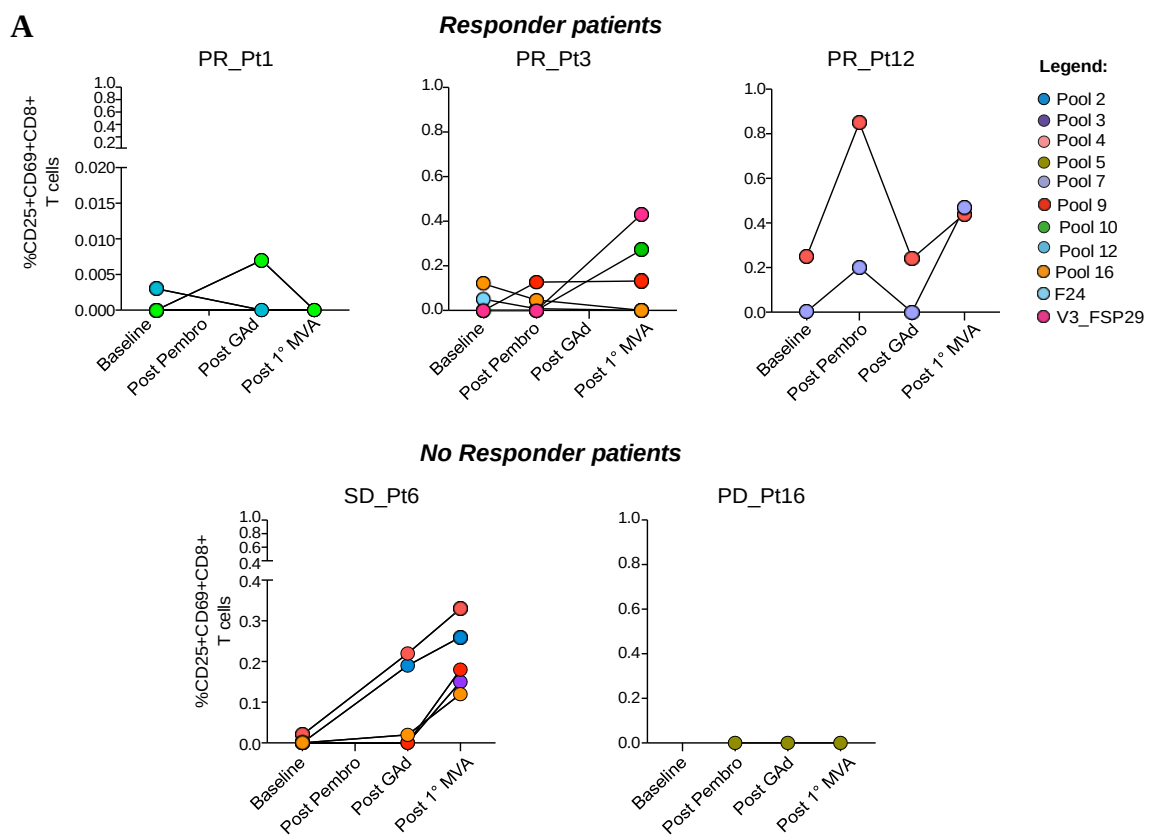


Fig. 2.24. Flowcytometry analysis of activated cells in partial responder and stable disease patients stimulated with individual peptide pools.

(A) Percentage of CD25+CD69+ CD8+ T activated cells were measured at different time point in Pt1 Pt3, Pt12 partial responders patients (upper panel) and no responder Pt6 and Pt16 (lower panel). Each colored dot represented a different peptide pool used for the stimulation.

To determine the immune phenotype status of activated neoepitope-specific CD8+ T cells, we exploited the expression of different surface markers, from stemness to memory markers, on gated CD25+CD69+CD8+ T cells by flow cytometry. Medium subtraction was performed

in all samples. The stemness populations of interest were: CD27+CCR7+CD45RA+ Naïve (T_{NAIVE}), CX3CR1+CD27+ transitory cells ($T_{TRANSITORY}$), CX3CR1+CD27- effector cells ($T_{EFFECTOR}$) (77), Tim3-CD95+ ($T_{EFFECTOR}$), CD62L+CCR7+Tim3-CD95+ stem-like (T_{STEM}) (71), CD27+CCR7+CD45RA- central memory 1 (T_{CM1}), CD27+CCR7-CD45RA- effector memory 1 (T_{EM1}), CD27-CCR7-CD45RA- effector memory 2 (T_{EM2}) (231).

CD8+ $T_{TRANSITORY}$ and T_{STEM} cells are crucial subsets for the replenishment and the self-renewal of exhausted T cells, leading to a sustain in the CD8+ T cell response against cancer or chronic infections (71). As reported in Fig. 2.25, our observations indicate that T_{NAIVE} cells are minimally detectable in patients classified as partial responders. Only PR_Pt12 showed an increase in post MVA boosting. In contrast, patient SD_Pt6, who exhibited stable disease, demonstrated a high baseline percentage of T_{NAIVE} cells, which decreased following GAd vaccination and subsequently increased again after MVA treatment when peripheral PBMCs were stimulated with a variety of peptide pools. Furthermore, the percentage of activated T_{STEM} CD8+ T cells followed a behavior similar to naïve, with an increase only in PR_Pt12 and SD_Pt6 following the boost with MVA vaccine in PBMCs stimulated with the majority of peptide pools (Fig. 2.25B). Moreover, the CD8+ $T_{TRANSITORY}$ cells were detected in PR_Pt12 and in the SD_Pt6 subject after the MVA injection (Fig. 2.25C). The percentages of CX3CR1+CD27- CD8+ $T_{EFFECTOR}$ cells in response to stimulation with some specific peptide pools were higher in the PR_Pt12 and SD_Pt6 after priming with GAd, these percentages were further enhanced in the PR_Pt3 and PR_Pt12 following MVA treatment, but they decreased in patients with SD after MVA (Fig 2.25D). Pool 10 and 3 induced an increase in the frequency of CD95+Tim3- $T_{EFFECTOR}$ after MVA vaccination in PR_Pt1 (Fig. 2.25E). In contrast, the FSP pools 9 and the single peptide V3_FSP29 for PR_Pt3 patient and, Pool4 elicited the highest frequency of this subset after pembrolizumab treatment in PR_Pt12. In contrast, SD_Pt6 patient showed a modest increase in CD95+Tim3- $T_{EFFECTOR}$ CD8+ T cells after the post-GAd vaccination, with an additional augmentation following the first MVA boost treatment (Fig. 2.25E). These findings suggest that the CD8+ T cell responses to the peptide pools varied across the different disease outcome groups. T_{NAIVE} and T_{STEM} augmented only in PR_Pt12 and SD_Pt6, whereas both CX3CR1+CD27- and CD95+Tim3- $T_{EFFECTOR}$ increase in all patients. These results suggest that, first patients exhibited distinct patterns of T_{STEM} , $T_{TRANSITORY}$ and $T_{EFFECTOR}$ CD8+ cells in response to the vaccination regimen and these differences in the magnitude and phenotype of the CD8+ T cell responses may contribute to the divergent clinical outcomes observed in these patients. Second, the most representative subsets after vaccination regimen are the

effector cells that have the unique ability to directly recognize and eliminate tumor cells through cytolytic mechanism and robust clonal expansion, important for the control of tumor progression (232).

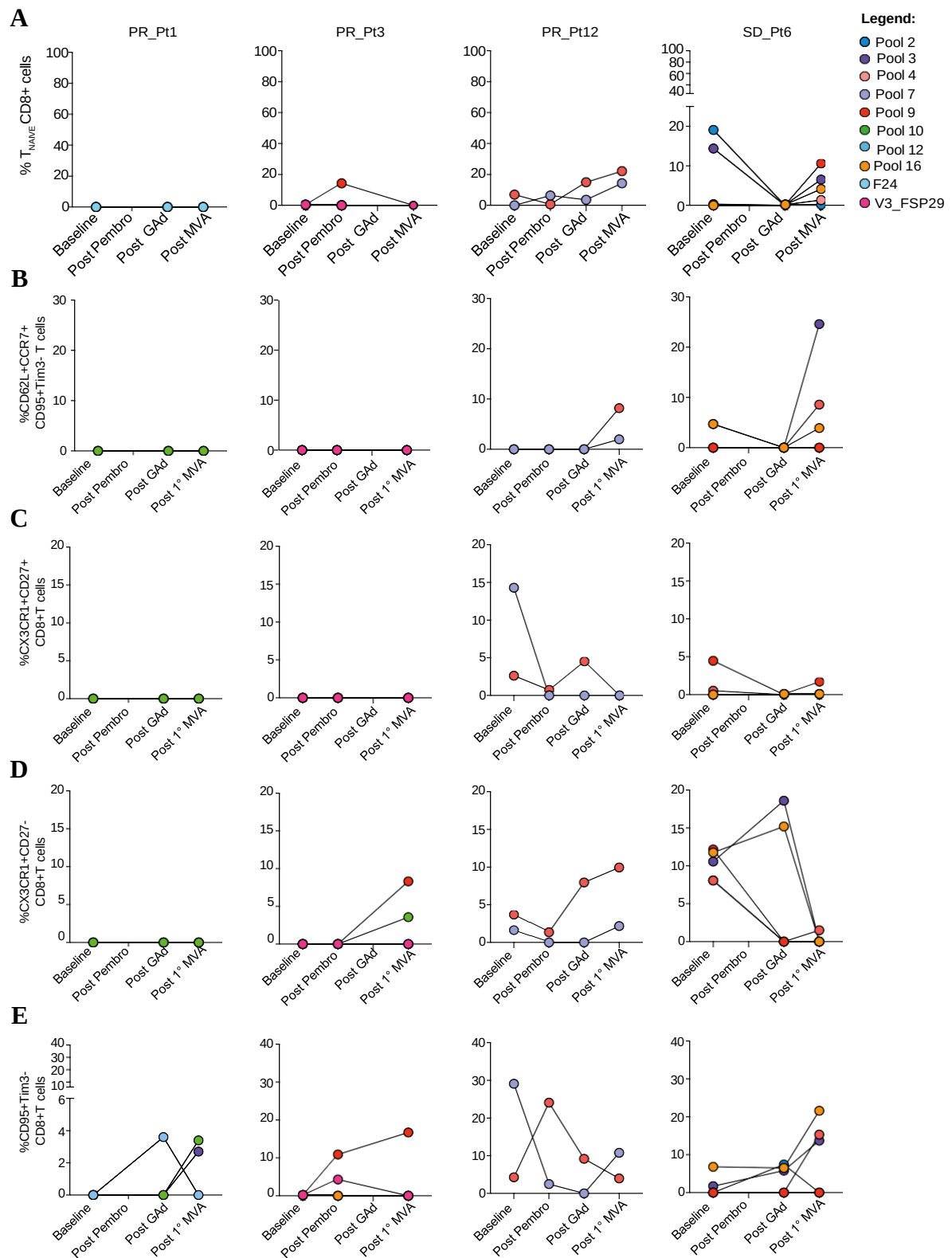


Fig. 2.25. Elucidation the immune phenotype of peptides activated CD8+ T cells following different clinical timepoints.

(A-E) Percentages of CD25+CD69+ CD8+ T (A) T_{NAIVE} , (B) CD62L+CCR7+Tim3-CD95+ T_{STEM} , (C) CX3CR1+CD27+ $T_{TRANSITORY}$, (D) CX3CR1+CD27- $T_{EFFECTOR}$ and (E) CD95+Tim3- $T_{EFFECTOR}$ cells were measured at different time point in patients. Each colored dot represented a different peptide pools used for the stimulation.

Then, we continued our analysis of the activated CD8+ T cells in terms of memory T cell differentiation. Medium subtraction was done in all condition analyzed (Fig2.26). The expression pattern of T_{CM1} mirrored the results of T_{STEM} , with an increase in the percentage of these subsets only in SD_Pt6 after vaccination with MVA with all peptide pools (Fig 2.26A). Furthermore, we noted an increase in T_{EM1} cells in patients PR_Pt1 and PR_Pt3 after vaccination, in details, with PR_Pt1 receiving GAd and PR_Pt3 receiving MVA. Notably, no T_{EM1} cells were detected in the stable disease SD_Pt6 (Fig 2.26B). Significant differences were also observed in the percentage of T_{EM2} cells. In responder patients (PR_Pt1, PR_Pt3, and PR_Pt12), there was a marked increase in T_{EM2} populations following MVA vaccinations across the majority of neoepitope pools. Conversely, in the stable disease SD_Pt6, there was an increase in T_{EM2} cells after GAd vaccination, followed by a decrease after MVA booster for most of the peptide pools (Fig2.26C).

Thus, the characterization of memory T cell subsets revealed a heterogeneous immune response among patients, influenced by both the specific neoepitope pools and the varying treatment regimens. For PR_Pt1 and PR_Pt3, there is the increase of T_{EM1} , whereas PR_Pt12 and SD_Pt6 are characterize by the expression of T_{STEM} . Although T_{EM1} and T_{STEM} are characterized by different markers (233), they share similar characteristics such as long - lifespan and self-renewal (41), rapid differentiation and cytokine responsiveness (234) (235). Consequently, patients expressing either T_{EM1} or T_{STEM} , could still have a long-lasting protective immune response. Finally, the significant increase in T_{EM2} populations, in all responder patients (PR_Pt1, PR_Pt3, and PR_Pt12) following MVA treatment, indicates that these patients may develop a more robust immune responses to vaccination (236). These observations align with findings in other studies that demonstrate the importance of T_{EM2} cells in mediating effective T cell responses against tumors. For instance, T_{EM2} cells are known to play a role in sustaining immune memory and promoting long-term protection against cancer recurrence (237) (238).

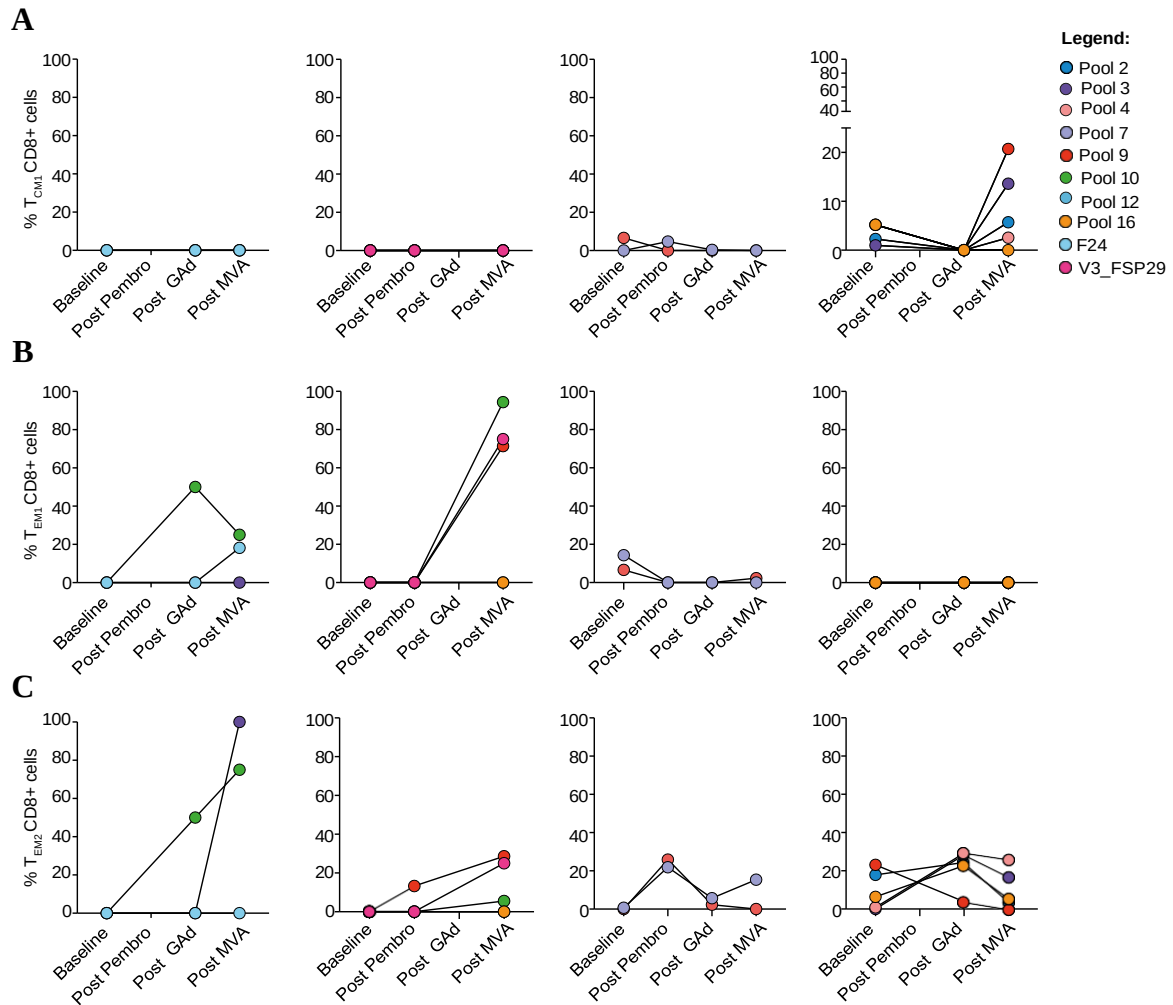


Fig. 2.26. Study of the memory phenotypes in responder and no responder patients stimulated with FPS pools following different vaccination protocol treatment.

(A-C) Percentages of CD25+CD69+ CD8+ T (A) T_{CM1}(B) T_{EM1}(C) and T_{EM2} cells were measured at different time point in patients. Each colored dot represented a different peptide pools used for the stimulation.

Cytokines and transcription factor profiles of CD8+ T cells with Nous209 vaccine

Cytokines and transcription factor profiles, obtained with flow cytometry, were analyzed using the SPICE (Simplified Presentation of Incredibly Complex Evaluations) software, which is designed for the analysis of complex datasets generated from polychromatic flow cytometry. This tool allows for the effective exploration and interpretation of intricate multivariate datasets, as detailed in the work by Roederer et al. (239).

PBMCs were defrosted and cultured with the specific peptides pool for 24h. After the incubation time, cells were stained for the detection of intracellular markers. The expression

levels of Granzyme B and IFN- γ , alongside Perforin, TOX and the transcription factor TCF-1 were analyzed in CD8⁺ T cells from both PR and SD patients at various treatment time points. As illustrated in the first panel Figure 2.27A, following medium subtraction, the expression of Granzyme B⁺, IFN- γ ⁺, Perforin⁺, TCF-1⁻, and TOX⁻ cells was observed in PR_Pt3 and PR_Pt12 at the post pembrolizumab time point, following stimulation with only one peptide pool. Whereas, in the second panel, Granzyme B⁺, IFN- γ ⁺, Perforin⁺, TCF-1⁺, and TOX⁻ cells were highly expressed following MVA treatment in PR_Pt1 and PR_Pt12, with stimulation with Pool 3 and F24 for PR_Pt1 and Pools 4 and 7 for PR_Pt12, respectively (Fig2.27A, second upper and lower panels). In contrast, the percentage of the same subset increased in PR_Pt3 when stimulated with Pool 12 and Pool 16 following pembrolizumab treatment (Fig 2.27A second middle panel). In PR_Pt1, the polyfunctional subset of Granzyme B⁺, IFN- γ ⁺, Perforin⁺, TCF-1⁻, and TOX⁺ cells expanded after F24 peptide stimulation at baseline and during the GAd vaccination time point. This subset exhibited further expansion post-MVA treatment when stimulated with Pools 10 and 16 in PR_Pt3. (Fig2.27A, third upper and middle panels). Of note, a significant increase in the percentage of Granzyme B⁺, IFN- γ ⁺, Perforin⁺, TCF-1⁺, and TOX⁺ cells was observed in PR_Pt1 and PR_Pt3 after administration of neoantigen vaccines, specifically both GAd and MVA treatments. Notably, stimulation with the F24 peptide and Pool 10 for PR_Pt1 (Fig2.27A, fourth upper panel), Pool 10 for PR_Pt3 (Fig2.27A, fourth middle panel) resulted in the highest percentages of this polyfunctional CD8⁺ T cell subset. Conversely, in PR_Pt12, no significant increase was detected in Granzyme B⁺, IFN- γ ⁺, Perforin⁺, TCF-1⁺, and TOX⁺ cells (Fig2.27A, lower fourth panel). These findings underscore the differential responses of CD8⁺ T cell subsets to various specific peptide pools and highlight the effect GAd and MVA therapies in enhancing polyfunctional T cell responses in cancer immunotherapy.

A

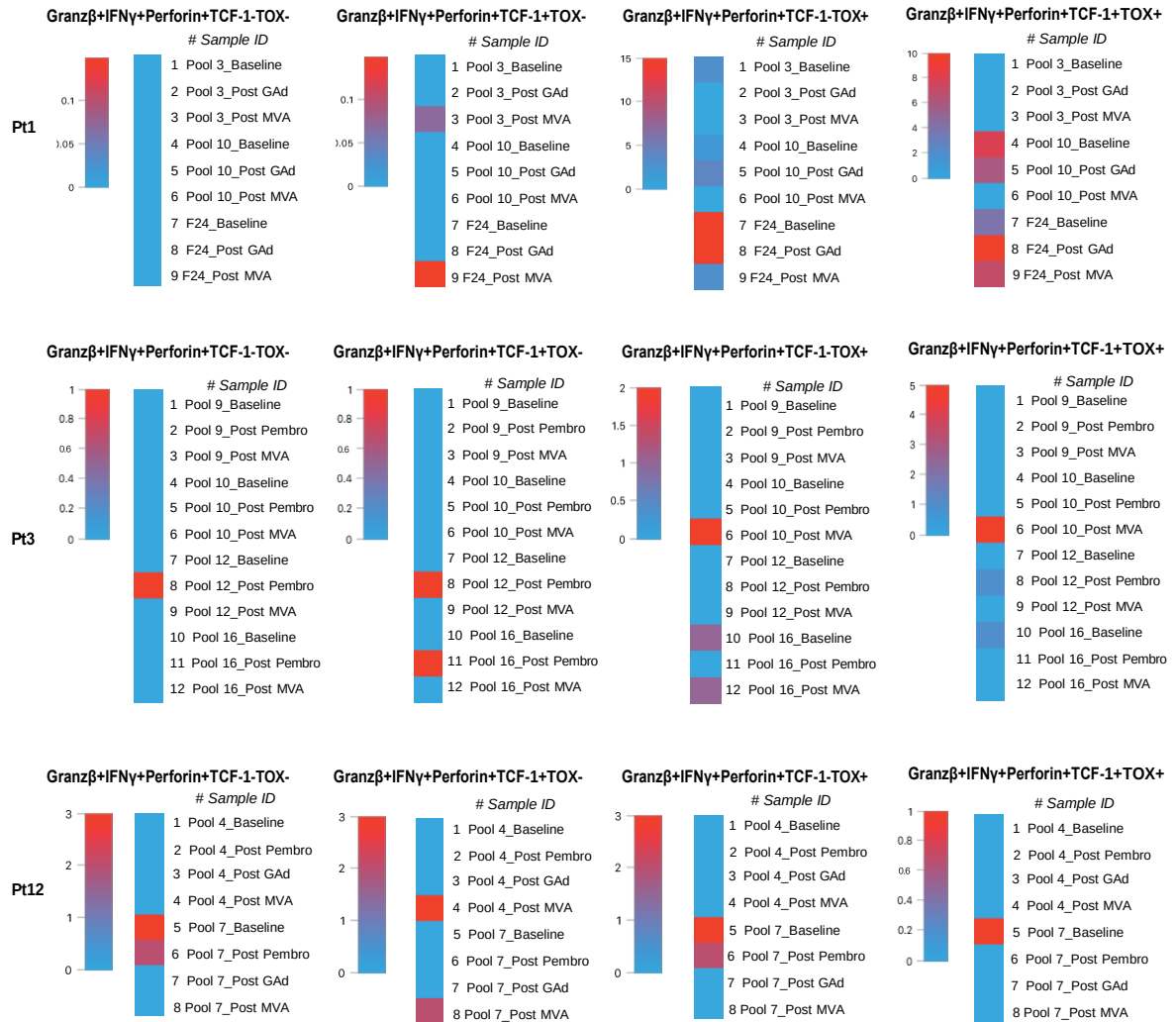


Fig. 2.27. SPICE Analysis in partial responder patients.

(A) Cool plots derived from SPICE analysis illustrate the expression of GranzymeB+,IFNγ+,Perforin+, TCF-1 +/- and TOX +/- in partial responder patients at different time points following stimulation with specific peptides pools. These plots are presented for three patients: PR_Pt1(**upper panel**) PR_Pt3 (**middle panel**) and PR_Pt12 (**lower panel**). Near each plot there is a numbered color legend that represents the expression levels, providing a clear visual reference for interpreting the data (from blue= low expression to red=high expression).

Next, we performed the same SPICE analysis in no responder patients (NR), respectively in the stable SD_Pt6 and progressive disease PD_Pt16. The expression of Granzyme B+, IFN-γ+, Perforin+ was found to be minimally detected in NR patients compared to PR patients. Only SD_Pt6 exhibited an increase in this subset following stimulation with Pool 2 and Pool 9 after GAd vaccination (Fig 2.28A, first panel). Notably, there was no expression of

Granzyme B+, IFN- γ +, Perforin+, TCF-1+, and TOX- cells detected across all conditions for SD_Pt6 (Fig 2.28A, second upper panel), whereas barely expression was detectable in Pt16 following MVA treatment after stimulation with Pool 5 (Fig 2.28A, second lower panel). In contrast, the population of Granzyme B+, IFN- γ +, Perforin+, TCF-1-, and TOX+ cells increased post GAd vaccination in SD_Pt6 when stimulated with Pools 3, 4, 9, 16, and Ly23.1 (Fig 2.28A, third upper panel). However, no expression of Granzyme B+, IFN- γ +, Perforin+, TCF-1-, and TOX+ cells was detected in PD_Pt16 (Fig 2.28A, third lower panel). Last, we found an increase in the percentage of Granzyme B+, IFN- γ +, Perforin+, TCF-1+, and TOX+ cells following stimulation with peptide pools 4, 16, and Ly23.1 after GAd vaccination (Fig. 2.28A, upper panel). In contrast, the progressive disease SD_Pt16 exhibited a markedly different immunological profile. In this patient, Granzyme B+, IFN- γ +, Perforin+, TCF-1+, and TOX+ cells were present in PBMCs following MVA vaccination (Fig 2.28A, lower panel). However, the immune response detectable in PD_Pt16 was lower when compared to SD_Pt6 and PR_patients. These findings suggest enhanced multi-functional cytotoxic T cell responses in PR patients, as these markers are indicative of an effective immune activity against tumors. Furthermore, this observation suggests that even in patients categorized as non-responders, certain immune responses may parallel those seen in partial responders, underscoring the complexity of immune dynamics across different disease states.

A

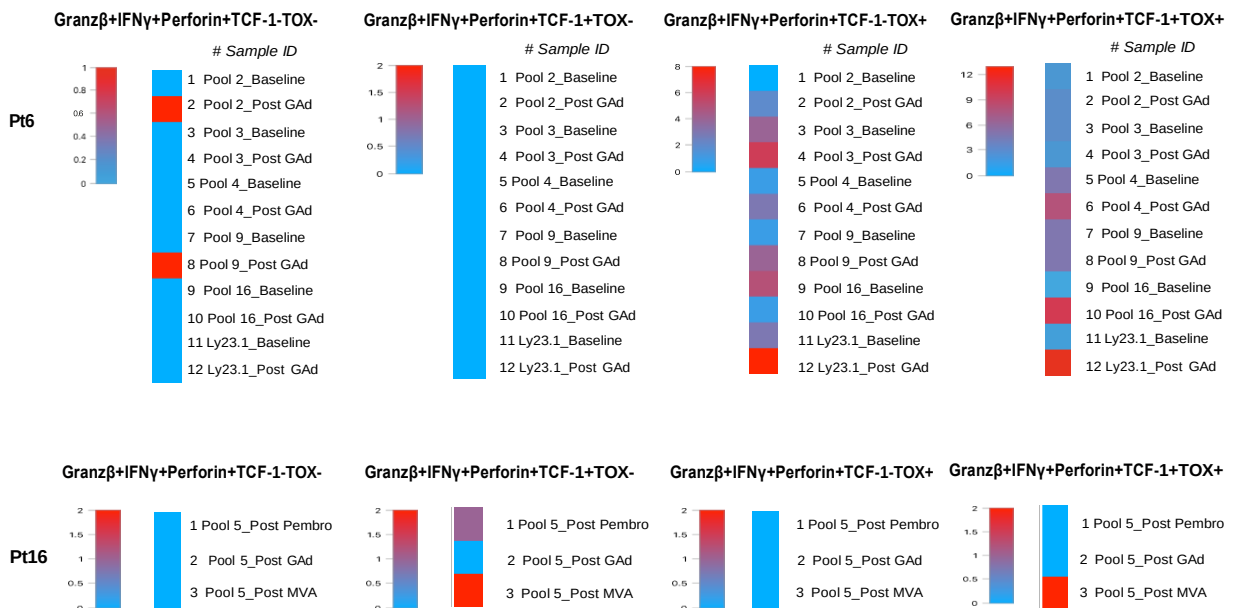


Fig. 2.28. SPICE Analysis in no responder patients.

(A) Cool plot derived from SPICE analysis illustrate the expression of GranzymeB+, IFN γ +, Perforin+, TCF-1 +/- and TOX +/- in no responder patients defined as stable disease patient, SD_Pt6 (**upper panel**) and progressive disease patient, PD_Pt16 (**lower panel**). Near each plot there is a numbered color legend that represents the expression levels, providing a clear visual reference for interpreting the data (from blue= low expression to red=high expression).

Nous-209 vaccine leads to an expansion and diversification of neoAg-specific TCR clonotypes in dMMR tumor patients

To investigate the variation in TCR repertoire upon treatment in different patients, we performed a bulk RNA-seq from the intratumorally TCR repertoire in matched pre- and on-treatment tumors in three patients whose pre- and post-treatment biopsies that were available (Pt1, Pt11, and Pt12). All three patients exhibited a partially durable response according to RECIST v1.1 evaluation criteria (Fig. 2.29A). Consistent with the findings in mice, the analysis of T cell infiltrates in all three patients revealed an increased number of TCR- β copies after Nous-209 treatment, indicating the expansion and diversification of clonotypes (Fig. 2.29A).

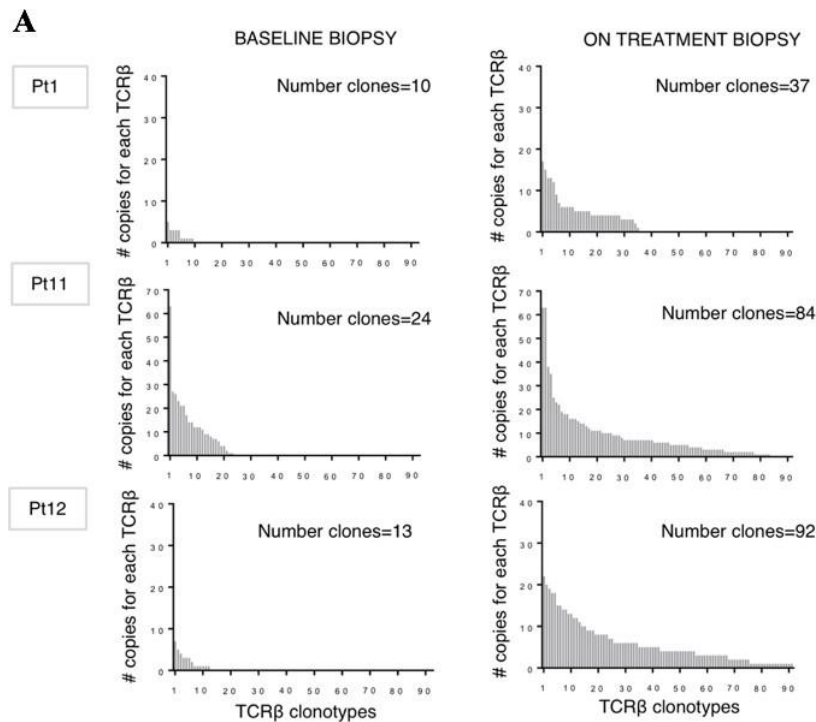


Fig. 2.29. Comparison of bulk RNA seq between baseline and on treatment biopsy in three patients.

(A) Expansion and diversification of TCR β repertoire in pre and post treatment biopsies in three patients with clinical response (PR).

The spread of TCR clonotypes was correlated with an increase in the estimated proportion of RNA derived from effector memory T cells, as determined by a novel algorithm for identifying gene signatures in tumors (Fig. 2.30A). To examine the migration of vaccine-induced CD8⁺ T cells from the peripheral circulation to the tumor, we focused on patient 1, who displayed a neoepitope specificity for CD8⁺ T cells induced by the vaccine. Specifically, the peptide carrying the mutation triggering the response, named F24, was one of the 56 FSPs shared between the vaccine and the baseline tumor biopsy, detected through next-generation sequencing (NGS) data analysis (Fig. 2.30B).

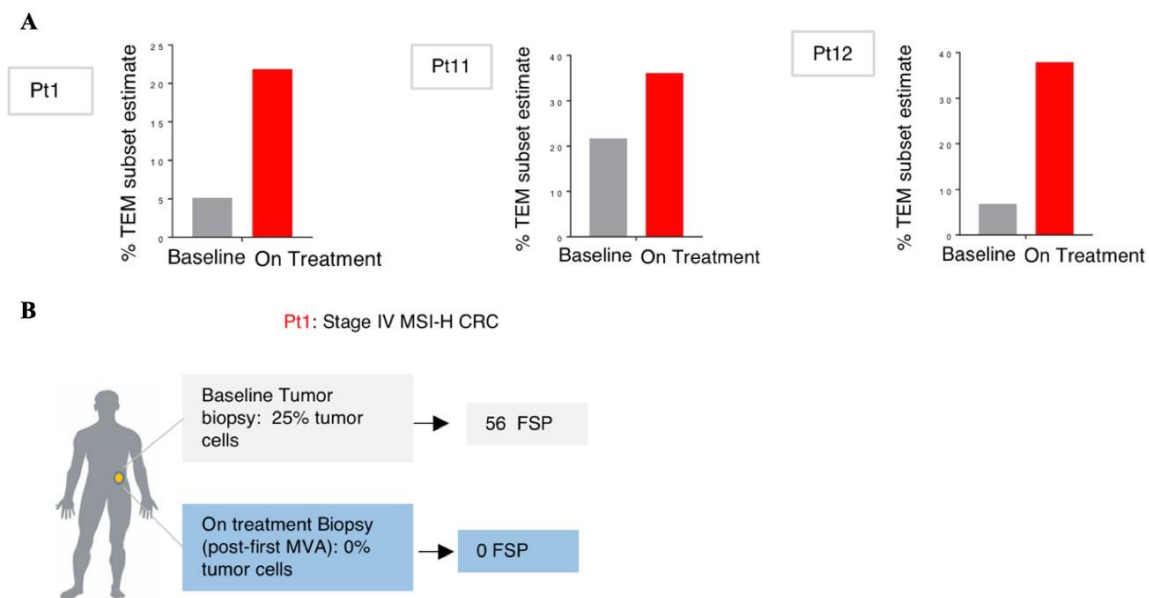


Fig. 2.30. Effector memory estimation in baseline and on treatment tumor biopsies plus the identification of shared peptide between the two biopsies.

(A) Estimated mRNA fraction of effector memory T (TEM) cells on tumor samples before and after treatment according to gene expression data. **(B)** Study of patient 1 (Pt1), a stage IV microsatellite instability–high (MSI-H) CRC, second line (2L) in PR. For this patient, baseline tumor biopsy and on-treatment biopsy were collected before the first pembrolizumab administration (week 1) and after first MVA (week 8), respectively.

The F24 mutation was identified in approximately 40% of tumor cells based on mutation clonality analysis. The F24 sequence comprises an 8-mer peptide predicted to be a potent binder of one of the patient's haplotypes, specifically IAKKRIKL, with HLA-B*08:01 and a predicted median inhibitory concentration (IC₅₀) of 36 nM. Following ex vivo and in vitro restimulation (IVS), although F24-specific CD8⁺ T cells were not detected in PBMCs collected after pembrolizumab treatment (Fig. 2.31A), the F24-specific CD8⁺ T cells were

found to be expanded after IVS of PBMCs collected after vaccination, as evident from the increased number of IFN- γ + SFCs (Fig. 2.31B).

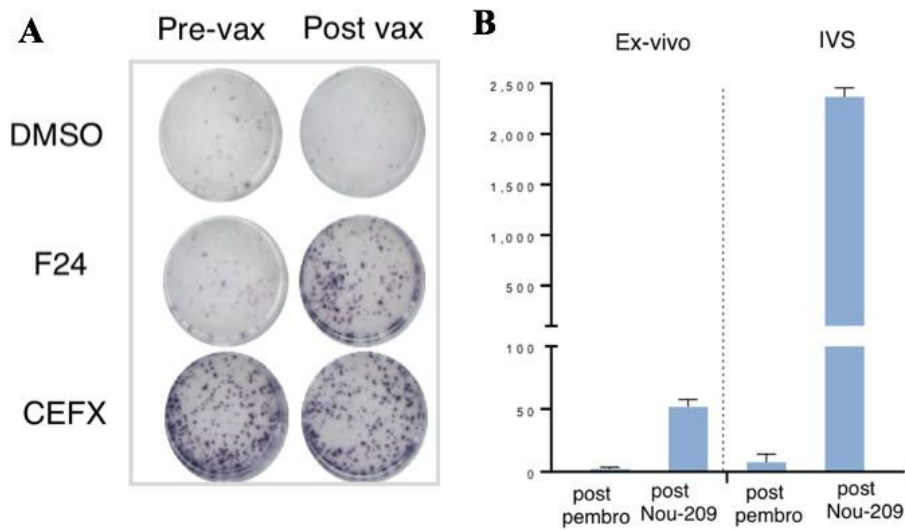


Fig. 2.31. Detection of F24 specific CD8+ T cells in PBMC pre- and post vaccination.

(A and B) T cell responses in Pt1 were measured by IFN- γ ELISpot assay performed ex vivo and after in vitro restimulation (IVS) with a peptide specific for one encoded FSP (FSP 24). Tested PBMC were collected after pembrolizumab (week 4) and after vaccination (week 7). DMSO and CEFX were used as negative and positive control, respectively.

Expanded F24-specific CD8+ T cells underwent TCR- β sequencing. In post-vaccine treatment tumor biopsy were identified through bulk RNA analysis at week 8 seven F24 FSP-reactive TCR- β clones that expanded following in vitro stimulation (IVS) tumor biopsy (Fig. 2.32, A and B). Among these, only one clonotype (TCR1) was present in the baseline biopsy and expanded after treatment, whereas the remaining six TCR clones were exclusively detected in the post-treatment biopsy (Fig. 2.32C). Collectively, these findings suggest that neoantigen-specific CD8+ T cells induced by the Nous-209 vaccine undergo expansion and diversification upon treatment, and successfully traffic and infiltrate the tumor microenvironment.

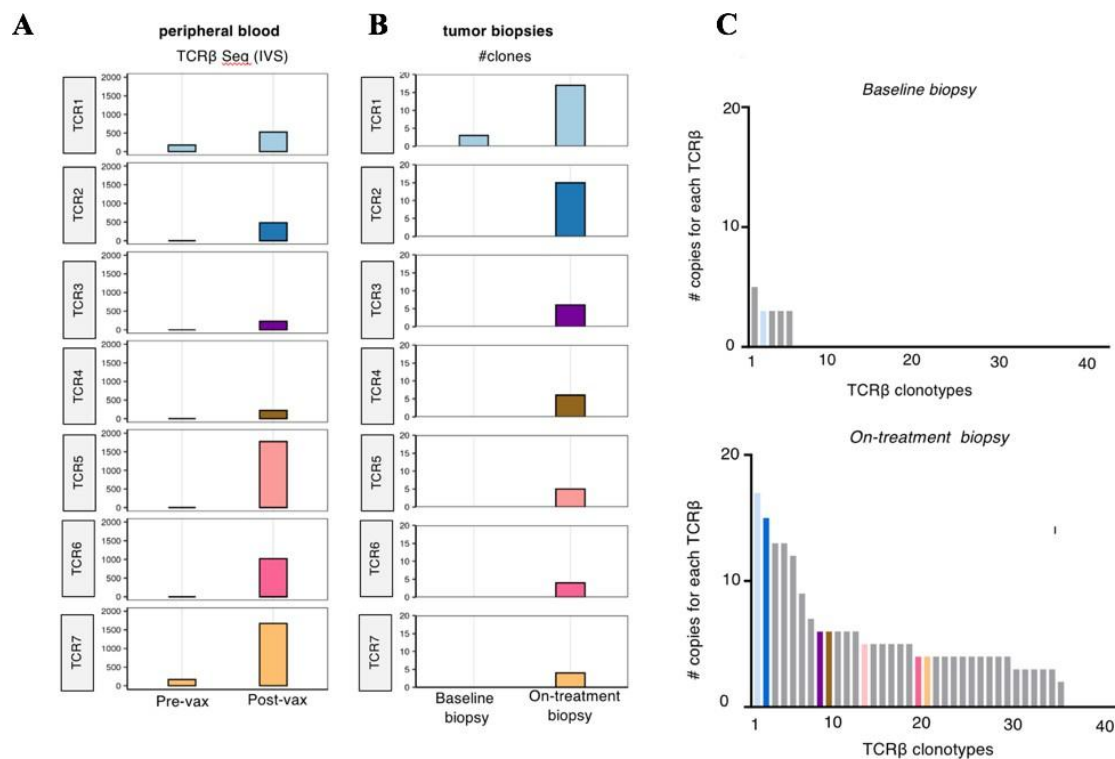


Fig. 2.32. TCR-β sequencing in peripheral blood and tumor of Pt1

(A) TCR-β sequencing of PBMCs stimulated in vitro with the F24 peptide. The abundance of seven TCR-β clonotypes shared among PBMCs stimulated in vitro with F24 peptide and the clonotypes in the on-treatment tumor biopsy, detected by RNA-seq, are represented. **(B)** Bar plots represent the abundance of the seven clonotypes analyzed at baseline and after treatment on the tumor biopsies. Only one TCR (TCR1) was also found in the baseline biopsy as already present in the tumor and expanded after treatment, whereas the other six clones were exclusively detected in the posttreatment biopsy. **(C)** Expansion and diversification of TCR-β repertoire in pre- and posttreatment tumor biopsies. The clonotypes detected on the tumor biopsies of Pt1 are shown. Each bar is a TCR-β individual clone; colored bars indicated seven TCR clonotypes specific for F24 FSP identified in **(B)**.

Analysis of F24 neoantigen-specific CD8⁺ T cells in partial responders treated with NOUS209

For certain patients, we could generate neoepitope specific tetramers. Although PR_Pt1 has response to peptide pool stimulation, this is still very low, so we decided to detect neoantigen specific CD8⁺ T cells via pentamer staining. To detect and characterize the expanded neoantigen-specific CD8⁺ T cells in patients after immunotherapy and vaccination combined treatment, PBMCs from the partial responder patient PR_Pt1 and no responder patient

(PD_Pt2) were analyzed through flow cytometry using the previously detected antigens through pentamer staining, the former was stained with the B08:01-IAKKRIKL-peptide pentamer, also named F24, whereas the latter with B07:02 HPRLPRTKW, also known as FSP26.6 peptide-pentamer. For PR_Pt1, PBMCs were available from all stages of the treatment, including baseline, post pembrolizumab (α -PD1), post-Gad, post-second MVA vaccinations and after six months. In contrast, for patient 2, only post-a-PD1 and post-1 MVA time points were available. As anticipated from previous results in mouse models, the frequency of neoantigen-specific CD8⁺ T cells significantly increased in patient 1 following the combination of anti-PD1 therapy and neoantigen vaccination (Fig. 2.33, A and B). In contrast, no neoantigen-specific CD8⁺ T cells were detected in no responder patient 2 (Fig. 2.33, C and D).

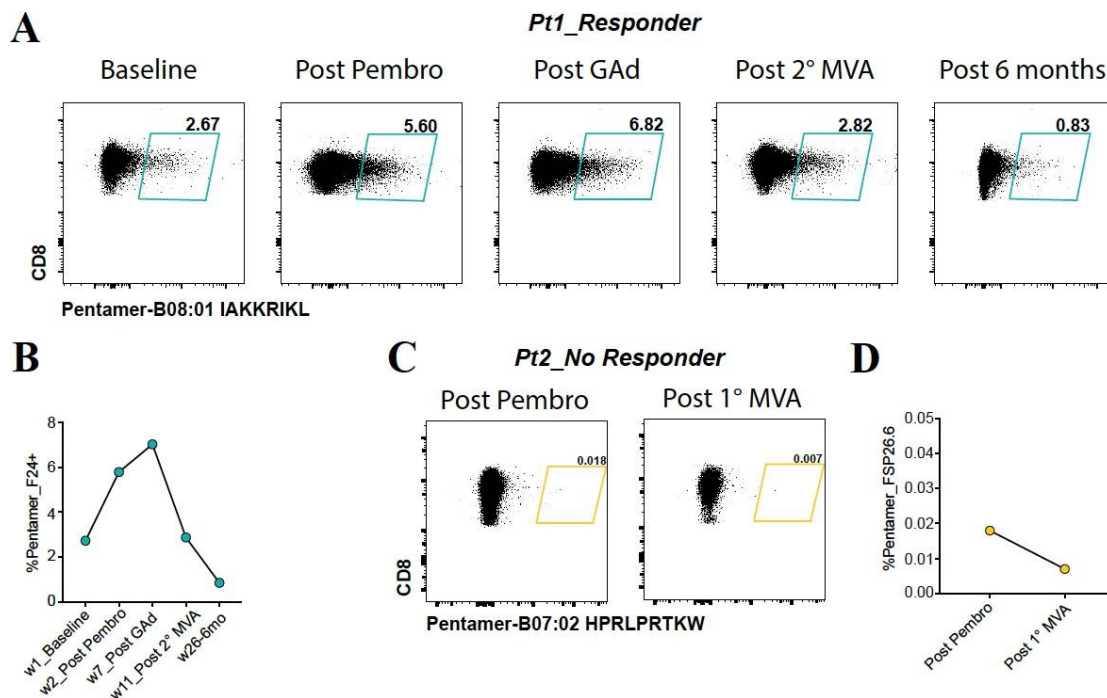


Fig. 2.33. Combination of a-PD1 therapy with neoantigen vaccination lead to an expansion of neoantigen-specific CD8⁺ T cells in PR patient

(A and C) Representative dot plots of F24+CD8⁺ T cells (A) and FSP26.6+CD8⁺ T cells (C) in PBMCs; (B) Percentages of F24+CD8⁺ T cells were measured in PBMCs at different time point in PR_Pt1. (D) Percentages of FSP26.6+CD8⁺ T cells were measured in PBMCs Post-Pembro and Post-1°MVA in Pt2.

To describe the immune-phenotypic properties of previously detected neoantigen-specific CD8⁺ T cells, we performed surface markers staining with fluorochrome labeled antibodies by flow cytometry. In particular, we focused our analysis on the previously aforementioned CD8⁺ T cells subsets, including the T_{STEM}, T_{TRANSITORY} and T_{EFFECTOR} (Fig.2.25). A notable

increase in the percentage of transitory neoantigen-specific CD8⁺ T cells was observed in patient 1 following the GAd vaccination, compared to both pembrolizumab monotherapy and the baseline timepoint (Fig.2.34B) The expression of memory markers revealed distinct behaviors of neoantigen-specific CD8⁺ T cells before and after treatment. Specifically, GAd vaccine induced the expansion of both naïve-like and stem-like F24-specific CD8⁺ T cells, as well as a small proportion of T_{EM1} cells. In contrast, T_{EM2} neoantigen-specific CD8⁺ T cells began to expand after the second MVA vaccination and reached a high percentage six months after the initiation of treatment (Fig 2.34C). These findings indicate that the combination of immunotherapies induced the generation and the expansion of transitory and stem-like cells, which can expand and generate new effector memory cytotoxic CD8⁺ T cells.

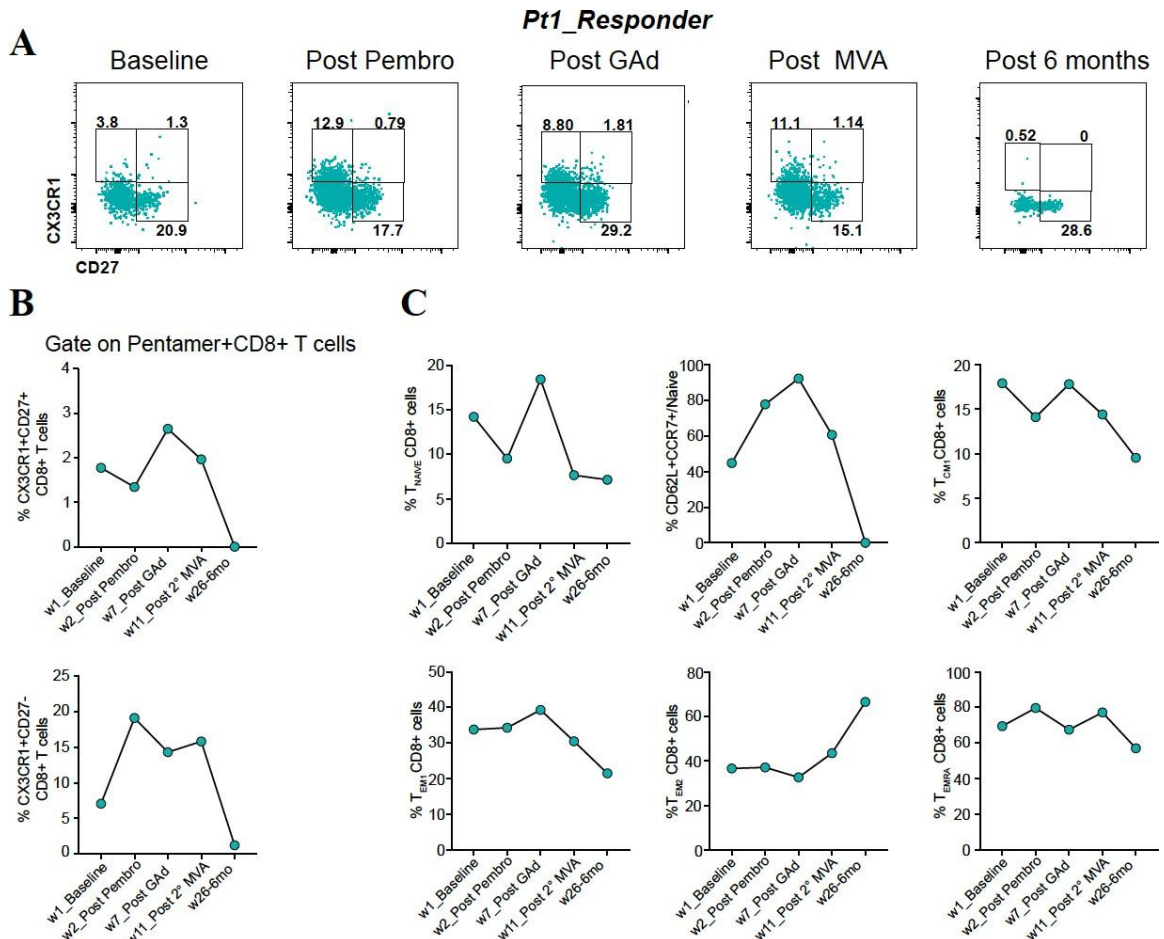


Fig. 2.34. Study of transitory, effector and memory subsets in neoantigen-specific CD8⁺ T cells after the different treatments.

(A) Representative dot plots of CX3CR1+CD27⁺ transitory and CX3CR1+CD27⁻ effector F24+CD8⁺ T cells **(B)** Percentages of CX3CR1+CD27⁺ transitory and CX3CR1+CD27⁻ effector F24+CD8⁺ T cells were measured in PBMCs at different time point in Pt1. **(C)** Percentages distinct memory subsets gated on F24+CD8⁺ T cells were measured in PBMCs at different time point in Pt1.

Single cells RNA sequencing analysis of neoepitope-specific CD8⁺ T cells from PR Pt1

To characterize the heterogeneity and the gene expression profiles of F24-specific CD8⁺ T cells before and after GAd immunization, a single cell RNA sequencing analysis (scRNA-seq) was conducted on sorted naïve CD8⁺ T cells as a control, and on sorted neoantigen-specific F24+CD8⁺T lymphocytes previously isolated through lympholyte density gradient from patient 1's PBMC. Cells were index-sorted into microwells and the transcriptome was profiled via scRNA-seq using Smart-Seq2 technology (221). After sequencing and quality control filtering, we obtained naïve (CD45RA⁺ CCR7⁺) cells from the subject after the Pembrolizumab treatment (n=20) and after the combined Pembrolizumab + neoantigen vaccine combined treatment (n=48). We also obtained CD8⁺Pentamer+(B08:01 IAKKRIKL) T cells after Pembrolizumab treatment (n=556) and after the combined treatment (n=504). A principal component analysis of the transcriptome data was performed and visualized as a Uniform Manifold Approximation and Projection (UMAP) plot (Fig. 2.35A). Clustering analysis led to the identification of seven clusters, naïve-like (cluster 1), effector (clusters 2 and 5), transitory CX3CR1+CD27⁺ (cluster 3) memory IL7R+KLRG1⁺ (cluster 4), memory stem-like (cluster 6) and low-quality cells (cluster 7) F24+CD8⁺ T cells. As expected, naïve cells from the Pembro and the combined treatment were found in the naïve-like cluster (90% and 88%, respectively, Fig. 2.35 B). Cells from the Pembro treatment predominantly displayed an effector phenotype (clusters 2 and 5, 45% of the cells), while 20% of the cells were classified as memory (cluster 4) and 13% as memory stem-like (cluster 6). In cells from the combined treatment, these phenotypes were found in reduced percentages: 37% of cells were effectors, 17% displayed a memory phenotype and 8% were memory stem-like. In contrast, the transitory CX3CR1+CD27⁺ phenotype (cluster 3), which was found in 6% of the cells from Pembrolizumab, experienced an increase (~ 3 Fold Change) in the combined treatment to 18% of the total F24 CD8⁺ T cells. These findings indicate that GAd vaccination, in combination with Pembrolizumab, was able to induce an expansion of the transitory cell subset: this result provides important insights into the immunological effects of the combined immunotherapy regimen, which may contribute to the enhanced anti-tumor efficacy observed when GAd vaccination is combined with pembrolizumab.

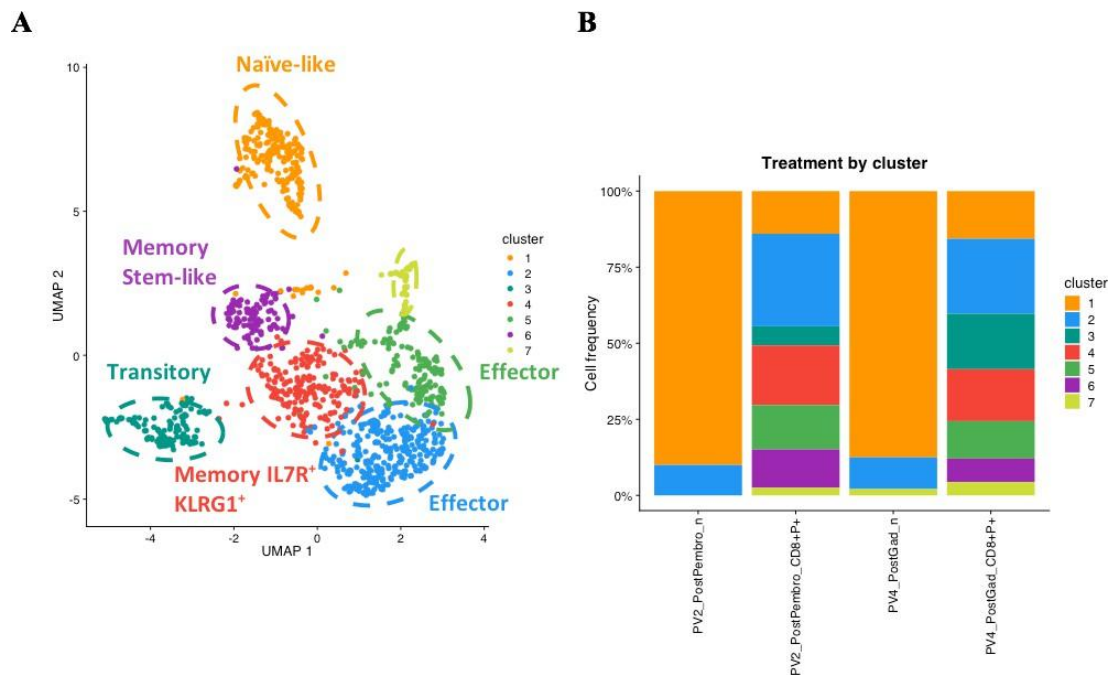


Fig. 2.35. Smart-Seq2 analysis of F24+CD8+ T cells in Pt1 before and after GAd treatment.

(A) UMAP visualization of clustering results with phenotype annotations. **(B)** Distribution of F24+ CD8+ T cell frequencies in each experimental condition, color-coded by cluster.

Naïve-like cells (cluster 1) and memory stem-like cells (cluster 6) were characterized by the expression of *IL7R*, *TCF7*, *CCR7*, *SELL* and *CD27*, whereas effectors cells that come from both cluster 2 and 5 have a high expression of *CX3CR1*, *GZMB1*, *KLRD1*, *TBX21* but lower expression of naïve genes as *CCR7*, *SELL* and *CD27*. While the distinct gene expression signatures of the identified cell clusters were evident, the transitory cells belonging to cluster 3 deserve particular attention. These cells exhibited a unique transcriptional profile, characterized by the co-expression of both naïve-like genes, such as *CD27* and *SELL*, as well as effector genes, including *CX3CR1*, *GZMB1* and *KLRD1*. The simultaneous expression of markers associated with both naïve and effector phenotypes suggests that the transitory cells in cluster 3 represent an intermediate or transitional state between these two functional states. Notably, differential gene expression (DEGs) analysis (Fig. 2.36A) highlighted a distinct pattern of gene expression for the transitory subset. These cells were characterized by the expression of *CD38*, a lymphocyte differentiation marker whose expression on differentiated CD8+ T cells is driven by mitotic and antigenic stimuli (240). We also observed the expression of *EZH2*, which promotes the expansion of memory precursor cells and their differentiation to functional memory (241). We detected expression of *CD74*, which

associates with MHCII, regulates antigen presentation and has been associated to accumulation of Tregs in tumors (242). HLA-DRA and HLA-DRB5, subunits of the MHCII, were also expressed: both play a central role in antigen presentation, leading to antitumor and cytotoxic activities (243). We also detected the expression of the inhibitory receptor TIGIT (244), which points to a close regulation of the transitory cell subset. This hybrid gene signature is intriguing and may indicate a dynamic and flexible cellular state that could play a pivotal role in the immune response following GAd immunization.

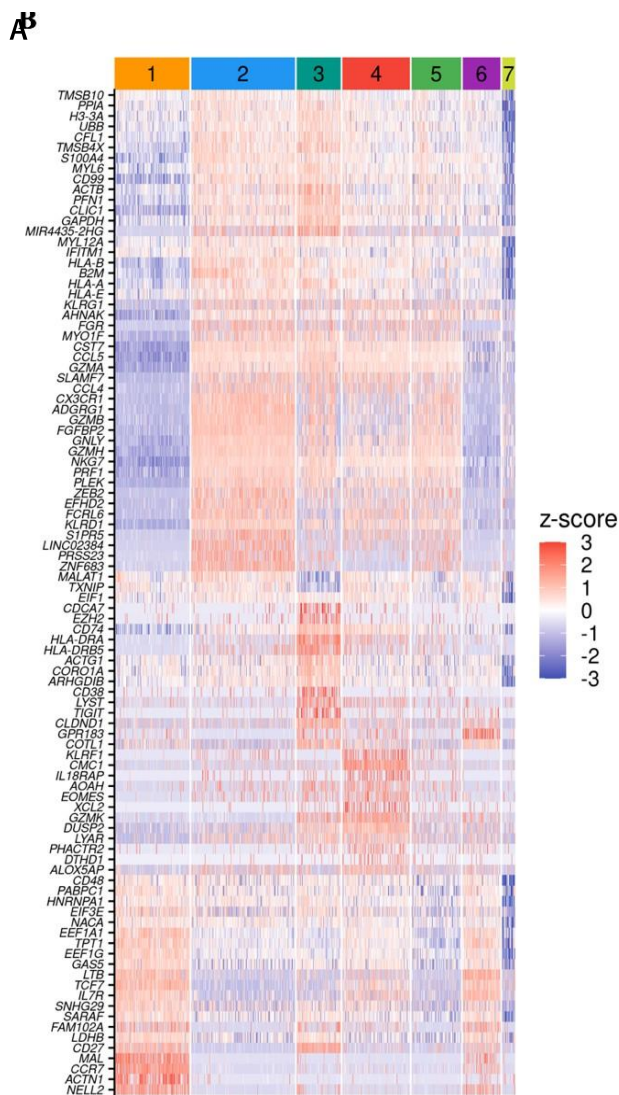


Fig. 2.36. Analysis of the genome expression in F24 specific CD8+ T cells via Smart-Seq2 technology.
(A)Heatmap of top 20 differentially expressed genes detected in each cluster through MAST.

Nous-209 vaccination expand F24-specific TCR clonotypes in PR_Pt 1

To investigate the relationship between the augmentation of transitory CD8⁺ T cells and the clonal expansion of F24-reactive cells following GAd immunization, we performed full-length paired $\alpha\beta$ -TCR sequencing analysis at single cell resolution using the Smart-Seq2 platform (221). We identified 476 unique TCR sequences (singletons) and 51 clonally expanded sequences (clones). The clonotypes displayed diverse degrees of expansion: 38 (74%) had a clone size of 2-9 cells, 11 (22%) were expanded into 10-37 cells, while 2 (4%) were found in more than 38 cells each. Our analysis of the TCR repertoire revealed distinct patterns across the identified cell clusters (Fig. 2.37A). Unique TCR clonotypes were predominantly found in the naïve-like and memory stem-like clusters (99% and 84% of the cells for which a TCR was reconstructed, respectively), suggesting these populations harbor a diverse TCR repertoire. 17 expanded clonotypes were detected in memory stem-like cells, effectors displayed 254 and 108 clones (for clusters 2 and 5, respectively), and 162 clones were found in memory cells. We detected 63 clones in the transitory cell cluster; of these, 8 were shared with the effector phenotypes, 3 with the memory phenotype and, 2 with the memory stem-like phenotype. This result in a PR Pt1 confirms the treatment-driven differentiation that we previously observed in the mouse model (Fig 2.37B). To elucidate whether the combined GAd+Pembrolizumab treatment induces clonal expansion of F24+CD8⁺ T cells, we examined the frequency distribution of TCR clones across the two treatments. Interestingly, we observed that the majority of F24+CD8⁺ T cell clones detected after pembrolizumab treatment were also present following GAd vaccination (33 clones, 64%). However, we also identified 11 clones that were detected only after the combined Pembrolizumab+GAd immunization (Fig 2.37C). Of these, 3 $\alpha\beta$ TCR sequences were found in 19, 13 and 10 cells respectively, while the other 8 $\alpha\beta$ TCR sequences were observed in up to 4 cells. These findings suggest that GAd vaccination drives a dual mechanism of clonal expansion: it promotes the expansion of pembrolizumab-induced clones, while also generating novel GAd-specific clones. These results indicate that the combination of pembrolizumab and Gad vaccination is an effective strategy for enhancing TCR clonotype expansion and diversification, potentially leading to a more robust and durable anti-tumor immune response. These results confirm what was previously found in the mouse experiments. Overall, our single-cell TCR analysis provides valuable insights into the clonal dynamics of F24-reactive CD8⁺ T cells in response to GAd vaccination, highlighting the

complex interplay between the expansion of pre-existing and de novo generated clones in shaping the anti-tumor immune landscapes.

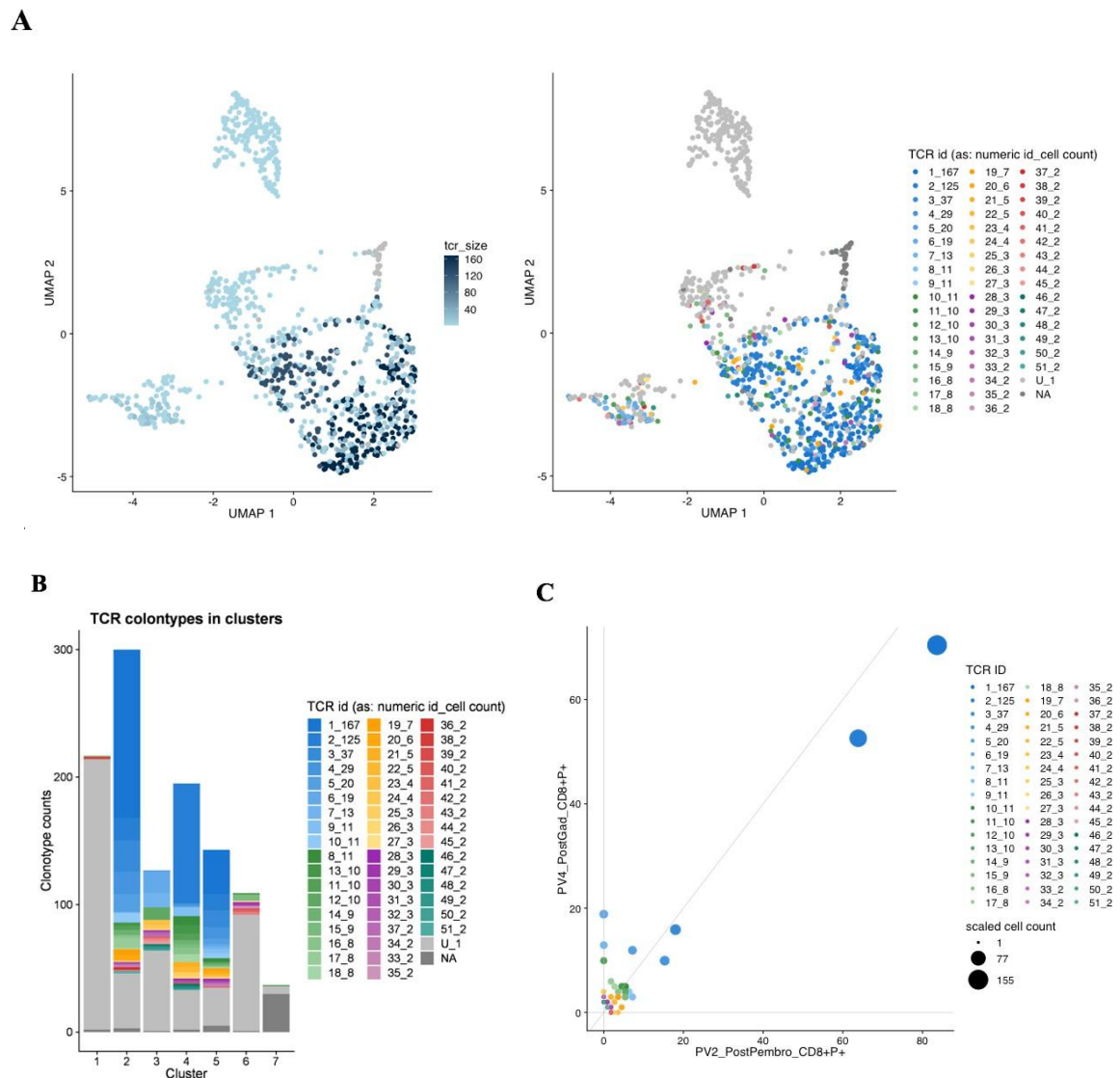


Fig. 2.37. Identification of expanded TCR clonotypes of F24+CD8+ T cells after pembrolizumab and GAd vaccination treatment.

(A) T cell receptor (TCR) clonotype size and distribution of each F24+CD8+ T cell projected onto UMAP
 (B) Quantification of F24+CD8+ T cell clonotypes in each cluster (C) Scatterplot of normalized cell frequencies for each TCR clonotype from Post Pembrolizumab treatment (x-axis) and Post GAd vaccination (y-axis).

Immune phenotype characterization of activated CD8+ T cells in melanoma patients treated with NOUS-PEV vaccination

In conjunction with the Nous209 vaccine protocol, a second vector-based personalized vaccine, referred to as NOUS-PEV, has been developed (245). This vaccine expresses 60 neoantigens (nAgs) and, similar to Nous209, has been employed in a heterologous

vaccination strategy that involves priming with GAd20 followed by three boosts with Modified Vaccinia Ankara (MVA), in addition to pembrolizumab treatment every three weeks (Fig 2.38A). Currently, NOUS-PEV is undergoing a phase Ib clinical trial (NCT04990479) targeting patients with metastatic malignant melanoma. However, the immune responses elicited by this vaccine remain poorly studied. To elucidate the immune phenotype, frozen PBMCs from two partial responder melanoma patients, PR_Pt1 and PR_Pt6, were thawed and stimulated for 48 hours with individual peptide pools. Following stimulation, the cells were labeled, and a multiparametric analysis was conducted via flow cytometry, focusing on the previously mentioned T cell subsets of interest (Fig.2.24, 2.25 and 2.26). As illustrated in Fig. 2.38 for both patients, we observed a notable increase in activated CD25+CD69+ CD8+ T cell populations following vaccination GAd treatment across all the peptide pools (Fig 2.38B). However, only PMBCs stimulated with Pool 3 continued to increase after MVA vaccination in PR_Pt6 (Fig 2.38B, right panel). These findings indicate a robust activation of the immune response and the expansion of neoantigen-specific CD8+ T cells, highlighting the potential of the NOUS-PEV vaccine in eliciting neoantigen-specific T cell responses in the context of metastatic melanoma.

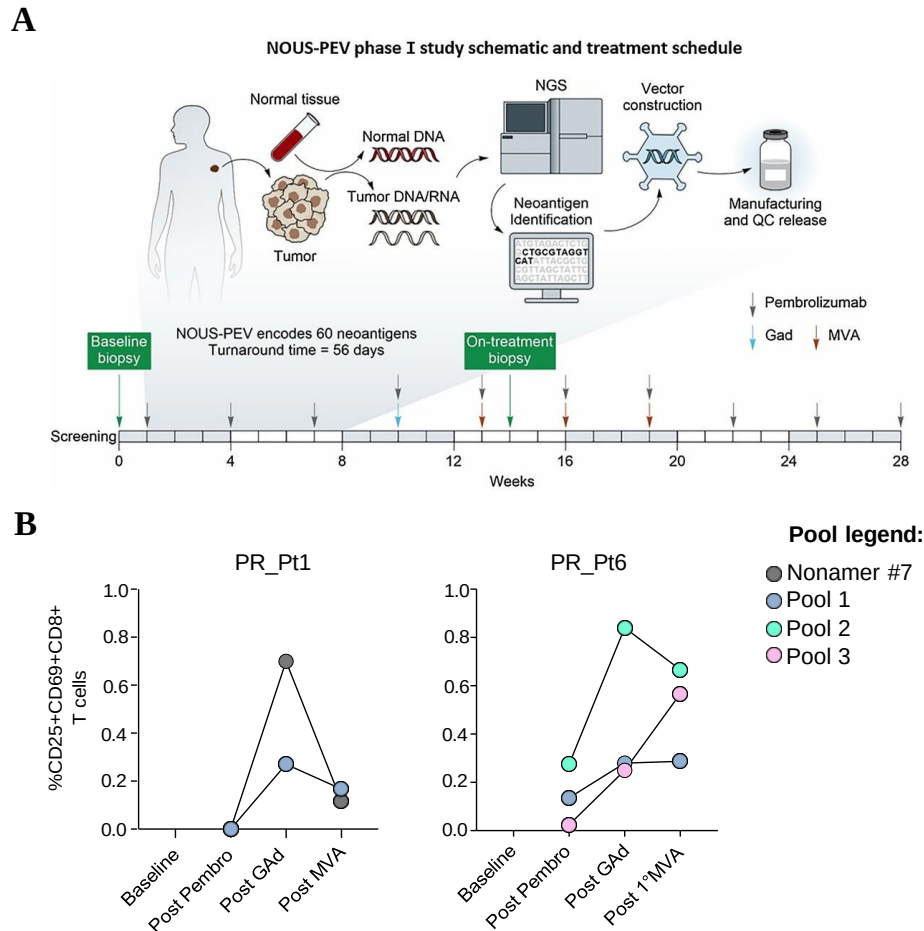


Fig. 2.38. Flowcytometry analysis of activated cells in partial responder stimulated with the pools reported in the legend.

(A) Clinical event timeline for two vaccinated patients from baseline to the latest time point; treatments include α PD-1 combined with GAd and MVA vaccine prime/boost administrations. Figure adapted from (245) **(B)** Percentage of CD25+CD69+ CD8+ T activated cells were measured at different time point in partial responders 1 and 6. Each colored dot represented a different peptide pools used for the stimulation.

In the characterization of the immune phenotype of neoantigen-specific CD8+ T cells, we observed an augmentation in the percentages of T_{NAIVE} CD8+ cells in PR_Pt1 following one nanomer stimulation (Fig2.39A). The behavior of T_{STEM} CD8+ T cells, characterized by the expression of CD62L and CCR7, varied depending on the peptide pool used for stimulation. In PBMCs stimulated with Pools 1 and 2, the frequency of T_{STEM} CD8+ T cells decreased following GAd vaccination but increased again after MVA treatment. Notably, stimulation with Pool 3 resulted in an increase of this subset after GAd vaccination. It is important to highlight that no stem-like CD8+ T cells were detected in patient PR_Pt1 (Fig 2.39B).

Moreover, a significant increase in CD8⁺ T_{TRANSITORY} cells was observed following MVA vaccination in both patients (Fig 2.39C). Conversely, CX3CR1+CD27⁻ T_{EFFECTOR} CD8⁺ T cells exhibited a decrease in both patients; specifically, in PR_Pt6, stimulation with peptide pools 1 and 2 after GAd vaccination resulted in a reduction of effector CD8⁺ T cells, and a similar result was found with pool 1 in Pt1. However, stimulation with Pool 3 led to an increase in this subset following GAd vaccination (Fig 2.39D). Whereas, CD95+Tim3⁻ T_{EFFECTOR} CD8⁺ T cells increased following MVA immunization in both patients (Fig 2.39E). These findings suggest a complex interplay between different CD8⁺ T cell subsets in response to vaccination, indicating that while transitory CD8⁺ T cells are enhanced, effector CD8⁺ T cells may be selectively reduced under certain conditions, probably these cells have already infiltrated the tumors. Moreover, the variability in stem-like CD8⁺ T cell responses highlights the need for a clear understanding of T cell dynamics in the context of neoantigen-targeted therapies.

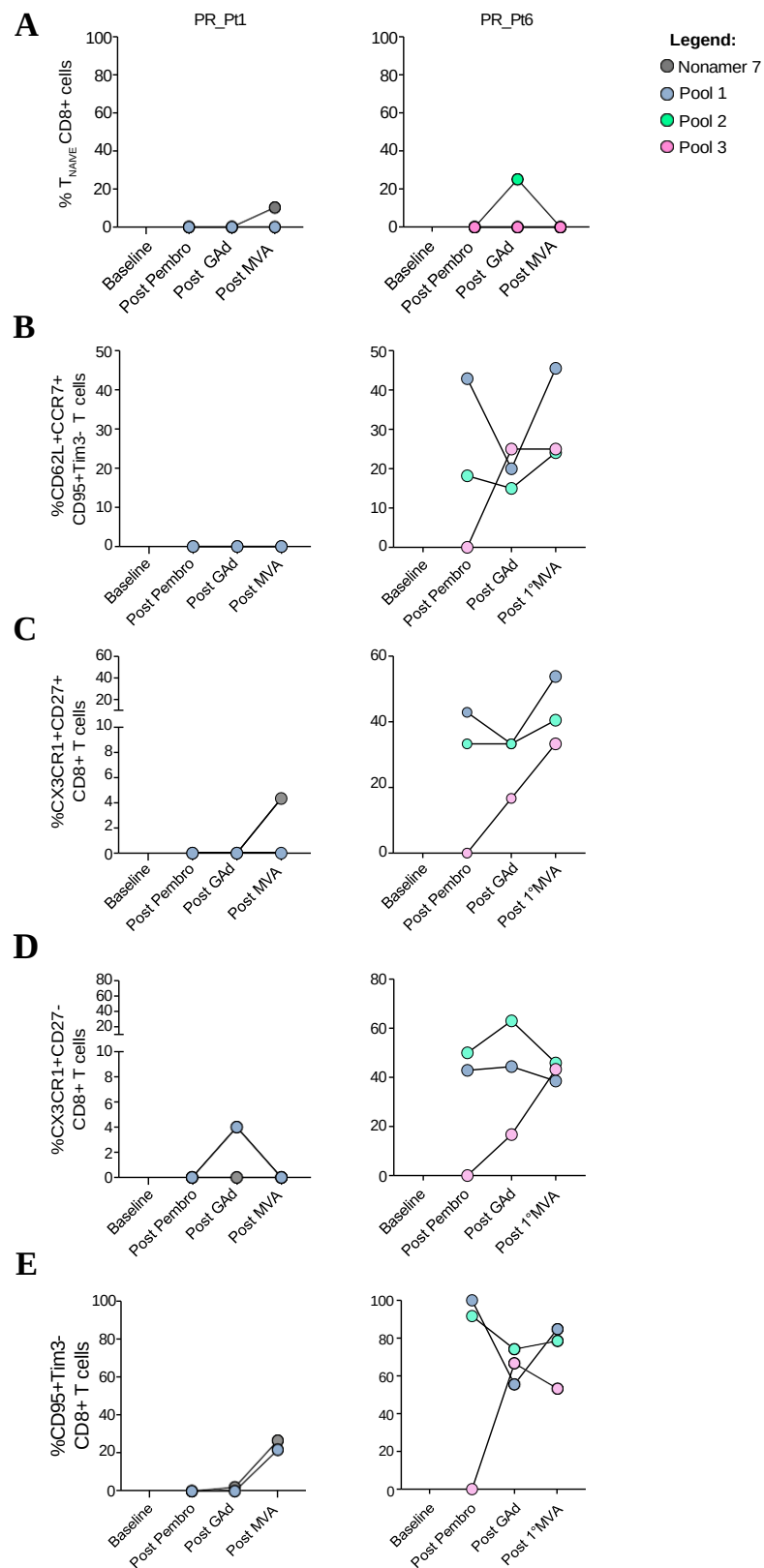


Fig. 2.39. Immune phenotype of peptides activated CD8+ T cells following different clinical timepoints. (A-E) Percentages of CD25+CD69+ CD8+ T (A) T_{NAIVE}, (B) CD62L+CCR7+Tim3-CD95+ T_{STEM} (C) CX3CR1+CD27+ T_{TRANSITORY}, (D) CX3CR1+CD27- T_{EFFECTOR} and (E) CD95+Tim3- T_{EFFECTOR} cells were

measured at different time point in PR treated with NOUS-PEV immunotherapies protocol. Each colored dot represented a different peptide pools used for the stimulation.

Subsequently, we analyzed the memory expression patterns of CD8⁺ T cells from partial responder patients at various time points following treatment, after stimulation with individual peptide pools. In PR_Pt1, a modest increase in the percentages of T_{CM1}, and T_{EM1} specific to neoepitopes was observed after the administration of the NOUS-PEV vaccination protocol (Fig 2.40A and B, first panels). However, the most significant finding was the notable increase in T_{EM2} cells following MVA treatment (Fig 2.40C, first panel). Conversely, the immune phenotype of PR_Pt6 exhibited greater complexity. CD8⁺ T_{CM1} cells decreased after GAd vaccination with Pool 2, but these cells were detected with pool 3 after MVA boost (Fig 2.40A, second panel). Furthermore, GAd vaccine elicited an augmentation of T_{EM1} with most peptide pools in PR_Pt1, but not in PR_Pt6 (Fig 2.40B, second panel). Finally, activated T_{EM2} cells increased after MVA vaccination when PBMCs were stimulated with pools 1 and 3, although a decrease was noted following stimulation with Pool 2 (Fig 2.40C, second panel). These findings indicate that T_{EM2} cells are elicited following MVA boosting, so we could hypothesize that the NOUS-PEV leads to a long-lasting protective immunity. However, we have analyzed only two patients, so it's difficult to draw some conclusions

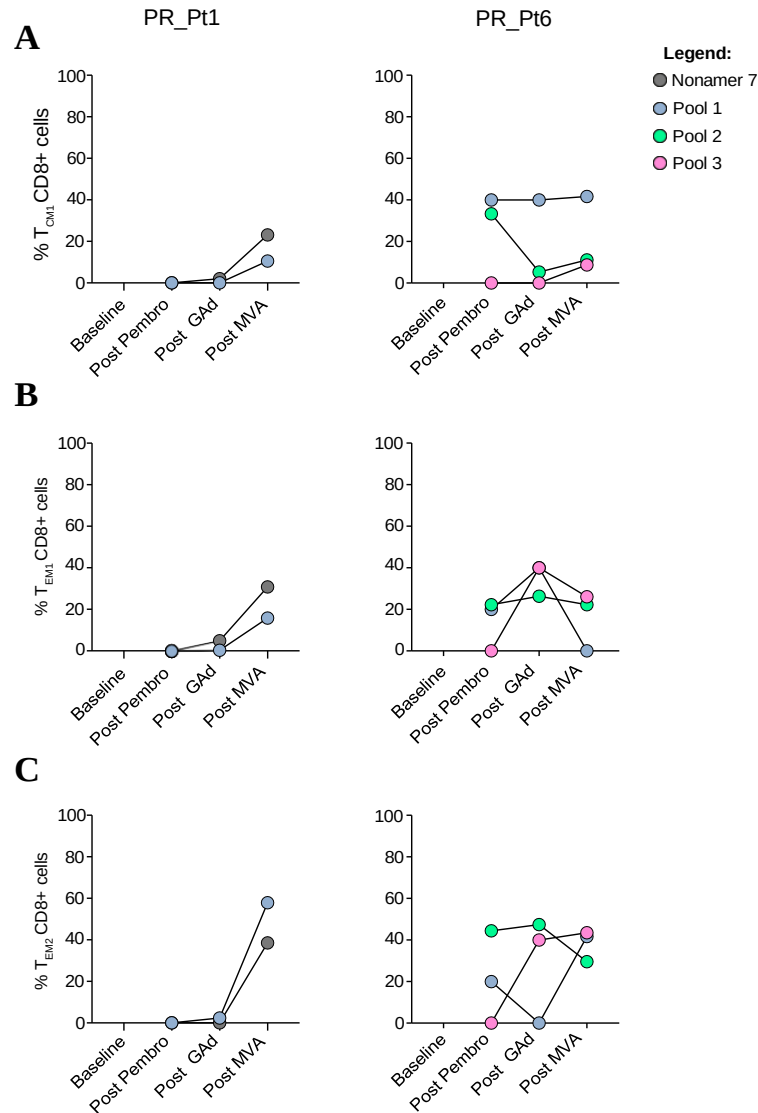


Fig. 2.40. Study of the memory phenotypes in responder patients stimulated with FPS pools following NOUS-PEV treatment protocol.

(A-C) Percentages of CD25+CD69+ CD8+ T (A) T_{CM1} (B) T_{EM1} (C) and T_{EM2} cells were measured at different time point in patients. Each colored dot represented a different peptide pools used for the stimulation.

Cytokines and transcriptional factor profile of FSP-specific CD8+ T cells in melanoma patients treated with NOUS-PEV vaccine.

After the analysis of the immune phenotype of the patients treated with NOUS-PEV protocol, we moved our attention to the study of the cytokine and transcriptional expression profile using the SPICE tools. PBMCs were thawed and cultured with a specific pool of peptides for 24 hours. Following this incubation period, the cells were stained to identify intracellular markers such as cytokines Granzyme B and IFN- γ , or Perforin, TOX and the transcription factor TCF-1, in CD8+ T cells derived from PR_Pt6. The analysis detected only one difference in cells stimulated with Pool 3, in Granzyme B+, IFN- γ +, Perforin+, TCF-1-, and

TOX- subpopulation after MVA boost and in Granzyme B+, IFN- γ +, Perforin+, TCF-1-, and TOX+ markers post GAd priming, whereas no expression was found in the other combination of markers (Fig 2.41A). These results are in accordance with similar findings obtained in partial responder patients treated with NOUS209 vaccine.

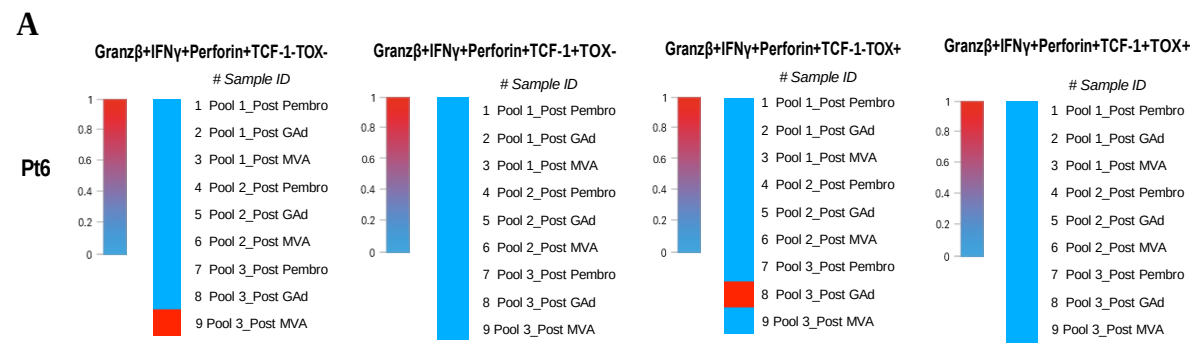


Fig. 2.41. SPICE Analysis in one partial responder patient Pt6.

(A) Cool plot derived from SPICE analysis illustrate the expression of GranzymeB+,IFN- γ +,Perforin+, TCF-1 +/- and TOX +/- in Pt6 patient. Near each plot there is a numbered color legend that represents the expression levels, providing a clear visual reference for interpreting the data (from blue= low expression to red=high expression).

DISCUSSION

The quantity and potency of neoepitope-specific CD8⁺ T cells are fundamental factors that drive effective antitumor responses. Conversely, for exhausted cells, TCF1⁺ CD8⁺ T_{STEM} cells can undergo clonal expansion and proliferation upon PD-1 inhibition, and serve as a reservoir to continuously generate and replenish the effector CD8⁺ T cells with cytolytic function (90). Recently, the growing interest in CD8⁺ T_{STEM} cells for T cell-based immunotherapies, such as adoptive T cell transfer or generation of chimeric antigenic receptor (CAR) T cells, is attributed to the association between the use of a less differentiated CD8⁺ T cell subsets and improved therapeutic outcomes (246, 247). Given the unique plasticity and self-renewal properties exhibited by CD8⁺ T_{STEM} cells, strategies aimed at expanding T cells to acquire stem-like properties may provide opportunities for the development of more effective immunotherapies in clinical cancer care, especially in patients with resistance to checkpoint blockade therapies (248). In the present study, we characterize neoepitope-specific CD8⁺ T cells in a murine CRC model. The MC38 cell line, known for its affinity to model hypermutated and microsatellite instability (MSI)-high CRC, was selected as a valid tumor cell line for the study (249). Our findings demonstrate that vaccination with GAd vector encoding tumor neoantigens, in combination with α PD-1, enhances the magnitude of neoantigen-specific CD8⁺ T cells and promotes their differentiation into stem-like Tcf1⁺ CD8⁺ T cell progenitors. scRNA-seq data from Adpgk-specific CD8⁺ T cells, induced by the vaccine in combination with α PD-1, revealed a broad dynamic of CD8⁺ T cell expansion and differentiation. The analysis highlights the accumulation of CD8⁺ T_{STEM} cells in tumor-draining lymph nodes, which was associated with memory T cell precursors in the spleen and CD8⁺ T effectors in tumor bed, suggesting a potential mechanism whereby specific CD8⁺ T progenitors' egress from the lymph nodes and further differentiate into effectors in the tumors. Based on these findings, it is plausible that the superior antitumor efficacy achieved by GAd and α PD-1 combination compared to α PD-1 monotherapy, is related to both the magnitude and transcriptional quality of CD8⁺ T cell population induced by the vaccine. The magnitude of the CD8⁺ T cell response, as measured by the number of neoantigen-specific CD8⁺ T cells, is a critical factor in determining the potency of antitumor response. Moreover, by using $\alpha\beta$ TCR sequences as a reliable molecular barcode to track Adpgk⁺-specific CD8⁺ T trajectories across tumor and draining lymph nodes, we confirmed that the same CD8⁺ T cell clones can be found in both

tissues in distinct differentiation stages. Our data support the role of CD8⁺ T_{STEM} cells as an active reservoir of progenitors of CD8⁺T effectors and subsequently exhausted cells. This process of commitment to tumor-specific CD8⁺ T cells begins in the draining lymph nodes before migrating into the tumor (250). Within the tumor microenvironment, as expected, chronic exposure to tumor antigens and the presence of multiple immunosuppressive signals lead to T cell exhaustion, a behavior confirmed by both trajectory inference and TCR clonal expansion. TCR clonotype analysis have demonstrated an increased inter- and intra-clonal heterogeneity of Adpgk⁺-specific CD8⁺ T cells in mice treated with the combination of GAd with α PD-1, as compared to mice receiving only α PD-1 monotherapy. So far, the best example of vaccines generating long-lived memory T cells with stem-like features and mediating lifelong protection is the yellow fever vaccine, based on a highly effective attenuated virus (251). The synergistic mechanism of action of vaccination combined with checkpoint blockade in cancer is supported by recent findings, demonstrating that intravenous vaccination based on neoantigen peptides and Toll-like receptor 7/8 agonists (SNP-7/8a) linked to nanoparticles promotes increased numbers of CD8⁺ T_{STEM} cells, associated with better tumor rejection in a prophylactic cancer model (252). Another key aspect of this study is related to the effect of the Ad vaccine in broadening the antigenic breadth and clonal diversity of CD8⁺ T cells in the context of cancer models (125). Our findings support the adenoviral vector platform as a promising approach promoting the generation of a diversified T_{STEM} cell reservoir, which may contribute to effective antitumor activity. According to results obtained in the preclinical mouse model, vaccination targeting shared FSP neoantigens (Nous209 vaccination protocol (214)) in patients with dMMR induced a potent and broad T cell response in the vast majority of patients. Broadening of the repertoire of T cells and an increase of T cells with an effector memory phenotype after vaccination were observed in three patients who responded clinically to the treatment. In one of these partial responder patients, we track neoantigen-specific vaccine-induced TCR clonotypes in both tumor biopsy and peripheral blood mononuclear cells (PBMCs) before and after GAd vaccination. The neoantigen of interest was previously identified by bulk RNAseq on patient tumor biopsy. ScRNAseq data from neoantigen-specific CD8⁺ T cells from partial responder patient PBMCs confirm the results obtained in mice experiments, in fact we observe that GAd vaccination induces a clonal expansion of neoantigen CD8⁺ T cells. In particular, we found an increase of transitory T cell cluster characterized by the coexpression of both naïve and effector gene signature after immunization with adenoviral vaccine. The expansion of T_{TRANSITORY} CD8⁺ T cell subset is of significant importance

because they have a high proliferation rate that allows them to self-renew and to produce CX3CR1- T_{CM1} cells, which are crucial to maintaining the T_{CM1} pool (77). In the context of tumor, the reservoir of T_{CM1} cells is crucial because they are circulating T cells capable of entering the lymph nodes and improving long-term antitumor responses (253). Furthermore, several patients exhibiting different disease outcomes have been immunophenotypically characterized. We found that in all responder patients, MVA boost vaccination induces an expansion of activated neoantigen-CD8+T cells which have an effector and effector memory phenotype, suggesting that the vaccination protocol expands effectors T cells that can kill tumor cells carrying specific neoAgs leading to the control of the tumor progression (254). Similar results were found in melanoma patients treated with the NOUS-PEV heterologous vaccination protocol, which is a protocol close to the Nous209 (245). However, the immunophenotypic patterns in both immunotherapies' protocols highlight a heterogeneous response of neoAgs specific CD8+ T cells. Different responses of CD8+ T cell can depend on different factors, like the clinical history of each patient, indeed subjects can present metastasis in the liver and peritoneal tissue. The correlation between cancer metastasis and incurable disease is significant, primarily due to the limited research on the interplay between the immune system and the metastasis in both preclinical and clinical models(255). Additionally, the heterogeneity of tumor types and stages presents considerable challenges that hinder the advancement of innovative therapeutic strategies (256). On the other hand, patients with Lynch syndrome represent ideal candidates for ICB therapy and neoantigen vaccination due to the germline pathogenic variants in the MMR genes that caused the disease and the increased number of mutations that lead to neoantigen formation (257).

Another important biological feature that influences tumor development and the response of the immune system is gender, in fact, sex hormones and sex chromosomes interact in different ways between males and females, with the immune system and the tumor microenvironment (258). Unfortunately, the results obtained from our patient immunophenotyping are limited by the availability of samples and the heterogeneous responses induced by stimulation with FPS. However, all these results suggest that neoantigen-specific CD8+T cells, induced by both Nous-209 and NOUS-PEV vaccination protocols, expand and diversify upon treatment, successfully traffic and infiltrate the tumor microenvironment to exert active antitumor activity.

Our data support the development of viral vector vaccines for neoantigen-based immunotherapies. Shared neoantigens are not common in tumor types other than MSI-high tumors, and a personalized approach is required. Related to the personalized approach, a

major challenge for clinical application in patients with metastatic cancer is the need for fast manufacturing and timely delivery of individually tailored vaccines to patients. This study has some limitations. First, there are some differences between the mouse model and the clinical trial in terms of treatment regimen and schedule. In mice, the vaccine was administered concomitant to α PD-1, whereas in the trial patients first received α PD-1 and then the vaccine. In patients, the vaccine was administered at the second injection of α PD-1 to establish a baseline immune response after α PD-1 treatment but before vaccination to evaluate vaccine-induced immune response. Unlike the clinical trial, in the mouse model, vaccine schedule did not include a MVA boost after GAd priming. Tumor growth is much faster in the mouse model and does not allow for the assessment of the MVA boost. However, the ability of MVA to improve the magnitude and quality of adenovaccine-induced T cells has been extensively demonstrated (259) and supports the use of the prime and boost regimen in patients. Second, the effect of expansion and diversification of the intratumoral TCR- β is reported in three patients, and it was only possible in one to track vaccine-induced neoantigen-specific TCRs in the tumor biopsy after treatment. Unfortunately, only a limited number of requested samples from the clinical trial were available and were of good enough quality for proper analysis. Additional clinical samples will be analyzed in the future to extend our data. In addition, the patient population of this study is heterogeneous in terms of both line of treatment (first and second line of treatment) and tumor type, and the number of patients is too low to allow comparison with historical data of α PD-1 efficacy. Overall, our findings indicate that Ad vector vaccines in combination with checkpoint inhibitor treatment can sustain the proliferation, expansion of neoepitope-specific T cell clones, and trafficking into the tumor both in mice and in patients with cancer. This vaccination platform encoding many different neoantigens is well suited to overcome α PD-1 therapy resistance by increasing polyclonal expansion and eliciting memory T cells against tumor rejection neoantigens, thus paving the way for more effective immunotherapy treatments.

MATERIALS AND METHODS

Study design

This is a multicenter, non-randomized, open-label phase 1 study designed to determine safety, immunogenicity, and preliminary efficacy of heterologous prime/boost GAd20-209-FSP/MVA-209-FSP vaccination schedule in combination with pembrolizumab, in subjects with second- or first-line unresectable or metastatic dMMR/ MSI-H CRC, gastric, or GEJ tumors (NCT04041310). The study is composed of two sequential cohorts, i.e., dose escalation and expansion, testing two dose levels, i.e., dose level 1 [GAd20-209-FSP: 1.88×10^{10} viral particles (vp); MVA-209-FSP: 1.65×10^7 infectious unit (ifu)] and dose level 2 (GAd20-209-FSP: 1.88×10^{11} vp; MVA-209 FSP: 1.65×10^8 ifu). The study started at the first pembrolizumab infusion (day 1 of week 1); GAd20-209-FSP prime was administered on the day of the second pembrolizumab infusion (week 4); MVA 209-FSP boosts were given at third, fourth, and fifth pembrolizumab infusions (weeks 7, 10, and 13, respectively).

In the dose escalation portion of the study, a 3 + 3 + 3 dose escalation design was applied in up to two sequential cohorts. In the dose expansion portion of the study, the expansion cohort was determined by logistic feasibility rather than statistical considerations. No formal sample size calculation was carried out. Safety monitoring committee was established to assess safety of both dose levels, to make a recommendation for recommended phase 2 (RP2D) of the vaccine, as well as recommendations concerning the continuation, modification, and termination of the trial. The severity and relatedness with vaccination or adverse effects were assessed by the medical team in each center and graded according to Common Terminology Criteria for Adverse Events (CTCAE) v5.0. Objective response rate (ORR) was assessed using RECIST v1.1. Immunogenicity was assessed using ex vivo ELISpot. Subjects were expected to attend several visits after screening (up to 28 days) in addition to vaccination visits. During the visits, the subjects underwent blood sampling and medical evaluation. Subjects also discussed with a doctor any potential adverse effects. Three subjects were vaccinated with dose level 1 of Nous-209 in combination with pembrolizumab treatment, not reporting any DLT. Subsequently, subjects were enrolled at dose level 2 of Nous-209 in combination with pembrolizumab. Nine subjects were vaccinated with dose level 2 of Nous-209 in combination with pembrolizumab treatment as of 29 September 2021, not reporting any DLT. Therefore, the high dose was declared as the RP2D.

Ethical statement

The study was approved by the Institutional Review Boards (IRBs) of MD Anderson University of Texas (protocol 2019-0651), Weill Cornell Medicine (protocol 19-08020684-02), John Hopkins (IRB 00220323), Roswell Park (MOD 00006973/P-457819), Karmanos Cancer (IRB 202000155), and City of Hope (IRB 19277/176947).

Vaccine vector generation

Production of GAd vaccine vector for preclinical studies has been performed as previously described (18). GAd-209-FSP and MVA 209-FSP vectors have been generated as previously described (19). For GAd, the transgenes were synthesized by GeneArt (Thermo Fisher Scientific) and subcloned via Eco R1–Not 1 restriction enzymes (New England Biolabs) into a shuttle plasmid containing the cytomegalovirus (CMV) promoter with two Tet Operator repeats and a bovine growth hormone (BGH) polyA. The expression cassettes were then transferred into the E1 deletion locus of pGAd20 plasmid by homologous recombination in BJ5183 cells. pGAd20 contains the genome of a gorilla Ad (serotype group C) deleted in E1 and E3 regions. For MVA-209, the transgenes were synthesized by GeneArt and sub cloned via Bam H1–Asc 1 restriction enzymes (New England Biolabs) into a shuttle plasmid under the control of the P7.5 promoter. In addition, the shuttle plasmid carries an enhanced green fluorescent protein (eGFP) expression cassette and sequences homologous to the deletion III locus of MVA, to allow insertion of both expression cassettes (eGFP and transgene) in this locus. Recombinant MVA vectors were obtained by homologous recombination in chick embryo fibroblast (CEF) cells. GAd-209-FSP and MVA-209-FSP clinical lots have been manufactured by Reithera s.r.l. (Rome, Italy). For Good Manufacturing Practice (GMP) production of GAd-209-FSP, the four GAd-20 vectors have been amplified in suspension M9 cells [human embryonic kidney (HEK) 293 derivative] and purified by anion exchange chromatography. Vector titers were determined by quantitative polymerase chain reaction targeted on CMV promoter sequence, and the four vectors were mixed to generate the GAd 209-FSP vaccine. Vector infectious titers were determined by hexon immunostaining on infected M9 cells. For GMP production of MVA-209-FSP, the four MVA vectors have been amplified in suspension AGE1.CR.pIX cells and, after purification, mixed to obtain the MVA-209-FSP vaccine. Infectious titer has been determined by immunostaining on infected

Vero cells with anti-vaccinia virus antibodies. Moreover, NOUS-PEV was designed and developed according to the following academic reference (245).

Mice

C57BL/6J female mice were obtained from Envigo and Charles River and used between 7 and 10 weeks of age. All the mice were housed in Molecular Bio technology Center (MBC) (Turin University) specific pathogen free (SPF) Animal Facility. Live animal experiments were done in accordance with the guidelines of Italian and European Veterinary Department.

In vivo treatments

Vaccines were administered via intramuscular injections in the quadriceps by delivering a volume of 50 μ l per side at 5×10^8 vp. For efficacy studies, anti-PD-1 antibody (PD-1, BioXcell, clone RMP114, catalog number BE0146) was administered twice a week until day 16 after start of treatment. The antibody was administered intra peritoneally at a dosage of 200 μ g. At the indicated time points, the draining inguinal lymph nodes, tumors, and spleens were harvested and analyzed by flow cytometry.

In vivo tumor progression

MC38 cells (2×10^5 ; C57BL/6 mouse colon adenocarcinoma, American Type Culture Collection) were subcutaneously injected as aforementioned. Before treatments start (day 0), animals were randomized (tumor size average per group, 70 to 100 mm³). Mice were sacrificed as soon as signs of distress or a tumor volume above 2000 mm³ occurred. Tumor growth was measured every 3 to 4 days. Tumor volume was calculated using the formula (tumor size W) $\times$ (tumor length L)²/2, where the length was the longer dimension.

Flow cytometry and index cell sorting on mouse model

The cells were harvested at different time points after immunization. Single-cell suspensions were generated, red blood cells were lysed with a hypotonic buffer, and cell number was determined. Tumor-infiltrating lymphocytes were enriched by Percoll gradient before labeling. Cell suspensions were prepared in phosphate-buffered saline (PBS)–0.5% bovine serum albumin (BSA) and 2 mM EDTA, and cells were labeled according to the experiment with CD8 (clone 53-6.7, reference 563332, BD), TCR (clone H57-597, reference 563221, BD), CD103 (clone M290, reference 562772, BD), CD69 (clone H1.2F3, reference 552879,

BD), CD44 (clone IM-7, reference 565480, BD), CD62L (clone MEL-14, reference 563252, BD), CD127 (clone SB/199, reference 562419, BD), KLRG1 (clone 2F1, reference 742199, BD), H-2Kb (clone AF6-88.5, reference 116507, BioLegend), H-2Db (clone REA619, reference 130-109-608, Miltenyi), PD-1 (clone J43, reference 67-9985-82, Invitrogen), and CD38 (clone 90, reference 11-0381-82, Invitrogen). Cells were then fixed with 1% paraformaldehyde. Fc receptors were blocked with the CD16/CD32 (2.4.G2) monoclonal antibody. Dead cells were stained with cell death dyes (eBioscience) according to the manufacturer's instructions. Phenotypic characterization of lymphocytes was performed using BD LSRFortessa X-20 and sorted with BD FACSAria III (Becton Dickinson). The limit of detection was set above 10 recorded cells. The data were analyzed with FlowJo 10.4.2 software.

Peptide stability assay

RMA-S cells (5×10^5) were plated in a 24-well plate, and each peptide (Dpagt1, Reps1, Adpgk, Irgq, Aatf, and Cpne1) was added with a final concentration of 30 M. The cells were incubated overnight at 37°C. Next, they were collected in a 50-ml tube, washed with $1 \times$ PBS, and plated in a 96-well plate. To monitor the relative stability of surface H2-Kb and H2-Db molecules, the cells were stained with H-2Kb (AF6-88.5) and H-2Db (clone REA619) antibody every 2 hours for 6 hours in total. Phenotypic characterization of lymphocytes was performed using BD LSRFortessa X-20 and sorted with BD FACSAria III (Becton Dickinson). The data were analyzed with FlowJo 10.4.2 software.

Single-cell RNA-seq

Dextramer-Adpgk⁺ CD8⁺ T cells were FACS-sorted from draining lymph nodes and tumors, harvested from nontreated tumor-bearing mice, and treated with PD-1 and with PD-1 combined with GAd. Neoepitope-specific CD8⁺ T cells were sorted into 384-well plates containing lysis buffer and immediately frozen. Single-cell library preparation was done as previously described (26). Libraries were pooled and sequenced [2×50 -base pair (bp) and 2×100 -bp paired end reads], 106 reads per cell, on the NOVAseq 6000 instrument.

For single-cell analysis of patient samples, thawed PBMCs were resuspended in complete RPMI 1694 media and counted. To detect neoantigen-specific CD8⁺ T cells, F24-pentamer staining was performed according to the manufacture instructions, whereas to characterize immune phenotype the following antibodies were used, CD3,CD8,CD4,CD45RA and CCR7.

After surface antibodies labeled, cells were washed, resuspended and DAPI was added to discriminate viable cells from nonviable cells and sorted with BD FACS Aria III.

Index single-cells sorting of F24 Pentamer+CD8+CD4-CD3+DAPI- cells was performed in a 384-well plate containing lysis buffer and immediately frozen. Single-cell library preparation was done as previously described (26). Libraries were pooled and sequenced on the NOVAseq 6000 instrument.

Single-cell RNA-seq processing

Single-cell sequencing files (base calls) were first demultiplexed using the bcl2fastq2 (v.2.20.0.422) software using default parameters for paired-end sequencing. The resulting FASTQ files were then aligned to the GRCm38 mouse genome (mice) and the GRCh38 (human) (mm10) genome using STAR (v.2.7.3a) (39, 40), and transcript-per-million (TPM) counts were obtained by processing the alignments with RSEM (v.1.3.1) (41). After quality controls, 1189 cells were selected for further analysis with R (v.3.6.3) (R Core Team, 2020). Only genes detected in at least 5% of the cells with a $\log_2\text{TPM} \geq 2$ were analyzed. Furthermore, mitochondrial and ribosomal genes were removed. Data were regressed through linear modeling, then scaled, and centered along mean values of the gene expression matrix. PCA was run on the scaled data, and using the elbow plot approach, the first 14 principal components were selected for the UMAP (42) and clustering procedures. Clusters were identified using the Louvain algorithm (43) with the following parameters for k, resolution, and number of iterations: $k = 35$, $r = 0.4$, and $i = 1000$ for mouse. Furthermore for human scRNAseq, the identification of the clusters was done using Louvain algorithm (43) with the following parameters for k, resolution, and number of iterations: $k=41$, $r=0.6$ and $i=1000$. Differentially expressed genes for each cluster and for each cluster subdivided by mouse response were obtained using MAST with default parameters (44). TCR sequences were reconstructed using TraCeR (45) with default parameters. The outputs of TraCeR include the assembled nucleotide sequences for both chains, the coding potential of the sequences, the estimation of TPM values, and the CDR3 sequences. Only chains with TPM values higher than 10 and 15 TPM, respectively, were kept for analysis. Clones were defined as different cells that shared identical - pairs. In addition, cells that shared CDR3 and sequence with other cells, but in which TraCeR failed to reconstruct the sequences, were associated to the respective clones. In the rare event of matches with multiple clones, the TCR was disregarded and not considered for further analyses. Cells with missing chains were not included in clones even if they shared a sequence with other cells. Supervised trajectory

inference was done using the slingshot R package (46). We defined the start and end points of the lineages as the naïve and exhausted cell populations, respectively.

Patient PBMC preparation

Peripheral blood mononuclear cells (PBMCs) from patients were isolated at different time points and cryopreserved at the clinical sites before shipment to the central laboratory for immunogenicity assessment. PBMC isolation and freezing procedures were performed within maximum 8 hours from blood collection to preserve PBMC functionality in immune-monitoring assays. 80 ml of peripheral venous blood from patients were collected through phlebotomy into 8 x10-ml lithium-heparin Vacutest blood collection tubes (Kima). Blood samples were diluted 2:1 with Hanks' balanced salt solution (HBSS), allocated into leucosep tubes, and centrifuged at 800g for 15 minutes without braking at 20°C. The cell suspension was harvest, washed one time with HBSS for 10 min at 800g, and then washed for two times with HBSS supplemented with 5% of fetal bovine serum (FBS) for 10 minutes at 250g at 20°. Cell pellets were resuspended and counted. After counting, PBMCs were frozen in freezing media composed of 10% of dimethyl sulfoxide (DMSO) and located in the -80°C for one to three days before the storage in liquid or vapor nitrogen (N₂) tanks. Once thawed the viability of the cells was around 90%

IFN- γ ELISpot assay

The frequency of IFN- γ -producing T cells was measured by ex vivo ELISpot-forming cell assay after antigen-specific stimulation. PBMCs were resuspended in R10, stimulated with a set of peptides designed to cover the 209 FSPs encoded by the vaccine, and arranged into 16 peptide pools (P1 to P16). Cells were plated at 2×10^5 cells per well in ELISpot plates (Human IFN- γ ELISpotPLUS kit, Mabtech) and incubated for 18 to 20 hours in the presence of the peptides in a 37°C ($\pm 1^\circ\text{C}$), humidified CO₂ incubator. At the end of incubation, the ELISpot assay was developed according to the manufacturer's instructions. Spontaneous cytokine production (background) was assessed by incubating PBMCs with the medium only plus the peptide diluent DMSO (negative control) (Sigma-Aldrich), whereas CEFX [a pool of known peptide epitopes for a range of human leukocyte antigen (HLA) subtypes and different infectious agents, namely, *Clostridium tetani*, coxsackievirus B4, *Haemophilus influenzae*, *Helicobacter pylori*, human Ad5, human herpesvirus 1, human herpesvirus 2, human herpesvirus3, human herpesvirus 4, human herpesvirus 5, human herpesvirus 6, human papillomavirus, JC polyomavirus, measles virus, rubella virus, *Toxoplasma gondii*,

and vaccinia virus (JPT Peptide Technologies)] was used as positive control. Results are expressed as SFC/106 PBMCs in stimulating cultures after subtracting the DMSO background. The cutoff values for a positive response were calculated on 20 healthy donors. A response was considered positive if (i) the number of SFC/106 cells was greater than 48 and (ii) higher than three times the background DMSO value. A subject is defined as a responder if reactivity to at least 1 of the 16 FSP peptide pools is induced after vaccination. Induction of an immune response is defined as a change from negative at baseline (after pembrolizumab) to positive at any of the time points collected after vaccination. In case of detection of a positive reactivity against one of the peptide pools at baseline, the vaccine is expected to determine an increase (of at least 80%) of the preexisting reactivity to at least 1 of the 16 FSP peptide pools, and in this case, such patients will be considered as responders.

In vitro expansion of neoantigen-specific T cells

For in vitro expansion of antigen-specific T cells, PBMCs were cultured in RPMI 1640 medium supplemented with l-glutamine, Hepes, penicillin/streptomycin (Gibco), and 10% heat-inactivated human serum (Defined, HyClone). Cells (4×10^6 per well) in 1-ml volume were stimulated in a 24-well plate with individual (4 g/ml) or peptide pool (each peptide at 4 g/ml) in the presence of interleukin-7 (IL-7) (330 U/ml; PeproTech). On day 3, low-dose IL-2 (10 U/ml; PeproTech) was added. Half-medium change and supplementation of IL-2 were performed at days 3, 7, and 10. After 12 days, PBMCs were collected and resuspended in complete medium without cytokines and peptides for overnight resting at 37°C. The day after, T cell response against F24 peptide was tested by IFN- ELISpot assay.

Tumor biopsy RNA-seq

For all the patients, appropriate IRB approval and written informed consent were obtained. Formalin-fixed paraffin-embedded tissue specimens before and after treatment together with a blood sample were collected from patient 1. DNA and RNA were extracted from each collected sample and sequenced at CeGaT GmbH. Raw exome and RNA-seq reads were aligned on the GRCh37 human genome using Burrows-Wheeler Aligner (BWA) mem (PMC2705234) and HISAT2 2.0.4 (PMC7605509) software, respectively. Multimapping reads were filtered out using SAMtools 0.1.19 (PMC7931819). Optical duplicates were marked using Picard's MarkDuplicates tool v1.14 (<https://broadinstitute.github.io/picard/>). DNA alignments were further optimized at regions around indels, and base scores were recalibrated after the optimization step using Genome Analysis Tool Kit (GATK) software

v3.4.46 (PMC2928508). Somatic variant calling of small indels was performed using Mutect2 (PMC2928508), VarScan 2 (PMC3290792), and Scalpel (PMC5507611) with default parameters. All frameshift mutations detected by at least one variant caller were considered. Analysis of clonality of detected mutations was performed by using allele-specific copy number analysis of tumors (ASCAT) software (PubMed identifier PMID: 20837533) to detect copy number variation in tumor exome sequencing data, followed by PyClone (PMC4864026) to estimate the clonality of each somatic mutation. All the parameters were kept to default except for the input of ASCAT that was created with a custom tool that filters only the SNPs with at least the 30% of mutation allele frequency in blood. The list of filtered SNPs was then passed to the AlleleCounter tool to prepare the input for ASCAT.

MHC-I binding prediction

Patient HLA class I haplotypes were determined by using the OptiType software (PMC4441069) on the exome sequencing performed on blood sample. MHC-I binding predictions on the positive peptide were performed using the consensus method included in Immune Epitope Database 2.17 (PMID: 16767078). HLA binders (8- to 10-mer) with a predicted $IC_{50} \leq 500$ nM were considered

TCR-sequencing analysis

TCR sequencing (TCR-seq) of TCR- chain was performed on IVS expanded T cells. Raw NGS data were analyzed with MiXCR 2.1.11 (PMID: 25924071) applying default parameters. The parameters were modified according to the manual to assess the TCR- chain repertoire in RNA-seq performed on tumor biopsies at baseline and after vaccination. The derived CDR3 sequences were further analyzed by tracking the expression of clonotypes that were shared between post-vaccination tumor biopsy and in vitro sample at week 7 stimulated with F24 peptide.

PBMCs peptide in vitro stimulation

Frozen PBMCs were defrosted in complete media composed of RPMI 1964 supplemented with 2,5 % Human serum Aurogene, 1% Penicillin-streptomycin, 1% L-Glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 0,1% 3-mercaptoethanol. Cells were centrifuged at 515g for 10 minutes at 4°C, then, the supernatant was removed, the pellet was resuspended with complete media and counted with Burker chamber. Once counted, PBMCs

(2×10^5 per well in triplicate) were plated in 96- well rounded-bottomed plates, stimulated with 1 ug/ml of the respective peptide pool, and incubated either for 16 and 48 hours at 37°C. Cells cultured only with media were used as the negative control. Whereas, as a positive control, PBMCs were stimulated with Dynabeads human T-activator CD3/CD28 (from Gibco, 1:1 bead: cell ratio) supplemented with human (h)IL-2 (from Novartis, 40 U ml⁻¹). To evaluate the effector and cytotoxic properties, cells were incubated with 5 ug/ml of Brefeldin overnight at 37°C with 5% CO₂.

Human Intra/Extracellular flow cytometry staining

After 16h of peptide and Brefeldin stimulation, PBMCs were labeled for intracellular cytokines transcription factors and surface markers, whereas after 48 hours cells were stained only with surface markers. Cells were washed with PBS, supplemented with 0.5% BSA and 2 mM EDTA (FACS buffer) and CD16/CD32 (Human Fc block) monoclonal antibody was used to block Fc receptors. Subsequently, cells were stained with Fixable Viability Stain Dye 450 in 1PBS for 20 min at 4 °C to discriminate viable from nonviable cells. To study the immune phenotype of the cultured PBMCs, they were marked with the following antibodies for multiparametric flow cytometry analysis: CD3 (clone UCHT1, reference 612896 BD), CD8 (clone SK1, reference 564116 BD), CD8 (clone SK1, reference 560179 BD) , CD4 (clone RPA-T4, reference 564724 BD), CD25 (clone 2A3, reference 740198 BD), CD69 (clone FN50, reference 565154 BD), CD137 (clone 4B4-1, reference 309814 Biolegend), CD137 (clone 4B4-1, reference 745256 BD), CD62L (clone DREG-56, reference 564742 BD), CD27 (clone M-T271, reference 560222 BD), CCR7 (clone I50503, reference 566602 BD), CD45RA (clone HI100, reference 742020 BD), CD45RA (clone HI100, reference 563031 BD), PD1(clone EH12.1, reference 564104 BD), TIM3 (clone 7D3, reference 565558 BD), CX3CR1(clone 2A9-1, reference 749357 BD), CD134 (clone ACT35, reference 563662 BD), CD107 (clone H4A3, reference 555801 BD), CD95 (clone DX2,reference 561633 BD) CXCR5 (clone RF8B2, reference 558113 BD), CD40L (clone TRAP1, reference 748983), CD19 (clone HIB19, reference 562440 BD), CD16 (clone 3G8, reference 562878 BD), CD56(clone NCAM16.2, reference 562751 BD), CD14 (clone MφP9, reference 563743 BD) TCF-1(clone S33-966, reference 567018 BD), Perforin (clone dG9, reference 12999442 Invitrogen), Granzyme β (clone GB11, reference 562462 BD), TNFα (clone Mab11, reference 560679 BD), IFNγ (clone B27,reference 557643 BD), TOX (clone NAN448B, reference 568356 BD), T-bet (clone O4-26, reference 568109 BD) and EOMES

(clone X4-83, reference 567170 BD). Surface staining was done in FACS buffer for 30 min at 4 °C. Then, cells were washed and fixed with 1% of paraformaldehyde in PBS. For the intracellular staining, after surface marker staining, cells were washed and fixed with the FoxP3 Fix/Permeabilization working solution and permeabilized with the permeabilization Buffer 1X, according to the manufacturer's instructions. Intracellular staining was performed for 30 minutes at room temperature in a Permeabilization Buffer. Then cells were washed two times and resuspended in FACS Buffer 1x for data acquisition. The flow cytometry acquisition was done on BD LSR Fortessa X-20 instrument (Becton Dickinson) and the data were analyzed with FlowJo 10.4.2 software.

Statistical analysis

Unpaired t test, or otherwise mentioned, was used for statistical analysis. SEM and P value were determined using Prism software (GraphPad Software Inc.). Symbols used: *P < 0.05, **P < 0.01, ***P < 0.001, and n.s. (not significant)

REFERENCE

1. M. Moser, O. Leo, Key concepts in immunology. *Vaccine* **28 Suppl 3**, C2-13 (2010).
2. M. F. Flajnik, M. Kasahara, Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat Rev Genet* **11**, 47-59 (2010).
3. G. Gasteiger *et al.*, Cellular Innate Immunity: An Old Game with New Players. *J Innate Immun* **9**, 111-125 (2017).
4. F. A. Bonilla, H. C. Oettgen, Adaptive immunity. *J Allergy Clin Immunol* **125**, S33-40 (2010).
5. K. Pieper, B. Grimbacher, H. Eibel, B-cell biology and development. *J Allergy Clin Immunol* **131**, 959-971 (2013).
6. J. Korzhenevich, I. Janowska, M. van der Burg, M. Rizzi, Human and mouse early B cell development: So similar but so different. *Immunol Lett* **261**, 1-12 (2023).
7. R. Carsetti, The development of B cells in the bone marrow is controlled by the balance between cell-autonomous mechanisms and signals from the microenvironment. *J Exp Med* **191**, 5-8 (2000).
8. W. Ise, T. Kurosaki, Plasma cell differentiation during the germinal center reaction. *Immunol Rev* **288**, 64-74 (2019).
9. L. Klein, M. Hinterberger, G. Wirnsberger, B. Kyewski, Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* **9**, 833-844 (2009).
10. J. Pongracz, K. Hare, B. Harman, G. Anderson, E. J. Jenkinson, Thymic epithelial cells provide WNT signals to developing thymocytes. *Eur J Immunol* **33**, 1949-1956 (2003).
11. B. Zhou *et al.*, Notch signaling pathway: architecture, disease, and therapeutics. *Signal Transduct Target Ther* **7**, 95 (2022).
12. Q. Shi *et al.*, Notch signaling pathway in cancer: from mechanistic insights to targeted therapies. *Signal Transduct Target Ther* **9**, 128 (2024).
13. M. M. Davis, P. J. Bjorkman, T-cell antigen receptor genes and T-cell recognition. *Nature* **334**, 395-402 (1988).
14. R. N. Germain, T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* **2**, 309-322 (2002).
15. M. G. Rudolph, R. L. Stanfield, I. A. Wilson, How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* **24**, 419-466 (2006).

16. E. Robey, B. J. Fowlkes, Selective events in T cell development. *Annu Rev Immunol* **12**, 675-705 (1994).
17. J. E. Smith-Garvin, G. A. Koretzky, M. S. Jordan, T cell activation. *Annu Rev Immunol* **27**, 591-619 (2009).
18. D. Masopust, J. M. Schenkel, The integration of T cell migration, differentiation and function. *Nat Rev Immunol* **13**, 309-320 (2013).
19. G. Iezzi, K. Karjalainen, A. Lanzavecchia, The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8**, 89-95 (1998).
20. N. D. Pennock *et al.*, T cell responses: naive to memory and everything in between. *Adv Physiol Educ* **37**, 273-283 (2013).
21. P. S. Linsley *et al.*, Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* **173**, 721-730 (1991).
22. D. Campillo-Davo, D. Flumens, E. Lion, The Quest for the Best: How TCR Affinity, Avidity, and Functional Avidity Affect TCR-Engineered T-Cell Antitumor Responses. *Cells* **9**, (2020).
23. M. P. Tan *et al.*, T cell receptor binding affinity governs the functional profile of cancer-specific CD8⁺ T cells. *Clin Exp Immunol* **180**, 255-270 (2015).
24. D. Campillo-Davo *et al.*, Rapid Assessment of Functional Avidity of Tumor-Specific T Cell Receptors Using an Antigen-Presenting Tumor Cell Line Electroporated with Full-Length Tumor Antigen mRNA. *Cancers (Basel)* **12**, (2020).
25. R. Ahmed, D. Gray, Immunological memory and protective immunity: understanding their relation. *Science* **272**, 54-60 (1996).
26. P. Golstein, G. M. Griffiths, An early history of T cell-mediated cytotoxicity. *Nat Rev Immunol* **18**, 527-535 (2018).
27. I. Voskoboinik, J. C. Whisstock, J. A. Trapani, Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* **15**, 388-400 (2015).
28. J. H. Russell, T. J. Ley, Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* **20**, 323-370 (2002).
29. A. M. Intlekofer *et al.*, Effector and memory CD8⁺ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* **6**, 1236-1244 (2005).
30. F. Raczkowski *et al.*, The transcription factor Interferon Regulatory Factor 4 is required for the generation of protective effector CD8⁺ T cells. *Proc Natl Acad Sci U S A* **110**, 15019-15024 (2013).

31. H. W. Mittrucker, A. Visekruna, M. Huber, Heterogeneity in the differentiation and function of CD8(+) T cells. *Arch Immunol Ther Exp (Warsz)* **62**, 449-458 (2014).
32. S. C. De Rosa, L. A. Herzenberg, L. A. Herzenberg, M. Roederer, 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med* **7**, 245-248 (2001).
33. T. van den Broek, J. A. M. Borghans, F. van Wijk, The full spectrum of human naive T cells. *Nat Rev Immunol* **18**, 363-373 (2018).
34. J. C. Nolz, G. R. Starbeck-Miller, J. T. Harty, Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions. *Immunotherapy* **3**, 1223-1233 (2011).
35. S. M. Kaech, W. Cui, Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* **12**, 749-761 (2012).
36. M. M. Opata, R. Stephens, Early Decision: Effector and Effector Memory T Cell Differentiation in Chronic Infection. *Curr Immunol Rev* **9**, 190-206 (2013).
37. F. Sallusto, D. Lenig, R. Forster, M. Lipp, A. Lanzavecchia, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712 (1999).
38. Y. Muroyama, E. J. Wherry, Memory T-Cell Heterogeneity and Terminology. *Cold Spring Harb Perspect Biol* **13**, (2021).
39. D. Hamann *et al.*, Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med* **186**, 1407-1418 (1997).
40. Y. D. Mahnke, T. M. Brodie, F. Sallusto, M. Roederer, E. Lugli, The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol* **43**, 2797-2809 (2013).
41. F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* **22**, 745-763 (2004).
42. N. S. Joshi *et al.*, Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* **27**, 281-295 (2007).
43. R. L. Rutishauser *et al.*, Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* **31**, 296-308 (2009).
44. L. Gattinoni, D. E. Speiser, M. Lichterfeld, C. Bonini, T memory stem cells in health and disease. *Nat Med* **23**, 18-27 (2017).

45. A. Lanzavecchia, F. Sallusto, Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol* **17**, 326-332 (2005).
46. Y. Zhang, G. Joe, E. Hexner, J. Zhu, S. G. Emerson, Host-reactive CD8⁺ memory stem cells in graft-versus-host disease. *Nat Med* **11**, 1299-1305 (2005).
47. L. Gattinoni *et al.*, Wnt signaling arrests effector T cell differentiation and generates CD8⁺ memory stem cells. *Nat Med* **15**, 808-813 (2009).
48. N. Lam, Y. Lee, D. L. Farber, A guide to adaptive immune memory. *Nat Rev Immunol*, (2024).
49. L. Gattinoni *et al.*, A human memory T cell subset with stem cell-like properties. *Nat Med* **17**, 1290-1297 (2011).
50. S. E. Henrickson *et al.*, T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol* **9**, 282-291 (2008).
51. L. Pace *et al.*, Regulatory T cells increase the avidity of primary CD8⁺ T cell responses and promote memory. *Science* **338**, 532-536 (2012).
52. A. C. Richard *et al.*, T cell cytolytic capacity is independent of initial stimulation strength. *Nat Immunol* **19**, 849-858 (2018).
53. A. Boissonnas *et al.*, Foxp3⁺ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. *Immunity* **32**, 266-278 (2010).
54. A. Scholer, S. Hugues, A. Boissonnas, L. Fetler, S. Amigorena, Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8⁺ T cell memory. *Immunity* **28**, 258-270 (2008).
55. D. Peng *et al.*, Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* **527**, 249-253 (2015).
56. X. Zhao, Q. Shan, H. H. Xue, TCF1 in T cell immunity: a broadened frontier. *Nat Rev Immunol* **22**, 147-157 (2022).
57. L. Pace, Temporal and Epigenetic Control of Plasticity and Fate Decision during CD8(+) T-Cell Memory Differentiation. *Cold Spring Harb Perspect Biol* **13**, (2021).
58. B. Youngblood *et al.*, Effector CD8 T cells dedifferentiate into long-lived memory cells. *Nature* **552**, 404-409 (2017).
59. S. M. Gray, R. A. Amezcua, T. Guan, S. H. Kleinstein, S. M. Kaech, Polycomb Repressive Complex 2-Mediated Chromatin Repression Guides Effector CD8(+) T Cell Terminal Differentiation and Loss of Multipotency. *Immunity* **46**, 596-608 (2017).

60. Y. H. Chen *et al.*, Asymmetric PI3K Activity in Lymphocytes Organized by a PI3K-Mediated Polarity Pathway. *Cell Rep* **22**, 860-868 (2018).
61. N. P. Restifo, L. Gattinoni, Lineage relationship of effector and memory T cells. *Curr Opin Immunol* **25**, 556-563 (2013).
62. J. G. Crompton *et al.*, Lineage relationship of CD8(+) T cell subsets is revealed by progressive changes in the epigenetic landscape. *Cell Mol Immunol* **13**, 502-513 (2016).
63. D. Herndler-Brandstetter *et al.*, KLRG1(+) Effector CD8(+) T Cells Lose KLRG1, Differentiate into All Memory T Cell Lineages, and Convey Enhanced Protective Immunity. *Immunity* **48**, 716-729 e718 (2018).
64. L. Pace *et al.*, The epigenetic control of stemness in CD8(+) T cell fate commitment. *Science* **359**, 177-186 (2018).
65. K. D. Omilusik *et al.*, Sustained Id2 regulation of E proteins is required for terminal differentiation of effector CD8(+) T cells. *J Exp Med* **215**, 773-783 (2018).
66. W. H. Hudson *et al.*, Proliferating Transitory T Cells with an Effector-like Transcriptional Signature Emerge from PD-1(+) Stem-like CD8(+) T Cells during Chronic Infection. *Immunity* **51**, 1043-1058 e1044 (2019).
67. J. C. Beltra *et al.*, Developmental Relationships of Four Exhausted CD8(+) T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. *Immunity* **52**, 825-841 e828 (2020).
68. J. Zhang, T. Lyu, Y. Cao, H. Feng, Role of TCF-1 in differentiation, exhaustion, and memory of CD8(+) T cells: A review. *FASEB J* **35**, e21549 (2021).
69. F. Gounari, K. Khazaie, TCF-1: a maverick in T cell development and function. *Nat Immunol* **23**, 671-678 (2022).
70. S. Verbeek *et al.*, An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* **374**, 70-74 (1995).
71. D. Zehn, R. Thimme, E. Lugli, G. P. de Almeida, A. Oxenius, 'Stem-like' precursors are the fount to sustain persistent CD8(+) T cell responses. *Nat Immunol* **23**, 836-847 (2022).
72. D. T. Utzschneider *et al.*, High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *J Exp Med* **213**, 1819-1834 (2016).

73. I. Siddiqui *et al.*, Intratumoral Tcf1(+)PD-1(+)CD8(+) T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* **50**, 195-211 e110 (2019).
74. T. Sekine *et al.*, TOX is expressed by exhausted and polyfunctional human effector memory CD8(+) T cells. *Sci Immunol* **5**, (2020).
75. O. Khan *et al.*, TOX transcriptionally and epigenetically programs CD8(+) T cell exhaustion. *Nature* **571**, 211-218 (2019).
76. K. Heim *et al.*, TOX defines the degree of CD8+ T cell dysfunction in distinct phases of chronic HBV infection. *Gut* **70**, 1550-1560 (2020).
77. C. Gerlach *et al.*, The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis. *Immunity* **45**, 1270-1284 (2016).
78. J. P. Bottcher *et al.*, Functional classification of memory CD8(+) T cells by CX3CR1 expression. *Nat Commun* **6**, 8306 (2015).
79. M. Lee, Y. Lee, J. Song, J. Lee, S. Y. Chang, Tissue-specific Role of CX(3)CR1 Expressing Immune Cells and Their Relationships with Human Disease. *Immune Netw* **18**, e5 (2018).
80. A. Gallimore *et al.*, Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* **187**, 1383-1393 (1998).
81. E. J. Wherry, J. N. Blattman, K. Murali-Krishna, R. van der Most, R. Ahmed, Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* **77**, 4911-4927 (2003).
82. C. M. Bucks, J. A. Norton, A. C. Boesteanu, Y. M. Mueller, P. D. Katsikis, Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* **182**, 6697-6708 (2009).
83. D. Zehn, E. J. Wherry, Immune Memory and Exhaustion: Clinically Relevant Lessons from the LCMV Model. *Adv Exp Med Biol* **850**, 137-152 (2015).
84. D. G. Brooks, D. B. McGavern, M. B. Oldstone, Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. *J Clin Invest* **116**, 1675-1685 (2006).

85. J. M. Angelosanto, S. D. Blackburn, A. Crawford, E. J. Wherry, Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol* **86**, 8161-8170 (2012).
86. M. J. Fuller, A. J. Zajac, Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* **170**, 477-486 (2003).
87. E. J. Wherry, T cell exhaustion. *Nat Immunol* **12**, 492-499 (2011).
88. A. Schietinger, P. D. Greenberg, Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends Immunol* **35**, 51-60 (2014).
89. J. Duraiswamy *et al.*, Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults. *J Immunol* **186**, 4200-4212 (2011).
90. M. Hashimoto *et al.*, CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu Rev Med* **69**, 301-318 (2018).
91. S. D. Blackburn *et al.*, Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* **10**, 29-37 (2009).
92. K. Zheng, X. Zheng, W. Yang, The Role of Metabolic Dysfunction in T-Cell Exhaustion During Chronic Viral Infection. *Front Immunol* **13**, 843242 (2022).
93. J. S. Yi, M. A. Cox, A. J. Zajac, T-cell exhaustion: characteristics, causes and conversion. *Immunology* **129**, 474-481 (2010).
94. E. J. Wherry *et al.*, Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* **27**, 670-684 (2007).
95. B. C. Miller *et al.*, Author Correction: Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol* **20**, 1556 (2019).
96. Z. Zeng, F. Wei, X. Ren, Exhausted T cells and epigenetic status. *Cancer Biol Med* **17**, 923-936 (2020).
97. M. A. Paley *et al.*, Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* **338**, 1220-1225 (2012).
98. Z. Wu *et al.*, CD83 expression characterizes precursor exhausted T cell population. *Commun Biol* **6**, 258 (2023).
99. T. Gebhardt, S. L. Park, I. A. Parish, Stem-like exhausted and memory CD8(+) T cells in cancer. *Nat Rev Cancer* **23**, 780-798 (2023).

100. M. M. Berrien-Elliott *et al.*, Checkpoint blockade immunotherapy relies on T-bet but not Eomes to induce effector function in tumor-infiltrating CD8⁺ T cells. *Cancer Immunol Res* **3**, 116-124 (2015).
101. C. Chong, G. Coukos, M. Bassani-Sternberg, Identification of tumor antigens with immunopeptidomics. *Nat Biotechnol* **40**, 175-188 (2022).
102. Z. Herceg, P. Hainaut, Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. *Mol Oncol* **1**, 26-41 (2007).
103. A. Peri *et al.*, The landscape of T cell antigens for cancer immunotherapy. *Nat Cancer* **4**, 937-954 (2023).
104. R. K. Bright, J. D. Bright, J. A. Byrne, Overexpressed oncogenic tumor-self antigens. *Hum Vaccin Immunother* **10**, 3297-3305 (2014).
105. L. Novellino, C. Castelli, G. Parmiani, A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother* **54**, 187-207 (2005).
106. M. A. Molina *et al.*, Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* **61**, 4744-4749 (2001).
107. V. Deubel *et al.*, Comparison of the enzyme-linked immunosorbent assay (ELISA) with standard tests used to detect yellow fever virus antibodies. *Am J Trop Med Hyg* **32**, 565-568 (1983).
108. L. Wayteck, K. Breckpot, J. Demeester, S. C. De Smedt, K. Raemdonck, A personalized view on cancer immunotherapy. *Cancer Lett* **352**, 113-125 (2014).
109. Y. Kawakami *et al.*, Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med* **180**, 347-352 (1994).
110. M. Hamieh *et al.*, Generation of Pure Highly Functional Human Anti-Tumor Specific Cytotoxic T Lymphocytes With Stem Cell-Like Memory Features for Melanoma Immunotherapy. *Front Immunol* **12**, 674276 (2021).
111. S. Yang *et al.*, Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. *Gene Ther* **15**, 1411-1423 (2008).
112. P. G. Coulie, B. J. Van den Eynde, P. van der Bruggen, T. Boon, Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer* **14**, 135-146 (2014).

113. Y. T. Chen *et al.*, A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* **94**, 1914-1918 (1997).
114. A. Gyurdieva *et al.*, Biomarker correlates with response to NY-ESO-1 TCR T cells in patients with synovial sarcoma. *Nat Commun* **13**, 5296 (2022).
115. D. Sommermeyer *et al.*, NY-ESO-1 antigen-reactive T cell receptors exhibit diverse therapeutic capability. *Int J Cancer* **132**, 1360-1367 (2013).
116. E. K. Moon *et al.*, Blockade of Programmed Death 1 Augments the Ability of Human T Cells Engineered to Target NY-ESO-1 to Control Tumor Growth after Adoptive Transfer. *Clin Cancer Res* **22**, 436-447 (2016).
117. M. Merhi *et al.*, Persistent anti-NY-ESO-1-specific T cells and expression of differential biomarkers in a patient with metastatic gastric cancer benefiting from combined radioimmunotherapy treatment: a case report. *J Immunother Cancer* **8**, (2020).
118. N. P. Restifo *et al.*, Enhancing the recognition of tumour associated antigens. *Folia Biol (Praha)* **40**, 74-88 (1994).
119. A. D. Waldman, J. M. Fritz, M. J. Lenardo, A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* **20**, 651-668 (2020).
120. C. C. Smith *et al.*, Alternative tumour-specific antigens. *Nat Rev Cancer* **19**, 465-478 (2019).
121. B. Heemskerk, P. Kvistborg, T. N. Schumacher, The cancer antigenome. *EMBO J* **32**, 194-203 (2013).
122. T. N. Schumacher, R. D. Schreiber, Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015).
123. Z. Zhang *et al.*, Neoantigen: A New Breakthrough in Tumor Immunotherapy. *Front Immunol* **12**, 672356 (2021).
124. N. Xie *et al.*, Neoantigens: promising targets for cancer therapy. *Signal Transduct Target Ther* **8**, 9 (2023).
125. A. M. D'Alise *et al.*, Adenoviral vaccine targeting multiple neoantigens as strategy to eradicate large tumors combined with checkpoint blockade. *Nat Commun* **10**, 2688 (2019).
126. Y. Zhu, Y. Qian, Z. Li, Y. Li, B. Li, Neoantigen-reactive T cell: An emerging role in adoptive cellular immunotherapy. *MedComm (2020)* **2**, 207-220 (2021).
127. R. Huang *et al.*, Adoptive neoantigen-reactive T cell therapy: improvement strategies and current clinical researches. *Biomark Res* **11**, 41 (2023).

128. L. Liu *et al.*, Concurrent delivery of immune checkpoint blockade modulates T cell dynamics to enhance neoantigen vaccine-generated antitumor immunity. *Nat Cancer* **3**, 437-452 (2022).
129. N. Hacohen, E. F. Fritsch, T. A. Carter, E. S. Lander, C. J. Wu, Getting personal with neoantigen-based therapeutic cancer vaccines. *Cancer Immunol Res* **1**, 11-15 (2013).
130. E. Tran, P. F. Robbins, S. A. Rosenberg, 'Final common pathway' of human cancer immunotherapy: targeting random somatic mutations. *Nat Immunol* **18**, 255-262 (2017).
131. S. Hutchison, A. L. Pritchard, Identifying neoantigens for use in immunotherapy. *Mamm Genome* **29**, 714-730 (2018).
132. T. N. Schumacher, W. Scheper, P. Kvistborg, Cancer Neoantigens. *Annu Rev Immunol* **37**, 173-200 (2019).
133. C. B. Blackadar, Historical review of the causes of cancer. *World J Clin Oncol* **7**, 54-86 (2016).
134. V. L. Cannataro, J. D. Mandell, J. P. Townsend, Attribution of Cancer Origins to Endogenous, Exogenous, and Preventable Mutational Processes. *Mol Biol Evol* **39**, (2022).
135. D. Hanahan, R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
136. J. Condeelis, J. W. Pollard, Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266 (2006).
137. K. Schumacher, W. Haensch, C. Roefzaad, P. M. Schlag, Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. *Cancer Res* **61**, 3932-3936 (2001).
138. C. G. Clemente *et al.*, Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77**, 1303-1310 (1996).
139. J. S. O'Donnell, M. W. L. Teng, M. J. Smyth, Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol* **16**, 151-167 (2019).
140. R. D. Schreiber, L. J. Old, M. J. Smyth, Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **331**, 1565-1570 (2011).
141. K. Takeda *et al.*, IFN-gamma is required for cytotoxic T cell-dependent cancer genome immunoediting. *Nat Commun* **8**, 14607 (2017).

142. M. Mandai *et al.*, Dual Faces of IFN γ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. *Clin Cancer Res* **22**, 2329-2334 (2016).
143. C. C. Chang *et al.*, Multiple structural and epigenetic defects in the human leukocyte antigen class I antigen presentation pathway in a recurrent metastatic melanoma following immunotherapy. *J Biol Chem* **290**, 26562-26575 (2015).
144. K. H. Song *et al.*, HDAC1 Upregulation by NANOG Promotes Multidrug Resistance and a Stem-like Phenotype in Immune Edited Tumor Cells. *Cancer Res* **77**, 5039-5053 (2017).
145. J. M. Taube *et al.*, Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* **4**, 127ra137 (2012).
146. D. S. Chen, I. Mellman, Elements of cancer immunity and the cancer-immune set point. *Nature* **541**, 321-330 (2017).
147. Z. Mei *et al.*, Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *Br J Cancer* **110**, 1595-1605 (2014).
148. H. Elomaa *et al.*, Prognostic significance of spatial and density analysis of T lymphocytes in colorectal cancer. *Br J Cancer* **127**, 514-523 (2022).
149. L. Rocamora-Reverte, F. L. Melzer, R. Wurzner, B. Weinberger, The Complex Role of Regulatory T Cells in Immunity and Aging. *Front Immunol* **11**, 616949 (2020).
150. N. Riaz *et al.*, Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell* **171**, 934-949 e916 (2017).
151. W. Fan, B. Yung, P. Huang, X. Chen, Nanotechnology for Multimodal Synergistic Cancer Therapy. *Chem Rev* **117**, 13566-13638 (2017).
152. I. Mellman, G. Coukos, G. Dranoff, Cancer immunotherapy comes of age. *Nature* **480**, 480-489 (2011).
153. R. Bai, W. Li, N. Du, J. Cui, Challenges of evaluating immunotherapy efficacy in solid tumors. *Chin J Cancer Res* **31**, 853-861 (2019).
154. A. Kallingal, M. Olszewski, N. Maciejewska, W. Brankiewicz, M. Baginski, Cancer immune escape: the role of antigen presentation machinery. *J Cancer Res Clin Oncol* **149**, 8131-8141 (2023).
155. D. H. Munn, V. Bronte, Immune suppressive mechanisms in the tumor microenvironment. *Curr Opin Immunol* **39**, 1-6 (2016).

156. E. A. Vasievich, L. Huang, The suppressive tumor microenvironment: a challenge in cancer immunotherapy. *Mol Pharm* **8**, 635-641 (2011).
157. L. Galluzzi *et al.*, Classification of current anticancer immunotherapies. *Oncotarget* **5**, 12472-12508 (2014).
158. N. E. Papaioannou, O. V. Beniata, P. Vitsos, O. Tsitsilonis, P. Samara, Harnessing the immune system to improve cancer therapy. *Ann Transl Med* **4**, 261 (2016).
159. E. J. Wherry, M. Kurachi, Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* **15**, 486-499 (2015).
160. G. J. Freeman *et al.*, Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* **192**, 1027-1034 (2000).
161. S. A. Rosenberg, J. C. Yang, N. P. Restifo, Cancer immunotherapy: moving beyond current vaccines. *Nat Med* **10**, 909-915 (2004).
162. P. S. Linsley *et al.*, Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med* **176**, 1595-1604 (1992).
163. F. Hirano *et al.*, Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* **65**, 1089-1096 (2005).
164. S. E. Strome *et al.*, B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res* **63**, 6501-6505 (2003).
165. Y. F. He *et al.*, Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine. *J Immunol* **173**, 4919-4928 (2004).
166. Y. Iwai *et al.*, Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* **99**, 12293-12297 (2002).
167. L. Sun *et al.*, Clinical efficacy and safety of anti-PD-1/PD-L1 inhibitors for the treatment of advanced or metastatic cancer: a systematic review and meta-analysis. *Sci Rep* **10**, 2083 (2020).
168. Q. Sun *et al.*, Immune checkpoint therapy for solid tumours: clinical dilemmas and future trends. *Signal Transduct Target Ther* **8**, 320 (2023).
169. A. Marchetti, A. Di Lorito, F. Buttitta, Why anti-PD1/PDL1 therapy is so effective? Another piece in the puzzle. *J Thorac Dis* **9**, 4863-4866 (2017).
170. J. Sunshine, J. M. Taube, PD-1/PD-L1 inhibitors. *Curr Opin Pharmacol* **23**, 32-38 (2015).

171. A. Parvez *et al.*, PD-1 and PD-L1: architects of immune symphony and immunotherapy breakthroughs in cancer treatment. *Front Immunol* **14**, 1296341 (2023).
172. T. O. Dang, A. Ogunniyi, M. S. Barbee, A. Drilon, Pembrolizumab for the treatment of PD-L1 positive advanced or metastatic non-small cell lung cancer. *Expert Rev Anticancer Ther* **16**, 13-20 (2016).
173. A. Rendon, A. Rayi, in *StatPearls*. (Treasure Island (FL) ineligible companies. Disclosure: Appaji Rayi declares no relevant financial relationships with ineligible companies., 2024).
174. J. Gong, A. Chehrazi-Raffle, S. Reddi, R. Salgia, Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *J Immunother Cancer* **6**, 8 (2018).
175. J. Weber *et al.*, Adjuvant Nivolumab versus Ipilimumab in Resected Stage III or IV Melanoma. *N Engl J Med* **377**, 1824-1835 (2017).
176. C. Robert *et al.*, Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med* **372**, 2521-2532 (2015).
177. S. Musetti, L. Huang, Nanoparticle-Mediated Remodeling of the Tumor Microenvironment to Enhance Immunotherapy. *ACS Nano* **12**, 11740-11755 (2018).
178. Y. Xu, J. Xiong, X. Sun, H. Gao, Targeted nanomedicines remodeling immunosuppressive tumor microenvironment for enhanced cancer immunotherapy. *Acta Pharm Sin B* **12**, 4327-4347 (2022).
179. S. A. Rosenberg, N. P. Restifo, Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62-68 (2015).
180. M. E. Dudley *et al.*, Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* **298**, 850-854 (2002).
181. H. W. Kessels, M. C. Wolkers, M. D. van den Boom, M. A. van der Valk, T. N. Schumacher, Immunotherapy through TCR gene transfer. *Nat Immunol* **2**, 957-961 (2001).
182. J. Maher, R. J. Brentjens, G. Gunset, I. Riviere, M. Sadelain, Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. *Nat Biotechnol* **20**, 70-75 (2002).
183. D. G. Song *et al.*, In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). *Cancer Res* **71**, 4617-4627 (2011).

184. R. C. Sterner, R. M. Sterner, CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* **11**, 69 (2021).
185. R. Rui, L. Zhou, S. He, Cancer immunotherapies: advances and bottlenecks. *Front Immunol* **14**, 1212476 (2023).
186. V. Janelle, J. S. Delisle, T-Cell Dysfunction as a Limitation of Adoptive Immunotherapy: Current Concepts and Mitigation Strategies. *Cancers (Basel)* **13**, (2021).
187. P. Zhang, G. Zhang, X. Wan, Challenges and new technologies in adoptive cell therapy. *J Hematol Oncol* **16**, 97 (2023).
188. M. Morotti *et al.*, Promises and challenges of adoptive T-cell therapies for solid tumours. *Br J Cancer* **124**, 1759-1776 (2021).
189. Z. Hu, P. A. Ott, C. J. Wu, Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nat Rev Immunol* **18**, 168-182 (2018).
190. C. Printz, FDA approves Gardasil 9 for more types of HPV. *Cancer* **121**, 1156-1157 (2015).
191. D. M. Harper, S. L. Vierthaler, J. A. Santee, Review of Gardasil. *J Vaccines Vaccin* **1**, (2010).
192. C. Printz, New immunotherapies show promise in some patients: physicians try to balance optimism with realistic expectations. *Cancer* **121**, 1155-1156 (2015).
193. R. H. Vonderheide, K. L. Nathanson, Immunotherapy at large: the road to personalized cancer vaccines. *Nat Med* **19**, 1098-1100 (2013).
194. A. Patel, H. L. Kaufman, M. L. Disis, Next generation approaches for tumor vaccination. *Chin Clin Oncol* **6**, 19 (2017).
195. L. A. Emens, Cancer vaccines: on the threshold of success. *Expert Opin Emerg Drugs* **13**, 295-308 (2008).
196. X. S. Liu, E. R. Mardis, Applications of Immunogenomics to Cancer. *Cell* **168**, 600-612 (2017).
197. M. Harndahl *et al.*, Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. *Eur J Immunol* **42**, 1405-1416 (2012).
198. M. Yadav *et al.*, Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature* **515**, 572-576 (2014).
199. E. Sasso *et al.*, New viral vectors for infectious diseases and cancer. *Semin Immunol* **50**, 101430 (2020).

200. B. Yang, J. Jeang, A. Yang, T. C. Wu, C. F. Hung, DNA vaccine for cancer immunotherapy. *Hum Vaccin Immunother* **10**, 3153-3164 (2014).
201. L. A. Rojas *et al.*, Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer. *Nature* **618**, 144-150 (2023).
202. F. Heil *et al.*, Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**, 1526-1529 (2004).
203. R. J. Malonis, J. R. Lai, O. Vergnolle, Peptide-Based Vaccines: Current Progress and Future Challenges. *Chem Rev* **120**, 3210-3229 (2020).
204. S. Walter *et al.*, Multi-peptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med* **18**, 1254-1261 (2012).
205. C. S. Shemesh *et al.*, Personalized Cancer Vaccines: Clinical Landscape, Challenges, and Opportunities. *Mol Ther* **29**, 555-570 (2021).
206. M. Sebastian *et al.*, A phase I/IIa study of the mRNA-based cancer immunotherapy CV9201 in patients with stage IIIB/IV non-small cell lung cancer. *Cancer Immunol Immunother* **68**, 799-812 (2019).
207. U. Sahin *et al.*, Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **547**, 222-226 (2017).
208. N. Tatsis *et al.*, Adenoviral vectors persist in vivo and maintain activated CD8⁺ T cells: implications for their use as vaccines. *Blood* **110**, 1916-1923 (2007).
209. H. C. Ertl, Viral vectors as vaccine carriers. *Curr Opin Virol* **21**, 1-8 (2016).
210. D. E. Zak *et al.*, Merck Ad5/HIV induces broad innate immune activation that predicts CD8⁽⁺⁾ T-cell responses but is attenuated by preexisting Ad5 immunity. *Proc Natl Acad Sci U S A* **109**, E3503-3512 (2012).
211. Z. Xiang *et al.*, Chimpanzee adenovirus antibodies in humans, sub-Saharan Africa. *Emerg Infect Dis* **12**, 1596-1599 (2006).
212. J. Alonso-Padilla *et al.*, Development of Novel Adenoviral Vectors to Overcome Challenges Observed With HAdV-5-based Constructs. *Mol Ther* **24**, 6-16 (2016).
213. K. M. Quinn *et al.*, Comparative analysis of the magnitude, quality, phenotype, and protective capacity of simian immunodeficiency virus gag-specific CD8⁺ T cells following human-, simian-, and chimpanzee-derived recombinant adenoviral vector immunization. *J Immunol* **190**, 2720-2735 (2013).
214. A. M. D'Alise *et al.*, Adenoviral-based vaccine promotes neoantigen-specific CD8⁽⁺⁾ T cell stemness and tumor rejection. *Sci Transl Med* **14**, eabo7604 (2022).

215. G. Sutter, B. Moss, Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc Natl Acad Sci U S A* **89**, 10847-10851 (1992).
216. G. Leoni *et al.*, A Genetic Vaccine Encoding Shared Cancer Neoantigens to Treat Tumors with Microsatellite Instability. *Cancer Res* **80**, 3972-3982 (2020).
217. W. Jia *et al.*, Colorectal cancer vaccines: The current scenario and future prospects. *Front Immunol* **13**, 942235 (2022).
218. A. M. D'Alise, E. Scarselli, Getting personal in metastatic melanoma: neoantigen-based vaccines as a new therapeutic strategy. *Curr Opin Oncol* **35**, 94-99 (2023).
219. A. Townsend *et al.*, Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**, 443-448 (1989).
220. M. Philip *et al.*, Chromatin states define tumour-specific T cell dysfunction and reprogramming. *Nature* **545**, 452-456 (2017).
221. S. Picelli *et al.*, Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* **9**, 171-181 (2014).
222. A. Subramanian *et al.*, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550 (2005).
223. B. C. Miller *et al.*, Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol* **20**, 326-336 (2019).
224. C. J. Luckey *et al.*, Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. *Proc Natl Acad Sci U S A* **103**, 3304-3309 (2006).
225. M. Sade-Feldman *et al.*, Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* **175**, 998-1013 e1020 (2018).
226. J. J. Milner *et al.*, Runx3 programs CD8(+) T cell residency in non-lymphoid tissues and tumours. *Nature* **552**, 253-257 (2017).
227. D. E. Speiser, P. C. Ho, G. Verdeil, Regulatory circuits of T cell function in cancer. *Nat Rev Immunol* **16**, 599-611 (2016).
228. L. H. Schwartz *et al.*, RECIST 1.1-Update and clarification: From the RECIST committee. *Eur J Cancer* **62**, 132-137 (2016).
229. E. A. Eisenhauer *et al.*, New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* **45**, 228-247 (2009).
230. A. I. Daud *et al.*, Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. *J Clin Invest* **126**, 3447-3452 (2016).

231. N. Brasu *et al.*, Memory CD8(+) T cell diversity and B cell responses correlate with protection against SARS-CoV-2 following mRNA vaccination. *Nat Immunol* **23**, 1445-1456 (2022).
232. H. Raskov, A. Orhan, J. P. Christensen, I. Gogenur, Cytotoxic CD8(+) T cells in cancer and cancer immunotherapy. *Br J Cancer* **124**, 359-367 (2021).
233. Y. Wang *et al.*, Stem cell-like memory T cells: The generation and application. *J Leukoc Biol* **110**, 1209-1223 (2021).
234. J. T. Chang, E. J. Wherry, A. W. Goldrath, Molecular regulation of effector and memory T cell differentiation. *Nat Immunol* **15**, 1104-1115 (2014).
235. L. Yi, L. Yang, Stem-like T cells and niches: Implications in human health and disease. *Front Immunol* **13**, 907172 (2022).
236. O. V. Orlova, D. V. Glazkova, E. V. Bogoslovskaya, G. A. Shipulin, S. M. Yudin, Development of Modified Vaccinia Virus Ankara-Based Vaccines: Advantages and Applications. *Vaccines (Basel)* **10**, (2022).
237. V. Barnaba, T Cell Memory in Infection, Cancer, and Autoimmunity. *Front Immunol* **12**, 811968 (2021).
238. J. Han, N. Khatwani, T. G. Searles, M. J. Turk, C. V. Angeles, Memory CD8(+) T cell responses to cancer. *Semin Immunol* **49**, 101435 (2020).
239. M. Roederer, J. L. Nozzi, M. C. Nason, SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* **79**, 167-174 (2011).
240. G. Shubinsky, M. Schlesinger, The CD38 lymphocyte differentiation marker: new insight into its ectoenzymatic activity and its role as a signal transducer. *Immunity* **7**, 315-324 (1997).
241. S. He *et al.*, Ezh2 phosphorylation state determines its capacity to maintain CD8(+) T memory precursors for antitumor immunity. *Nat Commun* **8**, 2125 (2017).
242. E. Bonnin *et al.*, CD74 supports accumulation and function of regulatory T cells in tumors. *Nat Commun* **15**, 3749 (2024).
243. R. Osuna-Gomez *et al.*, Effector Mechanisms of CD8+ HLA-DR+ T Cells in Breast Cancer Patients Who Respond to Neoadjuvant Chemotherapy. *Cancers (Basel)* **13**, (2021).
244. J. D. Worboys *et al.*, TIGIT can inhibit T cell activation via ligation-induced nanoclusters, independent of CD226 co-stimulation. *Nat Commun* **14**, 5016 (2023).

245. A. M. D'Alise *et al.*, Phase I Trial of Viral Vector-Based Personalized Vaccination Elicits Robust Neoantigen-Specific Antitumor T-Cell Responses. *Clin Cancer Res* **30**, 2412-2423 (2024).
246. G. Montacchiesi, L. Pace, Epigenetics and CD8(+) T cell memory. *Immunol Rev* **305**, 77-89 (2022).
247. S. Krishna *et al.*, Stem-like CD8 T cells mediate response of adoptive cell immunotherapy against human cancer. *Science* **370**, 1328-1334 (2020).
248. N. P. Restifo, M. J. Smyth, A. Snyder, Acquired resistance to immunotherapy and future challenges. *Nat Rev Cancer* **16**, 121-126 (2016).
249. M. Efremova *et al.*, Targeting immune checkpoints potentiates immunoediting and changes the dynamics of tumor evolution. *Nat Commun* **9**, 32 (2018).
250. S. J. Im *et al.*, Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* **537**, 417-421 (2016).
251. R. S. Akondy *et al.*, Origin and differentiation of human memory CD8 T cells after vaccination. *Nature* **552**, 362-367 (2017).
252. F. Baharom *et al.*, Intravenous nanoparticle vaccination generates stem-like TCF1(+) neoantigen-specific CD8(+) T cells. *Nat Immunol* **22**, 41-52 (2021).
253. M. Enamorado *et al.*, Enhanced anti-tumour immunity requires the interplay between resident and circulating memory CD8(+) T cells. *Nat Commun* **8**, 16073 (2017).
254. R. Houot, H. E. Kohrt, A. Marabelle, R. Levy, Targeting immune effector cells to promote antibody-induced cytotoxicity in cancer immunotherapy. *Trends Immunol* **32**, 510-516 (2011).
255. T. E. Kahkonen, J. M. Halleen, J. Bernoulli, Immunotherapies and Metastatic Cancers: Understanding Utility and Predictivity of Human Immune Cell Engrafted Mice in Preclinical Drug Development. *Cancers (Basel)* **12**, (2020).
256. H. Dillekas, M. S. Rogers, O. Straume, Are 90% of deaths from cancer caused by metastases? *Cancer Med* **8**, 5574-5576 (2019).
257. C. Therkildsen, L. H. Jensen, M. Rasmussen, I. Bernstein, An Update on Immune Checkpoint Therapy for the Treatment of Lynch Syndrome. *Clin Exp Gastroenterol* **14**, 181-197 (2021).
258. A. Clocchiatti, E. Cora, Y. Zhang, G. P. Dotto, Sexual dimorphism in cancer. *Nat Rev Cancer* **16**, 330-339 (2016).

259. L. Swadling *et al.*, A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci Transl Med* **6**, 261ra153 (2014).

EXPERIMENTAL ROLES AND TECHNICAL CONTRIBUTIONS

- Morena D'Alise from Nouscom designed and generated both adenoviral-based vaccines for mice and humans such as NOUS-209 and PEV, furthermore they conducted also ELISpot assays to measure antigen-specific T cell responses in human PBMCs and identified the peptides pools that Nadia Brasu used in the study.
- The peptides and the vaccines used in the study were designed by Nouscom.
- Nadia Brasu performed multiparametric cytofluorimetry for immune cell phenotyping in murine models and functional analysis of patient-derived PBMCs. Giuseppina Granato and Valentina Russo help with the tumor experiments *in vivo*.
- Valentina Russo executed all the single cell experiments and library preparations with the SMART-Seq2 protocol.
- Carlo De Intinis conducted the bioinformatics analysis using functions of R developed by Luca Petiti.

Denis Baev, Simonetta Guarrera, Caterina Parlato and Katiuscia Gizzi gave a huge technical contributions for the project.