Characters of neoantigens in cancer immunotherapy

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Abstract

Evidences have suggested that T cells that target mutation derived neoantigens are the main mediators of many effective cancer immunotherapies. Although algorithms have been used to predict neoantigens, only a handful of those are truly immunogenic. It is unclear which other factors influence neoantigen immunogenicity. Here, we classified clinical human neoantigen/neopeptide data based on their peptide-MHC binding events into three categories. We observed a conserved mutation orientation in anchor mutated neoantigen cohort after classification. By integrating this rule with existing prediction algorithm, we achieved improved performance of neoantigen prioritization. We solved several neoantigen/MHC structures, which showed that neoantigens which follow this rule can not only increase peptide-MHC binding affinity but create new TCR binding features. We also found neoantigen exposed surface area may lead to TCR bias in cancer immunotherapy. These evidences highlighted the value of immune-based classification during neoantigen study and enabled improved efficiency for cancer treatment.

Keywords: cancer immunology; major histocompatibility complex (MHC); vaccine; neoantigen prediction; antigen immunogenicity

Introduction

The immune system can sometimes recognize and destroy cancer cells[1]. CD8⁺ T cells are a major component in this process by both recognizing and destroying the target cells[2–5]. For T cells to react with tumor cells two components are required: major histocompatibility complex class I and possibly class II proteins (MHCI, MHCII) on the cancer cells and tumor derived antigenic peptides which bound to MHCs. T cells can then recognize such complexes, called pMHC epitopes (the combination of MHC and antigen), on the cancer cells as abnormal cells and target them for destruction. Thus, it is crucial to identify cancer antigens for immunotherapy.

During the last 25 years, great efforts have been made to identify tumor antigens[6]. These tumor antigens can be classified into two broad categories, self-antigens which are seldom expressed in normal tissues or expressed at much higher levels on the cancer cell versus normal tissues (like...
NY-ESO-1, MART-1) and nonself-antigens called neoantigens which are derived from somatic mutations in cancer cells[7]. Neoantigens are not presented by normal tissues, hence the immune system is not tolerant to them and views them, correctly, as foreign antigens and responds appropriately[8,9].

Currently, neoantigen identifying methods relies on next-generation sequencing (NGS) to provide formidable identification of cancer mutations, followed by predicting their theoretical binding for the patient’s HLA complex[10–14][15,16]. In brief, they compared tumor and normal DNA for identifying non-synonymous mutations and used peptide/MHCI binding prediction algorithms to find mutated peptides which could bind MHC to be potential effective neoantigens. Binding prediction could lighten the burden of immunotherapy by reducing the number of candidate neopeptides[7,10,12,16–19,19–22,22–25]. However, among neoantigens with actual MHC binding, only a small portion of them are immunogenic and have therapeutic effects[26,27]. It is likely that other features beyond MHC binding affinity could affect neoantigen immunogenicity.

It has been hypothesized that the TCR:pMHC ternary binding events may influence neoantigen immunogenicity. Yadav et al. and Fritsch et al. suggested that the side chain of neoantigen mutation point towards the T-cell receptor (TCR) would be more immunogenicity while Duan et al. thought that neoantigen substitutions at MHC anchor positions may be more important[7,18,28]. Recently, Capietto et al. also suggested that the MHC binding affinity of neoantigen corresponding wild type peptide is associated with predicting cancer neoantigens, with their mouse model study[29]. However, studies in this area are still limited due to scarce large human neoantigen datasets and neoepitope structures availability. Thus, systematic analyses based on large human neoantigen data and structural understanding of neoantigen immune properties are still needed.

Here, we attempted to determine different features between immunogenic neoantigens and noneffective neopeptides after mutation positional classification of human clinical neoantigens. We found that almost all immunogenic anchor mutated neoantigens in our datasets followed a unique mutation pattern (termed NP rule), rather than other patterns. Combining this rule with existing binding predictor could improve the neoantigen prioritization performance.

To provide structural insights of neoantigens with the NP rule, we solved several pMHC structures which follow this rule: the driver mutation derived KRAS G12D neoantigens in complex with
HLA-C*08:02 and the noneffective mouse DPAGT1 peptides in complex with mouse MHC H-2 Kb. We showed that neoantigens which follow the NP rule could generate new immune features for T cell recognition. The newly generated surface is necessary for anchor mutated cancer peptides to induce T cell response in vivo. It also suggested that the antigen exposed surface area might be an additional factor involves in neoantigen immunogenicity.

Results

Classification of cancer neoantigen/neopeptide data by mutation position

To study immune features of clinically relevant neoantigens arising from cancer mutations, we obtained HLA-I immunogenic (positive data, termed neoantigen) and noneffective (negative data, termed neopeptide) human clinical cancer neoantigen/neopeptide data from our NEPdb database (unpublished). These data come from 34 papers which contain neoantigen/neopeptide sequences and relevant HLAs as well as in vitro T cell assay reports and clinical tests[5,30,10–12,16,21,31–41,17,42,43,19,44–49,22,50–52].

The Immunogenic Neoantigen Dataset (IND) in this study, contains 128 entries (Fig. 1a; Supplementary Table 1). Neoantigens without T cell assay, minimal length or relevant HLA information were not recorded in the IND datasets. Melanoma and non-small cell lung cancer, with higher mutational burdens and more extensively studies, contained nearly 80% entries in this dataset.[24].

In this study, 11739 noneffective neopeptides with corresponding MHCIIs formed the Raw Noneffective Neopeptide Dataset (RNND) (Fig. 1a; Supplementary Table 2). All of these mutant peptides had been tested by T cell assays at least or tested in clinical trials, which were proven to be noneffective. The majority of them are not minimal peptides lengths (MHCI bind peptides that are predominantly 8-11 amino acid in length) known to present by MHCI. As we know, peptide-MHC binding is necessary albeit not sufficient for neoeptope immunogenicity. To process noneffective neopeptides into optimal length (9mer and 10mer) as negative control for further study, we used the NetMHCpan 4.0 algorithm for peptide-MHC binding prediction. This processed dataset is termed Noneffective Neopeptide Dataset (NND) and contains 2883 entries (Fig. 1a; Supplementary Table 3).
Neoantigen with strong binding affinity to MHC can form stable pMHC complex for T cell recognition and therefore induce T cell responses. Thus, neoepitope candidates could be prioritized through prediction algorithms to eliminate those peptides with weak binding affinity to MHCI. While the binding prediction algorithms have used for eliminating candidate neoantigens, the prediction accuracy of neoantigens capable of eliciting efficacious antitumor responses in patients remains quite low[53]. It is likely that features beyond MHC binding affinity involve in neoantigen immunogenicity. Recently, some studies found that the TCR:pMHC ternary binding events may influence immunogenicity[54,55]. However, the underlying immunological mechanisms of how the positional mutations affect cancer clinical outcome remains poorly defined.

In an attempt to answer above questions, we classified peptides from IND and NND based on the mutation position. To be specific, peptides from IND and NND were categorized into three categories: mutated at an anchor position (binds MHC intensively), MHC-contacting position (contacts MHC but provides less binding affinity) and TCR-contacting position (point towards TCR instead of MHC). The classification was performed by referencing the SYFPEITHI database[56], NetMHCpan antigen binding motif [24] and the Protein Data Bank (PDB) pMHC structures into account (see Methods). The classified information was recorded in Supplementary Table 1 and Supplementary Table 3.

We next calculated the percentage of neoantigens in different categories after classification. Immunogenic neoantigens were more likely to mutate at TCR-contacting region (Fig. 1b, c) and were less likely to change at MHC-contacting regions than those noneffective neopeptides (Fig. 1b, c). These results suggested that neopeptides with TCR-contacting position mutation rather MHC-contacting position will preferentially be immunogenic[7,28]. However, the percentage of anchor mutated neoantigens did not show significant differences compared with noneffective neopeptides (Fig.1c). It suggested that classification of anchor mutated peptides is not sufficient to distinguish immunogenic neoantigen from noneffective neoantigen candidates.

Anchor mutated neoantigens followed a conservative mutation pattern to acquire immunogenicity

We aimed to further characterize the intrinsic immunological properties of anchor mutated neoantigens beyond binding affinity. MHC molecules have many allelic variants with different binding properties of
their peptide binding cleft. Thus, the peptides recognized by different MHCs are very diverse, with allele-specific amino acid preferences. To investigate the preferential property of the anchor mutated peptide and its wild type counterparts in the context of HLAs, we set the cut-off frequency to be above 10% as HLA preferential amino acids at anchor positions as described previously[57] based on large peptide binding datasets from the IEDB[58] (Supplementary Fig. 1, Supplementary Table 1 and 3).

With the definition of “preference”, the anchor mutated neopeptide pairs (contain wild-type and mutant peptides) can be divided into 4 groups: non-preferential to non-preferential residues (mutated from wild-type counterparts with non-preferential amino acid to mutation with non-preferential amino acid, within the context of the certain HLA, abbreviated as NN), non-preferential to preferential residues (NP), preferential to non-preferential residues (PN) and preferential to preferential residues (PP) (Fig. 1d; Supplementary Table 4). Statistical analysis showed that the frequency of the immunogenic pairs with NP preferential change was significantly higher than those from the noneffective pairs (Fig. 1d, p=8.247e-06). Almost all (26 in 27) of the immunogenic pairs (from IND) appear to be classified into the NP group. In contrast, cancer neopeptides with HLA non-preferential property (the NN or PN groups) or those wild-type counterparts with HLA preferential property (the PN or PP groups) can hardly induce effective anti-tumoral response (Fig. 1e; Supplementary Table 4).

Next, we checked the exceptional case (the one which did not follow the NP rule in 27 cases). This neoantigen (MYADM R30W) from the MYADM protein has a mutation at C termini of the peptide that changes Arg to Trp. Of note, both wild type and mutant MYADM peptides can elicit self T cell responses[31]. This evidence suggested that, in this case, wild type peptide can also bind the patient’s HLA protein and eliciting T cell autoreactivity in vivo. Since both the MYADM neoantigen and its wild-type counterpart were reactive in this patient, this antigen should be excluded for further use.

Notwithstanding this exception, our observations suggested the NP rule of anchor mutated neoantigens is a conservative feature to assess T cells for reactivity against neoeptopes.

The NP rule of anchor mutated neoantigens thus can be treated as a binary variable (1= true NP, 0= false NP). To assess whether this variable can be used in neoantigen candidate prioritization, we tested the performance of the binary “NP” model and the combination model of the binary “NP” model with NetMHCpan 4.0 Rank% model (termed Com NP+B). Two existing prediction models, the NetMHCpan 4.0 (using NetMHCpan 4.0 Rank% score) and the DAI models (differential agreotoxic index, the difference of predicted binding affinity between the mutated epitope and its unmutated counterpart).
were also tested with our data as benchmark comparators. After taking the NP rule into account, the “Com NP+B” model achieved better performance compared with other three models (Fig. 1f, AUC=0.810). When comparing the performance of the four predictors, assessment was also done by 50-fold cross-validation (2/3rd random resampling) over the data to check whether the observed difference in average AUC differs significantly (Supplementary Fig. 2). Collectively, we proved that the NP rule can be taken as a predictive feature to improve neoantigen candidate prioritization.

**Anchor mutated neoantigens can generate new surface for T cell recognition**

Our results above indicated that the NP rule is a conservative feature in the immunogenic anchor mutated neoantigens. To understand how this rule forms the basis for acquired immunogenicity of anchor mutated neoantigens, we attempted to solve the structures of the typical neoepitopes: HLA-C*08:02 (C08) in complex with KRAS mutation derived neoantigens form a successful clinical case, reported by Tran et al[10]. In this case, objective regression of metastases was observed in a metastatic colorectal cancer patient after the infusion of HLA-C*08:02-restricted tumor-infiltrating lymphocytes (TILs) targeting KRAS G12D neoantigens. The KRAS G12D mutant 9mer peptide GADGVGKSA and 10mer peptide GADGVGKSAL (both mutated at position 3 from glycine to aspartic acid) can stimulate autologous HLA-C*08:02 reactive T cells, while their wild-type counterparts cannot[10]. These two neoantigens can be categorized in the anchor mutated group (Supplementary Table 1 and reference[59]) and follow the NP rule.

First, we attempted to get the protein complexes of HLA-C*08:02 (C08) in complex with four peptides: wild type KRAS 9mer GAGGVGKSA (wt9m); wild type KRAS 10mer peptide GAGGVGKSAL (wt10m); mutant KRAS G12D 9mer peptide GADGVGKSA (mut9m, mutation site is indicated with underline) and mutant KRAS G12D 10mer peptide GADGVGKSAL (mut10m). C08-mut9m and C08-mut10m complexes were successfully refolded. However, C08-wt9m complex and C08-wt10mer complex failed to refold even with ten-fold increase in concentration of the peptides. This result confirmed that the mutation from glycine to aspartic acid of these neoantigens is crucial to stabilize pMHC complex.

We next solved the C08-mut9m complex at 2.4 angstroms (Å) and the C08-mut10m complex at 1.9 Å (Supplementary Table 5). The electron density at the peptide region was unambiguous (Fig. 2a, b).
C08-mut9m complex and the C08-mut10m complex showed a conserved conformation except the peptide region (Fig. 2c, d). The smaller residues such as alanine and serine are preferably selected at peptide P1 and P2 position by the peptide binding motif of HLA-C*08:02 (Fig. 2e, Supplementary Fig. 1). This is due to the narrow cleft formed by several C*08:02 aromatic residues (Tyr7, Phe33, Tyr67, Tyr99, Tyr59, Tyr171, Tyr159 and Trp167) which limits the size of the P1 and P2 side chains that can bind at that site (Fig. 2f).

Generally, peptides use anchor positions at P2 and PΩ to occupy the B and F pockets of HLA class I molecules. However, the structures of the C08-mut9m and C08-mut10m, followed by the peptide binding analysis, revealed that KRAS G12D 9mer and 10mer peptides use unconventional P3 and PΩ sites as anchors to form stable complexes with HLA-C*08:02 (Fig. 2g, h; Supplementary Fig. 1). The B pocket of HLA-C*08:02 bind P3D via interactions with Arg97 and Arg156. The P3D side chain of C08-mut9m also forms an intra-chain hydrogen bond with P4G, while this hydrogen bond is absent in the C08-mut10m structure. Interestingly, it is apparent from the C08-mut9m structure that the anchor residue P3D could also provide TCR accessible surface by partially exposing of its charged side chain (Supplementary Fig. 3). This unusual phenomenon suggested that anchor mutation of some neoantigens can not only improve pMHC binding force but could also provide additional accessible surface for TCR interaction, under certain conditions, and therefore change the total strength of the TCR:pMHC complex.

At the PΩ anchor position, although the 9mer and 10mer peptides occupied the same F pocket with their PΩ residues, the P10L side chain from the 10mer peptide is buried more deeply than P9A in the 9mer structure (Fig. 2g, h). Meanwhile, instead of the upward residue P8S in mut9m peptide, the mut10m peptide uses P8S as an auxiliary anchor to bind the HLA protein via hydrogen bonds to Glu152 and Arg156. These interactions from the mut10m squeezed the P4 to P7 residues together with residue P3D (Fig. 2h). Although the mut9m and the mut10m have similar sequences with only one amino acid different at PΩ termini, the surface details are largely different after they engage with HLA-*08:02. We postulated that these different features from the mut9m and mut10m neoepitopes may respectively modulate the effective T cell recognition and activate different T cell repertoires (discussed below).

Noneffective anchor mutated neoantigens could not generate more differentiated TCR binding surface.
than their wild-type analogues, as they buried the anchor mutations into MHC pocket and can hardly contact with TCRs. However, structural analysis of immunogenic anchor mutated neoantigens suggested that these neoantigens can not only generate new immune features, but even create novel neoepitope surface. Thus, the necessity and conservation of NP rule in anchor mutated neoantigen might be explained by the generation of novel surface from mutant peptide, rather than the wild-type analogue, which could enable the boosting of neoepitope-specific response from T cells.

**Structural of non-therapeutic mouse neoantigen DPAGT1 V213L in complex with H-2 Kb**

We next determined two structures of non-immunogenic neopeptides with anchor mutation. Yadav et al described a neopeptide that can be presented by MHC but showed non-immunogenic property in vivo[7]. This mouse DPAGT1 V213L neopeptide contains a mutated C-terminal anchor residue that falls into the “preferential to preferential residues (PP)” group, with the changing of valine(V) to leucine(L).

Soluble mouse H-2 Kb in complex with mutant DPAGT1 V213L 8mer peptide (SIIVFNLL, termed mut8mL) and the wild type 8mer counterpart (SIIVFNLV, termed wt8mV) were separately expressed, refolded and purified for crystallization trials. Crystal diffraction data of Kb-wt8mV and Kb-mut8mL were processed to 2.4 Å and 2.5 Å resolution respectively (Supplementary Table 5) and provided electron density for each peptide (Fig. 3a).

The overall structure of the Kb-wt8mV complex closely resembles that of Kb-mut8mL with the exception of a slight difference at PΩ (P8) (Fig. 3b). The C-terminal PΩ residue acted as an anchor in both the Kb-wt8mV and the Kb-mut8mL complexes (Fig. 3b). Moreover, both PΩ valine and leucine were preferably selected by H-2 Kb (Fig. 3c). Both of the two PΩ residues formed hydrogen bonds with Kb Asp77, Tyr84, Thr143 and Lys146 (Fig. 3d, e). Although the side chain of leucine in the mut8mL peptide inserted more deeply into Kb than valine in the wt8mV peptide because of its longer side chain, these two peptides did not provide different TCR binding surface with relevant MHCs (Fig. 3b, d, e). These findings indicated that neopeptides with the “PP” rule cannot readily change the binding surface and therefore immunogenicity. The non-effectiveness across the neopeptides with PP rule might be explained by the pre-existence of the wild-type peptide-MHC complex in thymus, which can lead to negative selection of potential neopeptide restricted T cell repertoires. Considering that...
peptide-MHC binding is necessary for neoepitope immunogenicity, we did not further discuss the situation of those neopeptides with the “PN” or the “NN” rule.

Neoantigen exposed surface areas may affect T cell selection in cancer immunotherapy

Peptide antigen can form stable complexes with HLA by lying the peptide chain into the HLA antigen-binding cleft. Some antigens, called “featureless” antigens, have relatively small exposed surface areas (ESA) form side chains pointing towards the T cell receptor when they fill the peptide-binding cleft of HLAs[60]. Studies have indicated that featureless epitope are more likely to select relatively narrow TCR repertoires than epitopes with large exposed features in vivo[61]. We next examined the ESA features of the C08-mut9m and the C08-mut10m structures, by employing the PDBEPISA server. Of note, the ESA of two representative T cell epitopes were also calculated as benchmarks. One is the HLA-A2–M1, a viral antigen “M1” (M158-66 from the IAV) in complex with HLA-A*02:01(A2-M1, in Fig. 4a), which considered as a featureless epitope[62]. In contrast, a viral epitope HLA-A2-RT, which has a “reverse transcriptase peptide” (RT468-476 from HIV) in complex with HLA-A*02:01(called A2-RT, in Fig. 4a), is considered as largely exposed epitope[63]. After calculation, we found that the mut9m neoantigen has the smallest peptide ESA at 240 Å², even less than the well-known featureless M1 peptide (251 Å², in Fig. 4a-c). However, the mut10m neoantigen has relatively large ESA at 317 Å², which is comparable with the typical largely exposed antigen RT (330 Å², in Fig. 4a-c). These data suggested that the mut9m provides relatively less SEA than canonical T cell antigens. We thus postulated that the specific TCRs for C08-mut9m may be constrained in patients because of the featureless area available for specific recognition.

Studies have suggested that narrow TCR repertoires can recognize featureless epitope, because of the lack of TCR recognition modes[61,62,64]. To investigate the diversity of KRAS G12D neoantigen specific TCRs in clinical cases, we examined the TCR sequences of the restricted T cell repertoire targeting the C08-mut9m neoepitope (Fig. 4d, patients 3995 and 4095, both expressing HLA-C*08:02)[10,44]. Patient 3995 received ACT targeting KRAS G12D neoantigen and did not response. The transferred RK5 T cell repertoire (T cells expressed the RK5 TCR) was identified to recognize C08-mut9m neoepitope. Patient 4095 received a similar treatment and observed objective tumor regressions. In patient 4095, the RK1, RK3, RK4 T cell repertoires were verified to recognize...
the C08-mut9m while the RK2 recognized the C08-mut10m. All four T cell repertoires (RK1, RK3, RK4, RK5) with C08-mut9m restriction were identified biased usage with a public TCR pair (TRAV4/TRBV5-1) across different patients. The length and sequence of these TCRα chains was highly restricted, with the same TCR-V region and “CLVGDxDQAGTALIF” CDR3α motif among the four TCRs (Fig. 4d). The TCRβ chains was also restricted at TCR-V region but showed differences at CDR3β regions. Generally, the CDR1 and CDR2 loops of TCR can recognize the two conserved α-helices on the MHC, whereas the CDR3 loops mainly interact with the exposed peptide. Moreover, the CDR3β had proved to be the main factor that determines TCR bias (compared with CDR3α) in many cases, due to the greater sequence diversity in TCR repertoire and the extensive contact of peptide region[64]. However, in this case, similar CDR1, CRR2 and CDR3α sequences with C08-mut9m restriction were identified consistently from different patients. We thus speculation that these public CDR1, CRR2 and CDR3α regions are important in the C08-mut9m recognition, rather than the CDR3β regions[65]. In contrast, the C08-mut10m neoepitope did not observe dominant public TCRs in patient 4095 or across different individuals. Collectively, these data suggested that the featureless mut9m neoepitope can be recognized by T cells with public TCRs across different patients.

Previous reports have shown that the constrained TCR repertoires are associated with poor efficient to control viral infection[66,67]. However, the correlation between TCR bias and clinical outcome in cancer treatment is unclear. In an exploratory analysis, we examined the TCR bias and clinical performance in this case to address above question. The C08-mut9m restricted T cell repertoires with public TCR usage (RK1, RK3 RK4 and RK5) did not dominant presence in TILs or not show long-term persistence after infusion (Fig. 4d, detected after 39 and 266 days). However, the RK2 T cells with C08-mut10m restriction showed dominant persistent abilities both in tumor infiltrating stage and after cell transfer for 266 days. While we did not observe direct correlation between TCR diversity and clinical outcome due to the limitations of clinical data, we still observed short-lived T cell persistence in the presence of the featureless C08-mut9m neoepitope. This phenomenon might be explained due to the existence of cross-reactivity with self antigen and thereby leads to the suppression by regulatory effectors in vivo. We thus postulated that, outcomes of patients who receive more diverse adoptive T cells tended to be better than patients who receive constrained T cells in cancer immunotherapy.
Discussion

How the T cells recognize neoantigen as “non-self” is an important question in cancer immunotherapy.

In contrast to conventional pathogenic peptides that may totally different from self peptides, the neoantigens are single amino acid altered peptides compare with self peptides. Thus, differences between neoantigens and their wild-type counterparts and how neoantigen-MHC binding events involve in forming new TCR binding surface should be elucidated in neoantigen immunogenicity studies.

Efforts have devoted to discuss the complexity of neoantigen immunogenicity with their binding events. Different pieces of evidence led to different conclusions[68]. Fritsch et al. and Yadav et al. suggest that neoantigen are more commonly mutated at TCR-contacting position, however, Duan et al. thought that neoantigen substitutions at anchor may be more immunogenic[7,18,28]. Further, using mouse model, Capietto et al. showed that where the mutation is at an anchor residue, increased affinity relative to the corresponding wild-type peptide can influence neoantigen immunogenicity[29]. To elucidate the questions above, we assigned human neoantigen/neopeptide data into three different categories: mutations at TCR-contacting positions, mutations at MHC-contacting regions but not anchor positions and mutations at anchor positions. In our study, mutations occur more frequently at TCR-contacting positions rather than at MHC-contacting position in immunogenic dataset. It is possible that mutation characteristics, amino acid contact potentials and force-dependent interactions may affect the interactions in the TCR-contacting group[69,70]. For anchor mutated group, we showed that the NP rule in anchor mutated neoantigen is a more pervasive element of immunogenicity than previously understood. Also, we found the NP rule combined with binding predictor (NetMHCpan 4.0) that could contribute to prioritize neoantigen candidates. In a sense, the NP rule could be taken as a reflection of antigen binding property. This binary indicator can provide a direct understanding of the MHC binding difference between mutant peptides and their wild type counterparts. However, the uniqueness of the NP rule was not fully determined, since the test depends on how comprehensive the validation database is. More analyses to understand the NP rule are still needed with the data increasing.

We next performed structural studies to understand the NP rule. The molecular insight of anchor mutated neoantigens provided evidences that they can generate new surface and features for T cell
recognition but the wild-type cannot. In contrast, the anchor mutated neopeptides with PP rule reveal low immunogenicity in clinical treatment, reflecting that the most neoepitope restricted T cells might have been removed by negative selection. It also suggested that neoantigen exposed surface area (ESA) might be a factor to influence TCR diversity and clinical outcome based on our analysis. More experimental data and neoantigen-MHC structures are needed to fully understand the relevance of ESA and TCR diversity.

Our study showed three possible neoantigen binding models within the context of MHC (Supplementary Fig. 4). Model A represents the situation which mutation occurs at the TCR-contacting region and therefore directly towards to T cells. Model B represents the situation in which mutation occurs at MHC-contacting region (not including anchor sites) and therefore might be immunogenically irrelevant. Model C represents the situation which the mutation occurs at an anchor position. Anchor mutations may not change TCR-contacting surface but instead lead to de novo presentation. If the wild type allele prevented presentation in the thymus, self-reactive T cells would not have been selected against.

KRAS G12D mutations is indicative of poor prognosis with negative/poor response to standard cancer treatment. It is one of the most infamous driver mutations target leads to oncogenesis[71]. The neoantigen KRAS G12D in complex with HLA-C*08:02 is a typical case of a human driver mutation derived neoantigen-MHC structure which has been linked to clinical benefits. With these structures, further research could be undertaken to heighten the immunogenicity and stability of KRAS G12D-C*08:02 neoeptope, by taking modification of agonist peptides or by screening non-natural synthetic epitopes[72,73]. Alternatively, our structures could be used to design artificial receptors that bind mutant KRAS peptides based on synthetic biology means. Our findings reveal that immune-based classification is essential for neoantigen immunogenicity study The NP rule, which found in anchor mutated neoantigens, can be used to prioritize neoantigens. Further structural analyses indicated that newly generated surface as well as the ESA in neoantigens affected T cell activation and clinical outcome, suggesting that these factors could be considered in neoantigen discovery and design of future clinical trials.

Materials and Methods
Noneffective neopeptides dataset (NND) generation

In order to generate a minimal noneffective neopeptide dataset, binding prediction was done by NetMHCpan 4.0 Server[24] based on the data from noneffective neopeptide dataset (NND). Different length of mutant peptides from NND were input in NetMHCpan 4.0 Server with custom python scripts. The mutant peptides in 9- or 10-mer length with recommend cut-off (affinity < 500 nM) based on NetMHCpan 4.0 Server were considered to be binders and recorded in the Supplementary Table 3.

Differential agretopic index (DAI) score calculation

The calculation of DAI was described previously[74]. Briefly, peptide binding affinity with HLA was predicted for mutant and wildtype peptide pairs by NetMHCpan 4.0. The DAI score of each neoantigen pair was calculated by subtraction of its predicted IC50 binding affinity from the corresponding wild type counterparts.

Receiver operating characteristic (ROC) curve generation

The AUC value (the area under the ROC Curve) was calculated based on the different predictors listed. The ROC curve was plotted from the false positive rate (FPR) and true positive rate (TPR) values calculated by varying the cut-off value (separating the predicted positive from the predicted negative) from high to low.

Anchor, MHC-contacting and TCR-contacting positions determination

Identification of the anchor positions were based on the SYFPEITHI online database[56] and manually defined from NetMHCpan binding motif results[24]. Anchors were defined for each allele with the SYFPEITHI database definition and highest information content in NetMHCpan binding motif record. The anchor position for each entry was cross-validated based on solved HLA structures from Protein Data Bank (PDB). Thus, the combination of these tools can provide anchor position information for most HLA alleles in our datasets. We recorded the cross-validated result as the “consensus anchor”. The anchor position information was also recorded in IND and NND. Alleles without relevant
information above were recorded as “null” in the tables.

Peptide MHC-contacting and TCR-contacting positions was determined based on solved 9mer peptide-MHC complex. Briefly, positions from peptides which prove to be non-anchor position can be divided into MHC-contacting and TCR-contacting positions. With the pMHC structural model from PDB, the position on peptide which contact MHC was treated as MHC-contacting position. Contrary to MHC-contacting position, TCR-contacting position often harbor a residue with a side chain that points toward outside from the pMHC complex and may contact with TCR. The MHC contact and TCR contact region information was recorded in IND and NND. Alleles without relevant structure information above were recorded as “null” in the tables.

Peptide library and preferential HLA anchor position determination

The nonameric peptide library of 30 HLA alleles was obtained from IEDB database[58]. Sequence logos were generated base using the sequence logo generator[75]. Threshold for preferential amino acids at anchor position of each HLA alleles was set to include and above 10% based on the data from nonameric peptide library. Preferential information of amino acids at anchor position for each HLA allele was recorded in Supplementary Table 1 and 3.

Combined NP+Binding (Con NP+B) prediction model building

To combined the binary NP rule with existing prediction methods NetMHCpan 4.0, we took a logistic regression algorithm to model the prediction of immunogenicity. Analyses were performed using the R build-in function glm(), as below:

\[ \text{immunogenicity} \sim NP + \text{Binding prediction} \]

Anchor mutated neoantigen data was selected to train the model. After fitting, the performance of this model was shown by ROC curve.

To further test this model, we used 50 times resampling. Random resampling of the data (2/3rd resampling) was used for training. The AUC values were calculated by plotROC package. After iteration, the differences of AUC between four models were measured using paired t test in R software.
Protein Expression, refolding and purification

Inclusion bodies of HLA heavy chains and β2M was expressed as described previously[76]. Briefly, the DNA encoding MHC heavy chain (HLA-C*08:02, HLA-C*05:01 and H-2 Kb) and light chain (human β2M and mouse β2M) were synthesized (Idobioc) and cloned into pET-22(b) vector (Novagen). The vectors were transformed into the E. coli strain BL21 DE3 (Novagen). Transformants were selected from and selected on Lurian broth (LB) agar plates containing ampicillin. A single colony was selected and cultured in LB fluid medium with the antibiotics listed above at 37°C. Upon reaching an optical density OD600 of 0.6, expression was induced with the addition of 1mM IPTG. Incubation continued at 37°C for 5h. The cells were harvested by centrifugation and then resuspended in PBS buffer with 1 mM PMSF at 4°C. The cells were lysed, and the lysate was clarified by centrifugation at 10,000 g to collect inclusion bodies. Inclusion bodies were harvested and solubilized in 20 mM Tris (Vetec) pH 8.0, 8 M urea (Vetec), 1 mM EDTA (BBI life sciences), 1 mM DTT (Sinopharm chemical reagent) and 0.2 mM PMSF (Sinopharm chemical reagent).

Refolding was performed in the presence of MHC heavy chain, β2M and peptides as described previously[77]. Briefly, the resolubilized heavy chain (60 mg each) and light chain (25 mg each) in the presence of the corresponding peptide were added into 1 liter of refolding buffer [100 mM Tris (pH 8.4), 0.5 mM oxidized glutathione (BBI life sciences), 5 mM reduced glutathione (BBI life sciences), 400mM L-arginine (Vetec), 2mM EDTA (BBI life sciences)]. After 48h of refolding, the 1 L mixture was transferred into dialysis bag (Spectra) and dialyzed against 15 liters of 10 mM Tris buffer (pH 8.0) at 4°C for 24h.

Refolded proteins were purified by anion exchange chromatography with Q Sepharose HP (GE Healthcare) column then Mono Q column (GE Healthcare) and concentrated by tangential flow filtration using Amicon Ultra centrifugal filters (Merck). For desalination and purification, samples were loaded onto a Superdex 200 increase 10/300 GL column (GE Healthcare) for size exclusion chromatography. Chromatography was taken with BioLogic DuoFlow system (Bio-rad) at a flow rate of 1 ml/min. Peak analysis was performed using the ASTRA software package (BioLogic Chromatography Systems).
Crystallization, data collection, and processing

Purified pMHC complex were concentrated to 10 mg/ml for crystallization trials prior to screening using a series of kits from Hampton Research. Protein complex were crystalized by sitting drop vapor diffusion technique at 4 °C. Single crystals of C08-mut9m and C08-mut10m were obtained in the condition of 0.2 M ammonium acetate, 0.1 M HEPES (pH 6.5), 25% w/v polyethylene glycol 3,350. For the H-2 Kb complex, single crystal of Kb-8mV and Kb-8mL complex were obtained when 4% v/v Tacsimate (pH 6.0), 12% w/v Polyethylene glycol 3,350 was used as the reservoir buffer.

Crystals were transferred to crystallization buffer containing 20% (w/v) glycerol and flash-cooled in liquid nitrogen immediately. The diffraction data were collected at the Shanghai Synchrotron Radiation Facility (Shanghai, China) on beam line BL17U1/BL18U1/BL19U1, and processed using the iMosflm program[78]. Data reduction was performed with Aimless and Pointless in the CCP4 software suite[79]. All structures were determined by molecular replacement using Phaser[80]. The models from the molecular replacement were built using the COOT (Crystallographic Object-Oriented Toolkit) program[81] and subsequently subjected to refinement using Phenix software[82]. Data collection, processing, and refinement statistics are summarized in (Supplementary Table 5). All the structural figures were prepared using PyMOL (http://www.pymol.org) program. The atomic coordinates and structure factors for the reported crystal structures have been deposited on the Protein Data Bank (PDB; http://www.rcsb.org/pdb/).

Acknowledgements

We thank the staff from BL17U1/BL18U1/BL19U1 beamline of National Center for Protein Sciences Shanghai (NCPSS) at Shanghai Synchrotron Radiation Facility, for assistance during crystal data collection. We would like to thank Dr. Eric Tran (Providence Cancer Institute, USA) for his helpful comments and thank Chang-yi Ma (The Chinese University of Hong Kong, China) for advices on data analysis. This research was funded by the National Institutes of Health Grants AI018785 and AI135374 (to P.M.); the National Natural Science Foundation of China 31870728 and 31470738 (to L.Y.); the National Basic Research Program of China 2014CB910103 (to L.Y.); the Science Foundation of Wuhan University 2042016kf0169 (to L.Y.).
Author contributions

P.B., L.Y., and P.M. designed all the experiments; P.B. performed all experiments; P.B. collected neoantigen data; P.B., L.Y., P.M., J.W.K., M.W., Y.L., Y.Z., S.K.C., and J.X. analyzed neoantigen data; P.B., and Q.Z. determined crystal structures; P.B., L.Y., and P.W. analyzed crystal structures; P.B., L.Y., P.M., J.W.K., and S.K.C. wrote the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

References


Figure 1

<table>
<thead>
<tr>
<th></th>
<th>Immunogenic Neoantigen Dataset (IND)</th>
<th>Raw Noneffective Neopeptide Dataset (RNND)</th>
<th>Noneffective Neoantigen Dataset (NND)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entries</td>
<td>128</td>
<td>11,739</td>
<td>2,883</td>
</tr>
<tr>
<td>Anchor Mutated Peptides</td>
<td>29</td>
<td>N</td>
<td>428</td>
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<tr>
<td>Tumor Types</td>
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<td>11</td>
<td>10</td>
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<tr>
<td>HLA-A</td>
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<td>15</td>
<td>15</td>
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<td>HLA-B</td>
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<td>17</td>
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<td>HLA-C</td>
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<td>1,081</td>
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<td>References</td>
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<td>13</td>
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</table>

![Diagram b] (Immunogenic neoantigens and Noneffective neoantigens, showing percentages of various mutations)

![Diagram c] (Frequency distribution of mutations with significance markers)

![Diagram d] (P-value distribution with frequency comparison)

![Diagram e] (Comparison of N→P preferences)

![Diagram f] (ROC curve comparison for different models)
Figure 3
Supplementary Figure 1

HLA-A*01:01 (n=4661)  
HLA-A*02:01 (n=21172)  
HLA-A*02:05 (n=252)

HLA-A*03:01 (n=7467)  
HLA-A*11:01 (n=4850)  
HLA-A*24:02 (n=5683)

HLA-A*29:02 (n=5009)  
HLA-A*30:01 (n=610)  
HLA-A*30:02 (n=819)

HLA-A*31:01 (n=2000)  
HLA-A*32:01 (n=678)  
HLA-A*38:02 (n=2085)

HLA-B*07:02 (n=6573)  
HLA-B*08:01 (n=3955)  
HLA-B*15:01 (n=6349)

(continued on next page)
### Supplementary Table 1. Immunogenic Neoepitope Dataset (IND)

### Supplementary Table 2. Raw Noneffective Neoepitope Dataset (RNND)

### Supplementary Table 3. Noneffective Neoepitope Dataset (NND)

(see in separated EXCEL files)

### Supplementary Table 4. Classification of anchor mutated neoantigen based on MHC preference

<table>
<thead>
<tr>
<th>Anchor mutated neoantigen</th>
<th>N→N frequency</th>
<th>N→P frequency</th>
<th>P→N frequency</th>
<th>P→P frequency</th>
<th>Number of peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Immunogenic)</td>
<td>3.7% (1)</td>
<td>56.3% (28)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>27</td>
</tr>
<tr>
<td>(Non-effective)</td>
<td>27.78% (118)</td>
<td>65% (239)</td>
<td>6.82% (29)</td>
<td>9.41% (40)</td>
<td>425</td>
</tr>
</tbody>
</table>

Data come from Immunogenic Neoantigen Dataset (IND) and Noneffective Neoepitope Dataset (NND). Pecitides defined with anchor position were occulted in this analysis.
### Supplementary Table 5. Data collection and refinement statistics (molecular replacement).

<table>
<thead>
<tr>
<th></th>
<th>C2B-mut Ram</th>
<th>C2B-mut Ram</th>
<th>KS-smV</th>
<th>KS-smL</th>
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<tbody>
<tr>
<td>Resolution range</td>
<td>42.55 - 1.9</td>
<td>51.19 - 1.9</td>
<td>57.76 - 2.4</td>
<td>2.6 - 2.5</td>
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<tr>
<td>Space group</td>
<td>P 2 1 21 21</td>
<td>P 2 1 21 21</td>
<td>C 2 1 2</td>
<td>C 2 1 2</td>
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<tr>
<td>Unit cell</td>
<td>68.27 x 68.27 x 76.24</td>
<td>70.98 x 70.98 x 70.73</td>
<td>107.24 x 107.24 x 107.24</td>
<td>106.03 x 106.03 x 106.03</td>
</tr>
<tr>
<td>Total reflections</td>
<td>64,764 (63,233)</td>
<td>6,652,411 (5,656,324)</td>
<td>37,831 (3,734)</td>
<td>35,008 (3,358)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>32,460 (35,74)</td>
<td>33,288 (32,864)</td>
<td>19,013 (*860)</td>
<td>19,013 (*860)</td>
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<tr>
<td>Multiplicity</td>
<td>2.0 (2.0)</td>
<td>2.0 (2.0)</td>
<td>2.0 (2.0)</td>
<td>2.0 (2.0)</td>
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<tr>
<td>Completeness (%)</td>
<td>0.96 (0.98)</td>
<td>0.86 (0.88)</td>
<td>0.88 (0.88)</td>
<td>0.87 (0.96)</td>
</tr>
<tr>
<td>Mean Rmerge</td>
<td>7.15 (4.02)</td>
<td>9.26 (6.12)</td>
<td>14.58 (4.80)</td>
<td>11.50 (2.86)</td>
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<tr>
<td>Wilson B-factor</td>
<td>* 0.39</td>
<td>20.69</td>
<td>38.48</td>
<td>48.2</td>
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<tr>
<td>R-free</td>
<td>0.0462 (0.158)</td>
<td>0.03331 (0.1178)</td>
<td>0.02261 (0.1159)</td>
<td>0.02767 (0.1882)</td>
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<tr>
<td>R-merge</td>
<td>0.05787 (0.252)</td>
<td>0.04711 (0.1685)</td>
<td>0.03226 (0.1659)</td>
<td>0.039 * 0.2803</td>
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<tr>
<td>CC1/2</td>
<td>0.999 (0.993)</td>
<td>0.999 (0.993)</td>
<td>0.999 (0.978)</td>
<td>0.999 (0.944)</td>
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<td>CC*</td>
<td>0.999 (0.999)</td>
<td>0.999 (0.999)</td>
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<tr>
<td>Reflections used in refinement</td>
<td>31,550 (28,003)</td>
<td>33,288 (32,864)</td>
<td>18,991 (787)</td>
<td>16,922 (874)</td>
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<tr>
<td>Reflections used for R-free</td>
<td>19,013 (15,65)</td>
<td>20,01 (168)</td>
<td>18,991 (888)</td>
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<tr>
<td>R-work</td>
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<td>0.174</td>
<td>0.2194</td>
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<tr>
<td>R-free</td>
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<td>0.2634</td>
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<td>CC(work)</td>
<td>0.933</td>
<td>0.96</td>
<td>0.943</td>
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<td>CC(free)</td>
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<td>Protein residues</td>
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<tr>
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<td>0.93</td>
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<tr>
<td>Ramachandran allowed (%)</td>
<td>3.2</td>
<td>1.3</td>
<td>5.3</td>
<td>5.1</td>
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<tr>
<td>Ramachandran outliers (%)</td>
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<td>Rotamer outliers (%)</td>
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<td>Average B-factor</td>
<td>7.27</td>
<td>26.67</td>
<td>60.83</td>
<td>59.08</td>
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<tr>
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<td>50.95</td>
<td>55.2</td>
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<tr>
<td>solvent</td>
<td>24.02</td>
<td>33.81</td>
<td>&lt;7.71</td>
<td>&lt;7.71</td>
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*Statistics for the highest-resolution shell are shown in parentheses.*
**Figure Legends**

**Figure 1. Analyses of three datasets and immune-based data classification of Immunogenic neoantigen dataset (IND) and Noneffective neopeptides dataset (NND).**

**a,** Overview of the IND, RNND, and NND datasets.

**b,** Donut plots of percentage of 9mer peptide mutations categorized based on three different mutation locations: anchor mutation, MHC-contacting position and TCR-contacting position (immunogenic data, n=51; noneffective data, n=763).

**c,** Nonameric peptides mutation distribution of three different groups (mutated at anchor mutation, MHC-contacting position and TCR-contacting position) from IND and NND. The frequency of mutation distribution at TCR-contacting position and MHC-contacting position showed significant difference (TCR-contacting position, \( p=0.0378 \); MHC-contacting position, \( p=0.0027 \). \( n \) (immunogenic) =51; \( n \) (noneffective) =763. Fisher's exact test). The percentage of mutation distribution at anchor position did not show significant difference between immunogenic and noneffective data (ns, non-significant).

**d,** The frequency between anchor mutated IND and NND with significant difference in subgroup of non-preferential to preferential residues (N to P) change (\( p=8.247e-06 \), n=27 (immunogenic); n=425 (noneffective), Fisher's exact test). There was no difference between residue changes from wild type neopeptides to different types mutants: non-preferential to non-preferential residues (N to N), preferential to non-preferential residues (P to N) and preferential to preferential residues (P to P) of anchor mutated immunogenic data (from IND) and non-immunogenic data (from NND).

**e,** Pie charts represented the percentage of NP group and not NP group of anchor mutated datasets (n (immunogenic) = 27; n (noneffective) =425).

**f,** Receiver operator characteristic (ROC) curve showed the performance of different predictors (DAI score, binary NP rule, binding prediction (Rank\% scored by NetMHCpan 4.0), combination of NP rule + binding prediction (Com NP+B)) with anchor mutated data (data from IND and NND datasets, n (immunogenic) = 27; n (noneffective) =425). The AUC (Area Under the ROC Curve) was calculated for each predictive rule (\( \text{AUC}_{\text{DAI}} = 0.632 \); \( \text{AUC}_{\text{NP rule}} = 0.701 \); \( \text{AUC}_{\text{com NP+B}} = 0.810 \); \( \text{AUC}_{\text{Rank\%}} = 0.698 \)).

**Figure 2. Structural comparison of C08-mut9m and C08-mut10m complexes.**
a-b, Unambiguous 2Fo-Fc electron density maps of (a) KRAS G12D 9mer (GADGVGKSA, green) and (b) 10mer (GADGVGKSAL, yellow) peptides from solved pMHC complex structures. The underlined amino acids represented the mutation in the peptides.

c, Overlay of Cz traces (C08-mut9m, green; C08-mut10m, yellow). Differences in peptide conformation were observed.

d, Overlay of the KRAS G12D 9mer and 10mer peptides in the MHC binding groove.

e, Polar interactions at P1G and P2A positions within HLA-C*08:02 molecule showed in grey, 9mer peptide showed in green and 10mer peptide showed in yellow.

f, Aromatic residues (green) from HLA-C*08:02 accommodating P1 and P2 residues of KRAS G12D 9mer peptide.

g, The P3D and P9A side chains of mut9m peptide interact with HLA-C*08:02.

h, The P3D, P8S and P10LA side chains of mut10m peptide interact with HLA-C*08:02.

Figure 3. H-2 Kb presented DPAGT V213L wild type peptide wt8mV and mutant peptide mut8mL in a similar manner

a, Unambiguous 2Fo-Fc electron density maps of DPAGT1 wild type 8mer peptide (wt8mV peptide SIIVFNLV, magenta) and mutant 8mer peptide (mut8mL peptide SIIVFNLL, orange) from solved structures.

b, Overlay of the DPAGT1 wt8mV (magenta) and mutant mut8mL (orange) peptides.

c, Sequence logo based on the data from IEDB showing amino acid preferences for 8mer peptides bound to H-2 Kb. The peptide library was obtained from IEDB (n=4141).

d, Anchor residue P8V of wt8mV peptide interacts with H-2 Kb (grey).

e, Anchor residue P8L of mut8mL peptide interacts with H-2 Kb (grey).

Figure 4. Different peptide presentation patterns and peptide exposed surface areas (PESA) between C08-mut9m and C08-mut10m

a, Peptide exposed surface areas (PESA) of 4 pHLAs. PESA of A2-IAV M1 complex was calculated based on 2VLL (PDB ID). PESA of A2-HIV RT complex was calculated based on 2X4U (PDB ID).

b, Exposed surface areas (ESA) of individual residues at each position of four peptides within HLAs.
c. Mut10m exhibits a relatively large exposed surface area compared to A2-IAV M1 (the featureless benchmark), C08-mut9m and C08-mut10m. HLA backbone surface was shown in grey. IAV M1 (blue), mut9m (green) and mut10m (yellow) peptide surface in different colors.

d. Different T cell fate of KRAS G12D neoantigen restricted T cells reported by previous clinical studies. Rank in tumor sample represents the rank of restricted T cells in patient TILs before cell therapy. Rank in infusion products represents the rank of restricted T cells in ex vivo cell transfer products before cell transfer. Rank in blood represents the rank of restricted T cells in patients’ peripheral blood after cell transfer (d+ represents the day after cell transfer). The rank of T cells with same TCR pair was validated in one or three different metastatic tumor fragments from the patient. *, tested in three fragments; †, tested in one fragment; ND, not detected.

Supplementary Figure Legends

Supplementary Figure 1. 9mer peptides binding profile of 30 HLA alleles.
Sequence logos frequent amino acid binding profiles for 9mer peptides bound to each of 30 HLA alleles generated from peptide-binding matrices using the Seq2Logo. Peptide libraries were obtained from IEDB. The sample size of each allele was shown on the figure.

Supplementary Figure 2. The AUC values testing using a 50-fold cross-validation.
The 50-fold cross-validation (2/3rd random resampling) within the exploration set. Differences of AUC values were determined using paired T-test.

Supplementary Figure 3. Additional TCR accessible surface of mut9m neoantigen in complex with HLA-C*08:02.
P3D of C08-mut9m can provide additional TCR accessible surface. HLA-C*08:02 (grey), 9mer peptide (green) and P3D side chain (red) showed in different colors.

Supplementary Figure 4. The topology of different neoantigen-MHC binding models
Model A demonstrates the neoantigens with mutations at TCR-contacting positions that are presented by MHC. The blue circle represents wild type residues. The red triangle represents the mutant residues. Model B demonstrates the neoantigens with mutations at MHC-contacting positions. The red triangle represents the mutant residues compared to the wild type that would affect binding specificity. Model C demonstrates the peptides which have mutations at anchor position and are...
presented by MHC. The yellow rectangle represents wild type residues which cannot bind into the anchor position in the MHC peptide presentation groves and thus are not presented. The purple circle represents mutated residues which are preferential selected and bind better at the anchor position.