The landscape of T cell antigens for cancer immunotherapy

The landscape of T-cell antigens for cancer immunotherapy

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Abstract

The remarkable capacity of immunotherapies to induce durable regression in some patients with metastatic cancer relies heavily on T cell recognition of tumor-presented antigens. As checkpoint blockade therapy has limited efficacy, tumor antigens have the potential to be exploited for complementary treatments, many of which are already in clinical trials. The surge of interest in this topic has led to the expansion of the tumor antigen landscape with the emergence of new antigen categories. Nonetheless, how different antigens compare in their ability to elicit efficient and safe clinical responses remains largely unknown. Here, we review known cancer peptide-antigens, their attributes and the relevant clinical data and discuss future directions.

Introduction

Recent years have seen tremendous clinical benefit from cancer immunotherapy. However, although multiple immunotherapeutic modalities exist[25,26] (Box1), these largely converge to cytotoxic T cells (CTLs) targeting the tumor. T cells are activated through specific T cell receptor (TCR) – antigen interactions (Figure 1). V(D)J recombination can generate a huge diversity (up to ~10^{15}, theoretically) of T clonotypes in the thymus, each with its unique TCR (or two TCRs, in the case of ααβ clonotypes) [27]. This repertoire is further pruned by positive and negative selection processes, yielding ~10^6-10^{10} circulating clonotypes[27]. Each TCR can bind a particular (albeit unknown) set of antigens, thereby defining T cell specificity. T cell antigens are presented on two types of major histocompatibility complex (MHC) molecules, termed human leukocyte antigens (HLAs) in humans. MHC class I (MHC-I) molecules are expressed by all nucleated cells, whereas MHC class II (MHC-II) molecules are expressed by antigen presenting cells (APCs), epithelial cells and some tumors[28]. Peptides presented on MHC-I originate intra-cellularly primarily as proteasomal degradation products and are recognized by CD8+ CTLs, whereas the peptides that present on MHC-II are derived from exogenous or membrane proteins that are degraded by the endosomal/lysosomal system and recognized by CD4+ T cells. Overriding this principle is the process of cross presentation, whereby exogenously sourced peptides are presented on MHC-I mainly by $XCR1^+CD103^+$ type 1 dendritic cells (DC1s) [29] which then migrate to tumor-draining lymph nodes and prime T cells against tumor antigens[30] . Cross presentation is crucial for $CD8^+$ T cell priming and the maturation of tumor recognizing CTLs.

It is widely established that tumor immune-rejection is T-cell-mediated and the anti-tumor T cell response is antigen-specific[31]. Objective tumor regressions following antigen-selective and TCR-engineered adoptive cell transfer (ACT) treatments support these assertions[32,33].Genomic library screens have uncovered several prototypes of tumor rejection antigens, including mutation-derived antigens (called neoantigens) and cancer-germline antigens. The field has long recognized the archetypic distinction between tumor specific antigens (TSAs), which are exclusively presented on tumor cells, and tumor associated antigens (TAAs), which present on additional tissues as well. Advances in immunotherapy (Figure 2), and in the methods available for T cell antigen identification[25,34,35] (Figure 3), resulted in a surge of interest to identify and characterize tumor-presented T cell antigens, moving the field beyond classic TSA and TAA types, to previously unappreciated sources of cancer antigens, such as non-canonical and bacterial proteins[6,36,37]. The increased availability of sequencing data enabled the systematic exploration of cancer neoantigens, including the subgroup of recurrent (public) neoantigens[38].

Here we review the main classes of cancer T cell antigens known to date, discuss the attributes of effective cancer antigens and compare the different antigen classes with respect to

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- Please refrain from using italics for emphasis.
- Terms such as "while" and "since" should be used with their temporal meaning, appropriate alternatives are "although" and "whereas" instead of while and "given that" and "as" instead of since.

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these attributes. Finally, we summarize the clinical data that support the usefulness of each antigen class and give a critical overview of progress in the field.

Known classes of cancer-associated T-cell antigens

T cell antigens that permit efficient disease targeting are classified as TAAs or TSAs, depending on whether they are tumor exclusive. In this section we discuss an alternative, partly overlapping, classification that relies on the type of source protein. Accordingly, cancer antigens can be divided into self-antigens, originating from normal proteins that are differentially expressed in the tumor; genomic alteration-derived neoantigens, that are degradation products of tumor-specific mutated proteins; non-canonical antigens, arising from unannotated open reading frames (nuORFs), translation aberrations or post-translational modifications; and microbial antigens, derived from proteins encoded by tumor-infiltrating microbes such as bacteria and viruses (see also Figure 4).

Self-antigens

The precursors of tumor-associated *self-antigens* are non-mutated proteins that exhibit differential expression patterns in tumors. For instance, gp100[39], tyrosinase[40] and MART-1[41] are tissue-specific antigens expressed in melanoma. Tebentafusp, an immune-mobilizing monoclonal T cell receptor against cancer (ImmTAC) consisting of a soluble gp100-specific TCR fused to an anti-CD3 effector molecule, that yielded clinical benefit in patients with uveal melanoma [42] and was approved by the FDA, exemplifies the potential of self-antigen-targeting therapy. Given that tumors frequently share similar gene-expression patterns with their tissue of origin, the use of tissue-specific antigens in therapy is limited by the extent of collateral damage to the surrounding healthy tissue.

Cancer-germline antigens represent another class of self-antigens, which stem from proteins expressed only in germline tissues, i.e. fetal testes and ovaries, and trophoblast cells. Germline genes are epigenetically silenced by promoter methylation in most healthy tissues, excluding the immune sanctuaries of germ and placental trophoblasts. Yet, in many human cancers, promoter demethylation reactivates their expression. An analysis of 153 CG genes showed their highest aberrant expression is in skin, lung, liver and brain cancers[43]. cDNA expression library screens have greatly contributed to the identification of such antigens, including the X-chromosome-linked MAGE family of antigens and NY-ESO-1[44-46]. Cancer-germline antigens are less affected by central immune tolerance than other types of self-antigens due to their unique expression pattern[47], which together with their high prevalence in patients, makes them highly interesting immunotherapy targets. However, their expression in tumors was found to be heterogeneous due to their locally varying DNA-methylation status[48].

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Genomic alteration-derived neoantigens

Mutation-derived neoantigens feature cancer-distinct sequence aberrations encoded by somatic point mutations, frameshifts, or chromosomal aberrations. Non-synonymous mutations that result in aberrant proteins can lead to the generation of genuine tumor-specific antigens if their degradation results in HLA-binding neopeptides. Being the most abundant and simple form of mutations[49], non-synonymous point mutations are currently the best studied mutation-derived neoantigen precursors. Single amino acid changes may either alter the immunogenicity of an HLA-binding peptide[50] or, if they occur in anchor positions, turn a non-binding sequence into

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an HLA-binding one[51]. Alternatively, a mutated amino acid could give rise to a novel proteasomal cleavage site, thus allowing peptide processing and HLA loading[52].

Indirect but important evidence of the pivotal role neoantigens hold in immune-mediated tumor regression come from the observed association between tumor mutational burden (TMB) and immunotherapy response. The fact that high TMB stochastically increases the chance of neoantigen formation together with the central role of T cells in mediating tumor regression both in immune checkpoint blockade (ICB) and adoptive cell transfer (ACT), suggests the involvement of a neoantigen-driven T cell response. Independent studies, across multiple cancer types and immunotherapy modalities found improved clinical outcomes with increased TMB[7,21,22,53-57]. Recently, neoadjuvant anti-PD-1 administration to 12 patients with stage II-III mismatch repair deficient (i.e. high TMB) rectal adenocarcinoma achieved 100% complete clinical response rate, alleviating the need for standard chemoradiotherapy[58]. However, this association does not hold for all tumor types as shown in glioma, where TMB could not predict immunotherapy efficacy[59].

The advent of next-generation sequencing has allowed the systematic, unbiased survey of mutations from individual tumors[60]. These data, in turn, can guide antigen discovery[61-64] either through T-cell-based assays or HLA-peptidomics[25,34,35] (Figure 3). Data generated from extensive whole-exome sequencing-based screening raises the possibility that tumor-infiltrating lymphocyte (TIL) reactivities against mutation-derived neoantigens exist in the majority of cancers, not only in tumor types known to be amenable to immunotherapy[25]. Yet less than 2% of the screened mutations are recognized by T cells, and the associated neoantigens are almost universally unique to each patient (*private* neoantigens), greatly narrowing their applicability to the majority of patients[25].

In contrast, recurrent or *public* neoantigens derived from both point-mutations and larger genetic aberrations, although scarce, have also been identified[50,65,66]. Unlike functionally unimportant private passenger mutations, driver mutations are functionally important and tend to be more clonal[67]. Finally, cellular therapies or vaccinations against recurrent mutations can benefit many patients with the same tumor type , but also patients with different cancer types harboring the same recurrent mutation.

Among the known neoantigens derived from recurrent mutations are CDK4.R24C[3,68-70], KRAS.G12V/C/D[71-73], EGFR[74-79] and PIK3CA.H1047L[80]. HLA-peptidomics coupled with whole exome sequencing[81] to survey the landscape of recurrent neoantigens in melanoma discovered an (N)RAS.Q61K/HLA-A*01:01-derived neoantigen that elicits T cell reactivity and cross-reacts with the highly prevalent (N)RAS.Q61R variant[50] suggesting that patients with RAS.Q61 mutations and HLA-A*01:01 could benefit from cellular treatment. A similar methodology is Mutation-Associated Neoantigen Selected Reaction Monitoring **(**MANA-SRM**)**, an optimized immunoprecipitation and mass-spectrometry protocol for the detection of low-abundance neoantigens that was used to uncover several RAS and IDH2 derived recurrent neoantigens[82]. To date, the most clinically promising result for recurrent neoantigen targeting was achieved with the $KRAS^{G12D}$ mutation. Two patients, one with metastatic colorectal cancer and the other with metastatic pancreatic cancer, harboring this mutation on HLA-C*08:02 were successfully treated with TCR-transduced T cells against the mutation[32 83] , demonstrating the applicability of targeting recurrent neoantigens across different tumor types.

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Less frequent types of mutations, such as indels (insertions/deletions), translocations and inversions, may also give rise to neoantigens. Frameshift indel mutations were significantly associated with response to anti-PD-1 or anti-CTLA-4 in an analysis of three independent melanoma cohorts[84]. Further, the analysis indicated that frameshift mutations form a potentially more potent neoantigen landscape than an equivalent number of non-synonymous single nucleotide variations (nsSNVs)[84]. In mesothelioma, a cancer that generally exhibits low mutational burden but a high degree of large chromosomal rearrangements, the inter- and intra-chromosomal rearrangements were predicted to bind HLA molecules and were recognized by patient autologous TILs[85].

Finally, fusion genes, such as the BCR-ABL fusion in leukemia (Philadelphia chromosome)[86] and the EML4-ALK[87] fusion in non-small cell lung cancer (NSCLC) have been shown to generate T cell recognizable neoantigens [88,89]. Although large-scale structural variations (deletions, duplications, inversions and translocations) occur frequently and are a potential source for tumor neoantigens, existing tools for their prediction from whole-genome sequencing lack sensitivity, thereby limiting their usefulness[90,91]. An analysis of RNA-sequencing data from 9,624 TCGA samples across 33 cancer types, using the STAR-Fusion, Breakfast and EricScript algorithms for fusion calling, identified 25,664 fusion events[92]. Across the different cancer types, 1.5 neoantigens were predicted per fusion using NetMHCpan 4.0[93]. In contrast, analysis of two cohorts of patients with melanoma treated with ICB did not find fusion gene scores to positively correlate with survival, whereas the overall neoantigen score (nsSNVs, indels and fusion genes) did, raising questions regarding the role of fusion genes as tumor-rejection antigens[88].

Tumor antigens from non-canonical transcriptional and posttranscriptional aberrations

Accumulating evidence suggests that non-coding gene translation frequently occurs[94] and that anti-tumor immune responses can be directed against tumor antigens derived from non-coding regions[95-97]. By combining HLA peptidomics, RNA-sequencing and ribosomal-sequencing data[97], hundreds of shared and tumor-specific non-canonical HLA-presented peptides stemming from lncRNAs, pseudogenes, transposable elements, UTRs of coding genes and alternative open reading frames were uncovered. Yet, of the >500 antigens screened for immunogenicity, only one was recognized by autologous TILs and peripheral blood mononuclear cells. The low expression of non-canonical antigens and, hence, their limited availability for in-vivo cross-priming might underlie the low de novo T-cell responses detected. Antigen-specific T-cell responses have furthermore been observed against the intronic sequence N-acetylglucosaminyltransferase V gene (expressed in 50% of melanomas but not in healthy cells[95]), an incompletely spliced intronic region of gp100[98] and the 5' UTRs of c-akt oncogene[99]. . Examples for immunogenic, MHC-presented peptides arise from alternate reading frames include NY-ESO[100], HER2, telomerase reverse transcriptase (TERT), prostatic acid phosphatase (PAP) and nuORFs with non-AUG translation initiation sites[101-103]. Some nuORF neoantigens, such as the one stemming from a CUG-start-codon vascular endothelial growth factor (VEGF), were found to be cancer-specific, whereas others are expressed also in healthy tissue[102,103].

Translational reprogramming and impaired translational fidelity in cancer cells can give rise to non-canonically translated peptides and, potentially, novel immunogenic antigens[104]. Such neoantigens arise from translation malfunctions due to ribosome frameshifting during amino-acid deprivation[6], oxidative stress[105,106] or codon misreading by deregulated tRNAs[107]. Specifically, tryptophan shortage-induced ribosome frameshifting[6] was shown to lead to the

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presentation of novel trans-frame peptides on HLA molecules that are able to prime naïve T cells. Interestingly, patient samples with shared HLA alleles harbored identical frameshifting-aberrant peptides, suggesting that these peptides may be a recurring cancer feature. Aberrant protein translation by amino-acid deprivation are likely dynamic processes that depend on the tumor microenvironment and inflammatory and nutritional status, which puts into question their suitability for inducing anti-tumoral responses.

Finally, post-translational modifications (PTMs) can become deregulated in cancer cells, resulting in growth advantages[108] but also offering potential targets for cancer immunotherapy[109]. PROMISE, a computational pipeline for the detection of PTMs without enrichment has been used to identify numerous modified MHC-bound peptides with cancer-specific expression and their capacity to elicit a T-cell response[109]. However, it is yet to be determined if these PTM-derived antigens can elicit meaningful T cell responses for future cancer therapeutics.

Pathogen-derived tumor-associated antigens

Pathogen-derived tumor-associated antigens are remnants of bacterial or viral infections. If acute infections are not cleared properly, viruses can remain inside host cells and—due to the expression of oncogenic proteins, induced immunosuppression and disruption of the host genome—mediate a malignant transformation (reviewed in[110]). Pathogens that can directly drive cancer include *Helicobacter pylori* which induces gastric cancer[111] , human papilloma virus (HPV) which induces genital and head-and-neck cancers[112] , and hepatitis B and C viruses (HBV and HCV) which cause hepatocellular carcinoma[113], among others. The reported *de novo* T cell responses against such pathogens[114,115] make inducing a specific T cell response against pathogen-derived antigens a promising strategy to elicit immune responses against cancer cells while sparing healthy tissue, which lacks pathogenic antigen expression. This may be achieved through therapeutic cancer vaccines[116,117] or ACT[118].

For instance, peptides from different intratumoral bacteria were found to be presented on patients' HLA molecules and trigger antigen-specific immune responses in melanoma[37]. Accordingly, the knockout of β-microglobulin (B2M) or class II major histocompatibility complex transactivator (CIITA) caused a decrease in the number of HLA-I and HLA-II presented bacterial peptides. Antigens derived from other types of microbiome, such as the virome, may emerge either with an intrinsic ability to elicit T cell responses, or to cross-react with other tumor-associated antigens in a form of molecular mimicry. An example for this concept is the prophage-encoded antigen TMP1 which activates T cells that are reactive against PSMB4. The *Enterococcus hirea* strain 13144 carries the phage and is abundant in lung an renal cancers, with the presence of the prophage in human patients correlating with response to immunotherapy[119].Another possible source for viral element-derived antigens is the human endogenous retrovirus *ERVE-4* whose expression was associated with immunotherapy response in clear cell renal cell carcinoma[120] . Common oncogenic viruses have the potential to form widely applicable T-cell targets if the processing of persistently expressed oncoproteins intersects with prevalent-HLA binding. High-risk HPV strains are involved in ~5% of all human cancers, in particular, cervical and oropharyngeal malignancies. Moreover, immunogenic peptides have been identified deriving from the HPV-related cancer driver genes E6 and E7 that are restricted to the highly prevalent HLA-A*02:01 alleles[121,122]. Finally, the recent discovery of fungi in various tumors with distinct compositions[123] may suggest that

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fungi-derived antigens, whether they exist and able to elicit T cell reactivity, could be another layer of tumor antigens.

Tumor antigen attributes contributing to anti-tumor immunity

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The anti-tumor potency of any given antigen relies on a combination of attributes, some of which are unique to T cell targets (e.g., immunogenicity and effective cross-presentation), whereas others would be applicable to any form of targeted therapy (e.g., population-wide prevalence, disease specificity, clonality and functional significance). Determining the optimal combination of features is not a trivial task. For example, mutation-derived neoantigens are immunogenic but tend to be private, whereas self-antigens are widely applicable, but less immunogenic. Accumulating clinical experience provides invaluable insight into the usefulness of self-antigens and mutation-derived neoantigens. However, the therapeutic potential of newly explored antigen classes, such as non-canonical neoantigens and bacterial antigens remains unclear. In this section we discuss the various properties contributing to the therapeutic effectiveness of an antigen in terms of four main parameters- the prevalence of each antigen category in the patient population, the specificity of each category to tumor cells rather than somatic cells, the immunogenicity and clonality of different antigens (see also Figure 4).

Population-wide prevalence

The population-wide prevalence of an antigen is a strong determinant of its therapeutic utility. Some cancer antigens, such as the prototypic TSA exhibit high recurrence rates. Cancer-germline antigens display differential frequencies across tumor types and disease stages. The cancer-germline protein MAGE-A1, for example, is observed in less than 20% of primary malignant melanomas, 48% of metastatic melanoma cases, 25% of ovarian cancers, but only in 3.5% of leukemias[124]. Recurrent neoantigens derived from KRAS, NRAS, TP53, PIK3CA and BRAF are expected to be relevant to thousands of cancer patients yearly[38,50,80]. Importantly, as in all T cell antigens, therapeutic targeting of recurrent neoantigens depends on specific HLA-peptide composition which requires the combination of both a highly prevalent HLA and peptide.

The prevalence of non-canonical neoantigens ultimately depends on the robustness of the underlying generative process and on the ubiquity of the precursor protein across tumors. nuORFs originating from tissue-specific, cancer-germline or overexpressed transcripts may potentially be as prevalent in the population as their canonical counterparts[96,98,100]. In an analysis of ten tumor samples from different patients, about half of the detected nuORFs were shared between at least two samples, suggesting that nuORFs are valid precursors for recurrent antigens[36]. The same study validated two nuORF melanoma antigens whose source genes are highly overexpressed in 28% (a pseudogene) and 59% (lncRNA) of TCGA melanoma samples.

Recurrence of ribosomal frameshifting-derived neoantigens has also been reported. Specifically, tryptophan-shortage-induced ribosomal frameshifting in melanoma cells has been linked to prolonged IFNγ exposure[6], and immunopeptidomics of IFNγ-treated tumor samples with shared HLA alleles has revealed recurrent mis-translated peptides. Finally, the extent of pathogen-derived cancer antigens is currently unknown and should be addressed in future

studies. As every cancer type is characterized by its own unique microbial repertoire[125], the full landscape of bacterial antigens is likely to be immensely diverse, and together with viral and even fungi-derived antigens, remain to be elucidated.

Tumor specificity

For a cancer treatment to be tolerable, cytotoxicity must be confined to the tumor, and must be considerably less abundant in normal tissue. Despite other fundamental advantages, shared TAA fall short in this respect. Tissue-specific antigens derived from MART-1 and gp100 resulted in disease regression in 30% and 19% of melanoma patients, respectively, but also in significant immunotoxicities[126]. Lethal cardiac toxicities and cytokine release syndrome from on-target anti-MART1 effects were documented[127,128]. Conversely, toxicity resulting from the gp100 targeting soluble TCR product tebentafusp, used for uveal melanoma, is reasonably tolerated[42]. Keeping in mind the small scale of these trials, it should be noted that although a less avid anti-MART-1 TCR produced a weaker therapeutic effect (12% of patients exhibited tumor regression), it also did not induce toxicities[129]. In contrast, the unique expression pattern of cancer-germline antigens should in principle make them practically tumor specific. Not surprisingly, this class dominates TCR-T clinical trials, with NY-ESO-1 the most targeted antigen precursor (Table 1), with NY-ESO-1 TCR-T showing promise as both an effective and tolerable treatement[130,131]. In practice, some cancer-germline antigens do present outside of immune-sanctuaries, and in amounts that can induce fatal toxicities. In an anti-MAGE-3 TCR-T trial, for example, cross-reactivity toward MAGE-12 in the brain caused severe neurological sequela in three patients, resulting in the death of two of them[132].

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As they derive from somatic mutations that accumulate during tumorigenesis, neoantigens are the epitome of tumor-specific antigens. One major concern when targeting neoantigens is cross-reactivity toward the wild-type variant. The majority of discovered neoantigens exhibit point mutations at the TCR-exposed region of the neopeptide. Therefore, their HLA-anchoring region is expected to be similar to those of HLA complexes in normal tissues[133]. In this regard, frameshift-derived neoantigens are potentially superior to point-mutation neoantigens. Nevertheless, direct comparisons of mutant versus wild-type TCR reactivity frequently revealed sufficient mutant specificity (i.e., no observed wild-type reactivity even at supra-physiologic peptide concentrations)[50,80,134]. An understudied neoantigen-related concern is the prevalence of somatic driver mutations in aging normal tissues. The deep sequencing of non-cancerous esophageal and skin samples revealed a surprisingly high burden of cancer-associated mutations[135,136], with TP53 found to be mutated in ~37% of normal esophageal epithelium. Thus, caution is required even when targeting otherwise promising hotspot-derived neoantigens.

Although the disease-specificity of non-canonical antigens remains unexplored, it likely depends on the underlying process that generates them. For example, cancer-associated chromosomal abnormalities might increase the proportion of novel protein isoforms specifically in the tumor[137]. Similarly, overexpressed and cancer-germline precursor genes may yield differentially expressed non-canonical antigens even if the underlying generative process is not differentially activated in cancer cells[36,97,109]. Factors in the tumor microenvironment, such as IFNγ produced under inflammatory conditions, can potentiate local generative processes such as ribosomal slippage events or single amino-acid substitutions[6,138]. Whether non-canonical cancer antigens are therapeutically tolerable requires further investigation.

Oncogenic viruses exhibit tropism toward the tissues in which they drive oncogenesis. High-risk HPV strains, for example, mainly infect the mucosal epithelium of anogenital tissues (cervix, vagina, vulva, anus, penis) and the oropharynx. Hepatitis viruses chronically infect the liver. EBV is maintained in epithelial cells of the pharynx, B cells and NK cells. MCPyV infects skin cells[139]. Differential tropism likens viral antigens to tissue-specific or shared antigens, and if extra-tumoral infection is not widespread, off-tumor effects may be tolerable or at least manageable. For example, although non-cancerous liver tissue destruction is a concern with anti-hepatitis ACT, using it in liver transplant patients may be a viable option[140], given that ACT products that contain anti-viral specificities are considered safe[122,140-144]. Nevertheless, viral occupancy in normal tissues has not been sufficiently studied. For example, HCV RNA and antigens were detected extra-hepatically in the kidney, heart, pancreas, intestine, adrenal gland, lymph nodes and gallbladder of HCV-infected cadavers[145]. In the case of bacterial cancer antigens, it appears that the cancer microbiome is cancer-type specific, resulting in differential tropism depending on the cancer type. However, tumor and immune cells of the tumor microenvironment are infected by bacteria[125]. Moreover, the detected bacterial genera are not rare in non-cancerous tissues, and differential infection or antigen expression in tumor vs. normal tissues has not yet been established[37].

Antigen Immunogenicity

The immunogenicity of antigens can be broken down into three main variables: a) The functional avidity of reactive T cells to a certain antigen; b) the antigen level of expression or cell surface density on tumor cells; and c) its effective cross-presentation by DC1 cells taking up tumor material. Importantly, immunogenicity is best regarded as a potential rather than a constant trait. It is highly affected by the antigenic and inflammatory context within which the antigen is being presented and not an antigen autonomous trait. The importance of antigenic context is exemplified by domination of T-cell reactivities towards a handful of antigens and, as further discussed below, by clonal fraction.

Functional avidity is most commonly assayed *in vitro* by cytokine release, upregulation of activation markers or target cell lysis at varying peptide concentrations. Multiple factors affect functional avidity, including TCR affinity (measured as K_D, the ratio of the association and dissociation rates, k_{off}/k_{on}), cell-surface density and organization (which contribute to TCR avidity), and the functionality of co-stimulatory interactions (e.g., CD8:HLA, CD80/86:CD28, ICAM-1/LFA-1) and intracellular signaling pathways. Effective T-cell triggering is thought to require mid-range affinity, to allow both serial engagement and sufficient dwell time[146,147]. Although low-affinity TCRs will not induce adequate activation, affinities that are too high may result in anergy or deletion[148]. A K_D of ~5μM has been proposed as a TCR affinity threshold, above which $CD8^+$ T-cell function cannot be improved[149]. Moreover, according to a recent simulation analysis, TCRs with equal affinity may differ in the functional avidity they confer even when all other parameters are equal. Therefore, alternate (k_{on}, k_{off}) formulations should be considered to predict functional avidity[150].

Not surprisingly, lower affinity/avidity values are observed against tumor-associated self-antigens compared to viral antigens or neoantigens[151,152]. On average, TCRs against tumor-associated self-antigens have 10 times lower binding affinities than those against viral antigens (100μM vs. 10μM, respectively)[152]. A K_D of ~10μM has been suggested to best balance anti-tumor efficacy and autoimmune risk for tumor-associated self-antigens[153]. The unique expression pattern of cancer-germline antigens predicts that they will be less affected by negative selection than other self-antigens. However, this assertion has not been studied

systematically. Evidence from a mouse model supports the lack of central tolerance against cancer-germline antigens, and a naturally occurring anti-NY-ESO TCR (1G4) exhibits a K_D of 11μM, which is a low value for self-antigens[152,154]. Nonetheless, evidence exists for at least some level of expression of cancer-germline antigens in the thymus[152].

As opposed to the low affinity of TAAs, non-self-antigens –be they canonical, non-canonical, or pathogen-derived— are not being tolerized during thymic T cell repertoire development and thus are predicted to have higher affinity. Although TIL reactivities against bacterial and non-canonical specificities have been observed, the quality of these interactions has not been sufficiently characterized. Being foreign entities, one would assume high TCR efficiencies for bacterial tumor-presented antigens. Non-canonical peptides that are truly aberrant, and do not present in the thymus, may also be exempt from central negative selection. Lastly, high-affinity/avidity TCRs can be engineered even for non- or weakly-immunogenic antigens, using humanized model species through vaccination or *in-vitro* optimization strategies[126,155,156]. Targeting T-cell antigens with engineered antibodies or chimeric antigen receptors is a viable option, as highlighted by pre-clinical studies[157-160], as is using engineered TCRs, exemplified by a $KRAS^{G12D}$-directed TCR on the HLA-A*11:01 which was engineered to have a 10^6 higher affinity compared to the original naturelly-occuring TCR[161].

Apart from affinity, TCR signaling depends on the *number* of TCR-pHLA (peptide-HLA) interactions at equilibrium –a function of both the affinity of the interaction (as discussed above) and the density of pHLA ligands on the cell surface. This is a complex interplay: although long TCR-pMHC half-lives (TCR dwell time) result in impaired T-cell activation for low pMHC densities, these are non-restrictive at high antigen densities[162]. pMHC antigen density depends on the expression level of the precursor protein, its degradation rate, and the affinity of the peptide-HLA interaction itself. Sufficient presentation on APCs (e.g., cross-presentation) is required for T-cell priming, whereas the amount on tumor cells is important for their proper killing. High pHLA affinity contributes to both these processes, with an estimated threshold of 10nM required for tumor eradication[163,164].

In tumors, endogenously expressed HLA-I TAAs (derived from NY-ESO-1 and MAGE-1) were detected at ratios of 10-150 copies per tumor cell, using soluble TCR probes[165]. Quantitative immunopeptidomics reports indicate a wide range of tumor antigen densities[82,166]. This variation is because different peptide antigens from the same TAA precursor (PMEL) could have an order-of-magnitude difference in their number of presented copies in the same tumor cell line[166]. Based on the quantification of multiple cancer cell lines, neoantigens seem to present a few to several dozen copies per cell[82,157]. Many non-canonical proteins are defective, unstable and short lived[167]. Owing to their rapid degradation, they are estimated to generate MHC-I peptides 5-fold more efficiently per translation event[137]. However, to the best of our knowledge, the copy numbers that such individual antigens contribute has not been estimated[36,137]. Intracellular viral and microbial pathogens have evolved molecular mechanisms to decrease the presentation densities of their derived antigens[162,168] and thus might represent less ideal therapeutic targets.

Although direct HLA-I presentation on tumor cells is enriched for short-lived, rapidly degrading proteins[167,169], efficient cross-presentation requires the sufficient transfer of precursor proteins into presenting cells, thus favoring long-lived, stable, highly expressed substrates[170]. Dampened cross-presentation of unstable proteins has been postulated to effectively diminish the contribution of non-canonical antigens to anti-tumor immunity[167]. In a mouse model, insufficient cross-priming by a lowly expressed tumor-rejection antigen precluded tumor regression despite adequate presentation on tumor cells[171]. This could be

ameliorated by therapeutic enhancement of cross-priming through vaccination or by anti-CD40 administration. Such interventions mark an untapped opportunity to harness non-canonical and lowly expressed antigens (which are usually not considered for vaccine design) for cancer therapy.

Antigen clonality

In addition to the amount of a presented antigen, its *distribution across cancer cells*, i.e. its clonality, is also critical. As intratumor heterogeneity alongside TMB, are important determinants of anti-tumor immunity and responsiveness to immunotherapy[172], the immune system's ability to detect and eliminate antigen-bearing cells depends on their clonal fraction within the tumor[173]. Sub-clonal antigens that present on only a fraction of tumor cells are thought to facilitate tumor escape through the outgrowth of antigen-deficient cells. Thus, achieving effective immune control across all sites of a metastatic disease by targeting sub-clonal antigens is less likely. Indeed, the burden and fraction of clonal neoantigens correlate with response to ICB in lung cancer and melanoma[2]. Furthermore, in a mouse model of controlled intratumor heterogeneity, mixing together immune susceptible clones resulted in a polyclonal, immune-resistant tumor[174].

Tumor antigens vary significantly with regard to their clonality. Although self-antigens are generally considered clonal, the expression of cancer-germline antigens within tumors was found to be heterogeneous due to locally varying DNA methylation status[48]. Linked to clonality, the essentiality of the precursor protein to cancer survival also bears significance as reliance on functionally unimportant proteins facilitates evasion through elimination or down-regulation of the protein[175]. Although most mutation-driven neoantigens stem from passenger mutations, the subgroup of recurrent (public) neoantigens has the added value of functional relevance, and oftentimes also of clonality, making them superior therapeutic targets. However, evasion might ensue even when targeting clonal antigens that derive from functionally important proteins. For example, after initial regression of colorectal cancer metastases following KRAS-directed ACT, one metastasis recurred with HLA haplotype loss[32]. In another study, the metastasis of a primary tumor bearing an immunogenic BRAF neoantigen showed no trace of this oncogenic mutation in sequencing analyses[78]. Such reports exemplify that, much like with targeted therapies, and regardless of antigen clonality, combinatorial approaches to target multiple antigens, at once or sequentially, should be considered when possible.

Single-cell dissection of TIL specificities enables comparison of antigen classes

Coupled TCR and RNA single-cell sequencing have been used to map TIL specificities and transcriptional phenotypes in melanoma, lung, breast, colon, and rectal cancers[151,176-178]. Two of these studies showed that tumor-reactive cells occupy shared exhaustion/dysfunctional states, regardless of their target antigen class[151,176]. Furthermore, TILs targeting neoantigens and self-antigens could not be transcriptionally differentiated[151]. Conversely, bystander lymphocytes, including clones that target viral antigens, gravitated towards effector memory or tissue-resident memory phenotypes[151,178]. Prospective reactivity-testing of TILs matching a neoantigen-reactive transcriptional program, identified, in addition to neoantigen-specific cells, viral and shared-antigen specificities, as well as tumor-reactive orphan receptors[176]., Unlike for bystander anti-viral cells, the viral-specific clone in this case targeted an HPV-

derived antigen and originated from an HPV+ tumor. Transcriptomic patterns enriched for neoantigen-specific T cells correlated with pathologic response to ICB, indicating the functional importance of these newly defined phenotypes[178]. The above-mentioned work therefore marks different transcriptional programs for tumor-targeting versus non-tumor-targeting T cells but does not find distinguishing patterns between tumor-targeting T cells of different antigen classes. It is possible that functionally important differences would emerge when directly contrasting tumor-reactive clonotypes of different antigens classes. However, such analyses would require larger scale knowledge of TIL specificities.

Owing to the apparent transcriptional common ground of tumor-reactive TIL, one may expect immune-modulating approaches, such as ICB, to affect tumor-reactive T cells in similar manners, regardless of the antigen class that they target. How this modulation plays out may depend on additional factors, such as TCR avidity. Higher TCR avidities have been observed for neoantigens compared with self-antigens, as would be expected from differential thymic selection pressure for these two types of antigens[151]. Another study pointed to functional avidities of neoantigen-reactive TCRs that are on par with those of anti-viral TCRs[178], noting markedly higher neoantigen-specific avidities in ICB major pathologic responders (MPRs) compared to non-MPRs. Importantly, many tumor-reactive exhausted cells in these studies bear orphan TCRs. Whereas some of them probably target overlooked self, viral or mutation-derived neoantigens as demonstrated in ref. [178], we speculate that full delineation would reveal specificities also towards less studied classes of cancer-presented antigens, such as non-canonical neoantigens and bacterial peptides.

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Therapeutic utility of T cell antigens

The choice of antigen(s) is of utmost importance for the success of antigen-directed immunotherapies. Although arguments can be made as to the merits and drawbacks of each antigen class, one should focus on the accumulating clinical data alluding to their therapeutic efficacy. Self-antigens and mutation-derived neoantigens are the two antigen classes that have been studied most extensively, with demonstrated contributions to immune-mediated tumor control (see Table 1-2). Attempts have also been made to harness anti-viral T cell responses for cancer control (see Table 3). Newer antigen classes, such as non-canonical and bacterial-derived peptides, are yet to be tested. In this section we discuss the implications of key clinical findings relating to the different antigen classes.

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Clinical experience with self-antigens

As discussed above, the prevalence of self-antigens in normal tissues impacts both the efficacy and safety of treatment. The difficulty of balancing tolerance against these antigens (which must be overcome to reach clinical benefit) and excessive immune response (which could manifest in severe toxicity) lead to mixed results in clinical trials (Table 1). TCR-T therapy against a melanocyte-specific MART-1/HLA-A*02 antigen induced objective cancer regression in 12% of melanoma patients[129]. Higher-avidity TCR (DMF5) induced an improved response rate (30%), along with significant toxicities to normal melanocytes[126]. Similarly, targeting of gp100- and CEA-derived tissue-specific antigens using mouse-produced TCRs in melanoma and colorectal cancer patients, respectively, yielded disease remission but also substantial impairments to normal tissue[126,179]. Complete regression was reported with NY-ESO-1-selected $CD4^+$ ACT in a metastatic melanoma patient[180]. TCR-T utilizing affinity-

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enhanced TCRs against HLA-I restricted NY-ESO-1 and MAGE-4 CG antigens resulted in confirmed partial responses in ~50% of patients with an assortment of solid tumors, mainly consisting of synovial sarcoma[131,181,182]. In multiple myeloma, the same NY-ESO-1 TCR resulted in 70% complete or near-complete responses, however most responders relapsed within months due to immune-escape[183,184]. Repurposing of a naturally occurring high-affinity HLA-II restricted Treg-derived anti-MAGE-3/6 TCR conveyed objective response in 4/17 patients, including one complete response in a patient with cervical carcinoma[185]. Although manageable toxicity profiles were observed for the abovementioned trials with cancer-germline antigens, TCR enhancement and repurposing bear non-negligible risks for both off- and on-target adverse effects, as was exemplified by cardial and neurological lethal toxicities in TCR-T trials targeting MAGE-3 antigens[128,132]. Additional ACT trials targeting self-antigens both in solid cancers (AFP, CEA, tyrosinase, HER2, PRAME, WT1) and in leukemias (WT1, PRAME, HA-1) are currently ongoing.

The above results suggest that it is essential to improve current therapies targeting self-antigens. The success and subsequent FDA approval of the gp100 targeting product Tebentafusp is one key exampleA 69% response rate was reported for DMF5 TCR-T with concurrent anti-MART-1 dendritic cell vaccination[186]. Accumulating data suggest that, despite many disappointing clinical trials of other vaccines, self-antigens can be used for vaccination when potent delivery systems of high antigen loads and proper inflammatory stimuli are employed, such as in the case of mRNA vaccines (Box 2). In a phase I clinical trial involving 119 melanoma patients, inoculation with self-antigen-encoding intravenously administered liposomal RNA (RNA-LPX) vaccines and anti-PD-1 therapy elicited durable objective responses in checkpoint-inhibitor-treated patients with unresectable melanoma[187]. RNA-LPX vaccines encoding the cancer testis antigens NY-ESO-1 and TPTE (transmembrane phosphatase with tension homology), the melanoma tissue-specific antigen tyrosinase and the tumor-specific antigen MAGE-A3, induced strong CD4 and CD8 T-cell responses in the majority of patients, reaching low-double-digit percentages of circulating CD8 T cells. The perceived inefficiency of many therapeutic cancer vaccines may be explained, at least in part, by the requirement to not only induce potent antigen-specific T cell responses (as in the prophylactic setting), but also drive their efficient migration into the tumor and counteract various suppressive mechanisms that tumors impose.

Clinical experience with mutation-derived neoantigens

A number of studies have reported the expansion of neoantigen-specific T-cell populations following immunotherapy. ACT case studies in melanoma and head-and-neck cancer uncovered 8-750-fold increases in the frequency and long-term-persistence of mutation-reactive clones in responders' peripheral blood[188,189]. A 5-fold increase in peripheral blood neoantigen-specific T-cell reactivity was detected following anti-CTLA-4 therapy in a patient with metastatic melanoma [190]. Similarly, in a case study of metastatic melanoma, a patient exhibiting a durable clinical response to combination immunotherapy consisting of anti-PD-1 + IL-2-pathway agonist, was found to have tumors enriched with $CD8^+$ and $CD4^+$ neoantigen-specific T-cell clones prior to treatment[191]. The neoantigen-reactive, but not self-antigen-specific, clones transiently expanded in the blood, and modestly also within the tumor, during treatment. In the case of a patient with NSCLC who responded to anti-PD-1 treatment, a neoantigen-specific T-cell response could be detected in peripheral blood only after treatment

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initiation and increased 8-fold during treatment[21]. A rise in peripheral blood neoantigen-specific T-cell fractions was also noted in a patient with metastatic breast cancer, achieving complete durable regression following combination treatment with ACT, ICB and IL-2[192].

Several phase I and II clinical trials that specifically target nsSNVs for therapeutic vaccination (reviewed in[193] and summarized in Table 2) have reported promising initial results[194,195]. In one such phase I clinical trial, 13 melanoma patients were vaccinated with two mRNAs, each encoding five nsSNVs. These nsSNVs were predicted from whole-exome and RNA-sequencing data of healthy and tumor tissue. For neoepitope prioritization, MHC binding affinity, expression levels and frequency of the mutated allele were considered. T cell responses against multiple neoantigens and a reduction in the cumulative rate of metastatic events were observed in all patients[195]. Of the five patients with detectable lesions, two had objective responses, one a mixed response, one a stale lymph-node metastasis that was resected, and one a complete response when treated in combination with anti-PD-1 blockade. The other eight patients remained tumor-free over the whole follow-up period of 12-23 months. Vaccine-induced T cell responses were observed against 60% of 125 predicted neoepitopes, of which 68% were *de novo* and 32% pre-existing.

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In another personalized vaccine trial, patients were vaccinated with long-peptide vaccines (15-30 AA) and a poly-ICLC encoding up to 20 mutations per patient[194]. Of the six melanoma patients, four had no recurrence at 25 months. The two patients who experienced recurrence went on to complete tumor regression when treated with anti-PD-1. Vaccines induced 60% CD4 and 16% CD8 T-cell responses against the 96 predicted neoantigens, which were selected from whole-exome and RNA-sequencing data using NetMHCpan to assess MHC class I binding. Mutations in oncogenes were given the highest priority during epitope selection.

A small number of reports found that infusion products highly enriched for neoantigen reactivity induced significant disease regression. For example, in a case study of cholangiocarcinoma, treatment with TIL product consisting of 25% CD4+ cells directed at an ERBB2IP^{E805G}-derived neoantigen brought about initial disease stabilization. Re-infusion of a >95% neoantigen-specific preparation upon disease progression achieved tumor regression[33]. TIL products consisting of 23% and 35% neoantigen-reactive cells induced prolonged, complete regressions in breast and cervical cancer cases, respectively[188,192].

Concerning recurrent neoantigens, a phase I IDH1-specific peptide vaccine trial, involving 33 newly diagnosed patients with grade III-IV astrocytomas resulted in vaccine-induced immune-responses in 93.3% of patients[196]. Within this group of responders, a two-year progression-free rate of 0.82 was observed. A colorectal cancer patient treated with a TIL product highly selected (75%) for reactivity against a recurrent KRAS^{G12D}/HLA-C*08:02 neoantigen exhibited objective regression of multiple metastases[32]. One of the lesions progressed nine months after therapy and has been shown to have lost the HLA-allele presenting the neopeptide. In a case report of metastatic pancreatic cancer, TCR-T against the same neoantigen resulted in objective partial response of 72% in visceral metastases, that was ongoing at 6 months[83]. In a cohort of 12 patients with chemorefractory epithelial cancers, non-selected ACT treatment with p53-reactive TIL products resulted in only two partial responses. The infused TIL products contained low frequencies of p53-reactive cells, exhibiting an exhausted phenotype[197]. Anti-p53 TCR-T of a chemprofractory breast cancer patient resulted in objective tumor regression that lasted 6 months[197]. Unlike with self-antigens, no adverse off-target effects were reported in these highly antigen-specific ACT treatments. These studies provide direct proof of the clinical anti-tumor potency of mutation-derived neoantigens.

Clinical experience with viral antigens

HPV is etiologically implicated in the development of cervical and other anogenital cancers and is highly associated with oropharyngeal cancers.. Prophylactic vaccines against high-risk HPV types utilize L1 bearing viral like particles to induce neutralizing antibodies. These vaccines are estimated to prevent 90% of viral contraction and disease, however they are ineffective in the treatment of established infections, pre-malignant and malignant lesions[198]. In the therapeutic setting (Table 3), the aim is to induce a cellular response in the form of HPV-specific CTLs and Th cells, by presentation of HPV-derived antigens on APCs. The main oncogenic HPV proteins, E6 and E7, are usually targeted. Targeting of E2 may be beneficial for pre-cancerous lesions[199]. Live vector-based vaccines, peptide/protein-based vaccines, nucleic acid-based and whole cell vaccines have all been tried in the context of HPV (see Table 3, reviewed in[198]). These vaccines are generally safe, well-tolerated and are successful in inducing anti HPV cellular responses. However, the observed therapeutic effects are variable and oftentimes modest compared to mouse models.

ADXS11-001, a live, attenuated Listeria-monocytogenes-based vaccine, was evaluated in a phase II clinical trial in patients with relapsed/refractory cervical cancer previously treated with chemotherapy and/or radiotherapy. Combination therapy of ADXS11-001 and cisplatin achieved 17.1% overall response rate, and 38.9% overall survival at 12 months[200]. GLBL101c, a *Lactobacillus-casei*-based vaccine induced regression of cervical intraepithelial neoplasia 3 (CIN3, a pre-cancerous lesion) to CIN2 in 70% of the patients after 9 weeks of treatment[201]. In CIN2 patients the vaccine induced response in 22%, with complete remission achieved in 11% (2/19) of patients[202]. A phase II peptide vaccine trial queried whether combination therapy may improve upon ICB alone in HPV16+ cancer patients. An overall response rate of 33% was achieved for combination therapy, compared with 16-22% with anti-PD-1 alone[203]. The same vaccine achieved tumor regression in 43% of patients with advanced, recurrent, or metastatic cervical cancer, in a clinical trial combining vaccination and standard chemotherapy[204]. A clinical trial with DNA vaccine GX-188e in 64 CIN3 patients, induced 52% histopathological regression to ≤CIN1 at week 20 after treatment (67% at week 36). 73% of patients with proved histological regression showed HPV clearance at week 20 (77% at week 36)[205]. In a separate CIN2-3 DNA vaccine trial (pNGVL4a-CRT/E7), 30% (8/27) had histological regression[206].

ACT with HPV-reactive TIL products induced durable tumor regressions in 2/9 patients with HPV+ metastatic cervical cancer[207]. However, post-hoc dissection revealed that other types of tumor antigens dominated these infusion products[188]. Anti-E7 TCR-T therapy led to robust tumor regression in 6/12 patients, whereas anti-E6 TCR-T induced responses in 2/12 patients[141,142]. The experience with targeting other cancer-related viruses is currently limited but encouraging clinical responses have been observed[144,208]. Additional vaccine and ACT trials targeting cancer related viruses, such as HPV, EBV, MCPyV and HBV are currently ongoing.

Outlook

Recent years have witnessed great advances in charting tumor antigenic landscapes, including large-scale studies of genetically encoded neoantigens and the identification of new non-self-antigen classes stemming from aberrant transcription or translation. Although the therapeutic merit of cancer-associated T-cell antigens is well established, the intrinsic and contextual qualities defining effective tumor-rejection antigens are not yet fully understood. It has been argued that non-self-antigens are superior to self-antigens[209], and that clonal antigens are better

than sub-clonal ones[210]. However, even though tumor-antigens are usually discussed as discrete entities, the antigenic landscape within which they appear is bound to shape the immune-response they elicit[174]. For example, immunodominance may limit reactivity toward co-expressed antigens. Along with clonal fraction[173], cell-surface density may also attenuate antigenic potency and is influenced by peptide competition for HLA presentation. Processes affecting the peptide pool to increase competition may therefore drive immune evasion. The link between specific antigen traits and prototypic T cell states observed in tumors, such as exhaustion and stem-like phenotypes, also requires further elucidation. As with clonal heterogeneity[174], a richer antigen repertoire may not automatically imply more effective anti-tumor immunity . New classes of cancer antigens will undoubtedly be revealed in the future, illuminating the peptidome 'dark matter' piece by piece. Translationally, such discoveries are expected to provide new targets for immunotherapy, with further delineation of the cancer HLA-peptidome advancing our understanding of anti-tumor immunity. Moreover, it is increasingly recognized that antigen identity alone cannot predict tumor-targeting potential. Factors such as copy number and clonal fraction, as well as the identities of other antigens with which an antigen is presented (some of which may be immunodominant) may induce tolerance and significantly alter the observed immunogenicity[171,174,211,212]. A deeper understanding of the cancer immunopeptidome and dissection of the antigens at play during natural and treatment-induced anti-tumor immune responses will be crucial for improving immunotherapy.

Figures:

Figure 1 T cell antigens at the center of all immunotherapy modalities.

Different immunotherapy modalities all converge onto T cell recognition of tumor antigens: (1) Adoptive cell transfer of T cells specific for tumor cells according their TCR specificity. Patient are re-administrated with peripheral or intratumoral T cells previously isolated, *ex vivo* expanded and if aplicable, TCR-engineered. (2) Vaccines of different types (e.g., protein/peptide-, RNA- or DNA-based vaccine, DC vaccines) educate the immune system against specific tumor-presented antigens. Vaccines are taken up by local dendritic cells that migrate to secondary lymphoid organs, prime and activate T cells that mediate tumor immune attack. (3) Immune checkpoint blockade unleashed pre-existing T cell/tumor interactions. Monoclonal antibodies specific for inhibitory receptors prevent ligation and unleash halted immune attacks. Anti-PD-1/PDL-1 therapy is illustrated, but mechanisms hold true for other types of ICB as well. (4) Uni- and bispecific monoclonal antibodies (mABs) that are directed at a specific T cell antigen on tumor cells may either recruit T cells for attach or may release a cytotoxic cargo. DC: dendritic cell; PD-1: programmed cell death protein 1; PDL-1: programmed cell death protein 1 ligand 1; TCR: T cell receptor.

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Timeline – Advances and discoveries in tumor antigen research

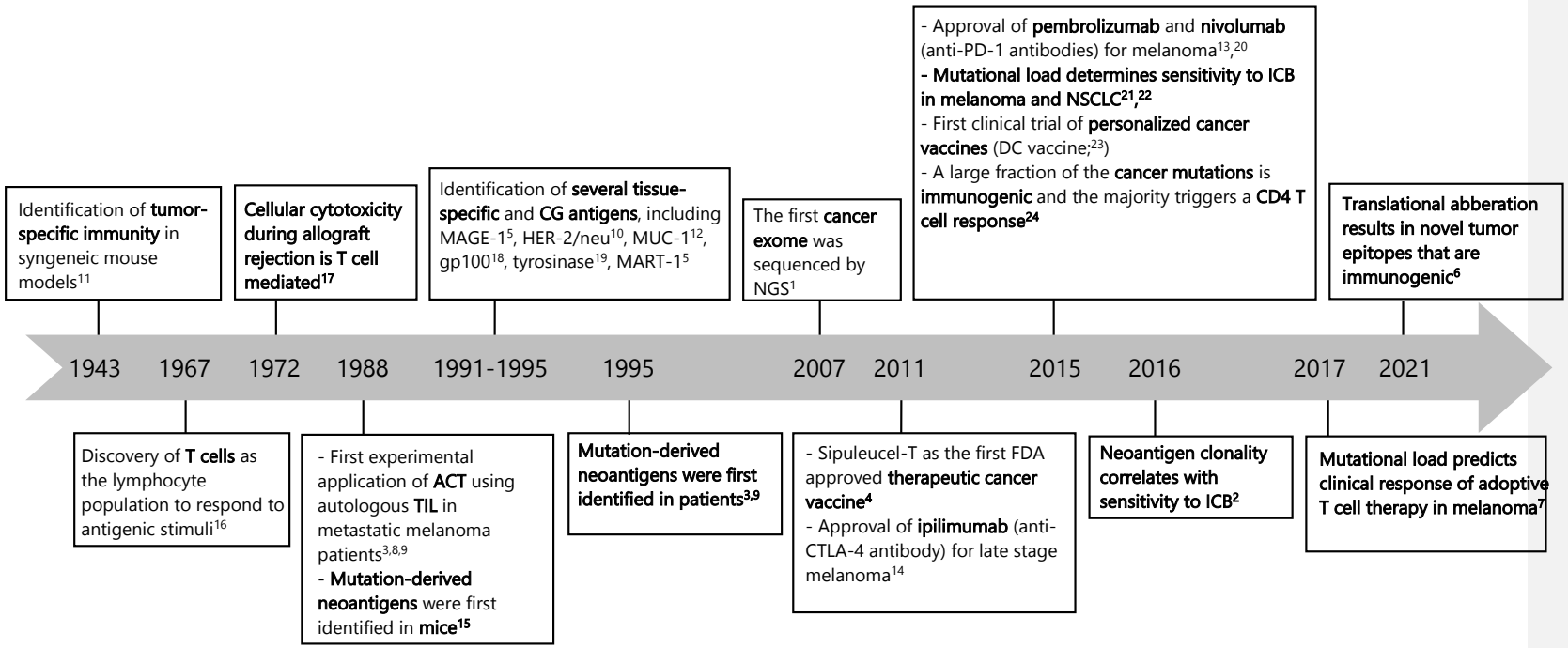

Figure 2 Advances and discoveries in tumor antigen research.
ACT: adoptive cell transfer; CG: cancer germline; CTLA-4: cytotoxic T-lymphocyte-associated Protein 4; DC: dendritic cell; FDA: food and drug administration; gp100: glycoprotein 100; HER-2: human epidermal growth factor receptor 2; ICB: immune-checkpoint blockade; MAGE-1: melanoma associated antigen 1; MART-1: melanoma antigen recognized by T cells 1; MUC-1: mucin-1; NGS: next generation sequencing; NSCLC: non-small cell lung cancer; PD-1: programmed death 1; TIL: tumor infiltrating lymphocyte

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Figure 3 Methods for the identification of cancer T-cell antigens. The three main routes for antigen-discovery: (1) prediction, (2) immunopeptidomics and (3) T-cell-based reactivity/expansion assays. Prediction algorithms (1), such as the popular NetMHCpan, receive as input protein/peptide sequences and a list of HLA-alleles of interest, and provide as output peptide/HLA pairs that are predicted to bind. In Immunopeptidomics (2) , HLA-peptide complexes are immunoprecipitated from cell lysates, and then the peptides are eluted and analyzed by mass-spectrometry to determine their sequences. This allows unbiased inquiry of the presented peptidome, regardless of immunogenicity. T-cell based assays (3) rely on in-vitro co-incubation of T cells with presenting cells that express the protein/HLA pairs of interest. Reactivity or expansion readouts are then utilized to confirm antigen-presentation. An added advantage with this approach is that antigen-reactive T cells may be isolated and further utilized. The flow of information between these methods aids in their improvement. Validated antigens from immunopeptidomics and T-cell based assays are used to train better prediction algorithms. Predictions, in turn, serve to narrow the search space in immunopeptidomics or T-cell based screens and add credibility to identified hits. Ag: antigen; HLA: human leukocyte antigen; PBMC: peripheral blood mononuclear cells

Figure 4 Tumor antigens recognized by T cells. Different classes of tumor-specific antigens, their potential to evade central tolerance, prevalence, tumor specificity and clonality. Overexpressed tumor- and tissue-specific antigens are ubiqitously present in tumor cells, however are shared with healthy tissues and thus have low tumor-specifity and are hampered by central tolerance. Cancer germline antigens are solely expressed in the germline and become re-expressed in tumor cells, providing them with a medium tumor specificicity and subjecting them to central tolerance. Viral- and bacterial-antigens stem from former oncogenic pathogen infection, which renders them highly tumor-specific with no expression in healthy tissue and a lack of central tolerance. Neoantigens arising from mutation, e.g. SNVs, INDELs or fusion genes arise from oncogenesis and are exclusively present in cancer cells, harbor a high tumor specificity and no central tolerance. Aberrant translation-or transcription-derived neoantigens are a result of malfunctional cellular transcription and translation machinery in cancer and not encoded by the genome. As a rather novel class of tumor-specific antigens, their prevalence and tumor-specificity largely remains to be explored. HBV: hepatitis B virus; HPV: human pappiloma virus

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Box 1: Immunotherapeutic strategies for induction and modulation of T cell responses

Adoptive cell therapy (ACT) includes the treatment of patients with their own, naturally occurring or genetically engineered tumor antigen-reactive T cells[213,214] Typically, resected neoplastic tissue obtained through biopsy or surgery is used to extract tumor-infiltrating lymphocytes (TILs), which are then massively expanded *ex vivo*. In addition, TCRs recognizing tumor antigens can be isolated and endogenous T cells can be genetically engineered to express these TCRs.

The relative ease with which the antigen component may be adapted, thereby providing an avenue for personalized treatment, along with its straightforward administration, makes vaccination an attractive strategy as well. Vaccination aims at de novo generation of tumor-antigen specific T cell responses as well as augmentation of existing T cell responses by delivery of tumor antigens to professional antigen presenting cells together with their proper activation via adjuvants. Evidence supporting the efficacy of vaccination in disease control is now emerging[215].

Another immunotherapeutic strategy approved in clinical practice, is immune-checkpoint blockade (ICB) which unleashes the T cell potential against the tumor. The leading ICB targets, PD-1 and CTLA-4, are both inhibitory co-receptors whose expression is upregulated in T cells on antigen-dependent TCR stimulation. An analysis of human specimens revealed significant

associations between increased CD8 CTL tumor infiltrates and response to PD-1 blockade[216]. The level of such infiltrates greatly increases in responders during treatment and correlates directly with reduction in tumor size. Expansion of T cell populations, broadening of the TCR repertoire and depletion of intra-tumoral regulatory T cells (Tregs) are also outcomes of clinical relevance with CTLA-4 blockade[217].

Box2: mRNA vaccines

mRNA vaccines were moved in the spotlight after the approval of prophylactic mRNA vaccines against COVID-19 during the pandemic era, although basic and translational research has shown the promise of therapeutic mRNA vaccines against various diseases including cancer in the last three decades (). Currently, multiple phase I and II clinical trials against various cancer types are being conducted to assess the potential of therapeutic mRNA vaccines with early signs of clinically relevant responses.

The elegant formulation of mRNA vaccines, an otherwise fragile and short-lived intermediate for intracellular antigen production, allows its efficient transfer to APCs through various routes of administration, which in turn help induction of high and durable antigen-specific cellular and humoral immune responses. mRNA vaccines combine various features of a desirable vaccine platform. *In vitro* transcribed mRNA can deliver molecularly defined antigens to be presented in the MHC class I and II context in an HLA-independent manner through expression of the whole antigen rather than selected HLA-restricted epitopes. Moreover, its intrinsic adjuvant activity obviates the need for an additional adjuvant. It also serves an ideal safety profile with immuno-pharmacologically optimized transient expression, the potential for repeated application, lack of genomic integration and anti-vector immunity. mRNA production scale can also be tuned easily and rapidly at good manufacturing practice level to provide the small doses required for a single patient as in the case of personalized cancer vaccines, or very large volumes as in the billion vaccine doses needed for global vaccination against COVID19.

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Table 1 Selected published clinical trials utilizing self-antigens as targets

Year of publication	Investigator /Sponsor	Clinicaltrials.gov identifier, phase	Indication	Platform /Treatment	Key results / Ref
2006	NIH	n/a, n/a	Metastatic melanoma	Adoptive transfer of MART-1 specific TCR engineered T cells	12% of the patients experienced tumor regression
2008	Fred Hutchinson Cancer Research Center	n/a, n/a	Metastatic melanoma	Adoptive transfer of NY-ESO-I specific CD4+ T cells isolated from blood	Complete regression on a single patient (case report)
2009	NIH	NCI-07-C-0174,I	Metastatic melanoma	Adoptive transfer of MART-1 and gp100 specific TCR engineered T cells	30% and 19% of the patients receiving MART-11 or gp100 TCRs, respective experienced objective antitumoral response along with toxicity
2011	NIH	NCT00923806, I	Metastatic colorectal cancer	Adoptive transfer of CEA specific TCR engineered T cells	All patients experienced decreases in serum CEA levels with one patient with objective regression along with toxicity

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2011	NIH	NCT00670748, I	Metastatic synovial cell sarcoma and melanoma	Adoptive transfer of NY-ESO-I specific TCR engineered T cells	~50% of patients with each indication experienced objective clinical responses
2014	Jonsson Comprehensive Cancer Center	NCT00910650, II	Metastatic melanoma	Combination of MART-1 peptide pulsed dendritic cell vaccine and adoptive transfer of MART-1specific TCR engineered T cells	69% of the patients experienced tumor regression
2015	Adaptimmune / GSK	NCT01352286, I/II	Multiple myeloma	Adoptive transfer of NY-ESO-I and LAGE-1 specific TCR engineered T cells	80% of the patients experienced objective clinical response
2017	NIH	NCT02111850	Metastatic cervical, urothelial, esophagial cancer , osteosarcoma	Adoptive transfer of MAGE-A3 specific TCR engineered CD4+ T cells	25% of te patients expressed regression of tumors, one cervical cancer patients exhibiting complete response
2018	Adaptimmune / GSK	NCT01343043, I	Metastatic or recurrent synovial sarcoma	Adoptive transfer of NY-ESO-I specific TCR engineered T cells	~50% of patients experienced objective clinical responses
2020	NCT02410733, I	Metastatic melanoma	Systemic mRNA **vaccine**	A majority of patients experienced	

			encoding for multiple self-antigens	rgressions in multiple target regions	

n/a not available

Table 2 Published clinical trials utilizing neoantigens as targets

Year of publication	Investigator /Sponsor	Clinicaltrials.gov identifier, phase	Indication	Platform /Treatment	Key results / Ref
2014	NIH	NCT01174121, I	Metastatic cholangio-carcinoma	**Adoptive transfer** of neoantigen specific (ERBB2IP E805G) $CD4^+$ T cells isolated from tumor	Decrease in target lesions with stabilization of disease, reinjection led to tumor regression, single patient report[33]
2015	Washington University	NCT00683670, I	Stage III or IV melanoma	Intravenous application of neoepitope peptide loaded DC **vaccine**	$CD8^+$ T cell responses and broadened antigenic breadth as well as clonal diversity[23]
2016	NIH	NCT01174121, II	Metastatic colorectal cancer	**Adoptive transfer** of neoantigen specific (KRAS G12D) $CD8^+$ T cells isolated from tumor	Regression of multiple lung metastases upon infusion of four different T-cell clonotypes[32]
2017	BioNTech	NCT02035956, I	Stage III or IV melanoma	Intranodal application of naked mRNA **vaccine** encoding for multiple neoepitopes	$CD8^+$ and especially $CD4^+$ T cell responses against multiple neoantigens, significant reduction of

					cumulative rate of metastatic events after vaccination[195]
2017	Dana-Farber Cancer Institute	NCT01970358, I	Stage III or IV melanoma	Subcutaneous application of peptide **vaccine** consisting of pooled mutated epitopes	Polyfunctional $CD8^+$ and especially $CD4^+$ T cell responses with durable memory response, recognition of autologous tumor, combination with anti-PD-1 therapy beneficial for clinical outcome[194,218]
2019	Immatics	NCT02149225, I	Glioblastoma	Intradermal application of peptide **vaccine** consisting of shared and mutated epitopes	$CD8^+$ and $CD4^+$ T cell responses against multiple shared and mutated epitopes[219]
2019	Dana-Farber Cancer Institute	NCT02287428, I/Ib	Glioblastoma	Subcutaneous application of peptide **vaccine** consisting of pooled mutated epitopes	Polyfunctional $CD8^+$ and $CD4^+$ T cell responses with enriched memory phenotype and augmented T cell infiltration to the tumor[220]
2020	Dana-Farber Cancer Institute /Neon Therapeutics /BioNTech US	NCT02897765, I	Advanced melanoma, non- small cell lung cancer, bladder cancer	Subcutaneous application of peptide **vaccine** consisting of pooled mutated	Durable $CD8^+$ and especially $CD4^+$ T cell responses with cytotoxic potential,

				epitopes combined with PD-1 blockade	observation of epitope spreading upon vaccination[221]
2020	NIH/Moderna	NCT03480152, I	Metastatic gastrointestinal cancer	Intramuscular application of LNP formulated mRNA **vaccine** encoding for multiple neoepitopes	$CD8^+$ and $CD4^+$ T cell responses against multiple mutated epitopes, small patient group (n=4), no objective clinical response[222]
2021	NCT/ University of Heidelberg	NCT02454634	Newly diagnosed glioma	Subcutaneous application of a single IDH1 (R132H) peptide **vaccine**	Vaccine induced $CD4\ T^+$ cell responses across multiple MHC alleles in over 90% of the patients[196]

Table 3 Published clinical trials utilizing viral antigens as targets with a focus on HPV

Year of publication	**Investigator /Sponsor**	**Clinicaltrials.gov identifier, phase**	**Indication**	**Platform /Treatment**	**Key results / Ref**
2014	n/a	n/a, n/a	Cervical intraepithelial neoplasia grade 3	Live anntenuated Lactobasillus casei expressing full length HPV16 E7 protein	70% of the patients pathological down-grade to CIN2 at week 9 of the treatment
2015	National Cancer Institute	NCT01585428, I	Metastatic HPV associated cervical cancer	Adoptive transfer of TILs with E6 and E7 specificity	2 out of 9 patients experienced durable tumor regression
2016	John Hopkins University	NCT00988559, I	Cervical intraepithel	DNA vaccine	30% of the patients

			ial neoplasia grade 2/3	encoding for HPV antigen E7	experienced histopathological regression
2018	Baylor College of Medicine	NCT02002182, II	HPV associated cervical cancer	Live attenuated Listeria monocytogenes engineered to secrete HPV proteins	vaccine and cisplatin combination achieved 17.1% overall response rate, and 38.9% overall survival at 12 months
2019	Isa Pharmaceuticals	NCT02426892, II	HPV16+ cancer types	Synthetic long-peptide encoding for HPV antigens E6/E7	Overall response rate of 33% in combination with anti-PD-1
2019	National Cancer Institute	NCT02280811, I/II	HPV16+ cancer types	Adoptive transfer of E6 specific TCR engineered T cells	2 out of 12 patients experienced objective tumor responses
2020	Isa Pharmaceuticals	NCT02128126, I/II	HPV16+ advanced, metastatic or recurrent cervical cancer	Synthetic long-peptide encoding for HPV antigens E6/E7	Tumor regression in 43% of the patients
2020	Genexine Inc.	NCT02139267, II	Cervical intraepithelial neoplasia grade 3	DNA vaccine encoding for HPV antigens E6/E7	52% of the patients experienced histopathological regression, 73% of which cleared HPV
2021	n/a	n/a, II	Cervical intraepithelial neoplasia grade 2	Live attenuated Lactobasillus casei expressing full length HPV16 E7 protein	Complete remission in 11% of the patients
2021	National Cancer Institute	NCT02858310, I	HPV associated epithelial cancers	Adoptive transfer of E7 specific TCR engineered T cells	50% of the patients experienced tumor regression with objective

	clinical response

n/a not available

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