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REVIEW

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# What's next in cancer immunotherapy? - The promise and challenges of neoantigen vaccination

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**ABSTRACT**

The process of tumorigenesis leaves a series of indelible genetic changes in tumor cells, that when expressed, have the potential to be tumor-specific immune targets. Neoantigen vaccines that capitalize on this potential immunogenicity have shown efficacy in preclinical models and have now entered clinical trials. Here we discuss the status of personalized neoantigen vaccines and the current major challenges to this nascent field. In particular, we focus on the types of antigens that can be targeted by vaccination and on the role that preexisting immunosuppression, and in particular T-cell exhaustion, will play in the development of effective cancer vaccines.

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## Introduction

The capacity of the immune system to recognize cancer cells has been known for over 100 years and is the basis of successful immune checkpoint inhibitor (ICI) immunotherapy. Tumors contain multiple sources of antigen that can be recognized by T cells and have the potential to be targets of novel vaccination strategies. For example, tumors aberrantly express, or over-express, proteins that are normally expressed developmentally or in a tissue-specific manner. These "tumour associated antigens (TAA)" such as MAGE-A1, were the subject of early vaccine trials[1]. However, vaccines targeting TAA have been largely ineffective, this is generally ascribed to the presence of tolerance mechanisms to these aberrantly expressed self-antigens.[2]

Cancers can also express tumor-specific antigens (TSA) which may arise from cancer-specific mutations. Somatic mutations become immunogenic, "neo"-antigens when they are expressed as proteins that are then displayed as peptide fragments to T cells via major histocompatibility complex (MHC) class I (MHC-I) or class II (MHC-II) molecules. Because each cancer will express a unique mutational signature, neoantigens are truly tumor specific. This specificity means that neoantigens are not subjected to preexisting tolerance mechanisms. Moreover, vaccination is unlikely to induce off-target autoimmunity. In this review we focus on neoantigen vaccination, an emerging personalized cancer immunotherapy. We discuss the outstanding questions and potential barriers to the implementation of this therapy approach, with specific reference to neoantigen targets and the role that prior immunity, in particular T-cell exhaustion, may play in limiting the efficacy of this approach.

## Pre-clinical mouse models set the template for neoantigen vaccines

In 2012 Castle and colleagues described the basic schema for the production and use of a neoantigen vaccine.[3] Using the B16F10 mouse model of melanoma, the authors sequenced tumor and normal tissue and, using bioinformatics approaches, predicted 50 high confidence neoantigens. Of these predicted neoantigens, 16 (32%) were immunogenic, with 60% of these being more immunogenic than non-mutated wild-type peptides.[3] Prophylactic vaccination with a single neoantigen led to complete protection in 40% of mice and delayed tumor growth in the remaining animals.[3] Matsushita and colleagues used a similar approach to identify neoantigens in a $Rag2^{-/-}$ murine methylcholanthrene (MCA) induced sarcoma model.[4] The authors were able to demonstrate T-cell mediated immunoediting of a defined neoantigen following transplantation into immunocompetent wild-type mice.[4] In an extension of the MCA sarcoma model, the same investigators identified two neoantigens that were the key targets of ICI-mediated tumor rejection.[5] Neoantigen vaccines containing these two antigens were equally as effective as ICI in causing regression of established tumors.[5] Together these early murine studies set the template for clinical neoantigen vaccine trials, demonstrating immunogenicity, protection, tolerability, and scalability.

## Establishing safety and immunogenicity in human clinical trials

The basic schema for the production of a personalized neoantigen vaccine (Figure 1) is little changed from the early mouse studies described above, and has been employed in multiple clinical trials. The safety and immunogenicity of neoantigen

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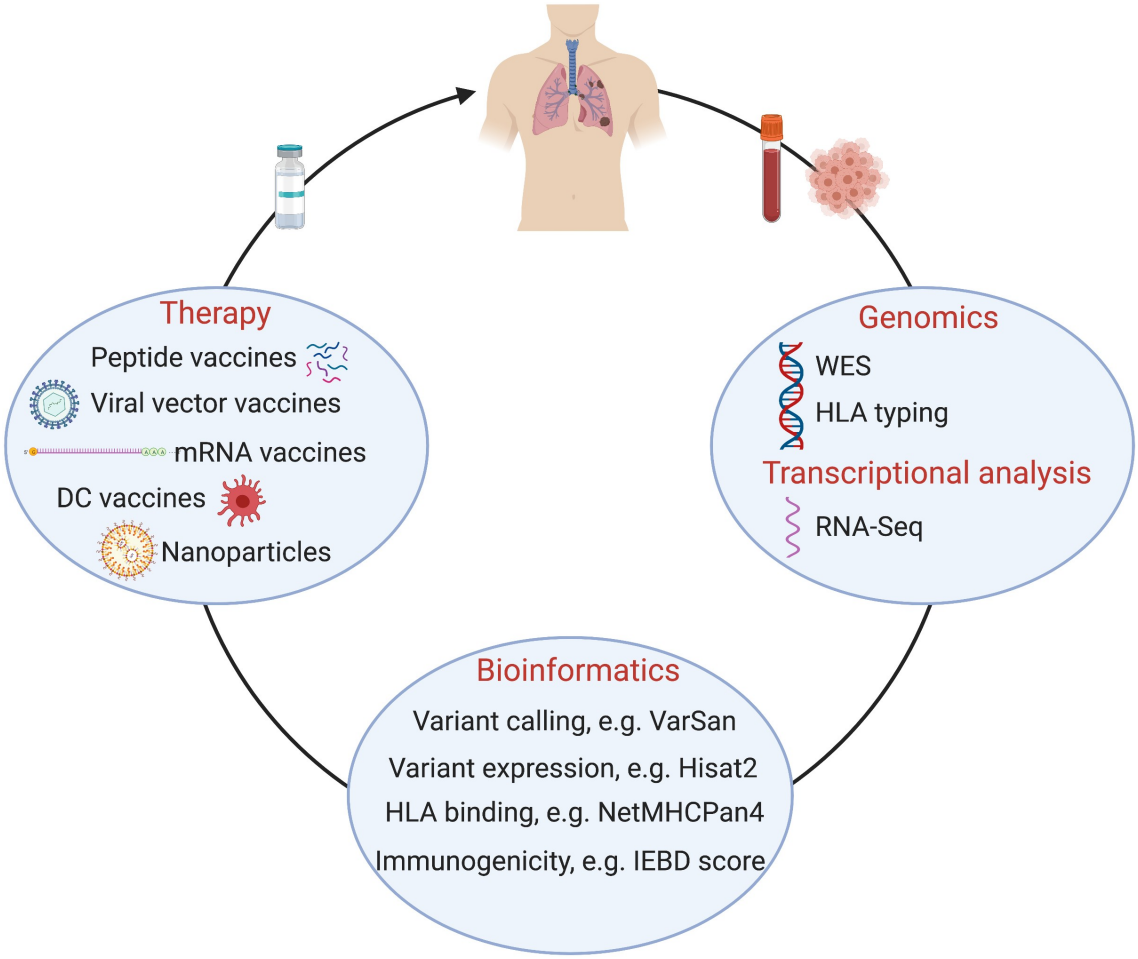


**Figure 1.** Neoantigen vaccine production. Blood and a sample of the patient's tumor are removed for DNA extraction and for the tumor, RNA extraction as well. DNA is used for WES (or WGS), and for normal DNA, HLA typing. Tumor specific somatic mutations are identified using a combination of variant callers which compare blood and tumor WES data. RNAseq analysis is employed to validate mutant allele expression. The patient's HLA is then used to predict MHC-I or MHC-II binding, using algorithms such as NetMHCPan4 and NetMHCIIpan4. Typically, other indicators of immunogenicity, such as the potential to engage with a TCR, location of the mutation to an anchor residue or DAI may also be employed to rank potential neoantigens. A series of potential neoantigens, typically 10–20, are then formulated for injection. Commonly peptide plus adjuvant approaches have been employed, because of the ease of manufacture, however other approaches such as mRNA vaccines, DC preparations and viral vectors have been, and are being developed. Abbreviations, DAI, differential agretopicity index; DC, dendritic cell; MHC, major histocompatibility complex; TCR, T-cell receptor; WES, whole exome sequencing: WGS, whole genome sequencing. Figure created with BioRender.com.

vaccines was demonstrated in three seminal studies in melanoma patients.[6–8] In the first study, neoantigen peptides with confirmed binding to HLA-A*02:01 were used in an autologous dendritic cell (DC) vaccine. DC vaccination led to the boosting of preexisting neoantigen responses and the generation of *de novo* responses to additional neoantigens.[6] Two other studies were subsequently published in the same issue of Nature. Ott and colleagues applied a peptide neoantigen vaccine approach to assess immunogenicity and safety in six melanoma patients. Each patient was vaccinated with up 20 predicted MHC-I restricted neoantigens. 60% of the neoantigens induced CD4 T-cell responses, 16% induced CD8 T-cell responses and 10% induced both CD4 and CD8 T-cell responses.[7] Four patients had no tumor recurrence at 25 months post-vaccination while two patients with recurrence, experienced complete tumor regression when treated with Pembrolizumab (anti-PD1).[7] Likewise, Sahin and colleagues demonstrated immunogenicity to 60% of mRNA encoded candidate neoantigens, and of these, 57% of the epitopes were recognized by CD4 T cells, 17% by CD8 T cells and 26% by both T-cell types.[8] Post vaccination, the rate of metastatic events was significantly reduced in this patient cohort.[8]

A series of additional phase I clinical trials, summarized in Table 1, have built on these early studies showing similar immunogenicity and safety profiles across a range of tumor types.[9–14] A recent clinical trial in 22 patients with different types of cancer demonstrated robust T-cell responses and tumor T-cell infiltration.[14] Expansion of neoantigen-specific T cells was also observed in four patients with metastatic gastrointestinal cancer following the administration of an mRNA concatemer vaccine containing up to 20 neoantigens.[13] Neoantigens were a mix of predicted and defined, pre-screened, neoantigens. T-cell responses were detected to 16% of neoantigens across 3 of the 4 patients, one patient failed to make detectable T-cell responses. Once again the majority of antigens, 59%, were recognized by CD4 T cells.[13] Notably there was no evidence that preexisting immunity to neoantigens was boosted by vaccination.

**Table 1.** Neoantigen types and use in clinical trials.

| Neoantigen class | Nature of the antigen | Most relevant tumors | Clinical trials |
|---|---|---|---|
| SNV, In-frame Indel | Single AA change or AA insertion/deletion, respectively | Most tumor types, lower prevalence in some tumors such as RCC | • Tumor, melanoma; Vaccine, peptide pulsed DC; Immunogenic, yes SNV[6]<br>• Tumor, melanoma, Vaccine, peptide plus adjuvant; Immunogenic, yes SNV and indel neoORF[7]<br>• Tumor, melanoma; Vaccine, mRNA; Immunogenic, yes SNV[8]<br>• Tumor, melanoma, NSCLC and bladder cancer; Vaccine, peptide plus adjuvant; Immunogenic, yes SNV and fusion neoORF[9]<br>• Tumor, glioblastoma; Vaccine, peptide plus adjuvant; Immunogenic, yes, TAA and SNV[10]<br>• Tumor, glioblastoma; Vaccine, peptide plus adjuvant; Immunogenic, yes SNV (non-dexamethasone treated patients)[11]<br>• Tumor, glioblastoma; Vaccine, DC tumor lysate followed by peptide plus adjuvant; Immunogenic, yes SNV[12]<br>• Tumor, gastrointestinal cancer; Vaccine, mRNA, Immunogenic, yes SNV and indel neoORF[13]<br>• Tumor, multiple; Vaccine, peptide plus GM-CSF adjuvant, Immunogenic, yes, class of neoantigen unclear, SNV, In-frame indel and Indel neoORF tested in pools[14] |
| Indel – frameshift (neoORF) | Expression of novel sequence | MSI-H tumors and RCC | • Tumor, melanoma, Vaccine, peptide plus adjuvant; Immunogenic, yes SNV and indel neoORF[7]<br>• Tumor, glioblastoma; Vaccine, peptide plus adjuvant; Immunogenic, no all patients that received neoORFs neoantigens also received dexamethasone, these patients also failed to make responses to SNV[11]<br>• Tumor, gastrointestinal cancer; Vaccine, mRNA; Immunogenic, yes SNV and indel neoORF[13]<br>• Tumor, multiple; Vaccine, peptide plus GM-CSF adjuvant, Immunogenic, yes, class of neoantigen unclear, SNV, In-frame indel and Indel neoORF tested in pools[14] |
| Fusion– In-frame | Expression of novel sequence at fusion site | AML, ALL, CML and sarcomas | • Tumor, synovial sarcoma, peptide plus adjuvant with IFN-α; Immunogenic, yes fusion peptides[15]<br>• Tumor, Ewing's sarcoma or alveolar rhabdomyosarcoma, Vaccine, peptide pulsed DC, other therapies include autologous T cells and IL-2. Immunogenic, yes fusion peptides[16] |
| Fusion– frameshift (neoORF) | Expression of novel sequence | AML, ALL, CML and sarcomas | • Tumor, Melanoma, NSCLC and Bladder Cancer; vaccine, peptide plus adjuvant; immunogenic, yes SNV and fusion neoORF[7]<br>• Also see<br>• Clinical trial number, NCT01885702, active, not recruiting. Colorectal cancer, DC vaccination with gene fusion frameshift-derived neoantigens<br>• Clinical trial number, NCT04998474, not yet recruiting. NSCLC, peptide vaccination with gene fusion frameshift neoantigens |
| Endogenous retroelement | Expression of novel sequence | RCC, low-grade glioma, testicular cancer | Not specifically described at Clinical.trials.gov |
| mRNA splice variants | Expression of novel sequence | AML, CMML, CLL, myelodysplastic syndrome | Not specifically described at Clinical.trials.gov |
| Post-translational splice variant | Expression of novel sequence | Identified in melanoma RCC, colon carcinoma and breast cancer | Not specifically described at Clinical.trials.gov |

Abbreviations. AA, amino acid; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; IFN-α, interferon-alpha; GM-CSF, granulocyte macrophage colony stimulating factor; RCC, renal-cell carcinoma; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; INDEL, insertion or deletion; MSI-H, microsatellite instability-high; Neo-ORF, neo (novel) open reading frame; NSCLC, non-small cell lung cancer; SNV, single-nucleotide variant; TAA, tumor associated antigen.

Finally, a combined neoantigen vaccine plus anti-PD1 phase 1b clinical trial in melanoma, NSCLC and bladder cancer patients demonstrated the induction of durable T-cell responses to candidate neoantigens at rates consistent with other studies; 52% of neoantigens in melanoma, 47% in NSCLC and 52% in bladder cancer.[9] Importantly, this study also noted epitope spreading to high-value neoantigens not included in the vaccine. The authors suggested that this was due to increased tumor cell death and the resultant antigen presentation.[9]

A key requirement for the broad applicability of neoantigen vaccines is that they can be used in all patient cohorts, irrespective of tumor mutational burden (TMB) and therefore neoantigen load. Tumors with a low TMB are also often "immunologically cold". These cold tumors are characterized by poor T-cell recruitment and resistance to immunotherapy. The efficacy of neoantigen vaccines in these tumors is therefore of considerable interest. To address this question, Hilf and colleagues trialed neoantigen vaccines in immunologically cold glioblastomas. Patients were first vaccinated from a library of shared TAAs, and then vaccinated with personalized neoantigen vaccines. The TAA vaccine elicited sustained CD8 T-cell responses, whilst the subsequent neoantigen vaccine generated predominantly CD4 T-cell responses.[10] In a second glioblastoma vaccine trial, patients were treated with neoantigen vaccines following surgical resection and radiotherapy. In this study neoantigen vaccination generated robust CD4 and CD8 T-cell responses and increased T-cell tumor infiltration.[11] Likewise, T-cell responses were identified to defined neoantigens in a single glioblastoma patient treated with an autologous DCs plus tumor cell lysate vaccine plus peptide neoantigen vaccine.[12] These early trials provide

support for the use of neoantigen vaccines, alone or in combination with ICI, across tumor types, including those with a low TMB.

Taken together, pre-clinical studies and early clinical trials have demonstrated safety and convincing T-cell responses to neoantigen vaccines. Some clinical trials have shown evidence of T-cell infiltration of tumors as well as killing of tumor cells. However, these single-arm trials were not powered to assess efficacy and larger phase II/III clinical trials are warranted. Here we now discuss some of the outstanding questions that remain to be addressed, with particular reference to the source and choice of neoantigen.

## What is the potential neoantigen pool?

Neoantigens can arise from several different mutational events within the cancer genome or can arise from non-templated events that occur post translation (Figure 2). Here we discuss what is known about each type of mutation and the potential utility of these antigens as vaccine targets.

### *Single nucleotide variants (SNVs) and indel derived neoantigens*

The bedrock of most clinical[6,7,8] and preclinical studies[5,17,18] are small somatic variations such as single nucleotide variants (SNVs) that lead to missense mutations, and small insertions or deletions (indels). SNVs and indels are the primary focus of most studies because they are readily detectable by whole-exome sequencing (WES). SNV induce immunogenic changes when they result in a mutation that changes an amino acid residue at an MHC anchor point, allowing an otherwise silent antigen to be presented, and/or when the amino acid substitution changes the topography of the MHC/peptide complex allowing novel T-cell recognition. Either mutation is capable of generating novel T-cell responses, however, there is no consensus on which if either of these events is the most immunogenic or protective when employed in a vaccine.

The protocols for selecting SNV derived neoantigens, whilst still evolving, are well understood, with most groups opting to prioritize well-expressed neoantigens with a predicted binding affinity of less than 500 nM. Others opt to include additional criteria and a recent report from the tumor neoantigen selection alliance (TESLA) recommended a selection process based on peptide "presentation" and "immunogenicity".[19] Presentation was defined as MHC-I binding affinity less than 34 nM (using the algorithm NetMHCPan4.0[20]), peptide MHC-I stability greater than 1.4 hours (using the algorithm NetMHCStabPan[21]) and antigen encoding RNA expression levels greater than 33 transcripts per kilobase million (TMP). Immunogenicity was defined as differential agretopicity index (DAI)[22,23] less than 0.1 and high foreignness,[24] greater than $10^{-16}$. This approach allows the many thousands of potential mutations in tumors with high TMB to be pruned to a manageable number, whilst maximizing the probability of selecting immunogenic, potentially protective, neoantigens. For cancers with a low TMB, this approach is likely to be too prescriptive, leading to the exclusion of most, if not all the potential neoantigens.

For some cancers, for example, clear-cell renal-cell carcinoma (ccRCC), the number of SNV mutations is low and SNV burden is not linked to successful ICI.[25] Vaccines targeting SNV neoantigen are unlikely to be useful in this patient cohort. However, ccRCC's, as well as DNA mismatch repair deficient, microsatellite instability-high (MSI-H) tumors, are rich in indel-induced frameshift mutations.[26] Frameshift mutations generate an entirely novel amino acid sequence. Neoantigens derived from frameshift mutations appear to be highly immunogenic because indel burden is associated with elevated and activated TILs[26–31] and because MSI-H tumors have high response rates to ICI immunotherapy.[32,33] Frameshift derived neoantigens are therefore likely to be highly effective vaccine targets.

Several neoantigen vaccine trials have sought to include indel-induced frameshift (neoORFs) mutations.[7,11,13,14] In the study by Keskin and colleagues, five patients were tested for T-cell responses post vaccination.[11] Of these three were negative which was attributed to the use dexamethasone during vaccination. The two remaining patients were vaccinated with SNV derived neoantigens only.[11] Cafri and colleagues detected responses to 6/18 SNV and 2/2 frameshift derived neoantigens from a single patient with gastrointestinal cancer.[13] Other patients in this study either did not receive neoORF antigens (2/4), or failed to make detectable responses to the vaccine (1/4).[13] In the third study, Ott and colleagues were able to demonstrate T-cell responses to neoORFs, some with high avidity, in 4 of 6 vaccinated melanoma patients.[7] Responses to neoORFs derived antigens were present within the CD4 and the CD8 T-cell compartment.[7] In the final study, Fang and colleagues tested response to neoantigens in pools and failed to record the immunogenicity of neoORF derived neoantigens.[14] These clinical trials were not designed to determine the relative immunogenicity of neoORF derived neoantigens, however they do suggest that neoORFs are worthy of consideration in future clinical trials.

### *Neoantigens derived from structural variants*

Large-scale chromosomal alterations such as insertions, deletions, inversions, duplications and translocations can generate frameshifted and in-frame fusion-derived neoantigens.[34,35] The number of structural variants per genome is typically lower than SNV and indels,[34,35] however these more complex genomic events are attractive vaccine candidates for several reasons. Neoantigens derived from these gene fusions events are likely to be entirely distinct from self and are predicted to be more immunogenic than SNVs.[35] Each mutation, especially frameshift mutations, will generate more potential neoantigens per event than an individual SNV.[35] In-frame gene fusion-derived neoantigens can be shared across patients.[35,36] Finally, because these genetic events often contribute to carcinogenesis, the mutations are common in driver genes[35,37,38] meaning that they are more likely to be clonally expressed and required for tumorigenesis. Both these latter features mean that vaccine-induced immunoediting may be reduced.

Thus, far there is little data on the use of neoantigens derived from structural variants in clinical trials and no consensus on the best methods for calling and validating these mutations. Whilst ICI therapy success broadly

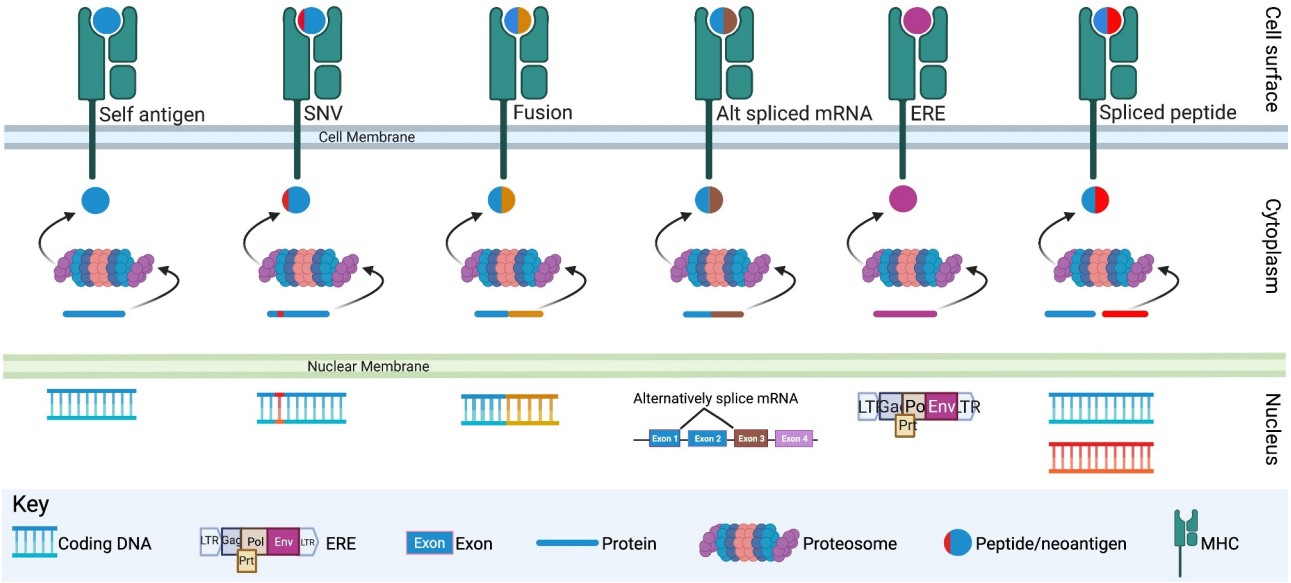


**Figure 2.** Potential tumor specific neoantigens. Neoantigens can arise from multiple sources within the tumor cells. Shown here from left to right are normal self-antigens and then, SNV that arise from non-synonymous, single nucleotide changes, to the tumor genome. Fusion neoantigens which arise from structural events within the genome such as translocations and inversions. Alternative mRNA splicing can also generate novel tumor specific neoantigens via events such as mutations at mRNA splice sites, intron retention or disruptions to the spliceosome. EREs are mobile genetic elements that may be aberrantly expressed in tumor cells and can serve as tumor specific neoantigens. Proteins from these DNA templated neoantigens are processed into linear peptides of between approximately 8–13 amino acids in the proteosome for presentation on the cell surface by MHC class I or MHC class II molecules. Spliced peptide neoantigens (far right) are non-templated and arise from the splicing of independent protein sequences within the proteosome which are then also presented by MHC molecules. Abbreviations, ERE, endogenous retroelement; MHC, major histocompatibility complex; SNV, single nucleotide variants. Figure created with BioRender.com

correlates with predicted SNV/indel burden, predicted fusion neoantigen load may not be a good indicator of successful ICI therapy.[35] Moreover, early vaccine trials targeting shared fusion neoantigens have been disappointing.[15,16] However, shared fusion neoantigens may be less immunogenic than private fusion neoantigens[35] which may explain the poor efficacy of these earlier trials. At least one study has demonstrated that fusion-derived neoantigens can be extraordinarily strong T-cell targets.[34] Ott and colleagues prioritized the selection of frameshifted, gene fusion derived, neoantigens in an anti-PD-1 plus neoantigen vaccine trial, and demonstrated responses to this class of neoantigens in at least one patient.[9] However, these authors did not report the number of fusion-derived neoantigens used, nor their relative immunogenicity compared to SNVs.[9] Therefore, it remains to be determined how valuable this class of neoantigen will be to vaccination regimes. However, the advent of improved neoantigen prediction methods[35,37,39–43] should facilitate any such study. As for SNV/indel neoantigens, the number of fusion neoantigens varies with tumor type[35] and therefore the utility of these targets is likely to be tumor dependent (Table 1).

## *Alternative neoantigen sources*

Neoantigens derived from mutational events are not the only tumor-specific antigen (TSA). TSA can arise from errors in the transcription of microsatellites, the mis-splicing of exons, from aberrant transcription of tumor-specific open reading frames or of endogenous retro-elements (EREs), including endogenous retroviruses. These alternative sources of neoantigens have been extensively and expertly reviewed by Smith and colleagues.[44]

TSA can also be derived from non-templated events including; post-translational modification,[45] such as phosphorylation,[46] or post-translational cis-splicing and trans-splicing of normal or mutated proteins.[47–50] The production of spliced peptides occurs through highly specific and reproducible processing within the proteosome.[51] MHC-I presented spliced peptides were first identified on the surface of tumor cells[49] and are expressed at sufficient levels and consistency to induce CTL responses.[49,52,53] Consequently, these non-templated neoantigens may be highly effective vaccine candidates, especially as they comprise a significant fraction of MHC-I presented peptides.[47,50] The role of these non-templated neoantigens has yet to be fully explored (Table 1) but it could be expected that these cancer antigens would be very different to self and therefore highly effective vaccine targets.

Theoretically the combination of sequencing and proteomics should be the gold standard for neoantigen identification, allowing validation of genomics approaches as well as the identification of non-templated neoantigens. Several groups have employed proteomics approaches to refine the search for neoantigens first identified by sequence analysis. Yadav and colleagues[54] combined WES with proteomics for neoantigen identification in CM-38 and TRAM-C1 mouse tumor models. In the TRAMP-1 model only 6 neoantigens were predicted by genomics approaches, none of which were confirmed by proteomics. In the CM-38 model, 170 SNV were predicted by sequence and bioinformatics approaches. Of these, only 7 of were also confirmed to bind to MHC-I by

mass spectrometry. Of these 7 neoantigens, 3 were immunogenic and protective when included in prophylactic or therapeutic vaccines.[54] Hilf and colleagues used proteomics to validate predicted neoantigens for inclusion into a neoantigen glioblastoma vaccine.[10] However, none of the 643 mutations, across 15 patients, could be identified on HLA class I or class II peptidomes meaning that predictions alone were used for vaccine production.[10] Therefore, the role of proteomics in vaccine design is yet unclear and may be most effective in the detection of non-templated antigens

### The impact of tumor heterogeneity on antigen selection

The mutational landscape of a tumor is not homogenous[55] meaning that genomic or proteomic neoantigen identification from a single biopsy is likely to be subject to sampling bias. This represents a potential barrier for successful neoantigen vaccination because vaccines that target poorly represented, sub-clonal, neoantigens could drive tumor escape through the process of immunoediting.[56] Therefore, neoantigen vaccines would ideally consist of clonal neoantigens, i.e., those that arose early in the tumorigenesis and are expressed by the majority of cells. Sampling bias will affect the identification of clonal antigens meaning that multiple samples may need to be taken from each patient.[57,58] This places significant logistical limits on neoantigen prediction and others have sought to use computational approaches for the identification of clonal neoantigens.[59]

Some classes of neoantigen, for instance gene fusions are more likely to be clonal because they often occur in driver genes.[35,37,38] Other neoantigens, for instance, spliced peptides may be less likely to be clonal. In an ideal world only clonal neoantigens would be included in a vaccine, however these antigens may be rare or difficult to identify. That clinical trials[9] and animal models[60] have shown vaccine mediated epitope spreading to non-vaccine targets implies that vaccination with sub-clonal antigens may be sufficient to cause spreading of the immune response to other targets, including clonal neoantigens. Clinical and pre-clinical studies will be required to determine the relative importance of targeting only the clonal neoantigens.

In summary there are many potential sources of neoantigens (Figure 2) and there is supportive evidence to presuppose that some of these antigens will be more protective than others. However, to date there have been no comprehensive studies that have sought to determine the most effective "class" of neoantigen, and it remains the case that most neoantigen trials have focused on small somatic variants, SNV and indels. In addition, the type of neoantigen available for vaccination will be dictated by the mutational landscape of the tumor (Table 1). Therefore, detailed studies assessing the relative immunogenicity of the different types of neoantigen will be critical to ensure an agile approach to vaccine design and ensure adaptability across patients and cancer types.

## Overcoming T-cell exhaustion

There are several potential impediments to successful neoantigen vaccination, in particular T cells specific for expressed neoantigens are likely to be under the influence of strong and ongoing immunosuppression. This suppression may be mediated by a number of nonspecific and specific factors, such as myeloid-derived suppressor cells[61] or regulatory T (Treg) cells,[62] respectively. Alternatively, this immunosuppression may be intrinsic to the neoantigen-specific T cells and manifested as T-cell exhaustion. Repeated T cell receptor (TCR) stimulation from chronic antigen exposure leads to T-cell exhaustion and is a feature of cancer and certain chronic viral infections. The exhausted T-cell (Tex) phenotype is characterized by the expression of inhibitory receptors (e.g., PD1, LAG3, 2B4, TIM3, CTLA4), impaired cytokine production (TNFα, IFNγ, and IL-2) and reduced proliferation.[5,63–65] T-cell exhaustion may therefore be one of the major barriers to neoantigen vaccination and so the plasticity of the Tex phenotype is of considerable importance.

### Can exhausted cells be re-invigorated?

A number of studies have linked the expression of ICI molecules within the TME to treatment efficacy, leading to the general view that ICI immunotherapy "re-invigorates" T-cell immunity by reversing the exhaustion of tumor resident T cells.[66–72] However, an increasing body of evidence suggests that T cells responding to ICI are not derived from the exhausted tumor-infiltrating lymphocyte (TIL) population. For example, T-cell proliferation and activation was seen in the blood and lymph nodes following ICI,[73] PD-1 blockade in the tumor draining lymph node (TDLN) has similar efficacy to that seen with systemic treatment[73,74] and PD-1/PD-1 L interactions in the TDLN, but not the tumor, correlates with prognosis in melanoma patients.[74] In addition, clonally expanded T cells detected in the tumor are predominantly derived from cells not present prior to αPD-1 therapy, whilst existing TILs did not expand and did not adopt a non-exhausted phenotype.[75] Taken together these data suggest that, at least for ICI therapy, treatment does not necessarily reverse the phenotype of exhausted T cells.

T-cell exhaustion is a continuum, and early on the pathway Tex cells can proliferate, produce chemokines and retain cytotoxicity.[66,76] These "early" exhausted cells have been variously called $Tex^{prog}$ or $Tex^{int}$ cells,[77] stem-like T cells[78] or precursor exhausted T cells.[79] Whilst the nomenclature varies, these stem-like T cells are commonly defined by expression of the transcriptional regulator, T-cell factor 1 (TCF1). Increasingly it is becoming clear that $TCF1^{+}$ Tex cells are the major targets of ICI.[67,69,80] Therefore, ICI therapy most likely acts on stem-like T cells within the tumor and as discussed above, at other sites such as the TDLN.

Whilst data from ICI studies suggest that early Tex cells can be reinvigorated, it is not clear how therapeutic neoantigen vaccines will perform in the face of existing T-cell exhaustion. In particular, whether vaccination can reverse established T-cell exhaustion and if so, how? Also, if vaccination recruits T cells from the naïve pool, is this impacted by ongoing immunosuppression? No direct study has determined that exhausted T cells can be reinvigorated by vaccination. However, circumstantial evidence does suggest this is possible. In a mouse MC-38 tumor model, therapeutic vaccine efficacy can be demonstrated despite neoantigen-specific T cells

expressing markers of T-cell exhaustion before vaccination.[54] In a mesothelioma clinical trial, improved response rates, in a DC plus allogeneic tumor lysate vaccine, were associated with a preexisting TCR repertoire and the expansion of the $PD1^{+}CD8^{+}$ population.[81] In addition DC-based neoantigen vaccines in melanoma patients recruit preexisting T cells and naïve T cells as determined by TCR sequencing.[6] It is not possible to determine from these studies if vaccination reversed the phenotype of individual Tex cells. However, they do suggest that vaccination is able to augment T-cell immunity even in the presence of an established tumor.

Overcoming T-cell exhaustion may require a combination of neoantigen vaccination plus ICI therapy. In a Lewis lung carcinoma (LLC) model, Li and colleagues demonstrated that dual ICI therapy (anti-PD1 plus anti-CTLA4) led to an expansion of neoantigen-specific T cells within the TME, and that these cells attained more stem-like features and gained effector functions.[82] Surprisingly, however, prophylactic vaccination followed by dual ICI therapy did not improve the efficacy of ICI therapy, nor limit the transition of neoantigen-specific T cells to exhausted phenotype.[82] In contrast however, Knuschke and colleagues, in a chronic retroviral mouse model, demonstrated that combined vaccination and anti-PD-L1 therapy led to recruitment of activated cells from the naïve T-cell pool, and enhanced the effector function of exhausted T cells.[83] Vaccination alone did not alter the phenotype of exhausted cells, resulting only in the expansion of effector T cells.[83] The scheduling of combination therapy may be critical to success. In the chronic retroviral model, ICI therapy and vaccination only synergized when given concurrently, not when staggered.[83] Likewise, in mouse cancer models (TC-1 or B16) Verma and colleagues demonstrated concurrent vaccination plus ICI therapy, but not staggered treatment, protected mice from tumor challenge.[84] Indeed, staggered therapy, where anti-PD1 was given prior to vaccination, lead to the expansion of a population of dysfunctional $PD1^{+}CD38^{hi}$ CD8 T cells and anti-PD-1 resistance. The authors concluded that the anti-PD -1 resistance was due to suboptimal priming in a tumor environment.[84] Notably, in the study by Li and colleagues,[82] PD-1 therapy was given after prophylactic vaccination.

While neoantigen vaccination can enhance both existing and preexisting immunity in clinical trials,[6] it is possible that exhaustion applies a limiting brake on the efficacy of this response, with some trials failing to demonstrate the boosting of existing responses.[13] More work is required to understand what types of Tex cells can be reinvigorated and if adjunct therapies such as ICI will be required for maximal efficacy. In particular, the scheduling of each treatment may be a key determining factor. These will be critical studies as most patients, given the time taken to make a neoantigen vaccine, will likely be treated with ICI therapy prior to vaccination.

### *Can choice of antigen bypass T-cell exhaustion?*

T-cell exhaustion need not be an impediment to neoantigen vaccination. The target antigens most impacted by T-cell exhaustion will be those that generate preexisting immunity, and these typically represent only 2–5% of the predicted neoantigens.[54,85,86] In contrast, as many as 40–60% of predicted neoantigens can stimulate *de novo* T-cell responses after vaccination.[7,8,10,11,60,87] These latter, novel, antigens should not have been subjected to preexisting immunosuppression. However, most clinical trials have not pre-screened for existing neoantigen responses for a number of reasons, not least of which is the low precursor frequency of cancer-specific T cells.[54,85,86] Pre-screening therefore places major costs, time and economic burden, on vaccine production whilst simultaneously targeting a T-cell population already under strong tumor mediated suppression. In contrast inducing de novo responses does not require pre-screening, should recruit novel specificities, and is not subjected to preexisting exhaustion. The relative merits of vaccinating against existing or novel T cell targets are summarized in Figure 3.

Until mechanistic studies have been performed showing conclusively that vaccination can reverse the phenotype of exhausted T cells or that targeting existing responses is more effective, the added burden of pre-screening could be avoided. Indeed, this strategy could improve vaccine efficacy via the inclusion of sub-dominant antigens that can augment vaccination when combined with ICI.[88] However, not all of these novel antigens will be naturally presented by the tumor, meaning that these vaccines are likely to need multiple targets to be effective.[6] Animal models and well controlled clinical trials will be essential to disentangle these questions to determine what neoantigen to target and how many candidate antigens are too many or too few.

## Is CD4 T-cell help required for vaccine efficacy?

To date neoantigen vaccines have focused predominantly on MHC-I restricted targets. This is largely because CD8 T cells are considered the main effector cell capable of killing tumor cells, but also because the prediction algorithms for MHC-I binding have in the past been considered superior to the MHC-II prediction algorithms. Despite this, and as discussed above, responses to predicted MHC-I restricted epitopes are frequently skewed to the CD4 T-cell compartment. Peptide vaccines typically employ long peptides (20–30 amino acids) variously called synthetic long peptides (SLP) or immunizing long peptides as these are more effective at inducing immune responses than shorter peptides encoding only the predicted minimal MHC-I epitope. The increased efficacy of SLP is believed to be due to the requirement for antigen processing and presentation. However, given the skewing of response to CD4 T-cells in neoantigen clinical trials, it is likely that the enhanced efficacy of SLP is due, at least in part, to the induction of CD4 T cells and their potential role as helper cells for CD8 T-cell activation.

A number of preclinical mouse studies have demonstrated the importance of CD4 T cells in tumor control.[60,89–93] Indeed in some models CD8 T cells alone are insufficient for vaccine mediated tumor rejection, with CD4 T-cell help required to augment CD8 T-cell priming and maturation.[94] Vaccines containing only MHC-II restricted neoantigens induced tumor protection and epitope spreading to unrelated MHC-I restricted epitopes.[60] Protection and epitope spreading was mediated via cross-priming, as anti-CD40 or anti-CD8 treatment abrogated vaccine efficacy.[60] Likewise adoptive transfer of neoantigen-

specific CD4 cells have shown efficacy in a patient with metastatic cholangiocarcinoma.[92] During the priming of antitumour responses, CD4 cells induce a transcriptional pathway in antitumor CD8 T cells that promotes the downregulation of inhibitory receptors including PD-1 and increases CD8 T-cell migration into the TME.[95] Enhanced CD8 responsiveness appears to be primarily mediated via DC-expressed CD70 and CD8 T-cell-expressed CD27[95]. When MHC-II peptides have been specifically linked with class I peptides, improved efficacy has been demonstrated in vaccine studies.[96] Finally, CD4 help may reverse or prevent CD8 T-cell exhaustion.[97]

With a few exceptions,[8,14] most neoantigen trials have not sought to specifically include MHC-II restricted neoantigens, however the data presented above suggest that targeting CD4 T cells may be critical for successful vaccinations. Optimal vaccination may even require that encoded MHC-I and MHC-II restricted antigens are contained within the same sequence. A number of improved MHC-II prediction algorithms have been developed in the past few years that have been trained using MHC-II peptide elution data[98–100] which is expected to improve neoantigen identification for these approaches.

## Does neoantigen presentation affect vaccine efficacy? – The role of specific HLA alleles

Neoantigens can be presented by any of the six HLA class I (HLA-A, HLA-B and HLA-C) molecules expressed by each patient. However, many trials focus on one or two common HLA alleles, either because the prediction algorithms are better trained for these molecules or because reagents for T-cell tracking, such as MHC-I tetramers, are readily available. For instance, the Rosenberg group has focused on HLA-A alleles[101] because these genes may be more highly expressed in melanoma.[102] Hilf and colleagues focused on HLA-A*02:01 and HLA-A*24:02[9]. Several other studies have focused on HLA-A and HLA-B alleles.[7,9] HLA-C alleles are typically expressed at lower levels than either HLA-A or HLA-B alleles and whilst not the overt target of neoantigen vaccines, neoantigens restricted by HLA-C alleles have been used in neoantigen-specific T-cell therapy.[103] The rationale for limiting the choice of antigens to specific HLA molecules and alleles in early clinical trials is understood; however, there are several reasons that choosing multiple MHC-1 molecules may improve efficacy. Targeting multiple alleles should limit individual allele silencing and limit peptide competition for the same restriction element, although different alleles may also compete for peptide.[104,105] Finally, higher HLA class I heterozygosity, as well as divergence at the peptide binding domain, correlates with increased survival post ICI therapy[106,107] suggesting that targeting multiple alleles will result in improved vaccine efficacy.

## Concluding remarks

Neoantigen vaccines hold great promise, particularly as an adjunct to other forms of immuno- or conventional-therapy. However, many outstanding questions remain to be addressed. Most pressing of these questions is the type and number of antigens to include in a vaccine, and how these will be identified and prioritized. Also important will be the role that ongoing immunosuppression places on vaccine efficacy, specifically if

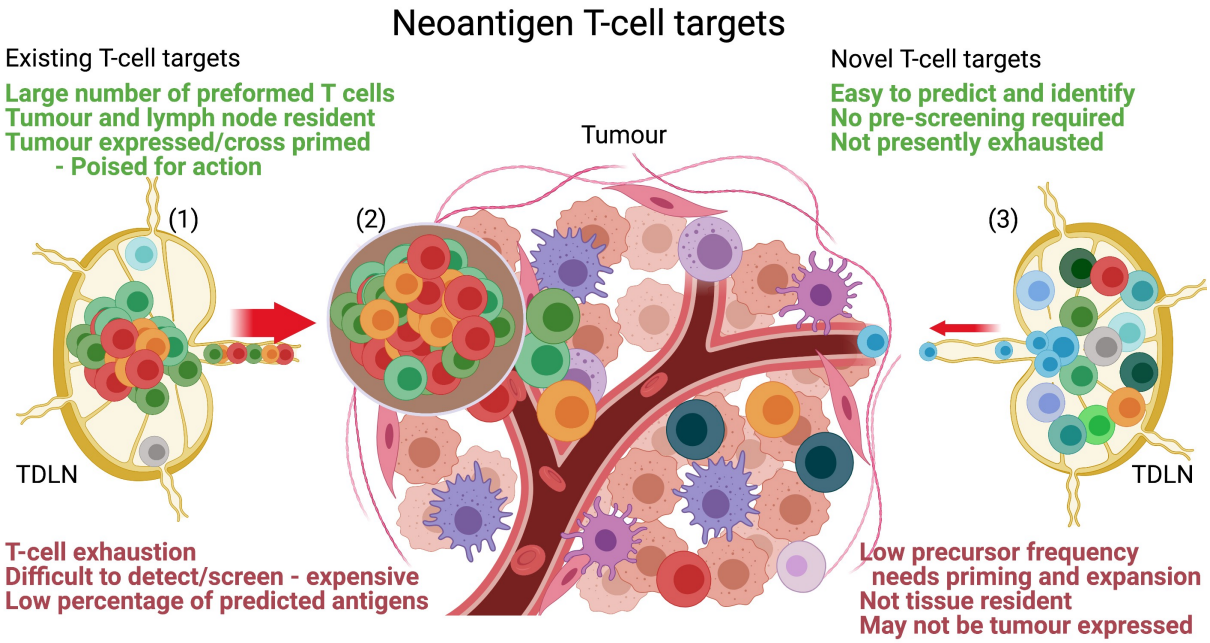


**Figure 3.** Neoantigen T-cell targets. Vaccine candidates can be derived from existing T-cell targets or from novel T-cell targets. In the former instance, T cells specific to these antigens have undergone clonal expansion and are prevalent in the TDLN (1) and the TME (2). Consequently, vaccination may rapidly recruit T cells into the tumor from the TDLN or reactivate extant TILs. However, targeting these rare antigens requires extensive pre-screening and existing T-cell exhaustion, if present, may be difficult to reverse. In contrast novel T cell targets are readily identifiable by epitope prediction algorithms and have the capacity to broaden the anti-tumor T-cell repertoire, as well as bypass existing T-cell exhaustion. However, T cells specific for novel targets have a low precursor frequency (3) and require priming and expansion prior to migration to the TME, meaning that therapeutic efficacy may be delayed. In addition, not all novel neoantigens will be expressed by the tumor. The relative value of targeting these two types of neoantigen is currently unknown. TDLN, tumor draining lymph node; TILs, tumor infiltrated lymphocytes; TME, tumor microenvironment. Figure created with BioRender.com.

T-cell exhaustion be reversed, minimized or circumvented. This is a critical, and unique, challenge to neoantigen vaccination that is not faced by conventional vaccines that target infectious agents. Much is to be learned in this space and it is likely that neoantigen vaccines will be most effective as an adjunct to other forms of therapy, in particular those that modulate preexisting immunosuppression.

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