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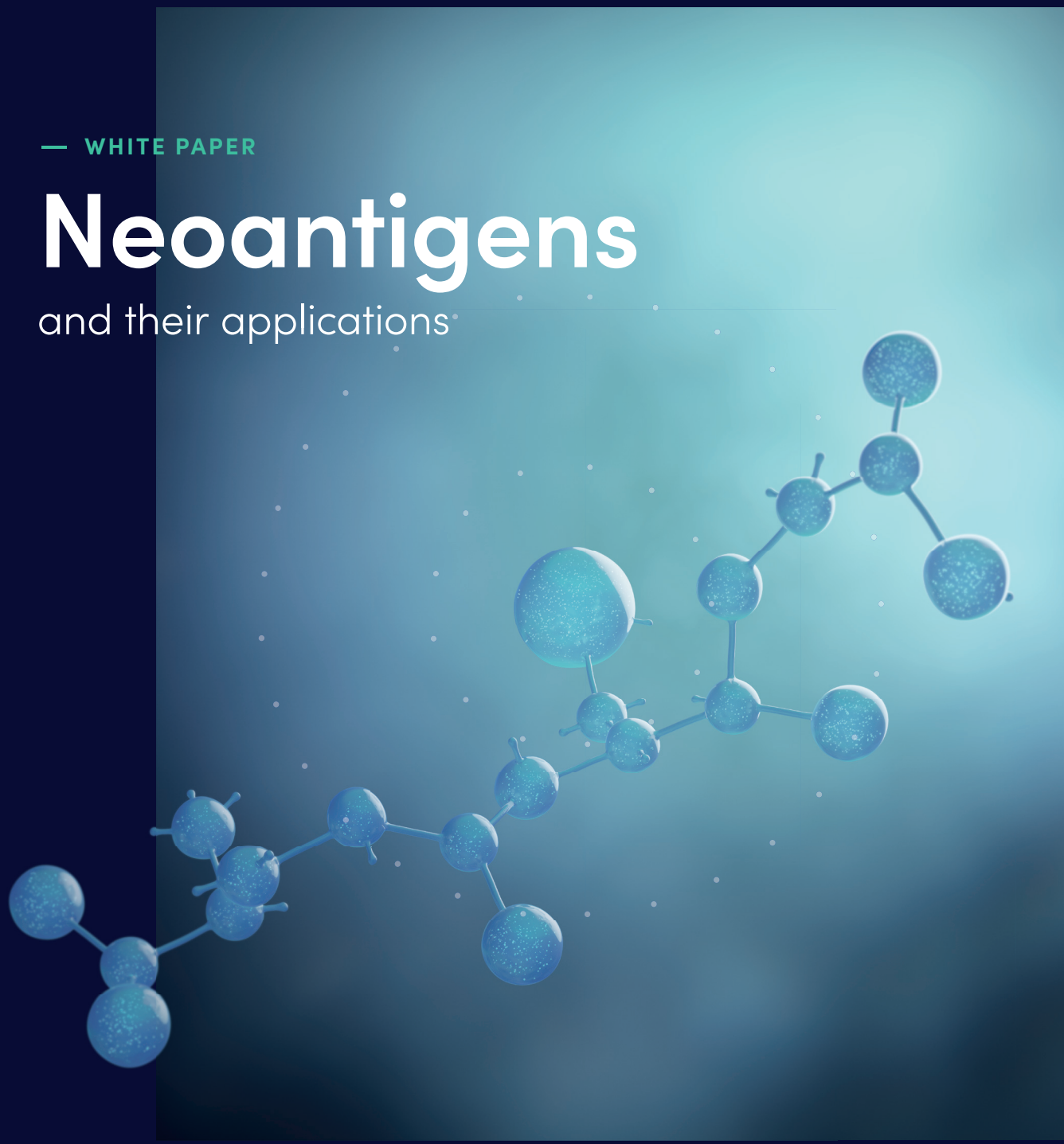
WHITE PAPER

Neoantigens

and their applications



01 Abstract

Tumor neoantigen peptides or tumor specific antigens (TSA) offer new possibilities in cancer immunotherapy. These peptides, which are found exclusively on tumor cells, are ideal targets for personalized medicine as anti-cancer vaccines or for *ex vivo* cell therapy. This introduces the need for rapid synthesis of multiple peptides manufactured and released under GMP conditions.

This white paper is a journey through the new developments in neoantigen research. It will guide you through the origins of neoantigens, explain the detailed steps of the immune response to these neoantigens, provide examples of how neoantigens are utilized in cancer vaccines and *ex vivo* therapies. The paper will also describe how to overcome the negative impact of check point inhibitors, demonstrate the achievements in neoantigen immunotherapy, and reveal new goals in personalized medicine.

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02 What are Neoantigens?

Cancer is known to exhibit uncontrollable cellular growth and immune escape mechanisms. This condition sporadically originates from normal somatic tissues either through spontaneous or environmentally induced accumulation of genetic mutations and epigenetic aberrations. These changes result in the production of modified protein molecules through protein misfolding, protein truncation, and/or abnormal post-translational modifications (1, 2, 3).

Antigens that are non-specifically present on tumor cells but also on normal cells, are called tumor-associated antigens (TAAs). Further genetic instability of cancer cells often leads to the occurrence of multiple mutations which can yield Tumor-specific neoantigens (TSNA's) that are found exclusively on tumor cells (4). In this case, for a given cancer, all patients will develop their own cancer mutation.

Somatic mutations can form tumor neoantigens, but realization depends on several factors:

A. if the mutated sequence can be translated into protein;

B. if the mutated protein can be processed into peptides and presented;

C. affinity between the mutated peptide and MHC molecules of the patients;

D. affinity of mutant peptide-MHC complex with T cell receptor (TCR)(3).

03 Neoantigen Immunogenicity

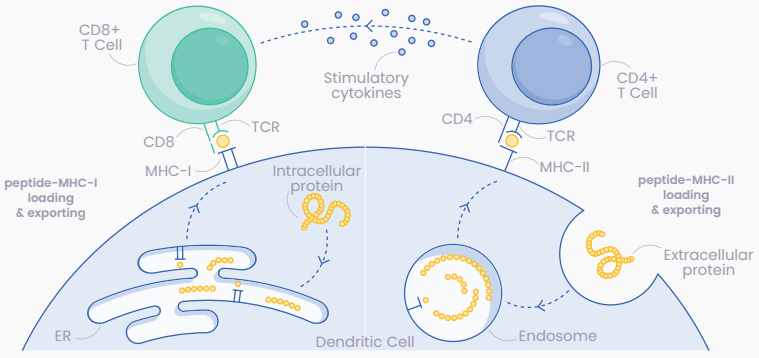
The adaptive immune system is composed of the humoral immune response and the cell-mediated immune response. In cell mediated immunity, the anti-cancer response is driven by T cells, antigen-presenting cells, and the release of cytokines and perforins. Professional antigen-presenting cells (APCs), such as dendritic cells (DCs), capture fragments of tumor cells and extract tumor protein antigens (1, 5). The tumor antigens are subsequently degraded into peptides and associated with the molecules of the major histocompatibility complex (MHC) at the surface of the dendritic cells (6, 7, 8). These dendritic cells then present the processed tumor antigens to the cognate naïve T cells (1).

There are two main T cells subsets that recognize these epitopes during antigen presentation, CD8+ T cells and CD4+ T cells. In CD8+ T cells, the CD8 glycoprotein acts as a coreceptor of the T cell Receptor (TCR). The TCR-CD8 complex binds to the Peptide-MHC class I molecule of the dendritic cell and can recognize 8–10 amino acid long peptides. Similarly, in CD4+ T cells, the CD4 glycoprotein acts as a coreceptor of its TCR. The T cell TCR-CD4 complex binds to the Peptide-MHC class II of the dendritic cell and is able to recognize longer peptides that are predominantly derived from both endosomal and ingested proteins through lysosomal digestion (1) (fig. 1).

Following peptide epitope recognition, MHC molecules associated with TCRs transmit the activation signal through their intracellular signaling domains. Once activated, both CD8+ and CD4+ T cells initiate a cascade of reactions that eventually leads to destruction of the target cell. CD8+ T cells

FIGURE 1. THE ROLE OF MHC-I AND -II IN T CELL ACTIVATION.

MHCs-I are present in almost every cell and involved in the surface presentation of peptides derived mostly from intracellular proteins. MHC-I is recognized by T cell receptors (TCRs) of CD8+ T cells. Conversely, class II MHCs are present only in professional antigen-presenting cells (e.g., dendritic cells) and involved in the surface presentation of peptides derived mostly from extracellular proteins. MHC-II is recognized by TCRs of CD4+ T cells. The recognition of displayed peptide-MHC complexes by the TCRs triggers T cells activation.



obtain cytotoxic abilities and migrate to the tumor. Tumor cells expressing on their surface the same peptide-MHC-I complex as the one which activates the CD8+ cells are recognized and eliminated by these latter. CD4+ helper T cells serve as key mediator of immune functions (5). Both T cell subsets are essential in controlling tumor growth.

CD4+ T cells can differentiate into one of several diverse functional subtypes in response to context-dependent signals which in turn allows them to provide 'help' to appropriate effector immune cells in their primary role as central coordinators of the immune response. CD4+ T cells primarily mediate anti-tumor immunity by providing help for CD8+ T cell lymphocyte and antibody responses, as well as via secretion of effector cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), and, under specific contexts, via direct cytotoxicity against tumor cells. CD4+ T cells are also indispensable for the induction of humoral responses against tumor antigens by providing help via CD40 ligand signaling to CD40 on B cells to drive their differentiation and maturation into affinity-matured, class-switched plasma cells. CD4+ T cells are a critical cornerstone of optimal anti-tumor immunity (10).

04 Neoantigens in Cancer Immunotherapy

The objective of a therapeutic cancer vaccine is to induce tumor regression, eliminate minimal residual disease, and establish lasting anti-cancer memory (11). Neoantigens are ideal targets for peptide-based and some cellular-based (*e.g.*, dendritic cells) cancer immunotherapies, given their highly immunogenic and exquisite tumor-restricted expression and lack of central tolerance against them (12)(fig. 2).

Main features of tumor antigens that influence their potential for therapeutic translation are their abundance on cancer cells and their immunogenicity (9). The larger the difference between the mutated sequence and the original coding sequence, the stronger the immunogenicity. For these reasons, identifying true tumor neoantigens is critical for developing neoantigen based anti-cancer vaccines.

05 Neoantigen Peptide Vaccines

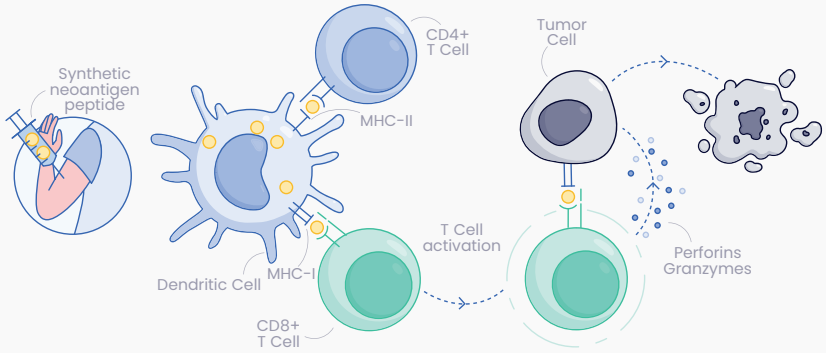
Vaccines can be made directly with single or multiple neoantigen peptides in combination with the appropriate adjuvant (8, 13). The advantages of immunizing with multiple neoantigen peptides are:

A. A robust immune response against at least some of the neoantigens.

B. A decrease in the possibility of tumors escaping the immune response by immunoediting, as they must downregulate multiple targets (14).

Since an individual-specific multitargeted vaccine requires production of many unique immunogens per patient in a critical raw material GMP environment, synthetic peptides are an attractive choice based on safety and relatively low cost.

FIGURE 2. NEOANTIGEN PEPTIDE VACCINES - MECHANISM OF ACTION.



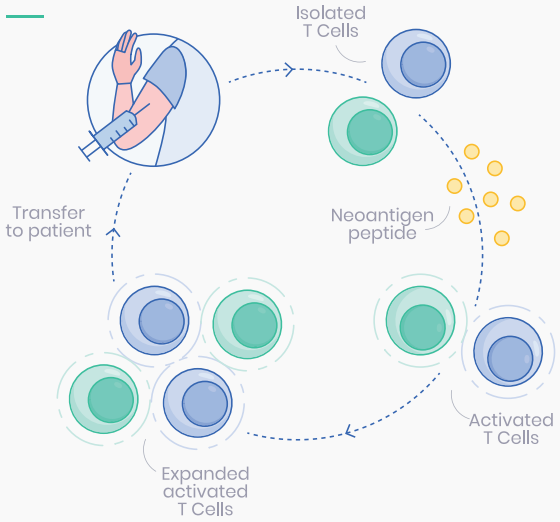
Synthetic peptide neoantigens are injected into a patient's body, captured, and presented by Dendritic cells to TCRs of CD4+ and CD8+ T cells. Naive CD8+ T cells are activated. CD8+TCRs are recognized by MHCs of mutated tumor cells displaying the same neoantigen peptide, which triggers the release of cytotoxic cytokines and the degradation of the tumor cell.

06 *Ex vivo* Cell Therapy

Cell separation and *ex vivo* expansion technologies provide the foundation for developing new cellular products for cancer treatment. *Ex vivo* procedures involve isolation of living cells or tissues from an organism, manipulation of these cells with functional material such as peptide neoantigens, and reintroduction back into the patient's body.

Adoptive T cell Therapy (ATC) is a type of *ex vivo* cell therapy that involves activation and amplification of a patient's autologous T lymphocytes *ex vivo* and then returning them back into the body to destroy the tumor (15). The advantages of ACT include the ability to overcome the impact of T cell-suppression in the tumor microenvironment by transferring a very large number (up to 10^{11}) of cells that exhibit antitumor activity (1)(fig. 3).

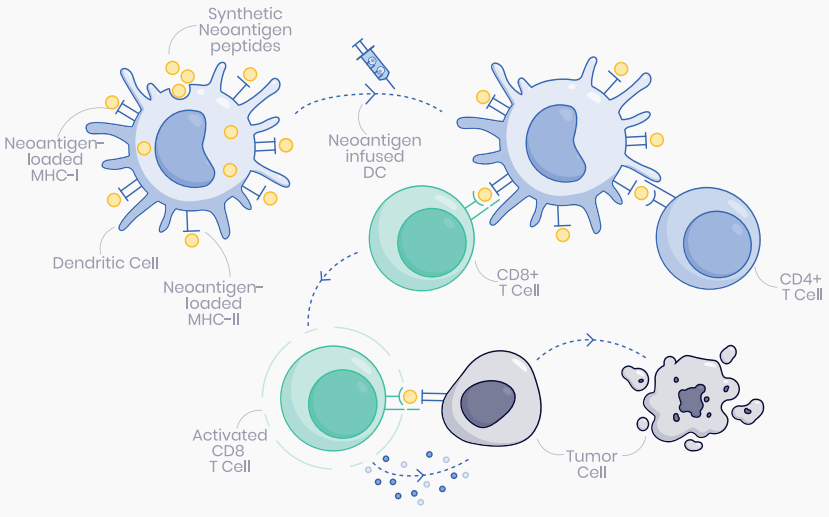
FIGURE 3. BASIC PROCEDURE OF NEOANTIGEN ADOPTIVE T CELL THERAPY.



Dendritic cells (DCs) are known as one of the most important players in the regulation of innate and adaptive immunity. Thus, *ex vivo* antigen-pulsed DC represents a potentially powerful tool to elicit T cell mediated responses against tumor-associated antigens (16). Several *ex vivo* strategies of antigen delivery and activation of DCs were proposed. Such technologies are based on the straight loading of required antigens or a single antigen into DCs *ex vivo*, accompanied by the external stimulus for DC maturation using several cytokines and ligands. The received autologous mature DCs can be administered back to the patient, resulting in lymphocyte activation. It may be possible that even whole tumor lysates could be loaded into DCs. Since isolation of DC as such from the blood is hampered by their scarcity, methods to generate *in vitro* large numbers of functional human DCs using either peripheral blood monocytes or CD34+ pluripotent hematopoietic progenitor cells (HPCs) were developed (16, 17). With the improvement of technologies and methods, it became possible to culture DCs *ex vivo* (18) (fig. 4).

DCs have shown great potential as a component or target in the landscape of cancer immunotherapy. Numerous clinical trials have demonstrated their efficacy and safety in cancer patients. It was shown however that the application of a DC vaccine alone is not sufficient and combination immunotherapy with recent advances, such as immune checkpoint inhibitors, should be employed to achieve a better clinical response and outcome (18).

FIGURE 4. DENDRITIC CELL-BASED THERAPY.



Dendritic cells externally loaded or pulsed with synthetic neoantigen peptides are injected back into the patient's body. Neoantigen is presented by the dendritic cells, which subsequently activates CD8+ and CD4+ T cells. TCR of CD8+ cytotoxic T cell is recognized by the mutated tumor cell displaying the same neoantigen peptide presented by its MHC. Tumor cells are destroyed by this cytotoxic CD8+ T cell.

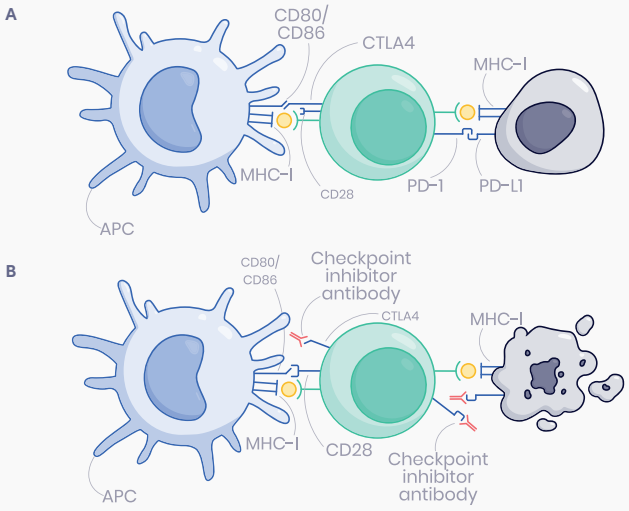
07 Checkpoint Inhibitors

While therapeutic efforts directed at targeting neoantigens have shown promising activity, there has been increasing recognition of the negative impact of immune escape mechanisms due to checkpoint inhibition.

Checkpoints on the surface of T lymphocytes act as molecular brakes during the immune response to maintain the balance of the immune system. Tumor cells can also express immunosuppressing molecules to achieve immune escape (3). Antibody-mediated blockage of immune checkpoints removes the inhibition of immune cells by tumor cells and achieves anti-tumor effect. Immune checkpoint inhibitors are therapeutic monoclonal antibodies against immune checkpoint molecules, such as programmed cell death protein-1 (PD-1), programmed cell death ligand-1 (PD-L1), and cytotoxic T lymphocyte antigen-4 (CTLA-4) (1, 20) (fig. 5).

Immune checkpoint inhibitors have revolutionized the treatment of cancer patients over the past decade (20). The era of personalized cancer immunotherapy combined with immune checkpoint inhibitors is expected to arrive circa 2030 (21).

FIGURE 5. INTERACTION OF TUMOR CELL, T CELL, AND ANTIGEN PRESENTING CELL (APC).



*MHC on the APC carries the same neoantigen as MHC on tumor cell. **A.** Cytotoxic abilities of T cell are blocked by immunosuppressing CTLA-4 molecule on T cell in complex with CD80/CD86 and PD-1-PDL1 complex on tumor cell to achieve immune escape. **B.** Antibody-mediated blockage of immunosuppressing molecules on T cell and tumor cell (CTLA-4 and PD-1, PD-L1) removes the inhibition of T cell resulting in tumor cell destruction by this cytotoxic T cell.*

08 Identification and Selection of Neoantigens

There are two critical factors that can greatly influence the identification of true neoantigens: the chosen screening methods to identify candidate neoantigens, and the evaluation of their immunogenicity (19).

8.1. Mutations

The first step in the design of a neoantigen is to identify tumor-specific and somatic mutations by comparing genomic differences between tumor tissues and normal cells. Mutation detection mainly includes whole-genome sequencing, non-synonymous mononucleotide variation (nsSNVs), total exome sequencing, and total transcript sequencing. Initial profiling of human tumors by whole exome and whole genome sequencing has been shown to detect high numbers (tens to hundreds) of non-synonymous mutations. Once the mutations have been identified, the expression level of the identified mutant allele is then verified and analyzed by RNA sequencing (22).

8.2. Neoantigen prediction

As an alternative to, or in synergy with *in silico* sequence-based predictions, liquid chromatography-mass spectrometry (LC-MS) can be used to directly interrogate the immunopeptidome or ligandome of tumor cells (23). MS-based immunopeptidomics is advantageous, since it is a direct analysis of the presented HLA-binding peptides so it substantially narrows down the list of candidate neoantigens to be screened. Another advantage is that this method results in fewer false positives

in comparison with *in silico* prediction. Although MS-based immunopeptidomics offers multiple advantages, this approach is hindered by technical limitations, such as the low sensitivity of MS (19).

8.3. **Immunogenicity (T cell recognition)**

The vast majority of selected candidate neoantigens identified in a tumor are not recognized by T cells. Thus, evaluation of the immunogenicity of candidate neoantigens using a variety of screening methods is critical to more precisely identify and select neoantigens suitable for clinical intervention (19).

The simplest approach available to analyze neoantigen immunogenicity relies on classically available immunological techniques such as IFN-γ release by ELISA or ELISPOT assays (19). However, these approaches have their limitations and are very time-consuming.

Stable peptide (p)MHC-T cell interactions are requisite for T cell cytotoxicity against cancer. However, identifying stable pMHC molecules is experimentally laborious and *in silico* predictions remain under scrutiny. Recently developed, the EZ MHC-I assay using the pMHC-I single chain trimer (SCT) molecule to enable a direct interrogation of the MHC ligandome predicted *in silico* or derived from patients' samples is designed to overcome the obstacles. Actual measurement of pMHC-I affinity and stability can potentially improve the reliability of predicted peptides (24). It is worth mentioning that immunological functional screening assays rely on the use of multiple synthetic peptides, with a purity of approximately 70% (19).

8.4. **Private and Public Neoantigens**

The vast majority of detected neoantigens are patient-specific, or "private". As mutations are acquired randomly, private neoantigens occur more frequently (although not exclusively) *in loci* non-essential for tumorigenesis and metastasis. They are termed "passenger" mutations. By contrast, mutations in some important driver oncogenes have been shown to occur in "hotspots" and are shared across multiple patients. When patients express both a shared mutation and a shared MHC molecule capable of presenting the encoded neoantigen in an immunogenic context, this pairing can be designated as a "public" neoantigen. Characterized public neoantigens generally occur in genes that play an important role in facilitating tumorigenesis or driving continued tumor growth, for instance the gain of function BRAF V600E mutation that confers a constitutive proliferative drive in melanoma. The prominent exception is TP53, a master orchestrator of cell cycle and DNA repair processes. TP53 is the most frequently mutated gene among all cancers, with TP53 mutations represented across at least 27 cancer types. As such, these targets may be more difficult for cancer cells to lose or silence without a concomitant loss of fitness (23).

09 Achievements and goals in cancer immunotherapy

Identification of neoantigens is essential for the development of anticancer therapeutic vaccines, clinical diagnosis of cancer, targeted immunotherapies, and biomarker discovery (25).

In recent years, the successes of cancer immunotherapy have been stimulated by major breakthroughs in melanoma (9). In fact, melanoma regression can now be achieved through the transfer of *in vitro* and *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) and through immune checkpoint therapy that targets regulatory pathways in T cells.

Personalized neoantigen vaccines have been demonstrated to induce strong T cell responses in solid tumors (15, 26). It was shown that neoantigen vaccines induced tumor rejection in mice bearing transplanted sarcomas and have an ability to generate neoantigen-specific T cells in glioblastoma in humans (12). Some therapeutic success of immune-based therapies was shown in blood malignancies. Adoptive transfer of autologous T cell products containing high fractions of neoantigen-specific T cells has generated tumor regression in a range of cancer types. *Ex vivo* expanded TIL therapy can deliver a significant reduction in tumor load in patients with epithelial tumors and melanomas (4). Combination of the inhibition of key immunosuppressing molecules with vaccine therapy has remarkable clinical effects on many types of cancers (20).

With the continuous development and wide cross-integration of biotechnology, immunology, materials science, chemistry, and artificial intelligence, additional neoantigens are likely to be identified and employed in tumor immunotherapy (27).

10 Conclusions

As neoantigens exhibit entirely novel amino acid sequences, their targeting through immunotherapy has shown no toxicities to normal tissues, as they are not subject to either pre-existing immune tolerance or are likely to generate autoimmunity. In addition, documented cases of off-target immune response against the wild-type non-mutated peptide are exceptionally rare (12). Recent advances in neoantigen research have accelerated the development and regulatory approval of tumor immunotherapies, including cancer vaccines, adoptive cell therapy and antibody-based therapies, especially for solid tumors (28). These therapies are thus viewed as a safe and effective approach to personalized medicine.

The rapid synthesis of multiple peptides is crucial to the further development of neoantigen personalized cancer vaccines. The great success of immunotherapy is paving the way for a new era in cancer treatment (29).

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