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Identification of neoantigens for individualised cancer immunotherapy

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Abstract

Cancer is characterised by an accumulation of somatic mutations. These can generate cancer-specific neoepitopes that are recognised by autologous T cells in the tumour-bearing host. As neoepitopes are not subject to central immune tolerance and not expressed in healthy tissues, they are attractive targets for therapeutic cancer vaccines. However, the vast majority of cancer mutations are unique for the individual patient. Harnessing the full potential of this rich source of targets requires individualised treatment approaches. A large body of computational algorithms and machine learning tools were developed for identification of mutations in sequence data, prioritisation of those likely to be recognised by T cells and the design of a tailored vaccine for every patient that is composed of multiple cancer mutations. The main scope of this review is to fill gaps in the integrated understanding of basic mechanisms of T-cell recognition of neoantigens and computational approaches for somatic mutation discovery and neoantigen prediction for cancer immunotherapy. We present a new classification distinguishing between guarding, restrained, and ignored neoantigens that is motivated by the key question of how neoantigens confer competent anti-tumour immunity in a given clinical context. Such a context-based differentiation will contribute to a framework connecting neoantigen science to clinical settings and medical peculiarities of cancer disease and will enable future neoantigen-dependent therapies to provide greater clinical benefit.

1 Introduction

Mutated gene products can act as tumour neoantigens when their peptide breakdown products are presented as neoepitopes **[G]** on major histocompatibility complex (MHC) **[G]** molecules of the patient and recognised by $CD4^+$ or $CD8^+$ T cells[1–4]. T cells recognising neoepitopes with high avidity have been shown to drive efficacy of cancer immunotherapies such as immune checkpoint blockade (ICB)[5–10] and adoptive T-cell transfer **[G]**[11,12] The

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Competing Interest

F.L. has nothing to disclose. B.S., M.L., Ö.T. and U.S. are inventors on patents related to some of the technologies described in this article. Ö.T. is shareholder and CMO at BioNTech SE. U.S. is co-founder, shareholder and CEO at BioNTech SE.

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number of somatic mutations in a tumour correlates with T-cell infiltration and is predictive for overall response rate and survival prolongation by immunotherapies across various cancer types[13].

Not every mutation gives rise to a neoantigen. It has been reported that spontaneously occurring neoepitope-specific T cells reflect only 1-2% of the mutations in an individual tumour[14,15]. Also, not all neoantigens are equal in their ability to mediate T-cell mediated tumour cell killing and an anti-tumour effect. This review is focussed on neoantigens of relevance. These are defined as somatic mutations in cancer cells that are recognised by T cells and do contribute to manifesting an anti-tumour effect.

As somatic cancer mutations are not expressed in healthy cells and as T cells that may recognise them are not subject to central immune tolerance, neoantigens are considered safe and potent targets for T-cell-based immunotherapies. Due to the random nature of occurrence, somatic cancer mutations are highly individual. Each cancer patient has a unique mutation profile and presents a unique composition of neoepitope/MHC complexes (called 'neoantigenome') on their cancer cells[16]. Therefore, the clinical use of cancer mutations calls for a truly individualised approach, which is associated with multi-faceted challenges.

While individualised cancer vaccines are the main scope of this review, predicting neoepitopes is also of interest for clinical applications in the cell therapy field, e.g. *ex vivo* stimulation of autologous T cells for enrichment of neoepitope specificities or cloning of neoantigen-specific TCRs (T-cell receptor) for T-cell reprogramming.

Engineering of an individualised cancer vaccine (Fig. 1) starts with the identification of tumour-specific non-synonymous **[G]** variants in protein-coding genes by comparing next-generation sequencing (NGS) data from the patient's tumour and healthy tissue. Multi-component computational pipelines assess the mutant peptide regions for binding to the patient's HLA (human leukocyte antigen) alleles and evaluate additional features of that mutated region (e.g. transcript expression level, clonality **[G]** and dissimilarity to self) that may contribute to the capability of a neoantigen candidate to induce potent and clinically meaningful anti-tumour T cells. Such data informs the selection of a tailored set of neoantigen candidates for on-demand production of a vaccine of unique composition for each patient[17–20].

Accurate identification of mutations and selection of relevant neoantigen candidates guided by biological knowledge are the rosetta stone for clinical success of individualised neoantigen vaccination. The need for algorithms to serve this purpose has created a new, fast-evolving and highly cross-disciplinary research field.

This review explains basic immunological mechanisms involved in the mode of action of neoantigen vaccines. It gives a comprehensive overview of currently used algorithms and computational pipelines to predict neoepitope candidates, the biological features they assess, and their implementation from the perspective of clinical translation. It proposes a novel concept for classification of neoantigens based on the clinical context for which they are of interest. Challenges met in clinical translation of neoepitope cancer vaccines when

computational approaches become a part of the highly regulated drug development process are discussed, as are future directions for the field.

2 Basic principles of neoantigen presentation, T-cell recognition and immunity

Neoantigen-specific T-cell immunity follows basic principles of T-cell priming, activation and effector function, which involve mechanisms occurring in two compartments, namely in the tumour and in lymphatic tissue (Fig 2).

2.1 Presentation of neoantigens

Like any other endogenous cellular protein, neoantigens expressed in cancer cells undergo proteasomal degradation into smaller peptides. The peptides are processed in the endoplasmic reticulum and are loaded onto MHC-I molecules **[G]**. The resulting peptide/MHC-I complexes including those, which harbour neoepitopes, are presented on the cancer cell surface for recognition by $CD8^+$ T cells. Tumour cells may express MHC-II molecules either constitutively (which is rarely the case) or upon induction by interferon (IFN)-γ[21]. MHC-II molecules preferentially present peptides originating from exogeneous proteins or peptides from endogenous proteins that accessed the secretory and endocytic compartments. Binding of a peptide to MHC-II has less stringent sequence and length requirements than binding to MHC-I. Therefore, the likelihood of mutant peptides to be presented on MHC-II and the diversity of neoepitope/MHC-II complexes is higher and the mutanome **[G]** is particularly poised for immune recognition by $CD4^+$ T-cells [22,23,20]. The same mutation can be presented by both MHC-I as well as MHC-II when the respective neoantigen accesses both processing pathways and when the patient has an MHC-I as well as an MHC-II allele that is capable of complexing the respective mutated peptide with sufficient affinity[19,20]

In addition to tumour cells, cancers harbour immune cell infiltrates, including dendritic cells (DCs), macrophages and B cells that can act as professional antigen-presenting cells (APCs). Antigen uptake by these APCs occurs by mechanisms such as macropinocytosis of soluble antigens, receptor-mediated uptake of apoptotic vesicles by DCs, phagocytosis of tumour cells by tumour-associated macrophages, or Fc-receptor-mediated uptake of immune complexes. Tumour-infiltrating APCs activate antigen specific memory $CD4^+$ and $CD8^+$ T cells that have previously undergone cognate priming.

However, neither cancer cells nor tumour-infiltrating APCs are capable of direct priming naïve T cells. Priming of naïve T cells occurs almost exclusively in lymph nodes (LN) through highly specialised LN resident DCs. These professional APCs either sample soluble neoantigens from the extracellular fluid drained by lymph vessels from the tumour tissue[24,25] or by active transfer from migratory APCs that had taken up the respective neoantigens in the peripheral tumour tissue[26–28]. Endocytosed antigens are processed and presented on MHC-II complexes for scanning by $CD4^+$ T cells. DCs are also capable of routing endocytosed antigens into cytosolic compartments for proteasomal degradation and presentation on MHC-I molecules. This process called ‘cross-presentation’ is critical for priming and stimulation of antigen-specific cytotoxic $CD8^+$ T cells[29,30].

2.2 Priming of neoantigen specific T cell responses

The priming, activation, expansion and subsequent fate of neoantigen-specific T cells is tightly controlled by fine-tuned mechanisms and affected by parameters, such as the density and stability of the peptide/MHC complexes on APCs, the precursor frequency and avidity of antigen-specific T cells, and the presence of costimulatory signals. The naïve T cell repertoire is shaped by central immune tolerance **[G]** established during thymic development, which involves the elimination of high-avidity autoreactive T cells that recognise MHC-I and MHC-II epitopes derived from germline-encoded self-antigens. As neoepitopes are non-self, they are not impacted by this mechanism. Naïve T cells recognising neoantigens remain susceptible to mechanisms of peripheral tolerance **[G]** which encompass clonal deletion[31], conversion to regulatory T cells[32] or induction of dysfunctional states such as anergy **[G]**[33] and exhaustion[34,35]. Priming of naïve T cells requires a high level of peptide/MHC complexes along with co-stimulation[36]. Once they have been primed in the LN and transitioned to the memory state, cognate activation of T cells can be achieved with much lower levels of neoantigen presentation. The quantity of peptide/MHC complexes i.e. the level of epitope presentation is a function of gene expression, affinity of peptide/MHC binding and stability of the peptide/MHC complex[37,38]. Accordingly, neoantigens that are expressed at a robust level in the tumour and provide neoepitopes of sufficiently high affinity to MHC-I or II have a higher likelihood for effective cross-presentation of endocytosed antigens and priming of naïve T cells. Below these critical levels, neoantigens will induce neither T cell immunity nor tolerance.

2.3 TCR diversity [G] and TCR degeneracy [G]

TCRs bind to peptide/MHC complexes by interacting with a few contact sites of the peptide's side chains that project out of the MHC groove (reviewed in[39,40]). The same neoepitope/MHC complex can engage T cells with diverse TCRs that may be composed of molecularly different TCR alpha and beta chains[20,41–44] (Fig 3a,b). TCR diversity is expected to be broader for neoepitopes that have a higher dissimilarity to MHC ligands derived from self-antigens[45], e.g. mutations that convert a non-binding peptide to a binding neoepitope, or novel open reading frames created by INDELs and gene fusions which alter more than merely a single amino acid. Also, higher dissimilarity to self is associated with a higher likelihood that potential high affinity binders were not deleted by central immune tolerance mechanisms.

Given the low affinity of a functional TCR/peptide/MHC interaction, a single TCR is able to bind various different peptide/MHC complexes, including epitopes that do not necessarily share sequence homology with each other and differ structurally[46–48]. Due to this TCR degeneracy, a mutant peptide may be recognised by cross-reactive memory T cells that were primed against an unrelated antigen e.g. from commensal bacteria or microbial pathogens[49,50]. Activation of T cells which were primed by an unrelated antigen is known as heterologous immunity **[G]** (Fig 3b,c).

2.4 Neoantigen driven immune effector mechanisms

Upon encountering antigens under conditions of co-stimulation, naïve $CD8^+$ and $CD4^+$ T cells are activated, expand by repeated cycles of cell division, leave the LN, and differentiate

into PD1$^+$ effectors and memory T cells that are capable of infiltrating tumours. In the presence of a favourable tumour microenvironment (TME), activated neoantigen-specific T cells exhibit their effector functions by recognition of their antigens on intratumoural APCs and tumour cells and may indirectly or directly contribute to tumour control[11,18,51–54].

CD4$^+$ and CD8$^+$ T cells collaborate in the eradication of tumours. While CD8$^+$ T cells exert direct cancer cell killing, neoantigen-specific CD4$^+$ T cells have a variety of effects that may promote profound inflammatory remodelling of the TME. CD4$^+$ T cells may also exhibit direct cytotoxicity against tumour cells expressing MHC-II[53,55–59] IFN-γ secretion by CD8$^+$ and CD4$^+$ T cells upon cognate antigen recognition induces an upregulation of MHC-I and II presentation on tumour cells and APCs which further sensitises recognition of neoantigens. Inflammation supports the cytotoxic activity of neoantigen-specific CD8$^+$ T cells. The killing of tumour cells and release of tumour antigens results in antigen spreading[51,60,61] and further re-stimulation and expansion of memory CD4$^+$ and CD8$^+$ T cells. The iteration of this sequence, called the cancer-immunity cycle[51] is counteracted by numerous immune suppressive mechanisms that have evolved to safeguard from autoimmunity[62,63].

The ultimate objective of a cancer vaccine is to re-ignite the cancer immunity cycle by priming of novel neoantigen-specific T cells or by activation of pre-formed ones, thus fostering a sustained adaptive anti-tumour immune response until tumour cells are completely eliminated.

Early evidence suggests that intratumour presentation of MHC-II neoepitopes is capable of stimulating and clonally expanding neoantigen-specific CD4$^+$ T cells with a FoxP3+ regulatory T-cell (Treg) phenotype[64]. The TCR repertoire of the intratumorally expanded oligoclonal Treg T-cell population overlaps with the repertoire of peripheral blood Tregs and differs markedly from the intratumoral TCR repertoire of the conventional FoxP3$^-$ CD4$^+$ T-cell population[64,65]. This suggests that intratumoral Treg cells are either specific for neoantigens that differ from those recognised by conventional CD4$^+$ helper T cells or that they are derived from a different T-cell pool. It is also not clear whether these neoantigen-specific Treg cells arise from suboptimal priming of naïve CD4$^+$ T cells driven by exposure to neoantigens under noninflammatory tolerogenic conditions[66] or are derived from already established cross-reactive Treg T-cell populations[67]. It is conceivable that neoantigen-specific Treg cells could attenuate anti-tumour immunity in an antigen-specific manner, regardless of their origin, and that a better understanding of their specificity could help to improve neoantigen prediction algorithms.

2.5 Immune surveillance [G], immune escape [G] and immune editing

Heterogeneity is a hallmark of cancer. The genetic evolution of tumours is driven by selection of clones with fitness advantage. The dynamic interplay between immune surveillance and tumour progression[50] results in primary and metastatic lesions of diverse clonal composition (Fig. 4).

Evasion from T-cell immunity does occur in the course of a tumour's natural evolution as well as under treatment and there are various mechanisms for tumours to escape immune surveillance.

Tumours may create an immuno-suppressive TME by up-regulating molecules such as PD-L1[68], TGF-beta[69] or by promoting expansion of regulatory T cells[70] that protect them from neoantigen-specific T cell immunity.

Further, tumour clones that are recognised by functional neoantigen-specific T cells may become subject to immune editing[50]. Selection of neoantigen-loss variants appears to occur frequently in immune-infiltrated tumours of treatment-naïve patients[71–73], yet rarely in tumours with insufficient immune cell infiltration[70,74]. Combining multiple neoantigens in a vaccine rather than relying on a single antigen mitigates the risk of escape by antigen loss.

Alternatively, cancer cell clones may be selected that have defects in the antigen-processing/-presentation machinery, such as loss of heterozygosity (LOH) **[G]** of MHC genes, downregulation and mutation of MHC molecules[75], dysfunction of the transporter for antigen presentation (TAP)[76] or mutations in the beta-2-microglobulin (B2M)[77]. These alterations disable cognate anti-tumour immunity at its roots and render tumours resistant to any treatment that is based on activity of neoantigen-specific T cells. This is where combination therapy comes in play with the objective of combining therapeutic vaccines with treatment modalities that have non-overlapping modes-of-action.

The risk for tumour immune escape is higher in metastatic disease. Each metastatic lesion can be viewed as independent island with its own immune microenvironment, immune escape strategies, evolution dynamics and neoantigenome[78–80].

ICB can indirectly contribute to eliciting new neoantigen-specific T-cells as neoantigens released from dying tumour cells are taken up by APCs and T cells are primed and undergo efficient activation and expansion under the effect of ICB. This process is called antigen spreading and plays an important role in broadening and enriching the repertoire of anti-tumour T-cell responses[60,61,81]. Patients may develop resistance to ICB by outgrowth of subclones that do not express the restrained neoantigens and thus are not recognised by ICB-mobilised T cells (Fig. 4).

3 A context-based classification of neoantigens

One way to categorise neoantigens is based on the type of somatic mutation that creates the altered epitope and defines its molecular characteristics (Box 1). Single nucleotide variations (SNVs) in coding regions are the mutation type that is best studied in clinical testing. An important future field is to develop discovery tools for neoantigens created by cancer-specific INDELs (insertions and deletions), fusion genes and splice variants that have a lower degree of similarity to self-antigens than SNV-derived neoantigens.

We propose an orthogonal classification of neoantigens (Fig. 5, Table 2) that is motivated by the key question how to identify relevant neoantigens that convey proficient anti-tumour immunity. We believe that the answer to this question may differ depending on the clinical

context and needs to consider that mechanisms and cancer life cycle effects that may drive the formation of neoantigen-specific immune responses are diverse. It is yet not clear why some neoantigens induce functional T-cell responses spontaneously and others need intervention to do so. Another unknown is whether we can learn from neoantigens targeted by the most abundant, immunodominant, prognostically favourable T-cell specificities in treatment-naïve patients or from those which are associated with deriving clinical benefit from ICB therapy in order to improve computational pipelines for neoantigen vaccine design. Also, while the term 'tumour rejection antigen' has been coined for relevant antigens that induce proficient immunity and several such neoantigens have been reported in mouse models, the context, within which those tumour rejections occurred, differ as do the implications those individual studies may have for vaccine design (Fig.6).

The classification below differentiates neoantigens based on the clinical setting in which they gain relevance. It is meant to provide a framework that guides neoantigen discovery and characterisation studies, and helps to structure and analyse new and available datasets to address gaps in our understanding and develop a tailored approach to define neoantigen candidate features for vaccine design and beyond.

3.1 Guarding neoantigens [G]

Tumours are subject to T-cell surveillance. Consequently, spontaneously occurring neoantigen-specific $CD4^+$ and $CD8^+$ T cells are found in patients with treatment-naïve cancers[15,41,50,82] Such neoantigens may have a guarding function by mediating early tumour rejection before a tumour becomes clinically apparent. Or they may decelerate tumour growth, inhibit metastatic dissemination and prevent recurrences after surgical removal of the primary tumour. The characteristic feature of guarding neoantigens is that their expression in the tumour is sufficient to drive clinically relevant anti-tumour immunity in the absence of immunotherapy. Guarding neoantigens may come in two flavours. Firstly, strongly antigenic ' supreme' neoantigens that are robustly expressed in tumour cells and form neoepitopes with extraordinarily high-affinity MHC binding[83] and stability (Fig 5). These features promote early onset priming and rapid and strong expansion of neoantigen-specific cytotoxic T cells that infiltrate and suppress the growth and metastatic dissemination of the primary tumour early in its life cycle before full manifestation of an immunosuppressive TME[83]. Guarding neoantigens are difficult to identify in human. The strongest evidence for the existence of such tumour rejection antigens comes from engraftment studies of very high mutational load mouse tumours with thousands of somatic mutations induced by UV irradiation or carcinogens. In these models, wild-type tumour cells expressing the respective neoantigens are rejected by naïve mice, whereas immune-edited tumour cell clones that have lost the respective neoantigens but express all other mutations grow aggressively[83–85]. Immunodominant neoantigens are derived from extremely rare mutational events and contribute to an improved clinical prognosis probably only in very high-mutational load tumours such as microsatellite instable cancers[86].

The second guarding neoantigen type is recognised by pre-established, cross-reactive memory T cells. Examples are neoantigens cross-recognised by T cells formed against gut microbiota, previously encountered pathogens or persistent viruses[50,87]. Neoantigen

recognition by heterologous T cells has two important effects. Firstly, memory T cells have a 50-fold lower functional activation threshold and respond faster as compared to naïve T cells[88]. Thus, cancer mutations with low MHC binding affinity, low peptide/MHC/complex stability or with low expression level that are incapable of priming naïve T cells may engage and expand pre-established cross-reactive memory T cells. Second, by definition pre-formed heterologous immunity is existent prior occurrence of tumour disease. Neoantigen recognition by cross-reactive, memory T cells early in the life cycle of a tumour may significantly shape the tumour-specific TCR repertoire towards high-affinity TCR binders, promote T-cell infiltration and growth inhibition of tumours, including those with low mutational load[41]. Heterologous immunity may explain that more than a quarter of neoantigen-specific T-cell responses identified by an unbiased screening with tumour-infiltrating lymphocytes (TIL) is directed against neoepitopes with low (>500 nM) predicted HLA binding affinity[15]. Accordingly, clinically relevant heterologous T-cell immunity against neoantigens is expected to be largely driven by memory T-cell repertoire and by the affinity of the TCR to the peptitde/MHC complex rather than by MHC binding affinity of the mutant peptide. Neopitopes that are able to stimulate a more diverse TCR repertoire, e.g. those with a higher dissimilarity to self-antigens[45] may be more likely to qualify for this subclass of cross-reactive neoantigens.

By definition, guarding neoantigens control the natural course of the disease and are associated with favourable prognosis of immunotherapy-naïve patients irrespective of the treatment. Only a few studies investigated the correlation between molecular neoantigen features **[G]** and favourable disease outcomes in suitable populations. One study showed that tumours of long-term survivors with pancreatic cancer in contrast to those from short-term survivors harbour neoepitope candidates displaying a composite quality feature of (i) sequence homology with pathogen-derived peptides and (ii) stronger predicted HLA binding affinity of the neoepitope relative to its wild-type[50] (differential agretopicity index **[G]**; DAI). Similarly, another study identified the mean DAI across all clonal mutations in a given tumour as predictor for increased survival in melanoma and lung cancer patients[89].

ICB treatment or neoantigen vaccination may further augment pre-existent T-cell responses against guarding neoantigens qualitatively or quantitatively. A potential disadvantage of guarding neoantigens is that they are targeted early in the course of disease and thus are at risk for early immunoediting **[G]**[41,72].

3.2 Restrained neoantigens [G]

Not all neoepitope-specific T cells that occur spontaneously in patients are fully functional. Neoantigen-specific T cells that are pre-existent but functionally impaired may require further invigoration to contribute to a favourable course of the tumour disease. This can be achieved by ICB therapy for which durable clinical responses have been shown to correlate with the expansion of neoepitope-specific T cells[5–7].

We designated targets that are recognised by ICB reinvigorated T cells as restrained neoantigens. While restrained neoantigens are capable of priming T-cell responses, their antigenicity is weaker compared to supreme neoantigens and the primed T cells are not proficient or not sufficiently expanded to prevent disease progression. T cells primed by

restrained neoantigens infiltrate tumours and recognise their targets on cancer cells and APCs but are outpaced by tumour growth and immunosuppressed by the established TME. Antigen-pulsed migratory DCs require several days to get from the tumour to LN-resident DCs[36] and priming of naïve T cells in lymphoid tissues requires a high level of neoantigen presentation. Thus, mutated peptides need to be robustly expressed in the tumour, exert high-affinity MHC binding and build stable peptide/MHC complexes to give rise to restrained neoantigens (Fig 5b).

In contrast to guarding neoantigens that are identified based on their prognostic **[G]** impact, restrained neoantigens are defined by their predictiveness **[G]** for the clinical benefit conveyed by immunotherapies such as ICB (Table 2). Datasets for studying restrained neoantigens and their specific features may e.g. come from randomised trials that compare ICB treatment to some non-T-cell-activating standard of care.

One study showed that all identified T-cell responses in patients responding to ICB were directed against clonal neoantigens[90]. The clonality of mutations and the number of predicted neoepitope candidates per mutation[91], the DAI[89] and sequence similarity to known pathogen epitopes combined with the ratio-based DAI[92] were found to be associated with clinical response to ICB. Oncogenic driver mutations **[G]** are typically clonal as they are critical for the survival of tumour clones and therefore less likely to be lost during immune editing. Neoantigen candidates derived from driver mutations were predicted more frequently in patients who responded to ICB[93]. In turn, patients with MHC alleles predicted to have poor presentation of driver mutations were shown to less likely respond to ICB[94]. SNVs[5,7,95], as well as frameshift mutations and gene fusions have been reported to act as restrained neoantigens[96–99].

Restrained neoantigens are discussed in this review in the context of ICB due to the clear association of their clinical effect with reactivation of impaired T cells. This principle can be extended to other immune-modulating therapies, e.g. T-cell homeostatic cytokines such as interleukin-2 (IL-2)[100], once they have been shown to convey clinical benefit through pre-existent yet functionally impaired neoantigen-specific T cells.

3.3 Ignored neoantigens [G]

Only a very small fraction of mutations in a given human cancer appear to induce spontaneously occurring T-cell response[14,15,101]. Similarly in mice, a substantial fraction of mutations identified by NGS in syngeneic tumours were not spontaneously immunogenic[23].

The lack of spontaneous immunogenicity does not mean that T cells against these ignored antigens would not be capable of contributing to tumour rejection. In fact, systematic immunogenicity studies in mice showed that 15-40% of the cancer mutations identified by NGS in murine tumours induce robust T-cell responses (with more $CD4^+$ than $CD8^+$ ones) when used as vaccine antigens[23,102]. A large portion of the induced immune responses were of high magnitude and resulted in shrinkage and rejection of established tumours, antigen spreading **[G]** and changes to the immunosuppressive environment[23]. Several clinical trials using individualised neoantigen vaccines in patients with high and low mutational load tumours such as melanoma, lung cancer, glioblastoma, ovarian cancer and pancreatic

cancer[19,20 103–108] (reviewed in[109,110]) showed up to 70% immunogenicity across all neoepitopes used in those vaccines. The vast majority of these immune responses were not detectable prior therapy and *de novo* induced by vaccination[19,20]. The relevance of vaccine-induced neoantigen immune responses was supported by detection of infiltrating vaccine-induced T cells in post-treatment biopsies, killing of autologous patient-derived tumour cell lines *in vitro,* and patients with shrinkage of tumour lesions, objective clinical responses or reduction of recurrences[20].

We propose the term ignored neoantigens for these mutant gene products that, although presented on MHC molecules, are incapable of eliciting a T-cell response and require vaccination to induce a clinically relevant T-cell response. We hypothesise that ignored neoantigens are characterised by a moderate level of neoepitope presentation which is below the threshold for priming of naïve T cells but above the level for recognition by memory T cells (Fig. 5). The purpose of a vaccine is to load LN-resident DCs with sufficient amounts of neoantigen to achieve priming. A substantial proportion of mutations encode neoantigens with either low expression and high MHC binding affinity, high expression and low binding affinity, or moderate expression and binding affinity. Thus, ignored neoantigens are a rich and complementary source of targets for neoantigen vaccines or cell therapy with individualised TCR-engineered T cells. Ignored neoantigens may be particularly relevant to stimulate poly-specific T-cell responses in patients with low mutational load tumours. Even though lower in frequency, guarding and restrained neoantigen candidates may be also highly relevant targets, to include into personalised vaccines.

As vaccine-induced T cells up-regulate PD1[20,111] even patients who are resistant or refractory to ICB monotherapy may benefit form combining vaccines and ICB and vaccines may expand the repertoire of pre-existent T cells for ICB[107] to include ignored neoantigens. Moreover, by counteracting immunosuppressive mechanisms, ICB may lower the neoantigen presentation threshold required for priming naïve T cells, thereby broadening T cell responses by antigen spreading[60,61,81].

4 Prediction of neoantigen candidates

4.1 Immunobiology driven approaches

The most basic prerequisite for an immune response is that the aberrant gene product that results from the somatic mutation is transcribed, translated, processed and presented on MHC molecules. Therefore, verification of expression and prediction of binding affinity to the patient's MHC alleles are the two key upfront elements of current neoantigen prediction computational pipelines **[G]** (Supplementary Table 1).

Beyond these, other potentially relevant biological features are implemented into algorithms to rank neoantigen candidates (Table 3).

These include features that may impact proficiency of a presented neoepitope candidate to activate T cells (dissimilarity to self-antigens tested by sequence homology queries) or its likelihood of immune escape by outgrowth of antigen-loss variants (such as clonality of the

mutation computed by DNA sequencing data analysis or driver mutations determined by database queries).

Whereas the features described below are based on sound rationales, how to weight each of them for prioritising neoantigen candidates for vaccine design is not established, in particular given that features have not been correlated with context-based neoantigen classes. Our own benchmarking studies of neoantigen features indicate that a critical mass of available datasets for accurate prediction of immunogenicity does not yet exist, and datasets are too diverse and not standardised. Most immunogenicity studies use datasets derived from testing of pre-existing T cell specificities. These T cells are most likely a mixed basket including T cells primed by the neoantigen itself or T cells that benefit from heterologous priming by unrelated antigens. The context-based classification provided in Chapter 3 may contribute to a framework that differentiates neoantigen candidates based on the clinical question asked and tailors data mining approaches accordingly.

4.1.1 Transcript Expression—The density of detected peptide/MHC complexes correlates with protein levels and transcript expression[112]. Tumour cell clones that express neoantigens derived from high abundance transcripts are efficiently cleared under ICB therapy[113] and downregulation of neoantigen candidates is an immune escape strategy[72]. Further, transcript abundance has been shown to compensate for low MHC binding affinity of a mutation [37]. In aggregate, these data support the notion that high transcript expression is associated with a higher likelihood of functional T-cell response. Therefore, various studies use gene expression to rank neoantigen candidates[15,20,23].

To quantify the expression of a mutation and its wild-type counterpart, both transcripts are searched in bulk RNA-seq data generated by NGS of RNA extracted from a tumour biopsy. Usually, expression analysis is performed only for the tumour tissue and not for the corresponding healthy tissue sample and tumour specificity of the mutation is confirmed by exome sequencing. Quantification of altered transcripts can be fast and reliable with tools such as kallisto, which pseudoaligns **[G]** reads against a reference transcriptome to detect the most likely transcript for each read[114].

4.1.2 MHC binding, stability and cell surface presentation—The capability of a mutation to bind to at least one of the MHC alleles of the patient in question is the most elementary requirement for T-cell recognition. Collaboration of antigen-specific $CD4^+$ and $CD8^+$ T cells is critical for efficient anti-tumour immunity[115]. Expression of a single MHC-I neoantigen alone is not sufficient and at least one additional MHC-II neoantigen is required for meaningful anti-tumour immunity in mouse tumour models [53]. Accordingly, an individualised vaccine should combine neoepitopes predicted to bind to MHC-I as well as MHC-II alleles of the patient.

Published tools that predict MHC binding affinity are trained on wet lab binding affinity data and/or eluted ligands detected by mass spectrometry. Recent benchmarking studies used ROC (receiver operating characteristic)-curve **[G]** analysis as the performance metric to assess prediction tools for MHC-I binding and presentation in humans[116] or for T-cell responses in mice[117]. NetMHCpan[118] and MHCflurry[119] achieved the best ‘area under

the ROC curve' (ROC-AUC) in these studies. Whereas these both tools are trained on binding affinity and eluted ligand data, MixMHCpred[120,121] is trained on eluted ligands only. MixMHCpred predicts the likelihood of a given peptide sequence to be presented on the cell surface and achieves higher ROC-AUCs in comparison to the MHC binding tools in a benchmark study using ROC-curve analysis on a larger dataset of experimentally verified MHC-I-binding epitopes[122]. All tools perform sufficiently well for enriching peptides with decent MHC-binding properties. The lack of ligand data for rare MHC alleles is a limitation and is addressed by tools such as NetMHCpan that use MHC sequence homology with more frequent MHC alleles to infer potential ligand preferences[123].

The stability of the neoeptiope/MHC complex has been proposed to be more important for immunogenicity prediction than the binding affinity, as higher stability may increase the probability of the complex being recognised by T cells[124]. Tools for stability prediction, e.g. NetMHCstabpan perform well to enrich for immunogenic mutations and will improve further with bigger training datasets becoming available [125,126]

Patients with a complete germline heterozygosity at MHC-I loci have a better survival upon ICB therapy than patients exhibiting homozygosity for one or more MHC-I genes[127], as a higher diversity of alleles increases the likelihood for a given neoantigen to find an allele to bind[127–129].

NetChop[130] or NetCTL[131] predict proteasomal cleavage and transport into the endoplasmic reticulum by the TAP protein complex **[G]**, which are prerequisites for an epitope to eventually be loaded onto an MHC-I molecule. However, as methods for predicting MHC presentation are trained on ligands eluted from MHC, which have gone through those earlier processing steps, the value of combining cleavage, transport and binding prediction tools is questionable.

4.1.3 Dissimilarity to self and similarity to pathogen-associated epitopes—

Dissimilarity to the non-mutated wild-type sequence (in particular if it is presented by one of the

patient's MHC alleles) and, more broadly, to the self-proteome, may lower the likelihood that the respective neoepitope is subject to immune tolerance and increases the likelihood for the presence high-affinity T cells in the T-cell repertoire[132,133]. One approach to leverage the dissimilarity hypothesis is to use alignment scores resulting from BLAST (Basic Local Alignment Search Tool) **[G]** searches against the non-mutated proteome as substitute for TCR binding energies[133]. Another approach uses a kernel similarity measure for the mutated and the corresponding wild-type epitope[132]. These metrics were reported as predictive for identifying neoepitopes derived from SNVs and may be even more so for frameshift INDELs or fusion genes.

Sequence similarity of a neoepitope to pathogen sequences may be associated with a higher likelihood for cross-reactivity with preformed T cells directed against frequently encountered pathogens (reviewed in[49]). A study characterising neoantigens in pancreatic cancer patients identified the combination of DAI and sequence similarity to pathogen-

associated epitopes as features for guarding neoantigens discriminating long-term and short-term survivors[50].

4.1.4 TCR recognition—Methods that address the TCR's interaction with the peptide/MHC complex are based on predicting the amino acid side chains of the TCR that will face the MHC-bound peptide[134] or the stability of the peptide-MHC complex which is associated with higher likelihood of TCR binding[135]. Exploratory approaches are underway that subject the amino acid sequence of the TCR to an artificial neural network **[G]** to predict the binding of a given TCR to the peptide/MHC complex[136], thus circumventing the use of structural modelling. Another approach claims to predict the most likely cognate peptide/MHC target of a TCR[137] based on the TCR sequence. However, these methods are not mature yet, operate at the limit of current computational algorithms, as the diversity of the MHC/peptide/TCR combination space is huge and the available experimental training data is not sufficient to train algorithms.

4.1.5 Mutation clonality and indispensibility—The clonal architecture of a cancer sample can be assessed by analysing the variant allele frequency **[G]** identified in a patient with PyClone[138] or SciClone[139] The robustness of the prediction depends on sample quality. A sample with low tumour content, for example, is unlikely to deliver an accurate clonal architecture. For robustness, multiple samples from the same tumour may be required, which is difficult to implement in a routine clinical setting.

Clonal and truncal mutations **[G]** may be preferable over subclonal and branched mutations **[G]**, as they allow to address tumour heterogeneity, and target tumour cells with potentially higher fitness and tumour-promoting function[140]. T-cell specificities that target a few high quality neoantigens may be sufficient to drive tumour control in treatment-naïve cancer patients and may be predictive for prolonged survival in ICB treated patients[90,141]. Neoantigens that exist prior to genome doubling will have higher variant allele frequencies than those generated after genome doubling. In NSCLC more than 70% of patients have whole genome doubling as an early clonal event[142].

The majority of driver mutations appear typically early in tumour evolution and have a high likelihood of being clonal[94,143]. Driver mutations per definition promote cancer cell fitness and are considered to be stable. Databases like COSMIC[144] or DriverDB[145] list known and functionally validated driver genes. While experimentally validated immunogenic driver mutations are rare[42,146], computational methods have been developed that allow screening for novel driver mutations[147].

Passenger mutations may also occur early and be clonal[148]. The designation 'passenger' is misleading as it implies that the respective mutated gene is dispensable and that the mutation does not provide an advantage for tumour cell survival and is prone to be lost during tumour evolution. Validation of a mutation to be a driver requires extensive experimental characterisation and proof that it transforms normal cells into tumour cells. Such studies are not undertaken for rare or unique mutations. As lack of evidence is not evidence of absence, mutations dubbed as 'passenger' may well provide a biological advantage in the setting of the individual cancer disease.

Computational analyses indicate that driver mutations are less frequently presented on MHC-I and MHC-II[128,129,149,150]. For inducing anti-tumour immunity, the degree of foreignness of the vaccine antigens may be much more relevant than their functional role in the cancer cell.

4.1.6 Loss of heterozygosity of essential gene products—Genes are usually present in two copies within the genome. If an essential gene is subject to LOH and generates a neoantigen from the remaining allele, the tumour cannot escape by neoantigen loss, as the remaining allele is required for tumour cell survival. Therefore, mutations in essential genes undergoing LOH may be particularly excellent targets for neoantigen vaccination[151]. LOH in coding regions can be reliably predicted from deep sequencing[152] and microarray data analysis[153]. Genetic knockouts and gene silencing studies have provided lists of about 1,600-2,500 genes that appear to be essential for cell survival[154–156] and may facilitate prioritisation of neoantigen candidates.

4.2 Deep learning based approaches

Artificial neural networks are inspired by biological neural networks. To predict binding of epitopes to MHC molecules, artificial neural networks that were trained on data from MHC binding assays are explored[119,136,157,158]. Neural networks trained on high quality immunopeptidome data from monoallelic cell lines show excellent performance for prediction of MHC-I and MHC-II binding[37,38,159]. Also, physico-chemical properties (for example energies of attraction and repulsion, hydrogen bond energies and confirmation energies) derived from three-dimensional (3D) structure models of peptide/MHC interactions are being used to train neural networks with early promising results[160]. Experimentally generated 3D structural data (e.g. crystal structures from X-ray refraction experiments) is limited in availability. Structure-based neoantigen prediction strategies may benefit from availability of broader experimental data or as *in silico* modelled 3D structures improve in accuracy for predicting MHC/ligand interactions.

Deep learning **[G]** models (Box 2) have led to critical breakthroughs in image analysis and speech recognition[161] and are now being explored for immunogenicity prediction. Deep networks use multil-ayer architecture to adapt to complex relationships within the training dataset. They have the potential to uncover patterns in peptide sequences that are missed by other machine learning algorithms or are not reflected in current biological hypotheses.

Such networks were published for MHC-I and MHC-II binding and ligand prediction[162]. The deep learning approaches EDGE and MARIA model the presentation of MHC-I or MHC-II epitopes, and use transcript abundance and flanking sequence as additional features[163,164]. Application of MARIA[164] to perform retrospective analysis of a dataset obtained from a melanoma neoantigen vaccine study showed that enrichment for neoepitopes that induced high-magnitude $CD4^+$ T-cell responses. EDGE[163] was used for neoepitope prediction in melanoma, gastrointestinal cancer and breast cancer and enriched for neoepitopes that expanded pre-existing $CD8^+$ T-cell responses (Table 4). DeepHLA combines the prediction of a MHC binding score and an immunogenicity score in one

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model[165]. Another approach subjects the amino acid sequence of the TCR to a deep artificial neural network to predict the binding of a given TCR to the peptide/MHC complex[136].

While deep learning algorithms show promising results to a certain extent, further maturation is required prior broad use. One obstacle is the lack of sufficiently large and standardised datasets with high quality T-cell response data and discrimination between datasets reflecting immunogenicity **[G]** versus antigenicity **[G]**. Another obstacle is that datasets have to be well curated and balanced, with comparable numbers of positive and negative training samples for the network to learn correct patterns[166]. Precise deconvolution of the allele-specific peptide/MHC binding patterns is critical for the use of pan-allelic data from mass spectroscopy experiments. Moreover, deep learning approaches often lack interpretability, making it difficult for the user to deduce critical biological features.

5 Challenges in Translation

5.1 Technological challenges

5.1.1 Biosamples as analytes—As heterogeneity is a hallmark of cancer, multiple biopsies of the same tumour lesion result in different molecular profiles[78,80] and neoantigen candidates identified in one metastatic lesion of a patient differ from those in a second metastatic lesion or the primary tumour[79].

The primary tumour, even if it is a historical and archived sample, may inform on clonal and truncal mutations and on the seed clones of disseminated metastatic lesions[140]. Metastatic lesions that are biopsied at a point of time close to the planned vaccination reflect the most recent status of the neoantigenome[167]. There is evidence that guarding neoantigens expressed in the primary tumour are lost from metastatic lesions[50]. Ignored neoantigens are not subject to selective pressure and likely to be more homogenously expressed and preserved across different lesions even in advanced disease, higher metastatic load and increasing immune suppressive mechanisms.

Many protocols are based on a single biopsy that may neither fully capture the heterogeneity of the probed individual tumour lesion, nor be representative in case of oligo- or multi-metastatic disease[168]. Thus, the resultant composite neoantigen vaccines may represent a minor proportion of the targets in a patient's lesion, generating mixed responses at best.

Multi-region or even multi-lesion sequencing would require additional invasive procedures and is difficult to implement into clinical practice routines[142,169]. This dilemma is not new: standard-of-care treatments such as tyrosine kinase inhibitors or checkpoint inhibitors, for which a companion diagnostic approach determines eligibility, rely on single biopsies. This dilemma could be overcome by computational algorithms that untangle the tumour heterogeneity and infer higher order organisation of tumours based on single biopsies.

Collection and storage conditions of biosamples may affect sequencing data. Fresh frozen samples deliver the best data quality but require complex logistics. Formalin-fixed, paraffin-embedded samples are broadly available, but the fixation process is associated with sequencing artefacts[170]. Biopsies are more convenient for patients than surgical resectates

but may yield insufficient amounts of analyte or even no tumour cells [171]. As trivial as these hurdles may sound, they are relevant in practise[172]. A less invasive method such as liquid biopsy, which analyses circulating tumour DNA (ctDNA) from the patients' blood, may provide a more comprehensive representation of multi-site disease and is easier to collect. While conceptually attractive the allele frequency of mutations in plasma DNA samples is often low and current technologies are limited to the detection of a predetermined set of mutations[173]. Further technological breakthroughs are required to enable a highly sensitive, unbiased identification of cancer mutations by liquid biopsies.

5.1.2 Mutation calling—The mutation calling process **[G]** begins with cleaning of sequencing reads, followed by sequence alignment **[G]** to a reference genome. The subsequent mutation calling has to distinguish accurately somatic variants from sequencing errors, sample preparation artefacts and germline mutations. Many software tools exists to address critical limitations of mutation calling. Commonly used tools (Table 5) vary in their ability to detect different mutation classes such as SNVs or INDELs, to handle tumour heterogeneity while maintaining acceptable levels of accuracy, and to deliver within an acceptable runtime. No single perfect solution exists and often the approach is to base the called mutations on the consensus of different tools[174]. Detection of SNVs is most advanced in terms of sensitivity and specificity, whereas these performance metrics are less favourable for e.g. INDELs[175,176] or fusion genes[177].

Tumour samples display a high degree of heterogeneity, e.g. with regards to clonality, somatic copy numbers and sample contamination with healthy cells[178–180]. Data generated by sequencing represents the average across all sampled cells. Therefore, the signal-to-noise ratio for the actual computational variant detection process is compressed. Heterozygous somatic SNVs in genes with a duplicated WT allele in a sample with only 30% purity can be a typical use case. In this example, the expected number of reads with the mutant variant would been reduced more than ten-fold to less than 5%, making the sequence change difficult to distinguish from noise.

The challenges that are posed by the intra- and inter-tumour heterogeneity call for improved approaches of integrating multiple data sources and variant types, as well as a structured reproducible workflow for analysing multiple samples from a single patient.

5.1.3 Dataset availability and quality—Setting parameters for neoantigen prediction algorithms and training them relies on the availability of well-curated datasets. Data integration and comparability is compromised by the lack of harmonised protocols for sequencing, mutation detection, neoantigen candidate prioritisation and immunogenicity testing. Immunogenicity datasets are often unbalanced, as the most likely immunogenic candidates are preferred for testing and the rules guiding candidate selection differ between studies. For many such datasets clear and consistent biological definitions are either not provided or not acknowledged. For example, depending on the question to be answered, meta-analyses pooling datasets of patients treated with different ICBs (e.g. blocking PD-1, PD-L1, CTLA4 or PD-1/CTLA4 in combination) or of datasets derived from patients in the adjuvant versus metastatic advanced setting is not advisable.

Furthermore, various methods are used for assessing neoantigen-specific T-cell responses, including IFN-γ ELISpot assays with or without prior expansion of T cells in cell culture, intracellular cytokine staining and flow cytometry and peptide/MHC multimers (reviewed in[181]). The T-cell assays differ with regard to their sensitivity, accuracy and which T-cell phenotype they detect. Consequently, depending on the assay, pre-existing responses may be missed due to low sensitivity and guarding or restrained neoantigens may falsely be classified as ignored neoantigens. For instance, an immunogenicity dataset may represent $CD8^+$ or $CD4^+$ T-cell responses or both, may describe neoantigen-specific T cells that occur spontaneously, upon ICB therapy or vaccination, or may have been obtained with non-comparable assay methods. Efforts to compile sets of neoantigens identified in different studies are often compromised by missing essential technical and biological information that had not been documented[182,183].

Long-term clinical outcome data differentiating efficacy endpoints such as objective response, progression-free survival, and overall survival from on-going clinical trials that study neoantigen vaccination, will be a valuable addition and could reflect anti-tumour efficacy of selected neoantigen candidates.

While there is some effort to achieve harmonised datasets, such as studies done by the TESLA (Tumour Neoantigen Selection Alliance) consortium[126], the many variables involved and the polymorphic nature and inter- and intra-patient variability of key biological determiners (TCRs, MHCs) will likely require several thousands or more data points rather than the few hundreds that are available nowadays. Algorithms that allow the creation of accurate prediction models while trained on relatively few data points and application of advanced techniques such as active learning[184] or transfer learning[185] are required.

5.1.4 Vaccine design—Vaccine design has two components: (i) selection of the technology platform, and (ii) selection of the set of individual neoepitope candidates to be delivered by this platform.

The molecular nature of MHC-presented epitopes derived from mutations allows combination or concatenation of multiple short sequences representing neoepitope candidates. Published clinical studies used between 2 and 20 mutations per individual vaccine[19,20,104]. Many vaccine formats would allow administration of dozens of mutations per patient. Thus, a vaccine can be designed to feature different complementary categories of neoepitopes e.g. MHC-I and MHC-II, clonal and subclonal, ignored spiked with a few restrained and guarding neoantigens. This mitigates the risk of betting on a biological hypothesis which may later prove to be wrong.

Vaccine technologies are still at an experimental stage and various formats are being explored in clinical studies for individualised as well as off-the-shelf cancer vaccines (for review of cancer vaccine formats see[186,187]).

The most frequently used vaccine formats are mixtures of 15-30 aa long peptides corresponding to the mutated sequences with poly-ICLC as adjuvant[19], and mRNA

formulations with intrinsic adjuvant activity which encode a string of multiple predicted neoepitopes[20].

Moreover, pre-clinical or clinical trials explore viral vectors[188–191] or DNA[192–194] in conjunction with various adjuvants. For each vaccine format, the need for adjuvant and the specific vaccination schedule (requirement and frequency of boosts after initial priming) need to be determined individually. It complicates learning exercises that the vaccine technology will affect substantially whether a neoepitope candidate is delivered in a way that its potential to induce an immune response is actualised.

The vaccine format impacts speed, scalability and costs of manufacturing that is probably the most critical element for the viable implementation of individualised vaccines into clinical practice. A vaccine technology that is synthetic and allows for fast production at low cost by an unsophisticated, robust, invariant, and GMP (good manufacturing practice)-compliant process is favourable.

Manufacturing individualised cancer vaccines requires a multitude of simultaneous, highly parallelised production campaigns, with each campaign representing a drug product for one individual. This is very different from the pure bulk-upscaling paradigm of manufacturing processes pursued in conventional pharmaceutical development. Suitable production technologies are required to be innovative, cost- and time-optimised and will benefit from. Emerging solutions for the mass production of customised products in the imminent future are potential enablers for individualised vaccine manufacturing. These may include full digitisation of production processes and autonomous cloud-controlled production plants that may arise based on advances in computational power, connectivity, human-machine interactions, robotics and innovative 3D technology enabling the building of parallelised miniaturised production lines at scale[195,196].

5.2 Challenges for clinical application

This review focuses on the process of getting from a patient sample to an injectable vaccine composed of a unique set of neoepitope candidates. There are further critical challenges to get such a vaccine into clinical development and on a sustainable path suitable for potential registration and implementation into clinical practice (reviewed in[16,197–199]).

As for any drug, clinical efficacy and superiority over standard of care have to be shown in randomised trials. What is different, though, is that each patient in the investigational arm receives a drug of distinctive composition that is manufactured on-demand during the ongoing trial via a standardised process rather than being ready and released before the trial has started. This paradigm shift from a drug-centred to a patient-centred approach requires regulatory approval not of a single compound but rather the process from sample acquisition to vaccine design and production[161,199,200] (Box 3).

One pertinent question is the most suitable clinical setting. Targeting patients with minimal residual disease has the advantage that immune-suppressive mechanisms are not firmly established and that turnaround time of a vaccine production is not a limiting factor. Efficient control of larger tumour loads, in contrast, may require combination

immunotherapies. Neoepitope vaccines are safe and well tolerated. Thus, combining them with other drugs carries low risk of added toxicities while leveraging synergistic modes of action. The combination of neoepitope vaccines with checkpoint inhibition keeps the repertoire of vaccine-induced T-cell specificities functional.

The most likely escape mechanisms from a strong multi-antigen T-cell response involve loss of the target antigen or of components of the antigen-presentation machinery. This can be addressed by combining the vaccine with approaches that do not depend on HLA-presented antigens, e.g. chimeric antigen receptor (CAR)-engineered T cells, antibodies or bispecific T-cell engagers.

6 Outlook

Boosted by technologies such as NGS, increased computing power and advanced algorithms the field of neoantigen identification has evolved enormously in the past decade. In parallel to the continuous progress and efforts invested in improving neoantigen prediction tools, clinical trials explore neoantigens as single or combinatorial targets for immunotherapy and generate data that contribute to an ever-clearer knowledge of the underlying science. ClinicalTrials.gov currently lists 61 clinical studies associated with the search terms “neoantigen AND vaccine”[201]. Reports from individualised neoantigen vaccination studies indicate early clinical activity signals of vaccines alone and in combination with PD(L)-1 blockade[19,20,104,106].

Further progress in NGS technologies[202] and mass spectrometry-based MHC ligandome analysis[121,203] will support the neoantigen field with higher resolution and lower noise. Technology advances will tap new neoantigen classes, for instance derived from non-coding and ‘dark matter’ regions of the genome and from non-canonical translation[204,205].

While the understanding of anchor and TCR-facing residues within T-cell epitopes is evolving[206,207], the potential of connecting neoantigen and TCR profiling datasets is not yet fully actualised. New prediction tools and studies incorporate TCR sequences and model the interaction of mutated peptide/MHC complexes with TCRs e.g. [136,137,208,209]. Structural analyses of the interaction between TCRs and their cognate neoepitopes will provide deeper insights into the structure-dependent mechanisms of mutation-specific T-cell recognition that cannot be inferred from the sequence alone[210]. The underlying complexity needs to be tamed with new artificial intelligence-based applications and by substantially increasing computing power. Standardised datasets generated by using well-thought through experimental designs and accurate and sensitive computational workflows and featuring biological and clinical information will shape a robust foundation for closing existent knowledge gaps. Approaches towards true translational medicine such as the proposed context-based differentiation between guarding, restrained and ignored neoantigens will contribute to a framework that connects neoantigen science to clinical settings and medical features and peculiarities of cancer diseases enabling future neoantigen-dependent therapies to provide greater clinical benefit.

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Finally, once the technology to pinpoint the relevant neoantigens for an individualised vaccine design at a given time point is optimised and paired with concepts to use sequencing data for early recognition of acquired resistance mechanisms of an individual tumour, adaptation of an individual's vaccine boosters to the dynamics of their disease over time is conceivable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1

Classification of neoantigens according to molecular characteristics

Somatic mutations that are the basis for the foreignness of cancer cells may result in protein sequences that depending on the type of mutation are altered in different ways (FIG BOX1). An important future field of development is to tap the various categories described below and others (e.g. non-coding regions of the genome) to broaden the discovery space out of which neoepitopes can be predicted.

SNVs

Single nucleotide variants (SNVs) represent the exchange of single nucleotides within the genome and are the most abundant mutation type in the majority of cancers[260]. Most SNVs generate neoantigens with a single amino acid substitution. In very rare cases, e.g. if a native stop codon is destructed, SNVs may create longer neoantigen sequences.

SNVs were the focus of the first efforts of predicting neoepitopes and their application in clinical trials. SNV burden is predictive for the clinical efficacy of ICB[8,261] and SNV-derived neoantigens were successfully targeted in individualised neoantigen vaccination trials, e.g. in melanoma and glioblastoma[19,20,104,106]

Whereas individualised treatment remains the mainstream approach for the broader patient population, efforts to discover shared SNVs that would enable standard clinical trial and development routes are being pursued. Several experimental studies report neoantigens that derive from shared mutations (Table 1). The common oncogenic mutations KRAS G12D in patients with metastatic colorectal cancer and other tumour types, and IDH1 R132H in glioma can trigger antigen-specific immune responses associated with tumour regression[42,52,108,146]. However, overall, the vast majority of shared SNVs are rare and in general confined to small subsets of patients.

INDELs

Insertions or deletions of nucleotides (INDELs) can result in neoepitopes, however those generated by frameshift INDELs may be longer and unrelated to known sequences and thus have a higher likelihood to be immunogenic[262].

However, INDEL mutations may introduce premature stop codons, which in turn can induce non-sense mediated decay (NMD) of the respective RNA. Of note, INDELs that are predicted to escape NMD, were shown to correlate better with clinical response to ICB as compared to INDELs in general or to SNVs[263]. Neoepitope candidates from INDELs show superior MHC binding capability as compared to SNVs. Tumour mutational burden analyses that include INDEL frameshift mutations correlate better with clinical response of melanoma patients to anti-PD1 or anti-CTLA4 than analysis based on SNV alone[96].

The high incidence of INDEL mutations in tumour entities with low SNV burden[96,264], may expand the application of neoepitope-based immune therapies to these tumour entities.

Fusion Genes

Intra- and inter-chromosomal rearrangements may join two unrelated genes to produce a fusion gene. A prominent example is the BCR-ABL1 fusion gene in chronic myelogenous leukaemia (CML), that is found in ~90% of CML patients[265]. Experimental evidence supports immunogenicity of this shared fusion (TABLE 1). The vast majority of fusion genes, however, appear to be individual[266].

In a head and neck cancer patient responding to PD-1 blockade, despite low SNV burden, a T-cell response against the DEK–AFF2 fusion gene was observed, while no neoantigens from other mutation classes were identified[99].

Overall, gene fusions are considered to be relatively rare events[267] and the immunotherapeutic utility of fusion gene-derived neoantigens is not fully grasped yet.

Splice Variants

Alternative splicing generates diversity and lineage-specificity by expression of multiple RNA and protein isoforms from one gene, is dysregulated in cancer cells and may generate neoepitope sequences[268–272]. If a somatic mutation in the respective gene results directly in its altered splicing, tumour specificity of the splice variant (which is a key defining criterion for a neoantigen) can be assumed. This may not be true for aberrant splice variants generated by other mechanisms such as cancer-associated epigenetic alterations. Here, physiologically expressed splice variants in a distinct cell lineage may be ectopically activated in cells of unrelated lineage.

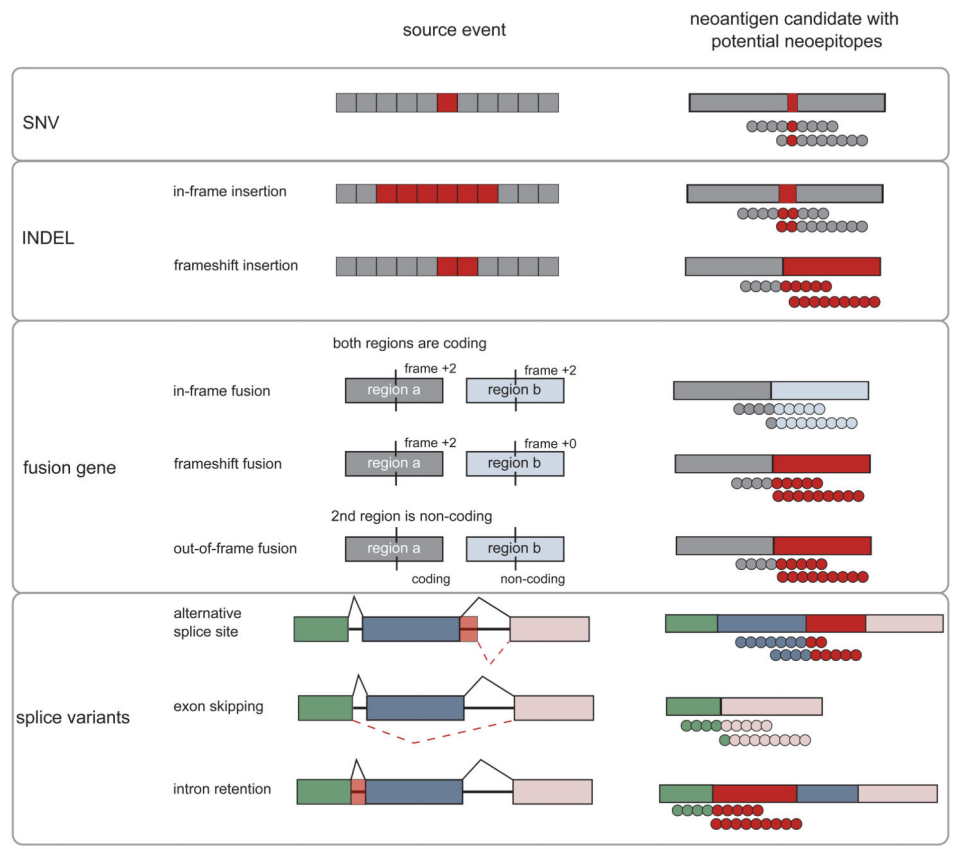

Figure Box 1. Mutation classes and neoantigen and neoepitopes derived thereof.

SNVs change a single amino acid. INDELs and fusion genes may be in-frame and preserve the original open reading frame or they may cause a frameshift, creating novel open reading frames downstream of the mutation site. Alternative splicing may occur by various mechanisms including the usage of alternative splice sites, skipping of exons or intron retention events. All of these classes may generate neoepitope sequences that are foreign to the immune system. Novel sequence regions derived from mutations are indicated in red.

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Box 2

Machine learning and deep learning.

Machine learning refers to the use of algorithms for learning patterns in data. Machine learning tasks may be divided into supervised (predicting a label) and unsupervised learning (pattern recognition). Deep learning describes a class of machine learning algorithms that employ deep neuronal networks mainly for supervised classification tasks. However, both regression as well as unsupervised learning can be performed with deep learning tools as well. Artificial neural networks are inspired by biological neuronal networks. In general, their architecture comprises an input layer, hidden layer(s) and an output layer. The input layer receives the data as numerical values. The association with weights and nonlinear transformation abstracts the input data during propagation across the hidden layers. Neural networks that support deep learning have more than one hidden layer, and the number of hidden layers defines the deepness of the network. The output layer provides the predicted class label. Training of a network involves comparison of the predicted label with the true label to calculate the loss function **[G]** that is optimised by updating the weights on the hidden layers. While deep learning is regularly applied in areas such as image processing, it is still in its infancy in fields in which the amount of high quality labelled data for the respective subject matter is insufficient; interpretation of neuronal networks is also not straightforward. Deep learning and its applications in biomedicine has been extensively reviewed elsewhere[166,273].

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Box 3

Principles of technical and analytical software validation

The entire neoantigen prediction process relies heavily on software and computerised systems. For the application of such systems in clinical studies and later in a pharmaceutical product, the technical validation of the systems is a regulatory requirement. Validation is the continuous process of demonstrating that a computerised system is fit for the intended use and part of a quality assurance program. Good Automated Manufacturing Practice[274] represents the industry standard for validation of automated systems, which includes any computer system, ranging from programmable calculators and embedded devices to super computers and any software running on those machines. GAMP reflects the requirements of the legislation called Good Manufacturing Practice (GMP), as is codified e.g. in the United States 21 CFR 210/211 or the European Union GMP guidelines. The basic validation process follows traditional software engineering practices, including detailed documentation of the requirements at different abstraction levels (e.g. user, functional and configuration), followed by documented qualification and testing of system components and finally the whole system. A key difference to pure software engineering is the inclusion of detailed risk evaluations at every stage, focusing on potential safety issues for the patient. Novel software engineering and computational concepts like agile development or cloud computing have been introduced into the GAMP framework in the recent years.

For analytical validation of the performance, robustness and repeatability of the process, the NGS process poses a challenge. A typical exome-sequencing experiment involves analysis of about 50 million genomic nucleotides as data points and random erroneous mutation calls will occur to some extent. Quality controls include demonstration of reproducibility and whole exome coverage. Optimised lab protocol and robust mutation calling algorithms are required to account for data obtained from low quality clinical samples. Machine learning based neoantigen prediction methods rely on the amount and quality of training data, which continuously and swiftly is growing. Increasing the size and quality of training datasets may improve the performance of such software tools even if the underlying algorithms are not substantially changed. Quality improvements on the fly may translate into better and more efficacious vaccines. As long as iterative improvements of the neoantigen prediction and individual vaccine design are not associated with safety concerns it is desirable to enable quality improvements updates within an ongoing clinical trial, a clinical development program or even once a product is approved. To this aim, a regulatory path must be defined, which allows the necessary degree of flexibility for process improvements while maintaining the safety properties of the product itself.

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Glossary

Adoptive T-cell therapy
Immunotherapy in which T cells are taken from the patient's tumour tissue or blood, expanded *in vitro* and then transferred back to the patient to support the immune system's natural fight against the cancer.

Alignment of NGS reads
Mapping sequencing reads to a reference genome to determine the genomic loci of origin.

Anchor residue
Position in a MHC epitope with specific amino acid preference.

Anergy
A hyporesponsive state in which an antigen experienced T cell is functionally impaired and does not adequately respond to cognate antigen exposure.

Antigen spreading
Expansion of an immune response to secondary epitopes, or other antigens that were not targeted by immunotherapy.

Antigenicity
Immune responses induced by vaccination as in the case of ignored neoepitopes.

Artificial neural network
Computing system which is inspired by biological neural networks and that applies tasks based on learned patterns.

BLAST
A tool to find local regions of similarity between biological sequences. It enables to compare a sequence of interest to a database of sequences and identify the sequences with highest local similarities.

Branched mutation
A mutation that occurs later during tumour evolution and is only present in a subset of tumour cells.

Cancer mutanome
Set of all non-synonymous somatic mutations occurring in a tumour.

Central tolerance
Thymic elimination of self-reactive T cells

Clonality
The fraction of tumour subclones that harbour a given mutation

Clonal mutation
A mutation that is present in all subclones of a tumour. In practise, the definition of clonal vs sublconal mutation is not standardised and depends on the experimental setting and bioinformatics tools as these provide a numeric estimation of clonality (e.g. PyClone[138]).

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Mutation calling process
Mutation calling strategy in which the overlap (consensus) from at least two different mutation callers is used to define the final set of mutational events.

Co-stimulatory signal
Secondary signal required to activate immune responses in the presence of antigen-presenting cells.

***de novo* immune response**
Vaccine induced antigen-specific T cell response that was not detectable prior vaccination. Used as opposed to the augmentation of a pre-existing T cell response.

Deep learning
Machine-learning methods using multi-layer models for feature extraction and pattern learning.

Driver mutation
A mutation that improves fitness of a tumour cell.

Differential agretopicity index (DAI)
Difference in MHC-I binding affinity between neoepitope and corresponding non-mutated peptide.

Guarding neoantigen
A neoantigen that drives a prognostically relevant tumour immunity in the absence of an immunotherapy

Heterologous immunity
Cross-reactive T cell immunity induced by an unrelated antigen, often pre-existent before tumour onset.

Ignored neoantigen
A neoantigen lacks intrinsic antigenicity but could serve as target for immunotherapy

Immune surveillance
A hypothesis that assumes that immune cells monitor, identify and eliminate pre-malignant or malignant cells in the body.

Immune escape
Mechanisms of tumour evolution allowing tumour cells to escape from a host's immune response.

Immunoediting
A hypothesis that describes the close interaction between tumour and immune system and transition between immune protection against tumour development and tumour outgrowth in three phases: elimination, equilibrium, and escape.

Immunogenicity
Induction of immune responses without vaccination as in the case of guarding and restrained neoepitopes.

Loss function

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A function that calculates how well or poorly an algorithm models the given data by comparing the predicted values to the actual values.

Loss of heterozygosity
A locus with two different alleles loses one of these two copies.

MHC/HLA
Cell surface proteins that present peptide fragments for recognition by T-cells. MHC is the general term; HLA is used in the human context only.

MHC-I
The MHC-I molecule is a protein complex formed by beta-2 microglobulin and an alpha chained encoded in the HLA-A, HLA-B, HLA-C locus in human. MHC-I is expressed on the cell surface of all nucleated cells and presents intracellularly synthesised peptides to $CD8^+$ T-cells. Antigen presentation has been reviewed in[211,212].

MHC-II
The MHC-II molecule is a protein complex formed by an alpha and a beta chain that are encoded in the HLA-DR, HLA-DP and HLA-DQ locus in human. MHC-II is mainly expressed on the cell of specialised antigen presenting cells and presents mainly extracellular peptides to $CD4^+$ T-cells. Antigen presentation has been reviewed in[211,212].

Neoantigen feature
A feature or algorithm that can be used to rank neoantigen candidates.

Neoantigen prediction pipeline
Computational tool for neoantigen prediction, starting with mutation calling or a set of called mutation and covering the translation into mutated peptide sequences and ranking of neoantigen candidates by a neoantigen feature.

Neoepitope
A major histocompatibility complex (MHC) bound peptide that arises from a tumour-specific mutation.

Non-synonymous mutation
A mutation that causes changes in the amino acid sequence of a protein.

Peripheral tolerance
Elimination or suppression of autoreactive T-cells or B-cell clones that escaped to the periphery.

Prognostic
Statement about the expected development of a disease based on its biology irrespective of a given treatment

Predictive
Statement about the expected response to therapy

Pseudoalignment
A pseudoalignment identifies the transcripts a RNA-seq read is most likely related to but does not specify how each nucleotide matches the reference like in a normal alignment.

Restrained neoantigen
A neoantigen that drives tumour immunity upon immune checkpoint inhibition

ROC curve
A graphical plot that reflects the quality of a classifier by showing the true positive versus false positive rate across varying thresholds.

Spontaneous tumour immunity
T cell responses induced spontaneously in the course of tumour growth

TCR degeneracy
The ability of a single TCR to recognise diverse peptide/MHC complexes.

TCR diversity
The ability of a single peptide/MHC complex to engage antigen-specific T cells with diverse TCR alpha / beta chains

TAP protein complex
Protein complex of TAP-1 (Transporter associated with antigen processing 1) and TAP-2 that imports peptides from the cytosol in to the endoplasmic reticulum.

Truncal mutation
A mutation that occurs early during tumour evolution.

Vaccine mediated immunity
T cell responses amplified or *de novo* induced by delivery and active exposure of the host to neoantigens.

Variant allele frequency
The fraction of sequence reads observed covering a mutation divided by the overall number of reads at that locus.

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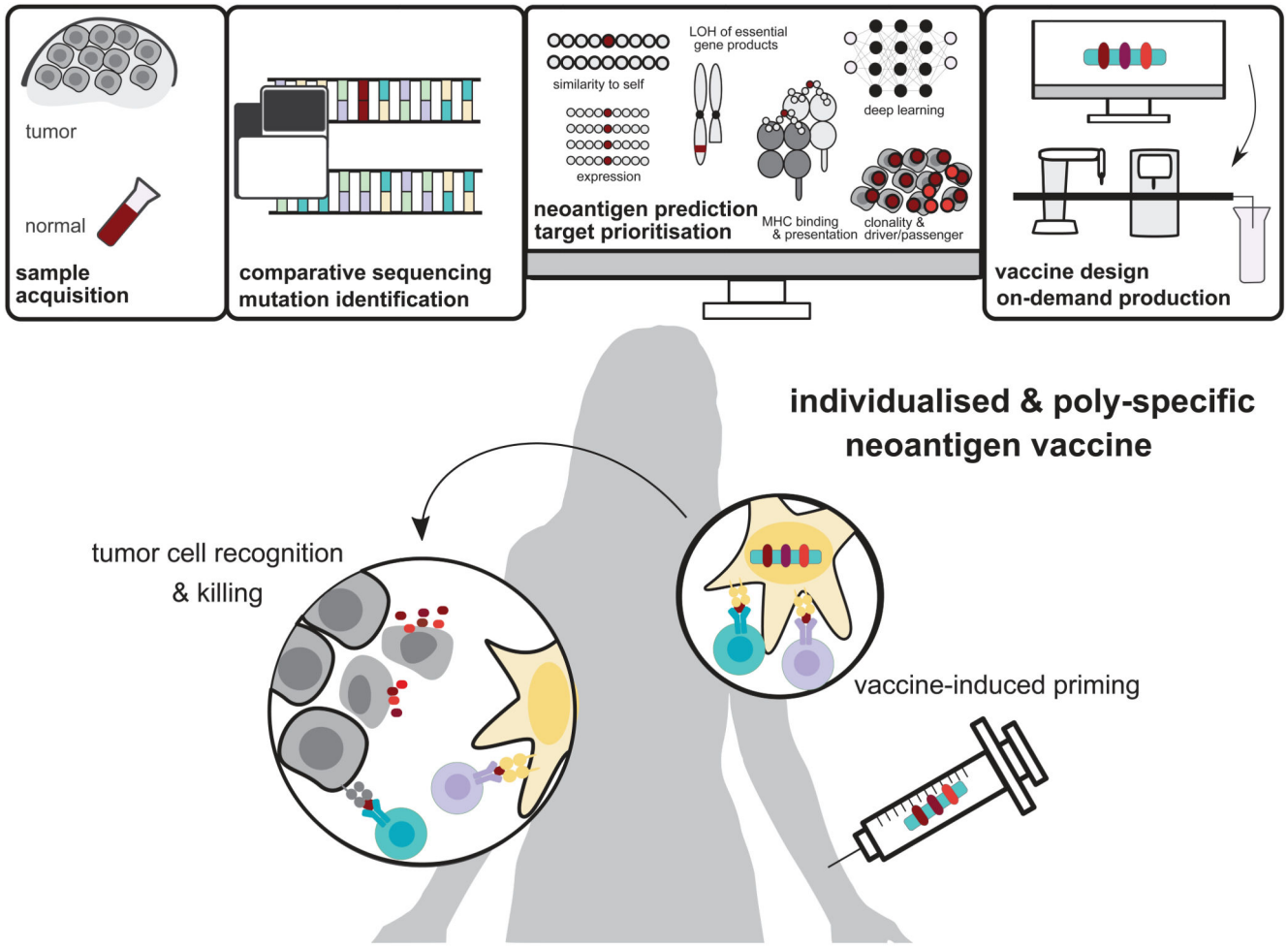

Figure 1. Engineering individualised neoantigen vaccines.

Next-generation sequencing of a patient's healthy tissue (e.g., PBMC, peripheral blood mononuclear cells) and tumour biopsies is performed. The sequencing data from tumour and normal DNA is compared to identify tumour-specific mutations. Mutations are prioritised as vaccine candidates based on their likelihood to elicit a T-cell response by computational methods such as MHC binding prediction, quantification of mutated transcript expression, clonality of the mutation and other features. Using the vaccine platform of choice (e.g. mRNA, long peptides) an individualised and poly-specific neoantigen vaccine is manufactured on-demand under GMP conditions. Neoantigen vaccination aims at restoring the cancer immunity cycle by inducing *de novo* T-cell responses that induce tumour killing and by supporting the shift from ignorance toward anti-tumour immunity. LOH: loss of heterozygosity, GMP: good manufacturing practice

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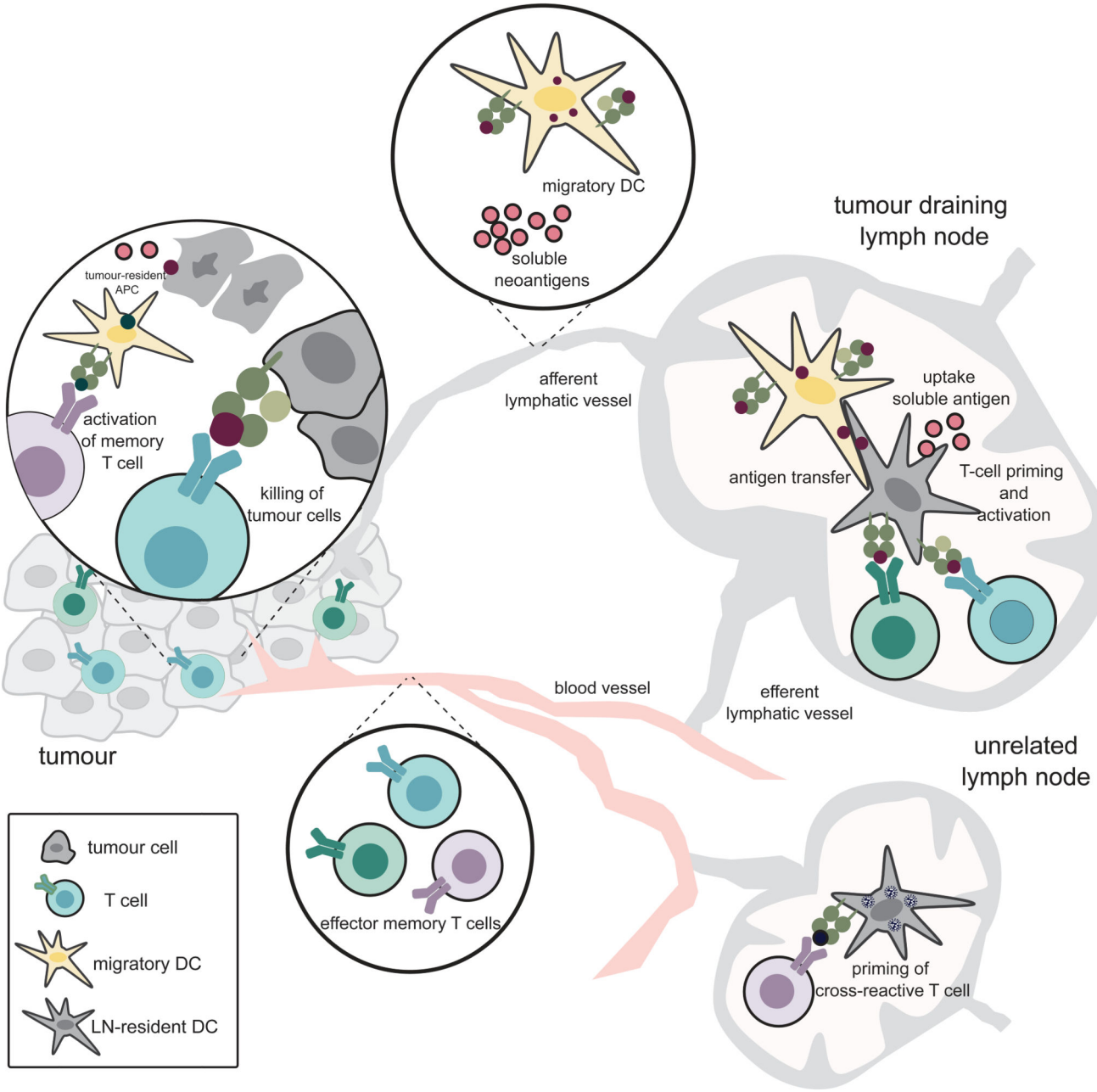

Figure 2. Mechanisms of neo-antigen-mediated tumour control.

Dying tumour cells release neoantigens that reach the draining lymph node either in a soluble form within the extracellular fluid or are transported from the tumour site by migratory antigen presenting cells (APCs). In the lymph node, highly specialised dendritic cells present the neoantigen on MHC-I or MHC-II molecules to naïve T cells for priming and activation. Activated neoantigen-specific $CD4^+$ and $CD8^+$ T cells leave the lymph node, enter the tumour and exert anti-tumour activities. APCs in the tumour microenvironment can activate antigen specific memory $CD4^+$ and $CD8^+$

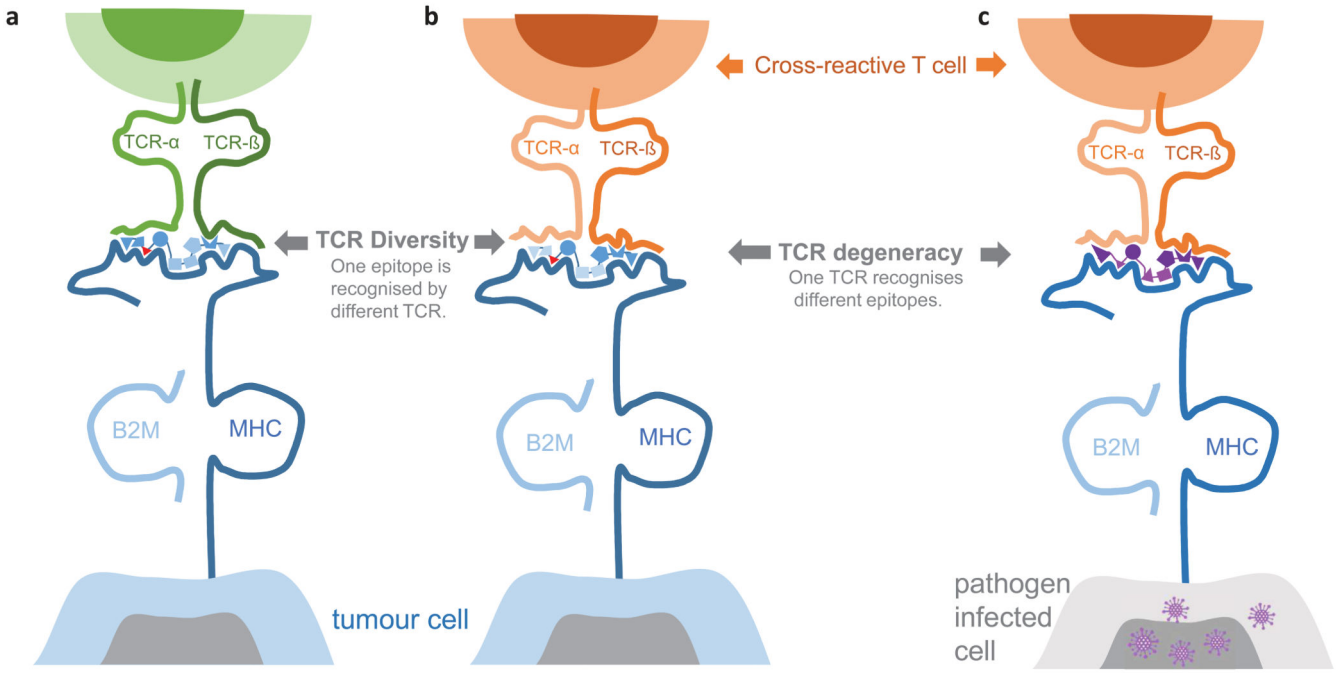

Figure 3. TCR diversity and degeneracy

A neoepitope (blue, mutant amino acid red) can be recognised by different T cells with molecularly different TCR alpha and beta chains (left, middle). The TCR/neoepitope contact residues (dark blue) may differ for individual T cells that recognise the same neoepitope. This is in particular the case for neoantigens resulting from a mutation that converts a non-binding wild type peptide into a binding mutant peptide. Also, a single TCR can recognise unrelated MHC peptide epitopes (right). For example, a T cell primed against a pathogen-derived epitope (purple) may cross-recognise a neoepitope presented on a tumour cell. MHC: major histocompatibility complex; TCR: T-cell receptor, B2M: Beta-2-Microglobulin.

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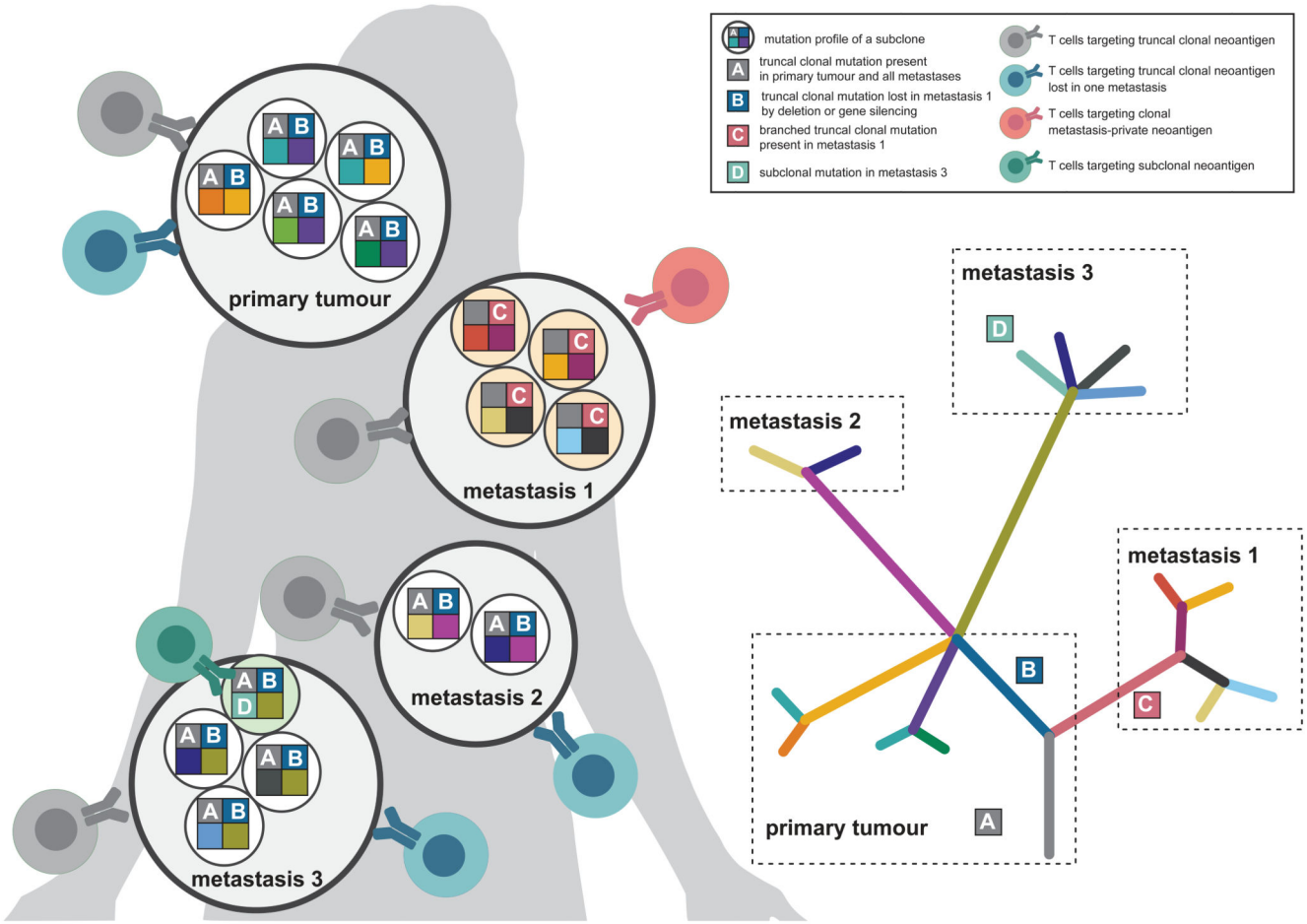

Figure 4. Factors affecting neoantigen recognition and evolution.

Each lesion (primary or metastasis) of an individual tumour disease consists of different subclones, each of which may contribute sets of different neoantigens to the patient's neoantigenome. Depending on whether the neoantigen is truncal clonal (neoantigen A), truncal clonal but lost in a metastasis by deletion or gene silencing (neoantigen B), clonal in a certain metastasis (neoantigen C) or specific for a certain subclone in a single metastasis (neoantigen D), neoepitope-specific T cells would target either all tumour cells (neoantigen A), all tumour cells of the lesions harbouring the neoantigen (neoantigen B), tumour cells of a distinct lesion (neoantigen C) or merely a single tumour subclone (neoantigen D).

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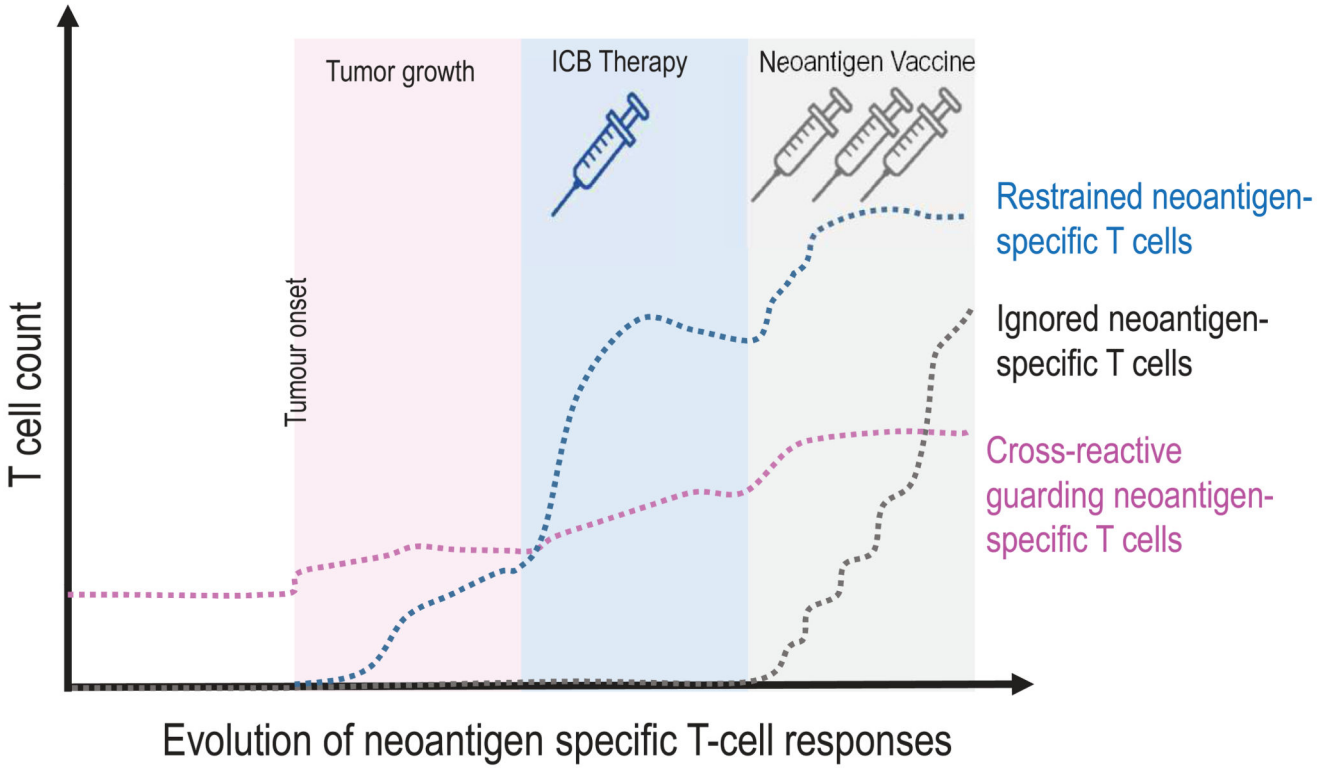

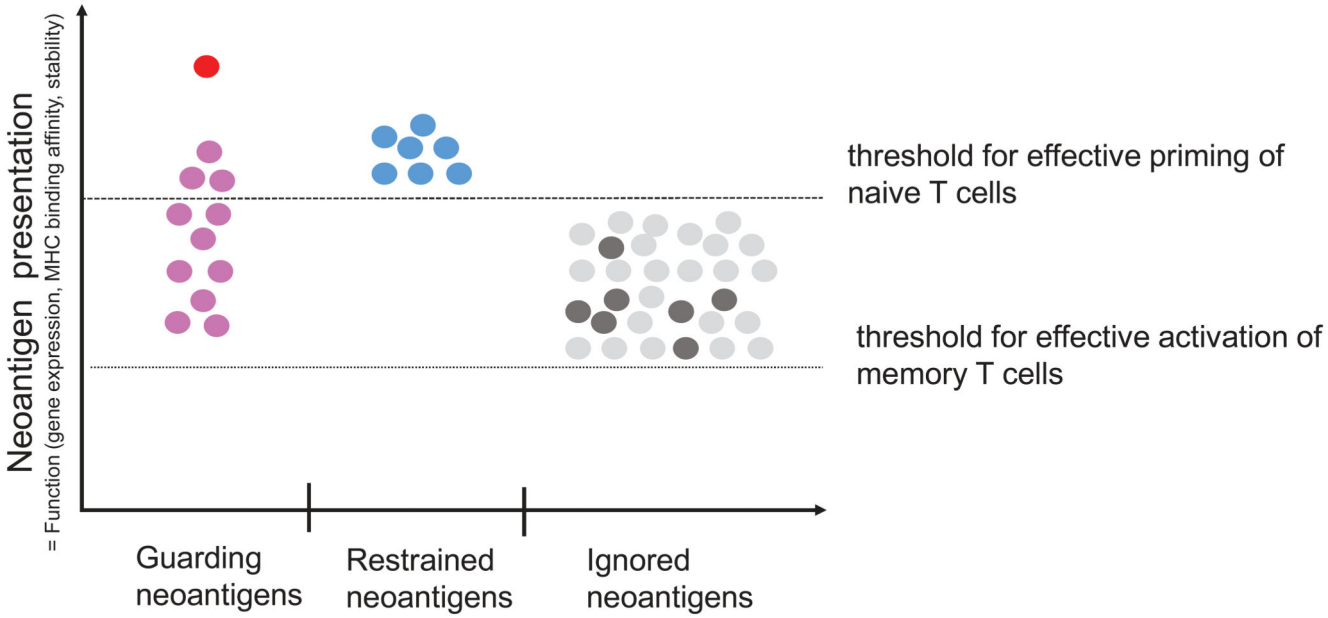

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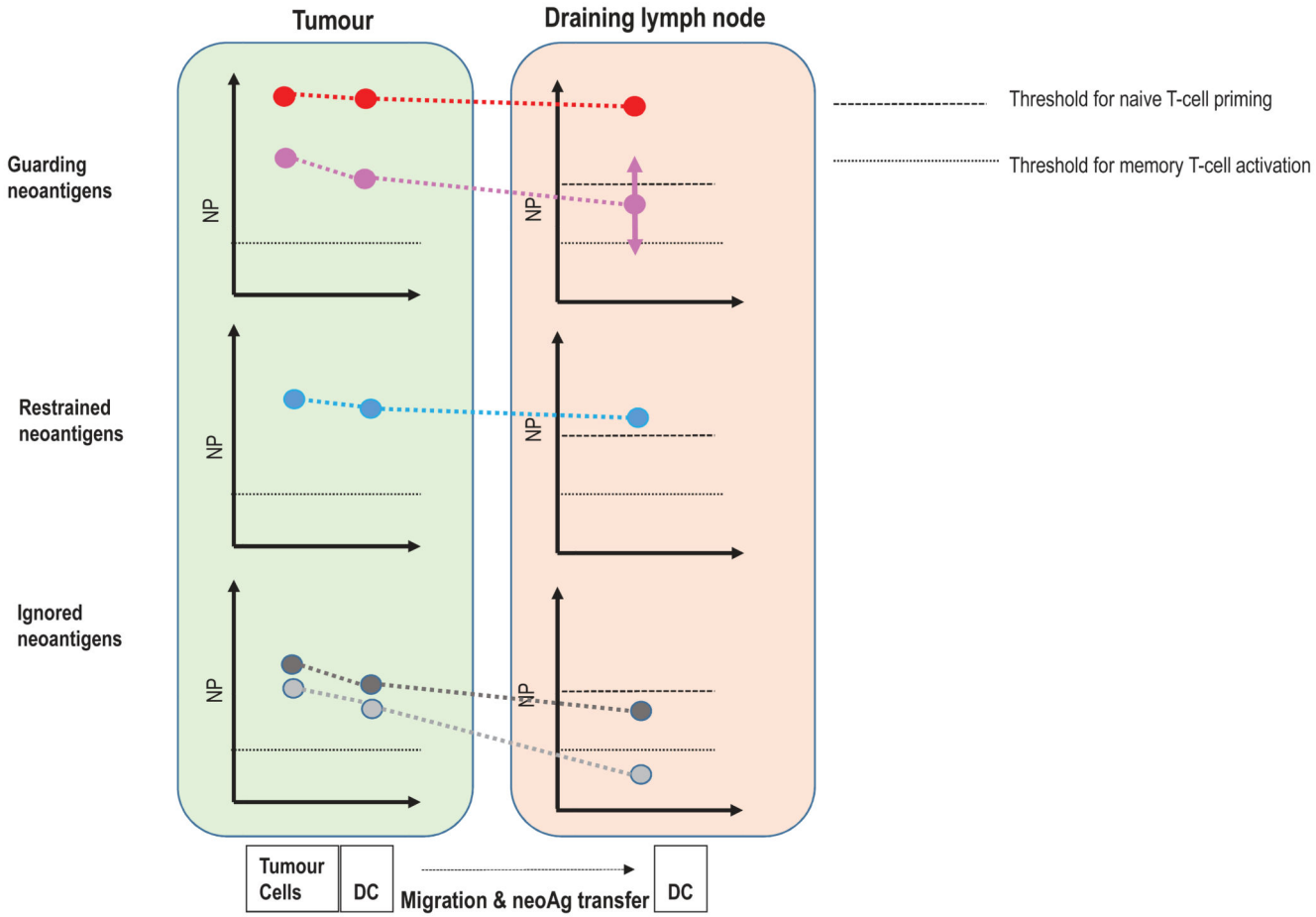

Figure 5. A context based classification of neoantigens

(**a**) The formation and evolution of neoantigen-specific T-cell responses depends on the clinical context. While ICB therapy boosts pre-existing T-cell responses, neoantigen cancer vaccines induce *de novo* responses or amplify preformed ones. (**b**) The robustness and level of a neoantigen presentation on LN-resident DCs defines the efficiency of cognate priming of T cells, while presentation on tumour-resident APCs and tumour cells activates primed cells at the tumour site. Neoantigen presentation is a function of expression level of the mutated protein, the binding ability of the mutated peptide to MHC and stability of the respective peptide/MHC complex. Memory T cell activation can be achieved with neoantigen presentation levels 50 fold lower than those required for priming of naive T cells. Dark red: supreme neoantigen; pink cross-reactive guarding neoantigen **(c)** Neoantigen-specific T cell responses are driven by the presentation of neoepitopes on tumour cells, on tumour-infiltrating APCs, and on DCs in the draining lymph node. Priming of naïve T cells in the lymph node requires substantially higher neoantigen presentation than is required for stimulation of memory T cells. Guarding neoantigens are either highly expressed with superior binding and stability of the respective neoepitope/MHC complex (red) or exploit cross-reactivity to heterologously primed memory T cells (purple). Restrained neoantigens exhibit robust expression and strong MHC binding affinity/stability and are able to prime and expand naive neoantigen-specific T cells in the lymph node. Ignored neoantigens require

a vaccine to generate neoantigen presentation levels in the LN that allow priming. As long as neoantigen presentation is moderate (e.g., low expression/high affinity MHC binding (dark gray) or high expression/low MHC binding (light gray), T cells can be activated for effector functions in the tumour. NP: level of neoepitope presentation, LN: lymph node, APC: antigen-presenting cell, ICB: immune checkpoint blockade

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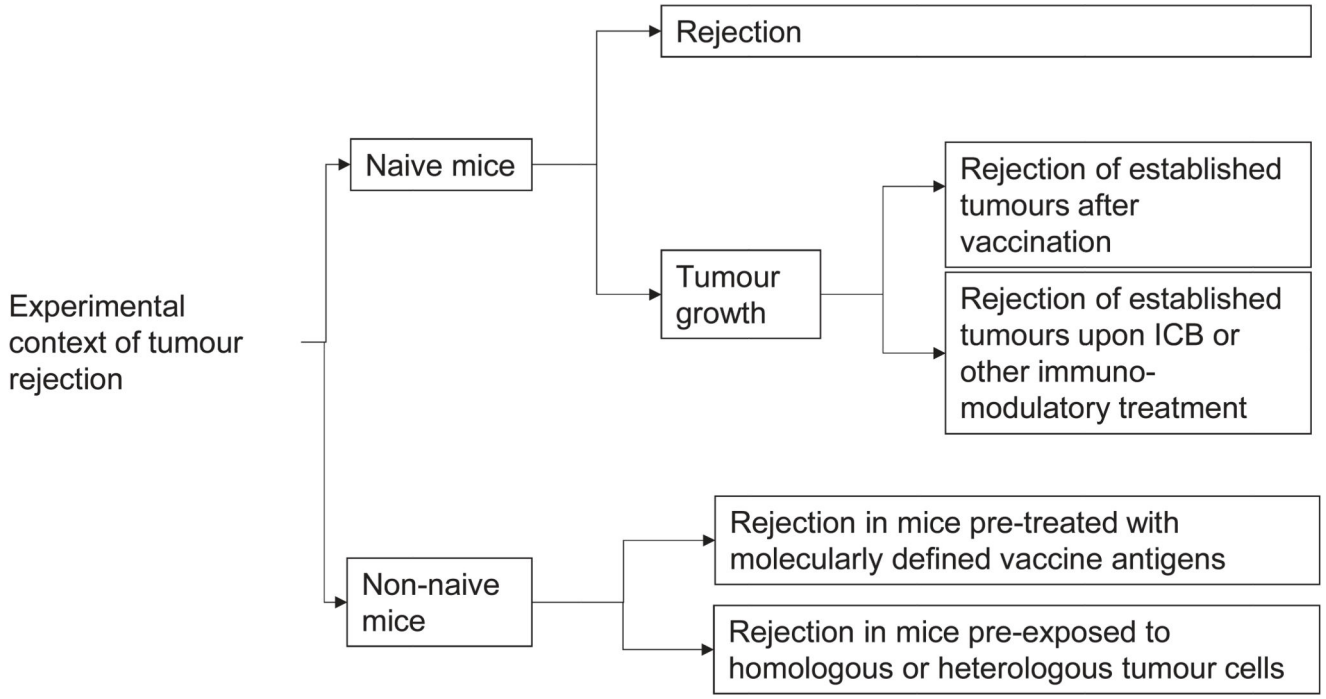

Figure 6. Discovery of tumour rejection antigens.

As the term "tumour rejection" and the conditions under which to assess it are not standardised, experimental mouse model setups are used that differ conceptually and provide answers to different questions. These include tumour challenge of naive mice[83,84] as well as of tumour- or vaccine-experienced mice[258,259] and assessment of rejection spontaneously[83,84] or upon various modalities of immunotherapy[6,23,53,102].

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Table 1

Shared neoantigens. Most frequent tumour type and mutation frequency in the respective tumour entity has been estimated using SNV data from TCGA. Index tumours are those, which are most frequently affected. MUT: mutation, RE: restriction element. MHC allele frequencies were derived from the NCBI dbMHC database[213].

Gene(s)	Mutation (MUT)	Reference	Restriction element (RE) c% of RE in European/North American population, Population with next highest % of RE and its % of RE)
SNVs			
KRAS	G12V	42	HLA-A*11:01 (4.2%, Oceania: 20.7%)
KRAS	G12D	42,108,146	HLA-A*02:01 (22.3%, South America: 22.1%), HLA-C*08:02 (-), HLA-DRB1*08:01 (1.2%, North Africa: 1.4%)
IDH1	R132H	52	HLA-DRB1*01:01 (4.3%, North-East Asia: 5.7%)
TP53	R175H	17,43	HLA-A*02:01 (22.3%, South America: 22.1%), HLA-DRB1*13:01 (2.37%, South India: 13.8%)
H3.3	K27M	214,215	HLA-A*02:01 (22.3%, South America: 22.1%), HLA-DR1 (-)
JAK2	V617F	216	HLA-A2 (-)
PIK3A	H1047L	217	HLA-A*03:01 (8.6%, South India: 8.0%)
BRAF	V600E	218	HLA-DQB1*03
CDK4	R24C	1	HLA-A2.1 (22.3%, South America: 22.1%)
CDK12	E928K	11	HLA-A*11:01 (4.2%, Oceania: 20.72%)
NRAS	Q61R	219	HLA-A*01:01 (10.0%, North Africa: 12.59%)
CTNNB1	F37S	220	HLA-A24+ (-)
INDELs			
GAS7	H225Y	11	HLA-A*02:01 (22.3%. South America: 22.1%)
NPM	4 bp hotspot insertion in exon 12	221	HLA-A*02:01 (22.3%. South America: 22.1%)
CALR	INDELs in exon 9	98	Mixed
TGFβRII	1bp deletion in poly$(A)_{10}$ tract (nucleotides 709-718)	222,223	HLA-A2+ (-), HLA-DPB1*03:01 (8.1%, Central America: 10.0%), HLA-DRB1*14:01 (23%, SouthEast Asia 13.4%)
Fusion Genes			
BCR-ABL	fusion protein b3a2	224–227	HLA-A3+ (-),HLA-DR+ (-)
dek-can	t(6;9)	226	HLA-DR53+ (-)
SYT-SSX	t(X; 18)(p11;q11)	228,229	HLA-A24+ (-)
pml/RAR alpha	-	230	HLA-DR11+ (-)

Gene(s)	Mutation (MUT)	Reference	Restriction element (RE) c% of RE in European/North American population, Population with next highest % of RE and its % of RE)
PAX-FKHR	t(2;13)	231	HLA-B7+ (-)
ETV6-AML1	t(12;21)	232,233	Mixed (-)

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Table 2
Classification of neoantigens by potential functional impact

	Guarding neoantigens		Restrained neo antigens	Ignored neoantigens
Characteristic features	Supreme neoantigens with a strong antigenicity driving early priming and rapid expansion of neoantigen specific cytotoxic T cells	neoantigens cross-recognised by pre-formed memory T cells induced by heterologous immunity	Neoantigens that are immunogenic in the immunotherapy-naïve host and induce PD1+ memory T cells which proliferate and expand under ICB	Neoantigens which do not induce a relevant immune response in the tumour-bearing host but are able to drive tumour immunity once memory effector T cells are induced by vaccination,
Frequency	Extremely rare	<2% of all mutations	<2% of all mutations	15-25% of all mutations
Examples in mice	DDX5[85], SPTBN2[83]	SIY[87]	LAMA4[6,53], ITGB1[53]	KIF18b[23,102]
Examples in human	n.a.	MUC16[50]	ATR[5]	RETSAT[19,20]
Clinical relevance	Prognostically relevant drivers of anti-tumour immunity in the immunotherapy-naïve host.		Inactive due to immunosuppression in immunotherapy-naïve host. Main drivers of clinical ICB activity.	No impact on tumour control in immunotherapy-naïve or ICB-treated host. Confer poly-specific anti-tumour T-cell control by broadening the repertoire of tumour-specific T cells upon neoantigen vaccination.

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Table 3

Hypothesis-driven neoantigen features and prediction algorithms. WT: wild-type

Name	Function	Input	Comment
MHC-I binding	MHC-I binding prediction	Neoantigen candidate sequence, MHC-I alleles	Several tools, e.g. netMHCpan[118]
MHC-II binding	MHC-II binding prediction	Neoantigen candidate sequence, MHC-II alleles	Several tools e.g. netMHCIIpan[234]
MixMHCpred score [120,235]	Prediction of cell surface presentation of MHC-I epitopes	Neoepitope candidate sequence, MHC-I alleles	Trained on eluted ligands
MixMHC2pred score [236]	Prediction of cell surface presentation of MHC-II epitopes	Neoepitope candidate sequence, MHC-II alleles	Trained on eluted ligands
Transcript expression [23,102]	Transcript expression in FPKM, RPKM or TPM	-	Sveral tools, e.g. kallisto[114]
Clonality [90]	The fraction of tumour clones in which a neoantigen is present	Number of reads that cover WT and mutated allele	Several tools, e.g. pyclone[138]
Differential Agretopicity Index (DAI)[89,206].	Difference in MHC-I binding affinities between mutated and WT peptide	MHC-I binding affinity of neoepitope candidate and corresponding WT	-
Self-Similarity [132]	Sequence similarity between mutated and WT peptide	Neoepitope candidate and corresponding WT sequence	Relevant for neoepitopes with similar MHC binding as WT peptide
Pathogen similarity [50]	Similarity to known pathogen epitopes	Neoepitope candidate sequence	BLAST search against pathogen-derived epitopes IEDB database
IEDB immunogenicity [237]	Summed up position-weighted enrichment of residues in immunogenic peptide sequences	Neoepitope candidate sequence	-
Neoantigen dissimilarity [133]	Dissimilarity of epitope sequence to self-proteome	Neoepitope candidate sequence	BLAST against WT proteome
PHBR-I [128]	Mutation presentation by multiple patient MHC-I alleles	Best MHC binding score for each MHC-I allele	-
PHBR-II [129]	Mutation presentation by multiple patient MHC-II alleles	Best MHC binding score for each MHC-II allele	-
Generator rate [238]	Mutation presentation by multiple neoepitopes	MHC-I binding affinities	-
Recognition potential [50,92]	Combinatorial score considering pathogen-similarity and differential MHC binding of mutated and WT epitope	Neoepitope candidate sequence, MHC-I binding affinities	-
Vaxrank [239,240]	Combinatorial score considering the presentation of a mutation by multiple epitopes and mutated transcript expression	Transcript expression of mutation, MHC-I binding affinities of neoepitope candidates related to a mutation	-
Priority score [241]	Combinatorial score considering MHC binding and expression	MHC-I binding affinity of neoepitope candidate and WT, transcript expression, variant allele frequency	-
Tcell predictor [242]	Random Forest Model considering several epitope features such as expression, hydrophobicity	Gene name, substitution, neoepitope candidate sequence	-
neoag [243]	Gradient Boosting Model considering epitope sequence intrinsic features	Neoepitope candidate sequence, corresponding WT sequence, variant position, MHC-I binding	Trained on a immunogenicity data set from mouse tumour models

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Table 4

Examples for neoantigen prediction algorithms that are based on neural network and deep learning based

Name	Short Description	Reference
HLAathena	Neural network for prediction of MHC-I epitope presentation	159
Neomhc2	Convolutional neural network for the prediction of MHC-II epitope presentation	38
EDGE	Deep learning model for prediction of MHC-I epitope presentation	163,163
MARIA	Multimodal recurrent neural network for predicting the likelihood of antigen presentation (MHC-II)	164
DeepHLA	Deep learning model combining binding and immunogenicity model (MHC-I)	165
Structure	Neural network on structural features that influence T-cell receptor (TCR) and peptide binding energies	160

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Table 5
State-of-the-art tools for mutation calling.

Software	Scope	Reference
Mutect / Mutect 2	Somatic SNVs + INDELs	244/176
Strelka / Strelka 2	Somatic SNVs + INDELs	245/175
VarScan 2	Somatic SNVs + INDELs. Germline variants. copy number variants	152
SomaticSniper	Somatic SNVs	246
RADIA	Somatic SNVs from DNA and matched RNA	247
FreeBayes	SNP calling	248
samtools / bcftools	Basic variant calling	249
Platypus	SNP calling	250
CaVEMan	Somatic SNVs	251
cgpPindel	Somatic INDELs	252
SvABA	Somatic INDELs	253
MuSE	Somatic SNVs	254
SMuFIN	Somatic SNVs + INDELs	255
GATK	All purpose toolkit. including germline genotyper	256
NeuSomatic	Deep Learning based somatic SNV detection	257